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Crosstalk in ferroptosis, immunity & inflammation

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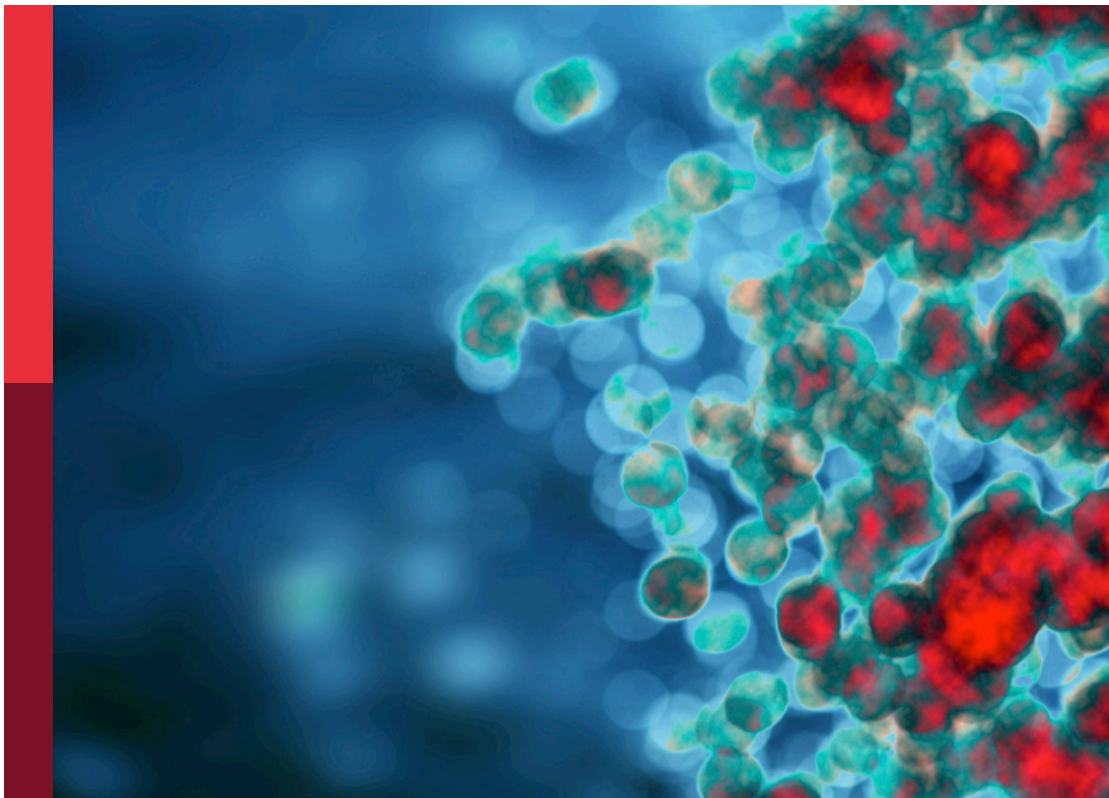

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Crosstalk in ferroptosis, immunity & inflammation

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Editorial: Crosstalk in ferroptosis, immunity & inflammation

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Editorial on the Research Topic
Crosstalk in ferroptosis, immunity & inflammation

Ferroptosis, an iron-dependent form of regulated cell death characterized by iron overload and lipid peroxidation, has gained significant attention because of its involvement in various pathological conditions, including cancer, neurodegenerative diseases, and inflammatory disorders (1, 2). The intricate interplay between ferroptosis, immune responses, and inflammatory pathways remains a key area of research, with implications for therapeutic strategies targeting these mechanisms. This Research Topic, "*Crosstalk in Ferroptosis, Immunity & Inflammation*," presents a collection of 24 articles that explore the multifaceted roles of ferroptosis across various disease contexts. By examining how ferroptotic processes intersect with immune responses and inflammatory pathways, these studies shed light on potential mechanisms underpinning a broad spectrum of conditions —from inflammatory and autoimmune disorders to cancer, neurodegeneration, metabolic diseases, and organ-specific injuries.

Ferroptosis in inflammatory and autoimmune diseases

Paper in this Research Topic discuss the pivotal role of ferroptosis in the pathogenesis of inflammatory diseases such as inflammatory bowel disease (IBD) (Xie et al.), systemic lupus erythematosus (SLE) (Zhao et al.), and lupus nephritis (Fan et al.). Iron metabolism dysregulation and oxidative stress-induced cell death contribute significantly to these pathologies. Investigations into immune imbalances, particularly T-cell-mediated responses, underscore the link between ferroptotic pathways and chronic inflammation in autoimmune disorders. Understanding these mechanisms offers potential therapeutic strategies to mitigate disease progression by targeting ferroptosis-related pathways. Furthermore, oxidative stress and lipid peroxidation—hallmarks of ferroptosis—have

been shown to amplify inflammatory responses in chronic diseases (3). The delicate balance between ferroptotic cell death and immune regulation is crucial for maintaining tissue homeostasis. Research suggests that inhibiting ferroptosis may protect against excessive inflammation, while the controlled induction of ferroptosis could be leveraged to eliminate hyperactive immune cells that contribute to autoimmune diseases (4).

Ferroptosis in cancer and tumor immunology

Ferroptosis is increasingly recognized as a key factor in tumor biology (5, 6). Xia et al. have implicated ferroptosis in cancers, including colorectal cancer, hepatocellular carcinoma (Yang et al.), and lung cancer (Gao and Zhang). Regulators such as SLC7A11 in ferroptosis emerges as promising targets for tumor therapy (7) (Jiang and Sun). Ferroptosis appears to synergize with immunotherapy to enhance antitumor responses, offering new perspectives for cancer treatment. The emerging role of long noncoding RNAs (lncRNAs) in regulating ferroptosis sheds light on novel molecular interactions influencing tumor progression (Chen et al.). Additionally, ferroptosis-based therapies may overcome resistance to traditional chemotherapies and improve patient outcomes by modulating the tumor microenvironment. One of the key mechanisms by which ferroptosis influences cancer progression is through its impact on immune cell infiltration and tumor-associated macrophages. Moreover, the release of damage-associated molecular patterns (DAMPs) from ferroptotic tumor cells can activate antigen-presenting cells, thereby modulating adaptive immune response. Harnessing ferroptosis offers exciting avenues for overcoming chemoresistance and improving the efficacy of immune checkpoint inhibitors.

Ferroptosis in neurodegenerative and neurological disorders

Ferroptosis has emerged as a key player in neurodegenerative diseases, contributing to neuronal damage through iron accumulation and oxidative stress. In Alzheimer's disease and Parkinson's diseases, iron accumulation and lipid peroxidation contribute to neuronal loss and cognitive decline. Additionally, ferroptosis is involved in neuroinflammation by modulating microglial activation, which in turn influences neuronal survival and disease progression. Emerging therapeutic strategies—ranging from antioxidant-based approaches to iron chelation—are being explored to protect neuronal integrity by targeting ferroptotic pathways. In addition to classical neurodegenerative disorders, ferroptosis also plays a critical role in sepsis-associated encephalopathy (SAE), a severe neurological complication of sepsis. Microglia-derived CXCL2 has been shown to promote neuronal ferroptosis in SAE by activating Jun, exacerbating cognitive dysfunction and neuronal loss. Inhibiting CXCL2 or Jun has demonstrated neuroprotective effects, suggesting that targeting ferroptosis could be a novel therapeutic strategy for mitigating SAE-induced brain injury (Yang et al.).

Ferroptosis in metabolic, renal, pulmonary, and cardiovascular diseases

Chronic metabolic disorders, including diabetes and obesity, are often accompanied by ferroptosis-driven tissue damage. Hyperglycemia-induced oxidative stress can lead to ferroptotic cell death in renal and vascular tissues, contributing to diabetic nephropathy and atherosclerosis. Understanding the metabolic regulation of ferroptosis could help in designing effective interventions for metabolic syndrome and related disorders (8). Additionally. Liu et al. have identified key ferroptosis-related genes involved in endometriosis pathogenesis, highlighting the potential for ferroptosis-targeting therapies to mitigate disease progression and immune dysfunction.

Kidney diseases is a typical metabolic disorder. Dysregulation of iron homeostasis during ferroptotic processes significantly influences progression of kidney diseases, including acute kidney injury and renal fibrosis (9, 10) (Long et al.). While alterations in lipid metabolism also modulate ferroptotic cell death. These findings highlight the need for further mechanistic studies to fully understand these pathways. In renal ischemia–reperfusion (I/R) injury, ferroptosis contributes to kidney damage, which is characterized by increased collagen deposition, α-SMA expression, and oxidative stress. Fer-1 treatment or ATF3 knockdown alleviated these changes while modulating Nrf2/HO-1 signaling. Additionally, exosomal miR-1306-5p release under hypoxia-reoxygenation promoted M2 macrophage polarization, linking ferroptosis to immune regulation in renal fibrosis (Tang et al.). Moreover, Li et al. 's study investigating the role of ferroptosis in acute kidney injury (AKI) revealed that GSTT1 and GSTM1 were significantly downregulated in the renal tubular cells of AKI patients and in cisplatin-treated mice. Notably, Gstm1/Gstt1 double knockout (DKO) mice, while phenotypically normal under baseline conditions, presented exacerbated kidney dysfunction, increased ROS levels, and severe ferroptosis following cisplatin administration, underscoring the contribution of GST enzymes to ferroptotic resistance in renal injury.

In chronic obstructive pulmonary disease (COPD), ferroptosis-mediated alveolar epithelial cell death exacerbates disease progression. Exposure to electronic nicotine delivery systems (ENDS) has been linked to worsening COPD features, including emphysema, mucus accumulation, inflammation, and fibrosis, all of which are associated with lipid dysregulation and ferroptotic injury (Han et al.). Beyond pulmonary diseases, ferroptosis also plays a crucial role in I/R injury, a pathological process affecting multiple organ systems, including the heart, brain, lung and kidneys. Ferroptotic cell death contributes to endothelial dysfunction, which in turn promotes vascular inflammation and atherosclerosis. In cardiovascular conditions such as heart failure and myocardial infarction, oxidative stress-induced ferroptosis of

cardiomyocytes accelerates disease progression, particularly in cases involving iron overload. Additionally, ferroptosis has been implicated in cerebral I/R injury, where it exacerbates neuronal damage and neuroinflammation (He et al.), further linking ferroptotic mechanisms to both cardiovascular and neurological pathologies. Given its broad impact, targeting ferroptosis represents a promising therapeutic strategy for mitigating I/R injury, reducing vascular complications, and improving outcomes in cardiopulmonary diseases.

Emerging perspectives and future directions

The rapid evolution of ferroptosis research has opened new therapeutic frontiers across various medical disciplines. Studies exploring anastasis—the recovery from ferroptotic stress—could unveil novel mechanisms of cellular resilience. Advanced bioinformatics analyses have identified key molecular signatures linking ferroptosis with immune infiltration, further reinforcing the importance of computational approaches in this field. Looking ahead, the development of reliable biomarkers, targeted inhibitors, and inducers of ferroptosis will be crucial. Moreover, integrating ferroptosis modulation with immunotherapy holds significant promise for clinical translation across a range of conditions, particularly in oncology and inflammatory diseases.

Conclusion

This Research Topic provides valuable insights into the crosstalk between ferroptosis, immunity, and inflammation across diverse disease models. By understanding its molecular mechanisms and interactions with immune responses, inflammation, and metabolic pathways, we can identify novel therapeutic targets and improve treatment strategies for a wide range of conditions. Future research should focus on ferroptosis-modulating strategies to enhance therapeutic efficacy, minimize disease progression, and improve patient outcomes. The findings presented in this Research Topic pave the way for future investigations and potential clinical applications, emphasizing the critical role of ferroptosis in immune regulation and disease pathogenesis.

Author contributions

YL: Investigation, Resources, Writing – original draft, Writing – review & editing. GW: Investigation, Writing – original draft. LF: Investigation, Writing – original draft. QF: Conceptualization, Visualization, Writing – review & editing. HZ: Conceptualization, Investigation, Writing – review & editing.

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Ferroptosis and hepatocellular carcinoma: the emerging role of lncRNAs

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Hepatocellular carcinoma is the most common form of primary liver cancer and poses a significant challenge to the medical community because of its high mortality rate. In recent years, ferroptosis, a unique form of cell death, has garnered widespread attention. Ferroptosis, which is characterized by iron-dependent lipid peroxidation and mitochondrial alterations, is closely associated with the pathological processes of various diseases, including hepatocellular carcinoma. Long non-coding RNAs (lncRNAs), are a type of functional RNA, and play crucial regulatory roles in a variety of biological processes. In this manuscript, we review the regulatory roles of lncRNAs in the key aspects of ferroptosis, and summarize the research progress on ferroptosis-related lncRNAs in hepatocellular carcinoma.

KEYWORDS

hepatocellular carcinoma, cell death, ferroptosis, lipid peroxidation, lncRNA

1 Introduction

Cancer has always been a worldwide health problem that perplexes physicians and patients. The American Cancer Society estimates that there will be approximately 2 million new cancer cases and 0.6 million cancer-related deaths in the United States in 2024 (1). With advances in medical treatment, the overall 5-year survival rate of cancer patients has increased from 49% in the 1970s to 69% in the 2010s. However, the survival rate of patients with liver cancer remains low at 22% (1). Hepatocellular carcinoma (HCC) is the main form of primary liver cancer, accounting for 90% of liver cancers (2, 3). The molecular mechanism of HCC is complex and is related to susceptibility genes and viral and nonviral risk factors. Common risk factors for HCC include hepatitis B virus (HBV), hepatitis C virus, and fatty liver disease, which can cause chronic hepatitis and lead to cirrhosis and eventually to HCC (4). Moreover, the persistence of cirrhosis leads to a high recurrence rate of HCC (2). Despite significant advances in the treatment and management of HCC, the

overall prognosis of patients remains unsatisfactory. Precisely targeted therapies are required to eliminate tumor cells and minimize side effects on healthy cells.

Killing tumor cells is an important part of tumor treatment. In multicellular organisms, cell death is an important mechanism that helps maintain the cell balance while renewing cells. Cell death can be divided into programmed cell death (PCD) and accidental cell death, and PCD can be classified as apoptosis, necroptosis, autophagy, pyroptosis, ferroptosis, or entosis (5). Ferroptosis is a type of PCD that is characterized by iron-dependent lipid peroxidation. Morphologically, when cells undergo ferroptosis, mitochondria exhibit shrinkage, reduced numbers of cristae, and an increased membrane density (6). Mitochondria are important organelles involved in energy metabolism and biosynthesis. Reactive oxygen species (ROS) are intermediate products of mitochondrial respiration and are highly oxidative. Mitochondria of cancer cells characteristically produce more ROS, which facilitate the progression of cancer by promoting the expression of oncogenes and related signaling pathways (7–9). Compared with that in healthy cells, iron metabolism in cancer cells is often altered towards iron accumulation, and iron overload promotes cancer development (10). Therefore, targeting cancer cell ferroptosis may be a potential cancer therapy with few side effects.

Non-coding RNA (ncRNA) refers to RNA that is transcribed but is not translated, and ncRNAs account for 98–99% of the eukaryotic genome (11). Although they do not encode proteins, ncRNAs affect the expression of genes and proteins in different ways and participate in various physiological and pathological processes. Long non-coding RNAs (lncRNAs) are the largest subgroup of ncRNAs with lengths >200 nt. At present, tens of thousands of lncRNAs have been identified and divided into the following five categories: sense, antisense, bidirectional, intergenic, and intronic lncRNAs (12, 13). Recent studies have shown that lncRNAs are involved in the regulation of iron overload, ROS production, and other important ferroptosis-related processes (14–16). Therefore, targeting ferroptosis-related lncRNAs may be a promising therapeutic approach for treating cancers such as HCC.

In this manuscript, we review the research progress on the key links and mechanisms of ferroptosis, summarize the regulatory effects of lncRNAs on ferroptosis, and explore the potential mechanisms of lncRNA-mediated ferroptosis and their significance in HCC. We believe that this review will help further understand the role of ferroptosis-related lncRNAs in HCC and provide novel strategies for the treatment of this malignancy.

2 Ferroptosis and HCC

Ferroptosis was initially discovered as a unique form of cell death that was induced by erastin and RSL3 in RAS-expressing cancer cells; this type of cell death did not have the characteristics of apoptosis, nor could it be reversed by caspase inhibitors (17). The term 'ferroptosis' was first used in 2012 by Dixon et al. to describe a form of cell death that was characterized by intracellular iron dependency and was distinct from other types of PCD; the term was finally recommended by the Nomenclature Committee on Cell Death (NCCD) in 2018 (18, 19). Mechanistically, ferroptosis is triggered by iron-dependent lethal levels of lipid peroxidation, resulting in characteristic mitochondrial changes and necrosis-like changes in cells (17). The solute carrier family 7 member 11 (SLC7A11)/glutathione (GSH)/glutathione peroxidase 4 (GPX4), ferroptosis suppressor protein 1 (FSP1)/$CoQ_{10}H_2$ and GTP cyclohydrolase-1 (GCH1)/tetrahydrobiopterin (BH_4) axes constitute the main intracellular ferroptosis defense system and inhibit lipid peroxidation through strong reducing products (20). When these ferroptosis defense axes are impaired, intracellular iron overload leads to the generation of large amounts of ROS via the Fenton reaction, which promotes lipid peroxidation and ferroptosis (21). In addition, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), p53, AMPK, nuclear factor E2-related factor 2 (Nrf2)/HO-1, hypoxia, and other signaling pathways are involved in the regulation of ferroptosis (6).

The liver is the primary site for iron storage and metabolism, as well as an essential organ for lipid and amino acid metabolism (22). In the liver, excessive iron storage or metabolic disorders can lead to abnormally high iron levels within cells and an increased production of ROS. Abnormal lipid metabolism may cause excessive accumulation of lipids within cells, creating a lipotoxic environment (23). Meanwhile, abnormal amino acid metabolism can affect the synthesis of antioxidants such as glutathione, reducing the cell's resistance to oxidative stress. Consequently, this high metabolic state makes hepatocytes prone to ferroptosis under specific conditions, contributing to the development of various liver diseases. In recent years, increasing number of studies have shown that ferroptosis plays a role in the occurrence, progression, and treatment of HCC. Iron overload is a risk factor for HCC (24). Liang et al. analyzed TCGA cohort data of HCC patients and showed that more than 80% of ferroptosis-related genes were differentially expressed between HCC tissues and peritumoral tissues, and a prediction model including 10 ferroptosis-related genes such as *GPX4* and *SLC7A11* was established to predict the prognosis of HCC patients (25). Ferroptosis is an important mechanism of radiotherapy-induced tumor cell death (5). The regulation of important factors involved in ferroptosis (such as SLC7A11) can promote ferroptosis and ionizing radiation sensitization in HCC cells (26, 27). Currently, the first-line chemotherapy agents for HCC are sorafenib and lenvatinib (4). Sorafenib, a multiple-target tyrosine kinase inhibitor, has been shown to induce ferroptosis in various solid tumors, such as HCC, melanoma, and colon carcinoma (28–30). The tyrosine kinase inhibitor lenvatinib is noninferior to sorafenib in treating HCC (4, 31). Lenvatinib can inhibit SLC7A11 and GPX4 by inhibiting fibroblast growth factor receptor-4 (FGFR4), leading to ferroptosis in HCC cells (32). Ferroptosis is also closely related to immunotherapy for HCC. Immunotherapy aims to change the tumor microenvironment (TME) and inhibit tumor progression. Liu et al. established a ferroptosis and epithelial-mesenchymal transition (EMT)-related prognostic model (FEPM) to predict the prognosis of HCC patients. According to this model, the expression of ferroptosis suppressor genes such as *GPX4*, *SLC7A11*, *FTH1*, and *SCD*, was increased in the high-FEPM group, and

immunosuppressive cells such as Tregs were highly enriched, resulting in a worse prognosis of patients (33). In another study, KEGG pathway analysis revealed that erastin-induced signaling cascade could affect Th17 cell differentiation and IL-17 signaling pathway in HCC (34). Single-cell RNA sequencing revealed that APOC1$^+$ macrophages were abundant in HCC tissues, and expressed the M2 macrophage marker CD163, which plays a tumor-promoting and immunosuppressive role (35). APOC1 inhibition can promote the transformation of tumor-associated macrophages (TAMs) from the M2 to the M1 phenotype through the ferroptosis pathway. APOC1 inhibition was shown to reduce the expression of GPX4, SLC7A11, and Nrf2, and iron overload-induced ROS could also promote M1 polarization and inhibit HCC (36). Based on the important role of ferroptosis in the occurrence, progression, and treatment of HCC, targeting ferroptosis could be a potential direction for HCC treatment. In the following subsections, we discuss the effects of lncRNAs on ferroptosis at different stages and the function of ferroptosis-associated lncRNAs in HCC (Figure 1).

3 LncRNAs and ferroptosis

3.1 LncRNAs and iron metabolism

Iron is an essential trace element for human body and participates in a wide range of biological processes, such as cellular respiration, energy metabolism, oxygen transport, and DNA synthesis (10). Normal intracellular iron metabolism maintains intracellular iron homeostasis and cell function. Cells acquire transferrin (TF)-bound ferric (Fe^{3+}) iron from the circulation through a transferrin receptor (TFR1). Ferric iron is subsequently reduced to ferrous (Fe^{2+}) iron in endosomes by metalloreductases, such as six-transmembrane epithelial antigen of prostate 3 (STEAP3), and is transported to the cytoplasm via divalent metal transporter 1 (DMT1) to participate in intracellular biological processes (37). Fe^{2+} that is not involved in biological processes is excreted from the cell by ferroportin 1 (FPN1) or binds to ferritin for storage. FPN1 is the only known intracellular iron exporter, and hepcidin is a major regulator of FPN1 (38). Excess intracellular Fe^{2+} can be oxidized by ferritin to Fe^{3+}, and Fe^{3+} is stored in a complex with ferritin light chain (FTL) or ferritin heavy chain 1 (FTH1) (30). Nuclear receptor coactivator 4 (NCOA4) can bind to ferritin, promote ferritinophagy, and release iron stored in ferritin (39). Abnormal iron metabolism can lead to increased intracellular free iron levels and iron overload (Figure 2).

Lu et al. reported that the lncRNA plasmacytoma variant 1 (PVT1) could inhibit miR-214, and in acute ischemic stroke (AIS) with cerebral ischemia/reperfusion (I/R) injury, PVT1 silencing could promote miR-214 expression, suppress TFR1 and ferritin levels, and reduce ferroptosis (40). Mechanistically, miR-214 could bind to PVT1 and TFR1, and PVT1 silencing promoted the binding of miR-214 to the 3' untranslated region (3'UTR) of TFR1 and reduced its expression. He et al. reported that the lncRNA related to iron metabolism (LncRIM) could directly bind to neurofibromatosis 2 (NF2), activate the Hippo pathway and activate Yes-associated protein 1 (YAP1) to promote the expression of TFR1 and DMT1 and to promote breast cancer cell ferroptosis (41). Studies have shown that FPN1 has an iron chaperone protein poly(rC)-binding protein 2 (PCBP2)-binding domain and that iron-loaded PCBP2 binds to FPN1 to promote intracellular iron export (42). Xiang et al. reported that the lncRNA MAF transcription factor G antisense RNA 1 (MAFG-AS1) could promote PCBP2 stabilization and iron export in bladder urothelial carcinoma (BUC) cells (43). MAFG-AS1 expression is regulated by the transcription factor MAFG and can also promote MAFG transcription via positive feedback. MAFG-AS1-MAFG inhibition promotes ferroptosis and sensitivity to cisplatin in BUC cells.

3.2 LncRNAs and lipid peroxidation

Phospholipids (PLs) are one of the important components of the cell membrane and are composed of a head group and two fatty

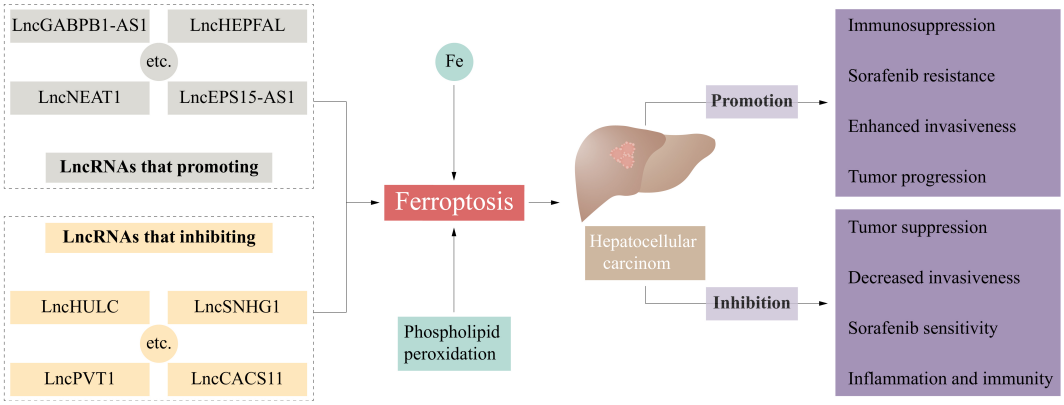

FIGURE 1
Ferroptosis-related lncRNAs influence the occurrence and development of hepatocellular carcinoma. CASC11, cancer susceptibility candidate 11; EPS15, epidermal growth factor receptor pathway substrate 15; GABPB1, GA-binding protein B1; HULC, highly upregulated in liver cancer; LncRNA, long non-coding RNA; PVT1, plasmacytoma variant 1; SNHG1, small nucleolar RNA host gene 1.

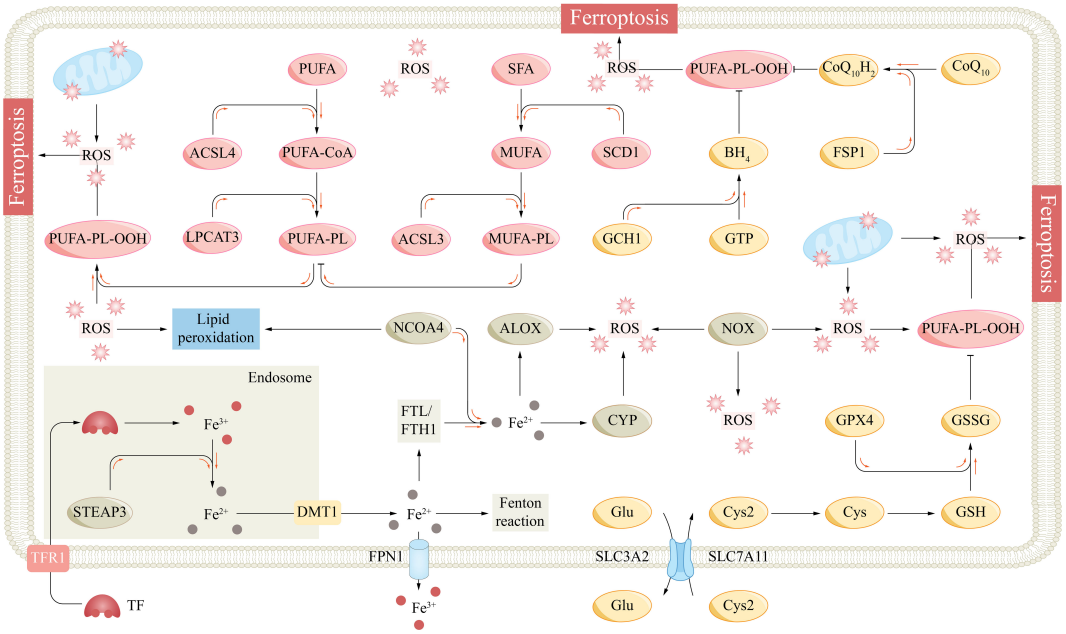

FIGURE 2
The fundamental mechanism underlying ferroptosis. ACSL3/4, acyl-CoA synthetase long-chain family member 3/4; ALOX, arachidonate lipoxygenase; BH4, tetrahydrobiopterin; CoQ10, coenzyme Q10; CYP, cytochrome p450 family; Cys, cysteine; Cys2, cysteine; DMT1, divalent metal transporter 1; Fe^{2+}, ferrous; Fe^{3+}, ferric; FPN1, ferroportin 1; FSP1, ferroptosis suppressor protein 1; FTH1, ferritin heavy chain 1; FTL, ferritin light chain; GCH1, GTP cyclohydrolase-1; Glu, glutamate; GPX4, glutathione peroxidase 4; GSH, glutathione; GSSG, glutathione disulfide; GTP, guanosine triphosphate; LPCAT3, lysophosphatidylcholine acyltransferase 3; MUFA, monounsaturated fatty acid; NCOA4, nuclear receptor coactivator 4; NOX, NADPH oxidase; PL, phospholipid; PLOO, phospholipid peroxyl radical; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SCD1, stearoylCoA desaturase-1; SFA, saturated fatty acid; SLC7A11, subunit solute carrier family 7 member 11; STEAP3, six transmembrane epithelial antigen of the prostate 3; TF, transferrin; TFR1, transferrin receptor 1.

acyl chains. Different fatty acyl chains, such as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs), link the head group at the *sn1* and *sn2* sites, enriching phospholipid diversity (44). Free PUFAs, such as epinephrine and arachidonic acid, are processed by acyl-coenzyme A synthetase long-chain family member 4 (ACSL4), lysophosphatidylcholine acyltransferase 3 (LPCAT3), and other enzymes to bind to the *sn2* site to form PUFA-PLs and are closely associated with the fluidity of cell membranes (45). However, PUFAs are lipids that are most susceptible to peroxidation, which leads to ferroptosis. Lipid peroxidation is caused by strong oxidants that attack the carbon–carbon double bonds (C=C) of lipids (46). PUFA-PLs contain bis-allylic groups and are easily oxidized to form phospholipid radicals (PL•), which subsequently form phospholipid peroxyl radicals (PLOO•) with oxygen molecules (20). Importantly, PLOO• can seize a hydrogen atom from the bis-allylic group of another PUFA-PL, leading to the formation of another PL• and the transmission of lipid peroxidation (47). Under iron overload conditions, a large amount of ROS produced by the Fenton reaction induce nonenzymatic peroxidation of PUFAs (48). Some iron-dependent oxygenases, such as arachidonate lipoxygenase (ALOX) and cytochrome P450 (CYP) family proteins, can cause lipid peroxidation (Figure 2) (49, 50).

Several lncRNAs have been shown to regulate ACSL4. In high-glucose-induced diabetic retinopathy, the highly expressed lncRNA zinc finger antisense RNA 1 (ZFAS1) promoted ferroptosis in human retinal endothelial cells (hRECs). ZFAS1 acted as a sponge for miR-7–5p to competitively regulate the expression of ACSL4 and drive lipid peroxidation in hRECs (51). Sun et al. reported that the lncRNA taurine-upregulated gene 1 (TUG1) interacted with serine/arginine splicing factor 1 (SRSF1) to regulate ACSL4 mRNA stability and inhibit ACSL4-mediated ferroptosis and acute kidney injury (52). Jin et al. reported that the lncRNA HOX transcript antisense RNA (HOTAIR) could directly bind to up-frameshift 1 (UPF1), competitively inhibit UPF1-ACSL4 binding and ACSL4 degradation, and positively regulate ACSL4-dependent neuronal ferroptosis (53). LncRNA small nucleolar RNA host gene 1 (SNHG1) knockdown was shown to regulate the miR-16–5p/ACSL4 axis to suppress hyperglycemia-induced ferroptosis in diabetic nephropathy (54).

Stearoyl-CoA desaturase 1 (SCD1) is a key enzyme of lipid metabolism that is capable of catalyzing the conversion of SFAs into MUFAs. SCD1-catalyzed MUFAs can replace PUFAs and inhibit ferroptosis in an ACSL3-dependent manner (55). Luo et al. reported that the lncRNA LINC01606 was highly expressed in colon cancer and regulated SCD1 expression through competitive inhibition of miR-423–5p, which promoted MUFA production and ferroptosis inhibition (56). In addition, the positive feedback loop of SCD1–Wnt/β-catenin–IGHM enhancer 3 (TFE3) signaling promoted LINC01606 expression, which continuously increased the MUFA levels and protected colon cells against ferroptosis. Lai et al.

reported that the exosomal lncRNA SRY-box transcription factor 2 overlapping transcript (SOX2-OT) could promote the progression of ovarian cancer cells through the miR-181b-5p/SCD1 axis (57).

3.3 LncRNAs and the ferroptosis defense system

Under physiological conditions, cells inhibit lipid peroxidation through several antioxidant systems. The subunit solute carrier family 7 member 11 (SLC7A11)/glutathione (GSH)/glutathione peroxidase 4 (GPX4) axis is a major ferroptosis defense system, and its inactivation can lead to severe ferroptosis (58). GPX4 is the major lipid peroxide reductase that reduces PL-OOH to nontoxic PL-OH, and its activity is dependent on GSH and selenium (59). GSH is a tripeptide composed of glutamate, glycine, and cysteine and is the main endogenous nonprotein antioxidant. GSH is reduced to oxidized glutathione (GSSG) by GPX4 to drive the reduction of PL-OOH, and this process is inhibited when the GSH concentration is insufficient. Cysteine is the major rate-limiting precursor for GSH synthesis, and most cells need to obtain cysteine by extracellular transport, except for tissues (such as the liver) that can synthesize cysteine through the transsulfuration pathway (60). Extracellular cysteine is transported by the cystine/glutamate reverse transporter (X_C^-). Its heavy chain SLC7A11 transports extracellular cystine and excretes intracellular glutamate at a 1:1 ratio; subsequently, cystine is reduced to cysteine by NADPH and participates in GSH synthesis (58, 61). The SLC7A11-GSH-GPX4 axis constitutes a major PL-OOH detoxification system (Figure 2).

Various lncRNAs have been demonstrated to regulate the SLC7A11/GSH/GPX4 axis. The lncRNA T-UCR Uc.339 was shown to inhibit miR-339 and promote SLC7A11 expression, also promoting lung adenocarcinoma growth and metastasis (62). The lncRNA HEPFAL can regulate the ubiquitination of SLC7A11 and affect its function (63). The lncRNA double homeobox A pseudogene 8 (DUXAP8) can regulate SLC7A11 palmitoylation and protect HCC cells (64). The lncRNA ovarian tumor domain containing 6B antisense RNA1 (OTUD6B-AS1) can promote GPX4-mediated ferroptosis and increase the radiosensitivity of colorectal cancer cells (65). The lncRNA LINC01134 inhibits ferroptosis and promotes oxaliplatin resistance of HCC by promoting Nrf2-GPX4 binding (66). The lncPVT1/miR-214 axis can also promote GPX4 expression and the proliferation of liver cancer cells (67).

The ferroptosis suppressor protein 1 (FSP1)/ubiquitin (CoQ_{10}) and GTP cyclohydrolase-1 (GCH1)/tetrahydrobiopterin (BH_4) axes defend cells against ferroptosis in parallel with the SLC7A11/GSH/GPX4 axis. CoQ_{10} is synthesized in mitochondria and is involved in electron transport in the mitochondrial respiratory chain (68). CoQ_{10} can be transformed into oxidized and reduced states, and its fully reduced form, $CoQ_{10}H_2$, has strong antioxidant activity. FSP1 is a NAD(P)H-dependent CoQ_{10} reductase, and the FSP1/CoQ_{10} axis consumes NAD(P)H and promotes the reduction of CoQ_{10} to $CoQ_{10}H_2$, which directly consumes lipid free radicals (69). BH_4 also has strong antioxidant properties and can directly reduce lipid free radicals. GCH1 can catalyze the conversion of guanosine triphosphate (GTP) to BH_4 (70). Dihydroorotate dehydrogenase (DHODH) can also couple the oxidation of dihydroorotate (DHO) to orotate (OA) to reduce CoQ_{10} to $CoQ_{10}H_2$ in the mitochondrial inner membrane (71). Mao et al. reported that DHODH could inhibit ferroptosis in parallel with GPX4 and FSP1 (71). However, Mishima et al. reported that the ferroptosis effect of DHODH appeared to be small and environment dependent, with high concentrations of DHODH inhibitors also inhibiting FSP1 and inducing ferroptosis mainly through FSP1 inhibition (Figure 2) (72).

LINC02587 expression is elevated in various cancers and is linked to a poor prognosis (73–75). Wang et al. reported that LINC02587 silencing led to the inhibition of the CoQ10/FSP1 axis and ferroptosis promotion in glioma cells (75). Similarly, high expression of LINC01133 is associated with a poor prognosis in cancers (76). Wang et al. reported that overexpression/knockdown of LINC01133 resulted in increased/decreased expression of FSP1, respectively, but did not affect that of GPX4. An RNA pulldown assay revealed that LINC01133 and FSP1 could bind to FUS to form the LINC01133-FUS-FSP1 complex, which regulated the expression of FSP1 mRNA (77). There are no reports on lncRNA regulation of the GCH1/BH_4 axis, and this is a potential research direction. The circRNA circLRFN5 was shown to suppress paired related homeobox 2 (PRRX2)-mediated GCH1 expression in glioblastoma (78). A study also reported that the lncRNA USP30 −AS1 was coexpressed with GCH1 in breast cancer (79).

3.4 LncRNAs and mitochondria

Mitochondrion is an important organelle of eukaryote, which is involved in biological processes such as energy production, material metabolism, signal transduction, etc (80). Mitochondria play important roles in ferroptosis. Mitochondria are the center of iron metabolism and iron balance, participating in the storage, transportation, and excretion of iron within cells. Iron within mitochondria is involved in the formation of iron-sulfur clusters, heme, and mitochondria ROS (mitoROS) (81). As the core of the iron-porphyrin structure, iron is a crucial component of enzymes such as catalase and peroxidase. Iron ions participate in the electron transport of the mitochondrial respiratory chain, promoting the progress of redox reactions (82). Intracellular ROS are mainly produced by mitochondria. The tricarboxylic acid (TCA) cycle is a key metabolic pathway in mitochondria, connecting the metabolism of glucose, lipid, and amino acids, and providing energy for cells (83). Changes in the levels of certain intermediates in the TCA cycle, such as isocitrate and 2-oxoglutarate, can affect the accumulation of lipid ROS and the progress of ferroptosis (84). The electron transport chain (ETC) is a series of complexes located on the inner membrane of mitochondria, which generate ATP by transferring electrons, providing energy for cells, and this process produces ROS (84). Additionally, there is a free iron pool within mitochondria that participates in the accumulation of mitoROS (85). Functionally normal mitochondria can also resist oxidative stress and lipid peroxidation through antioxidant substances. The GSH-GPX4

axis within mitochondria plays an important antioxidant role. In addition, dihydroorotate dehydrogenase (DHODH) is an antioxidant parallel to GPX4, which reduces CoQ_{10} to $CoQ_{10}H_2$ and can inhibit ferroptosis when GPX4 is inactivated (71). However, another study showed that the inhibitory effect of DHODH on ferroptosis is minimal and is related to FSP1 (72). Finally, mitophagy can alleviate intracellular oxidative stress and inhibit ferroptosis by removing aged or damaged mitochondria (86).

Various lncRNAs play important roles in the regulation of mitochondrial function. A study based on public databases has identified eight lncRNAs (AC023090.1, AC099850.4, AL365361.1, CYTOR, ACSL6-AS1, LHFPL3-AS2, LINC02362, and MSC-AS1) that potentially affect mitochondrial function and ferroptosis in HCC through cell cycle pathways (87). Zhao et al. found that the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) plays a role in mitochondrial gene reprogramming in HCC. RNA reverse transcription-associated trap sequencing (RAT-seq) revealed that MALAT1 interacts with multiple sites on mitochondrial DNA. MALAT1 function as an epigenetic messenger to coordinate the functions between the nucleus and mitochondria. Although they did not examine ferroptosis-related indicators, knocking down MALAT1 can inhibit mitochondrial autophagy and induce apoptosis (88).

4 Ferroptosis-related lncRNAs and HCC

As potential biomarkers, lncRNAs play crucial roles in the genesis, progression, and metastasis of HCC, which renders them viable options for assessing tumor prognosis based on their expression. In recent years, several ferroptosis-related lncRNA models have been constructed to predict the prognosis of patients with HCC (87, 89–97). Nevertheless, predictive models, particularly those based on RNA-sequencing and retrospective bioinformatics data analysis are based on retrospective bioinformatics data analyses and necessitate additional experimental validation to ensure their reliability. Xu et al. established a ferroptosis-related 9-lncRNA model (LINC00942, LINC01224, LINC01231, LINC01508, CTD-2033A16.3, CTD-2116N20.1, CTD-2510F5.4, DDX11-AS1, and ZFPM2-AS1) to predict HCC prognosis and the immune response, and the expression of these lncRNAs was increased in the high-risk group, which was correlated with the suppression of HCC cell ferroptosis (98). CTD-2033A16.3, LINC01231, and LINC01508 knockdown led to increased intracellular Fe^{2+} and malondialdehyde (MDA) levels in the HCC cell line HUH-7. In the high-risk group, the numbers of Tregs, follicular helper T cells, and M0 macrophages were increased, and the immune tolerance of HCC patients was enhanced, which provided potential targets for immunotherapy in HCC patients. Xie et al. established a model that comprised 16 m6A-methylated and ferroptosis-associated lncRNAs (99). RNA N6-methyladenosine (m6A) methylation modification specifically targets the sixth nitrogen of adenine (100). m6A plays a crucial role in the splicing, transport, stability, and degradation of ncRNAs and is strongly associated with tumorigenesis and ferroptosis (101, 102). These lncRNAs are associated with ferroptosis and are methylated at m6A, which significantly increases the utility of the model in accurately predicting the clinical stage, prognosis, immune infiltration, and hot and cold tumor subtypes in HCC. A study based on multiomics data revealed that the lncRNA MALAT1 was highly expressed in HCC and was associated with various tumor signatures, including high cell cycle, DNA damage repair, mismatch repair, and homologous recombination signature feature scores and a low ferroptosis feature score (103). Although knocking down MALAT1 has been shown to induce ferroptosis in endometriosis, there is currently no research exploring the mechanism by which MALAT1 regulates ferroptosis in HCC (104).

PVT1 is upregulated in various cancers and promotes cancer progression (105). He et al. reported that PVT1 acted as a competing endogenous RNA (ceRNA), competitively binding to miR-214–3p, inhibiting its binding to GXP4, and promoting GPX4 expression. Ketamine treatment inhibited the expression of PVT1 and GPX4 and induced ferroptosis in HCC cells (67). Similarly, the lncRNA HLA complex group 18 (HCG18) is overexpressed in HCC, particularly in sorafenib-resistant HCC cells (106, 107). As a ceRNA, HCG18 suppressed miR-450b-5p and concomitantly increased the expression of GPX4. Inhibition of HCG18 enhanced the accumulation of MDA and ROS in HCC cells, thereby increasing their sensitivity to sorafenib (106). Chen et al. discovered that high expression of the lncRNA cancer susceptibility candidate 11 (CASC11) in HCC contributed to its resistance to sorafenib. CASC11 directly interacted with SLC7A11 mRNA, increasing its stability and half-life. CASC11 silencing resulted in the elevation of Fe^{2+}, ROS, and MDA levels in HCC cells, which potentiated the therapeutic impact of sorafenib (108). LncRNA NRAV is highly expressed in different HCC cell lines, affecting the expression of GPX4, SLC7A11, and ACSL4 and inhibiting ferroptosis. Functional experiments revealed that NRAV can bind to miR-375–3p, promoting the expression of SLC7A11. However, the specific mechanism remains unclear (109). Zhang et al. reported that the expression of HEPFAL in HCC was significantly reduced, and HCC cells with lower HEPFAL expression had greater invasion and migration abilities. Overexpression of HEPFAL in HCC cells increased the intracellular Fe^{2+} and ROS levels and the sensitivity of HCC cells to erastin-induced ferroptosis (63). Mechanistically, HEPFAL overexpression led to the ubiquitination of SLC7A11, reducing its stability and half-life, but had no significant effect on FSP1. In addition, HEPFAL may inhibit the PI3K/Akt/mTOR signaling pathway, thereby inhibiting GPX4 expression. In another study, lncRNA sequencing revealed a significant increase in the expression of URB-AS1 in HCC tissues, which was positively correlated with the tumor size, grade, and resistance to sorafenib (110). Sorafenib-resistant HCC cells overexpressed URB-AS1 and exhibited decreased levels of free iron, ROS, and lipid peroxidation. Under hypoxic conditions, hypoxia-inducible factor-1α (HIF-1α) is activated, and URB-AS1 is upregulated. URB-AS1 colocalized and interacted with ferritin in the cytoplasm, promoting its punctate distribution and liquid–liquid phase separation. This led to the inhibition of NCOA4-mediated ferritinophagy and a decrease in the level of free iron. Long-term sorafenib treatment may induce

hypoxia within the HCC tumor microenvironment by decreasing microvascular density, which leads to the activation of HIF-1α (111). This HIF-1α activation may enhance the resistance to ferroptosis and sorafenib by activating the URB-AS1 promoter. Yuan et al. reported the regulatory effect of the lncRNA ferroptosis-associated lncRNA (lncFAL, NONHSAG111059.1) on the FSP1/CoQ_{10} pathway (112). LncFAL originates from the plexin B2 (PLXNB2) gene and is modified by YTH N6-methyladenosine RNA-binding protein 2 (YTHDF2) from pre-lncFAL in an m6A-dependent manner. High-density lipoprotein-binding protein (HDLBP) binds to lncFAL and promotes its stabilization. Stable lncFAL remains inactive in regulating the expression of GPX4 or FSP1 mRNA, yet it effectively competes with Trim69 to hinder its binding to FSP1. This competitive inhibition suppresses the ubiquitination-mediated degradation of FSP1 and ferroptosis in HCC cells.

Nrf2 is a crucial transcription factor in regulating cellular antioxidant responses (113). Under physiological conditions, Nrf2 binds to its negative regulator Kelch-like ECH-associated protein 1 (Keap1), which makes Nrf2 inactive, and intracellular Nrf2 is maintained at low levels. However, during oxidative stress, Nrf2 dissociates from Keap1 and binds to the antioxidant response element (ARE) in the nucleus, thereby activating diverse downstream antioxidant pathways (114). Nrf2 plays a pivotal role in regulating various ferroptosis-related factors, including GPX4, SLC7A11, and intracellular free iron (58). The overexpression of the lncRNA LINC01134 is positively associated with a poor prognosis in HCC patients. Mechanistically, LINC01134 facilitates the recruitment of Nrf2 to the GPX4 promoter, which leads to the upregulation of GPX4 expression and the development of resistance to oxaliplatin (66). Peroxiredoxin 5 (PRDX5) is a nonselenium-dependent peroxidase that has been demonstrated to be a binding partner of Nrf2 (115). GABPB1 serves as an activating subunit of Nrf2, and the promoter region of PRDX5 contains binding sites for GA-binding protein (GABP) (116). Overexpression of GABPB1 in HCC cells attenuates ROS and MDA levels, protecting cells from ferroptosis. GABPB1-AS1 is the antisense lncRNA of GABPB1. Qi et al. discovered that erastin treatment potentiated the expression of GABPB1-AS1 and prolonged its half-life. Consequently, GABPB1-AS1-caused suppression of GABPB1 translation resulted in reduced expression of PRDX5 and triggered ferroptosis in HCC cells (117).

Fanconi anemia complementation group D2 (FANCD2), a pivotal nuclear protein that is integral to DNA damage repair, serves as a critical negative regulator of ferroptosis by modulating iron metabolism and lipid oxidation, thereby protecting cells from ferroptosis-induced death (118, 119). A comprehensive database analysis demonstrated that FANCD2 was overexpressed in various tumors, including HCC, and exhibited a positive correlation with immune cell infiltration ($CD4^+$ and $CD8^+$ T cells, B cells, macrophages, etc.) and immune checkpoints (PD-L1, CTLA-4, etc.) (120). Upstream lncRNA and miRNA analysis suggested that LINC00511 and particularly DUXAP8 might function as ceRNAs in the miR-29c-3p/FANCD2 axis, promoting FANCD2 expression and contributing to HCC progression. Another study showed that DUXAP8 was significantly elevated in HCC tissues, was positively correlated with SLC7A11 levels, and was associated with a poor prognosis in patients with HCC. DUXAP8 silencing led to the inhibition of HCC progression and an increase in its sensitivity to sorafenib. DUXAP8 can promote the palmitoylation of SLC7A11 and enhance its stability through the lysosomal pathway (64). Glucose-6-phosphate dehydrogenase (G6PD) is a crucial enzyme within the pentose phosphate pathway that plays a pivotal role in the promotion of NADPH production and the preservation of cellular redox homeostasis (121). SNHG1 serves as a ceRNA and is overexpression in HCC cells, effectively sponging miR-199a-5p/3p and subsequently upregulating FANCD2 and G6PD, which ultimately leads to the inhibition of ferroptosis (122).

Epidermal growth factor receptor (EGFR) belongs to the ErbB receptor family and is involved in cell proliferation and division. Aberrant activation of EGFR is a significant mechanism underlying tumorigenesis and cancer progression (123). Epidermal growth factor receptor pathway substrate 15 (EPS15) is an important substrate of the EGFR pathway. In HCC, EPS15 exhibits high expression levels, which are correlated with tumor cell progression and invasiveness. EPS15-AS1 is an antisense lncRNA of EPS15 that is downregulated in HCC. Increasing the expression of EPS15-AS1 can suppress EPS15 and EGFR, thereby inhibiting the activity of HCC cells (124, 125). In addition, the overexpression of EPS15-AS1 suppresses EGFR and aldo-keto reductase family 1 member B1 (AKR1B1), resulting in elevated levels of free iron and lipid peroxidation in HCC cells. Notably, this inhibitory effect of EPS15-AS1 can be mitigated by the ferroptosis inhibitors ferrostatin-1 and deferasirox (124). AKR1B1 belongs to the aldose reductase superfamily and is involved in the reduction of diverse aldehydes. AKR1B1 is overexpressed in a range of tumors, including HCC (126). Research has demonstrated that EGFR1 inhibition can lead to the upregulation of AKR1B1 and subsequent suppression of the *de novo* GSH synthesis (127). However, the mechanism underlying the upregulation of AKR1B1 remains unclear and requires further investigation.

Zhang et al. identified the role of the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) in ferroptosis. NEAT1 is a downstream gene of the tumor suppressor p53. In HCC, erastin treatment promoted the binding of p53 to the NEAT1 promoter and increased the expression of NEAT1 (16). Myoinositol oxygenase (MIOX) is a non-haem iron oxygenase that can catabolize myoinositol to D-glucuronate, and its upregulation can lead to a decrease in the cellular antioxidant capacity and an increase in ROS production (128). NEAT1 competitively binds to miR-362–3p and reduces the interaction between miR-362–3p and MIOX, which promotes MIOX expression and the accumulation of Fe^{2+} and ROS.

Activating transcription factor 4 (ATF4) belongs to the cAMP response element-binding protein (CREB) family and is involved in diverse biological processes, such as oxidative stress, endoplasmic reticulum stress, and cell death (129). ATF4 targets numerous ferroptosis-related genes, exerting both promoting and inhibitory effects (129–131). Guan et al. discovered that the lncRNA highly upregulated in liver cancer (HULC) influenced the miR-3200–5p/ATF4 axis via a ceRNA mechanism; the study revealed that the suppression of HULC led to a decrease in ATF4 expression, subsequent inhibition of GPX4 and promotion of ferroptosis in HCC cells (132). In summary, multiple ferroptosis-related lncRNAs are involved in regulating the occurrence, progression, treatment,

and drug resistance of HCC, and thus, their study provides a promising potential direction for the treatment of HCC (Table 1; Figure 3).

5 Ferroptosis-related LncRNA and HCC tumor heterogeneity

Tumor heterogeneity is a salient feature of malignancies like HCC. As cancer progress, cancer cells undergo numerous rounds of division and proliferation, triggering molecular biological or genetic alterations that give rise to disparities in growth rate, invasiveness, drug responsiveness, prognosis, and tumor immune heterogeneity (133, 134). Ferroptosis-related lncRNAs play a pivotal role in HCC tumor heterogeneity. For example, NEAT1 has been found to enhance erastin-induced ferroptosis in HCC via the miR-362–3p/MIOX axis (16). In contrast, NEAT1 exhibits high expression levels in non-small cell lung cancer and lung adenocarcinoma, where it acts as an inhibitor of ferroptosis (135, 136). This suggests that ferroptosis-related lncRNAs may contribute to HCC tumor heterogeneity by modulating ferroptosis. However, there are currently limited studies on the differences between ferroptosis-related lncRNAs in HCC and other cancers where ferroptosis is a key mechanism. Future studies will endeavor to unveil the precise mechanisms of these lncRNAs in HCC tumor heterogeneity, thereby offering novel perspectives and approaches for the diagnosis, therapy, and prevention of HCC.

6 Conclusion

Ferroptosis, a unique type of PCD, has attracted widespread attention in tumor research in recent years. An imbalance in iron metabolism, lipid metabolism, and the antioxidant defense axis underlies the main process of ferroptosis, which is delicately regulated at the transcriptional, translational, and posttranslational levels. Alterations in iron metabolism are frequently observed in the pathogenesis of tumors. Tumor cells are often characterized by abnormal processes of iron acquisition and utilization, which satisfy their demands for rapid growth and division. This abnormal iron metabolism not only is closely related to the occurrence of ferroptosis but also may influence the tumor growth, invasion, and metastasis. Therefore, targeting ferroptosis in tumor cells appears to potentially be a treatment approach with fewer side effects on healthy cells.

Although lncRNAs do not encode proteins, they play pivotal roles in diverse cellular processes as functional RNAs. In this review, we describe the regulatory roles of lncRNAs in various aspects of ferroptosis and detail the functions of ferroptosis-related lncRNAs in HCC. LncRNAs play crucial regulatory roles in major aspects of ferroptosis, including iron metabolism, lipid metabolism, and antioxidant ferroptosis defense axes. LncRNAs can directly target the expression and stability of crucial ferroptosis-associated factors, such as GPX4 and SLC7A11, or modulate the expression of ferroptosis regulators, such as p53 and Nrf2. More commonly, lncRNAs function as molecular sponges for miRNAs, exerting

TABLE 1 Mechanism of lncRNAs affecting ferroptosis in hepatocellular carcinoma.

LncRNA	Effect	Mechanism	References
PVT1	Inhibit	Inhibiting the miR-214-3p-GPX4 axis and promoting GPX4 expression	(67)
HCG18		Inhibiting the miR-450b-5p-GPX4 axis and promoting GPX4 expression	(106)
CASC11		Directly binding to SLC7A11 mRNA, increasing its stability and half-life	(108)
NRAV		Inhibiting the miR-375-3p-SLC7A11 axis and promotingSLC7A11 expression	(109)
URB-AS1		Promoting punctate distribution and liquid-liquid phase separation of ferritin, suppressing NCOA4-mediated ferritinophagy, and reducing intracellular free iron	(111)
LncFAL		Inhibiting the binding of Trim69 and FSP1 and the ubiquitination degradation of FSP1	(112)
LINC01134		Promoting Nrf2-GPX4 axis and GPX4 expression	(66)
DUXAP8		Suppressing the miR-29c-3p-FANCD2 axis and enhancing FANCD2 expression; promoting the palmitoylation and stability of SLC7A11	(64, 120)
SNHG1		Inhibiting miR-199a-5p/3p and promoting the expression of FANCD2 and G6PD	(122)
HULC		Suppressing the miR-3200-5p/ATF4 axis and enhancing the expression of ATF4 and GPX4	(132)
NEAT1	Promote	Inhibiting the miR-362-3p-MIOX axis and promoting MIOX expression	(16)
HEPFAL		Promoting ubiquitination of SLC7A11, reducing its stability and half-life, and suppressing GPX4 through the PI3K-Akt-mTOR pathway	(63, 110)
GABPB1-AS1		Inhibiting the translation of GABPB1 and the expression of Nrf2	(117)
EPS15-AS1		Inhibiting EPS15 and AKR1B1, suppressing the *de novo* synthesis of GSH.	(124)

AKR1B1, aldo-keto reductase family 1 member B1; Akt, protein kinase B; AS1, antisense RNA 1; ATF4, activating transcription factor 4; CASC11, cancer susceptibility candidate 11; DUXAP8, double homeobox A pseudogene 8; EPS15, epidermal growth factor receptor pathway substrate 15; FANCD2, fanconi anemia complementation group D2; FSP1, ferroptosis suppressor protein 1; GABPB1, GA-binding protein B1; G6PD, glucose-6-phosphate dehydrogenase; GPX4, glutathione peroxidase 4; HCG18, HLA complex group 18; HULC, highly upregulated in liver cancer; LncFAL, ferroptosis-associated lncRNA; LncRNA, long non-coding RNA; MIOX, myo-inositol oxygenase; mTOR, mammalian target of rapamycin; NCOA4, nuclear receptor coactivator 4; NEAT1, nuclear paraspeckle assembly transcript 1; Nrf2, nuclear factor E2-related factor 2; PI3K, phosphatidylinositol-3 kinase; PVT1, plasmacytoma variant 1; SLC7A11, solute carrier family 7 member 11; SNHG1, small nucleolar RNA host gene 1.

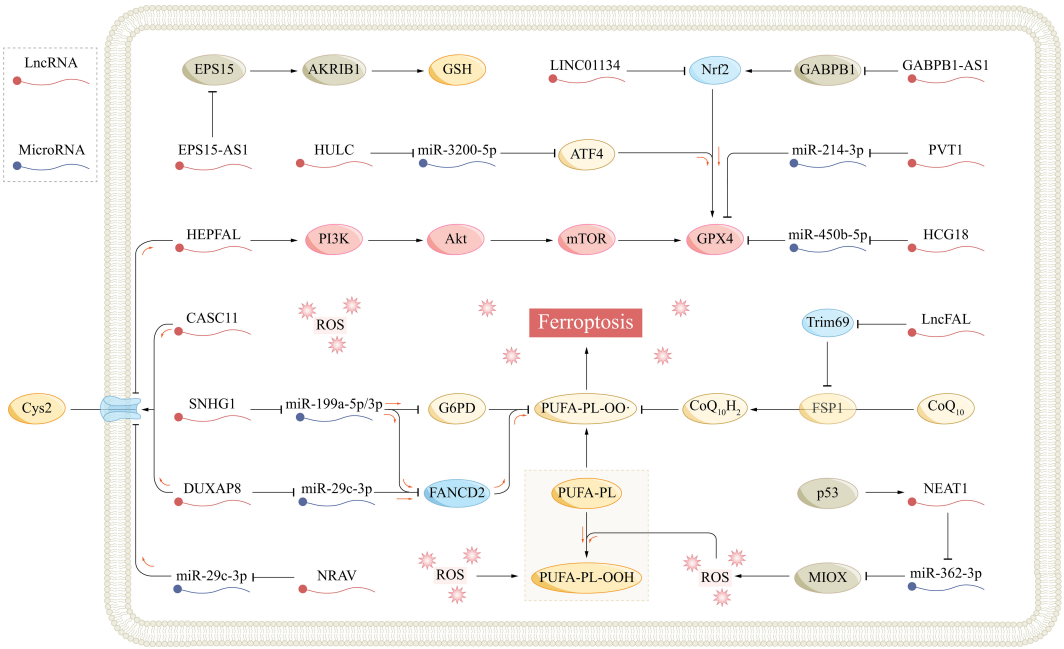

FIGURE 3
The role of ferroptosis-related lncRNAs in hepatocellular carcinoma. AKR1B1, aldo-keto reductase family 1 member B1; Akt, protein kinase B; AS1, antisense RNA 1; ATF4, activating transcription factor 4; CASC11, cancer susceptibility candidate 11; CoQ10, coenzyme Q10; Cys, cysteine; Cys2, cysteine; DUXAP8, double homeobox A pseudogene 8; EPS15, epidermal growth factor receptor pathway substrate 15; FANCD2, fanconi anemia complementation group D2; Fe^{2+}, ferrous; FSP1, ferroptosis suppressor protein 1; FTH1, ferritin heavy chain 1; FTL, ferritin light chain; GABPB1, GA-binding protein B1; G6PD, glucose-6-phosphate dehydrogenase; Glu, glutamate; GPX4, glutathione peroxidase 4; GSH, glutathione; GSSG, glutathione disulfide; HCG18, HLA complex group 18; HULC, highly upregulated in liver cancer; LncFAL, ferroptosis-associated lncRNA; LncRNA, long non-coding RNA; MIOX, myo-inositol oxygenase; mTOR, mammalian target of rapamycin; NCOA4, nuclear receptor coactivator 4; NEAT1, nuclear paraspeckle assembly transcript 1; Nrf2, nuclear factor E2-related factor 2; PI3K, phosphatidylinositol-3 kinase; PL, phospholipid; PUFA, polyunsaturated fatty acid; PVT1, plasmacytoma variant 1; ROS, reactive oxygen species; SLC7A11, solute carrier family 7 member 11; SNHG1, small nucleolar RNA host gene 1.

regulatory functions through the lncRNA–miRNA–mRNA pathways (Table 1). Ferroptosis-related lncRNAs play roles in the occurrence, progression, metastasis, and drug resistance of HCC by regulating the levels of free iron, ROS, and MDA in HCC cells. Notably, in related studies, inhibiting ferroptosis in HCC cells consistently accelerated the progression of HCC and led to its resistance to sorafenib, whereas promoting ferroptosis suppressed HCC and increased its sensitivity to sorafenib. Furthermore, several predictive models for clinical prognosis, staging, and immune infiltration in HCC have been centered on ferroptosis-related lncRNAs. According to these models, the lncRNAs in the high-risk group tended to consistently suppress ferroptosis. These results highlight the role of ferroptosis in HCC, demonstrating the potential of targeting ferroptosis in HCC therapy and the feasibility of using ferroptosis-related lncRNA models for predicting HCC prognosis.

Despite the encouraging results achieved, there are still numerous limitations and challenges in the current research. While several models have been constructed through database studies, the lncRNAs utilized in these models are inconsistent, and the predicted patient populations differ, which somewhat restricts their reliability and comparability. More importantly, the experimental validation of these lncRNA markers is still insufficient, making it difficult to precisely determine their functions and impacts in actual patient samples. Further laboratory studies and clinical trials are needed to verify the specific mechanisms of action of these lncRNAs in HCC patients. Additionally, the clinical use guidelines for these clinical prognosis prediction models also require further clarification. We need to understand the applicability, predictive accuracy, and potential limitations of these models in different patient groups. Finally, the roles of serval ferroptosis-related lncRNAs in HCC cell lines have been preliminarily identified, but their accuracy in predicting HCC patient prognosis and immune regulation remains a critical research direction in the future. Currently, while we primarily rely on techniques like gene knockout and overexpression to assess the function of lncRNAs, these approaches may not comprehensively simulate their authentic role in physiological or pathological settings. Furthermore, the intricate nature of lncRNAs poses challenges in thoroughly deciphering their intricate interactions with other biomolecules such as mRNAs and proteins.

Overall, the specific mechanisms of action of ferroptosis-related lncRNAs are crucial for understanding the pathogenesis of HCC. These lncRNAs may have close associations with the expression of ferroptosis-related genes, signaling pathways, or iron metabolism. By studying these mechanisms in depth, we can gain a better understanding of the pathogenesis of HCC, which will provide new insights for the development of treatment strategies. Furthermore, regulating the expression or function of ferroptosis-related lncRNAs offers new potential targets and methods for the treatment of HCC. By modulating these lncRNAs, we can influence biological processes related to HCC, such as proliferation, invasion,

PCD, and immunity. In future research, we still need to focus on the following aspects: 1) Develop more precise and accurate methods for studying the functions of lncRNAs, for instance, utilizing single-cell sequencing technology can provide us with a deeper understanding of the expression patterns and functions of lncRNAs in individual cells. Additionally, through high-throughput screening and validation techniques, we can more rapidly identify crucial lncRNAs that are related to HCC. 2) Deeply investigate the specific mechanisms of action of ferroptosis-related lncRNAs in HCC, including their interactions with genes, proteins, or signaling pathways, and how these interactions affect the occurrence and development of HCC. 3) Develop specific regulatory methods targeting these lncRNAs, such as inhibitors or activators, and evaluate their effectiveness and safety in the treatment of HCC. 4) Conduct more preclinical and clinical studies to validate the feasibility and effectiveness of these lncRNAs as therapeutic targets. This includes testing the effectiveness of these treatment methods in animal models and evaluating their safety and efficacy in human clinical trials. By deeply studying the mechanisms of action of ferroptosis-related lncRNAs and developing specific therapeutic methods targeting them, we have the potential to provide new insights and strategies for the treatment of HCC.

Author contributions

HC: Writing – original draft. ZH: Writing – original draft. JS: Investigation, Writing – review & editing. XS: Investigation, Writing – review & editing. QM: Investigation, Writing – review & editing. YL: Investigation, Writing – review & editing. ZR: Investigation, Writing – review & editing. XL: Investigation, Writing – review & editing. RM: Writing – review & editing. YW: Writing – review & editing. DL: Writing – review & editing.

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Conflict of interest

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Inhalation of nicotine-containing electronic cigarette vapor exacerbates the features of COPD by inducing ferroptosis in βENaC-overexpressing mice

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Introduction: Chronic obstructive pulmonary disease (COPD) is currently listed as the 3rd leading cause of death in the United States. Accumulating data shows the association between COPD occurrence and the usage of electronic nicotine delivery systems (ENDS) in patients. However, the underlying pathogenesis mechanisms of COPD have not been fully understood.

Methods: In the current study, bENaC-overexpressing mice (bENaC mice) were subjected to whole-body ENDS exposure. COPD related features including emphysema, mucus accumulation, inflammation and fibrosis are examined by tissue staining, FACS analysis, cytokine measurement. Cell death and ferroptosis of alveolar epithelial cells were further evaluated by multiple assays including staining, FACS analysis and lipidomics.

Results: ENDS-exposed mice displayed enhanced emphysema and mucus accumulation, suggesting that ENDS exposure promotes COPD features. ENDS exposure also increased immune cell number infiltration in bronchoalveolar lavage and levels of multiple COPD-related cytokines in the lungs, including CCL2, IL-4, IL-13, IL-10, M-CSF, and TNF-α. Moreover, we observed increased fibrosis in ENDS-exposed mice, as evidenced by elevated collagen deposition and a-SMA+ myofibroblast accumulation. By investigating possible mechanisms for how ENDS promoted COPD, we demonstrated that ENDS exposure induced cell death of alveolar epithelial cells, evidenced by TUNEL staining and Annexin V/PI FACS analysis. Furthermore, we identified that ENDS exposure caused lipid dysregulations, including TAGs (9 species) and phospholipids (34 species). As most of these lipid species are highly associated with ferroptosis, we confirmed ENDS also enhanced ferroptosis marker CD71 in both type I and type II alveolar epithelial cells.

Discussion: Overall, our data revealed that ENDS exposure exacerbates features of COPD in bENaC mice including emphysema, mucus accumulation, abnormal lung inflammation, and fibrosis, which involves the effect of COPD development by inducing ferroptosis in the lung.

KEYWORDS

fibrosis, ENDS (electronic nicotine delivery systems), COPD (chronic obstructive pulmonary disease), ferroptosis, lipid dysregulations

Introduction

Chronic obstructive pulmonary disease (COPD) is a primary leading cause of death with limited treatment options, accounting for more than 3 million deaths worldwide annually (1). The phenotypes of COPD are characterized by limitations in expiratory airflow, emphysematous destruction of the lungs, chronic bronchitis, and inflammation of the lung tissue. Patients with COPD experience a variety of symptoms including shortness of breath, wheezing, and/or a chronic cough (2). Cigarette smoking is the major risk factor for COPD. While cigarette smoking in the U.S. has decreased over the past 50 years, there has been a dramatic increase in the usage of electronic nicotine delivery systems (ENDS) as substitutes (1). As ENDS are perceived as safer than traditional cigarettes, they are often used to assist in smoking cessation. The risk of cigarette smoking and its contribution to chronic lung diseases has been well characterized. However, the impact of ENDS on COPD development remains poorly understood (3). Therefore, a thorough understanding of the effects of ENDS usage on chronic lung conditions is essential.

ENDS include vape pens, hookah pens, and electronic cigarettes (e-cigarettes or e-cigs), which transport noncombustible vaporized nicotine products to the lungs (4). The common components of e-cigarette liquid include nicotine, glycerin, propylene glycol, flavorings, and other ingredients. When heated, the nicotine-containing e-cigarette liquid generates vapor, which will be inhaled into the lungs. Vaporization of e-liquid generates toxic compounds similar to cigarette smoke. (Rahman) Reactive aldehydes, such as formaldehyde and acrolein, are common products of the thermal decomposition of propylene glycol and glycerol, the major vehicle components of e-liquids (5). Notably, reactive aldehydes generated from ENDS have been noted in much greater concentrations than recommended occupational safety standards (6). Accumulating data suggest that e-cigarette vapor induces oxidative stress and inflammation *in vitro* (7, 8) and *in vivo* (9, 10). For example, e-cigarette liquid alone caused morphology changes in lung epithelial cells, by initiating a stress phenotype and inflammatory response (9). As a major component of ENDS, nicotine, the addictive component in cigarettes and e-cigarettes, inhibits mucus hydration (11) and induces pro-inflammatory dendritic cell responses (12). Similar results were evidenced in mice where e-cigarette vapor induced airway responses similar to cigarette smoke, including airway inflammation, tissue remodeling, and enhanced mucin expression following four months of exposure (10). These findings suggest the ability of nicotine-containing e-cigarettes could induce physiological changes akin to human COPD.

As the primary risk factor for COPD, cigarette smoking is widely used to study the pathogenesis of COPD in various animal models (13). Despite the recent popularity of ENDS, there are limited investigations aimed at elucidating the effects of e-cigarettes on the development and progression of COPD (4). Therefore, there is an unmet need to study the effects of ENDS, e-cigarettes in particular, on COPD progression utilizing the proper animal model with pre-existing phenotypes of COPD. In this study, we utilized the βENaC mouse, a transgenic line that exhibits defective airway mucus clearance due to the overexpression of the epithelial sodium channel non-voltage gated 1, beta subunit (*Scnn1b)* (14–16). βENaC mice have been used in the investigation of respiratory diseases including COPD and cystic fibrosis (17, 18). Because this strain of mice has symptoms of emphysema and mucus obstruction (17), we postulated that ENDS could exacerbate the phenotypic response of COPD following acute e-cigarette vapor exposure. This study was to evaluate the effects of nicotine-containing e-cigarette vapor in the development and progression of a COPD phenotype including 1) inflammatory response in the lung tissues, 2) mucus accumulation within the bronchioles, 3) small airway fibrosis, and 4) destruction and degradation of alveolar structure; and also investigate the potential mechanisms underneath.

Materials and methods

Animals and genotyping

Male and female βENaC were purchased from The Jackson Laboratory (congenic C57BL/6J background, Stock No. 006438, Bar Harbor, ME). Mice were housed in a specific pathogen-free facility on a 12-hour light/dark cycle with free access to water and a standard chow diet throughout treatment. All animal procedures

described were approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia State University. To have enough animals for this study, βENaC mice were bred with wild-type C57BL/6J mice to produce an appropriate number of hemizygous βEnaC for our studies. Before weaning age, animals were genotyped to determine the presence of *Scnn1b* overexpression. Briefly, tail snips were collected and lysed in Proteinase K overnight at 56°C in preparation for DNA extraction. DNA was extracted by Dneasy Blood and Tissue kit (Qiagen, Foster City, CA) in preparation for polymerase chain reaction (PCR). PCR was performed using primers specific for *Scnn1b* gene expression as suggested (Forward: CCTCCAAGAGTTCAACTACCG; Reverse: TCTACCAGCTCAGCCACAGTG) (16). To verify the expression of *the Scnn1b* gene, samples were run on a 4% agarose gel containing ethidium bromide. Mice identified as possessing *Scnn1b* overexpression were tagged as βENaC mice for future studies.

ENDS exposure to βENaC mice

At 12 weeks of age, mice (n=10 per group) were randomized into either the ENDS exposure group or the control group (sham air). Mice in the ENDS group were exposed to e-cigarette vapor for 1 hour twice daily at a frequency of 5 times per week for a total duration of 10 days as shown in Figure 1A. Mice were placed in a smoking chamber attached to an inExpose Smoking Robot (SCIREQ, Montreal, QC, Canada) with an attached e-cigarette accessory (ECX JoyeTech E-Vic Mini, SCIREQ, Canada) for the administration of vaporized S brand e-cigarette liquid containing nicotine salt at 50 mg/ml or sham air (control). During vapor administration, an optimal vapor/air ratio of 1:6 was obtained based on our previous publication (19). After 10 days of ENDS exposure, animals were euthanized within 24 hours after the last e-cigarette vapor exposure using CO_2. Bronchoalveolar lavage (BAL) fluid and lung tissues were collected for analysis as described below.

Bronchoalveolar lavage fluid and cytokine measurements

After the mice were euthanized, a tracheostomy was performed, and the lungs were immediately flushed with 1 mL ice-cold phosphate-buffered saline (PBS) twice for collection of BAL fluid. Blood was collected via the abdominal aorta, allowed to sit for 30 min to promote coagulation, and centrifuged at 5,000 rpm for 10 min for isolation of serum. Total cells from the BAL were centrifuged and counted using Countess™ II Automated Cell Counter (Invitrogen, Carlsbad, CA) as previously described (16). Differential immune cell count (5,000 cells/slide) was performed on Shandon cytospin slides (Thermo Shandon, Pittsburgh, PA) stained with Diff-Quik (Dade Bering, Newark, DE). The BAL supernatant and serum samples were stored at -80°C until analysis. Expression of inflammatory cytokines, proteases, and growth factors was assessed in BAL fluid via Mouse XL Cytokine Array (R&D Systems, Minneapolis, MN) following the manufacturer's instructions to determine the presence of a localized inflammatory response within the lung.

Morphometric assessment

In order to determine the effects of ENDS exposure on the development of emphysema, we did a morphometric assessment according to the previous protocol (17, 20). Briefly, the mouse lungs were inflated with 1% low melting-point agarose to 25 cm of fixative pressure. After 48 hours of fixation, lungs were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Mean linear intercepts were determined and calculated (21).

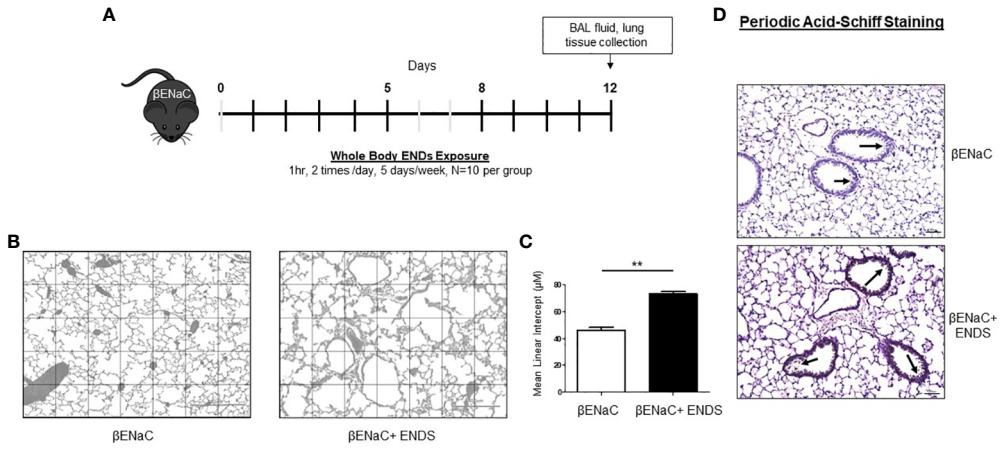

FIGURE 1
ENDS exposure promoted emphysema and mucus accumulation in βENaC mice. **(A)** Experimental schematic of whole-body ENDS exposure in βENaC mice. **(B)** ENDS exposure caused emphysematous phenotype changes in βENaC mice. Representative images of H&E-staining in lung tissues of sham air (left) (n=4) and ENDS-exposed (right) (n=4) mice. **(C)** Quantitative morphometric analysis of the alveolar septum of the lung. **(D)** Representative images of Periodic Acid-Schiff staining in lung sections of βENaC mice with or without ENDS exposure. All data were presented at mean ± SEM (student t-test, ** $p< 0.01$).

Immunohistochemical, immunofluorescent, and histology analysis

The mouse lungs were inflated with 1% low melting-point agarose to 25 cm of fixative pressure and then fixed with 4% neutral buffered formalin for 48 hours. Later, these tissue samples were embedded in paraffin and sectioned into 4 μm sections using a rotary microtome. For immunohistochemical analysis, tissue sections were dewaxed with xylene and rehydrated with graded concentrations of ethanol, followed by antigen retrieval in 10 mM citric acid solution (pH = 6) for 20 min using a pressure cooker. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. After blocking with 5% Bovine serum albumin (BSA) for 30 min, the slides were incubated with the primary antibodies against the following proteins at 4°C overnight: α smooth muscle actin (α-SMA) 1:500 (Ab5694; Abcam); Von Willebrand Factor (vWF) 1:300 (A008229; Agilent); CD71 (Ab84036, Abcam), proSP-C (AB3786, Sigma), HOPX (11419-AP, Proteintech). After washing, the sections were incubated with the appropriate HRP polymers and developed with 3–3′ diaminobenzidine solution (DAB substrate kit; Vector Laboratories). After counterstaining with hematoxylin, the slides were dehydrated and mounted with a mounting medium. For immunofluorescent staining, after incubated with primary antibodies, the sections were incubated with fluorescent secondary antibody Goat anti-Mouse IgG 488 (A11001, Thermo Scientific), Goat anti-Mouse IgG 594 (A11005, Thermo Scientific), Goat anti-Rabbit IgG 488 (A11008, Thermo Scientific), Goat anti-Rabbit IgG 594 (A11012, Thermo Scientific) for 1h at room temperature. Then the sections were mounted with ProLong Diamond Antifade Mounting with DAPI (P36962, Thermo Scientific).

For Hematoxylin and Eosin (H&E) staining, the paraffin slides were baked at 60°C for 2 hours, then dewaxed with xylene and rehydrated with graded ethanol solutions. Next, the slides were incubated with Mayer's Hematoxylin for 10 min and washed under tap water for 10 min. Slides were then immersed in Eosin for 30 sec and washed under tap water, after which, the slides were dehydrated and mounted with a mounting medium.

Periodic-acid Schiff (PAS; Sigma Aldrich, Saint Louis, MO) staining was used to evaluate mucus accumulation and immune cell number. Masson's Trichrome (Sigma, St. Louis, MO) and Picro-Sirius Red (Abcam, Cambridge, MA) staining were used to assess collagen deposition in lung tissue. All stainings were performed following the manufacturer's instructions. All images were taken at 20x magnification using Keyence Fluorescent Microscope (Keyence, Itasca, IL).

Cell culture

Human airway epithelial cells (16HBE) were a gift from Dr. Pierre Massion (Vanderbilt University) and maintained in DMEM media. 16HBE cells were grown in 1% penicillin/streptomycin with 10% fetal bovine serum. Human Aortic Endothelial Cells (HAEC) were obtained from Invitrogen (Invitrogen, Carlsbad, CA) and cultured in endothelial cell growth medium supplemented with low serum (2% V/V FBS) (PromoCell; Heidelberg, Germany), and both cells were maintained in a cell culture incubator at 37°C and 5%p CO_2. 16HBE cells were plated at 30,000 cells/well in 6-well plates. Then cells were treated with ENDS liquid (50 mg/ml) or ENDS liquid without nicotine for 48 hours and harvested for analysis of apoptosis by flow cytometry or western blot. Similarly, HAEC cells were cultured in 6-well plates at a density of 2.0×10^5 cells/well, starved overnight, and treated with 50 mg/ml ENDS liquid with nicotine or ENDS liquid without nicotine for 24 hours or 1 mM H_2O_2 for 12 hours (positive control) and then subjected to Annexin V/PI apoptosis analysis by flow cytometry.

Annexin V/PI apoptosis detection with flow cytometry

The cells were detached with accutase solution and washed with PBS and later binding buffer, followed by resuspension in binding buffer at 10^6 cells/ml. The cell suspensions were incubated with 5 μL of FITC-conjugated Annexin V (V13242, Thermo) for 15 min at room temperature without exposure to light, followed by washing and resuspension in binding buffer. Propidium Iodide (Sigma Aldrich, Saint Louis, MO) was added followed by incubation for 5 min. The cells were acquired using LSRFortessa flow cytometer (BD Biosciences, Durham, NC), and the data was analyzed by FlowJo software (BD, Durham, NC).

TUNEL assay for apoptosis detection

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using VasoTACS *In Situ* Apoptosis Detection Kit (4826–30-K, Trevigen, Gaithersburg, MD) following the manufacturer's instructions. In brief, after rehydration, tissue slides were incubated with Proteinase K Solution for 20 min at room temperature, followed by quenching for 5 min. 50 μL of Labeling Reaction Mix were added to the tissues and the slides were incubated at 37°C for 60 min, after which the reaction was terminated by adding the stop solution. The slides were covered with Strep-HRP Solution for another 10 min and developed using TACS Blue Label solution. After counterstaining by Red Counterstain C, slides were dehydrated and mounted with the mounting medium.

Western blot

Cells were lysed with lysis buffer (RIPA buffer from Thermo Scientific, 89900, 1X Halt protease inhibitor cocktail from Thermo Scientific, 78430, and 1X Halt phosphatase inhibitor cocktail from Thermo Scientific, 78426). Mouse tissues were mixed with lysis buffer (300ul of lysis buffer per 5mg piece of tissue) and homogenated with a homogenizer. Cell debris was pelleted for 10 min at 12,000 x g at 4C. After protein concentration

quantification by BCA assay (Thermo Scientific 23225), an equal amount protein of each sample was separated using SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were treated with 5% BSA in PBS-T (PBS with 0.1% Tween 20) blocking buffer for at least 1 h at room temperature, then incubated with primary antibody diluted in blocking buffer overnight at 4C. Anti-ACSL4 antibody (Abcam, ab155282, 1:5000 dilution); anti-GAPDH antibody (CST #2118, 1:1000 dilution); Anti-CD71 (Abcam, Ab84036, 1:1000 dilution), anti-Ferritin antibody (Abcam, Ab75973, 1:1000 dilution). Following three 10 min washes in PBS-T, the membrane was incubated with HRP conjugated secondary antibodies, goat anti-rabbit (Thermo Scientific, 65–6120, 1:5000 dilution), or goat anti-mouse IgG antibody (Thermo Scientific, 62–6520, 1:5000 dilution) in blocking buffer for 1 hour at room temperature. Following three 5 min washes in PBS-T, membranes were incubated with Chemiluminescent Substrate (Thermo Scientific, 34580), and bands were visualized using Autoradiography Film (Denville Scientific, E3012).

Bodipy staining for peroxidation of polyunsaturated fatty acids

Since the peroxidation of polyunsaturated fatty acids (PUFAA) has emerged as a key driver of oxidative damage to cellular membranes leading to ferroptosis (22), we explored the effects of ENDS exposure on peroxidation of PUFA *in vitro*. 16HBE cells were treated with 50ng/ml of ENDS liquid for 24hs or 1uM of RSL3 (positive control) for 6 hours. After the treatments, cells were washed three times for 10 minutes in PBS, and then fixed in 4% paraformaldehyde for 5 minutes. Next, cells were incubated in 0.1mg/ml BODIPY 581/591 (Invitrogen C10445) in DMSO for 30 minutes in the dark, followed by three times of washes. Cells were then mounted with Prolong gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI). Images were taken by Keyence Fluorescent Microscope (Keyence, Itasca, IL).

Mass-spectrometry-based lipidomics analysis

Lipidomic analysis on serum samples was performed at The Harvard Center for Mass Spectrometry according to our previous publication (23). Briefly, 200 µl of serum samples from mice with either ENDS exposure or normal control was mixed with lung tissue was homogenized with 2 mL of pre-cold methanol (MX0486–1, Sigma), then mixed with 4 mL of chloroform (A452–1, Sigma). The mixtures were combined with 1.2 mL of HPLC grade water (WX0001–1, Sigma), and centrifuged at 3000 rpm for 10 min at 4°C. The bottom layer (chloroform phase) was collected for lipidomics analysis using LC-MS on an Orbitrap Exactive (Thermo Scientific) in line with an Ultimate 3000 LC (Thermo Scientific). Each sample was analyzed in positive and negative modes, in the top 5 automatic data-dependent MS/MS modes. Flow rate was set to 100 µL/min for 5 min with 0% mobile phase B, then, switched to 400 µL/min for 50 min, with a linear gradient of mobile phase B from 20% to 100%. The column was then washed at 500 µL/min for 8 min at 100% mobile phase B followed by re-equilibrated at 0% mobile phase B, 500 µL/min for 7 min. For positive mode runs, buffers for mobile phase A contain 5 mM ammonium formate, 0.1% formic acid, and 5% methanol in water, and, while buffers for mobile phase B are made up of 5 mM ammonium formate, 0.1% formic acid, 5% water, and 35% methanol in Isopropanol. For negative runs, buffers consisted of 0.03% ammonium hydroxide and 5% methanol in water for mobile phase A, and, for mobile phase B, 0.03% ammonium hydroxide, 5% water, and 35% methanol in isopropanol. Each of the spectra for each lipid identified with Lipidsearch software (version 4.1.16, Mitsui Knowledge Industry, University of Tokyo) was manually examined for the presence of the head group characteristic fragment, and for the side chain's fragment if present. Intensity signals are represented as the area of the parent ion. Integrations and peak quality were curated manually before exporting and analyzing the data in Microsoft Excel. Each ID was based on the fragments found in the literature and compiled in the Lipidsearch database.

Data analysis

In the case of only two groups, two-tailed *t*-tests were performed to assess the differences. Prior to analysis, we confirmed that the data met the assumptions of normality and homogeneity of variances using appropriate tests (Shapiro-Wilk test for normality and Levene's test for homogeneity of variances). All analyses were carried out using Prism7.0 software (GraphPad Software Inc., San Diego, CA). Values are represented as mean ± SEM. Levels of significance are designated as $p < 0.05$ unless otherwise indicated.

Results

ENDS exposure causes alveolar enlargement and destruction in β ENaC mice

The β ENaC mice have been used as a mouse model for induction of COPD (24–26). The βENaC mice, after exposure to cigarette smoke, display key pulmonary abnormalities of COPD, including inflammation, emphysema, and destruction of alveolar tissue, thus are used to study the molecular pathogenesis of COPD (25). One of the features of emphysema is the enlargement of alveolar spaces associated with the destruction of alveolar walls (27). To determine whether ENDS exposure can induce COPD, the β ENaC mice were given whole-body ENDS exposure as indicated (Figure 1A). At the end of the treatment, the mice were sacrificed, and lungs were collected for further pathological analysis. We observed that ENDS-exposed mice displayed impaired lung structure integrity with alveolar enlargement, compared with

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control mice (Figure 1B). We further quantified the air space size and found that the free distance between gas exchange surfaces was significantly increased in the lungs of ENDS-treated mice, indicating that ENDS caused lung emphysema in βENaC mice (Figures 1B, C). As mucus accumulation is another feature of COPD, we then assessed mucus content using PAS staining. The results showed that ENDS exposure increased the deposition of mucus in airways compared with the control group (Figure 1D). Altogether, our results suggest ENDS exposure causes the pathogenesis of COPD in β ENaC mice.

ENDS exposure enhances inflammation response in β ENaC mice

COPD is characterized by lung inflammation, which is a critical factor leading to progressive and irreversible airflow obstruction (28). Therefore, we next determined the presence of immune cells in BAL from mice with or without ENDS exposure using the Diff-Quik staining. Clearly, ENDS exposure increased the total number of immune cells including macrophages and neutrophils in BAL (Figures 2A, B). We then performed a cytokine array assay to measure cytokine production. Elevated levels of multiple cytokines including CCL2, IL-4, IL-13, IL-10, M-CSF, and TNF-α were found in the lungs of ENDS-exposed mice (Figures 2C–H). Interestingly, individual cytokines such as CCL-2, IL-4, IL-10, and IL-13 have been indicated to contribute to macrophage infiltration, M2 macrophage polarization, and pulmonary fibrosis (29–31). Thus, these results indicate ENDS exposure leads to inflammation response in the lung by enhancing immune cell number as well as cytokines productions in the βENaC mice.

ENDs exposure elevates fibrosis in small airway

Lung fibrosis with excessive deposition of collagen in airways was identified in patients with COPD (32, 33). It has been shown that lung fibrosis promotes airway obstruction thus exacerbating COPD (34). Therefore, we examined whether ENDS affected lung fibrosis during COPD progression. We first assessed the deposition of collagen by Picro-Sirius red staining (Figure 3A) and Masson's Trichrome staining (Figure 3C) using lung tissues from mice with or without exposure to ENDS. The results showed that exposure to ENDS elevated total collagen deposition in βENaC mice within the airway and lung parenchyma compared with the control (Figures 3A–D). Myofibroblasts, the major player in collagen synthesis, is a well-known hallmark of fibrosis. We therefore examined the existence of myofibroblasts by staining α-SMA, a widely used myofibroblast maker. The results demonstrated that ENDS exposure increased the α-SMA$^+$ myofibroblast population (Figures 3E, F). Thus, these data indicate that exposure to ENDS enhances collagen deposition and the number of collagen-producing myofibroblasts, revealing that ENDS exposure promotes fibrosis in the lung.

ENDS exposure induces cell death of endothelial and epithelial cells in the lung

Next, we try to elucidate how ENDS could promote COPD in the lung. Cell death is reported to be a major contributor to the pathogenesis of COPD and there is increased evidence showing enhanced levels of cell death of alveolar epithelial and endothelial

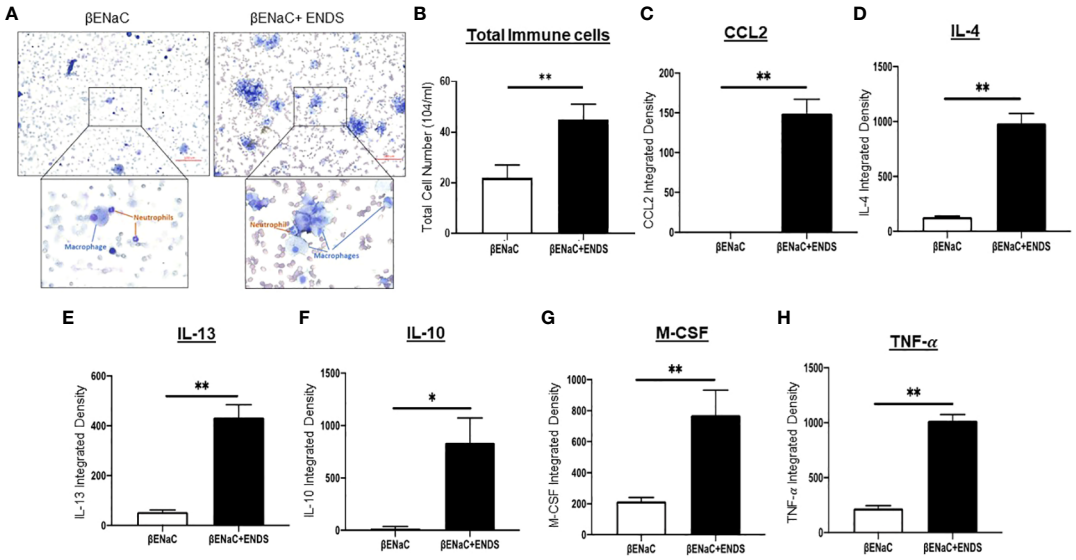

FIGURE 2
ENDS exposure enhanced inflammation response in β ENaC mice. **(A)** Representative images of Diff-Quik staining of BAL from βENaC mice with or without ENDS exposure (n=3 per group). **(B)** Quantitative analysis of cell number of BAL from βENaC mice with or without ENDS exposure. **(C–H)** Quantification of levels of cytokines/chemokines using Mouse XL Cytokine Array in BAL fluid of βENaC mice with or without ENDS exposure (n=3 per group). *p<0.05; **p<0.01.

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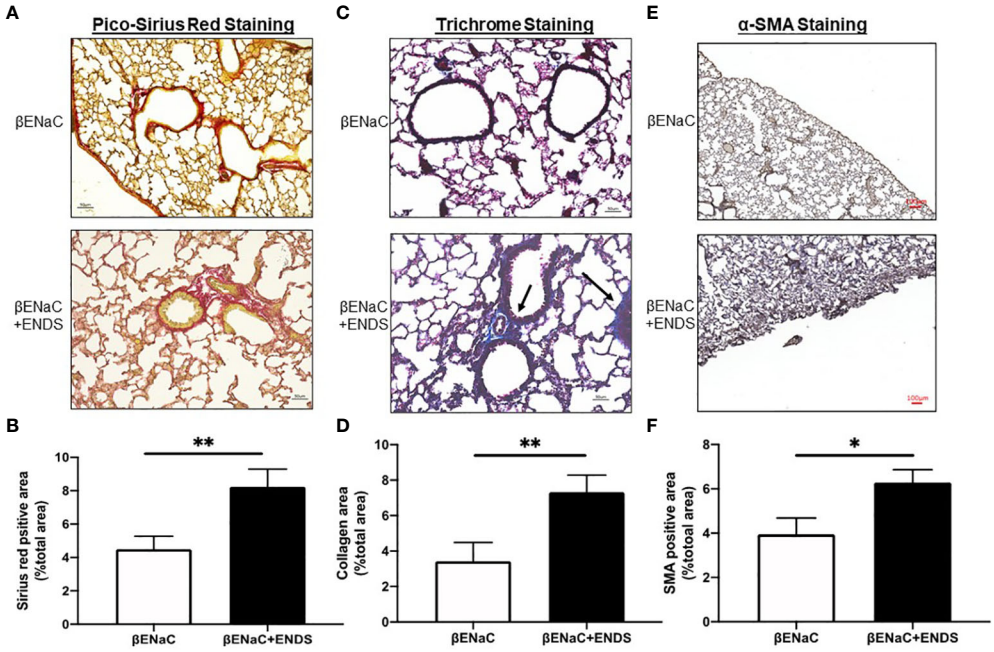

FIGURE 3
ENDS exposure promoted fibrosis in the small airway. **(A)** Representative images of Pico-Sirius Red staining in lung sections from βENaC mice with or without ENDS exposure (n=3 per group). **(B)** Quantitative analysis of Sirius Red staining as in **(A)** (n=3 per group). **(C)** Representative images of Masson's Trichrome staining in lung sections from βENaC mice with or without ENDS exposure (n=3 per group). and **(D)** Quantitative analysis of Trichrome staining as in **(C)** (n=3 per group). **(E)** Representative images of α-SMA immunohistochemistry staining in lung sections from βENaC mice with or without ENDS exposure. **(F)** Quantitative analysis of α-SMA staining as in **(E)** by Frida software. The positive staining areas were calculated as percentages of the total area, using five randomly selected sections per mouse, a total of 5 mice in each group. * $p<0.05$; ** $p<0.01$.

cells in the lungs of COPD patients (35, 36). Therefore, we examined total cell death levels in lung tissues using TUNEL staining. The results demonstrated an elevated TUNEL staining of endothelial cells and airway epithelial cells in ENDS-exposed mice compared with control mice, respectively (Figures 4A, B, 5A, B). We observed that the number of endothelial cells, stained by von Willebrand Factor (vWF), a widely used biomarker for vascular endothelial cells, was significantly diminished in ENDS-exposed mice compared with the control mice (Figures 4C, D). To confirm this data *in vitro*, we employed the human aortic endothelial cells (HAEC) and tested the effects of ENDS on endothelial cells *in vitro*. As shown in Figure 4E, the cell viability was significantly decreased in the nicotine-containing ENDS liquids treated group (57%, Figure 4E bottom right) compared with the control group (82%, Figure 4E top left). Interestingly, ENDS liquid without nicotine also induced remarkable cell death (75%, Figure 4E). On the other hand, ENDS liquid with nicotine and without nicotine significantly led to cell death compared with the control group in airway epithelial cells (16HBE) (Figures 5C, D).

ENDS exposure induces lipid dysregulation and ferroptosis in mice

We noticed that the level of cell death detected by TUNEL staining (either apoptosis or necrosis) does not fully match with lung tissue destruction by ENDS as shown in histological staining. We, therefore, questioned whether there are other formats of cell death mechanisms contributing to tissue mass loss during the progression of ENDS-induced COPD. Besides apoptosis, ferroptosis has drawn intensive attention recently as a critical player in COPD pathogenesis and growing evidence has shown involvement of epithelial cell ferroptosis in COPD pathogenesis (37, 38). Ferroptosis is also reported to induce the release of pro-inflammatory cytokines, leading to COPD-related airway remodeling and emphysema (37), which matches our observation of the elevated level of pro-inflammatory phenotype in ENDS-exposed mice. So we speculated ENDS exposure might induce ferroptosis. We performed a mass-spectrometry-based lipidomics analysis using serum samples collected from mice with or without ENDS exposure. The result demonstrated that relative abundances of more than 50 lipid species were altered after ENDS exposure (Figure 6A). We identified increases in the level of 9 species of TAGs and 9 major phospholipid classes (with the total number of detected molecular species of 34 distributed between the following major classes: 22 species; phosphatidic acid (PA); lysophosphatidylcholine (LPA) 7 species; phosphatidylethanolamine (PE), 2 species; phosphatidylinositol (PI) 1 species; phosphatidylserine (PS); 1 species, phosphatidylcholine (PC); 1 species) (Figure 6A). Furthermore, we measured the expression of biomarkers of ferroptosis using mouse tissues and found ENDS increased expression of ferroptosis-specific markers including Transferrin Receptor/CD71 (39) and ACSL4 (40) and reduced the expression of GPX4 (Figure 6B), the loss of which is reported required for

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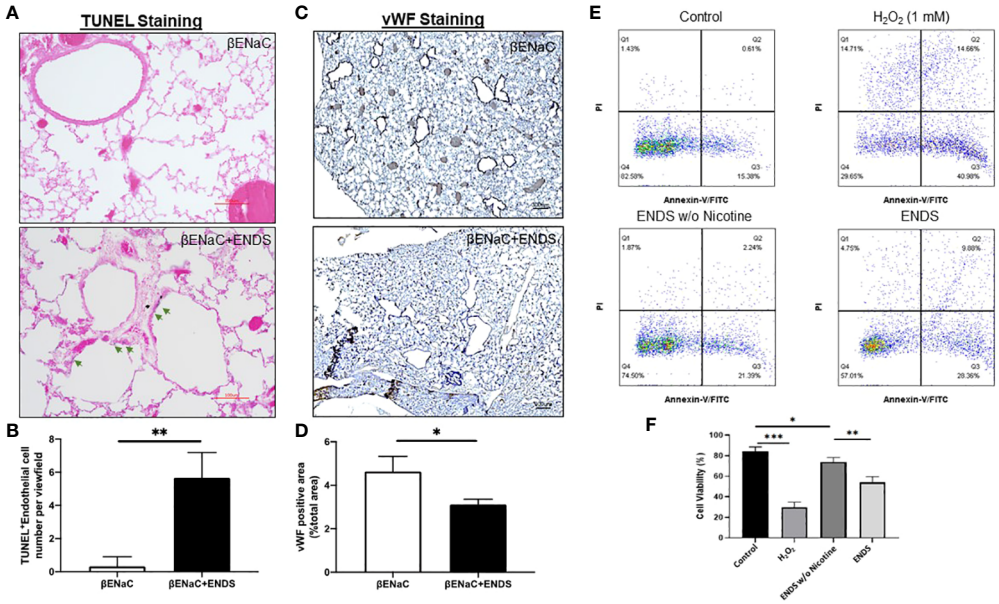

FIGURE 4
ENDS exposure caused lung vascular injury by inducing endothelial cell death. **(A)** Representative images of H&E and immunochemical staining of TUNEL (TdT-mediated dUTP nick end labeling, blue; counterstained by Red Counterstain C, red) in lung sections from βENaC mice. Green arrowheads indicate TUNEL-positive cells. **(B)** Quantitative analysis of TUNEL staining as in **(A)** (n=3 per group). **(C)** Representative images of vWF (von Willebrand Factor) immunohistochemical staining of lung tissue from βENaC mice with or without ENDS exposure. **(D)** Quantitative analysis of vWF staining as in **(C)** by Frida software. The positive staining areas were calculated as percentages of the total area, using five randomly selected sections per mouse, a total of 5 mice in each group. **(E)** Annexin V/PI apoptosis analysis in human aortic endothelial cells (HAEC). HAEC were treated with ENDS w/o nicotine (bottom left) and ENDS with nicotine (bottom right) for 24h or 1 mM H_2O_2 (top right) for 12 hours as positive control and then processed for Annexin V/PI apoptosis analysis by flow cytometry. **(F)** Quantification of Annexin V/PI assay. The cell viabilities were quantified as the Annexin V/PI double-negative cell percentages of total acquired cells. Columns and error bars represent means ± SEM, n=4 *p<0.05; **p<0.01; ***p<0.0001.

ferroptosis (41). Furthermore, IHC staining of CD71 showed ENDS-treated mice have elevated levels of CD71 in the lungs (Figure 6C, Supplementary Figure 1A). Then we treated the human bronchial epithelial cell line (16HBE) with or without ENDS (RSL3 as a ferroptosis positive control) and measured ferroptosis by determining the amount of lipid peroxides in cellular membranes using the BODIPY-C11 probe. The data showed that ENDS-treated 16HBE has increased BODIPY-positive ferroptosis cells (Figure 6D).

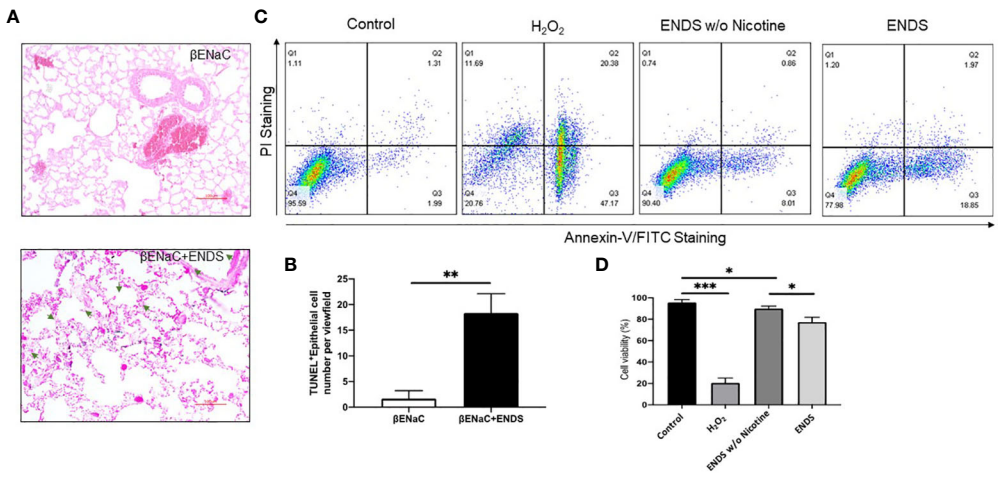

FIGURE 5
ENDS exposure caused lung epithelial cell apoptosis. **(A)** Representative images of immunochemical staining of TUNEL (TdT-mediated dUTP nick end labeling, blue; counterstained by Red Counterstain C, red) in lung sections from βENaC mice. Green arrowheads indicate TUNEL-positive cells. **(B)** Quantitative analysis of TUNEL staining as in **(A)** (n=3 per group). **(C)** Annexin V/PI apoptosis analysis in human lung epithelial cells (16HBE). The 16HBE cells were treated with, ENDS w/o nicotine and ENDS for 24h or 1 mM H_2O_2 for 12 hours as positive control and then processed for Annexin V/PI apoptosis analysis by flow cytometry. **(D)** Quantification of Annexin V/PI assay. The cell viabilities were quantified as the Annexin V/PI double-negative cell percentages of total acquired cells. Columns and error bars represent means ± SEM, n=4 *p<0.05; **p<0.01; ***p<0.0001.

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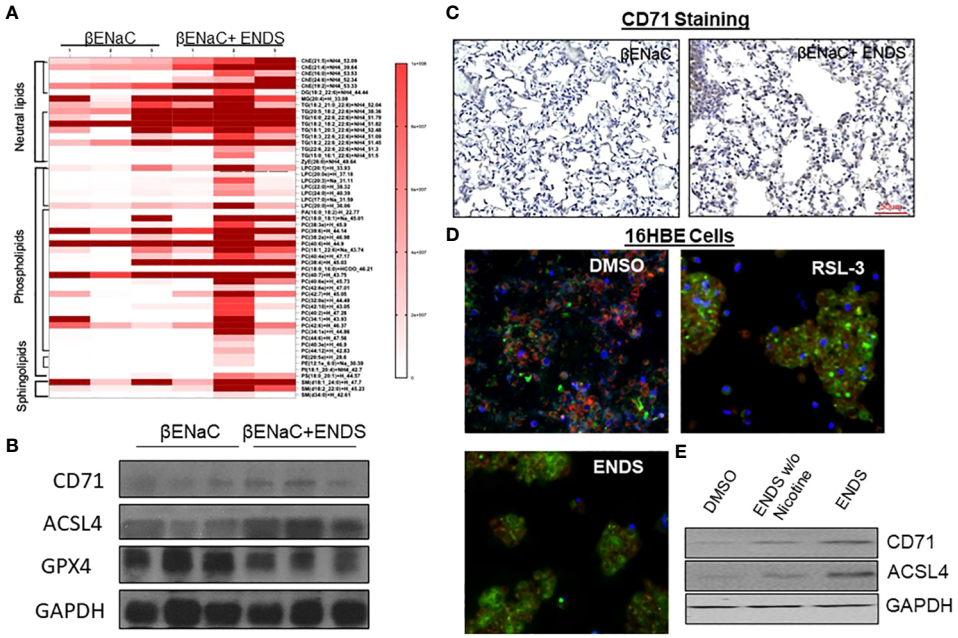

FIGURE 6
ENDS exposure induced ferroptosis of epithelial cells. **(A)** Mass-spectrometry-based lipidomics analysis using tissues collected from mice with or without ENDS exposure (n=3 per group). **(B)** Western blot determination of expression levels of ferroptosis-specific markers Transferrin Receptor/CD71 and ACSL4 using mouse tissues with or without ENDS exposure (n=3 per group). **(C)** Representative images of CD71 immunohistochemical staining of lung tissues from βENaC mice with or without ENDS exposure. **(D)** BODIPY-C11 assay determining the number of lipid peroxides in cellular membranes in 16HBE cells treated with DMSO (control), RSL-3(Positive control), or EDNS. **(E)** Western blot determination of expression levels of CD71 and ACSL4 in 16HBE cells treated by DMSO or ENDS or ENDS without Nicotine.

The western blot results further supported that ENDS increased the expression level of CD71 and ACSL4, confirming ENDS could induce ferroptosis in bronchial epithelial cells (Figure 6E). In order to verify the impact of ENDS on ferroptosis in epithelial cells, we performed immunofluorescent staining with ferroptosis biomarker, CD71. We found ferroptotic type I alveolar epithelial cells ($CD71^{+}HOPX^{+}$) and ferroptotic type II alveolar epithelial cells ($CD71^{+}$proSP-C^{+}) are both elevated after ENDS exposure (Figures 7A, B, Supplementary Figures 1B, C), suggesting that ENDS induced ferroptosis in epithelial cells, which is partially responsible for alveolar degradation and destruction.

Discussion

Cigarette smoking is considered the major risk factor for the development of lung diseases such as COPD and lung cancer in the developed world. Based on the assumption that ENDS vapor contains fewer toxins than conventional cigarette smoke, they were initially considered to be safer (42, 43). While ENDS have a less detrimental effect in terms of environmental pollutants (44) and production of carcinogens (45), the health effects of ENDS usage on chronic lung conditions, such as COPD, and associated public health recommendations remain scant. Recent data classified ENDS aerosol as unsafe (43, 46). By February of 2020, the Centers for Disease Control and Prevention reported approximately 3,000 hospitalizations associated with ENDS or vaping, product use–associated lung injury (EVALI) (47). Given the clinical health issues of ENDS in lung pathobiology, our current study was to investigate the effects of ENDS on COPD progression.

The correlation between ENDS use and COPD occurrence has been reported in human patients (48). However, how ENDS exposure induces COPD has not been fully understood. Our study demonstrates that ENDS exposure promotes COPD in mouse models. ENDS triggered inflammation response in βENaC mouse. ENDS exposure increased immune cell number within BAL fluid and cytokine/chemokine productions. Most notably, CCL2, also known as monocyte chemotactic/chemoattractant protein 1 (MCP1), is a soluble factor in driving monocytic infiltration of tissues during inflammatory processes (49). In a previous study, CCL2-producing macrophages are highly activated in patients with COPD, which is associated with increased monocyte migration (49). Therefore, it is possible that ENDS exposure could cause an increase in monocyte infiltration, which may be worth further investigation. Additionally, significant increases in IL-4, IL-13, IL-10, and well-known anti-inflammatory cytokines, were also observed in response to ENDS exposure. IL-4 and IL-13 are known to coincide with the switching of macrophages toward the M2-like phenotype and IL-10 is the typical cytokine released predominately by M2 macrophage. Multiply research groups have reported an alteration of immunophenotypes of macrophages toward M2 phenotype in COPD subjects, with cytokine production skew toward an M2 profile in blood and BAL samples from patients, suggesting M2 macrophages phenotype is a critical player in COPD disease progression (50–52). It is putative that ENDS might be involved in the regulation of M2 macrophages

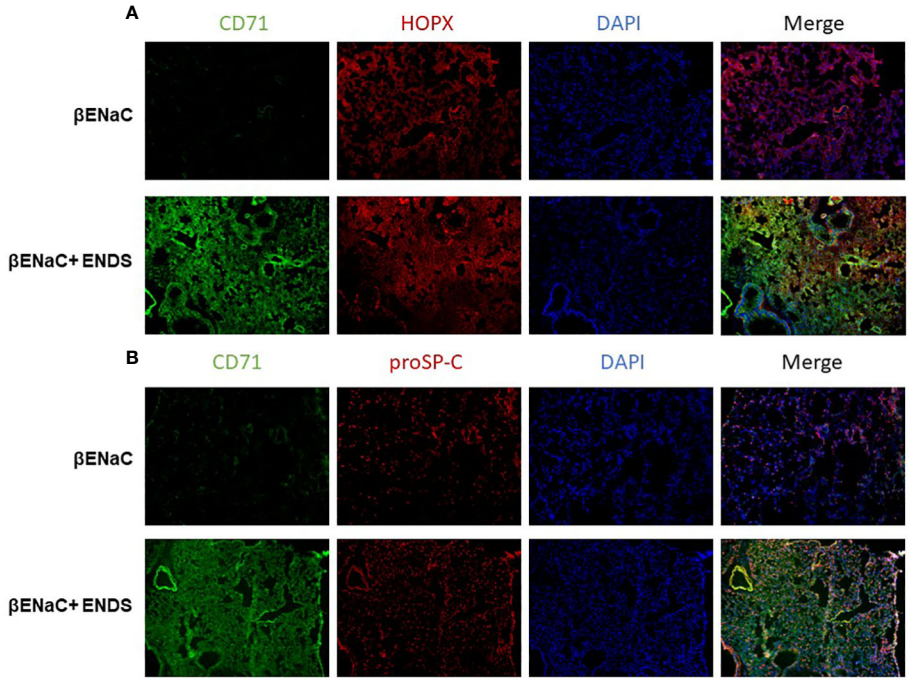

FIGURE 7
ENDS exposure induced ferroptosis of type I and type II alveolar epithelial cells *in vivo*. **(A)** Representative images of CD71 and HOPX co-immunofluorescent staining of lung tissues from βENaC mice with or without ENDS exposure. **(B)** Representative images of CD71 and proSP-C co-immunofluorescent staining of lung tissues from βENaC mice with or without ENDS exposure.

during COPD. Uncovering the effect of ENDS on macrophage regulation could be of great importance to deepen our current understanding of how ENDS promotes COPD.

IL-10 also appears to function as a regulator of fibrotic processes (53). We premised that ENDS exposure might cause a pro-fibrotic response. We found ENDS exposure caused collagen deposition and a significant increase in the expression of α-SMA within the lung tissues (Figure 3E), suggesting activation of fibrotic myofibroblasts. However, we did not uncover the detailed mechanism by which ENDS contributes to lung tissue fibrosis, which is worth further investigation.

While the majority of research focus is on the epithelial lining within the airway, recent evidence suggests endothelial dysfunction plays an essential role in the oxidative stress response to ENDS vaping (54). Here, we validated the effects of ENDS exposure on the cell viability of both endothelial cells and epithelial cells. Cell viability was significantly decreased following treatment with ENDS containing nicotine (Figures 4F, 5C). Interestingly, ENDS liquid without nicotine also had an effect on the induction of apoptosis of endothelial and epithelial cells, indicating other components in ENDS besides nicotine might also play a role in triggering apoptosis of endothelial and epithelial cells. Consistent with our findings, Itsaso et al. demonstrated that the way ENDS exposure in mice induces features of COPD is through a nicotine-dependent manner (10). Another recent study found that chronic ENDS exposure without nicotine adversely affects the vascular endothelial network by promoting oxidative stress and immune cell adhesion (54). One of our future works will focus on elucidating which component(s) besides nicotine in ENDS could lead to apoptosis of endothelial and epithelial cells.

It has been reported that traditional cigarettes smoke can induce ferroptosis which leads to COPD pathogenesis (38, 55, 56). However, it is not clear whether ferroptosis is involved in ENDS-related COPD. Our study is, to the best of our knowledge, is the first study to report the association between electronic cigarette and ferroptosis. Additionally, our discovery of ENDS-inducing ferroptosis in a novel mechanism of how ENDS promotes COPD would provide a new targeting strategy to treat ENDS-related COPD. Our mass-spectrometry-based lipidomics analysis on mouse serum demonstrated that relative abundances of more than 50 lipid species were altered after ENDS exposure (Figure 6A). There is substantial evidence supporting a strong correlation between ferroptosis susceptibility and the abundance of PUFA-lipids, including TAGs and phospholipids (57–59). In particular, phosphatidylcholines (PC), the most abundant class of lipids among those that were increased after ENDS exposure, are reported to trigger ferroptosis once oxidized (60, 61). Phosphatidic acid (PA) plays a pivotal role in lipid signaling and membrane dynamics, which are crucial in ferroptosis as PA can influence membrane fluidity and the formation of lipid peroxides (62). Lysophosphatidylcholine (LPC) is known to act as a pro-inflammatory mediator and can modulate cell membrane integrity, thereby contributing to the oxidative stress seen in ferroptosis (63). Phosphatidylethanolamine (PE) is particularly significant in ferroptosis; PE-bound polyunsaturated fatty acids (PUFAs) are substrates for lipid peroxidation, leading to the

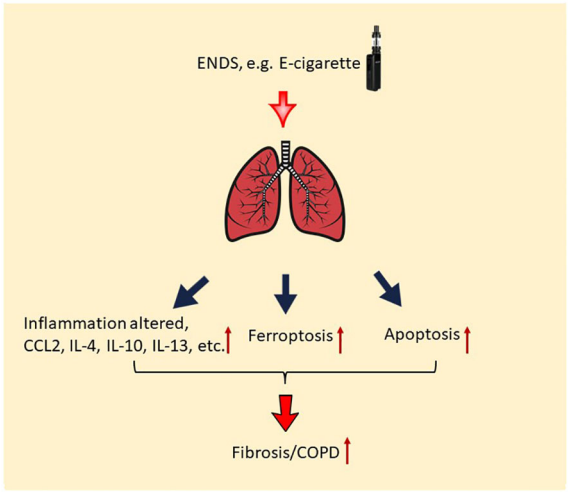

FIGURE 8
Potential molecular mechanism of the effect of ENDS on COPD. ENDS exposure increases levels of multiple COPD-related cytokines in the lungs, including CCL2, IL-4, IL-10, IL-13, and IL-10; and ENDS promotes fibrosis in the lung. Moreover, ENDS exposure triggers apoptosis of pulmonary endothelial cells and epithelial cells and ferroptosis of epithelial cells. Overall, abnormal lung inflammation, fibrosis, and cell death of endothelial/epithelial cells caused by ENDS suggest that ENDS exposure exacerbates features of COPD.

generation of lipid hydroperoxides that drive ferroptotic cell death (64, 65). We further verified that both type I and type II alveolar epithelial cells are prone to ferroptosis after ENDS exposure using immunofluorescent staining with mouse tissues. The molecular mechanism of ferroptosis on alveoli epithelial cells is still under investigation. There is a high correlation between ferroptosis and inflammatory response reported. We observed increased levels of proinflammatory cytokines which support macrophage infiltration. Therefore, a reasonable speculation is the macrophage could induce ferroptotic cell death of epithelial cells which might further drive COPD.

We did not find all the ferroptotic lipid species as in the previous publication (66, 67), this inconsistency might be due to the sample preparation, limits of mass spectrometry detection, or the specificity to ENDS exposure. However, due to the limitation of the genetic background of ENaC mice, further investigation is needed to elucidate the impact of chronic ENDS exposure with or without nicotine on the endothelium in other genetic background mice. In addition, the vulnerability of gender to COPD due to ENDS exposure also needs to be addressed.

Our study's findings have significant implications for human health, particularly regarding the use of electronic nicotine delivery systems (ENDS) among individuals with chronic obstructive pulmonary disease (COPD). As ENDS usage rises, understanding its impact on COPD is crucial, and our results suggest that ENDS may worsen COPD symptoms and accelerate disease progression. This challenges the view of ENDS as a safer alternative to traditional tobacco products, especially for those with respiratory conditions. Therefore, our findings support the need for stringent ENDS regulations, including comprehensive testing and standardized guidelines for manufacturing and marketing, alongside public health campaigns to educate users about the potential risks of ENDS, emphasizing that they are not necessarily safe for individuals with compromised lung health.

It is essential to acknowledge the limitations of our study to provide a balanced perspective on our findings. One limitation of our study is the extrapolation of data from mouse models to human COPD progression, as differences in respiratory systems and immune responses between species can affect the applicability of these findings to humans. Further research with human subjects is needed to validate our results and understand their implications for human health. Another limitation is the potential variability in ENDS devices and liquids, with different products having diverse designs, power settings, and compositions, potentially leading to varying levels of exposure to harmful substances. Our study focused on specific ENDS products, which may not represent the full range available to consumers. Future research should examine a broader array of ENDS products for a more comprehensive health impact assessment.

Conclusions

Taken together, our results demonstrate that nicotine-containing ENDS, in the form of e-cigarette vapor, causes the exacerbation of features of COPD in βENaC-overexpressing mice. Specifically, acute exposure to nicotine-containing ENDS vapor results in mucus accumulation, overproduction of multiple cytokines, deposition of ECM, and fibrosis (Figure 8). Last but not least, ENDS exposure induces cell death in epithelium and endothelium in the lung of βENaC-overexpressing mice (Figure 8).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Georgia State University IACUC Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HH: Conceptualization, Writing – original draft. MM: Conceptualization, Methodology, Writing – original draft. GP: Data curation, Writing – original draft. YY: Methodology, Writing – original draft. JQ: Formal analysis, Project administration, Writing – review & editing. JY: Conceptualization, Data curation, Funding acquisition, Methodology, Writing – review & editing. Z-RL: Data curation, Supervision, Writing – review & editing. XJ: Data curation, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1429946/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Quantification of histopathological findings on ENDS exposure induced ferroptosis **(A)** Quantification of CD71 immunohistochemical staining of lung tissues from βENaC mice with or without ENDS exposure (n=3 per group). **(B)** Quantification of CD71 and HOPX co-immunofluorescent staining of lung tissues from βENaC mice with or without ENDS exposure (n=3 per group). **(C)** Quantification of CD71 and proSP-C co-immunofluorescent staining of lung tissues from βENaC mice with or without ENDS exposure (n=3 per group). Columns and error bars represent means ± SEM, *$p<0.05$; **$p<0.01$.

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Ferroptosis: a novel mechanism of cell death in ophthalmic conditions

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Ferroptosis, a new type of programmed cell death proposed in recent years, is characterized mainly by reactive oxygen species and iron-mediated lipid peroxidation and differs from programmed cell death, such as apoptosis, necrosis, and autophagy. Ferroptosis is associated with a variety of physiological and pathophysiological processes. Recent studies have shown that ferroptosis can aggravate or reduce the occurrence and development of diseases by targeting metabolic pathways and signaling pathways in tumors, ischemic organ damage, and other degenerative diseases related to lipid peroxidation. Increasing evidence suggests that ferroptosis is closely linked to the onset and progression of various ophthalmic conditions, including corneal injury, glaucoma, age-related macular degeneration, diabetic retinopathy, retinal detachment, and retinoblastoma. Our review of the current research on ferroptosis in ophthalmic diseases reveals significant advancements in our understanding of the pathogenesis, aetiology, and treatment of these conditions.

KEYWORDS

ferroptosis, metabolic pathway, eye diseases, therapy, corneal injury

1 Introduction

The pathology of ophthalmic diseases involves various mechanisms of cell death, including apoptosis, necrosis, and autophagy (1, 2). It is specifically regulated by iron levels and the antioxidant enzyme glutathione peroxidase 4 (GPX4), unlike apoptosis's caspase-dependent mechanism, necrosis's inflammatory cell rupture, and autophagy's dual role in both cell survival and death (3). Each form of regulated cell death (RCD) exhibits distinct morphological features and is controlled by unique biochemical, genetic, and functional processes. Studies have shown that pharmaceutical agents modulate apoptosis, necrosis, and autophagy within ocular cells by targeting pathways such as inflammation, oxidative stress, and endoplasmic reticulum stress, leading to positive therapeutic effects (4).

The key membrane protein xCT related to ferroptosis was discovered as early as the 1980s (5). Subsequently, over the course of more than 20 years, the classic inducers of ferroptosis, erastin, which can kill RAS-mutated BeJLR cells, were also discovered in 2003 (6). However, it was not until 2012 that researchers such as Dixon defined this type of cell death as ferroptosis. Dixon and colleagues identified a form of programmed cell death (PCD) reliant on iron metabolism, particularly in cancer cells (7). The term "ferroptosis" was coined to describe a new form of non-apoptotic cell death activated by the small molecule erastin, which was found to induce cell death through iron-dependent lipid peroxidation.

This phenomenon is observed not only in mammals but also in plants, protozoa, and fungi, including *Magnaporthe oryzae* and *Caenorhabditis elegans* (8, 9). Emerging evidence suggests that ferroptosis significantly regulates the progression of numerous systemic diseases (Figure 1) (10–17).

It is widely believed that ferroptosis primarily results from an overload of intracellular reactive oxygen species (ROS) dependent on iron, combined with a reduction in the detoxifying activity of GPX4, leading to an imbalance in ROS production and clearance (7). When the cell's antioxidant defenses are overwhelmed and cannot neutralize the excess ROS, ferroptosis is triggered within the cell, with the subsequent peroxidation reactions being pivotal to its onset (18). Consequently, numerous molecules and signaling pathways involved in iron metabolism and peroxidation reactions are pivotal in the modulation of ferroptosis.

This review delves into the latest developments and future prospects in the research of ferroptosis in corneal injury, glaucoma, age-related macular degeneration, diabetic retinopathy, retinal detachment, and retinoblastoma. This study investigates the metabolic interplay between ferroptosis and ophthalmic pathology, aiming to establish a foundation for further exploration of the mechanisms and preventive strategies of ferroptosis in ophthalmic diseases.

2 Characteristics of ferroptosis

Iron ions play a pivotal role in metabolism in the ocular region, serving as essential micronutrients for a wide array of biological activities (19). They contribute significantly to the light conversion process in photoreceptor cells in the retina, participate in the electron transport chain within mitochondria, and are crucial for the activity of enzymes such as cytochrome oxidase (20). Excess iron ions produce ROS through the Fenton reaction, and iron overload and ROS accumulation are involved in the pathophysiological mechanisms of ferroptosis (21). Ferroptosis is a form of programmed cell death triggered by excessive iron accumulation and lipid peroxidation-induced damage. Signaling pathways and molecules involved in the regulatory mechanism of ferroptosis include iron metabolism, cysteine metabolism, GPX4 inactivation, polyunsaturated fatty acid (PUFA) synthesis, nuclear factor E2-related factor 2 (Nrf2), p53, heat shock proteins (HSPs), iron-regulated inhibitor-1 (FSP1), AMP-activated protein kinase (AMPK) activation, and nicotinamide adenine dinucleotide phosphate (NADPH), etc. We will classify and introduce ferroptosis-related molecules from signaling, execution, regulation and other functions.

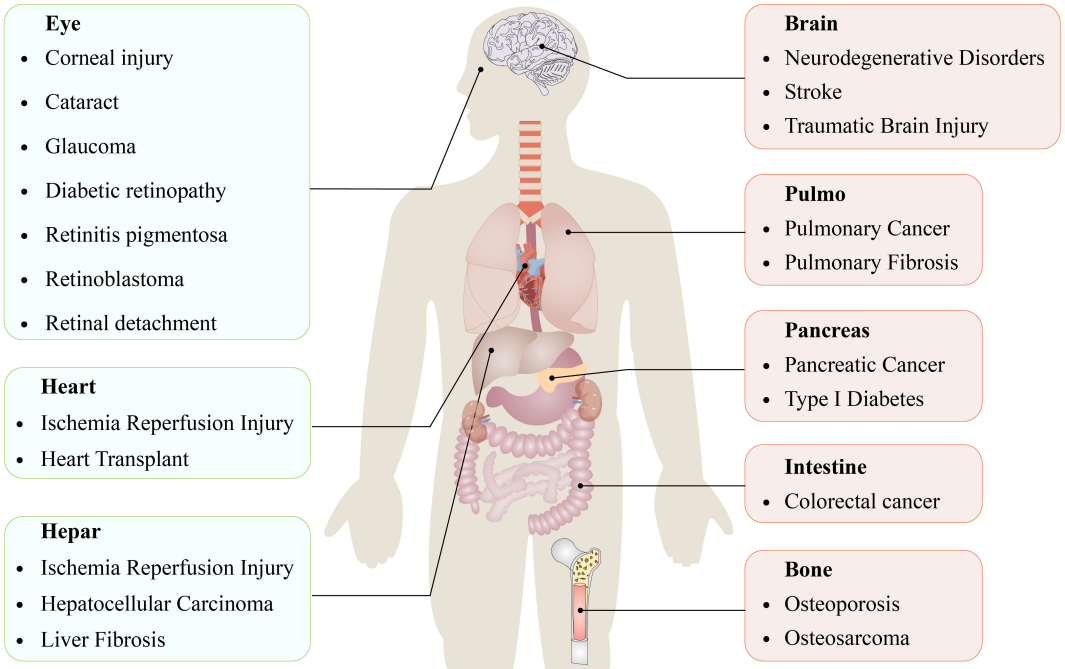

FIGURE 1
Ferroptosis is involved in the occurrence and development of various diseases in organs and tissues.

2.1 Signaling

2.1.1 Iron metabolism

Iron metabolism is crucial in ferroptosis, influencing the accumulation of lipid peroxides (22). Iron uptake, iron transport across cell membranes, and iron storage profoundly influence the regulation of ferroptosis (23). Organisms carefully maintain a delicate balance to maintain iron homeostasis. The acquisition of iron through transferrin receptor 1 (TFR1) is crucial for ferroptosis; this receptor facilitates the transport of ferric ions (Fe^{3+}) to endosomes, where they are converted to ferrous ions (Fe^{2+}) (Figure 2). Divalent metal transporter 1 (DMT1) releases Fe^{2+} from endosomes into the labile intracellular iron pool. Excess iron is then sequestered within the cytoplasm in the form of ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1) (24). Disruptions in the normal expression or function of these proteins

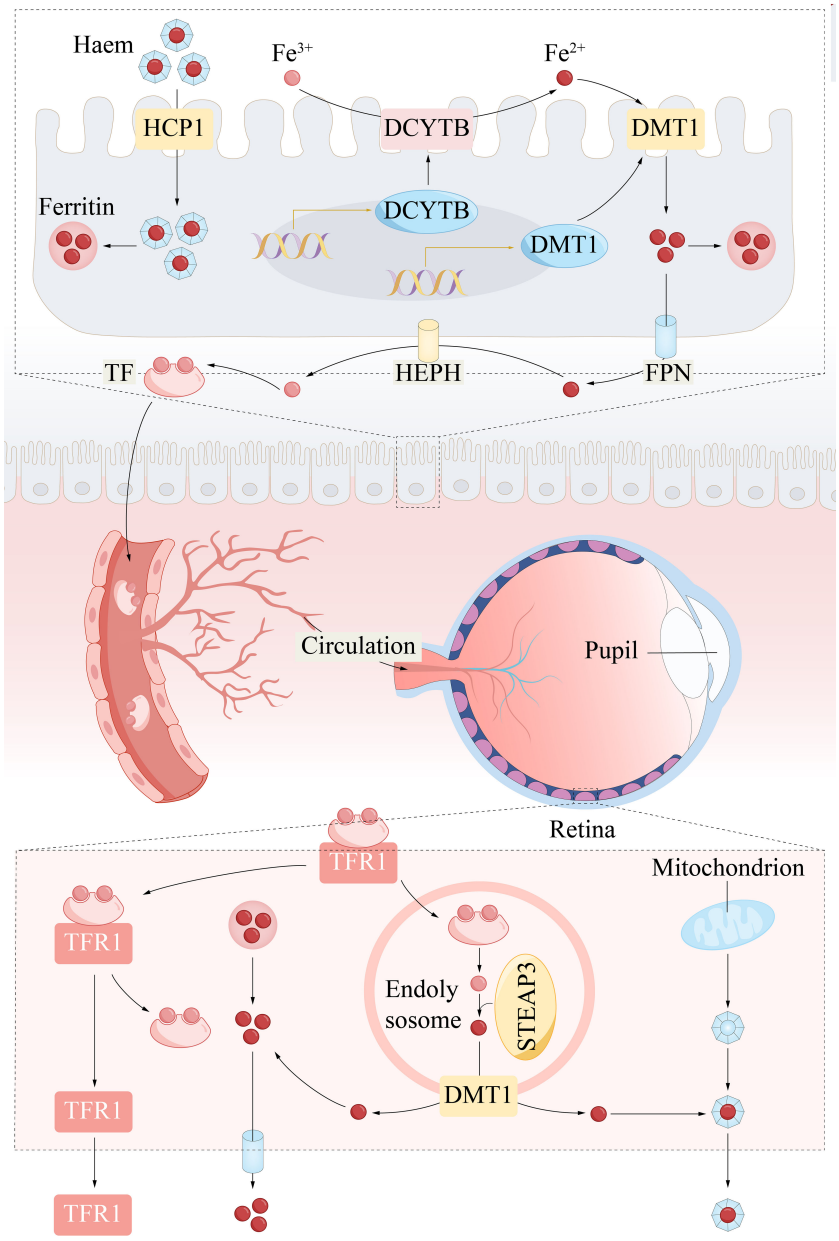

FIGURE 2
Systemic circulation of iron and iron metabolism. Iron ions are absorbed from the gut and reach the eyeball as blood circulates throughout the body. Specifically, Fe^{3+} is reduced to Fe^{2+} by DCYTB in the intestinal lumen. DMT1 then transports Fe^{2+} into enterocytes. HCP1 also incorporated heme iron into duodenal enterocytes. HIF2α (not shown, in the nucleus) binds to hypoxia response elements (HRE) in the regulatory regions of the DMT1 and DCYTB promoters and is therefore post-transcriptionally regulated. Intracellular iron storage is controlled by ferritin, whereas Fe^{2+} is exported into the blood via FPN and simultaneously oxidized to Fe^{3+} by hephaestin. Plasma transferrin captures and distributes Fe^{3+} to the retina. Cells take up iron via TFR1 -mediated endocytosis of holotransferrin. In retinal cells, DMT1 transports Fe^{2+} into the cytoplasm. Fe^{2+} can shuttle to mitochondria for heme biosynthesis. DCYTB, duodenal cytochrome B; DMT1, divalent metal transporter 1; FPN, ferroportin; HEPH, hephaestin; STEAP3, six transmembrane epithelial antigen of the prostate 3; TF, transferrin; TFR1, transferrin receptor 1.

related to iron metabolism can lead to an increase in intracellular iron levels, resulting from metabolic dysregulation (25). Under conditions of iron surplus, ferroportin 1 (FPN1) is notably expressed on the brush border membrane of renal proximal tubule cells, indicating its potential role in the excretion of excess iron into the urinary space (26). Hepcidin, which is predominantly produced in the liver, plays a crucial role in governing the release of iron by FPN1 in macrophages (27).

Iron plays a crucial role in the visual process, particularly in phototransduction, where it acts as a cofactor for key enzymes involved in the regeneration of rhodopsin, converting light signals into neural signals. Additionally, iron is involved in maintaining the antioxidant balance of the retina, helping to eliminate free radicals and protect the retina from oxidative damage (28). However, disruptions in iron metabolism can lead to iron deposition and lipid peroxidation, which may have a negative impact in age-related macular degeneration (29). Therefore, the balance of iron ions is vital for retinal health, and excessive light exposure may cause iron metabolic disorders and harmful oxidative stress, damaging retinal cells.

2.2 Execution

2.2.1 GPX4 inactivation

The glutathione peroxidase (GPX) family comprises various isoforms, including GPX1 through GPX8, with GPX4 playing a particularly crucial role in the context of ferroptosis (Figure 3) (30). GPX4, a selenoprotein in mammals, is essential for the repair of lipid peroxidation within cells (31). It facilitates the conversion of the reduced form of glutathione (GSH) to its oxidized counterpart (GSSG) and transforms toxic lipid hydroperoxides (L-OOH) into nontoxic lipid alcohols (L-OH), thereby catalysing the breakdown of hydrogen peroxide (H_2O_2). This process is vital for safeguarding the cell membrane's structure and function against oxidative stress and damage (32).

GPX4 plays a crucial role in protecting retinal cells from oxidative stress and ferroptosis damage. It catalyzes the reduction of lipid peroxides by GSH, thereby protecting the cell membrane from oxidative damage, which is essential for maintaining retinal health (33). In ophthalmic diseases such as diabetic retinopathy, the downregulation of GPX4 may lead to increased oxidative stress,

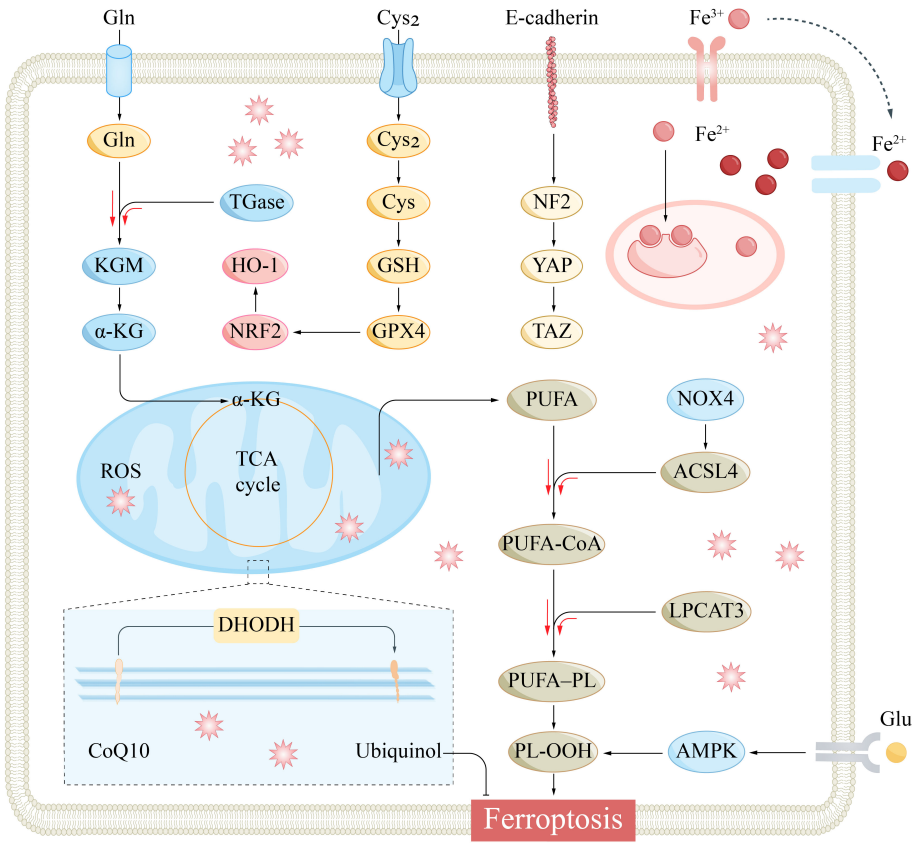

FIGURE 3
The main signaling pathways that regulate ferroptosis. Specifically, Cysteine metabolism, Iron metabolism, GPX4 inactivation, PUFA synthesis, Hippo pathway, AMPK, and NRF2 are involved in the regulatory mechanisms of ferroptosis. α-KG, α-ketoglutarate; ACSL4, acyl-CoA synthetase long-chain family member 4; AMPK, AMP-activated protein kinase; CoQ10, coenzyme Q10; Cys2, cystine; DHODH, dihydroorotate dehydrogenase; Gln, glutamine; Glu, glutamate; GPX4, glutathione peroxidase 4; GSH, glutathione; HO-1, heme oxygenase-1; KGM, konjac glucomannan; LPCAT3, lysophosphatidylcholine acyltransferase 3; NF2, Neurofibromin 2; NOX4, NADPH oxidase 4; Nrf2, nuclear factor erythroid 2-related factor 2; PL-OOH, phospholipid hydroperoxide; PUFA, polyunsaturated fatty acid; PUFA-PL, phospholipid containing polyunsaturated fatty acid chain; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; YAP, yes-associated protein.

subsequently affecting the function and survival of retinal photoreceptor cells (34).

Agents that induce ferroptosis, such as RAS-selective lethal 3 (RSL3) and erastin, lead to an increase in lipid ROS levels (35). While erastin impacts GSH levels, RSL3 does not substantially alter GSH levels during ferroptosis (36). Subsequent investigations revealed that RSL3 specifically targets the GPX4 molecule, inhibiting its activity through covalent binding and resulting in the accumulation of lipid peroxides (37). Yang et al. noted that cells with diminished GPX4 expression are more susceptible to ferroptosis, whereas those with elevated GPX4 levels exhibit increased resistance (38). Consequently, GPX4 serves as a significant regulator of ferroptosis, and modulating its expression can either enhance or suppress the occurrence of ferroptosis (16).

2.2.2 PUFA synthesis

PUFAs play a significant physiological role in the ophthalmic environment, particularly in terms of retinal health and visual function. PUFAs are crucial components of the cell membrane, essential for maintaining the fluidity and integrity of the retinal cell membrane (39). They are also involved in regulating lipid metabolism in the retina, playing an important role in promoting growth and development, immune modulation, and antioxidation.

The molecular structure of PUFAs features vulnerable carbon –carbon double bonds, making them susceptible to a process known as lipid peroxidation, a key event in ferroptosis (40). Two pivotal enzymes in the lipid remodeling process are acyl-CoA synthase long-chain 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), which are crucial for the production of PUFAs during ferroptosis (Figure 3) (41). The deletion or silencing of these genes can curtail the synthesis of PUFAs, thereby impeding the progression of ferroptosis (42, 43). Conversely, an abundance of arachidonic acid and other PUFAs can exacerbate the ferroptosis response when triggered by an inducer, as it accelerates the formation of peroxide lipids within the cell (40). This result highlights the significance of PUFAs as targets for plasma membrane peroxidation during ferroptosis.

Additionally, the enzyme lipoxygenase (LOX) facilitates the peroxidation of PUFAs, and decreasing LOX expression has been shown to mitigate ferroptosis, particularly that induced by erastin (40, 42). Given that free PUFAs serve as substrates for lipid peroxidation, their concentration and cellular distribution can influence the extent of lipid peroxidation and, consequently, the intensity of ferroptosis (42). Understanding the dynamics of PUFA localization and concentration presents an alternative avenue for modulating ferroptosis.

Some PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), play a crucial role in visual development and retinal function. DHA is the most abundant omega-3 fatty acid in retinal photoreceptors and is essential for maintaining the structure and function of photoreceptor cells (44). EPA helps to reduce inflammatory responses and decrease the risk of age-related macular degeneration and other eye diseases. In addition, omega-3 PUFAs may also mitigate oxidative damage to ocular tissues through antioxidant mechanisms, protecting the retina from light-induced damage (45).

Future research may explore the possibility of modulating enzymes involved in the synthesis of phospholipids containing PUFAs as a strategy to either initiate or prevent ferroptosis. This approach could reveal novel regulatory mechanisms and potential therapeutic targets within the complex interplay of lipid metabolism and cell death pathways.

2.3 Regulation

2.3.1 Cysteine metabolism

Cysteine metabolism plays a pivotal role in ferroptosis, serving as a critical regulatory hub for the synthesis of GSH, the primary antioxidant defense against the peroxidation of lipids within the cell membrane. The cystine/glutamate antiporter System Xc- is a complex composed of two subunits, the light chain (SLC7A11) and the heavy chain (SLC3A2), which are linked by disulfide bonds (46). This transporter plays a vital role in cellular antioxidant defense mechanisms. It operates by taking up cystine while exporting glutamate in an equimolar exchange (47). Once internalized, cystine is converted to cysteine, which is a critical component in the assembly of GSH. GSH, under the influence of GPX4, facilitates the conversion of harmful lipid peroxides into harmless fatty alcohols, thereby acting as a pivotal cellular antioxidant (Figure 3) (48).

In this biochemical cascade, GSH acts as a reducing agent, with GPX4 being the central enzyme that catalyses the reduction of lipid peroxides and thereby suppresses ferroptosis (49). Nonetheless, the rate of GSH production is contingent upon the availability of its precursor, cysteine, making System Xc- a crucial regulatory element. Compounds such as erastin, which are known to induce ferroptosis, impede GSH synthesis by directly targeting System Xc- and curbing cysteine uptake. A subsequent deficiency in GSH can result in the accumulation of detrimental peroxides, leading to protein and cell membrane damage and ultimately triggering ferroptosis (7).

Cysteine metabolism plays a crucial role in the ophthalmic environment, particularly in maintaining the health and function of the retina. Cysteine is the rate-limiting precursor for the biosynthesis of GSH, which is one of the primary antioxidants within cells and essential for protecting retinal cells from oxidative stress and ferroptosis. In the retinal pigment epithelial cells and photoreceptor cells, GSH helps to neutralize light-induced ROS, which are continuous byproducts of the visual cycle (50). One of the hallmarks of ferroptosis is the accumulation of lipid peroxides, and GSH, in its reduced form, aids in maintaining the integrity of the cell membrane, preventing lipid peroxidation.

2.3.2 Nrf2

The Nrf2 pathway is integral to the cellular response against ferroptosis, acting as a master regulator of antioxidant and detoxification processes. In a 2016 study, Sun and colleagues showed that the transcription factor Nrf2 can modulate ferroptosis through the P62-KEAP1-NRF2 signaling axis (51) (Figure 3). Upon activation, Nrf2 enhances the sequestration of iron and reduces its cellular uptake, thereby curtailing ROS

generation and exerting an inhibitory effect on ferroptosis (52). Furthermore, Nrf2 suppresses ferroptosis by upregulating the expression of genes pivotal for the metabolism of iron and ROS, including quinone oxidoreductase 1 (NQO1) and haem oxygenase-1 (HO-1) (53). Additionally, stimulation of the p62-KEAP1-NRF2 cascade can lead to the upregulation of System Xc-, which in turn augments the transport of cystine and glutamate. This accelerated transport aids in the elimination of accumulated lipid peroxides (54), contributing to the overall antioxidant response and cellular defense against ferroptosis.

Nrf2 mitigates oxidative stress-induced damage to ocular tissues by activating antioxidant enzymes that eliminate harmful reactive oxygen species and free radicals. It suppresses the production of inflammatory mediators, reducing ocular inflammatory responses, while also promoting the repair of damaged cells and the regeneration of retinal tissue, maintaining the integrity of ocular structures (55). Nrf2 also regulates cellular metabolic pathways, affecting the synthesis and breakdown of fatty acids, influencing the progression of metabolic diseases, and providing protective effects on retinal neurons, reducing neurodegeneration, and preserving visual function (56). Furthermore, it inhibits pathological angiogenesis, safeguarding vision, and is involved in the regulation of ferroptosis, the clearance of damaged organelles and misfolded proteins, thereby maintaining the stability of the intracellular environment (57). These integrated functions underscore the essential role of Nrf2 in maintaining ocular health and visual function.

2.3.3 P53

The tumor suppressor p53, which is activated by various stress signals, has been implicated in regulating ferroptosis (58). By repressing the SLC7A11 gene at the transcriptional level, p53 contributes to the onset of ferroptosis by hindering the uptake of cysteine, an essential amino acid for GSH synthesis, thereby promoting ferroptosis (54). The role of p53 in inducing ferroptosis is associated with a specific mutational mechanism involving the substitution of three lysine residues with arginine. These mutations created an acetylated mutant form of p53 known as p533KR, which significantly suppresses the expression of SLC7A11 without affecting other recognized p53 target genes, such as those linked to cell cycle regulation, apoptosis, or ageing (59).

System Xc- is a crucial transporter for cystine and glutamate, forming a heterodimeric complex with the subunits SLC7A11 and SLC3A2 (60). Consequently, p53 impedes cystine uptake by System Xc- through the downregulation of SLC7A11. This reduction in cystine uptake leads to a decrease in cystine-dependent glutathione peroxidase (GPX) activity, which weakens the cell's antioxidant defenses (61). As a result, lipid ROS levels are increased, triggering ferroptosis within the cell (62). This regulatory pathway underscores the importance of p53 in modulating the cellular susceptibility to ferroptosis and provides a potential therapeutic target for interventions aimed at preventing or treating conditions associated with this form of cell death.

P53 plays a critical protective role in ophthalmic diseases by monitoring cellular DNA damage and stress signals. When retinal cells undergo photic damage, hypoxia, or metabolic stress, P53 is swiftly activated to induce cell cycle arrest, thereby allowing time for DNA repair (63). If the damage is too severe, P53 initiates the programmed cell death process to prevent the proliferation of damaged cells and potential tumor formation.

Moreover, P53 is involved in regulating cellular metabolism, influencing the progression of eye diseases such as diabetic retinopathy by affecting blood sugar balance and fatty acid oxidation (64). The activity of P53 is also linked with neuroprotection in age-related macular degeneration and glaucoma (65). In these conditions, it fosters the expression of stress-resistant molecules to shield retinal neurons from degeneration.

2.3.4 HSPs

HSPs are critical in modulating the cellular response to ferroptosis, serving as a network of molecular chaperones that assist in the preservation of cellular integrity under oxidative stress. HSPs form an evolutionarily conserved family of molecular chaperones that fulfil a variety of immunological functions (66). These functions include mitigating cellular stress, exhibiting antioxidant activity, modulating immune responses, and facilitating antigen presentation. Through these functions, HSPs confer cellular resilience to various modes of cell death, including ferroptosis (67). One member of this family, heat shock protein B1 (HSPB1), also known as HSP25 or HSP27, safeguards the integrity of the actin cytoskeleton. This function is achieved by mitigating ferroptosis through a reduction in iron uptake, thereby curtailing subsequent oxidative damage (68).

HSPB1 also regulates the expression of TFR1 (69). Furthermore, the protein kinase C-mediated phosphorylation of HSPB1 results in the upregulation of FTL and FTH1 expression (68). This increase in ferritin expression reduces the concentration of intracellular ferrous ions and the production of lipid ROS (70).

The HSPB1 pathway, shown to counteract erastin-induced ferroptosis in cancers like cervical, prostate, and osteosarcoma, reduces iron and ROS levels (68, 71). Additionally, another member of the HSP family, heat shock protein A5 (HSPA5), also known as binding immunoglobulin protein (BIP) or 78 kDa glucose-regulated protein (GRP78), is localized to the endoplasmic reticulum. HSPA5 can bind to GPX4, thereby significantly bolstering the cell's antioxidant defenses (72).

HSPs play the role of protectors in ophthalmic diseases, participating in the maintenance of cellular proteostasis, especially when faced with damaging conditions such as oxidative stress, inflammation, heat shock, ultraviolet radiation, and metabolic stress. HSPs like HSP27, HSP70, and HSP90 can prevent protein aggregation and cellular dysfunction by promoting the proper folding of damaged proteins, repairing, or clearing misfolded proteins. In retinal diseases, HSPs help protect photoreceptor cells from light damage and slow the progression of age-related macular degeneration (73). In glaucoma and neurodegenerative eye diseases, the neuroprotective role of HSPs is particularly important, as they can protect retinal ganglion cells from mechanical stress or disease-related neurotoxic damage (74, 75). This intricate interplay between HSPs and cellular antioxidant systems presents a promising avenue for therapeutic intervention in conditions where ferroptosis contributes to pathology.

2.3.5 FSP1

A comprehensive genome-wide screening study revealed the role of FSP1 in regulating ferroptosis. Interestingly, the FSP1-CoQ10-NAD(P)H pathway operates as a significant intracellular antioxidant mechanism (76). This complements the traditional GSH-GPX4 pathway to collaboratively suppress phospholipid peroxidation and inhibit ferroptosis.

Specifically, FSP1 facilitates the reduction of CoQ10 to Ubiquinol using NAD(P)H (77). This reaction subsequently captures lipid peroxyl radicals, thereby halting the propagation of phospholipid peroxidation. In parallel, the GPX4 and glutathione systems aid in neutralizing phospholipid hydroperoxides and free radicals, further dampening peroxidative processes. In instances of GPX4 deficiency, the FSP1-CoQ10-NAD(P)H pathway steps up as the primary mechanism for combating ferroptosis.

In 2019, research conducted by Bersuker and colleagues indicated that the deletion of FSP1 resulted in tumor cells losing their resistance to the iron regulatory compound RSL3, concomitant with a marked increase in intracellular iron levels (78). FSP1, known for its myristoylation, contributes to the composition of numerous cellular membranes, and any modification of its myristoylated sites can impair its ferroptosis-inhibiting capabilities (79).

Mechanistically, FSP1 exerts its effects through the quinone oxidoreductase activity associated with NADH, converting ubiquinone to ubiquinol, which in turn inhibits lipid peroxidation and ferroptosis (80). Ubiquinol can directly neutralize lipid-derived free radicals, thereby halting the process of lipid autoxidation. Alternatively, it can indirectly cease lipid autoxidation through the regenerative oxidation of α-tocopherol free radicals (78).

FSP1 plays an integral role in the repair processes of the cornea and retina, facilitating the healing of damaged tissues by promoting cell adhesion and migration (81). Following a corneal injury, the deposition of FSP1 can provide a temporary substrate for epithelial cell regeneration, thereby expediting wound closure and repair.

Moreover, FSP1 significantly contributes to the functionality of RPE cells. It assists in maintaining the polarity and integrity of RPE cells, which are vital for the health and function of photoreceptor cells. In vascular diseases such as diabetic retinopathy, FSP1 may play a role in regulating the growth of abnormal blood vessels and scar formation (82). It influences the remodeling of the extracellular matrix and angiogenesis processes through interactions with growth factors and cell surface receptors. FSP1 may also have a role in age-related macular degeneration, affecting the formation of neovascularization and scar tissue beneath the retina (81).

This intricate regulatory network underscores the multifaceted role of FSP1 in modulating the cellular susceptibility to ferroptosis, providing novel insights into the complex interplay between cellular metabolism and iron homeostasis in the context of oxidative stress and cell death.

2.3.6 AMPK

AMPK serves as a pivotal sensor within the cell, monitoring energy metabolism and orchestrating a variety of metabolic pathways to maintain energy homeostasis (83). The activation of AMPK has been implicated in both the promotion and suppression of ferroptosis (84). The seemingly contradictory effects of AMPK on ferroptosis may stem from the intricate interplay of environmental factors and the degree of AMPK phosphorylation at specific sites (85–87).

Moreover, AMPK is recognized for its role in governing mitochondrial stability, which is integral to cellular health and the regulation of cell death pathways (88). The activation of AMPK triggers the degradation of ferritin, an iron-storage protein, leading to increased ROS levels and the initiation of ferroptosis (89, 90). A study identified AMPK as an upstream modulator of ferroptosis, and the depletion of AMPK sensitizes cells to this form of death (91). Conversely, another study reported that AMPK becomes activated under glucose-deprived conditions and that the AMPK signaling cascade curbs lipogenesis, thereby reducing the propensity for ferroptosis (Figure 3) (92).

In addition, AMPK is also involved in the regulation of mitochondrial biogenesis and inflammatory responses, both of which are significant factors in the development of eye diseases. For instance, the activation of AMPK can promote the process of mitochondrial ferroptosis, assisting in the replacement of dysfunctional mitochondria, which is crucial for maintaining the health of ocular cells (93). At the same time, AMPK can also inhibit the mTORC1 pathway, a key regulatory factor for cell growth and proliferation, and its inhibitory effect helps to control the progression of eye diseases (94). Therefore, the regulatory role of AMPK has potential application value in ophthalmic treatment and can serve as a new strategy for treating eye diseases.

2.3.7 NADPH

NADPH is a vital coenzyme in cellular redox homeostasis and plays a crucial role in ferroptosis by functioning as the primary reducing equivalent to counteract oxidative stress. It functions as a coenzyme for glutathione reductase, is integral to maintaining intracellular GSH levels (95). The influence of well-established ferroptosis initiators, including erastin, RSL3, and FIN56, has been explored across diverse cell lineages, notably those derived from human osteosarcoma (96). These studies consistently reported a significant decrease in the levels of intracellular NAD(H) and NADP(H), alongside the detection of ROS formation (97).

Current research shows NADPH donates electrons to cytochrome P450 via P450 oxidoreductase (POR), supplying them with electrons (98). This electron transfer may precipitate lipid peroxidation through several proposed mechanisms, either by facilitating the dehydrogenation of PUFAs or by impeding the reduction of Fe^{3+} to Fe^{2+} (99, 100).

These findings suggest that NADPH not only is central to the cellular redox balance but also plays a critical role in regulating ferroptosis, potentially by influencing iron metabolism and lipid peroxidation (101). Understanding the nuances of the role of NADPH in these processes could provide novel perspectives on the modulation of ferroptosis and its implications in various pathophysiological conditions.

Besides, NADPH is a key coenzyme for the synthesis of lipids and cholesterol within cells, which is crucial for the membrane

structure and function of retinal cells. In ophthalmic diseases, the synthesis and utilization of NADPH may be affected, leading to dysfunction of retinal cells (102). Therefore, maintaining the levels and functionality of NADPH is essential for protecting vision and treating ophthalmic diseases (103). Supplementing NADPH or enhancing its synthetic pathways may help improve retinal health and delay the progression of ophthalmic diseases.

2.3.8 GCH1

A laboratory investigation revealed that GCH1, an essential enzyme in the biosynthesis of folates, can prevent ferroptosis (104). This protective effect is believed to be mediated by its metabolic products, tetrahydrobiopterin (BH4) and dihydrobiopterin (BH2), which are crucial for cellular redox homeostasis and the maintenance of nitric oxide synthase function (105).

Complementary lipidomic profiling revealed that cells with elevated GCH1 levels contain two PUFA chains that seem to shield phosphatidylcholine phospholipids from peroxidative damage. These PUFA chains may contribute to the cell's defenses against oxidative stress by stabilizing the cell membrane and preventing the propagation of ROS. However, the precise biochemical pathways and molecular interactions that underlie this protective mechanism are not yet fully understood (106).

In the retina, the activity of GCH1 is particularly important for protecting photoreceptor cells from light damage (107). Photoreceptor cells are particularly susceptible to oxidative damage due to their continuous exposure to light. the function of GCH1 is potentially important for maintaining retinal health and preventing certain ophthalmic diseases, such as age-related macular degeneration and certain types of retinal degenerative diseases.

Further research is needed to elucidate the detailed mechanisms by which GCH1 and its metabolites modulate lipid peroxidation and ferroptosis. Understanding these processes could provide valuable insights into the development of novel therapeutic strategies for diseases in which ferroptosis plays a significant role, such as neurodegenerative disorders, ischemic injury, and certain types of cancer.

3 Ferroptosis and other forms of cell death

Ferroptosis is a unique form of regulated cell death that relies on iron and sets itself apart from other forms such as apoptosis, programmed necrosis, and pyroptosis (108–110).

From a morphological perspective, ferroptosis is characterized by specific features, including the atrophy of mitochondria, a significant reduction and shriveling of mitochondrial cristae, an increase in membrane density, and instances of membrane rupture (111, 112). Notably, the nucleus often appears normal, and an increase in cell membrane density occurs. In contrast, apoptosis is characterized by a decrease in cell volume, chromatin condensation, nuclear fragmentation, the preservation of a complete membrane structure, and the formation of apoptotic bodies (113). Programmed necrosis is identified by an increase in cell size, simultaneous rupture of the plasma membrane, the generation of substantial cell debris, and, frequently, the formation of necrosome structures (114, 115). Pyroptosis involves the swelling of cells, the formation of pores on the plasma membrane, and the release of inflammatory mediators (116).

Biochemically and immunologically, ferroptosis is characterized by a significant reduction in the granular membrane potential, elevated levels of intracellular iron ions, a substantial increase in ROS levels, and intensified lipid peroxidation (117). It is also characterized by the release of damage-associated molecular patterns (DAMPs) and the promotion of an inflammatory response (118, 119). In apoptotic cells, DNA is cleaved into 180–200 base pair fragments, the mitochondrial membrane potential decreases significantly, phosphatidylserine flips from the inner to the outer membrane, and no endolysosomal release or inflammatory response occurs (119).

Programmed necrosis is characterized by the release of endolysates and DAMPs, which contribute to inflammation (120). In pyroptotic cells, inflammatory bodies such as the NLRP3 inflammasome can be observed (121). During pyroptosis, inflammatory caspases activate and cleave GSDMD, releasing its N-terminal fragment (GSDMD-N), which forms pores in bacterial membranes leading to their death without damaging the host cell (119).

These distinct characteristics of each form of cell death underscore the complexity of cellular demise and the importance of accurate identification for both diagnostic and therapeutic purposes. Understanding the biochemical and immunological signatures of ferroptosis, in particular, may provide novel avenues for intervention in diseases where this form of cell death plays a significant role.

3.1 Ferroptosis and apoptosis

Apoptosis, a form of PCD, is intricately governed by genetic mechanisms (122). It plays a vital role in maintaining tissue homeostasis, orchestrating immune and defense functions, and managing cellular damage associated with tumorigenesis (123).

A growing body of research indicates a close relationship between ferroptosis and apoptosis (124, 125). Under specific conditions, cells can transition from apoptosis to ferroptosis (126). This interplay can amplify the cellular susceptibility to apoptotic signals (127). Furthermore, the tumor suppressor protein P53, known for its role in halting the cell cycle and inducing apoptosis in tumor cells, can also trigger ferroptosis under certain circumstances (128). Both *in vivo* and *in vitro* investigations have shown that cells treated with metal organic network encapsulated with p53 plasmid (MON-p53) exhibit pronounced signs of ferroptosis and apoptosis. This dual action not only curbs tumor proliferation but also extends the lifespan of mice bearing tumors (129). Consequently, the convergence of ferroptosis and apoptosis pathways represents a promising avenue for therapeutic intervention in cancer treatment.

This emerging paradigm underscores the complexity of cell death mechanisms and highlights the potential for novel strategies

that harness the interplay between ferroptosis and apoptosis (130). Targeting this combined pathway could represent a more comprehensive approach to managing tumor growth and potentially other diseases in which these cell death mechanisms are implicated. Future research is likely to explore the nuances of this interaction and develop targeted therapies that leverage the synergistic effects of ferroptosis and apoptosis.

3.2 Ferroptosis and programmed necrosis

Programmed necrosis is characterized by a cellular morphology resembling necrosis and involves a signaling pathway akin to apoptosis (131). Moreover, ferroptosis has been observed to coexist with programmed necrosis, particularly in the demise of neurons following hemorrhagic stroke (132). Studies have reported an increase in the mRNA levels of both ferroptosis and programmed necrosis markers during haem chloride-induced cell death (133).

In 2017, research conducted by Müller et al. (134) proposed that ferroptosis and programmed necrosis are complementary mechanisms of cell death. Within this framework, ACSL4, a key enzyme in lipid metabolism, was utilized as an indicator of ferroptosis, while mixed lineage kinase-like protein (MLKL), a protein involved in the execution of necrosis, served as a marker for programmed necrosis (135, 136). These findings indicated that a deficiency in ACSL4 resulted in the upregulation of MLKL, which sensitized cells to ferroptosis (137). This result suggests a compensatory relationship between the two pathways, where the suppression of one cell death mechanism can lead to the activation of the other.

Wulf Tonnus et al. (138) reported that NADPH, a critical coenzyme in cellular redox reactions, can diffuse freely between cells, potentially influencing both ferroptosis and programmed necrosis. The hypothesis posits that the consumption of NADPH during programmed necrosis may sensitize neighboring cells to ferroptosis (139). When cells undergo either programmed necrosis or ferroptosis, intracellular NADPH levels are reduced, potentially rendering adjacent cells more vulnerable to alternative cell death pathways (138). This perspective provides a more integrated view of how ferroptosis relates to and intersects with other PCD mechanisms, highlighting the complexity and interconnectivity of cell death processes.

3.3 Ferroptosis and autophagy

Autophagy, a process that relies on the lysosome for the degradation of cellular components, has been implicated in a spectrum of pathological conditions, including infectious diseases, immunological disorders, metabolic syndromes, and malignancies (140–142).

Recent investigations have revealed a novel role for autophagy in cancer cell death, showing that autophagy can initiate the breakdown of ferritin, consequently triggering ferroptosis (143). In fibroblasts and cancer cells, this form of iron-mediated autophagy has been shown to increase intracellular iron levels through the degradation of ferritin, further promoting ferroptosis (144).

Moreover, the deletion or inhibition of genes such as Atg5 and Atg7, which are essential for autophagy, has been reported to curtail ferroptosis induced by erastin (145, 146). This process is achieved by reducing both intracellular iron levels and the extent of lipid peroxidation (147). These discoveries shed light on the intricate relationships and cross-regulatory mechanisms between ferroptosis and autophagy, providing new insights into the complex network of cell death pathways and their interdependence (144).

4 Ferroptosis and ophthalmic disorders

Ferroptosis has been implicated in the progression of numerous diseases. Ferroptosis is particularly prevalent in ophthalmology and may be associated with corneal disease in the anterior segment, uveal disease in the middle segment, and retinal disease in the posterior segment of the eye (Figure 4). By exploring the regulatory mechanisms of ferroptosis, including the metabolism of iron ions, the production of reactive oxygen species, and the balance of antioxidant systems, we can obtain a better understanding of its role in ophthalmic diseases (Table 1).

4.1 Ferroptosis and corneal injury

Corneal injuries are medical conditions characterized by the disruption of the protective functions and structural integrity of the corneal epithelium due to various factors (223). This disruption can result in partial or complete loss of corneal epithelial cells. Clinical manifestations may include widespread punctate defects or erosions on the corneal surface, along with continuous detachment and damage to the epithelium (224). Additionally, varying levels of inflammation affecting the ocular surface may be present (225). In severe instances, such injuries can lead to pathological alterations in the corneal stroma, potentially impacting visual acuity (226).

Ferroptosis research, triggered by corneal injury, is attracting medical research interest. Particularly in cases of corneal trauma, damage to the corneal epithelial layer is notably prevalent. When the cornea sustains an injury, it initiates a series of intricate biochemical reactions, with oxidative stress being a particularly prominent factor (151, 227).

GPX4, an essential player in ferroptosis, is integral to maintaining the oxidative balance, ensuring cell survival, and facilitating the wound healing process in corneal epithelial cells (228). Research conducted by Sakai et al. (148) reported that a reduction in GPX4 levels, achieved through the transfection of GPX4 siRNA into the human corneal epithelial cell line HCEC, could lead to an increase in lipid peroxidation products such as lactate dehydrogenase (LDH) and 4-hydroxynonenal (4-HNE), thus triggering ferroptosis. Furthermore, the application of Fer-1, an inhibitor of ferroptosis, was found to mitigate cellular damage associated with the knockout of the GPX4 gene (229). Additional

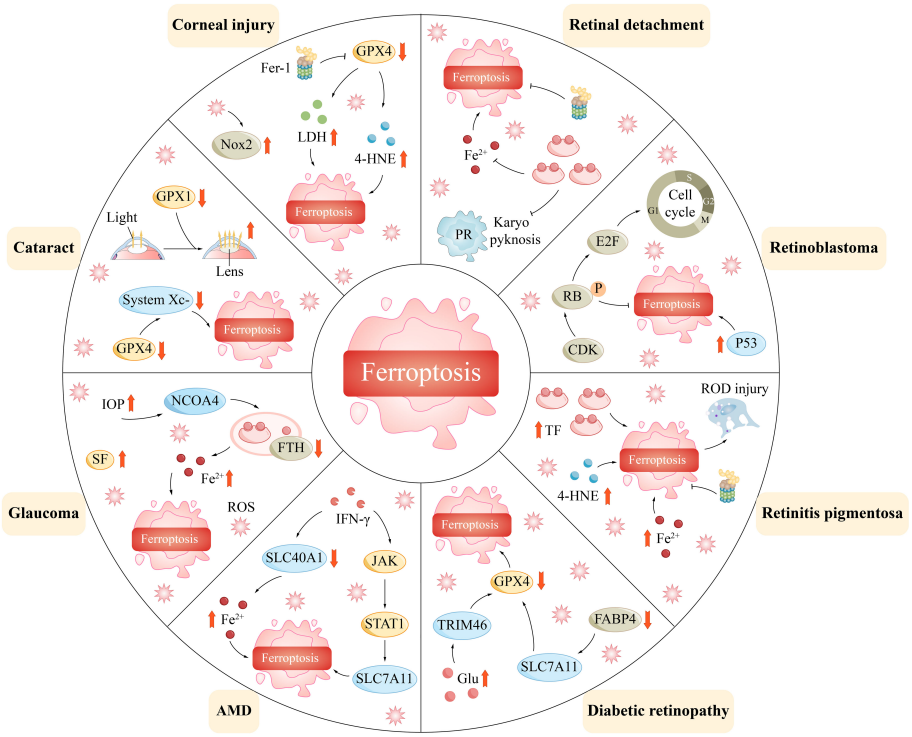

FIGURE 4
Ferroptosis mediates a variety of ophthalmic disorders.

TABLE 1 The mechanism of ferroptosis-related targets in ophthalmic diseases.

Ophthalmic diseases	Ferroptosis-related targets	Study subject	Mechanism	Reference
Corneal injury	GPX4	HCECs, C57BL/6 mice	Downregulation of GPX4 can promote the production of LDH and lipid peroxidation products such as 4-HNE	(148)
	Lipid ROS	Male ICR mice	High levels of ROS can upregulate the expression of NOX2, NOX4, VEGF, and MMPs	(149)
	Lipid ROS	HCECs	Accumulation of ROS leads to the upregulation of ferroptosis promoters and the downregulation of ferroptosis inhibitors	(150)
	GPX4	HCE-S cells	After exposure to Phenylarsine oxide, the expression of GPX4 in HCE-S cells is significantly reduced, indicating that Phenylarsine oxide induces ferroptosis mediated by lipid peroxidation in HCE-S cells	(151)
	GSH;GPX4	Male SD rats	siVEGF-TIL can inhibit oxidative stress, inflammation, and the expression of VEGF in vitro, and effectively maintain corneal transparency in vivo, accelerate epithelialization, and inhibit corneal neovascularization	(152)
	Ferrous ion	HCE-T cells	Exposure to CSE induces lipid peroxidation and the accumulation of ferrous ions within the lysosomal compartments	(153)

(Continued)

TABLE 1 Continued

Ophthalmic diseases	Ferroptosis-related targets	Study subject	Mechanism	Reference
Cataracts	SIRT6	Male SD rats	Melatonin inhibits ferroptosis through the SIRT6/p-Nrf2/GPX4 and SIRT6/COA4/FTH1 pathways	(154)
	Nrf2, ferrous ion	Female C57BL/6J mice	Nrf2 may affect ferritin degradation by decreasing the expression of NCOA4	(155)
	GSK-3β/Nrf2	Female C57BL/6J mice	Targeting the balance of GSK-3β/Nrf2 can alleviate ferroptosis in LECs	(156)
	GPX4	LECs	Inhibition of IncRNA MEG3 by interacting with PTBP1 promotes the decay of GPX4 messenger RNA, accelerating cellular vitality and inhibiting ferroptosis	(157)
	GPX4	Male C57BL/6J mice, SD rats	Astaxanthin-mediated targeting of GPX4 may alleviate damage to human LECs by inhibiting ferroptosis and improving oxidative stress	(158)
	GPX4	ARCC patients	GPX4 downregulation enhanced LEC ferroptosis and decreased viability via RSL3 in SRA01/04 cells	(159)
	Hippo pathway	LECs	Downregulation of KRAs might restrain ferroptosis and affect Hippo pathway in cataract	(160)
	System Xc-, GPX4	LECs	System Xc inhibitor Erastin and GPX4 inhibitor RSL3 can induce ferroptosis in human LECs in vitro and in mouse corneal epithelium in vitro.	(161)
	Ferrous ion	LECs	The iron released from heme by HMOX1 may play a key role in increasing the sensitivity of human LECs to erastin	(162)
	GPX-1	C57BL/6 mice	Lipid peroxides are substrates for GPX-1, and lenses with elevated levels of GPX-1 activity can resist the cytotoxic effects of H2O2	(163)
Glaucoma	MAPK;SLC7A11;GPX4	Male SD rats	SB202190 can inhibit ferroptosis by regulating SLC7A11/GPX4 pathways, protecting retinal ganglion cells	(164)
	GPX4;FSP1;Nrf2	Male New Zealand rabbits	Overexpression of mitochondrial GPX4 rescued artesunate-induced lipid peroxidation and ferroptosis. FSP1 and Nrf2, which are also inhibited by artesunate.	(165)
	GSH, ROS	RGC-5 cells	GGT1 inhibits autophagy in RGC-5 cells by targeting GCLC, further suppressing the occurrence of cellular ferroptosis	(166)
	NCOA4-FTH1	Male C57BL/6 mice	The NCOA4-FTH1-mediated iron metabolism disorder and ferroptosis play a role in glaucomatous RGCs	(167)
	GPX4;System Xc-	Male Wistar rats, Male C57BL/6J mice	Treatment with ONC leads to a significant downregulation of GPX4 and system Xc- in rat retinas, accompanied by increased levels of peroxidized lipids and iron.	(19)
	Serum ferritin	Korean patients	Higher serum ferritin levels are associated with an increased likelihood of glaucoma	(168, 169)

(Continued)

TABLE 1 Continued

Ophthalmic diseases	Ferroptosis-related targets	Study subject	Mechanism	Reference
AMD	Lipid ROS	ARPE-19;hRPE cells	Necrostatin-1 can inhibit the activation of RIPK1/RIPK3 and the accumulation of lipid ROS	(170)
	Ferrous ion	C57BL6 mice;ARPE-19 cells	Knocking down ZIP8 can significantly inhibit the ferroptosis induced by sodium iodate-induced oxidative stress in RPE cells	(171)
	Nrf2	661W cells;C57BL/6 mice	Melatonin inhibits the GSK-3β/Fyn-dependent nuclear translocation of Nrf2	(172)
	GPX4;FTH1	C57BL/6 mice	Overexpression of PEDF can upregulate the expression of GPX4 and FTH1	(173)
	Lipid ROS	ARPE-19;hTERT RPE-1 cells	Increasing the intracellular concentration of POS or DHA can enhance the susceptibility of the RPE to oxidative stress damage	(174)
	Ferrous ion, Hydroxyl radicals	ARPE-19	HIF exacerbates oxidative stress-induced ferroptosis in RPE cells	(175)
	Mitochondrial ROS	ARPE-19, C57BL/6J mice	CircSPECC1, mediated by m6A modification and sponging miR-145-5p, resists oxidative stress injuries and maintains lipid metabolism in RPE	(176)
	KEAP1, NRF2, Ferrous ion	C57BL/6J mice	Overexpression of HO-1 leads to a significant increase in Fe^{2+} levels in photoreceptor cells, promoting the excessive production of ROS	(177)
	Lipid peroxidation	ARPE-19, OMM-1	Ommochrome extract seem to be promising regarding protection against lipid peroxidation in healthy ocular cells	(178)
	Ferrous ion	C57BL/6J mice	Chac1 plays a key role in ferroptosis induced by oxidative stress by regulating GSH depletion.	(179)
	Nrf2	ARPE-19	GBE pre-treatment attenuates pro-oxidant stress and protects human RPE cells from oxidative injury by modulating ERK1/2-Nrf2 axis	(180)
	FTH1, GPX	C57BL/6J mice	Deregulated LCN2-iron axis triggers oxidative damage and lipid peroxidation in RPE cells	(181)
	Lipid ROS	C57BL/6J mice	CaPB effectively prevented the degeneration of RPE, significantly rescuing retinal structure and visual function	(182)
	Nrf2, SLC7A11, Ferrous ion	Male C57BL/6 mice, ARPE-19 cells	Nrf2-SLC7A11-HO-1 leads to RPE cell death and subsequent photoreceptor degeneration through the accumulation of ferrous iron ions and fatal oxidative stress	(183)
	GPX4, FSP1	HRPEpiC, ARPE-19 cells, Male C57BL/6J mice	Ferrostatin-1 significantly ameliorated the compromised GSH-GPX4 and FSP1-CoQ10-NADH signaling	(81)
	L-Ft	Male C57BL/6J mice	IVT FAC induced iron accumulation in Müller glia and myeloid cells, and the formation of lipidperoxidation products	(184)

(Continued)

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TABLE 1 Continued

Ophthalmic diseases	Ferroptosis-related targets	Study subject	Mechanism	Reference
	SLC40A1, System Xc-, Ferrous ion, GPX4	ARPE-19 cells	IFN-γ raises Fe^{2+} by inhibiting the iron exporter SLC40A1 and induces GSH depletion by blocking the System Xc- transporter.	(185)
	GSH	ARPE-19 cells	GSH depletion induces ferroptosis	(186)
	ferrous ion, ACSL4, System Xc-	C57BL/6J mice	Iron overload, GSH depletion, and mitochondrial damage cause the production of ROS, which, together with the activation of ACSL4, promote lipid peroxidation and thus induce ferroptosis.	(187)
	SLC7A11, Ferrous ion, Nrf2, GPX4	ARPE-19 cells, C57BL/6J mice	SLC7A11 overexpression induced resistance to erastin or RSL3-induced ferroptosis	(188)
	Ferrous ion, SLC7A11, GPX4	661W cells;Male SD rats	Light exposure significantly induced changes, including iron accumulation, mitochondrial shrinkage, GSH depletion, increased MDA, and decreased SLC7A11 and GPX4.	(189)
DR	GPX4, FTH1, ACSL4, TFRC	HRMECs	Under high glucose conditions, ferroptosis is associated with increased levels of ROS, lipid peroxidation, and iron content	(190)
	HMOX1	C57BL/6J mice	HMOX1 expression correlated with M2 macrophage infiltration and ferroptosis	(191)
	ROS	SD rats	Inhibition of AQP4 inhibits the ferroptosis and oxidative stress in Muller cells by downregulating TRPV4	(130)
	Ferrous ion, ROS	ARPE-19 cells	USP48 overexpression deubiquitinated SLC1A5 to elevate cell proliferation and suppress ferroptosis and oxidative stress	(192)
	ACSL4, TFR1, SLC7A11, GPX4, Ferrous ion	hRMECs	Overexpression of PIM1 inhibited the inflammatory response, oxidative stress, cell migration, and tube formation potential in hRMECs, whereas elevated tight junction protein levels.	(193)
	GPX4-YAP	DR patients, Male C57BL/6 mice, hRCECs	Pipecolic acid may impede the progression of DR by inhibiting the YAP-GPX4 signaling pathway	(194)
	GPX4, SLC7A11, ACSL4, FTH1, NCOA4	661W cells, Male C57BL/6J mice	The expression of GPX4 and SLC7A11 was downregulated, while the expression of ACSL4, FTH1, and NCOA4 was upregulated in the 661W cells cultured under HG conditions and in the photoreceptor cells in diabetic mice.	(195)
	SLC7A11, GPX4	hRMECs	Ferroptosis-related genes are significantly enriched in processes of ROS metabolism, iron ion reactions	(196)
	GPX4, FTH1, Ferrous ion	ARPE-19 cells, C57BL/6J mice	Sestrin2 inhibits ferroptosis by inhibiting STAT3 phosphorylation and ER stress	(197, 198)
	Nrf2, GPX4	ARPE-19 cells	Maresin-1 inhibits high glucose induced ferroptosis in ARPE-19 cells by activating the Nrf2/HO-1/GPX4 pathway	(199)
	GPX4, FSP-1	Male C57BL/6 mice, ARPE-19 cells	1,8-Cineole ameliorates DR by inhibiting RPE ferroptosis via PPAR-γ/TXNIP pathways	(200)

(Continued)

TABLE 1 Continued

Ophthalmic diseases	Ferroptosis-related targets	Study subject	Mechanism	Reference
	Ferritin	ARPE-19 cells	BECN1, HERC2, ATG7, and BCAT2 might regulate ferritinophagy to influence its development and progression	(201)
	NRF2, GPX4, Ferrous ion	HRECs	Amygdalin treatment inhibited ferroptosis and oxidative stress in HG-stimulated HRECs by activating the NRF2/ARE signaling pathway	(202)
	GPX4	ARPE-19 cells	Ferrostatin-1 alleviates tissue and cell damage in DR by improving the antioxidant capacity of the Xc–GPX4 system	(203)
	FTH1	Tg (hb9: GFP) zebrafish	ACR could directly activate ferroptosis and impairs peripheral neurogenesis	(204)
	GPX4, SLC7A11, Ferrous ion	hRMVECs	25-hydroxyvitamin D3 inhibits oxidative stress and ferroptosis in retinal microvascular endothelial cells induced by high glucose through down-regulation of miR-93	(205)
	GPX4, GSH, Ferrous ion	DR patients	Compared with the normal group, GPX4 and GSH concentrations were significantly lower, and LPO, Fe^{2+}, and ROS concentrations were significantly higher in DR patients	(206)
	ACSL4	Male SD rats	Glia maturation factor-β induces ferroptosis by impairing chaperone-mediated autophagic degradation of ACSL4 in early DR	(207)
	SLC7A11, GPX4	Male C57BL/6 mice, ARPE-19 cells	Downregulation of FABP4 alleviates lipid peroxidation and oxidative stress in DR by regulating peroxisome proliferator-activated receptor y-mediated ferroptosis	(208)
	Nrf2, GPX4	ARPE-19 cells	Astragaloside-IV alleviates high glucose-induced ferroptosis in RPE cells by disrupting the expression of miR-138-5p/ Sirt1/ Nrf2	(209)
	GSH, Ferrous ion	ARPE-19 cells	Downregulation of Circular RNA PSEN1 ameliorates ferroptosis of the high glucose treated RPE cells via miR-200b-3p/cofilin-2 axis	(210)
	GPX4	HRCECs	TRIM46 aggravated high glucose-induced hyper permeability and inflammatory response in human retinal capillary endothelial cells by promoting IκBα ubiquitination	(211, 212)
	SLC1A5	ARPE-19 cells	miR-338-3p targeted the 3' untranslated regions (3'UTR) of SLC1A5 for its inhibition and degradation, and high glucose downregulated SLC1A5 by upregulating miR-338-3p in RPE cells.	(213)
RP	SLC7A11, GPX4, P53	661 W cells	Fructus Lycii and Salvia miltiorrhiza Bunge extract attenuate oxidative stress-induced photoreceptor ferroptosis	(50)
	ROS	ARPE-19 cells	TXNIP-Trx-TrxR redox pathway may participate in RPE dysfunction	(214)

(Continued)

TABLE 1 Continued

Ophthalmic diseases	Ferroptosis-related targets	Study subject	Mechanism	Reference
	NRF2, GPX4, SLC7A11	rd10 mice, C57BL/6J mice	Qi-Shen-Tang alleviates retinitis pigmentosa by inhibiting ferroptotic features via the NRF2/GPX4 signaling pathway	(33)
	Ferritin, Ferrous ion	rd10 mice	RP are associated with altered iron homeostasis regardless of the primary insult	(215)
RB	GPX4, SLC7A11	A375, SK-Mel-28, and FO-1 cells	Hyperforin Enhances HO-1 Expression Triggering Lipid Peroxidation in BRAF-Mutated Melanoma Cells and Hampers the Expression of Pro-Metastatic Markers	(216)
	GPX4, FTH1, ACSL4	Y79 cells, Y79/DDP cells	USP14 might suppress the DDP resistance in RB by mediating ferroptosis	(217)
	Hippo pathway	Y79 cells, SO-RB50 cells, RB3823 cells	Expression of YAP suppresses cell proliferation and elevates the sensitivity of chemotherapy in RB cells through lipid-peroxidation induced ferroptosis	(218)
	GPX4, FTH1, SLC7A11, P62	ARPE-19, Y79 cells, Male BALB/c nude mice	Nuclear translocation of CTSB induces lysosomal stress, which eventually leads to ferroptosis	(219)
	FTH1, NCOA4	Y79 cells, WERI-Rb-1 cells	The anticancer potential of an itaconate derivative in RB cell relies on ferritinophagy-mediated ferroptosis	(220)
	P53	TKO cells	Deletion of RB genes enhanced ferroptosis	(221)
RD	Transferrin, Ferrous ion	RD patients, Male Long-Evans rats	TF decreases both apoptosis and necroptosis induced by RD	(222)

ACSL4, Acyl-CoA synthetase long-chain family member 4; AMD, Age-related macular degeneration; ARCCs, Age-related cortical cataracts; Chac1, GSH-specific γ-glutamylcyclotransferase 1; CSE, Cigarette smoke extract; DHA, Docosahexaenoic acid; DR, Diabetic retinopathy; FABP4, Fatty acid binding protein 4; FTH1, Ferritin heavy chain-1; GBE, Ginkgo biloba extracts; GCLC, Glutamate cysteine ligase catalytic subunit; GGT1, Gamma-glutamyl transpeptidase 1; GPX4, Glutathione peroxidase 4; HCECs, Human corneal epithelial cells; HCE-S, Human corneal epithelial cells; HIF, Hypoxia-inducible factor; HMOX1, Heme oxygenase 1; HRCECs, Human retinal capillary endothelial cells; IFN-γ, Interferon-gamma; KEAP1, Kelch-like ECH-associated protein 1; LECs, Lens epithelial cells; L-Ft, Ferritin light chain; MDA, Malondialdehyde; MMPs, Matrix metalloproteinases; Nrf2, Nuclear factor erythroid 2-related factor 2; ONC, Optic nerve crush; PEDF, Pigment epithelium-derived factor; POS, Photoreceptor outer segments; RB, Retinoblastoma; RD, Retinal detachment; RGC-5, Retinal ganglion cells; RGCs, Retinal ganglion cells; ROS, Reactive oxygen species; RP, Retinitis pigmentosa; RPE, Retinal pigment epithelium; SD, Sprague–Dawley; VEGF, Vascular endothelial growth factor.

studies have indicated that mice with partial knockout of GPX4 exhibit slower corneal wound healing after epithelial injury than their wild-type counterparts, underscoring the significance of GPX4 in the repair process following corneal injury.

Corneal alkali burns represent an exceedingly severe form of corneal chemical injury that inflicts substantial damage on the ocular surface, with a particular emphasis on the cornea. Addressing this type of injury is complex, and the prognosis is often unfavorable, frequently resulting in a significant visual impairment or even blindness. During corneal alkali burn injury, ROS production is substantially increased, which can increase the expression of genes such as Nox2, Nox4, VEGF, and MMP. This upregulation contributes to further corneal damage and encourages the development of new blood vessels (149). Wang et al. (150) reported that ferroptosis, induced by lipid peroxidation, plays a pivotal role in the injury caused by alkali burns, along with ROS. The pathological changes linked to ferroptosis can be partially reversed by treatment with Fer-1. However, due to the hydrophobic nature of Fer-1, its direct application in clinical settings is not feasible. Consequently, Wang et al. (150) have engineered a liposomal delivery system for Fer-1 (Fer-1-NPs) to enhance its bioavailability and augment its therapeutic effects on corneal cloudiness and neovascularization.

In addition to the presence of ferroptosis during the process of corneal alkali burn, excessive ROS and reactive nitrogen species (RNS) may also be produced in corneal inflammation or corneal injury caused by trauma (230). These substances can promote the release of iron ions and lipid peroxidation, thereby triggering ferroptosis (231). Furthermore, corneal surgery or infection may also induce similar oxidative stress reactions, which can subsequently impact the metabolism of iron ions and the cellular redox state.

The collective findings from these studies highlight the critical role of ferroptosis in corneal injury and suggest that its inhibition could be beneficial for corneal preservation (152, 232). The development of pharmaceuticals based on the understanding of ferroptosis offers promising avenues for treating corneal injuries (233). Moreover, these investigations provide a substantial theoretical framework and empirical data that can inform the creation of novel therapeutic approaches for treating corneal

injuries. Overall, an in-depth understanding of the ferroptosis mechanism is anticipated to pave the way for more efficacious treatment strategies for corneal injuries, providing renewed hope to those suffering from keratopathy.

In summary, the study of ferroptosis in the context of corneal injury represents a multifaceted and significant area of research. A thorough investigation of the roles played by key elements, such as GPX4, ROS, and lipid peroxidation, in the ferroptosis process, coupled with the development of potent ferroptosis inhibitors, can yield innovative strategies for treating corneal injuries. These research outcomes are also likely to serve as valuable references for addressing other related medical conditions. As scientific and technological advancements continue to progress, further breakthroughs and advancements in the field of corneal injury treatment will occur.

4.2 Ferroptosis and cataract

Cataract, a prevalent eye disorder, is identified by clouding of the ocular lens, which impairs visual acuity (234). This ailment can impact either a single eye or both simultaneously. Affected individuals often report a range of symptoms, including but not limited to, hazy vision, heightened nearsightedness, the occurrence of double vision, the appearance of light halos, photophobia, and diminished visual capacity in low-light conditions (235). Such symptoms can significantly impact daily activities, such as driving and reading, and can also hinder the ability to discern faces. Patients with critically impaired vision have an elevated risk of falls and an increased propensity for depressive symptoms (236). Cataract accounts for half of the global blindness cases and is responsible for 33% of visual impairments worldwide (237).

Recent scientific investigations have revealed that the ageing process can disrupt the redox equilibrium of the lens, leading to a decrease in the activity of enzymes linked to the glutathione synthesis pathway (238). This change results in a reduction in GPX activity, an increase in the accumulation of ROS, and an increase in lipid peroxidation byproducts (239). These discoveries point towards the potential involvement of ferroptosis in the genesis of cataracts.

In a study by Reddy and colleagues (163), a noted increase in the scattering of light within the nuclei of lenses from mice that were genetically deficient in GPX1 was documented. Over time, these mice exhibited signs of age-related cataracts, a phenomenon not observed in the wild-type control group. Wei et al. (161) reported that lens epithelial cells are susceptible to ferroptosis when exposed to inducers of this process. The level of GSH was identified as a pivotal determinant of cell vulnerability. Additionally, Wei et al. (161) observed that the sensitivity of lens epithelial cells to ferroptosis inducers increases with the extent of cellular aging.

Hydroxyl radicals are highly reactive and can attack PUFAs in the cell membrane, initiating lipid peroxidation (240). This process leads to damage and dysfunction of the cell membrane structure, impacting the normal physiological activities of lens cells. Apart from lipids, hydroxyl radicals can also target proteins and nucleic acids, resulting in protein cross-linking and DNA damage (241). These damages can impair the cell's repair mechanisms and the stability of genetic information, ultimately influencing cell survival. Damage to lens cells can cause the accumulation and precipitation of proteins within the cells, diminishing the transparency of the lens and contributing to the development of cataracts.

Further analysis of the transcriptome revealed that in older cells, GPX4 expression was downregulated, System Xc- function was suppressed, and genes involved in iron ion uptake were upregulated, while genes responsible for iron ion export were downregulated. These findings indicate that ferroptosis could be instrumental in the progression of cataracts (154). Elucidating the relationship between ferroptosis and cataract development may pave the way for innovative preventative strategies and therapeutic approaches for cataract treatment.

4.3 Ferroptosis and glaucoma

Glaucoma encompasses a collection of eye disorders linked to abnormal levels of intraocular pressure (242). This condition has the potential to inflict damage on the optic nerve, leading to a reduction in the visual field and a loss of vision (243). The clinical manifestations of glaucoma can include a range of symptoms such as a decrease in visual acuity, discomfort or pain in the eyes, moderate enlargement of the pupils, redness and swelling of the ocular area, and nausea (244). Typically, glaucoma first affects peripheral vision; without timely intervention, it may progress to the central visual field, potentially resulting in irreversible blindness (245).

Recent scientific findings suggest that iron ions could be instrumental in injury to retinal ganglion cells (RGCs). Lin et al. (168) documented a positive correlation between increased levels of serum ferritin and the incidence of glaucoma. This correlation implies that disruptions in iron metabolism might be intertwined with the pathogenesis of the disease. Building on this information, Yao et al. (167) showed that pathological increases in intraocular pressure (IOP) can disrupt iron homeostasis, leading to an excessive accumulation of iron ions (Fe^{2+}) in the retina, particularly affecting the RGC layer. This overabundance of iron ions compromises the retinal redox balance, triggering ferroptosis in RGCs. The administration of deferiprone, an iron ion chelator, has been found to significantly reduce iron ion levels in the retina, effectively inhibiting RGC death.

Moreover, comprehensive studies have indicated that pathologically elevated IOP might also expedite the degradation of ferritin by enhancing NCOA4-mediated ferritin autophagy (246). This process leads to the release of a substantial amount of previously bound iron ions into the cell. Once ferritin is degraded, the iron ions it had sequestered are released, intensifying the oxidative stress response and causing harm to RGCs. Knockdown of the NCOA4 gene can effectively prevent ferritin degradation and curtail the release of iron ions, thereby significantly diminishing iron ion levels and mitigating RGC damage (247).

The aforementioned research suggests that ferroptosis can contribute to the demise of RGCs through diverse pathways, thereby influencing the advancement of glaucoma. Recent studies have revealed that the reduction of GPX4 expression triggers

ferroptosis in nerve cells, indicating that ferroptosis may further aggravate the progression of glaucoma by targeting optic nerve damage (248).

These insights not only illuminate the potential role of iron ions in the development of glaucoma but also present a novel perspective for therapeutic approaches (249). Regulating iron homeostasis, especially by reducing iron ion accumulation due to pathological IOP, could represent a groundbreaking strategy for glaucoma management (250). Future research may explore the regulatory mechanisms of iron metabolism and investigate how these pathways can be targeted by pharmaceuticals or other treatments. These investigations could lead to the development of more effective treatment plans for glaucoma patients. Collectively, these studies may pave the way for innovative preventative measures and therapeutic interventions for glaucoma with the potential to slow or even prevent the progression of this debilitating eye disease.

4.4 Ferroptosis and age-related macular degeneration

Age-related macular degeneration (AMD) is a complex ocular disorder that occurs with ageing and is characterized by a deterioration in the macula structure and function (251). The macula, which is situated at the retina's core, is pivotal for sharp central vision (252).

Recent in-depth examinations of retinal pigment epithelial (RPE) cells revealed that interferon-γ (IFN-γ) can trigger ferroptosis in these cells, contributing to the progression of AMD (185). The process is intricate and includes several stages: it starts with the suppression of the iron transporter SLC40A1, which results in an increase in intracellular iron (Fe^{2+}) levels; this step is followed by the obstruction of System Xc−, which leads to a depletion of GSH within the cell. This process also involves the upregulation of acyl-CoA synthetase long-chain family member A (ACSLA) and 5-lipoxygenase (5-LOX), which promote lipid peroxidation (253). Finally, the downregulation of SLC7A11, GPX4, and GSH occurs through the activation of the JAK1–2/STAT1/SLC7A11 signaling pathways, stimulating ferroptosis (185).

In addition to IFN-γ, the pigment N-retinol-N-retinoethanolamine (A2E), a distinctive fluorophore within RPE cells, has also been implicated in ferroptosis (254). Through comprehensive transcriptome profiling, Scimone and colleagues noted the upregulation of A2E expression and the concomitant downregulation of SLC7A11 in H-RPE cells upon blue light exposure. This change curtailed the biosynthesis of GSH, further propagating ferroptosis in RPE cells.

Ferroptosis impacts not only RPE cells but also traditional AMD models (255). Liu and associates (256) analyzed sodium iodate (SI)-induced AMD cellular models and discovered that SI, while not influencing GPX4 activity, could deplete intracellular GSH, liberate Fe^{2+} from liposomes, increase intracellular ROS production, and increase lipid aggregation, consequently precipitating ferroptosis in ARPE-19 cells. Lee et al. (257) also detected that SI could provoke the generation of mitochondrial ROS, promoting ferroptosis in RPE cells.

Tang et al. (183) reported that ferroptosis induced by SI in RPE cells is linked to the Nrf2/SLC7A11/HO-1 pathway, with heightened HO-1 expression precipitating the upregulation of the TFR and the downregulation of SLC40A1, culminating in iron ion aggregation. The liposomal accumulation of Fe^{2+} can then increase HO-1 expression, perpetuating ferroptosis. The suppression of HO-1 or the application of the HO-1 inhibitor ZnPP has been shown to ameliorate the detrimental effects of ferroptosis on the morphology and functionality of RPE cells and photoreceptors (258).

Ferroptosis in photoreceptors also contributes significantly to the aetiology of AMD, particularly the dry form (50). Within the visual cycle, the regeneration of 11-cis-retinoic acid retinol (atRAL) between photoreceptors and the RPE is crucial for sustaining visual acuity (259). ATP-binding cassette transporter A4 (ABCA4) facilitates the transport of atRAL from the outer segment discs of photoreceptors to the cytoplasm, while all-trans retinol dehydrogenase 8 (RDH8) catalyses the conversion of atRAL back to all-trans retinol (260). In mouse models deficient in the Abca4 and Rdh8 genes, a deficiency in the clearance of photoreceptor outer segment discs, thinning of the retinal photoreceptor layer postlight exposure, the accumulation of atRAL in the retina, diminished GSH levels, aberrant expression of the lipid metabolism-related proteins COX2 and ACSLA, and heightened lipid peroxidation—indicators of ferroptosis—are observed (187). Subsequent studies have validated that atRAL can incite ferroptosis in the cone cell line 661W by impeding System Xc−, increasing Fe^{2+} levels, and amplifying mitochondrial ROS production.

Tang et al. (189) confirmed these findings, showing that light exposure can precipitate the degeneration of retinal photoreceptor cells through ferroptosis in 661W cells and male Sprague–Dawley rats. Illumination significantly diminishes the viability of photoreceptor cells and induces pro-ferroptosis alterations, such as iron buildup, mitochondrial condensation, GSH depletion, increased malondialdehyde (MDA) levels, and reduced expression of the SLC7A11 and GPX4 proteins.

The decrease in glutathione and GPX4 may result in the retinal cells' ineffectiveness at efficiently eliminating lipid peroxides, thereby impairing the visual function of these cells and worsening AMD (81). The abnormal build-up of ROS and other oxidative stress molecules inflicts damage on the retinal cells, influencing the transmission and processing of visual signals (261). Moreover, as AMD is a neurodegenerative disease, ferroptosis could potentially hasten the progression of AMD by advocating the demise of nerve cells.

Ferristatin-1 has been shown to attenuate the impact of light on ferroptosis, ameliorate photoreceptor atrophy and ferroptosis, curb neuroinflammation, and protect the retinal structure and function from the effects of light (51, 189). Collectively, these insights provide innovative avenues for leveraging the mechanisms of ferroptosis in the prevention and treatment of retinal degeneration diseases, including AMD.

4.5 Ferroptosis and diabetic retinopathy

Diabetes mellitus is widely acknowledged as the primary cause of diabetic retinopathy (DR) (262). Persistently high blood sugar

levels inflict damage on retinal vascular endothelial cells, resulting in a spectrum of retinal lesions, such as microaneurysms, hard exudates, cotton-wool spots, neovascularization, vitreous proliferation, macular oedema, and, in extreme cases, retinal detachment (263).

Recent research has revealed the crucial role of ferroptosis in the progression of DR, where it contributes to the disease by impairing the integrity of capillary endothelial cells. Uric acid, a significant biomarker for type 2 diabetes, influences serum uric acid levels through the TRIM46 gene, which exhibits increased expression in diabetic individuals (264). Under high-glucose conditions, the expression of TRIM46 in human retinal capillary endothelial cells (HRCECs) increases and correlates with the extent of cell death (212). Studies have indicated that TRIM46 overexpression facilitates the ubiquitination and subsequent degradation of GPX4 in HRCECs, thereby promoting ferroptosis.

Ferroptosis affects not only the microvasculature but also RPE cells. Research by Liu and colleagues has shown that in diabetic rat models, hyperglycemia induces the secretion of GMFB by Müller cells, and elevated levels of GMFB can impair retinal function by altering the quantity and connectivity of RPE cells (207). GMFB is capable of hindering lysosomal assembly and increasing alkalinity, which diminishes lysosomal functionality. This reduction in function impedes the autophagic degradation of ACSLA, leading to its accumulation and exacerbating lipid peroxidation in ARPE19 cells. This process results in a decrease in mitochondrial activity and the eventual induction of ferroptosis.

In addition to GMFB, fatty acid binding protein 4 (FABP4) has been shown to alleviate lipid peroxidation and oxidative stress associated with DR by modulating PPARγ-mediated ferroptosis (208). FABP4 is instrumental in sustaining the glucose and lipid balance and serves as an independent prognostic marker for patients with type 2 diabetic retinopathy.

PPARγ, a crucial modulator of fatty acid storage and glucose metabolism, regulates the expression of the FABP4 gene Conversely, FABP4 can initiate the ubiquitination and subsequent proteasomal degradation of PPARγ. In a streptomycin-induced diabetic mouse model, a marked increase in the expression of both the FABP4 and PPARγ proteins was observed in retinal tissue. Suppressing FABP4 has been shown to reverse the downregulation of the SLC7A11 and GPX4 proteins, thereby mitigating ferroptosis in RPE cells (208).

Furthermore, Tang and colleagues confirmed that RPE cells subjected to high-glucose conditions undergo ferroptosis (209). Persistent hyperglycemia intensifies oxidative stress, precipitating structural and functional alterations in retinal neurons (265). This degeneration characterizes the early neural damage in DR, potentially resulting in vision loss and other visual impairments. Oxidative stress induced by iron overload may also contribute to the manifestation of macrovascular complications of diabetes, including atherosclerosis and cardiovascular diseases (266). Within the context of DR, these changes could cause damage to and dysfunction of the retinal microvasculature. Therefore, inhibiting ferroptosis to curtail oxidative damage in the retina is a novel therapeutic approach for the management of diabetic retinopathy.

In summary, the pathogenesis of diabetic retinopathy involves a complex interplay of factors, including ferroptosis, uric acid metabolism, GMFB, and FABP4. These findings provide new insights into the intricate mechanisms underlying DR and present potential targets for future therapeutic interventions.

4.6 Ferroptosis and retinitis pigmentosa

Retinitis pigmentosa (RP), an inherited disorder, results in progressive degeneration of the retina, leading to night blindness, retinal atrophy, pigment deposition, and constriction of the visual field, potentially culminating in total blindness (267). The advancement of RP significantly diminishes the quality of life for those affected, underscoring the importance of investigating its underlying pathological processes to identify effective therapeutic approaches (268).

In the study of RP, researchers have focused on the expression levels of genes involved in iron metabolism, as well as the levels of iron and oxidative damage within the retina. In the rd10 mouse model, which exhibits rapid progression of retinal degeneration, a notable increase in the expression of several key proteins related to iron transport and storage, such as transferrin, ceruloplasmin, ferritin, and the transferrin receptor, was observed (215). Concurrently, an increase in the total iron content and the amount of iron bound to ferritin within the retina was observed. Moreover, the level of 4-hydroxy-2-nonenal (4-HNE), an indicator of lipid peroxidation, was significantly elevated, suggesting a strong correlation between excess iron and retinal damage (215).

Further research has established a connection between ferroptosis—the form of regulated cell death associated with iron metabolism and oxidative stress—and cell death induced by sodium iodate in ARPE-19 cells, highlighting the relevance of ferroptosis to the progression of RP (269). This discovery provides a novel perspective on the pathogenesis and potential treatment of this disease.

Mitochondrial dysfunction can result in the leakage of iron ions from the mitochondria into the cytoplasm. These iron ions react with ROS to generate hydroxyl radicals via the Fenton reaction (270). The accumulation of iron ions coupled with the production of hydroxyl radicals escalates the level of oxidative stress (271). Notably, oxidative stress is a pivotal factor in the onset of RP, which can inflict damage on photoreceptor cells, leading to cellular dysfunction and death (272). Moreover, the oxidative stress and cellular damage brought about during the process of ferroptosis can trigger inflammatory responses. The subsequent release of inflammatory mediators further impairs retinal cells, exacerbating the pathologic progression of RP (273).

Subsequent studies using RP models have revealed the efficacy of various iron homeostasis regulators in mitigating ferroptosis in photoreceptor cells and in protecting against photoreceptor degeneration. Notably, agents, such as zinc deferoxamine (274), the iron-chelating drugs (275) VK28 and VAR10303, the iron chelator deferiprone (276), and the ferroptosis inhibitors deferoxamine and hepstatin-1 (183), have all been proven to be effective in slowing ferroptosis. These findings offer a fresh scientific

rationale for targeting iron homeostasis in therapeutic strategies for RP and propose innovative avenues for its management.

In conclusion, maintaining iron homeostasis is crucial in the pathogenesis of RP, with alterations in iron metabolism-related genes and iron levels being intricately linked to retinal damage. The utilization of iron chelators and inhibitors of ferroptosis represents innovative therapeutic avenues for RP and is anticipated to emerge as a promising treatment strategy in the future. These insights not only enhance our understanding of the pathological mechanisms of RP but also pave the way for the development of novel therapeutics, providing hope for more efficacious treatment solutions for individuals with RP.

4.7 Ferroptosis and retinoblastoma

Retinoblastoma (Rb), a malignant neoplasm originating from the precursor cells of the retinal photoreceptors, predominantly affects children under the age of three (277). This tumor presents with a variety of clinical signs and symptoms, which may affect one or both eyes and include conjunctival hyperemia, ocular oedema, corneal swelling, vitreous body turbidity, increased intraocular pressure, and ocular misalignment (278).

Delving into the pathogenesis of Rb, genetic alterations, particularly mutations in the p53 gene and the loss of the RB1 gene, have been pinpointed as pivotal for disease development. The p53 gene, known for its tumor-suppressive properties, modulates the phosphorylation of the RB protein by influencing cyclin-dependent kinases (CDKs), thereby regulating the E2F family of transcription factors, which are integral to the progression of the cell cycle. Disruptions in this regulatory system due to p53 mutations or RB1 gene deletions can lead to the abnormal activation of genes that drive the cell cycle and heightened vulnerability to ferroptosis (221).

Ferroptosis, an emerging form of regulated cell death, is closely linked to disruptions in iron metabolism and increased oxidative stress (279, 280). A significant challenge in Rb treatment is the potential for tumor cells to develop resistance to chemotherapy drugs. However, recent research has indicated that inducing autophagy-dependent ferroptosis can effectively counteract drug resistance in retinoblastoma cells. Itaconic acid derivatives have been shown to enhance ferroptosis by stimulating autophagy, thereby diminishing the activity of caspases, key mediators of apoptosis, and reversing the efficacy of chemotherapeutic agents such as carboplatin, etoposide, and vincristine (220).

By inducing selective ferritinophagy, a process that specifically degrades ferritin within the cell, the reserves of intracellular iron ions can be diminished, thereby heightening the cell's sensitivity to ferroptosis. 4-Octyl itaconate (4-OI) is a metabolite capable of inducing ferritinophagy-dependent ferroptosis (281). Future studies could explore the possibility of 4-OI inducing ferritinophagy-dependent ferroptosis in Rb.

Furthermore, the therapeutic potential of ferroptosis has been noted in the treatment of other malignancies. In the context of advanced hepatocellular carcinoma (HCC), the RB protein has been shown to facilitate ferroptosis when exposed to sorafenib, thereby hastening the oxidative necrosis of cancer cells (282).

By synthesizing these findings, modulating ferroptosis is evidently a novel therapeutic strategy in oncology. By meticulously controlling the onset of ferroptosis, tumor cell resistance to chemotherapeutic agents can be significantly diminished and the death of cancer cells can be expedited. This approach is particularly pertinent to Rb, given that it is commonly diagnosed in early childhood, necessitating treatments with heightened sensitivity and responsiveness.

Consequently, the modulation of ferroptosis introduces a novel therapeutic perspective for Rb and may also offer novel insights into the treatment of other types of cancer. A deeper understanding of the molecular underpinnings of ferroptosis, coupled with the development of targeted modulators, holds promise for the delivery of more efficacious and targeted treatment regimens for patients afflicted with Rb. This area represents an auspicious frontier with the potential to bring about transformative advances in cancer therapy.

4.8 Ferroptosis and retinal detachment

Retinal detachment (RD) is a significant ophthalmological condition characterized by the separation of the retina from the underlying RPE and choroid (283). The formation of retinal tears or holes is a prevalent cause of this detachment, which, if neglected, can result in permanent vision impairment (284).

Contemporary medical research has detected an increase in iron concentrations and total iron-binding capacity within the vitreous and subretinal fluid as the duration of retinal detachment in patients increases (285). An examination of retinal tissue sections from individuals with RD revealed substantial iron accumulation within the RPE. This accumulation implies a potential correlation between aberrant iron ion levels and the pathogenesis of RD.

According to more in-depth experimental investigations, the administration of transferrin can efficiently mitigate the accumulation of iron ions in the RPE of rodent models of RD (285). This intervention also prevents the nuclear condensation of photoreceptor (PR) cells, thereby providing substantial protection against PR cell damage and reducing the incidence of ferroptosis (222). These findings indicate that iron ions may be partially responsible for PR cell damage during the onset of RD.

The discovery of the role of iron in RD opens the possibility of employing iron chelators as a novel therapeutic approach to diminish the damage associated with RD. The strategic use of iron chelators post-RD could reduce PR cell damage and enhance postoperative visual rehabilitation in patients. This approach introduces a novel avenue of research for the management of the RD prognosis.

The RPE cell layer provides support and nourishment for photoreceptor cells, and the integrity of its tight junctions is crucial for the structure and function of the retina (286). Ferroptosis could disrupt these tight junctions, leading to fluid permeation between the retinal layers, which could compromise the stability of the retina (287). RPE cells are responsible for phagocytosing and digesting the membrane discs of the outer segments of photoreceptor cells, a critical component of the visual

cycle. Ferroptosis could possibly affect the phagocytic capability of RPE cells, disrupting the normal renewal of photoreceptor cells. This could potentially hinder the reattachment process and impede visual recovery (170).

In conclusion, iron ions are pivotal contributors to the pathophysiology of RD. The use of iron chelators is a promising therapeutic strategy for RD management. Subsequent studies may further elucidate the interplay between iron metabolism and the development of RD, as well as the prospective therapeutic utility of iron chelators in treatment protocols. This line of research could not only enhance the therapeutic outcomes for RD patients but also offer innovative treatment paradigms for a spectrum of ophthalmic conditions.

5 Therapeutic approaches targeting ferroptosis in ophthalmic disorders

Recently, significant progress has been achieved in the study of ferroptosis, revealing a diverse array of drugs associated with this process. These drugs include compounds derived from natural sources to synthetic small-molecule inhibitors and inducers. These discoveries provide fresh insights for mechanistic studies and potential therapeutic strategies for ferroptosis treatment.

In corneal injury, reducing the levels of GPX4, an antioxidant enzyme in corneal epithelial cells, leads to increased lipid peroxidation and ferroptosis. Fer-1, which inhibits ferroptosis, has been shown to decrease cell damage due to reduced GPX4 levels, suggesting its utility in corneal recovery. Fer-1 has been shown to partially ameliorate the associated pathology, especially in severe corneal injuries such as alkali burns, where ferroptosis significantly impacts tissue damage, as reported by Wang et al. (150). Given the hydrophobic properties of Fer-1, which can hinder its clinical use, researchers have developed a liposomal formulation (Fer-1-NPs) to improve its therapeutic efficacy. This development implies that drugs targeting ferroptosis could offer novel treatment options for corneal injuries, potentially enhancing treatment outcomes for keratopathy and similar conditions.

Cataract treatment can benefit significantly from understanding the association with ferroptosis, which affects the lens's redox balance and the activity of enzymes in the glutathione pathway (239). Consequently, these changes lead to decreased GPX activity along with the accumulation of ROS and lipid peroxidation byproducts. In a study, mice deficient in GPX1 exhibited signs of age-related cataracts attributed to heightened light scattering within the lens nucleus (163). Additionally, lens epithelial cells are susceptible to ferroptosis, and the level of GSH plays a crucial role in determining their vulnerability (161). Gaining insights into this connection could pave the way for innovative preventative strategies and therapeutic interventions for cataract management.

In glaucoma, the physiological function of RGCs is influenced by iron ions and ferritin. Elevated serum ferritin levels are associated with the risk of glaucoma, and increased IOP can lead to the excessive accumulation of iron in the retina, triggering ferroptosis in RGCs (167, 168). Deferiprone, an iron chelator, has been reported to reduce iron levels and protect RGCs. Moreover, high IOP may induce ferritin degradation, resulting in the release of iron ions and exacerbating RGC injury. However, the inhibition of ferritin degradation by knocking down the NCOA4 gene attenuates RGC damage caused by high IOP (167). These findings indicate that targeting ferroptosis could serve as a novel therapeutic strategy for glaucoma and may offer innovative options for slowing disease progression.

Tang and colleagues developed an innovative iron chelator known as CaPB to address the issue of elevated iron levels in RPE cells and Bruch's membrane in patients with AMD (182). This compound is characterized by its high biocompatibility and minimal cytotoxic effects, enabling it to selectively target and reduce intracellular bivalent iron concentrations. Additionally, CaPB mitigates oxidative stress by suppressing the expression of specific genes, thus potently inhibiting ferroptosis. This discovery suggests that the modulation of ferroptosis could emerge as a novel therapeutic approach for the treatment and prevention of AMD, providing a promising avenue for the development of new pharmaceutical interventions.

Ferroptosis plays a significant role in the progression of DR by impairing retinal endothelial cells and is associated with the upregulation of the TRIM46 gene in diabetic patients (212, 264). Inhibiting the effect of this gene on GPX4 degradation could alleviate this condition. Furthermore, interventions targeting FABP4 in PPARγ-mediated ferroptosis may decrease the oxidative stress and lipid peroxidation linked to DR (208, 209). These findings indicate that therapies concentrating on ferroptosis pathways could represent novel treatment options for diabetic retinopathy, underscoring the potential of molecular interventions in combating diabetic complications.

RP studies have highlighted the significance of iron metabolism-related genes and oxidative damage in the retina. Therapies targeting iron homeostasis, including zinc deferoxamine, VK28, VAR10303, deferiprone, and ferroptosis inhibitors such as deferoxamine or hepstatin-1 (183, 274–276), have shown promise in slowing photoreceptor cell degeneration in individuals with RP. These findings underscore the importance of iron management in treating RP and suggest innovative approaches to address this degenerative eye disease, potentially leading to more effective treatments for affected individuals.

Targeting ferroptosis in Rb represents a pioneering therapeutic approach. Central to Rb are genetic mutations in the p53 and RB1 genes, which heighten cell susceptibility to ferroptosis (221). Inducing ferroptosis through autophagy has the potential to overcome drug resistance in Rb cells, and itaconic acid derivatives can enhance chemosensitivity to agents such as carboplatin and etoposide (220). The role of the RB protein in promoting ferroptosis has also been observed in HCC, indicating the broader applicability of this strategy.

Elevated iron levels in the vitreous and subretinal fluid of RD patients suggest a link to RD pathogenesis. In rodent models, transferrin administration reduced iron accumulation in RPE cells, prevented photoreceptor cell damage, and decreased ferroptosis, indicating the role of iron in cell damage during RD (222). The potential use of iron chelators post-RD could protect

photoreceptor cells and enhance visual rehabilitation, suggesting a novel therapeutic strategy.

Targeting ferroptosis for the treatment of ophthalmic diseases has emerged as a significant area of research. However, current targeted therapies face several challenges. For instance, the cornea's structure complicates drug delivery, and traditional eye drops have low bioavailability due to factors such as tear dilution and blinking. Nanomedicine drug delivery systems offer an effective strategy for addressing the challenges of ocular drug delivery. By enhancing corneal penetration, achieving sustained drug release, and improving bioavailability, nanomedicine could potentially enhance the therapeutic effect of treatments for eye diseases, thereby improving patients' quality of life.

The eye's immune system is distinct from that of other body parts and possesses a unique immune privilege. This means that the eye can avoid triggering a robust immune response, which is crucial for protecting the eye from damage. However, it also implies that therapeutic drugs need to be specially designed (combining targeted drugs with immunosuppressants) to prevent unnecessary inflammation or immune reactions.

Currently, there is limited clinical research on targeting ferroptosis for the treatment of ophthalmic diseases, with most studies focusing on animal experiments. Given the differences between disease models and human biology, understanding the absorption, distribution, metabolism, and excretion of drugs in the human eye is vital for effective treatment.

Finally, as scientists deepen their research on the mechanism of ferroptosis, we anticipate a more comprehensive understanding of ferroptosis as research progresses. This will likely result in more therapeutic targets for patients to choose from in clinical settings.

6 Conclusion

Since ferroptosis was identified in 2012, researchers have expressed a burgeoning interest in understanding its association with various ophthalmic conditions. This manuscript delves into the regulatory mechanisms of ferroptosis and their potential impacts on eye diseases. However, numerous aspects of how ferroptosis is implicated in the regulation of ophthalmic diseases remain largely unknown. Further research is essential to explore the mechanisms and roles of ferroptosis in conditions such as corneal injury, glaucoma, cataract, age-related macular degeneration, diabetic retinopathy, retinal detachment, and retinoblastoma.

The role of cell type and sensitivity in ferroptosis is intricate and tissue-specific. In the context of ocular diseases, this implies that ocular cells may be more prone to ferroptosis, which deviates from its manifestation in other tissues such as tumor cells and neurons. For instance, the retina, the light-sensitive tissue of the eye, contains a large number of neurons and glial cells. In conditions like AMD and certain types of retinal degenerative diseases, retinal cells are more susceptible to the effects of ferroptosis. Ferroptosis drives the progression of these diseases by affecting these cells' redox balance.

Due to its high energy consumption and highly oxidative physiological characteristics, the retina forms an environment particularly susceptible to oxidative stress. Photoreceptor cells require a substantial amount of energy to maintain the transmission of visual signals. Mitochondria are extremely active in these cells, generating energy while also producing reactive oxygen species (ROS), which heighten oxidative stress.

Moreover, the eyes are directly exposed to light, especially high-energy visible light and ultraviolet rays. Such exposure can promote the generation of ROS and cause oxidative damage to retinal cells. Given that ferroptosis is a form of cell death closely linked to oxidative stress, its significance is even more pronounced in the eyes.

The focus of future research on ferroptosis in ophthalmic diseases involves uncovering its molecular mechanisms, including the regulation of iron ion metabolism and lipid peroxidation. Understanding these mechanisms is critical for identifying key factors in the development of ophthalmic diseases. This knowledge can aid in discovering new therapeutic targets and biomarkers, thereby enabling early diagnosis and monitoring of disease progression.

As our understanding of the molecular mechanisms of ferroptosis deepens, researchers will endeavor to develop new drugs that can target these pathways. These include small molecule inhibitors and inducers, as well as exploring the application of gene editing technology and stem cell therapy in the treatment of ophthalmic diseases. These strategies may offer new approaches to treatment, particularly when conventional treatments prove ineffective.

Furthermore, the advancement of non-invasive diagnostic technology and interdisciplinary collaboration will be pivotal to advancing research on ferroptosis. Non-invasive techniques can empower doctors to intervene at an early stage of the disease, while interdisciplinary collaboration can harness the expertise of professionals from diverse fields to collectively address complex issues in the treatment of ophthalmic diseases. This collaboration can optimize treatment strategies and ultimately deliver more effective treatment plans to patients.

The current manuscript does have certain limitations. For instance, our exploration of ophthalmic diseases is restricted to eight specific types: corneal injury, cataract, glaucoma, AMD, DR, RP, RB, and RD. This does not encompass all ophthalmic diseases potentially related to ferroptosis, such as Graves' ophthalmopathy (288) and myopia (289). Our literature search was confined to English-language studies, thus excluding research in other languages, including Chinese. The review may inevitably incorporate lower-quality studies, potentially impacting the reliability of the conclusions. There may also be publication bias in the conclusions, as studies with negative or non-significant results may not have been published, possibly skewing the review towards supporting the role of ferroptosis in ophthalmic diseases. Given these limitations, future research should address a broader range of diseases, adopt more rigorous study designs, and conduct more comprehensive investigation into mechanisms.

In summary, ferroptosis is considered a significant form of cell death in ophthalmology. A more comprehensive understanding of ferroptosis may reveal new targets for the diagnosis and treatment

of ophthalmic diseases, offering promising avenues for future therapeutic advancements.

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Exploring the relationship between anastasis and mitochondrial ROS-mediated ferroptosis in metastatic chemoresistant cancers: a call for investigation

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Ferroptosis induces significant changes in mitochondrial morphology, including membrane condensation, volume reduction, cristae alteration, and outer membrane rupture, affecting mitochondrial function and cellular fate. Recent reports have described the intrinsic cellular iron metabolism and its intricate connection to ferroptosis, a significant kind of cell death characterized by iron dependence and oxidative stress regulation. Furthermore, updated molecular insights have elucidated the significance of mitochondria in ferroptosis and its implications in various cancers. In the context of cancer therapy, understanding the dual role of anastasis and ferroptosis in chemoresistance is crucial. Targeting the molecular pathways involved in anastasis may enhance the efficacy of ferroptosis inducers, providing a synergistic approach to overcome chemoresistance. Research into how DNA damage response (DDR) proteins, metabolic changes, and redox states interact during anastasis and ferroptosis can offer new insights into designing combinatorial therapeutic regimens against several cancers associated with stemness. These treatments could potentially inhibit anastasis while simultaneously inducing ferroptosis, thereby reducing the likelihood of cancer cells evading death and developing resistance to chemotherapy. The objective of this study is to explore the intricate interplay between anastasis, ferroptosis, EMT and chemoresistance, and immunotherapeutics to better understand their collective impact on cancer therapy outcomes. We searched public research databases including google scholar, PubMed, relemed, and the national library of medicine related to this topic. In this review, we discussed the interplay between the tricarboxylic acid cycle and glycolysis implicated in modulating ferroptosis, adding complexity to

its regulatory mechanisms. Additionally, the regulatory role of reactive oxygen species (ROS) and the electron transport chain (ETC) in ferroptosis has garnered significant attention. Lipid metabolism, particularly involving GPX4 and System Xc- plays a significant role in both the progression of ferroptosis and cancer. There is a need to investigate the intricate interplay between anastasis, ferroptosis, and chemoresistance to better understand cancer therapy clinical outcomes. Integrating anastasis, and ferroptosis into strategies targeting chemoresistance and exploring its potential synergy with immunotherapy represent promising avenues for advancing chemoresistant cancer treatment. Understanding the intricate interplay among mitochondria, anastasis, ROS, and ferroptosis is vital in oncology, potentially revolutionizing personalized cancer treatment and drug development.

KEYWORDS

anastasis, ferroptosis, chemoresistance, mitochondria, chemoresistance, EMT

1 Introduction

Chemoresistance promotes cancer stemness by facilitating the release of exosomes containing peptides, nucleic acids, and various small molecules in multiple cancer types. This process converts drug-sensitive cells into cancer stem cells (1, 2). These cells represent a distinct subpopulation within tumor tissues, exhibiting self-renewal capabilities and giving rise to diverse tumor cell phenotypes (3). Chemoresistance could induce tumor heterogeneity (4–7), contributing significantly to tumor progression (8, 9). Chemoresistance can be modulated by the ferroptosis (9).

Cell death is a critical event within the physiological milieu and ferroptosis represents a significant kind of iron-dependent regulated cell death (RCD), distinct from established mechanisms such as apoptosis, pyroptosis, necrosis, and autophagy (10, 11). Ferroptosis is characterized by the generation of ROS and iron ion-dependent lipid peroxidation. Within cellular environments, ferroptosis is mediated by compounds like erastin or rat sarcoma virus oncogene homolog-selective lethal 3 (RSL3), which inhibit the expression of glutathione peroxidase 4 (GPX4) (12, 13). GPX4 is a selenoprotein that specifically catalyzes the conversion of lipid peroxide into lipids using glutathione. Consequently, reduced GPX4 levels disrupt the cellular oxidation balance. The Fenton reaction between ferrous ions and endogenous hydrogen peroxide generates hydroxyl radicals, which, in conjunction with polyunsaturated fatty acids, undergo multi-step free radical chain reactions, resulting in the formation of lipid peroxides (14). In a complete cellular system, ROS can stimulate mitochondria to open the permeability transition pore, amplifying the ROS signal through positive feedback. This positive feedback loop can be potentially regulated by mitochondria (15, 16). Consequently, when mitochondrial membrane integrity is compromised, the regulatory mechanism becomes impaired, leading to increased ROS production. After lipid peroxidation, modifications in the cell membrane is observed subsequently cause the generation of cytotoxic byproducts including malondialdehyde and 4-hydroxynonanaldehyde are generated, ultimately leading to the induction of ferroptotic cell death (17, 18).

When the term “anastasis” was introduced in 2012, it was associated solely with the reversal of apoptosis. This study explore novel cellular recovery phenomenon involving the reversal of ferroptosis in addition to anastasis, chemoresistance in cancer research.

Ferroptosis is a kind of programmed cell death reported in diseases including cancers (19, 20). Although the regulatory mechanisms and implications of ferroptosis continue to be elucidated, its potential resembles that of apoptosis in either augmenting cell death during cancer therapy or attenuating cell death to safeguard susceptible cells such as neurons, cardiomyocytes, and hepatocytes at the time of tissue damage (21).

For a significant duration, cell death was predominantly categorized into two main types: programmed cell death (PCD), exemplified by apoptosis mediated by cysteine aspartate-specific proteinase (caspase), and non-programmed cell death modalities such as necrosis (22–24) (Table 1). However, with advancements in molecular research within cell biology, an expanding repertoire of PCD modalities, including autophagy-dependent cell death, pyroptosis, and more recently, ferroptosis and cuproptosis, have been elucidated (47–49). Ferroptosis, a novel variant of programmed non-apoptotic cell death exerts a crucial regulatory influence on cancer progression. Growing evidence indicates ferroptosis induction as a promising avenue for cancer therapy. In recent years, several experimental reports described the role of mitochondria in various regulated cell death (RCD) processes, encompassing apoptosis, necrosis, pyroptosis, and ferroptosis (Figure 1). Thus, this study of ferroptosis reversibility offers novel

Abbreviations: Fsp-1, fibroblast-specific protein 1; RFP, a red fluorescent protein gene; GFP, green fluorescent protein; CDKs, Cyclin dependent Kinases.

TABLE 1 History of classification and differences of cell death mode implicated in cell physiology.

S.No	Mode of Cell Death	Morphological characteristics	Core regulators	History	References
01	Necrosis	Cell volume expands, leading to organelle swelling and disintegration of the endoplasmic reticulum. Subsequently, the cells undergo self-dissolution, resulting in the release of cellular contents, often accompanied by inflammation in the surrounding tissues.	TNF, Toll, RIP, RIP1, MLKL, NPEPL1, COL4A3BP etc.	**Kerr et al., 1972** *shrinkage necrosis*- Observed in adrenal cortices of rats treated with prednisolone **Wyllie et al., 1973** *shrinkage necrosis- observed in* healthy neonatal rats **Crawford et al., 1972** Observed in developing vertebral arches of fetal rats.	(25–31)
02	Apoptosis	The cell membrane remains undamaged, with a decreased volume, leading to the formation of apoptotic bodies. There is no autolysis of the cells, no release of cellular contents, and no associated inflammatory response.	p53, Bax, Bcl-2, Caspases etc.	**Sydney Brenner, H. Robert Horvitz, and John E. Sulston -2002** *Nobel Prize* – for demonstrating the role of genes in modulating tissue and organ development and programmed cell death.	(32–35)
03	Autophagia	Formation of bilayer membrane structure with no changes of cell membrane.	ATG5, ATG7, Beclin 1, TOR, PI3K etc.	**Christian de Duve-1963** Gave the name autophagy **Yoshinori Ohsumi-2016** *Nobel Prize* – for demonstrating autophagy in yeast and the role of genes involved in autophagy	(36–40)
04	Ferroptosis	The cell membrane remains intact, but there is a decrease in mitochondrial volume characterized by densely packed mitochondrial membranes, a reduction or disappearance of mitochondrial cristae, and the occurrence of ruptures in the outer mitochondrial membrane.	GPX4, VDAC2/3, Ras, FANCD2, TFR1, p53, CISD1, SLC7A11, HSPB1 etc.	**Dolma et al., 2003** *Erastin*- Kill tumor cells with Ras gene mutation; Irreversible by caspase inhibitor. **Yang et al.,2008** *RSL3, RSL5*- Irreversible by iron-chelate agent DFOM and Vitamin E; ROS abnormality. **Dixon et al.,2012**- Ferroptosis official nomination- Caspase-independent; RPL8, IREB2, ATP5G3, CS, TTC35, ACSF-mediated mechanism; controlled by iron-chelate agent and antioxidant.	(19, 41–46)

insights for potential therapeutic interventions aimed at modulating cell death and survival through its reversible nature.

Mitochondria play dual roles as key contributors to oxidative phosphorylation and primary generators of intracellular ROS (50, 51). Furthermore, they function as central hubs for various metabolic and signaling pathways, particularly fatty acid metabolism (52). Several studies have demonstrated that mitochondrial iron content, assessed through selective fluorescent iron indicators or electron paramagnetic resonance, constitutes a substantial portion, ranging from 20% to 50%, of the total cellular iron, depending on cell type (53–55). Mitochondrial iron plays a critical role in the formation of iron-sulfur clusters and heme synthesis. Notably, redox-active iron pools within mitochondria contribute to the accumulation of mitochondrial (MitoROS) (56–58). A distinctive feature of ferroptosis is its ability to evoke specific immune responses. During ferroptotic cell death, intracellular components are released into the extracellular milieu, triggering an immune reaction (59, 60). This response subsequently activates neighboring immune cells, such as macrophages, and facilitates the recruitment of additional immune cells to the site. It's noteworthy that other forms of RCD may also induce immune responses, although the nature of the immune response varies depending on the type of RCD involved (61). Induction of ferroptosis in cells using erastin result in the reduced mitochondrial size compared to normal cells (62, 63).

In this review, we described a comprehensive exploration of the complex relationships and interactions between mitochondria, ROS, and ferroptosis-mediated chemoresistance and subsequently described the need for investigating the interplay of anastasis, EMT and mitochondrial ROS-mediated ferroptosis, and comparative efficacy of anticancer immunotherapeutics in metastatic chemoresistant cancers. This study is beneficial to several oncologists, molecular biologists, clinicians to develop personalized cancer treatment strategies to promote novel drug development.

1.1 Literature search

We undertook a significant literature analysis, sourcing data from different databases including Pubmed, Medline, eMedicine, Scopus, Google Scholar, the National Library of Medicine (NLM), and ReleMed. Our focus encompassed published reports and articles investigating the role of mitochondria, ROS, anastasis, ferroptosis-mediated chemoresistance, and immunotherapeutics in metastatic cancers.

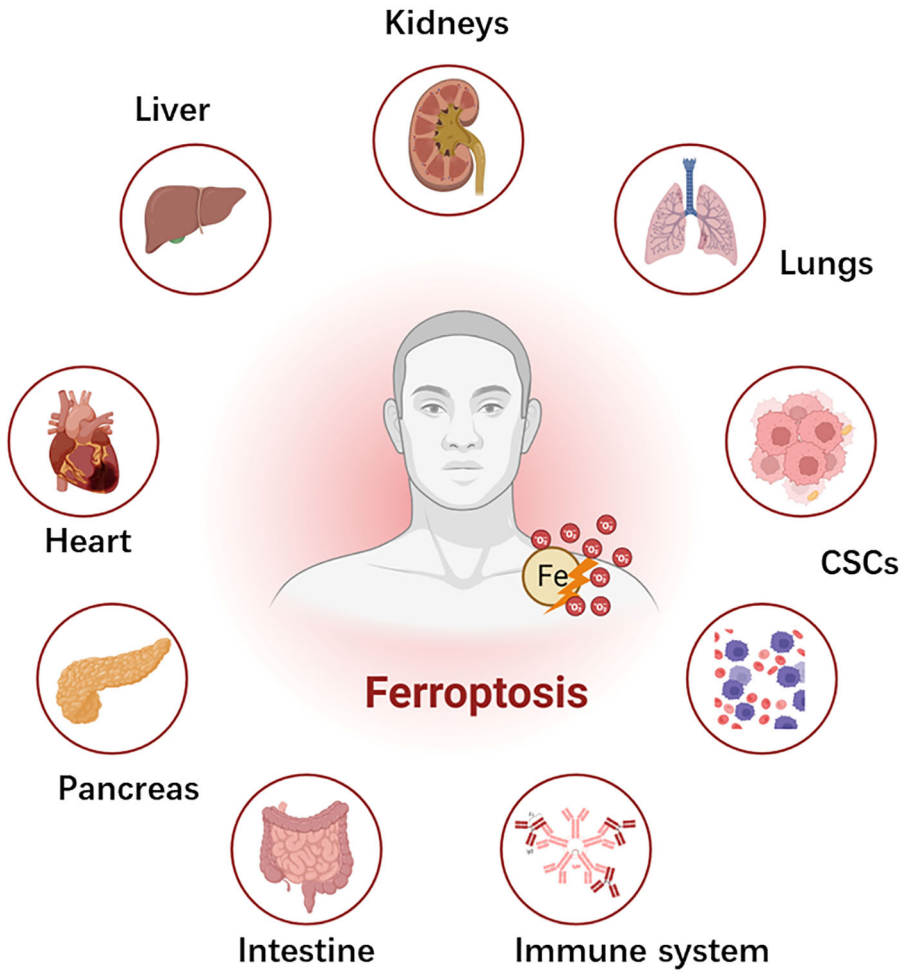

FIGURE 1
Schematic depiction of ferroptosis and systemic toxicities. Ferroptosis plays a pivotal role in several diseases across different organs. Ferroptosis has significant implications in the pathophysiology of chemoresistance and the development of cancer stem cells (CSCs), which yet require future studies in chemoresistant cancers (?). In the liver, it contributes to the development and progression of hepatocellular carcinoma, liver fibrosis, and ischemia-reperfusion injury. Within the cardiovascular system, it is implicated in ischemia reperfusion injury, transplantation, and atherosclerosis. Gastrointestinal system disorders, including gastric cancer and colorectal cancer, are influenced by ferroptosis. In the pancreas, it is associated with pancreatic cancer and type I diabetes mellitus. In the kidney, ferroptosis is involved in acute kidney injury, ischemia-reperfusion injury, and clear cell renal cell carcinoma. Lung diseases, such as lung cancer and acute lung injury, typically have ferroptosis-mediated pathophysiology. Hematological malignancies in the blood system and the suppression of T- cell immunological function in the immune system are also associated with ferroptosis.

2 Recent reports on intrinsic cellular iron metabolism & ferroptosis

Iron in dietary sources primarily exists as Fe (III) form, which undergoes reduction to Fe (II) within the intestinal lumen facilitated by brush-border membrane ferrireductase, duodenal cytochrome b (DCYTb), and other iron-reducing enzymes (64, 65). The resulting Fe (II) is then transported across the apical membrane of small intestinal epithelial cells (IECs) via divalent metal transporter 1 (DMT1/SLC11A2) and is subsequently sequestered within the labile iron pool (LIP) (66, 67). Intracellular Fe (II) is exported from IECs into the bloodstream via basolateral membrane ferroportin (FPN/SLC11A3), promoted through poly(rC)-binding proteins, and re-oxidized to Fe (III) by hephaestin (HEPH) (68, 69). Fe (III) is then bound to transferrin (TF) to form transferrin-bound iron (TBI) complexes, enabling circulation for transport to various tissues and organs. Within cells, TBI binds to transferrin receptor 1 (TfR1) and undergoes endocytosis, where Fe (III) is released into the cytoplasm, reduced back to Fe (II) by six-transmembrane epithelial antigen of prostate (STEAP3), and transported via DMT1/SLC11A2. A portion of cytoplasmic Fe (II) is stored in ferritin (FT) as the Fe^{2+}-FT complex, while the remainder contributes to the LIP. Heme also plays a significant role in iron metabolism, entering cells through heme carrier protein 1 (HCP1) and feline leukemia virus subgroup C receptor 2 (FLVCR2), with subsequent release of Fe (II) catalyzed by heme oxygenase 1 (HO-1) for transport to the LIP (69–72). In addition, non-transferrin-bound irons (NTBI) in the cytoplasm of different tissues are transported to the cells through different NTBI transporters such as Zrt/Irt-like protein 8/14 (ZIP8/14, SLC39A8/SLC39A14), L-type and T-type calcium channels after which they are stored in the LIP (73–75). The process of detachment and release of Fe (II) from FT in Fe^{2+}-FT is referred

to as ferritinophagy, and it is regulated by nuclear receptor coactivator 4 (NCOA4). FT has heavy chain (FTH) and light chain (FTL) subunits. NCOA4 binds specifically to FTH1 and induces Fe^{2+}-FT degradation, which separates FT from Fe (II) and thus allows Fe (II) release (76–80). A portion of intracellular Fe (II) enters the mitochondria through the MFRN1 and MFRN2 channels where they are then involved in the synthesis of heme and iron-sulfur cluster (ISC). ISC is an important inorganic cofactor that is widely engaged in a variety of biological functions including electron transfer which is the fundamental basis for cellular ATP production and protein synthesis. Meanwhile, recent studies have shown that ISC is tightly associated with DNA regulation (81–83).

Mitochondrial Ferritin (FtMt) is a protein responsible for iron storage within cell mitochondria, sharing structural and functional similarities with cytoplasmic H-ferritin (84, 85). FtMt expression is confined to the mitochondria of the central nervous system and select oxygen-dependent tissues. Studies have indicated that erastin induces upregulation of Voltage-Dependent Anion Channel (VDAC) proteins on the mitochondrial membrane, thereby triggering ferroptosis (86–88). However, investigations on FtMt-SY5Y cells treated with erastin have shown unchanged levels of VDAC2 and VDAC3, suggesting a specific protective mechanism of FtMt against ferroptosis (89, 90). Upon erastin treatment, there is an upregulation of NADPH oxidase (NOX) proteins, critical for ROS generation in ferroptosis (91, 92). Interestingly, erastin-treated cells overexpressing FtMt exhibit a minimal increase in NOX2 levels, indicating inhibition of ROS generation by FtMt during ferroptosis (92, 93). Additionally, the mitochondrial glutaminolysis-tricarboxylic acid cycle-electron transport chain (TCA-ETC) axis plays a pivotal role in initiating ferroptosis. Within this pathway, ROS generation leads to ferroptosis through lipid peroxidation (94, 95) (Figure 2).

Dolma et al. (45) reported a compound within the erastin class that exhibited selective cytotoxicity against tumor cells specific for Ras gene mutations. This compound induced a distinct mode of cell death not previously documented, which was unaffected by caspase inhibitors. Subsequently, Yang et al. (46) identified two additional erastin analogs, RSL3 and RSL5, and demonstrated that changes in ROS levels could induce cell death. This process could be counteracted by iron chelators such as deferoxamine and vitamin E. Dixon et al. (10) formally coined the term “ferroptosis” to elucidate that this novel form of cell death relies on iron but is independent of the caspase activity. This study delineated the ferroptosis regulation by multiple genetic factors and elucidated its mechanistic association with the cystine/glutamate antiporter (System Xc-) (Table 1).

ROS encompasses a heterogeneous group of oxygen-containing reactive molecules, including superoxides, peroxide radicals,

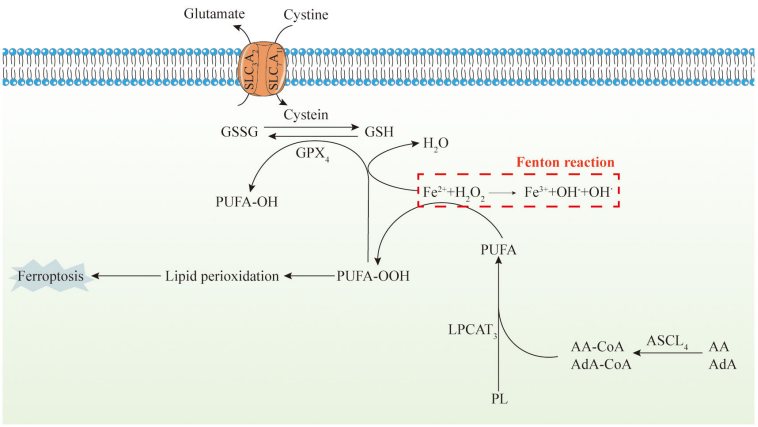

FIGURE 2
Mitochondria, iron metabolism, and ferroptosis. The diagram depicts the intricate series of events involved in iron metabolism within the human body, alongside ferroptosis, a form of regulated cell death. Initially, dietary iron, predominantly in the Fe(III) state, undergoes a sequence of conversions and transport processes. These processes include its reduction to Fe(II) within the intestinal environment, uptake by small intestinal epithelial cells, sequestration within the labile iron pool, extracellular release facilitated by ferroportin, and subsequent re-oxidation mediated by hephaestin. Subsequently, iron complexes with transferrin (TF), form transferrin-bound iron, which circulates in the bloodstream. TBI is internalized by cells via endocytosis, where it undergoes reduction to Fe(II) and enters the cellular cytoplasm. A fraction of the Fe(II) is stored within ferritin, while the remaining is reincorporated into the LIP. Heme, another significant iron source, follows a comparable pathway. Additionally, non-transferrin-bound iron from various tissues penetrates cells and is sequestered within the LIP. Furthermore, an additional reservoir of LIP is replenished through ferritinophagy, regulated by nuclear receptor coactivator 4. Intracellular free Fe(II) gains access to mitochondria via MFRN1 and undergoes subsequent biochemical transformations, ultimately leading to the onset of ferroptosis. Furthermore, ferroptosis is characterized by its dependence on iron and lipotoxicity. It operates by suppressing the activity of the lipid repair enzyme glutathione peroxidase 4 (GPX4), resulting in the accumulation of lipid hydroperoxides. Genetically, multiple genes modulate ferroptosis. Unlike other types of cell death characterized by extracellular manifestations, ferroptosis primarily unfolds intracellularly. This leads to distinctive cellular alterations, including reduced mitochondrial size, heightened membrane density, damaged cristae, fragmentation of the outer membrane without compromising the cell membrane, and minimal changes in nuclear morphology without chromatin condensation. Biochemically, the deficiency in peroxidation repair capacity primarily originates from the impairment of the phospholipid peroxidase GPX4. This deficiency triggers the acquisition of reactive iron and the oxidation of phospholipids containing polyunsaturated fatty acids (PUFA), ultimately inducing ferroptosis. The decrease in intracellular antioxidant capacity exacerbates lipid ROS accumulation, and cause cellular ferroptosis. Glutathione peroxidase is influenced by various pathways, including the XC −/GSH/GPX4 system, and the ACSL4/LPCAT3/15-LOX and FSP1/CoQ10/NAD(P)H pathways.

hydroxyl radicals, and alkoxyl radicals (96–98). Intracellular ROS are predominantly generated during ATP production within various cell types, representing normal metabolic byproducts of the mitochondrial respiratory chain. Furthermore, enzymes such as NADPH oxidase contribute to ROS production via enzymatic reactions (99, 100).

Polyunsaturated fatty acids (PUFA) constitute integral components of cell membrane phospholipid bilayers, crucial for maintaining membrane fluidity (101). Intracellular iron (II) (Fe (II)) participates in the Fenton reaction with hydrogen peroxide (H_2O_2), yielding hydroxyl radicals (OH•) that subsequently involved in lipid peroxidation reactions. Fe (II) itself catalyzes lipid peroxidation, promoting the formation of ROS such as PUFA-OOH, thereby causing cellular membrane damage (102–108).

The fundamental mechanism underlying ferroptosis involves the higher ROS generation, which leads to lipid peroxidation and disruption of cellular redox homeostasis, ultimately results in cell death that has significant implications in oncology. Despite the existence of various upstream regulatory pathways, it is widely acknowledged that the activity of glutathione peroxidase 4 (GPX4) directly or indirectly influences the onset of ferroptosis (13, 109–111).

3 Recent updated molecular reports on mitochondria's role in ferroptosis and cancers

The principal morphological hallmark of ferroptosis is the changes in mitochondrial architecture and function, distinguishing it from other modes of cell death (112). Mitochondria undergoing ferroptosis display distinct alterations in morphology. Upon treatment with erastin, cells lack the characteristic morphological features typically associated with the absence of nuclear condensation, fragmentation, and necrotic lysis characteristic of necrosis; the lack of cellular crumpling, chromatin condensation, and apoptotic vesicle formation typical of apoptosis are changes associated with mitochondria-mediated ferroptosis. In response to erastin treatment, cells exhibit a distinct set of alterations characterized by a higher mitochondrial potential and membrane density. Furthermore, there is a reduction or disappearance of mitochondrial ridges, accompanied by the rupture of the mitochondrial outer membrane. These distinctive alterations serve as discernible criteria for distinguishing ferroptosis from apoptosis, necroptosis, and autophagy (113–118). These morphological alterations should be explored in various chemoresistant cancers.

3.1 The effect of tricarboxylic acid cycle and glycolysis on ferroptosis

The tricarboxylic acid (TCA) cycle and glutamine metabolism play integral roles in ferroptosis. While the precise metabolic pathways remain elusive, evidence suggests a close association between TCA cycle metabolites, enzymes, and ferroptotic cell death (119–121). In cellular contexts, blocking the cystine/glutamate antiporter (System Xc (-)) prevents the reverse transport of cystine and glutamic acid, thereby reducing cystine uptake, and causing intracellular ROS accumulation, lipid peroxidation, and initiation of ferroptosis (122). Elevated extracellular glutamate levels impede System Xc (-) function, consequently promoting ferroptosis.

Hence, diminishing glutamine (Gln) availability or inhibiting glutamine metabolism profoundly attenuates ferroptosis. Without adequate glutamine, mitochondrial damage and cell death induced by cystine starvation or erastin treatment are impeded (123, 124). Thus, glutamine is necessary for ferroptosis, and glutamine synthase (GLS) is a key regulatory factor for glutamine decomposition. GLS is divided into two isoforms: GLS1 and GLS2. GLS1 is mainly located in the cytoplasm, while GLS2 is located in the mitochondria (125–127). GLS1 and GLS2 catalyze the conversion of Gln to Glu (128, 129). Tal Hirschhorn et al. (130) found that ferroptosis can be prevented by drugs or gene inhibition of mitochondrial subtype GLS2. GLS2 has previously been shown to have activity consistent with tumor inhibition. GLS2 is a transcriptional target of p53 and upregulates during p53-dependent ferroptosis (131). Suzuki S et al. (132) found that GLS2-mediated glutamate can be converted into glutamic acid in the presence of aspartate aminotransferase (GOT) and glutamate dehydrogenase 1 (GLUD1). α - KG increases the production of ROS to downregulate the antioxidant defense function of cells, increase their sensitivity to iron-dependent cell death, and thus promote iron-dependent cell death. Downstream products of α-KG, such as succinic acid and fumaric acid, can also enhance cysteine depletion and cause cell ferroptosis (133). Several enzymes in the TCA cycle are necessary for iron-mediated ferroptosis caused by cystine starvation or erastin therapy, such as fumarate hydroxylase (FH), aconitase, and citrate synthesis (CS) (134–137). Therefore, reducing glutamine or blocking the glutamine decomposition typically impairs CDI ferroptosis. Without glutamine, cysteine starvation or erastin treatment cannot induce mitochondrial damage and subsequent cell death (123, 124).

Glycolysis serves as a crucial pathway for cells undergoing anaerobic respiration to generate energy. In tumor cells, metabolic rates are typically increased to sustain their rapid and uncontrolled proliferation. Metabolic shift is one of the significant physiological activities occurring in tumor cells to foster uncontrolled proliferation. Mainly, malignant cells rely on glycolysis to fulfill their energy demands while maintaining redox homeostasis to prevent ferroptosis (119, 138, 139). Ibtissam et al. (140) described that glycolytic cells exhibit significant inhibition of mitochondrial OXPHOS activity, mitigating ROS stress. However, this inhibition is reversible, and when glycolysis is suppressed, metabolic reconfiguration toward OXPHOS elevates cellular ROS levels, subsequently disrupting iron homeostasis and promoting lipid peroxidation, thus rendering tumor cells more sensitive to conventional chemotherapeutic agents and ferroptosis inducers. Hence, it is crucial to develop novel ferroptosis inducers to modulate the mitochondrial OXPHOS mainly to inhibit the development of CSCs in TME.

The altered function of VDACs may contribute to glycolytic alterations. Specifically, the opening of VDACs enhance mitochondrial substance exchange, subsequently increasing aerobic respiration efficiency (141, 142). VDACs, comprising VDAC1, VDAC2, and VDAC3 isoforms typically facilitate ATP, adenosine diphosphate, and mitochondrial-cytoplasmic exchanges (141, 142). Studies have indicated (143–145) that VDAC closure by microtubule proteins in a quiescent state restricts respiratory substrate influx into mitochondria, promoting glycolysis in cancer cells (Warburg effect), and this mechanism is yet to be studied in chemoresistant cancer cells. Yagoda et al. (146) revealed that cells activated by the RAS-RAF-MEK pathway can bind to certain VDAC ligands, subsequently enhancing susceptibility to erastin, a pivotal factor in cancer cell development. This heightened sensitivity prevents and reverses VDAC blockade by cytoplasmic free microtubule proteins, leading to VDAC opening and subsequent non-apoptotic cell death. Furthermore, intracellular free microtubulin abundance inhibits VDAC conductivity. N. DeHart et al. (86) described a novel mechanism of cell killing induced by erastin and erastin-like compounds, where the reversal of microtubulin-dependent VDAC inhibition fosters mitochondrial hyperpolarization and oxidative stress, culminating in mitochondrial dysfunction, dissipation of mitochondrial membrane potential ($\Delta\Psi m$), and cell death. These mitochondrial mechanisms pertinent to the ferroptosis in chemoresistant tumor cells in other different cancer types should be explored typically by several preclinical studies.

3.2 Regulatory role of ROS and electron transport chain in ferroptosis

Mitochondria is the primary site for oxidative respiration within cells, where ETC activity not only facilitates ATP production but also plays a crucial role in generating ROS necessary for triggering cellular ferroptosis (147–149). For instance, glucose-dependent mitochondrial bioenergetic processes involve the transfer of electrons from complexes I and III in the ETC to molecular oxygen, resulting in ROS generation during catabolism (150–153). ROS, including hydrogen peroxide, superoxide anion, hydroxyl radicals, and peroxynitrite, are predominantly produced via mitochondrial metabolism and act as mediators of intracellular signaling pertinent to various forms of cell death including in tumor cells (16).

Electron leakage from ETC complexes I and III generates $O2^{\cdot-}$, which is consequently converted to H_2O_2 by disproportionation in the presence of superoxide dismutase. H_2O_2 reacts with Fe^{2+} to form hydroxyl radicals, initiating a chain reaction with PUFAs to induce cell death via the production of PUFA hydroperoxides (PUFA -OOH) (154). Studies by Feng et al. (155) demonstrate that COX7A1, a cytochrome C subunit, inhibits mitochondrial autophagy and promotes susceptibility to ferroptosis in NSCLC cells by modulating the TCA cycle, ETC complex IV activity. This mechanism should be explored in several other cancer types.

Additionally, the inhibition of ETC mitochondrial complexes I, II, III, and IV attenuates ROS accumulation and ferroptosis induced by cysteine starvation or erastin et al. (134). Inhibition of mitochondrial complex III function with S3QEL significantly reduces lipid peroxidation and ferroptosis induced by cysteine starvation, highlighting the crucial role of superoxide produced by complex III in cysteine starvation-induced ferroptosis (156). The implications of cysteine depletion-induced ferroptosis in CSCs should be explored vividly by exploring the different cell signaling mechanisms.

Furthermore, ETC activity could influence the regulation of energy sensor adenosine 5'-monophosphate (AMP) and ROS levels. Mitochondrial electron transfer via the ETC complex generates a proton concentration gradient that powers ATP synthesis via ATP synthase. ATP production inhibits AMP-dependent protein kinase (AMPK) activity (157), promoting cellular ferroptosis by decreasing phosphorylation of acetyl-coenzyme A carboxylase (ACC) and increasing lipid synthesis, thereby influencing cellular susceptibility to ferroptosis (157).

3.3 Lipid metabolism and GPX4/system Xc^- in ferroptosis and cancers

Lipids form the structural basis for cell membranes and organelles (mitochondria, endoplasmic reticulum), with lipid metabolism closely linked to cellular ferroptosis sensitivity via multiple pathways (47). System Xc- functions as a reverse cystine/glutamate transporter, comprising the transmembrane transporter proteins SLC3A2 and SLC7A11 (xCT). It transports intracellular glutamate and extracellular cystine, with intracellular cystine being converted to cysteine. Cysteine plays a crucial role in glutathione synthesis, primarily existing as reduced glutathione (GSH) and oxidized glutathione (GSSG), dynamically balanced within cells (158–161). GPX4, a selenoprotein, plays a pivotal role in maintaining this balance by catalyzing the conversion of glutathione to GSSG (122, 162). It effectively reduces phospholipid peroxides, decomposes H_2O_2 to water with GSSG, and inhibits arachidonic acid activation, thereby suppressing lipid peroxidation and ROS generation (22–24). As we discussed above, PUFAs are major substrates for lipid peroxidation during ferroptosis, damaging membrane structure and function. Enzymes involved in binding PUFA to phospholipids, such as acyl coenzyme A synthetase long-chain family member 4 (ACSL4), are integral to ferroptosis. ACSL4, besides being a sensitive indicator of iron-dependent cell death, regulates lipid metabolism by linking free fatty acids to CoA linkage, ultimately exchanged for phospholipids (163). ACSL4 specifically binds to substrates like arachidonic acid (AA) and adrenergic acid (ADA) (164) leads to the generation of fatty acyl CoA esters, esterified by lysophosphatidylcholine acyltransferase 3 (LPCAT3) into phospholipids like phosphatidyl ethanolamine (AA-ETA), predominantly found in the endoplasmic reticulum. Subsequent oxidation of AA-PE and ADA-PE by 15-lipoxygenase (ALOX15) produces lipid hydroperoxides, and other signaling molecules promoting ferroptosis (162, 165, 166). Recent studies highlight alternative pathways to ferroptosis, indicating that ACSL4/LPCAT3/15-LOX or p53/SLC7A11/12-LOX may cause lipid peroxidation during this process (167, 168). This underscores the complexity of ferroptosis regulation, offering potential therapeutic avenues for modulating cell death pathways to ameliorate chemoresistant-cancers (Figure 3).

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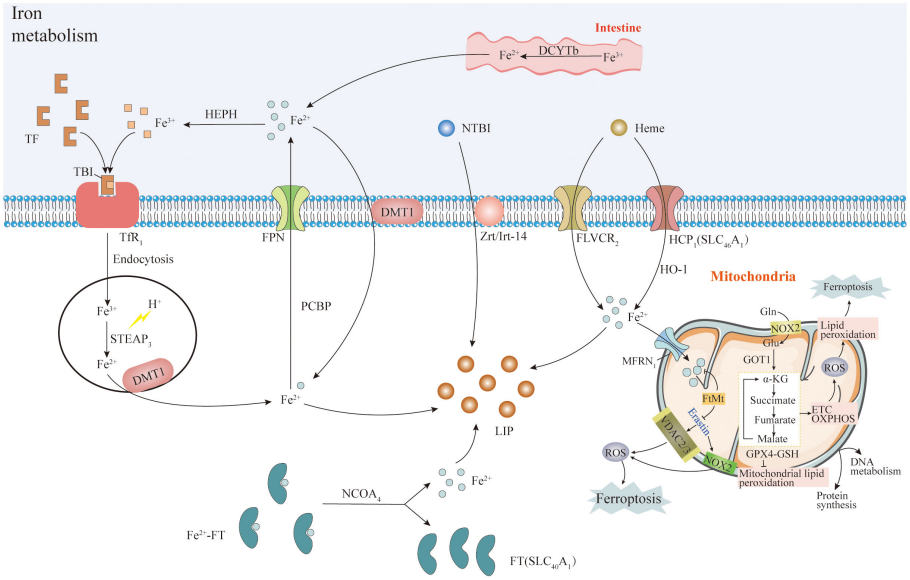

FIGURE 3
System Xc- and lipid metabolism. System Xc- is a transmembrane transporter composed of SLC3A2 and SLC7A11 (xCT) proteins. It transports intracellular glutamate and extracellular cystine, which is converted into cysteine. Cysteine plays a pivotal role in synthesizing the antioxidant glutathione. Glutathione exists mainly in reduced (GSH) and oxidized (GSSG) forms, maintained in balance by various mechanisms. GPX4, a selenoprotein, converts glutathione to GSSG and efficiently reduces phospholipid peroxides while decomposing H_2O_2. GPX4 also inhibits arachidonic acid activation and lipid peroxidation (ROS). Long-chain acyl-CoA synthetase 4 (ACSL4) activates arachidonic acid and adrenic acid into their CoA forms, promoting the activation of polyunsaturated fatty acids (PUFA) mediated by LPCAT3. Intracellular Fe(II) undergoes the Fenton reaction with H_2O_2, generating hydroxyl radicals (OH·), contributing to lipid peroxidation and ROS production, causing cell membrane damage. Note: The complete pathways explanation was given in subheading 3.

4 Need for investigating the interplay between anastasis, ferroptosis, and chemoresistance and metastasis

Although both ferroptotic and apoptotic modes of cell death processes can be reversed, the underlying mechanisms facilitating these cell recovery phenomena remain to be fully elucidated in distinct cancer types. Primarily, both modes of cell death are typically different suggesting that different mechanisms may be required to halt the respective cell death mediators and repair the distinct types of damage for cell recovery. For instance, apoptosis involves the activation of proteases that can cause cleavage of distinct functional proteins, along with DNases like endonuclease G and DNA fragmentation factors, resulting in genomic destruction (169–171). Upon cessation of apoptotic stimulus, the cells that are undergoing recovery could induce upregulation of heat shock proteins and XIAP to impair stimulation of caspases, whereas the ICAD/DFF45 could cause inhibition of DNase activity, and PARP (a DNA repair enzymatic protein) to foster genome repair (172–174). In contrast, ferroptosis is characterized by the accumulation of ROS beyond the cell's redox balance, which can cause lipid peroxidation followed by cell death (175). Administration of compounds like glutathione (GSH) or Fer-1 can facilitate ferroptosis reversal, potentially by enhancing GPX4 activity to mitigate ROS accumulation (176), while Fer-1 acts as a ROS scavenger to eliminate excessive cytosolic and lipid ROS, which could confer to the redox homeostasis and enabling cell recovery (177, 178).

Though glutathione (GSH) and Fer-1 are considered as inhibitors of ferroptosis, not all ferroptosis inhibitors possess the capacity to facilitate its reversal. For instance, compounds like aminooxyacetic acid (AOA), the iron chelator deferoxamine (DFO), dopamine, and vitamin C are known to inhibit ferroptosis initiation but do not promote cell recovery once ferroptosis has initiated. This limitation may stem from their targeting of upstream pathways involved in ferroptosis initiation, instead of conferring to the modulation of other downstream signaling pathways responsible for cell death. DFO functions as an iron chelator, depleting iron levels and hindering the iron-dependent accumulation of lipid ROS (19). Similarly, AOA inhibits transaminase activity, thereby blocking glutamine metabolism to alpha-ketoglutarate implicated predominantly involved in fatty acid synthesis (179). Dopamine prevents GPX4 degradation, while vitamin C scavenges free radicals in the aqueous phase (180–182). Previous studies (178) on the ferroptosis reversal through GSH and Fer-1 implicate regulators linked to GPX4 activity and lipid peroxidation as potential compounds that can induce ferroptosis reversibility. Unlike GSH and Fer-1, which act on mechanisms associated with removing lipid ROS, these inhibitors predominantly target the initiation of lipid peroxidation. This suggests potential differences in the regulation of preventing reversal of ferroptosis that warrant further investigation.

Several previous studies described the reversal of apoptosis when the removal of apoptotic stimuli is induced *in vitro* and *in vivo* studies (172–174, 178, 183–188). In contrast, another study (178) reveal that simply removing ferroptosis-inducing stimuli,

including 'erastin' from HT-1080 cells or 'erastin/glutamate' from HT-22 cells, is insufficient to cause recovery of these ferroptosis-initiated cells. In this study (178), authors demonstrated the need to explore the possibility that certain types of dying cells with robust redox balance restoration capabilities may recover without the supplementation of GSH or Fer-1, a possibility that requires further investigation.

Ferroptosis can be reversed but raises fundamental inquiries that remain unanswered. For instance, can ferroptosis reversal occur within live animals? Tracking ferroptosis *in vivo* presents technical challenges, as cells recovered from ferroptosis appear morphologically identical to healthy non-ferroptotic cells. During the reversal of ferroptosis or apoptosis, cells encounter distinct forms of cellular damage. The existence of a common master regulator or signaling pathway triggering anastasis remains to be determined. Additionally, the broader physiological & therapeutic implications of this cellular recovery process require further exploration. Ongoing investigations aim to address these questions and identify the molecular regulators involved in these pathways. Understanding the signaling mechanisms and compounds that modulate ferroptosis and the reversal of ferroptosis could generate new approaches to investigate and comprehend the cell recovery process of anastasis (178).

Chemotherapy is a widely used treatment strategy aimed to ameliorate cancer cell proliferation (189, 190); however, some cells can survive chemotherapy, leading to cancer recurrence and metastasis (191–193). Even though various mechanisms, such as alterations in chemotherapeutic metabolism, changes in gene expression, metabolic reprogramming, stemness development, and changes in cell death pathways are significant influential factors which have been implicated in chemotherapy resistance (194–196), however, the precise underlying mechanisms remain unclear.

Apoptosis, a key mechanism targeted by chemotherapy, involves the activation of caspases, a group of cysteine proteases, ultimately leading to cell death. Previously, activation of executioner caspases was thought to be irreversible, marking a "point of no return" in apoptosis (197–199). Previous reports revealed anastasis process, wherein cells could refrain from apoptotic stress although executioner caspase activity is evident (174, 184, 200). Anastasis, or cellular survival following stress-induced activation of executioner caspases, has been documented in a subset of mammalian cell lines following exposure to various chemical stressors and chemotherapeutic agents (172, 173, 184, 201–204).

Studies on different cancer cell types, including breast (108, 205–208), melanoma (117), cervical, and ovarian cancers (209), have shown that anastasis confers new traits upon cancer cells, such as increased drug resistance and migration (172, 173, 201–204, 210). For instance, anastatic breast and cervical cancer cells are associated with enhanced chemoresistance and migration capabilities (203). Similarly, melanoma cells surviving executioner caspase activation caused through the upregulation in the expression of transient tBid or exposure to the dacarbazine (a chemotherapeutic drug) demonstrate elevated cell migration *in vitro* and increased metastasis *in vivo*.

According to previous reports (202), a lineage tracing system was employed to identify and isolate cells undergoing executioner caspase activation and their progeny. These studies suggest that anastatic colorectal cancer cells exhibit a higher metastasis, and chemoresistance due to increased upregulation in the expression of cIAP2 and NF-κB activity. According to this study, NF-κB activation and cIAP2 expression conferred by chemotherapeutics are crucial for anastasis (202). Exposure to chemotherapeutic drugs triggers NF-κB activation and upregulation of cIAP2 expression in colorectal cancer cells, promoting anastasis. Therefore, activated NF-κB and cIAP2 establish a positive feedback loop within anastatic cells, further enhancing migration and metastasis (202).

Thus, anastasis cause colorectal cancer cells with heightened migratory potential. Similar observations of higher metastasis following survival from stress-induced executioner caspase activation were evident in various tumor types, including cervical (173), breast (203), melanoma (202), and ovarian cancers (204), suggesting that a higher migration may represent a typical phenotypic alteration associated with anastasis. Furthermore, apoptosis and executioner caspases are typically involved in mediating the metastasis of lymph nodes (211–213). However, anastatic cells maintained enhanced migration independently of apoptotic cells, suggesting that executioner caspase activity is dispensable for sustaining increased migratory capacity in anastasis (214, 215). This observation aligns with previous reports on melanoma cells (202).

Various cancer cells exhibit increased migration following anastasis, with diverse underlying molecular mechanisms. Inhibition of TGF-β signaling moderately mitigated anastasis-induced migration in HeLa cells following brief ethanol exposure (173). Another report showed that the blocking of nuclear export reversed heightened migration in anastatic breast cancer cells (203). Similarly, a previous study (202) concluded that melanoma cells surviving executioner caspase activation resulted in augmented motility through the JNK hyperactivation.

Ru Wang et al., 2013 (216) revealed that cIAP2 enhances migration in anastatic colorectal cancer cells in an NF-κB-dependent manner, emphasizing role of cIAP2 as a positive regulator of migration. According to previous reports, NF-κB has a significant role in proliferating cancer cells and promotes migration or metastasis by modulating the downstream signaling cascades including STAT3 and MMPs (217–219). cIAP2 has previously been associated with drug resistance in pancreatic cancer, colorectal cancer (220, 221), and oral squamous cell carcinoma (222, 223), further supporting its role in mediating chemoresistance. A positive feedback loop between cIAP2 and NF-κB fostered levels of cIAP2 expression and NF-κB activity in anastatic cells, enhancing their migratory potential. Thus, cIAP2/NF-κB signaling and anastasis are critical regulators of post-chemotherapy metastasis (216). Additionally, Ru Wang et al., 2013 revealed (216) demonstrated a cIAP2-dependent increase in chemoresistance in anastatic colorectal cancer cells.

Studies on *Drosophila notum* epithelium demonstrated that (224) the ability of stressed cells to undergo anastasis may be relied upon executioner caspase activation. By inhibiting caspase-3 and 7, cIAP2 can foster a higher executioner caspase activity (225, 226), thereby promoting anastasis. Molecular mechanisms governing the anastasis regulation through cIAP2, NF-κB, and

regulators which were already investigated in previous studies warrant further elucidation in future research for several other chemoresistant cancers.

5 Integrating ferroptosis with chemoresistance

Drug-resistant cancer cells, along with neighboring stromal cells, may create a protective environment, allowing vulnerable cancer cells to evade ferroptosis and chemotherapy. For example, cancer-associated fibroblasts (CAFs) could foster a reduction in cisplatin accumulation, subsequently conferring resistance to both chemotherapy and ferroptosis (21). Ferroptosis is intricately controlled by a multifaceted network involving epigenetic, pre-transcriptional, post-transcriptional, and post-translational modifications (227). Tumor cells, characterized by heightened sensitivity to signals regulating cell survival and death, consequently cause increased susceptibility to ferroptosis. Various factors that promote tumor growth, the buildup of iron, enhanced lipid synthesis, or undergoing epithelial-to-mesenchymal transition (EMT), can augment susceptibility of tumor cells to ferroptosis. Additionally, numerous chemotherapeutic agents elicit anti-cancer efficacy through the induction of oxidative stress, disrupting the anabolism of genetic components thereby synergizing with ferroptosis to ameliorate cancers. Nonetheless, the precise targeting of tumor cells by promoting ferroptosis induction remains a challenging task in current oncological strategies (9). Tumor drug resistance encompasses several intricate mechanisms, with disruption of redox homeostasis emerging as a pivotal contributor. Tumor cells exhibit greater resistance to oxidative stress by modulating ROS generation, thereby acquiring drug resistance (228). Ferroptosis typically linked with mitochondrial oxidative stress and ROS production, plays a significant role. Alterations in oxidative stress regulation can modulate ferroptosis, consequently influencing tumor cell sensitivity to chemotherapeutics. This association depends upon a nuanced balance between lipid peroxidation and the accumulation of ROS promote ferroptosis, thereby suppressing tumor proliferation; as we discussed the reduction of lipid peroxidation and ROS levels facilitates tumor cell survival, promoting resistance to anti-tumor agents. The dynamic interplay between ROS generation and scavenging mechanisms lies at the core of this relationship, which require future studies pertinent to several chemoresistant cancers (9).

As we discussed previously, chemotherapy remains a significant therapeutic modality treating numerous solid malignancies, but the challenge of chemoresistance significantly limits its effectiveness (229). Interestingly, a significant link has been observed between resistance to cisplatin and resistance to ferroptosis within tumor environments. Malignant cells resistant to cisplatin often exhibit increased expression of genes associated with ferroptosis resistance (230–232). For instance, cisplatin-resistant cancer cells overexpress the Wnt pathway membrane receptor, frizzled homolog 7 (FZD7), leading to activation of TP63 and subsequently enhanced GPX4 expression (233, 234). Various cancer types develop cisplatin resistance by upregulating system Xc– expression and maintaining high levels of GSH (235–237). Recent studies indicate that cisplatin treatment could induce ferroptosis in tumor cells, suggesting the significant efficacy of chemotherapy in triggering ferroptosis (238, 239).

CAFs have been implicated in reducing cisplatin accumulation, thereby promoting resistance to both chemotherapy and ferroptosis by supplying glutathione (GSH) and cysteine to ovarian cancer cells (240). Another study described that upregulation of miR-4443 in NSCLC cells, and undergoes exosomal transfer to sensitive cells, and enables upregulation of FSP1 expression. This elevated FSP1 expression is crucial to evade ferroptosis. Conversely, CD8+ T cells induce the generation of IFN-γ subsequently reduces system Xc– activity in cancer cells, thus fostering reduction in cisplatin resistance through modulation of the JAK/STAT pathway (240).

The significant interplay between chemoresistance and the underlying ferroptosis mechanisms is yet to be explored vividly to develop novel therapeutic modalities against ferroptosis-resistant tumor cells (239). Combinatorial administration of GPX4 inhibitors and system Xc– inhibitors & cisplatin has been shown to overcome chemotherapy resistance, enhancing the anti-cancer response in various tumors (238, 241, 242). However, these mechanisms require future studies in several other chemoresistant cancer types.

Chemoresistance specific to the anti-glioma agent, temozolomide (TMZ) is typically linked to resistance to ferroptosis induced by the overexpression of system Xc– and GSH (243, 244). Moreover, cancer cells can alter the iron homeostasis by lipocalin-2, subsequently causing ferroptosis resistance to 5-fluorouracil (245). The phenotypes of chemoresistant tumors closely mimic resistance to ferroptosis, particularly focusing on crucial targets such as GPX4, system Xc–, FSP1, and NRF2 (6, 246–249). Hence, it is crucial to profile these ferroptosis targets in chemoresistant tumors (Figure 4).

Yet, the adaptive reaction to chemotherapeutics includes the activation of MTDH gene, promoting the mesenchymal phenotype of tumors and augmenting chemoresistance. However, diminishing the expression of GPX4 and system Xc– can increase susceptibility to ferroptosis. Thus it has been demonstrated that the ferroptosis resistance emerges as a new rationale underlying tumor recurrence following chemotherapy (21).

The surgical intervention combined with adjuvant multi-drug chemotherapy is another strategy of therapeutic modality for patients diagnosed with pancreatic ductal adenocarcinoma to enhance survival rates. However, the persistence of gemcitabine resistance in PDAC cells, continues to challenge clinicians (251). Recent insights suggest a correlation between gemcitabine resistance (252, 253) and ferroptosis resistance, primarily driven by the hyperexpression of system Xc–. Notably, inhibiting system Xc– in PDAC cells leads to glutathione (GSH) depletion *in vitro*, triggering ferroptosis and restoring sensitivity to both gemcitabine and cisplatin (254).

Changes in apoptosis-regulating genes are common in cancer, promoting activation of pro-proliferation and pro-survival pathways, leading to resistance against apoptosis. Contrary to apoptosis irreversibility, emerging evidence suggests its potential reversibility, termed anastasis, enabling cells to evade apoptotic signals even at advanced stages, challenging chemotherapy development (178, 250). It is crucial to explore the multifaceted role of survival mechanisms, metastasis, EMT, and DNA damage repair in facilitating anastasis. It

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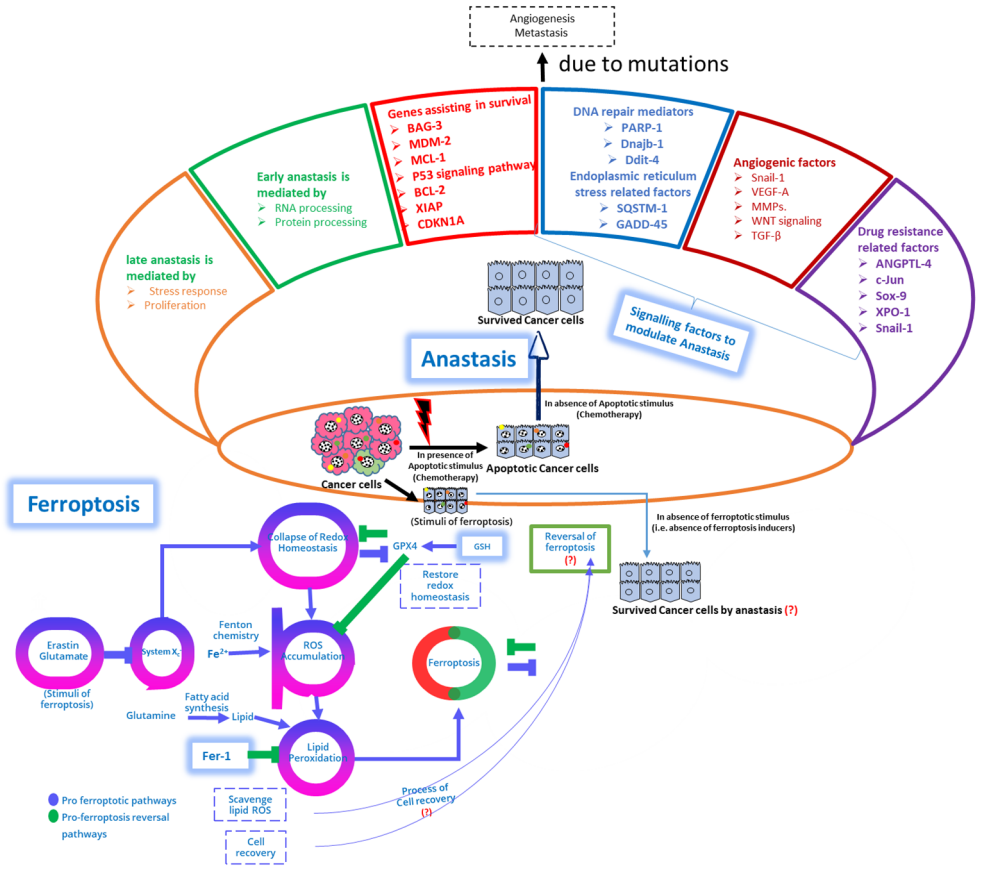

FIGURE 4
Schematic depiction of genomic variations within genes regulating apoptosis are evident in cancer and contribute to the activation of pro-proliferation and pro-survival pathways, leading to apoptosis resistance. Upper Panel: While apoptosis has traditionally been regarded as irreversible, emerging evidence suggests its potential reversibility, termed anastasis, wherein cells can escape apoptotic signals even after reaching advanced stages. Anastasis poses challenges for chemotherapy development and utilization. This figure demonstrates the multifaceted role of survival mechanisms, metastasis, epithelial-mesenchymal transition (EMT), and DNA damage repair in facilitating anastasis, and the mechanistic interplay between ferroptosis reversal and anastasis to modulate chemoresistance, (?) emphasizing its implications for drug resistance and therapeutic strategies. Lower Panel: A proposed model illustrates the reversal of ferroptosis, and the interplay between pro-ferroptosis reversal pathways and pro-ferroptosis pathways is discussed. The schematic representation depicts structural and molecular changes during anastasis, showing cancer cells recovering from apoptosis by activating anastasis and acquiring mesenchymal features and molecular alterations promoting survival, metastasis, and angiogenesis. Genes associated with anastasis-mediated drug resistance, including *ATF3, c-FOS, c-JUN, INBHA, SNAIL-1, ANGPTL4*, and *SOX-9*, are highlighted. Cells recovering from apoptosis through anastasis exhibit pronounced structural changes, alter focal adhesion kinases, activate genes related to the actin cytoskeleton, and elevate proteins associated with chemoresistance. In addition, the interplay between the anastasis, and mitochondrial-ROS modulated ferroptosis proposed mechanisms should be explored to demonstrate the underlying mechanisms pertinent to chemoresistance with anastasis, and ferroptosis reversal (178, 250).

also explores the interplay between ferroptosis reversal and anastasis in modulating chemoresistance, highlighting implications for drug resistance and therapeutic strategies. Future studies are warranted to examine ferroptosis reversal, and the interaction between pro-ferroptosis reversal and pro-ferroptosis pathways. The schematic representation Figure 4 delineates structural and molecular changes during anastasis, with cancer cells activating anastasis to recover from apoptosis, acquiring mesenchymal features, and molecular alterations promoting survival, metastasis, and angiogenesis. Genes linked to anastasis-mediated drug resistance, such as ATF3, c-FOS, c-JUN, INBHA, SNAIL-1, ANGPTL4, and SOX-9, are emphasized (178, 250). Cells recovering from apoptosis via anastasis undergo notable structural changes, altering focal adhesion kinases, activating genes associated with the actin cytoskeleton, and increasing chemoresistance-associated proteins. Additionally, exploring the interplay between anastasis and mitochondrial-ROS modulated ferroptosis mechanisms can elucidate underlying mechanisms of chemoresistance with anastasis and ferroptosis reversal (178, 250)

Intricate interplay pertinent to the anastasis, chemoresistance, and ferroptosis resistance in tumors require studies related to therapeutic efficacy of several therapeutic strategies. The recurrence of tumors following chemotherapy may, in part, be attributed to ferroptosis resistance. This underscores the significant chemotherapeutic efficacy of combinatorial regimens. In this context, targeting the tumor microenvironment (TME) and metabolic pathways to enhance ferroptosis sensitivity in malignant cells could emerge as a significant therapeutic strategy for overcoming the challenges posed by chemoresistance (21).

Emerging evidence indicates that ferroptosis can mitigate acquired resistance to several targeted therapies, including

lapatinib, erlotinib, trametinib, dabrafenib, and vemurafenib (255–257). Notably, tumor cell lines resistant to these targeted drugs often exhibit markers of EMT and display heightened susceptibility to ferroptosis (255–257). EMT, characterized by the loss of epithelial characteristics and acquisition of mesenchymal properties, is known to confer chemoresistance in tumor cells, driven by transcription factors such as TWIST1 and ZEB1. For instance, β-elemene has been shown to sensitize KRAS mutant colorectal cancer cells to cetuximab through a ferroptotic mode of cell death while blocking EMT (257). Thus, ferroptosis could modulate chemoresistance to the chemotherapeutics and appear intricately linked to EMT processes.

Additionally, agents targeting PD-1 and PD-L1 garnered approval from the FDA for treating various malignancies. Anti-PD-L1 antibodies have been observed to foster lipid peroxide-mediated ferroptosis in proliferating cancer cells, with the efficacy of anti-PD-L1 antibody therapy being compromised by ferroptosis inhibitors (258). Combining anti-PD-L1 antibodies with ferroptosis inducers has shown significant efficacy in inhibiting tumor growth, attributed to the release of interferon-γ by cytotoxic T cells, which activates STAT1 and inhibits xCT expression, thereby promoting ferroptosis (258). Implicating ferroptosis inducers that stimulate STAT1 activation may overcome tumor resistance to immunotherapy, providing a wide range of potential for the clinical implementation of ferroptosis inducers in combination with immune checkpoint inhibitors (ICIs) (9).

6 Integrating anastasis with EMT and chemoresistance and immunotherapeutics

Cancer stem cells (CSCs) pose significant challenges for developing new therapies due to their strong association with tumor relapse and chemoresistance (259, 260). Cells that are undergoing anastasis exhibit resistance to various apoptotic phases. Previous reports in cancer metastasis demonstrated that cells recovering from apoptotic phase show a higher CD44 expression, a key marker that signifies chemoresistance, and these cells exhibit properties similar to CSCs. Additionally, the apoptosis reversal induces epigenetic modifications in the gene promoters related to CD44 and CD24 genes (201), further contributing to their stem-like characteristics. It is crucial to examine functional role of anastatic mechanisms for the formation and maintenance of CSCs by the prospective studies. Anastasis harnesses conventional prosurvival and pro-metastatic factors to impair the apoptosis process. Actuation of EMT and the related EMT modulators is considered as crucial aspect to modulate cell survival and for maintaining stemness (261, 262). For instance, among these modulators, SOX-9, a gene involved in tumor growth, angiogenesis, differentiation, and survival, plays a critical role. SOX-9 is also linked to chemoresistance against gemcitabine, underscoring its significance in chemoresistance and the need for exploring its role in anastasis subsequently to identify targeted therapeutic strategies to overcome these challenges (263, 264).

Understanding the molecular signatures of anastasis through preclinical or clinical studies is a significant aspect in cancer research (172). Anastasis was observed in mouse models where primary murine liver cells and NIH3T3 mouse embryonic fibroblast cells were exposed to ethanol to induce apoptosis. Following the treatment, cells were subjected to washing and allowed to recovery phase in apoptosis by exposing them into a fresh media (172, 184). Transcriptomic analysis from the cells obtained from recovery phase of this apoptosis revealed a significant upregulation of genes linked to survival pathways; these genes are BCL-2 family members (BAG-3, BCL-2, and MCL-1), XIAP, MDM2. In addition, the genes related to heat shock proteins including Dnajb1, Hsp90aa1, Hspa1b, and Hspb1 were found to be upregulated in this recovery phase (172, 184) (Figure 4). Notably, residual apoptotic markers dissipated during early phase of anastasis initiation. Inhibiting key survival factors such as Hsp90, MDM2, XIAP, and BCL-2 significantly impaired anastasis, highlighting the significant dependency on these cellular survival mechanisms (172, 184).

Cancer cell survival is not solely dependent on anti-apoptotic gene expression. It also relies on genes involved in regulating cell cycle, and DNA repair; In addition, the survival of these proliferating cancer cells relies on gene expression pertinent to angiogenesis, migration, and TGF-β modulation, and these genes are reported to be upregulated at the time of early anastasis phase (172). Previous reports on HeLa cells recovering from ethanol-induced apoptosis revealed significant increases in c-Fos, c-Jun, Klf4, and Snail-1 expression (Figure 4). These findings imply that the upregulated genes at the recovery phase and post-apoptosis induction are primarily linked to proliferation and pro-survival signaling (173). On the contrary, genes activated during late anastasis predominantly affect actin cytoskeleton rearrangement, and ribosome biogenesis (Figure 4) (173, 200). At the time of anastasis, the involvement of several survival factors includes MDM2 revealing that the p53 signaling implications include CDKs, expression of E2F and cell cycle factors (265). Other investigations into the AP-1 transcription factor actuation, which includes c-Jun and c-Fos; these genes are significantly involved in mediating pro-apoptotic and pro-survival factor expression. Hence, their actuation during anastasis offers insights into the balance between pro-survival and pro-apoptotic signaling which is yet to be explored vividly (266–268).

Many proteins that play roles in promoting cell proliferation and preventing apoptosis are also critical for recovering cells from death, which indicates that cancer cells can trigger survival mechanisms under stress conditions, resulting in significant molecular transformations that restore the cell to its normal state (173, 183). During anastasis, structural changes such as actuation of focal adhesion kinases and reorganization of the actin cytoskeleton (173, 183). Due to these changes, recovered cancer cells undergo migration. Furthermore, Snail-1 expression is significantly increased during the initial anastasis phase. When Snail-1 is silenced using shRNA, the recovery process, including migration, is impaired, leading to increased PARP-1 cleavage and sustained activation of cell death pathways (173, 183). Early phase of cell recovery involves the coordinated actuation of TGF-β signaling,

which is crucial for the survival of the cells followed by migration. Additionally, MMP-9, MMP-10, MMP-13, VEGF-A, and Angpt-l4 are other EMT and angiogenic factors that could play a significant functional role in promoting the cell recovery from cell death (173, 183).

Furthermore, EMT plays a crucial role in modulating the process of metastasis, anastasis, and stemness, making it a promising target for combinatorial drug design (81) (250, 269). Salinomycin, mocetinostat, and metformin could effectively target EMT and they were studied significantly for their potential to overcome chemoresistance when used alongside drugs like doxorubicin, 5-FU, and gemcitabine (270–276). Trichostatin A and vorinostat are HDAC inhibitors that can inhibit EMT by downregulating E-cadherin expression and modulating the expression of HIF-1α and NF-κB (277–280). In cancers with EGFR mutations, diindolylmethane (DIM) and its analogs have demonstrated potential in preventing EMT and metastasis, suggesting their suitability for use in combinatorial regimens along with DNA-damaging agents (281, 282). Combinatorial regimen of DIM with camptothecin can prevent drug-induced EMT in the murine models associated with Apc-floxed colorectal cancers (283). Recurrent administration of therapies such as chemo/radio-therapies can cause side effects by co-activating DNA repair followed by cell survival and EMT pathways, which are crucial in anastasis. For instance, the nuclear translocation of nucleases pertinent to apoptosis such as AIF and Endo-G are evident at the time of anastasis when observed during reversal of ethanol-mediated DNA damage (283).

Critical involvement of DNA damage response (DDR) proteins during anastasis yet require future studies for several other cancers. DDR proteins, such as ATM, ATR, and DNA-PK, are known to play pivotal roles in detecting and repairing DNA damage, thereby maintaining genomic stability. Their activation during anastasis suggests a potential mechanism by which cells recover from near-death states and restore proliferation (184). Given the established link between robust DNA repair mechanisms and poor cancer prognosis (284), it is imperative to further investigate how these pathways contribute to anastatic processes and chemoresistance. Elucidating these mechanisms may reveal new therapeutic targets to improve cancer treatment outcomes, particularly in tumors that exhibit high levels of DNA repair activity. Studies should focus on understanding the specific DDR pathways (284) involved in anastasis and their impact on the efficacy of chemotherapeutic agents, potentially leading to the development of novel strategies to overcome resistance.

Cancer cells exert chemoresistance when administered recurrent cytotoxic chemotherapeutics to several cancer types, and EMT plays a pivotal role in mitigating drug susceptibility (270, 285–287). At the time of treating cancer cells with DNA-damaging agents, specifically epithelial-origin cancer cells start expressing mesenchymal markers. As previously discussed, EMT is closely associated with survival factors, in addition, the drug-induced EMT leads to a significant activation of survival factors such as NF-κB, c-FLIP, survivin, as well as AKT. Notably, markers pertinent to early apoptosis were found to occur alongside EMT and cell survival proteins. Moreover, EMT modulator vimentin was shown to hinder process of apoptosis (283). EMT is significantly higher in drug-induced metastasis than in spontaneous metastasis. For instance, autochthonous breast cancer murine model was studied where transgenic mice with a Cre recombinase construct driven by the Fsp-1 promoter (288) which would express Cre recombinase in mesenchymal lineage cells undergoing EMT. This system was designed so that when the Fsp-1 promoter was actuated, the Cre recombinase could induce knock out of RFP in another transgenic construct that included GFP (288). Consequently, cells expressing GFP indicated EMT activation. According to this study, spontaneous lung metastasis had few GFP-positive cells, whereas cyclophosphamide-treated mice had significant GFP expression in metastatic lung nodules (288). GFP-positive cells confined to the regions of metastasis exhibited a higher chemoresistance than the GFP-negative cells (288). These findings highlight the complexity of drug-induced EMT and *in vivo* actuation of anastasis.

As previously discussed, NF-κB plays a critical role in promoting cell survival, making it a key target in emerging cancer therapies. Bortezomib, for instance, inhibits NF-κB by preventing the degradation of IκB, thereby suppressing NF-κB signaling pathways. This therapeutic approach in combinatorial regimen with temozolomide and paclitaxel resulted in promising results (289). Furthermore, several NF-κB inhibitors such as curcumin, BMS-345541, and bindarit exhibit significant efficacy (290). These compounds could be particularly effective when used as combinatorial regimen with DNA-damaging drug molecules to counteract anastasis, a process through which cells survive and recover from apoptosis phases. This combination strategy aims to enhance the efficacy of cancer treatments by targeting the molecular mechanisms involved in evading apoptosis and chemoresistance. Further research and clinical trials are essential to validate these findings and optimize combination therapies for better clinical outcomes.

7 Immunotherapy treatment with ferroptosis and chemoresistance

Previous studies reported the combinatorial regimen of immunotherapeutics along with novel ferroptosis modulators against cancers. Anti-cancer immunity is higher with GPX4 inhibitors against triple-negative breast cancers (TNBCs) specifically LAR subtype. The synergistic effect of GPX4 inhibition and PD-1 blockade demonstrates a higher efficacy compared to individual therapeutic interventions *in vivo* settings (291). In glioma, wherein ferroptosis serves as the predominant mechanism of programmed cell death (PCD), it fosters an immunosuppressive tumor microenvironment facilitated by tumor-associated macrophages, thereby promoting malignant progression and unfavorable clinical outcomes. Targeting ferroptosis inhibition is suggested as a promising strategy to augment the immune checkpoint blockade (ICB) efficacy (292).

In melanoma, a predictive model termed the Ferroptosis Score (FPS) was developed utilizing 32 ferroptosis-related genes to prognosticate cancer outcomes. Elevated FPS levels are indicative of a more dynamic tumor immune microenvironment and

enhanced responsiveness to immune checkpoint blockade (ICB), such as PD-1 blockade (293). BEBT-908, an inhibitor targeting PI3K and HDAC, induces immunogenic ferroptotic cell death in cancer cells, augmenting ICB therapy efficacy by upregulating MHC class I expression and activating IFN-γ signaling (294). Statins facilitate immunotherapy by inducing ferroptosis in non-small cell lung cancer cells while inhibiting PD-L1 expression (295). Inhibition of Ferroptosis Suppressor Protein 1 (FSP1) significantly induces ferroptosis in cancer cells and promotes immune cell infiltration, including dendritic cells, macrophages, and T cells. Combinatorial therapy with immunotherapy and FSP1 inhibition typically mitigates hepatocellular carcinoma burden *in vivo* (296). Inflammation-associated ferroptosis biomarkers positively correlate with PD-L1 expression, high microsatellite instability, tumor mutational burden, and ICB response rates (297). Short-term methionine starvation synergistically promotes ferroptosis with CD8+ T cells by stimulating CHAC1 transcription, enhancing ICB therapy. Combined treatment involving intermittent methionine starvation, system xc- inhibition and PD-1 blockade exhibits potent anti-tumor effects (298). Ferritin Light Chain (FTL) promotes cancer cell ferroptosis in glioblastoma, polarizing tumor-associated macrophages towards the M2-type, thus fostering a pro-tumor TME. Inhibiting FTL represents a promising strategy to sensitize glioblastoma to PD-1 blockade (299). However, further investigations are warranted to evaluate the efficacy of these ICBs in combination with ferroptosis modulators against chemoresistant cancers (21).

8 Conclusions and future prospects

Recent advancements in cancer research and development have significantly shifted the focus from merely identifying anti-cancer compounds to unraveling the intricate molecular mechanisms underpinning cell survival and drug resistance. Current studies described the molecular signaling pathways between tumors and their microenvironment, examining how these interactions affect cellular survival and resistance to therapy-induced apoptosis. This review highlights various cellular mechanisms that enable cancer cells to evade apoptosis and acquire oncogenic phenotypes. Two pivotal concepts emerging from this research are mitochondria-modulated ferroptosis in cancer cells, anastasis, which introduces a novel perspective on cancer-related therapies by fostering novel investigations on chemotherapeutics or immunotherapeutics to mitigate chemoresistant cancers.

As previously highlighted, ferroptosis is characterized by iron-dependent toxicity, excessive ROS accumulation, and consequent lipid peroxidation, disrupting cellular redox balance and culminating in membrane damage, ultimately triggering cell death (10, 102, 300). Excessive oxidative stress induces irreversible damage to mitochondrial function and integrity, leading to energy depletion and ferroptosis (88). Ferroptosis manifests distinct morphological, genetic, and biochemical features when compared to apoptosis, necrosis, and autophagy (12, 108, 301). Numerous studies have implicated ferroptosis in diverse biological processes and pathogeneses, prominently in cardiovascular diseases, brain lesions, renal impairment, and various malignancies, where it plays a pivotal regulatory role within the tumor microenvironment (63, 302–307).

Additionally, cancer cells exhibit a higher demand for iron metabolism compared to normal cells, rendering them more susceptible to ferroptosis induction (305, 308). Research further suggests a close nexus between ferroptosis and the inhibition of tumor cell growth. Activation of ferroptosis can impede cancer cell proliferation, thereby influencing the efficacy of tumor immunotherapy and patient prognosis (309–313). Consequently, targeting or inducing ferroptosis in cancer cells is emerging as a novel therapeutic strategy (314). This comprehensive review elucidated the intricate interplay between ferroptosis, mitochondria, and iron metabolism, underscoring the potential translational impact of these findings in oncology.

In prostate cancer (PCa), it has been proposed that the ferroptosis initiators erastin and RSL3 could profoundly inhibit PCa cell progression without adverse events (315–317). In pancreatic cancer, gemcitabine is the primary treatment, albeit with unsatisfactory efficacy (167). Heat shock protein family A5 (HSPA5) has been closely linked to the prognosis of gemcitabine-treated patients (318). Zhu et al. (318) suggested that HSPA5-GPX4 pathway modulation to contribute to chemoresistance in pancreatic cancer cells against gemcitabine, and blocking of HSPA5 or GPX4 gene expression potentially reversing this resistance. Ferroptosis has also been implicated in this process.

In hepatocellular carcinoma (HCC), sorafenib remains the principal chemotherapeutic drug. Sun et al. (319) found that sorafenib could induce HCC cell death by increasing oxidative stress. However, the ferroptosis inhibitor ferrostatin-1 could counteract this process, indicating the significance of ferroptosis in the mechanism of action of sorafenib. Furthermore, a combination of erastin, sorafenib, and haloperidol could induce a higher intracellular iron concentration, inducing the Fenton reaction and excessive ROS production, ultimately leading to HCC cell ferroptosis (320).

In TNBC, Ding et al. (321) demonstrated that the inhibitor DMOCPTL ubiquitinated GPX4 through EGR1 regulation, inducing TNBC cell ferroptosis. The fundamental mechanism of action and clinical value of ferroptosis remain incompletely understood. Indeed, potential valid ferroptosis-related biomarkers warrant further investigation. Moreover, it remains unclear in clinical treatment whether inducing ferroptosis in tumor cells would impair liver and kidney functions in patients. Developing ferroptosis-inducing agents capable of selectively eliminating chemoresistant tumor cells while sparing normal cells remains an area requiring further exploration. Basic research and clinical translation of ferroptosis encounters myriad uncertainties and obstacles. As foundational research on ferroptosis progresses, the clinical application of ferroptosis inducers and inhibitors may become viable. Thus, research endeavors focused on ferroptosis hold considerable potential to confer substantial benefits to cancer patients with chemoresistance in the future by exploring the interplay between ferroptosis, anastasis, and chemoresistance. Specifically, can the reversal of ferroptosis manifest *in vivo* within live animals? Tracking ferroptosis *in vivo* poses technical challenges,

given the morphological similarity between cells recovered from ferroptosis and healthy non-ferroptotic cells. Additionally, while cells undergoing reversal of ferroptosis or apoptosis must address distinct forms of cellular damage, it is plausible that they may share a common master regulator or signaling molecule initiating the process of anastasis. Moreover, elucidating the long-term physiological, pathological, and therapeutic aspects of anastasis modulators remains imperative (178).

The fundamental mechanism underlying ferroptosis involves the interplay of iron metabolism and lipid peroxides. The identification of FSP1 as a negative regulator expands our understanding of ferroptosis. Extensive investigations into ferroptosis have unveiled numerous potential therapeutic targets for combating chemoresistant tumors. These discoveries hold promise for the development of novel drugs and treatment modalities targeting these specific pathways. Presently, resistance to conventional chemotherapy, targeted therapy, or immunotherapy agents represents a major challenge in the management of refractory tumors or recurrent malignancies. Given the ubiquitous nature of chemoresistance mechanisms, tumors that exhibit resistance to one drug often display varying degrees of resistance to others within the same class, with some even demonstrating multidrug resistance, significantly compromising treatment efficacy and patient survival. Multiple studies have demonstrated that ferroptosis can enhance tumor cell death synergistically with anti-tumor drugs, thereby augmenting drug efficacy and potentially reversing drug resistance, offering a promising therapeutic avenue for patients with drug-resistant tumors. Notably, tumor cells exhibit heightened sensitivity to iron overload and ROS accumulation compared to normal cells, rendering targeting of iron ions a feasible approach to enhance treatment efficacy and mitigate side effects.

Integration of cross-disciplinary technologies, such as nano-vehicle agents (322) combined with ferroptosis, may represent a more tailored approach to tumor treatment. However, several challenges must be addressed before clinical application. Firstly, while numerous *in vitro* methods exist for assessing ferroptosis and anastasis, the development of sensitive and accurate *in vivo* evaluation techniques remains elusive. Secondly, the specificity and safety profile of compounds targeting ferroptosis regulators must be significantly evaluated in preclinical and clinical settings to minimize adverse effects. Lastly, further elucidation is warranted regarding which tumor types or characteristics, such as specific gene mutations, are particularly susceptible to ferroptosis. Nonetheless, comprehensive investigations into the molecular signaling underlying ferroptosis, anastasis, and tumor drug resistance hold considerable significance to revolutionize tumor diagnosis and treatment, offering new avenues for therapeutic intervention (9). Continued research is warranted to develop personalized combinatorial therapeutic regimens against cancers to modulate these molecular signaling that optimize therapeutic efficacy and overall survival.

Author contributions

YC: Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. CL: Writing – original draft, Formal analysis, Data curation. NB: Writing – original draft, Validation, Project administration, Data curation, Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. SE: Writing – review & editing, Project administration, Methodology, Investigation. ME: Writing – review & editing, Methodology, Investigation, Formal analysis. YF: Writing – original draft, Project administration, Methodology. XY: Writing – original draft, Software, Resources. BB: Writing – review & editing, Validation, Project administration. MH: Visualization, Resources, Project administration, Funding acquisition, Writing – review & editing, Supervision, Methodology. ZL: Validation, Software, Resources, Project administration, Methodology, Data curation, Conceptualization, Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Formal analysis.

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Conflict of interest

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Targeting ferroptosis: a new therapeutic opportunity for kidney diseases

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Ferroptosis is a form of non-apoptotic regulated cell death (RCD) that depends on iron and is characterized by the accumulation of lipid peroxides to lethal levels. Ferroptosis involves multiple pathways including redox balance, iron regulation, mitochondrial function, and amino acid, lipid, and glycometabolism. Furthermore, various disease-related signaling pathways also play a role in regulating the process of iron oxidation. In recent years, with the emergence of the concept of ferroptosis and the in-depth study of its mechanisms, ferroptosis is closely associated with various biological conditions related to kidney diseases, including kidney organ development, aging, immunity, and cancer. This article reviews the development of the concept of ferroptosis, the mechanisms of ferroptosis (including GSH-GPX4, FSP1-CoQ1, DHODH-CoQ10, GCH1-BH4, and MBOAT1/2 pathways), and the latest research progress on its involvement in kidney diseases. It summarizes research on ferroptosis in kidney diseases within the frameworks of metabolism, reactive oxygen biology, and iron biology. The article introduces key regulatory factors and mechanisms of ferroptosis in kidney diseases, as well as important concepts and major open questions in ferroptosis and related natural compounds. It is hoped that in future research, further breakthroughs can be made in understanding the regulation mechanism of ferroptosis and utilizing ferroptosis to promote treatments for kidney diseases, such as acute kidney injury(AKI), chronic kidney disease (CKD), diabetic nephropathy(DN), and renal cell carcinoma. This paves the way for a new approach to research, prevent, and treat clinical kidney diseases.

KEYWORDS

ferroptosis, iron metabolism, acute kidney disease, chronic kidney disease, natural compounds

1 Introduction

Cell death is a finely regulated process that occurs through different molecular pathways. Apoptosis is an active, programmed mode of cell death, while necrosis is classically defined as uncontrolled, accidental cell death (1). In recent years, various forms of cell death, including programmed necrosis, ferroptosis, pyroptosis, and mitochondria permeability transition-regulated necrosis, have gained attention (2, 3). The term "ferroptosis" was introduced by Dixon et al. in 2012 to describe a cell death form induced by the small molecule erastin (4). It inhibits cysteine import, leading to glutathione (GSH) depletion and glutathione peroxidase 4 (GPX4) lipid peroxidase inactivation. Lipid peroxidation is a downstream feature of ferroptosis, where the accumulation of lipid peroxidation products and reactive oxygen species (ROS) generated by iron metabolism lead to membrane integrity loss through unknown mechanisms (5). Ferroptosis exhibits distinct cellular morphology and function compared to necrosis, apoptosis, and autophagy. It does not display the typical morphological features of necrosis, such as swollen cytoplasm and organelle or cell membrane rupture, nor does it show the characteristic features of traditional apoptosis, such as cell shrinkage, chromatin condensation, and formation of apoptotic bodies (6, 7). Morphologically, ferroptosis is mainly characterized by mitochondrial shrinkage, increased membrane density, and reduction or disappearance of mitochondrial cristae, differentiating it from other cell death modes (8).

In recent years, there has been an increasing recognition of the importance of non-apoptotic cell death mechanisms in elucidating the molecular processes that regulate cell death. However, under normal physiological conditions, these alternative mechanisms of regulating cell death largely remain unknown (8). In this review, we focus on ferroptosis, a form of regulated cell death dependent on iron and involving lethal, iron-catalyzed lipid damage. Ferroptosis is significantly controlled by lipid repair systems including GSH and GPX4, as well as pharmacological interventions, and relies on various pro-survival enzyme reactions (9). Therefore, ferroptosis relies on the interplay of iron, sulfhydryl, and lipid metabolism pathways, with renal tissues particularly susceptible to oxidative-reductive imbalances (10, 11). Recent studies have confirmed the significant role of ferroptosis in the pathophysiology of various kidney diseases, emerging as a new focus of research in the field of renal fibrosis (12). Renal tubules are essential components of the kidney and are vulnerable to damage from factors such as hypoxia, toxins, metabolic disorders, and aging. In response to injury, renal tubular epithelial cells undergo morphological changes and secrete bioactive molecules, driving interstitial inflammation and fibrosis, ultimately leading to the development of chronic kidney disease (CKD) and end-stage renal disease (ESRD) (13, 14). Although specific targeted therapy for renal fibrosis is currently lacking, recent studies suggest that inhibiting ferroptosis may alleviate renal fibrosis. Researchers are investigating the mechanisms by which ferroptosis regulates renal fibrosis in the hope of developing new treatment strategies to delay disease progression, reduce the incidence of ESRD, and lower mortality rates (15–17). Additionally, there is increasing evidence indicating that ferroptosis plays a crucial role in the development of various other kidney diseases, including acute kidney injury (AKI), diabetic nephropathy (DN), renal cell carcinoma, polycystic kidney disease, among others (18–21).

However, there are still gaps in our understanding of the triggering, execution, and propagation mechanisms of ferroptosis in kidney diseases that require further research. This article summarizes current research on ferroptosis, its potential mechanisms, and its role in the progression of various kidney diseases, aiming to provide insights and information for the prevention and treatment of these devastating diseases (Table 1).

2 Overview of ferroptosis

The discovery process regarding ferroptosis began in 2001 when TAN et al. observed that exogenous glutamate inhibits cystine uptake through the cystine/glutamate antiporter system, depleting GSH and inducing an increase in ROS levels and intracellular Ca2+ influx, causing a form of programmed cell death in neuronal cells distinct from apoptosis (22). In 2003, Dolam et al. identified a compound, Erastin, through screening compounds with genotype-selective properties, that exhibited selective lethal effects on cells with RAS gene mutations. Erastin induces a non-apoptotic cell death pathway in RAS gene mutant cells (23). Subsequently, in 2008, Yang et al. identified two new compounds, Ras-selective lethal compound (RSL)3 and RSL5, which induced a non-apoptotic form of cell death similar to Erastin (24). In 2013, Dixon et al. discovered that Erastin triggered an iron-dependent, non-apoptotic form of cell death, and officially named this cell death mechanism "ferroptosis". Ferroptosis differs from apoptosis, necrosis, and autophagy: morphologically, there are no chromatin condensation and marginalization as in apoptosis, cytoplasmic and organelle swelling as in necrosis, and double-membrane-wrapped vesicles as in autophagy. A unique morphological feature of ferroptosis is that mitochondria appear smaller than normal, with increased membrane density (25). Further research has shown that Erastin and similar ferroptosis inducers induce cell death by reducing the synthesis of GSH, leading to the inactivation of GPX4. This results in increased lipid peroxidation, ultimately causing cell death. On the other hand, ferroptosis inducers such as RSL3 do not affect the concentration of GSH in cells, instead, they directly bind to GPX4, leading to increased lipid peroxidation and subsequent cell death (26). Cysteine is the rate-limiting substrate in the biosynthesis of reduced GSH in a biological system. It is either taken up by cells in its oxidized form (cystine) through the cysteine/glutamate antiporter (Xc- system) and the sodium-dependent neutral amino acid transporter B(0)AT1 (SLC6A19), or in its reduced form, cysteine, entering cells through neutral amino acid transporters, or being produced through transsulfuration from endogenous sources (27, 28). GSH is the most abundant reducing agent in mammalian cells, playing a crucial role in iron-sulfur cluster biogenesis. It also serves as a cofactor for various enzymes including glutathione peroxidase (GPX) and glutathione S-transferase (29). Genetically, it has been demonstrated that the GSH synthesis, the Xc- system, and GPX4 all contribute to

TABLE 1 Comparison of characteristics of different types of programmed cell death.

Types of cell death	Ferroptosis	Apoptosis	Necroptosis,	Pyroptosis,	Autophagy
Biological characteristics	Inhibition of system X_c^- and GPX4, reduced cysteine uptake, depletion of GSH, iron accumulation, lipid peroxidation	Nucleosomal DNA fragmentation, Caspase activation, mitochondrial membrane potential decrease	Decreased ATP levels, activation of RIP1 and RIP3	Activation of caspase-1-dependent inflammasome and release of pro-inflammatory cytokines	Formation and degradation of autophagosomes,
Morphological features	Cell swelling, increased mitochondrial membrane density, outer membrane rupture, reduced or lost mitochondrial cristae	Membrane blebbing, cell shrinkage, nuclear fragmentation	Plasma membrane rupture, cell swelling, organelle disarray, chromatin condensation	Plasma membrane rupture, organelle swelling, nuclear condensation	Formation of double-membrane lysosomes
Regulatory genes	ATP5G3, GPX4, Nrf2, RPL8, RAS,SLC7A11	Bcl-2, Bax, Bak, Caspase, P53, Fas	RIPK1,RIPK3, MLKL	Caspase-1, Gasdermins, NLRP3	mTOR, LC3, ATG5,Beclin 1, ULK1,AMPK
Inducers	Erastin, Glutamate, SAS, SRS, Sorafenib, Artemisinin	Apoptosis protein A, Hypoxia, Fasl, Staurosporine, UNC5B	TNFα, Fasl, TWEAK	ZnO-NPs, Ivermectin	Rapamycin, Simvastatin, Valproic acid, Thapsigargin
Inhibitors	Ferrostatin-1, Liproxstatin-1, Deferoxamine, VitE, U0126, DFO, CHX	NAIP, CTX1,c-IAP1/2, XIAP, ILP-2, Survivin, Z-VADFMK	Necrostatin-1, NSA, Kongensin-A	Necrosulfonamide	BafilomycinaA1,3-MA, LY294002, wortmannin, Spautin-1
Inflammatory features	Pro-inflammatory	anti-inflammatory	Pro-inflammatory	Pro-inflammatory	anti-inflammatory

protecting cells from death under various oxidative stress conditions, especially under conditions leading to sulfhydryl depletion, including the inhibition of Xc- system activity (30). With the establishment of the roles of GSH synthesis, the Xc- system, and GPX4 in ferroptosis, we can now contextualize all these early studies within the framework of iron-dependent cell death.

The concept of ferroptosis was first proposed by Dixon et al (31) in 2012 as an iron-dependent form of cell death characterized by intracellular ROS accumulation, distinct from apoptosis. Before the introduction of the term "ferroptosis", relevant inducers had already been discovered. In 2003, Dolma et al. first discovered a new compound, erastin, capable of killing tumor cells with mutant RAS oncogenes (23, 32). The cell death did not involve changes in the nucleus or activation of caspase-3. Subsequently, Yang et al. discovered another compound, the RSL3, which can induce this form of cell death. The cell death caused by these two compounds was later confirmed to be ferroptosis (24, 33).

As a form of RCD, ferroptosis differs from apoptosis, necrosis, autophagy, and other forms of cell death. Morphologically, cells undergoing ferroptosis exhibit significant mitochondrial contraction, increased mitochondrial membrane density, loss of mitochondrial cristae, and rupture of the mitochondrial outer membrane (8); Biochemically, inhibiting the cystine/glutamate antiporter system, known as system Xc on the cell membrane leads to a decrease in the activity of GPX4. This inhibition directly or indirectly results in intracellular depletion of GSH. Consequently, GPX4 is unable to utilize GSH to convert lipid hydroperoxides into lipid alcohols, leading to an inability to effectively clear ROS and lipid reactive species generated by lipid membrane damage, disruption of the mitochondrial electron transport chain, and potentially by iron release from iron-containing enzymes. This cascade eventually induces cell death, known as ferroptosis (8, 34). A pathway map of the current mechanism of ferroptosis is summarized in Figure 1.

2.1 The mechanism of ferroptosis

2.1.1 The GPX4/GSH signaling pathway in ferroptosis

GSH is the primary antioxidant involved in intracellular antioxidant stress, participating in numerous essential cellular metabolic activities such as the removal of ROS, DNA and protein synthesis, and signal transduction. Severe oxidative stress can cause damage to cellular lipids, proteins, DNA, and even lead to cell death. Oxidative stress can be induced in two main ways: directly increasing ROS levels or impairing antioxidant defense systems. Among the members of the GPX family, GPX4 acts as an inhibitory protein in lipid peroxidation. It reduces lipid hydroperoxides to lipid alcohols, preventing ROS accumulation and playing a role in inhibiting cell ferroptosis (35). GPX4's activity depends on system xc, a widely distributed amino acid antiporter in the phospholipid bilayer consisting of a light chain subunit (solute carrier family 7A11, *SLC7A11*) and a heavy chain subunit (solute carrier family 3 member 2, *SLC3A2*). System xc can mediate the exchange of extracellular cystine and intracellular glutamate across the cell membrane. Cysteine, derived from cystine, is a rate-limiting substrate for the synthesis of the antioxidant GSH (36). Therefore, the import of cystine through this transporter is crucial for GSH production and oxidative protection. Ferroptosis is primarily caused by an imbalance between the generation and degradation of intracellular lipid ROS within cells.

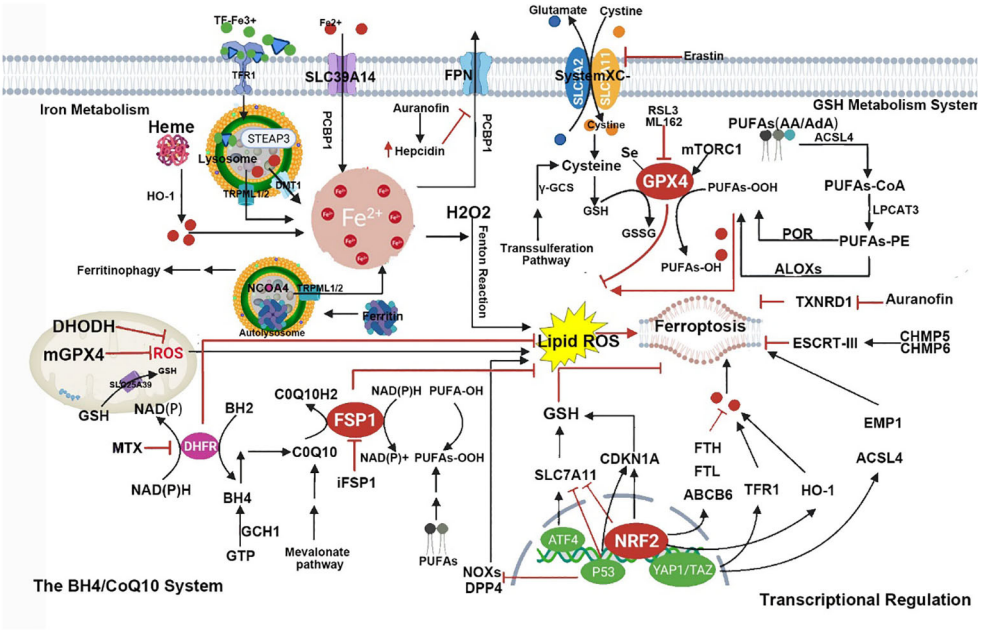

FIGURE 1
This figure shows the metabolic pathways involved in iron-dependent cell death. Iron-dependent lipid peroxidation drives iron death at the cellular level. Several aspects of iron metabolism, such as absorption, storage, and utilization, play important roles in regulating iron death. Additionally, activation of long-chain fatty acid CoA ligase 4 (LACS4), lysophosphatidyltransferase 5 (LPLAT5), lipid oxidase (LOX), or NADPH oxidase (NOX) in the lipid metabolism pathway promotes lipid peroxidation and iron death. The classic iron death suppression pathway involves the cysteine-glutamate reverse transporter (Xc-system), which induces the biosynthesis of GSH by facilitating cysteine (Cys) uptake. Using GSH as a cofactor, GPX4 reduces phospholipid hydroperoxides to their respective alcohols. The peroxidation of phospholipids can also be suppressed by the iron death inhibitor factor 1 (FSP1)-coenzyme Q10 (CoQ10) system. Furthermore, iron death is regulated by iron metabolism, including absorption, transport, storage, and utilization of iron. At the cellular level, non-heme iron enters cells through transferrin receptor 1 (TFR1)-mediated iron uptake by transferrin (TF) binding, or iron uptake independent of TF mediated by solute carrier family 39 member 14 (SLC39A14, also known as zinc transporter ZIP14). Additionally, iron engulfment mediated by heme degradation and nuclear receptor coactivator 4 (NCOA4) increases the labile iron pool (LIP), making cells more sensitive to iron death via the Fenton reaction. FPN, ferritin, Glu represents glutamate; GSSG, oxidized glutathione; HO1, heme oxygenase 1; KEAP1, kelch-like ECH-associated protein 1; NRF2, nuclear factor E2-related factor 2; PUFA, polyunsaturated fatty acid; PUFA-CoA, polyunsaturated fatty acid-coenzyme A; PUFA-PL, phospholipid containing polyunsaturated fatty acids (PUFA); and STEAP3, metalloreductase STEAP3.

Studies have shown that inhibiting system X_c^- using erastin and sulfasalazine in cancer cells cultivated in covered dishes results in a unique form of iron-dependent cell death known as ferroptosis (31). The small molecule erastin inhibits system X_c^-, impeding GSH absorption. GSH is a necessary cofactor for *GPX4* activity. Consequently, GPX4 activity decreases, reducing cellular antioxidant capacity, leading to lipid peroxidation accumulation and inducing oxidative cell death, known as ferroptosis. (1S,3R)-RSL3, also known as *RSL3*, and *ML162* (also known as DPI7) can deactivate GPX4, triggering ferroptosis in cells (33).

2.1.2 Ferroptosis and polyunsaturated fatty acids

Ferroptosis is characterized by lipid peroxidation, a process regulated by the system xc−/GPX4/GSH signaling pathway. Another condition for cells to undergo ferroptosis is the presence of polyunsaturated fatty acids (PUFAs), including arachidonic acid (AA) and docosapentaenoic acid. PUFAs contain easily extractable bis-allylic hydrogen atoms, making them prone to lipid peroxidation, which is necessary for executing ferroptosis (37). Therefore, the abundance and localization of PUFAs determine the extent of lipid peroxidation occurring in cells, thereby influencing the degree of ferroptosis's action. Free PUFAs serve as substrates for the synthesis of lipid signaling mediators, but they must be esterified into membrane phospholipids and undergo oxidation to become signals for ferroptosis. In cells undergoing ferroptosis, the AA is significantly depleted, and lipid fragments derived from AA are detected in the conditioned media of $GPX4^{-/-}$ mouse embryonic fibroblast cultures (34). Long-chain acyl CoA synthetase 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) encode enzymes involved in incorporating AA into membrane phospholipids. The absence of ACSL4 and LPCAT3 can prevent ferroptosis induced by GPX4 inhibitors RSL3 and ML162 (38). This indicates that the execution of cell ferroptosis in the presence of highly oxidative PUFAs such as AA can only occur after the direct or indirect (i.e., induced by GSH depletion) inactivation of GPX4 (35). Existing research suggests that this could be another potential point of regulation for ferroptosis, by modulating the enzymes involved in the biosynthesis of membrane phospholipids containing PUFAs to trigger or block ferroptosis (39, 40). The summary of metabolism and cell signaling in ferroptosis is shown in Figure 2.

2.1.3 Iron metabolism and ferroptosis

Iron is an essential trace element in the human body, playing multiple important biological roles, including inducing ATP

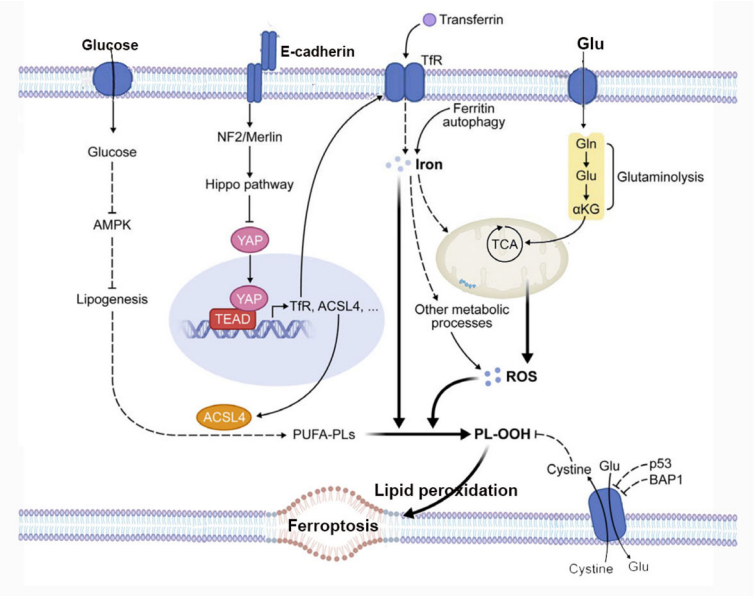

FIGURE 2
The figure depicts the regulation of ferroptosis by multiple metabolic events (such as lipogenesis, autophagy, and mitochondrial TCA cycle) and signaling pathways (such as E-cadherin-NF2-Hippo-YAP pathway, glucose-regulated AMPK signaling, and p53 and BAP1 tumor suppressor function). See text for details. TfR, transferrin receptor; PL-OOH, phospholipid hydroperoxide; PUFA-PL, phospholipid with polyunsaturated fatty acid chain; ROS, reactive oxygen species; TCA, mitochondrial TCA cycle; Gln, glutamine; Glu, glutamate; αKG, α-ketoglutarate.

production, participating in DNA and hemoglobin synthesis, and many other physiological activities (41). Due to its ability to accept and donate electrons, the accumulation of ferrous ions can lead to oxidative damage and even cell death (42). In mammalian cells, the absorption pathways for non-heme and heme iron involve various transport proteins or receptors, providing iron for subsequent lipid peroxidation processes. Elevated intracellular iron levels, particularly high ferrous ions levels, can lead to lipid peroxidation (43). Cellular iron homeostasis is closely related to the absorption, storage, circulation, and utilization of iron (44). In general, extracellular Fe3+ ions first bind to transferrin (TFR), and then enter the cell through the transferrin receptor 1 (TFR1) for storage in the form of the ferritin complex (primarily ferritin) (45).

Fe^{3+} ions are reduced to Fe^{2+} ions and then transported and stored in the cellular iron pool, while excess Fe^{2+} ions are stored in ferritin (46). Ferritin is a complex of iron storage proteins consisting of ferritin light chain and ferritin heavy chain 1 (FTH1). In cases of disrupted iron metabolism, low expression of FTH1 and overexpression of TFR1 often lead to excessive accumulation of Fe^{2+} ions, inducing the production and accumulation of large amounts of ROS through the Fenton reaction, ultimately promoting cell ferroptosis (47). With increased iron supplementation, tissue iron concentrations rise, potentially exceeding the body's binding capacity, leading to the formation of non-transferrin-bound iron (NTBI). Organs such as the liver and kidneys are sensitive to iron, and their absorption and clearance of iron differ from the reticuloendothelial system, possibly causing tissue iron deposition and iron overload (48). During the iron cycling process, NTBI and certain unstable ferrous species are prone to oxidation and reduction through Fenton and Haber-Weiss reactions, generating hydroxyl radicals (·OH). These radicals can damage large molecules such as lipids, proteins, and nucleic acids, causing oxidative stress (49). The products of lipid peroxidation chain reactions exhibit high biological activity (50). It can damage DNA, proteins, and enzyme activity, serving as molecular signals activating pathways that lead to cell death (51). Lipid peroxidation plays a driving role in ferroptosis and can be accomplished through non-enzymatic or enzymatic reactions. Compared to saturated fatty acids and monounsaturated fatty acids, PUFAs are more prone to lipid peroxidation and ferroptosis (52). The formation of PUFA coenzyme A derivatives is a necessary condition for initiating ferroptosis. Enzymes involved in regulating PUFA biosynthesis in membrane phospholipids can either trigger or prevent ferroptosis (52). Furthermore, studies have found that altering the intracellular iron content can change the sensitivity of cells to ferroptosis. Increasing transferrin and transferrin receptor-1 can boost cellular iron levels, thereby promoting ferroptosis (53).

TFRC is the gene encoding the transferrin receptor. The transferrin receptor is essential for cellular uptake of transferrin-iron complexes. Silencing the *TFRC* encoding gene can effectively prevent ferroptosis induced by erastin or cysteine deficiency. Changes in the transcription of iron-regulatory genes such as *IREB2*, *FBXL5*, *TFRC*, *FTH1*, and *FTL* affect the sensitivity to erastin-induced ferroptosis, with this sensitivity positively correlating with intracellular iron abundance (54). So far, the mechanism of iron ions in ferroptosis remains incompletely understood. While the independent redox action of iron ions cannot be entirely ruled out, the most plausible explanation for chelators preventing ferroptosis is by inhibiting iron ions from donating electrons to oxygen to generate ROS (55). Iron ions are

essential for the accumulation of lipid peroxides and the execution of ferroptosis. Therefore, the sensitivity of iron-induced cell death is influenced by iron intake, output, storage, and turnover. The Regulation of systemic iron homeostasis was shown in Figure 3. Iron metabolism was shown in Figure 4.

2.1.4 Ferroptosis and necroinflammation

Cellular ferroptosis triggers the innate immune system by releasing damage-associated molecules associated with inflammation. Immune cells stimulate inflammatory responses by recognizing the mechanisms of different patterns of cell death mechanisms (56). The ferroptosis inhibitor Ferrostatin-1 can avoid the exacerbation of kidney damage after ferroptosis by blocking the release of necrosis-related alarmone IL-33 and other chemokines and cytokines, thus preventing macrophage infiltration. This indicates the significant relationship between ferroptosis and inflammatory responses (57). When renal tubular epithelial cells undergo ferroptosis or other forms of RCD, their cellular contents are released in the form of DAMPs. These DAMPs bind to different molecular receptors on other cells in the interstitium, leading to the generation of an immune response known as necroinflammation, causing further damage via inflammatory reactions. This process contributes to further inflammation and damage due to the necroinflammatory response (58). Among the numerous damage-associated molecules released by necrotic cells, high-mobility group box 1 protein (HMGB1) receives significant attention. HMGB1 is a non-histone nuclear protein. When tissues or organs are damaged, it is released from damaged cells into the extracellular space or directly as a component of necrotic cell debris. It serves as a danger signal recognized by the immune system, thereby initiating an inflammatory response (59, 60). Current research indicates that HMGB1 can activate the TLR4-MyD88 signaling pathway (61) by binding to Toll-like receptors (TLRs), mainly TLR4. This activation leads to the phosphorylation of p38 MAPK, mediating the activation of mitogen-activated protein kinases (MAPK) and subsequent nuclear transcription. This process promotes the release of more inflammatory factors, thus exhibiting a pro-inflammatory effect. Past research has indicated that HMGB1 is a key regulatory factor in ferroptosis since HMGB1 translocation requires ROS-dependent signaling (62, 63). Ye et al. (64) conducted a study showing that in HL-60/NRASQ61L cells, the ferroptosis inducer erastin increases ROS levels, facilitates cytoplasmic translocation of HMGB1, and promotes cell death. The downregulation of HMGB1 reduces ROS generation induced by erastin and iron-mediated cell death in HL-60/NRASQ61L cells.

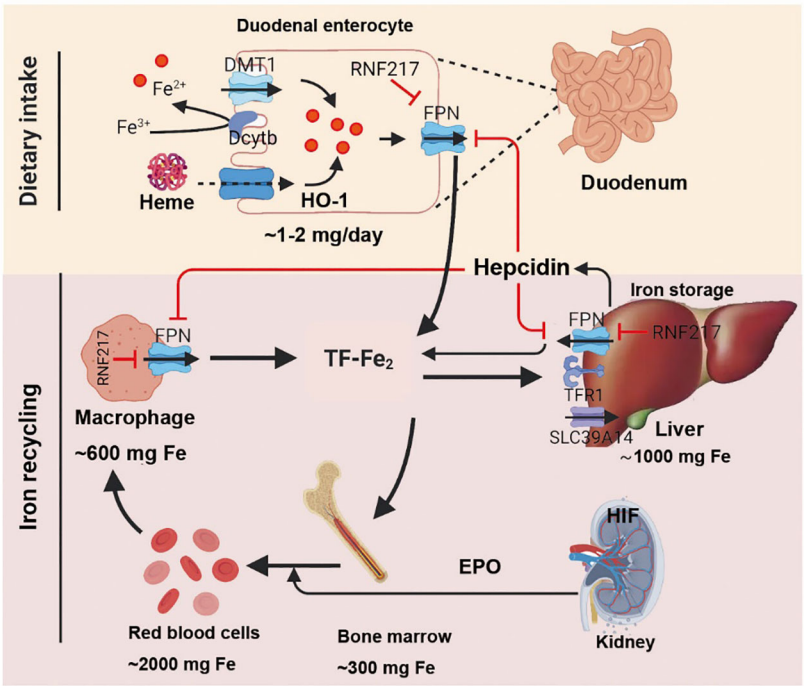

FIGURE 3
After intake of iron, Fe3+ is reduced by dcytb and then transported into enterocyte through DMT1. Dietary heme is absorbed by unknown mechanism and degraded in enterocyte by HO-1. Once exported by FPN, Fe3+ binds to transferrin (diferric transferrin, TF-Fe2), travels to tissues, and largely utilized in new red blood cells. Macrophage degraded senescent RBCs to recycle iron. Once needed, EPO, released by kidney, promotes erythropoiesis by HIF signaling pathway. The iron utilization of erythroid marrow and its recycling by macrophages represent the major iron circulation. Excess iron can be stored in hepatocytes through TFR1-mediated TF-Fe2 or SLC39A14-participated non-transferrin-bound iron (NTBI). The release of iron from enterocyte, red blood cells, and macrophages is precisely controlled by FPN, the body's sole iron exporter, to maintain a relatively stable iron level. The peptide hepcidin, the master regulator of systemic iron homeostasis, is a circulating hormone synthesized by the liver. Recently, we identified RNF217 as a novel E3 ligase for mediating FPN degradation. Dcytb, duodenal cytochrome b; DMT1, divalent metal transporter 1; EPO, erythropoietin; FPN, ferroportin; TFR1, transferrin receptor 1; HO-1, heme oxygenase 1; HIF, hypoxia induced factor; RBCs, red blood cells; NTBI, non-transferrin-bound iron.

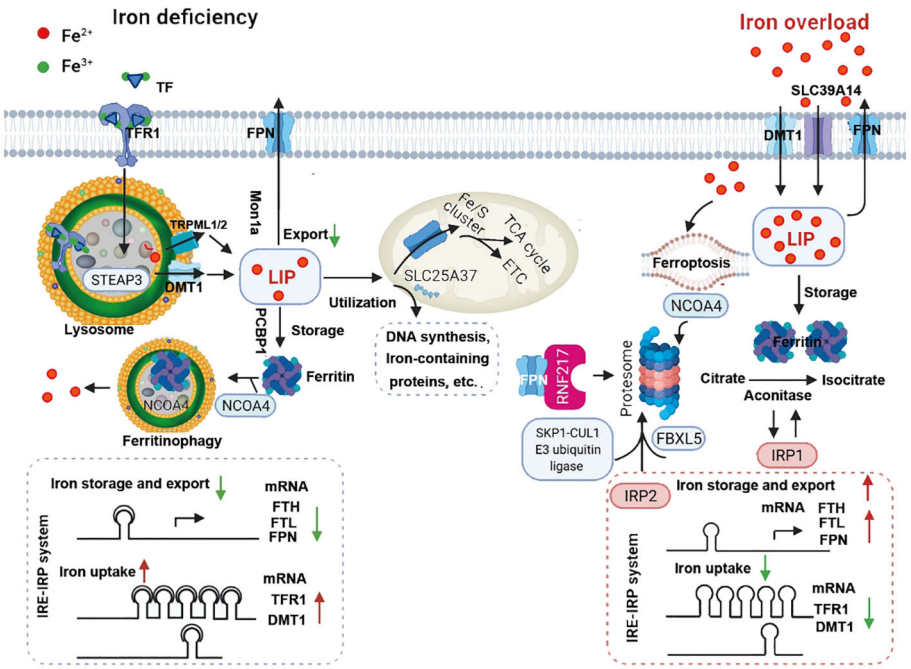

FIGURE 4
Under iron-deficient conditions (left), the majority of iron is bound to transferrin (TF), which binds to the transferrin receptor 1 (TFR1) at the cell surface followed by receptor-mediated endocytosis, resulting in ferric iron being released from TF and reduction to ferrous iron by an lysosomal reductase such as STEAP3. The ferrous iron is then transported into the lysosomal membrane by DMT1 and TRPML1/2, where it becomes part of the labile iron pool in the cytosol. Labile iron can be stored in the iron-storage protein ferritin or used to synthesize heme and iron-sulfur clusters in the mitochondria or in the cytosol. Iron can also be exported from the cell by the body's sole iron exporter, ferroportin (FPN). In addition, the IRE/IRP system regulates the expression of iron-related proteins such as TFR1, ferritin and FPN, upregulating TFR1 and DMT1 expression and downregulating FPN and FTH/FTL expression. During iron overload (right), hepcidin expression is upregulated by either the canonical bone morphogenetic protein (BMP)/SMAD pathway or by IL-6-pSTAT3 inflammatory signaling, which in turn limits iron absorption by increasing FPN degradation. In response to excess iron, BMP6, together with HJV, activates type 1 (Alk2/3)and type 2 (BMPR2, ACVR2A) BMP serine threonine kinase receptors to phosphorylate R-SMAD (receptor-activated SMAD), leading to activation of BMP/SMAD signaling pathway. High concentration of TF-Fe2 interact with TFR1, resulting in forming complex of TFR2/HJV/HFE to enhance the BMP/SMAD signaling in regulating hepcidin. TMPRSS6 inhibits BMP/SMAD signaling by cleaving HJV. The IRP system not only downregulates iron uptake-related genes such as TFR1 and DMT1 expression, it also upregulates FPN and FTH/FTL expression. IRP2 mediated by SKP1-CUL1 E3 ubiquitin ligase and NCOA4 are degraded, while IPR1 works as aconitase to convert citrate to isocitrate due to conformational change. RNF217 is a recently identified E3 ligase that regulates the degradation of FPN. ACVR2A, activin receptor type-2A; ALK, activin receptor-like kinase; BMP6, bone morphogenetic protein 6; BMPR2, bone morphogenetic protein receptor type 2; DMT1, divalent metal transporter 1; EPO, erythropoietin; ERFE, erythroferrone; ETC, electron transport chain; FBXL5, F-box/LRR-repeat protein 5; FPN, ferroportin; FTH, ferritin heavy chain; FTL, ferritin light chain; JAK, Janus kinase; LIP, labile iron pool; NCOA4, nuclear receptor coactivator 4; NTBI, non-transferrin-bound iron; HJV, hemojuvelin; IL-6, interleukin 6; IRE, iron-responsive elements; IRP, iron-regulatory proteins; SLC39A14, solute carrier family 39 member 14; SMAD4, SMAD family member 4; SMAD7, SMAD family member 7; STAT3, signal transducer and activator of transcription 3; STEAP3, six-transmembrane epithelial antigen of prostate 3; TCA cycle, tricarboxylic acid cycle; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2; TMPRSS6, transmembrane protease serine 6; TRPML1/2, Mucolipin TRP channel 1/2;UTRs, untranslated regions.

This suggests that the ferroptosis inducer erastin acts as an activator for HMGB1 cytoplasmic translocation and release and that HMGB1 is a crucial regulatory point in executing ferroptosis.

2.2 Other regulatory pathways and significance of ferroptosis

2.2.1 Regulation by System X_c^-

X_c^- is a cystine/glutamate antiporter protein that plays a crucial role in regulating cellular redox homeostasis (65). The system X_c^- consists of two subunits: SLC7A11 and SLC3A2, functioning to import cystine into the cell in exchange for glutamate (66). Cystine is then converted to cysteine, and cysteine serves as a precursor to synthesize GSH, an important intracellular antioxidant (67). The interaction mechanism between ferroptosis and the system X_c^- involves the regulation of cellular redox balance. The normal function of the system X_c^- is crucial for maintaining intracellular redox balance. When the system X_c^- is dysfunctional or inhibited, the levels of cysteine and GSH inside the cell decrease, leading to an exacerbation of intracellular oxidative stress, resulting in the accumulation of lipid peroxides and ultimately triggering ferroptosis (68, 69). Therefore, restoring or maintaining the normal function of the system X_c^- can help in maintaining intracellular redox balance, inhibiting the occurrence of ferroptosis, and potentially have therapeutic effects for various diseases, such as cerebral ischemia-reperfusion injury (CIRI) (70, 71).

2.2.2 FSP1/CoQ10/NADPH pathway

Ferroptosis suppressor protein 1(FSP1) is a protein capable of participating in iron-sulfur cluster modifications, transferring iron-sulfur clusters from mitochondria to target proteins (72). Coenzyme Q10 (CoQ10) is an antioxidant that can reduce the generation of free radicals and diminish the occurrence of oxidative stress reactions (73). Nicotinamide adenine dinucleotide phosphate (NADPH) is a reducing coenzyme that provides reducing power and participates in many metabolic pathways (74). Recent research indicates that FSP1 in the FSP1/CoQ10/NADPH pathway can protect mitochondria from oxidative stress damage during ferroptosis processes by regulating the synthesis and transfer of iron-sulfur clusters; CoQ10 reduces the generation of free radicals directly, thereby reducing the occurrence of oxidative stress reactions. NADPH provides reducing power, reducing the severity of oxidative stress reactions, thereby protecting cells from damage caused by oxidative stress reactions (72, 74). In conclusion, the FSP1/CoQ10/NADPH pathway regulates ferroptosis processes through various mechanisms, protecting cells from damage caused by ferroptosis. This discovery provides a new perspective and approach to the treatment of ferroptosis (74).

2.2.3 DHODH pathway

Dihydroorotate dehydrogenase (DHODH) is a mitochondrial inner membrane enzyme that plays a role in important metabolic pathways such as cytochrome P450, purine synthesis, and fatty acid metabolism (75, 76). Research indicates that the DHODH pathway can protect cells from oxidative stress and mitochondrial damage through various mechanisms, including downregulating ROS levels, maintaining mitochondrial membrane potential, and inhibiting cell apoptosis (77, 78). DHODH inhibits ROS production and increases mitochondrial membrane potential, thus preventing cells from undergoing mitochondrial permeability transition and apoptosis, safeguarding cells from oxidative stress and mitochondrial damage. This provides a novel approach and pathway to shield cells from ferroptosis (79, 80). The regulatory role of mitochondria in ferroptosis shown in Figure 5.

2.2.4 GTP cyclohydrolase 1 (GCH1)/ tetrahydrobiopterin (BH4)

The GCH1/BH4 pathway is an intracellular enzymatic pathway involved in various crucial cellular metabolic processes (81, 82). GCH1 is the rate-limiting enzyme for BH4, and the GCH1/BH4 pathway can regulate redox reactions and cell apoptosis, thereby protecting cells from the impact of oxidative stress and mitochondrial damage (83, 84). Recent research indicates that the GCH1/BH4 pathway also plays a critical role in ferroptosis, where GCH1 increases BH4 synthesis, decreases ROS levels, mitigates oxidative stress, and promotes cell survival. Furthermore, BH4 can regulate multiple cellular signaling pathways and metabolic pathways, further reducing oxidative stress and cell death, thereby protecting cells from the damage caused by ferroptosis (82, 83, 85).

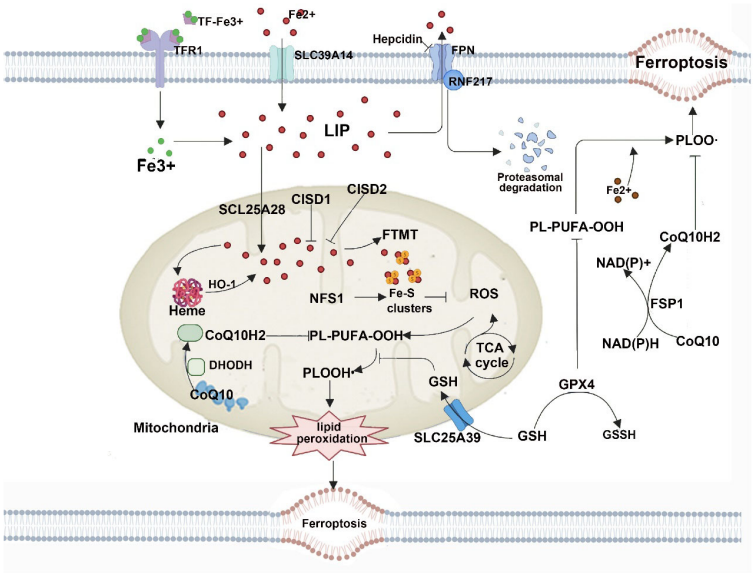

FIGURE 5
Mitochondria host a wide range of key metabolic processes (such as the tricarboxylic acid (TCA) cycle) and are a major source of reactive oxygen species (ROS). Separate mitochondria-localized defense systems have evolved to prevent mitochondrial lipid peroxidation and ferroptosis. For example, either the mitochondrial version of phospholipid hydroperoxide glutathione peroxidase 4 (GPX4) or dihydroorotate dehydrogenase (quinone), mitochondrial (DHODH) can specifically detoxify mitochondrial lipid peroxides. Moreover, the mitochondria-specific form of ferritin (FTMT) protects mitochondria from iron overload-induced oxidative injury, and mitoNEET (also known as CISD1) suppresses ferroptosis by limiting mitochondrial iron uptake. CoQ10, coenzyme Q10; FPN, ferroportin; FSP1, ferroptosis suppressor protein 1; GSH, glutathione; GSSG, glutathione disulfide; HO1, haem oxygenase 1; LIP, labile iron pool; PL-PUFA-OOH, polyunsaturated fatty acid-containing phospholipid hydroperoxides; PLOO·, phospholipid peroxyl radical; RNF217, E3 ubiquitin protein ligase RNF217; SLC25A39, probable mitochondrial glutathione transporter SLC25A39; SLC39A14, solute carrier family 39 member 14; TF, transferrin; TFR1, transferrin receptor protein 1.

2.2.5 Others

Currently, there are multiple endogenous defense pathways in cells to counteract ferroptosis, including *GSH-GPX4*, *FSP1-CoQ1*, *DHODH-CoQ10*, *GCH1-BH4*, as well as *MBOAT1/2* (86, 87). Lang et al. revealed through whole-genome CRISPR activation screening that MBOAT1 and MBOAT2 are novel inhibitors of ferroptosis (88). They suppress ferroptosis by reshaping phospholipids, a mechanism independent of *GPX4* or *FSP1* (88). The research also uncovered that transcription of *MBOAT1* and *MBOAT2* is upregulated by estrogen receptor (ER) and androgen receptor (AR), respectively. Inducing ferroptosis in combination with ER or AR antagonists significantly inhibits the growth of ER-positive breast cancer or AR-positive prostate cancer, offering a novel therapeutic approach for cancers with specific genetic backgrounds. Additionally, Interleukin 4 Induced Protein 1 (IL4i1) is an extracellular matrix enzyme that metabolizes tryptophan and its metabolites to regulate the intracellular redox balance (89, 90). Recent studies indicate that IL4i1 plays a crucial role in ferroptosis by modulating cellular redox balance through the metabolism of tryptophan and its derivatives. This helps in reducing ROS levels, mitigating oxidative stress, and protecting cells from ferroptosis-induced damage (91). The relationship between ferroptosis and IL4i1 is intricate, necessitating further in-depth exploration of their interactions and regulatory mechanisms to offer new insights and avenues for ferroptosis treatment.

2.3 The relationship between ferroptosis and other programmed cell death responses

Ferroptosis is intricately linked to biological processes, including autophagy, endoplasmic reticulum stress, and inflammation, among others, and may be involved in the reciprocal regulation of recalcitrant diseases. Therefore, a deeper exploration of the relationship and regulatory effects between these processes can offer a solid foundation for disease treatment.

2.3.1 The relationship between ferroptosis and autophagy

Appropriate autophagy has evolved into a pro-survival response for cells, but excessive autophagy, especially selective autophagy, and impaired lysosomal activity may promote cellular ferroptosis (92). The degradation of ferritin can be completed through ferritinophagy, a selective autophagy process mediated by Nuclear Receptor Co-activator 4 (NCOA4). Knocking out NCOA4 can inhibit ferritin degradation, preventing ferroptosis caused by free iron in fibroblasts and pancreatic cancer cells, thereby directly linking autophagy to ferroptosis (92). The autophagy-dependent lysosomal degradation of ferritin also enhances artemisinin-induced ferroptosis in cancer cells, which is another mechanism during ferroptosis that leads to ferritin degradation (93). Clockophagy is a recently discovered form of selective autophagy. *ARNTL* is a circadian rhythm transcription factor that inhibits the transcription of Egln2 and activates the survival transcription factor HIF1A, thereby inhibiting ferroptosis. Targeting this novel ARNTL-EGLN1-HIF1A pathway may enhance the anticancer activity of ferroptosis inducers (94).

2.3.2 Ferroptosis and endoplasmic reticulum stress

Dixon et al. found that Erastin induces endoplasmic reticulum stress by activating the PERK-eIF2α-ATF4-CHOP pathway and upregulating the expression of the apoptotic protein PUMA, indicating that endoplasmic reticulum stress may be involved in ferroptosis (95). Furthermore, HSPA5 is a molecular chaperone associated with endoplasmic reticulum stress that can bind to GPX4 to inhibit protein kinase-induced degradation of GPX4, thereby suppressing ferroptosis in pancreatic cancer cells. Chen et al. (96) identified elevated levels of ATF4 in human glioblastoma, and pharmacological or genetic inhibition of System X_c^- can attenuate ATF4-induced cancer cell proliferation. Additionally, ATF4 promotes tumor-mediated neurotoxicity and tumor angiogenesis, which can be alleviated by ferroptosis inducers such as Erastin and RSL3. Therefore, inhibition of ATF4 may be an effective target for reducing tumor growth by sensitizing cancer cells to ferroptotic cell death.

2.3.3 Ferroptosisis and inflammation

Unlike immunologically silent apoptosis, ferroptosis is immunogenic, as cells undergoing ferroptosis release cell contents including DAMPs and alarm proteins due to plasma membrane rupture, amplifying cell death and triggering a cascade of inflammation-related responses (97). In a mouse model of crystal-induced acute kidney injury (AKI), inhibitors of ferroptosis suppressed the expression of pro-inflammatory cytokines and the infiltration of neutrophils into the damaged tissue (98). Kang et al. (99) demonstrated that Gpx4 expressed in myeloid cells plays a crucial role in lipid peroxidation, inflammasome activation, and release of DAMPs in the setting of sepsis, with Gpx4 deletion leading to increased lethality in sepsis conditions. Qi et al. (100) found that in a mouse model of nonalcoholic steatohepatitis (NASH) induced by methionine/choline deficiency (MCD) feeding, levels of inflammatory cytokines including TNF-α, IL-1β, and IL-6 protein increased significantly after treatment with the ferroptosis inducer RSL3. However, mice fed with an MCD diet and treated with sodium selenite (a GPX4 activator) showed elevated hepatic GPX4 levels, reduced lipid peroxidation, and decreased severity of NASH.

3 Ferroptosis inducers and inhibitors

Ferroptosis inducers and inhibitors act by modulating key mechanisms in the ferroptosis pathway mentioned above to respectively promote or inhibit iron-dependent cell death (24, 74, 98, 101–108). Inducers mainly include (1) small molecules and drug inducers targeting iron metabolism: such as Erastin, Temozolomide (TMZ), and small molecules like MMRi62. (2) Small molecules and drug inducers targeting lipid metabolism: such as the anti-cancer

drug sorafenib, and inhibitors of cardiolipin oxidation like XJB-5–131 and JP4–039. (3) Small molecules and drug inducers targeting the GSH/GPX4 axis: such as RSL3 and RSL5, ML162, DPI7, and DPI10. (4) Small molecules and drug inducers targeting the FSP1/CoQ-related pathway: NDP4928, FIN56, etc. (5)Small molecules and drug inducers targeting other pathways include brequinar, dexamethasone, etc. Inhibitors primarily include (1) Small molecule inhibitors that lower iron levels, such as ciclopirox olamine (CPX), deferiprone (DFP), and deferasirox (DFX). (2) Small molecule inhibitors used to reduce lipid peroxidation include Ferrostatin-1 (Fer-1), α-Tocopherol (Vitamin E), SRS15–72B, SRS15–72A, SRS16–80, and SRS16–86, among others. (3) Small molecule inhibitors affecting the GSH/GPX4 axis: such as β-mercaptoethanol (β-ME), 2-amino-5-chloro-N, 3-dimethylbenzamide (CDDO), a triterpenoid compound, etc. Next, let's summarize a few representative drugs from the list.

3.1 Ferroptosis inducers

Erastin, first reported in 2003 (102), was the earliest discovered ferroptosis inducer. It was later confirmed that Erastin binds to and blocks the transport of cysteine by the cystine/glutamate antiporter, leading to intracellular depletion of GSH and triggering iron-dependent cell death. Affinity purification and mass spectrometry analysis have shown that Erastin interacts with voltage-dependent anion channel 2 (VDAC2) (103), reducing the permeability of VDAC2 to the reduced form of nicotinamide adenine dinucleotide (NADH), altering its ion selectivity, and disrupting mitochondrial respiratory chain oxidative phosphorylation (104).

3.2 Ras selective lethal compound 3

Ras-selective lethal small molecule 3 (RSL3) was first reported in 2008 (24), and it wasn't until 2014 that Yang et al. discovered that GPX4 is the target protein of RSL3, revealing this key pathway molecule (105). RSL3 directly inhibits GPX4, leading to an imbalance in the intracellular redox system and triggering ferroptosis. Alongside RSL3, RSL5 was discovered to induce iron-dependent cell death by targeting VDAC (24).

3.3 The inhibitor of ferroptosis inhibitor 1

Bersuker et al. discovered that Apoptosis-Inducing Factor Mitochondria-Associated 2(AIFM2) can reduce cellular sensitivity to iron-dependent cell death by decreasing Coenzyme Q10 (CoQ10) level, thereby exhibiting an anti-ferroptotic effect independent of GPX4 (106). It was consequently renamed Ferroptosis Suppressor Protein 1 (FSP1). Meanwhile, Doll et al. found that the inhibitor of FSP1, iFSP1, can induce selective ferroptosis in GPX4 knockout cells overexpressing FSP1. As a novel inducer of ferroptosis, the mechanisms of iFSP1 are worthy of further exploration (74).

3.4 Ferroptosis inhibitors 1(Fer-1)

Fer-1 is a ferroptosis inhibitor obtained through high-throughput screening. It captures lipid peroxides through its lipophilic properties, downregulating prostaglandin-endoperoxide synthase 2 (PTGS2), upregulating GPX4, and nuclear factor erythroid 2-like 2 (NFE2L2) (107). Linkermann et al (98) discovered a third-generation Fer compound, SRS-16–86, which has superior plasma stability and stronger inhibition of ferroptosis compared to Fer-1.

2.5 Liproxstatin-1(Lip-1)

Lip-1 is a specific inhibitor of LPO, and its mechanism of action in clearing LPO is similar to that of Fer-1. Lip-1 easily stays within the lipid bilayer, and the free radicals formed after clearing LPO can be reduced by other antioxidants in the body through targeted contact. Research indicates that the aromatic amine structure is essential for Liproxstatin class compounds to reduce peroxides (108).

2.6 Others

Other ferroptosis inhibitors include iron chelators like deferoxamine (DFO), ciclopirox olamine (CPX), and antioxidants like vitamin E. They primarily function by reducing iron levels and inhibiting oxidative stress to prevent cell death (109–111).

4 Ferroptosis and kidney disease

Ferroptosis-related diseases that can present throughout the human lifespan are present in Figure 6.

4.1 Ferroptosis and DN

The pathogenesis of DN involves multiple factors, including dysregulation of glucose and lipid metabolism, oxidative stress, accumulation of advanced glycation end-products (AGEs), inflammation, activation of the innate immune system, genetic susceptibility, activation of the renin-angiotensin system, and miRNAs (112–114).

Recently, researchers have identified hub genes (FPR3, C3AR1, CD14, ITGB2, RAC2, and ITGAM) associated with ferroptosis in diabetic kidney disease through gene differential expression analysis in patients. Non-coding genes (hsa-miR-572, hsa-miR-29a-3p, hsa-miR-29b-3p, hsa-miR-208a-3p, hsa-miR-153–3p, and hsa-miR-29c-3p) and transcription factors (HIF1α, KLF4, KLF5, RUNX1, SP1, VDR, and WT1) that interact with these hub genes may also be relevant to diabetic kidney disease (115). This further deepens our understanding of the molecular mechanisms involved in the pathogenesis of diabetic kidney disease. In patients with type 2

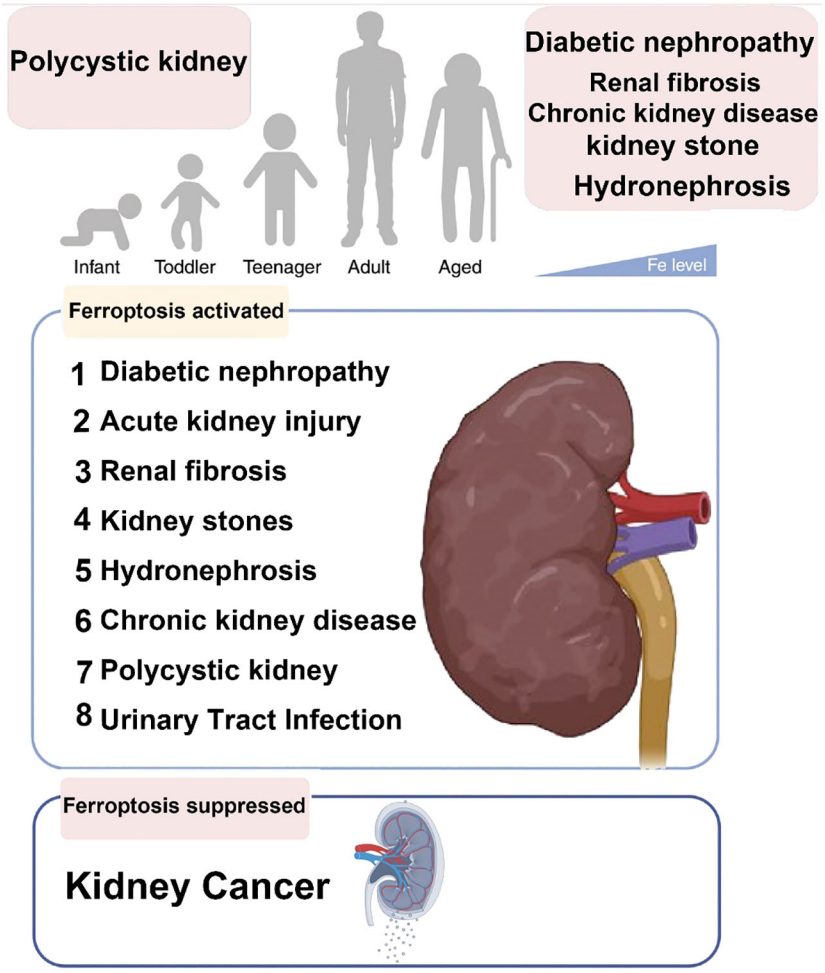

FIGURE 6
Ferroptosis-related diseases that can present throughout the human lifespan.

diabetes kidney disease, ferritin levels are elevated, along with significantly increased ROS and MDA levels. The expression of ferroptosis-related proteins ACSL4, PTGS2, and NOX1 is elevated, while GPX4 levels are reduced. In a diabetic kidney disease model, it was observed that the renal tissue shows a significant increase in iron ion content, elevated levels of MDA and 4-hydroxynonenal (4-HNE), decreased GSH, markedly decreased FTH1 expression, significantly increased TFR-1 expression. Transmission electron microscopy revealed mitochondrial membrane rupture, fragmentation, and mitochondrial cristae fragmentation and disappearance. Treatment with Ferrostatin-1 improved the iron overload, accumulation of lipid peroxidation, and antioxidant levels associated with ferroptosis. Additionally, there was a significant decrease in urinary protein, urinary creatinine, and urinary protein/creatinine ratio. This leads to significant reductions in urinary protein, urinary creatinine, and urinary protein/creatinine ratio, along with improvements in glomerular changes, tubular epithelial degeneration with loss of brush border, tubular luminal dilation, folding and fracturing of the tubular basement membrane, renal fibrosis area, and collagen content associated with diabetic kidney disease (116, 117). In high glucose-induced models of renal tubular epithelial cells, mesangial cells, and podocytes, ferroptosis phenomena were also observed. Furthermore, in tubular cells stimulated with TGF-β1, a decrease in GSH concentration and enhanced lipid peroxidation, which are characteristic changes of ferroptosis, were observed.

Ferrostatin-1 was able to alleviate TGF-β1-induced ferroptosis in these cells (117). The above data indicate that ferroptosis contributes to the exacerbation of diabetic kidney injury and kidney fibrosis. Inhibiting ferroptosis may help improve the structure and function of the kidney. Ferroptosis plays a significant role in the advancement of diabetic kidney disease.

4.1.1 The involvement of ferroptosis in the mechanism of DN

Research suggests that ferroptosis leads to the development of DN by inducing injury to renal tubules (117–120), glomeruli (121–126), and kidney fibrosis (127, 128). Tubular injury is a key factor in the development of DN, as high glucose levels trigger renal tubular cell iron overload, reduced antioxidant capacity, excessive ROS production, and lipid peroxidation (129). Animal studies have shown that in the kidneys of mice induced with DN models by

streptozotocin and diabetic (db/db) mice, there is an increase in iron content, particularly in the renal tubules. The inhibitor of acyl-CoA synthetase long-chain family 4 (ACSL4), rosiglitazone, improves renal function in DN model mice, reducing lipid peroxidation products and iron content. These effects are related to the alleviation of ferroptosis (118). Mesangial cells in the glomerulus are a special type of smooth muscle cell located between the capillary loops of the glomerular capillaries. Their injury is a fundamental pathological change in DN (123).

Recent studies have indicated that ferroptosis is involved in renal fibrosis in DN (117, 118). The latter represents the ultimate pathological change in DN (130). In patients with DN, serum ferritin, and lactate dehydrogenase release both increase (124); In kidney biopsy specimens, the expression of xCT and GPX4 mRNA decreases (119). A low iron diet or iron chelators can delay the progression of DN in rats (131). Recent studies have suggested that inhibiting ferroptosis may be a novel approach to exploring the progression and treatment of DN (117–119, 124, 131). In conclusion, ferroptosis is involved in the pathogenesis of DN, and targeting the inhibition of ferroptosis holds promise as a new pathway for treating DN.

4.1.2 Targeting the inhibition of ferroptosis for the treatment of DN

Exploration has been conducted on inhibiting ferroptosis as a therapy for DN. Current research indicates that certain active ingredients from natural plant ingredients can target the inhibit ferroptosis, thereby attenuating diabetes-induced tubular and glomerular damage as well as renal fibrosis, ultimately serving as a potential treatment for DN (132–134). Studies suggest that certain drugs or bioactive substances can mediate the occurrence and development of DN by modulating ferroptosis (135). Nobiletin (136) upregulates NRF2, enhancing the antioxidant stress capacity in the kidneys of diabetic mice. Increased expression of FTH-1 and downregulation of TFR-1 help ameliorate iron deposition in the kidneys of diabetic mice. Moreover, pretreatment with nobiletin can reverse mitochondrial morphological changes induced by diabetic ferroptosis, delaying the progression of DN. The ACSL4 inhibitor rosiglitazone (Rosi) can also alleviate diabetic kidney damage by inhibiting ferroptosis (135). The role of certain traditional Chinese herbs in regulating ferroptosis is also noteworthy. Gimatinib inhibits podocyte ferroptosis by modulating the mmu_circRNA_0000309/miR-188–3p/GPX4 signaling axis, thus improving diabetic kidney damage (137). Berberine can significantly improve the levels of ROS and GSH in podocytes induced by high glucose, upregulate NRF2 expression, and thereby alleviate podocyte ferroptosis (138). Furthermore, Aloe-emodin can significantly improve oxidative stress responses, downregulate the expression of HMOX-1 and NRF2, and inhibit ferroptosis levels (139). HMGB1 regulates mesangial cell ferroptosis induced by high glucose through the NRF2 pathway (124). Upregulation of Prdx6 expression mediated by Sp1 can alleviate oxidative stress and ferroptosis, preventing podocyte damage in DN (126). Furthermore, certain endogenous active peptides like salicin-β can promote high glucose-induced ferroptosis in HK-2 cells by regulating gene expression of antioxidant systems (GPX4 and SLC7A1) and iron metabolism regulatory systems (FTH-1 and TFR-1) (129). These studies suggest that in the future, regulating DN from the perspective of iron cell death may provide new insights for the treatment of diabetic kidney disease.

4.1.3 Targeting ferroptosis to improve tubular damage in DN

Ferroptosis mediates tubular injury in DN, suggesting that inhibiting tubular injury ferroptosis may provide a therapeutic approach for DN. Licorice may exert therapeutic effects on diabetes and its complications through anti-inflammatory or antioxidant mechanisms (140). Glabridin, a flavonoid extracted from the natural plant component licorice, can promote tubular epithelial cell survival by increasing the activity of superoxide dismutase (SOD) and GSH in NRK-52E cells, upregulating the expression of GPX4, SLC7A11, and SLC3A2, reducing malondialdehyde and iron concentrations, lowering TfR1 expression to inhibit ferroptosis in DN. Similarly, the flavonoid calycosin, which also possesses antioxidant and anti-inflammatory properties, prevents high glucose-induced cell ferroptotic damage by upregulating the GSH/GPX4 pathway in human renal proximal tubular epithelial cells (HK-2 cells), reducing LPO, and inhibiting the expression of nuclear receptor coactivator 4 (NCOA4) (141). Umbelliferone (142) and platycodin D (143) protect renal tubules by inhibiting iron cell ferroptosis in HK-2 cells and blocking cell damage induced by high glucose. Therefore, the above-mentioned compounds may exert therapeutic effects on DN by inhibiting ferroptosis in renal tubular cells.

4.1.4 Targeting ferroptosis to improve glomerular injury in DN

Mesangial cells are a special type of smooth muscle cell distributed between the capillary loops of glomerular capillaries. Their injury is a basic pathological change in DN renal damage (123). *In vitro*, experimental results demonstrate that the ferroptosis inducers erastin and high glucose both induce ferroptosis in the mesangial cells of the glomeruli. High glucose and erastin significantly induce LDH release, promote the expression of ACSL4, cyclooxygenase 2, and NADPH oxidase 1, and decrease GPX4 levels. Conversely, iron chelators reverse the glucose-induced LDH release and alterations in ferroptosis-related genes in mouse mesangial cells, indicating that high glucose can induce ferroptosis in mesangial cells (124). High glucose can induce ferroptosis in podocytes, leading to podocyte injury (126). Berberine in high glucose-induced podocytes inhibits ROS production, promotes GSH generation, upregulates the expression of nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), GPX4, and podocin, and decreases the levels of cyclooxygenase 2 and ACSL4. This alleviates podocyte cytoplasmic membrane foaming and mitochondrial shrinkage under high glucose conditions. By activating the Nrf2/HO-1/GPX4 pathway, it inhibits ferroptosis in podocytes, thereby exerting its renal protective effect (144). Glycyrrhizic acid primarily exerts renal protective effects by upregulating GPX4 and inhibiting ROS production to inhibit high glucose-induced iron cell death in podocytes (145). In conclusion, the active ingredients of the aforementioned natural plant

ingredients may improve glomerular damage caused by DN by inhibiting ferroptosis.

4.1.5 Targeting ferroptosis to improve renal fibrosis in DN

Studies indicate that ferroptosis is involved in renal fibrosis in DN, which is the final pathological change in DN (130). In a diabetic rat model induced by a high-sugar diet and streptozotocin, Sirius Red staining revealed that liquiritigenin improved kidney function, and inhibited renal interstitial fibrosis. This is related to its promotion of SOD and GSH activity, upregulation of GPX4, SLC7A11, and SLC3A2 expression, reduction of malondialdehyde content and iron concentration, as well as downregulation of TFR1 expression. These findings indicate that liquiritigenin improves renal fibrosis in DN by inhibiting ferroptosis (131). In db/db diabetic mouse models, hesperetin can upregulate GPX4 expression, inhibit LPO and NCOA4 expression, and suppress collagen deposition in renal tissue. This indicates that hesperetin may reduce collagen deposition and renal fibrosis by inhibiting ferroptosis (140). The above-mentioned compounds may improve renal fibrosis in DN by inhibiting ferroptosis.

Since the involvement of ferroptosis in the pathogenesis of DN was identified in 2020, scholars have started to explore drugs targeting the inhibition of ferroptosis for the treatment of DN. Some active ingredients from natural plant components can alleviate tubular and glomerular injury as well as renal fibrosis induced by high glucose and diabetes by selectively inhibiting ferroptosis, thereby serving as a treatment for DN. Although exploratory experimental studies currently indicate that ferroptosis is involved in the pathology of DN and can be targeted and intervened pharmacologically, the detailed mechanism still needs to be elucidated. The broad application prospects also require further elucidation. In conclusion, ferroptosis plays a significant role in the progression of DN, and targeting ferroptosis is a promising therapeutic approach for treating DN. It is a potential treatment approach with hopeful prospects.

4.2 Ferroptosis and AKI

AKI has always been a severely debilitating disease worldwide, and it remains a focus of clinical research (146). In recent years, the incidence and mortality rate of AKI has been on the rise. AKI has been associated with acute changes in kidney function and long-term prognosis, including progression to CKD, cardiovascular disease, persistent function, and even death (147, 148). AKI is caused by a variety of factors. Prerenal AKI refers to AKI caused by inadequate renal perfusion. AKI caused by renal parenchymal injury is named based on the location of the injury (glomerular, tubular, or interstitial) (149). The pathophysiological response to AKI may determine whether kidney function is recovered or progresses to CKD. Regeneration of tubular epithelial cells promotes recovery, whereas interstitial fibrosis and loss of renal capillaries are associated with progression to CKD (150). Experimental models have demonstrated that AKI can lead to chronic damage of renal parenchyma, resulting in CKD, indicating that early intervention may impact long-term outcomes (151). However, the specific mechanisms of AKI occurrence and development of AKI are not yet clear. Currently, there are no effective treatment methods to prevent the occurrence of AKI.

4.2.1 AKI caused by ischemia-reperfusion injury

In ischemia-reperfusion injury-induced AKI, apoptosis has long been considered the primary mechanism of cell death. However, using apoptosis-related inhibitors has not been effective in blocking the occurrence of AKI (152). On the contrary, using ferroptosis inhibitors such as liproxstatin-1 has been shown to alleviate tissue damage caused by ischemia-reperfusion and significantly protect kidney function (153). Pannexin-1 is a member of the ATP-release pathway protein family. Research (154) has shown that silencing pannexin-1 can promote the expression of the intracellular antioxidant enzyme HO-1 and inhibit ferroptosis through the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, thereby reducing ischemia-reperfusion injury in the kidneys. Irisin is an exercise-induced hormone that can improve mitochondrial function and reduce the production of ROS. Research (155) has found that treatment with irisin can significantly alleviate the inflammatory response, endoplasmic reticulum stress, and oxidative stress in mice with renal ischemia-reperfusion injury. Its mechanism of action may be related to the upregulation of GPX4 expression. Quercetin is a natural flavonoid compound known for its pharmacological properties such as antioxidant, anti-inflammatory, and anti-aging effects. Research (156) has shown that quercetin can inhibit ferroptosis in renal tubular epithelial cells by downregulating the expression of activating transcription factor 3 gene, leading to a significant increase in the expression of SLC7A11 and GPX4. This conclusion has been validated by studies on the regulatory role of microRNAs (miRNAs, miR) on ischemia-reperfusion-induced renal injury in rats (157). Ischemia-reperfusion induced upregulation of miR-182–5p and miR-378a-3p, leading to activation of ferroptosis in kidney injury through downregulation of GPX4 and SLC7A11. Therefore, ferroptosis may be the primary pathway through which ischemia-reperfusion injury triggers AKI.

4.2.2 AKI caused by cisplatin

Cisplatin is a widely used anti-tumor drug, and its main adverse effect is severe nephrotoxicity (158). Researchers have long been striving to elucidate the mechanisms underlying cisplatin-induced nephrotoxicity to better utilize this therapeutic drug. GPX4 is significantly downregulated in cisplatin-induced AKI, while ferroptosis biomarkers 4-hydroxynonenal and malondialdehyde are upregulated. This indicates that ferroptosis plays an important role in cisplatin-induced AKI (159). The Vitamin D receptor agonist paricalcitol can prevent cisplatin-induced AKI by reducing lipid peroxidation and reversing GPX4 downregulation (160). This is similar to the mechanism of mangiferin in treating cisplatin-induced AKI (159). Conversely, overexpression of myo-

inositol oxygenase in proximal renal tubules can exacerbate ferroptotic damage in the kidneys of mice treated with cisplatin (161). Ras homolog enriched in brain 1 (Rheb1), a GTPase, plays a crucial role in regulating cell growth, differentiation, and survival in the brain. Research (162) found that Rheb1 can prevent cisplatin-induced ferroptosis in renal tubular cells by maintaining mitochondrial homeostasis. Fumarate esters, an oral small molecule drug, have been found in a study (163) to prevent ferroptosis through its antioxidant action via Nrf2 and improve AKI. Furthermore, research (164) has demonstrated that mice with knockout of the ferritin heavy chain gene exhibit more severe kidney injury after cisplatin injection compared to control mice. Indicating the crucial protective role of the ferritin heavy chain as a significant iron metabolism-related protein in renal tubular damage. The above research results also indicate that the use of iron chelators such as deferoxamine or iron suppressors like Ferristatin-1 can significantly alleviate cisplatin-induced acute AKI (161, 164).

4.2.3 Folic acid-induced AKI

The folic acid-induced acute kidney injury (AKI) model is commonly regarded as an excellent model for replicating human AKI. Studies have shown that in mice pre-treated with the ferroptosis inhibitor Ferrostatin-1, folic acid-induced intracellular lipid peroxidation and tissue damage were markedly reduced, leading to improved kidney function (165). FG-4592, a hypoxia-inducible factor-prolyl hydroxylase inhibitor, has been found to elevate intracellular GSH levels and decrease iron accumulation when administered as a pre-treatment (166). Its protective mechanism primarily involves the activation of the intracellular antioxidant enzyme Nrf2, thereby inhibiting folic acid-induced renal cell ferroptosis and slowing down fibrosis progression. Studies have also indicated that nuclear receptor subfamily 1 group D member 1 (NR1D1) can stimulate ferroptosis by directly binding to ROR response elements and repressing the transcription of SLC7A11 and HO-1. Consequently, targeting and inhibiting NR1D1 may restrain ferroptosis, thereby ameliorating folic acid-induced AKI in mice (167). Notably, nuciferine, the primary bioactive compound isolated from lotus leaf, can prevent iron accumulation and lipid peroxidation in folic acid-induced AKI by enhancing intracellular GSH and GPX4 levels, ultimately inhibiting ferroptosis (166). These research findings collectively underscore the significant role of ferroptosis in folic acid-induced AKI.

4.2.4 AKI caused by rhabdomyolysis

The causes of rhabdomyolysis include factors such as trauma, drugs, toxins, and infections, with AKI being a serious complication of rhabdomyolysis. Research (168) indicates that the Fe^{2+} directly induced by myoglobin metabolism may lead to lipid peroxidation in proximal tubule epithelial cells, which could be an important pathogenic mechanism of rhabdomyolysis-induced acute AKI. Guerrero-Hue et al (169) discovered that curcumin, as a potent antioxidant, can inhibit ferroptosis in kidney cells. The mechanism may involve the inhibition of the Toll-like receptor 4/NF-κB signaling pathway and the activation of intracellular HO-1, which reduces the myoglobin-mediated inflammation and oxidative stress response. The study also found that the use of iron chelator-1 significantly improved renal function in glycerol-injected mice.

4.2.5 Other models of AKI

Aristolochic Acid I (AAI) is an important metabolite of aristolochic acid, which has been found to have significant nephrotoxicity. Research has shown that it can significantly decrease the levels of intracellular GSH while simultaneously upregulating the expression of 4-hydroxynonenal and Fe^{2+}. Iron chelators such as deferoxamine mesylate and ferroptosis inhibitor ferrostatin-1 have been found to significantly alleviate the cell toxicity induced by aristolochic acid I. The Nrf2/HO-1/GPX4 antioxidant signaling pathway may be an important intervention target for preventing drug-induced AKI containing AAI. AKI (170). Alpha-lipoic acid is a natural antioxidant with the ability to scavenge free radicals and chelate toxic metals. Research (171) has found that it can effectively mitigate cobalt-induced ferroptosis in the kidneys due to metal implants in the human body. The above research results indicate that ferroptosis is widely involved in the occurrence and development mechanisms of various types of AKI.

4.2.6 Targeting ferroptosis for the treatment of AKI

Given that ferroptosis is extensively involved in the occurrence and development mechanisms of various types of AKI, targeting the ferroptosis pathway may be a novel strategy for preventing and treating AKI. This strategy mainly includes iron chelation therapy, targeting iron metabolism-related proteins, lipophilic antioxidants, and direct inhibitors of ferroptosis (172). The overall strategy of iron chelation therapy is to reduce the unstable iron pool, minimize the production of ROS, and thus prevent lipid peroxidation caused by excessive iron overload. In addition to iron chelators, cellular iron depletion can also be achieved by targeting iron metabolism-related proteins. Research has found that increasing the iron regulator hepcidin level in the bloodstream can induce the degradation of ferroportin 1 and promote ferritin expression, effectively restoring iron homeostasis and reducing the generation of ROS (148). Overactivation of intracellular Nrf2 can not only promote the production of a series of downstream antioxidant enzymes but also increase the level of GSH, effectively inhibiting the progression of renal ischemia-reperfusion injury in the early stages (173). HO-1, as an intracellular antioxidant protective enzyme, plays a good role in preventing AKI in various animal injury models induced by ischemia-reperfusion, cisplatin, and lipopolysaccharide (174). Iron-regulatory proteins such as ferritin-1, as representatives of ferroptosis inhibitors, primarily inhibit iron-dependent cell death by interfering with lipid peroxidation (174). However, the *in vivo* use of ferritin-1 is limited by its stability and lower effectiveness. Therefore, there is an urgent need to develop a safer, more stable ferroptosis inhibitor that can be used for treating human diseases in clinical settings.

4.2.7 Natural plant compounds regulate ferroptosis to intervene in kidney injury

Natural plant components offer unique advantages in the prevention and treatment of kidney injury. Compounds such as glycyrrhizic acid, astragaloside IV, ginsenoside Rg1, and dioscin, derived from licorice, astragalus, ginseng, and yam, respectively, possess natural properties that nourish qi, nourish yin, invigorate the spleen, and benefit the lungs. Similarly, compounds like paeoniflorin and curcumin, sourced from peony and turmeric, are known for their natural properties that promote blood circulation and remove blood stasis. Furthermore, compounds such as acteoside and emodin, derived from natural plant sources, are recognized for their heat-clearing and detoxifying effects. These natural components align with the pathogenesis and treatment principles of acute kidney injury (AKI). In recent years, there have been numerous reports on the use of natural plant compounds to regulate ferroptosis and alleviate kidney injury, showcasing targeted therapy and significant efficacy (Tables 2, 3).

(1) Flavonoids: Baicalein mainly exists in the roots of Scutellaria baicalensis and Scutellaria lateriflora. being one of the flavonoids with the highest content in Scutellaria baicalensis. It has effects such as reducing cerebral vascular resistance, anti-inflammatory, and antibacterial properties (175). Research has found that baicalein has significant anti-ferroptosis activity. It markedly inhibits GPX4 degradation, and lipid peroxidation, and enhances cellular resistance to ferroptosis. In the AKI model induced by polymyxin B (PMB), baicalein reduces P53 acetylation levels, inhibits ferroptosis, and ultimately alleviates AKI (175). Isoliquiritigenin is an isoflavone compound found in licorice, with various pharmacological effects such as anti-tumor, antioxidant, and anti-inflammatory properties. It can inhibit the expression of HMGB1 and NCOA4 induced by lipopolysaccharides (LPS), suppress the accumulation of free iron in renal tubular epithelial cells, alleviate mitochondrial damage in renal tubules, enhance the expression levels of GPX4, and provide certain protective effects on kidney function (176). Chrysanthemin-3-glucose has antioxidant and anti-tumor effects. It significantly reduces levels of Fe^{2+}, ROS, MDA, and ACSL4 in AKI mice and damaged renal tubular epithelial cells By activating the AMPK pathway. It also increases GPX4 and GSH levels, effectively inhibiting ferroptosis and alleviating kidney damage (177).

(2) Saponins: Astragaloside IV, an active component of Astragalus, activates the PI3K/AKT and Nrf2 signaling pathways, reduces oxidative stress, enhances GPX4 and Nrf2 expression, reduces iron accumulation, inhibits ferroptosis induced by Aflatoxin, significantly improves kidney damage, and protects kidney cells (178). Ginsenoside Rg1, a compound found in Panax ginseng, exhibits a positive therapeutic effect on kidney diseases.

TABLE 2 Reagents that modulate ferroptosis (Ferroptosis inhibitors).

Reagents	Targets	Impact on ferroptosis
Ferrostatin-1	Lipid peroxidation	Inhibition of lipid peroxidation
Liproxstatin-1	Lipid peroxidation	Inhibition of lipid peroxidation
Vitamin E	Lipid peroxidation	Inhibition of lipid peroxidation
SRS 16–86、SRS 11–92	Lipid peroxidation	Inhibition of lipid peroxidation
Troglitazone, Pioglitazone, Rosiglitazone	ACSL4	Inactivation of ACSL4
Deuterated polyunsaturated fatty acids (D-PUFAs)	Lipid peroxidation	Inhibition of lipid peroxidation
XJB-5–131	Lipid peroxidation	Inhibition of lipid peroxidation
Butylated hydroxytoluene, butylated	Lipid peroxidation	Inhibition of lipid peroxidation
hydroxyanisole	Lipid peroxidation	Inhibition of lipid peroxidation
Ferrostatins, liproxstatins	Lipid peroxidation	Inhibition of lipid peroxidation
CDC, PD-146176, AA-861, zileuton	Lipoxygenase	Inhibition of lipid peroxidation
Selenium	Selenoproteins	Inhibition of lipid peroxidation
Deferoxamine, cyclipirox, deferiprone	Intracellular iron	Decreases cellular iron
Vildagliptin, alogliptin, and Linagliptin	DPP4	Blocks DPP4-mediated lipid peroxidation
Irisin	GPX4	Upregulates GPX4
Melatonin	System Xc and GPX4-	Upregulates system Xc and GPX4-
Vitexin	System Xc and GPX4-	Upregulates system Xc and GPX4-
Isoliquiritigenin	System Xc and GPX4-	Upregulates system Xc and GPX4-
Vitamin A	Lipid peroxidation	Blocks lipid peroxidation
Paricalcitol	GPX4	Upregulates GPX4
Pachymic acid	Nrf2,GPX4, SCL7A11, and HO-	Upregulates of Nrf2, GPX4, SCL7A11 and HO-1
Rheb1	Maintains mitochondrial homeostasis	Maintains mitochondrial homeostasis
Quercetin	System Xc and GPX4-	Upregulates system Xc and GPX4-
Artesunate	Lipoxygenases	Blocks lipid peroxidation

TABLE 3 Reagents that modulate ferroptosis (Ferroptosis inducers).

Reagents	Targets	Impact on ferroptosis
Sulphasalazine	System X_c^-	Prevents cystine import, causes GSH depletion
Sorafenib	System X_c^-	Prevents cystine import, causes GSH depletion
Glutamate	System X_c^-	Prevents cystine import, causes GSH depletion
Erastin and its analogs	System X_c^- VDAC2/3-	Prevents cystine import, causes GSH depletion
RSL3	GPX4	Covalent inhibitor of GPX4 that causes accumulation of lipid hydroperoxides
ML162	GPX4	Covalent inhibitor of GPX4 that causes accumulation of lipid hydroperoxides
$FINO_2$	GPX4	Covalent inhibitor of GPX4 that causes accumulation of lipid hydroperoxides
FIN56	CoQ10 and GPX4	Depletes CoQ10 via SQS-mevalonate pathway and causes a decrease in GPX4 protein abundance
BSO, DPI2, cisplatin	GHS	GHS deletion
Statins (e.g., cerivastatin, simvastatin)	HMGCR	Blocks CoQ10 biosynthesis
Trigonelline, brusatol	Nrf2	Nrf2 inhibition
Siramesine, lapatinib	Ferroportin, Transferrin	Upregulates cellular iron
Brequinar	DHODH	Inhibits DHODH
iFSP	FSP1	Inhibits FSP1
BAY 87-2243	Mitochondrial respiratory chain	Inhibits mitochondrial respiratory chain
Andrographolide	Lipid peroxidation	Promotes lipid peroxidation
Toosendanin	Lipid peroxidation	Promotes lipid peroxidation
Gliotoxin	ROS, lipid peroxidation	Promotes lipid peroxidation
Arsenic trioxide	ACSL4	ACSL4 activation
Manganese	ROS, lipid peroxidation	Promotes lipid peroxidation
Fatostatin	GPX4	Inhibits GPX4 expression
Legumain	GPX4	Facilitates chaperone-mediated autophagy of GPX4
Dihydroartemisinin (DHA)	Ferritin, iron overload	Increases cellular iron
Artemisinins	Iron overload	Increases cellular iron

It promotes the expression of FSP1, reduces cellular level of Fe^{2+}, ferritin heavy chain, and MDA, increases GPX4, and GSH, inhibits lipid peroxidation and cell ferroptosis, enhances the vitality of renal tubular cells, and alleviates AKI in septic rats (179). Diosgenin saponin, as the basic raw material for synthesizing various steroidal hormones, is present in natural plants such as Dioscoreaceae and Fabaceae. It possesses pharmacological properties such as anti-inflammatory, anti-tumor, and antioxidant effects. Research indicates that it can alleviate kidney tissue lesions and mitochondrial damage in mice, reducing inflammatory responses (180); Furthermore, by promoting Nrf2 expression and activating HO-1, it significantly reduces ROS levels and MDA content in the kidneys of AKI rats, increases levels of GSH, GPX4, and other anti-ferroptosis proteins, markedly reduces the number of apoptotic cells in the kidneys and renal tubular epithelial cells of model rats, and promote the expression levels of pro-apoptotic proteins, thereby protecting the kidneys (181). Paeoniflorin possesses pharmacological properties such as analgesic, sedative, vasodilatory, antipyretic, and anti-inflammatory effects. It plays a beneficial role in protecting kidney diseases such as AKI and DN. *In vitro* experiments have shown that paeoniflorin can upregulate the expression levels of SLC7A11, thereby inhibiting ferroptosis in renal tubular epithelial cells, providing a potential therapeutic strategy for protecting kidney tissues (182).

(3) Alkaloids: Nelumbine, a bioactive alkaloid isolated from lotus leaf, exhibits strong antioxidant properties and targets the suppression of folate-induced ferroptosis-related renal pathological changes by regulating the levels of GPX4, SLC7A11, and FSP1. It also improves inflammation, cell infiltration, and kidney function impairment in renal tubular epithelial cells (166). Protopine is a substance extracted from the fresh or dried aerial parts of motherwort. By activating the Nrf2 pathway, it disrupts iron accumulation, lipid peroxidation, and ferroptosis, preventing the downregulation of GSH and GPX4 levels, thus ameliorating cisplatin-induced AKI (183).

(4) Polyphenols: Rhein, as one of the main components of rhubarb, has been proven to possess antibacterial and antioxidant effects. Recent research has shown that rhein can alleviate changes in the renal microstructure of model rats, and decrease the expression of apoptosis-related proteins, thereby reducing cell apoptosis, and counteracting the damage caused by oxidative stress to the kidneys (184). Further research reveals that rhein can alleviate endoplasmic reticulum stress induced by H/R, upregulate GPX4 and SLC7A11 to mitigate lipid peroxidation, reduce renal ferroptosis, and protect against AKI (185). Curcumin is a lipophilic polyphenol extracted from the rhizomes of ginger and turmeric plants. It possesses renal protective properties by inhibiting the TLR4/NF-κB signaling pathway, activating HO-1 to suppress myoglobin-induced inflammation and oxidative stress. This action helps improve lipid peroxidation, decreased antioxidant capacity, and myoglobin-induced

ferroptosis in renal tubular cells, thereby alleviating kidney damage (169). Tiger cane glucoside is derived from the dried rhizomes of the Polygonum cuspidatum, a plant in the Polygonaceae family. It possesses various pharmacological activities such as anti-inflammatory and antioxidant effects, and has a protective effect on AKI (186). It significantly reduces the excessive production of free iron, ROS, and MDA induced by cisplatin, alleviates GSH depletion, and distinctly reverses ferroptosis in renal tubular epithelial cells, thereby offering protection to the kidneys (187).

(5) Triterpenoids: Centelloside is a triterpenoid compound isolated from gotu kola, possessing anti-inflammatory and antioxidant properties. Research has shown that centelloside selectively regulates the Nrf2 pathway, increasing the expression of Nrf2, GPX4, and SLC7A11 in a kidney injury model, inhibiting oxidative stress and ferroptosis, alleviating LPS-induced vacuolation and glomerular mesangial expansion in kidney tissues, to reduce AKI (188). Poria acid has anti-renal interstitial fibrosis and can improve renal pathological damage. It activates the Nrf2 signaling pathway, decreases MDA and cyclooxygenase-2 (COX-2) expression, increases GSH, GPX4, and SLC7A11, inhibits blood creatinine and urea nitrogen retention caused by renal ischemia-reperfusion injury, thus ameliorating renal pathology (189). In conclusion, ferroptosis is involved in the pathogenesis of AKI, and natural plant components can alleviate ferroptosis, and reduce AKI progression by targeting the inhibition of GPX4 degradation and lipid peroxidation. However, there is currently no reported evidence on whether traditional Chinese medicines and herbal formulations can also alleviate AKI by inhibiting ferroptosis. With the advancement of science and technology, further exploration of the deep mechanisms of natural plant components in regulating AKI ferroptosis can be carried out through various techniques such as mass spectrometry analysis, metabolomics, and genomics.

In summary, ferroptosis, as a novel form of cell death, is involved in the pathogenesis of various diseases. It plays an indispensable role in the pathogenesis of AKI. Due to the incomplete understanding of the specific mechanisms of ferroptosis in AKI and the high mortality rate associated with AKI, exploring therapeutic targets for AKI from the perspective of ferroptosis may represent a novel direction with great potential for development. In recent years, there has been an increasing amount of research on using natural plant compounds to inhibit ferroptosis and alleviate kidney damage. Natural plant components are complex, and future studies could focus on enhancing the activity of GPX4 and System X_c^-, inhibiting lipid peroxidation, and targeted regulation of the Nrf2 signaling pathway to reduce ferroptosis in the kidney. However, most studies have focused on the active ingredients of natural plant compounds, failing to fully harness the complex effects of these compounds. Additionally, the long-term therapeutic efficacy of active ingredients from natural plant compounds has not been fully evaluated yet. This review focuses on intervening in AKI by targeting ferroptosis and summarizes the targets in traditional Chinese medicine for regulating ferroptosis in AKI. However, there is currently limited research on complex formulas, and the mechanisms of intervening in AKI through ferroptosis have not been deeply explored. In the future, natural plant compounds could enhance the therapeutic effects on ferroptosis in AKI by targeting pathways, receptors, inhibitors, activators, and other methods. This could further promote the development of traditional Chinese medicine in the future.

4.3 The relationship between ferroptosis and renal fibrosis

Renal fibrosis is the ultimate common process and main pathological manifestation of CKD caused by different etiologies, characterized primarily by the activation of myofibroblasts. The transforming growth factor-β (TGF-β)/Smad signaling pathway is a major pathway in renal fibrosis, and it is closely related to ferroptosis (190). TGF-β is a major pro-fibrotic factor, and various factors can promote its secretion, such as angiotensin II, hypoxia-inducible factor 1, mitogen-activated protein kinase, and high glucose (190). Research indicates that TGF-β1 modulates renal injury by stimulating downstream Smads, where Smad3 is a key mediator that promotes renal fibrosis, Smad2, and Smad7 have renal protective effects, and Smad4 exhibits both promotion of fibrosis and inhibition of inflammation through different mechanisms (191). additionally, Smads also interact with other signaling pathways such as the mitogen-activated protein kinase and nuclear factor κB pathways to positively or negatively regulate renal inflammation and fibrosis (192). TGF-β secreted by tumor vascular endothelial cells can induce differentiation of endothelial cells and adjacent fibroblasts into myofibroblasts through both autocrine and paracrine mechanisms (193). Renal tubular epithelial cells can release exosomes containing TGF-β mRNA to activate fibroblasts, thereby promoting renal fibrosis following AKI (194). Research has confirmed that the pro-fibrotic factor Wnt-1-induced signaling protein 1 (WISP-1) may mediate renal fibrosis by enhancing autophagy mediated by TGF-β1 (195, 196). The physiological functions of iron include participating in the mitochondrial respiratory chain and hemoglobin synthesis. Both iron excess and deficiency are detrimental to metabolic homeostasis. Iron excess can lead to the generation of free radicals, and it may result in organelle stress and disruption of cellular structural integrity. Renal cells are susceptible to the effects of iron overload, and tissue iron deposition can lead to oxidative damage and pathological reactions, including fibrosis and inflammatory reactions (197). Ferroptosis is a non-traditional form of RCD characterized by iron overload and lipid peroxidation. Erastin, Sorafenib, RSL3, and FIN56 are common inducers of ferroptosis, while deferoxamine, Ferrostatin-1 (Fer-1), and Liproxstatin-1 (Lip-1) are common inhibitors of this process. Obstruction of the ureter leads to ferroptosis in renal tubular epithelial cells, which in turn causes the secretion of various

fibrogenic factors and affects interstitial fibroblasts in a paracrine manner, promoting their proliferation and differentiation (128). However, the specific mechanism of fibrogenic factor secretion during ferroptosis in renal tubular epithelial cells remains unclear. In TGF-β1-stimulated renal tubular epithelial cell injury, the expression of SLC7A11 and GPx4 is decreased, while Fer-1 can alleviate this change (198).

Inhibition of ferroptosis to alleviate renal fibrosis: Studies indicate that targeting ferroptosis specifically with certain inhibitors can mitigate kidney damage and renal fibrosis: (1) In a 5/6 nephrectomy-induced CKD rat model, deferoxamine alleviates kidney injury and fibrosis by regulating iron metabolism and the TGF-β1/Smad3 pathway (199). (2) Lip-1 significantly attenuates renal collagen protein deposition and expression of fibrotic factors in a unilateral ureteral occlusion (UUO) mouse model, thereby alleviating renal fibrosis. Moreover, it reduces the activation of surrounding fibroblasts by inhibiting the paracrine secretion of fibrosis-promoting fibrotic factors in human renal proximal tubule cells (HK2) (200). (3) Fer-1 and deferiprone can inhibit iron-dependent cell death in renal tubular epithelial cells, thereby reducing kidney injury and fibrosis induced by UUO or ischemia-reperfusion (128). (4) Berberine reduces lipid peroxidation and inhibits ferroptosis by activating adenosine monophosphate-activated protein kinase in a renal ischemia-reperfusion mouse model, thereby alleviating kidney fibrosis (201). (5) Irisin can inhibit ferroptosis induced by Erastin/RSL3 and fibrosis stimulated by TGF-β1 in primary renal tubular epithelial cells. Irisin also significantly alleviates tubular epithelial cell injury and fibrosis by inhibiting Smad3 phosphorylation and suppressing the expression of Nox4 (a downstream regulator of ferroptosis) in the UUO mouse model, thus blocking Smad3-mediated ferroptosis (202). Furthermore, Balzer et al. established an adaptive repair and maladaptation (fibrosis) kidney regeneration model by titrating ischemic injury doses (203). Through detailed biochemical and histological analysis of a maladaptive/pro-fibrotic cluster of proximal tubules, they discovered that cell necroptosis/ferroptosis is a vulnerable pathway in these pro-fibrotic cells. Pharmacological targeting of cell necroptosis/ferroptosis could promote cell adaptive repair and improve fibrosis. The above studies all indicate that targeting ferroptosis for treatment may prevent renal fibrosis in CKD patients, which shows promising potential applications.

4.3.1 Ferroptosis and fibrosis induced by kidney stones

Kidney stones are a common urinary system disorder, with an incidence rate as high as 14.8% and a recurrence rate of up to 50%. 0.8% to 17.5% of kidney stone patients also suffer from CKD. Research has found that genes related to renal interstitial fibrosis are significantly upregulated in patients with kidney stones. Calcium oxalate (CaOx) crystals can adhere to and aggregate on the renal tubular epithelial cells, causing damage to the tubular epithelial cells and endothelial-mesenchymal transition, ultimately leading to renal fibrosis (204, 205). Research has shown that in mice with high oxalate urine and oxalate-stimulated HK-2 cells, there is a significant increase in ROS levels (206), indicating that ferroptosis may be involved in the formation of kidney stones. Furthermore, recent studies have found that CaOx crystals can induce ferroptosis in renal tubular epithelial cells, leading to damage of the tubular epithelial cells and kidney injury. Research has found that exposing renal tubular epithelial cells to various concentrations of CaOx crystal solution results in a significant increase in the expression of ferroptosis-related proteins such as p53, ACSL4, transferrin receptor (TRC), and TF as the concentration of CaOx crystals increases. Conversely, the expression of ferroptosis-inhibiting proteins, solute carrier family 7 member 11 (SLC7A11), and GPX4 decreases relatively. Additionally, due to the stimulation by CaOx crystals, mitochondrial function is impaired and cannot exert antioxidant capabilities, leading to the accumulation of ROS within cells, ultimately inducing ferroptosis. The use of a ferroptosis inhibitor, Ferrostatin-1, can suppress ferroptosis, thereby alleviating damage to renal tubular epithelial cells (207). Furthermore, Song et al (208) found in a cell model of oxalate-induced kidney stones that CaOx crystals can induce ferroptosis through autophagy, thereby exacerbating kidney damage. BECN1 is a molecule that regulates autophagic activity. Experiments have shown that overexpression of BECN1 significantly increases levels of malondialdehyde (MDA), Fe^{2+}, and ROS in cells, promoting the occurrence of ferroptosis induced by CaOx crystals. Nuclear receptor coactivator 4 (NCOA4) is a widely expressed intracellular protein that can mediate ferritinophagy to control the release and storage of iron within cells. Studies have shown that knocking out the NCOA4 gene inhibits ferritin degradation and even reverses the effects of BECN1. Other studies have demonstrated that ferroptosis not only participates in cell damage caused by kidney stones but also plays a role in the process of kidney injury repair. Persistent damage to renal tubular epithelial cells can lead to the accumulation of inflammatory proximal tubular cells. These inflammatory proximal tubular cells can significantly downregulate GSH metabolic genes, making cells more prone to ferroptosis, resulting in repair failure after kidney injury, ultimately promoting kidney fibrosis and the progression of AKI to CKD (209). Although ferroptosis is currently believed to be associated with the progression of kidney fibrosis induced by kidney stones, the specific mechanisms remain to be further explored.

4.3.2 Ferroptosis and renal hydronephrosis

Renal hydronephrosis refers to the obstruction of urine drainage from the renal pelvis, leading to an accumulation of urine and increased pressure within the kidney, resulting in the dilation of the renal pelvis and calyces, renal parenchymal atrophy, and a decline in kidney function. Research has found that renal hydronephrosis not only leads to kidney damage but also induces renal fibrosis. Moreover, the severity of renal fibrosis is positively correlated with the severity of renal hydronephrosis (210, 211). At the same time, research indicates that ferroptosis is involved in the process of renal fibrosis caused by renal hydronephrosis. Zhang et al (200) found that in a mouse model of UUO-induced renal hydronephrosis, renal tubular epithelial cells underwent ferroptosis. Additionally, *in vitro* studies suggest that the ferroptosis inhibitor Lip-1 can alleviate renal fibrosis and

extracellular matrix deposition. The mechanism may be that Lip-1 inhibits the secretion of pro-fibrotic factors by renal tubular epithelial cells, ultimately suppressing the activation of fibroblasts surrounding the renal tubules. Smad3 is a common signaling protein that can directly promote the expression of pro-fibrotic genes as a transcription factor. Recent studies have revealed that in the mouse model of renal hydronephrosis induced by UUO, the expression of the ferroptosis biomarker GPX4 significantly decreases, accompanied by abnormal activation of Smad3, thereby promoting fibrosis. Additionally, quercetin, as an inhibitor of Smad3 phosphorylation, may inhibit renal interstitial fibrosis in UUO mice by inhibiting Smad3-mediated ferroptosis (202), further confirming the close relationship between ferroptosis and renal fibrosis induced by renal hydronephrosis.

4.3.3 Ferroptosis and CKD

CKD affects 8% to 16% of the global population (212). CKD is defined as a clinical syndrome caused by various factors, characterized by a glomerular filtration rate <60 ml·min-1·(1.73 m2)-1, urine protein >30 mg/d, or the presence of kidney damage markers for more than 3 months. The most common causes are diabetes and hypertension. Zhou et al. (128) found typical ferroptosis characteristics in a CKD mouse model, with decreased expression of GPX4 and increased abundance of 4-HNE. Treatment with Fer-1, which inhibits ferroptosis, reduced kidney damage and fibrosis in the mice. In addition, Jin and Chen (142) found that in diabetic kidney disease, umbelliferone inhibits ferroptosis by activating the nuclear factor E2-related factor 2 (NRF-2)/heme oxygenase-1 (HO-1) pathway, significantly improving kidney damage and reducing ROS generation. In Wang et al.'s study (213) on a rat CKD model, typical ferroptosis characteristics were observed in the CKD group, including increased iron content, oxidative stress, and lipid peroxidation. Further investigation revealed the upregulation of NCOA4 expression and downregulation of FTH1 and FTL expression in residual kidney tissue. Treatment with DFO reversed these phenomena, indicating that ferroptosis in CKD is associated with iron overload caused by ferritin autophagy. Moreover, cellular autophagy is closely related to the progression of CKD to renal fibrosis (214).

CKD is the common outcome of various primary and secondary kidney diseases, characterized by renal interstitial fibrosis and progressive decline in kidney function (215). Studies have shown that the majority of CKD patients experience varying degrees of iron metabolism and lipid metabolism disorders. Disruptions in iron metabolism lead to iron deposition in the kidneys, thereby inducing ferroptosis; disruptions in lipid metabolism result in lipid deposition in the renal parenchyma, promoting lipid peroxidation, further inducing ferroptosis (216). Furthermore, iron-mediated epithelial cell death promotes the secretion of various pro-fibrotic factors such as TGF-β, CTGF, etc., inducing proliferation and differentiation of interstitial fibroblasts, ultimately leading to renal fibrosis. Nait et al. (217) found that the iron chelator deferoxamine can inhibit pathways such as TGF-β1/Smad3, inflammatory response, and oxidative stress, thereby alleviating renal fibrosis in CKD rats. Disruption of iron metabolism and lipid metabolism in CKD can lead to ferroptosis, aggravating renal damage. Targeted therapies focusing on inhibiting ferroptosis may play an important role in protecting renal function in CKD patients.

The above studies indicate that the progression of CKD is closely associated with ferroptosis. By inhibiting ferritinophagy to reduce intracellular free iron levels, thereby suppressing the occurrence of ferroptosis, may emerge as a therapeutic target for CKD.

4.3.4 Targeted inhibition of ferroptosis to alleviate renal fibrosis: key mechanisms

GPX (Glutathione Peroxidase) is a selenoprotein antioxidant enzyme that converts hydrogen peroxide to water (H2O) using oxidized GSH as a substrate. GPX consists of 8 members, among which GPx4 is one of the most important antioxidant enzymes in mammals, regulating the occurrence and progression of ferroptosis (218–220). Renal tubular epithelial cells preferentially oxidize fatty acids as an important energy source (221).

Under normal circumstances, there is a dynamic balance between fatty acid synthesis and oxidation, preventing intracellular lipid accumulation. However, blocking fatty acid oxidation (FAO) in renal tubular epithelial cells can promote intracellular lipid deposition during the fibrotic process. TGF-β can reduce FAO and enhance lipid accumulation associated with renal fibrosis. Research has found that TGF-β1-stimulated renal tubular epithelial cells exhibit increased lipid peroxidation associated with renal failure, a process that can be reversed by GPx4 (153). Leonarduzzi et al. (222) observed that a lack of GPx4 can promote the production of TGF-β1, thereby exacerbating fibrosis, while upregulation of GPx4 can reverse this change. Therefore, the deficiency in GPx4 may be involved in the occurrence and development of renal fibrosis. Conversely, elevation of GPx4 can weaken the activation of the nuclear factor κB pathway, thus alleviating renal fibrosis (223). Interleukin-6 is a pleiotropic cytokine positively correlated with renal fibrosis. Overexpression of GPx4 in fibroblasts can inhibit ultraviolet A radiation-induced release of interleukin-6 (224). In renal biopsy tissue studies of CKD patients and mice models including UUO and renal ischemia-reperfusion injury, downregulation of GPx4 and upregulation of 4-hydroxynonenal were observed. This study demonstrates the potential role and mechanism of ferroptosis in renal tubular epithelial cell death in renal fibrosis. The above conclusions all indicate that GPx4 plays a protective role in the development of renal fibrosis. On the other hand, Lip-1 can inhibit the downregulation of GPX4 expression, and reduce iron deposition and lipid peroxidation, thereby inhibiting ferroptosis. Gong et al (225) found that Erastin promotes the differentiation of fibroblasts into myofibroblasts by increasing lipid peroxidation and inhibiting GPx4 expression, while Fer-1 inhibits ferroptosis and fibrosis by suppressing lipid peroxidation and enhancing GPx4 expression. The above studies all confirm that GPx4 is a key substance for targeted inhibition to alleviate ferroptosis and renal fibrosis. This holds important implications for the specific treatment of renal fibrosis in the future, but further research is still needed to reveal the mechanisms of certain key steps in this process.

In summary, we can observe that in recent years, there has been a considerable amount of evidence supporting the role of ferroptosis in renal fibrosis. This article primarily reviews the main signaling pathways and regulatory factors involved in regulating ferroptosis, the role of ferroptosis in renal fibrosis, and potential therapeutic strategies that interfere with ferroptosis in the treatment of renal fibrosis. While progress has been made, there are still some issues that need to be addressed, including (1) Ferroptosis is not an isolated event and appears to be closely related to other forms of cell death. Besides ferroptosis, necrosis, apoptosis, pyroptosis, autophagy, and other cell death pathways have also been observed in renal fibrosis. Do these cell death mechanisms interact with each other during the fibrosis process? Therefore, studying the potential antagonistic or synergistic effects of ferroptosis in the context of kidney disease is necessary; (2) Currently, research on ferroptosis is based on various disease models, and the impact of ferroptosis on renal fibrosis under physiological conditions is not yet clear; (3) Although regulatory factors of ferroptosis such as ROS, GPx4, GSH, and iron metabolism have been described, they do not constitute suitable sensitive biomarkers for monitoring ferroptosis. Finding simple and reliable biomarkers, especially in the context of kidney disease, remains a challenge; (4) Although the connection between ferroptosis and AKI has been extensively explored, research on the relationship between ferroptosis and renal fibrosis as well as CKD remains limited. Most relevant studies conducted so far have utilized *in vitro* cell culture models or animal models of kidney disease. However, the use of different disease models leads to variations in research outcomes, posing a challenge for the translation of research on the relationship between ferroptosis and renal fibrosis into clinical applications.

4.4 Polycystic kidney disease (PKD) and urinary tract infections

PKD is an autosomal dominant genetic kidney disease characterized by the progressive development of cysts in renal epithelial cells, ultimately leading to end-stage renal disease (ESRD). Studies have shown that the mRNA, protein content, and activity levels of antioxidant enzymes such as GPX and superoxide dismutase are downregulated in PKD, resulting in an exacerbation of lipid peroxidation reactions, implicating oxidative stress in the growth of PKD cysts. The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a cAMP-activated ATP-gated chloride ion channel expressed in the apical membrane of various epithelial cells, plays a significant role in mediating the efflux of glutathione (GSH) in renal cell lines. In proximal tubule cells, CFTR-mediated reactive oxygen species (ROS)-induced cell death has been observed (226). Simos et al. (227) demonstrated that increased ROS in the cytoplasm activate transmembrane protein 16F (TMEM16F), leading to outward chloride ion flux and disruption of membrane phospholipids. This disruption results in the translocation of phosphatidylserine from the cytoplasm to the extracellular space, potentially leading to sustained lipid peroxidation of the membrane lipids and ultimately cell death, with CFTR playing a synergistic role in this process. Schreiber et al. (228) found that membrane phospholipid peroxidation activates renal TMEM16A, thereby stimulating CFTR. The ferroptosis inhibitor Fer-1 shows promise in preventing the growth of renal cysts, with related clinical trials currently underway. The aforementioned findings suggest that ferroptosis may be one of the pathogenic mechanisms of PKD, and inhibitors of ferroptosis offer a new perspective for developing treatment strategies for PKD (229). Other animal models in chronic kidney disease (CKD) research also provide functional evidence of iron exposure in kidney injury. For instance, in proteinuric CKD rats, restricting iron intake can reduce proteinuria, glomerular iron deposition, and glomerulosclerosis. In the unilateral ureteral obstruction (UUO) animal model, the administration of deferoxamine (DFO) has been shown to alleviate tubulointerstitial fibrosis. Iron chelators can specifically reduce the deposition of iron in the lysosomes of proximal tubules (127). Kidney biopsy specimens from CKD patients reveal the accumulation of iron in renal tubular epithelial cells and infiltrating macrophages. Iron-induced cell death, serving as a trigger, may contribute to the transition from acute kidney injury (AKI) to CKD through sustained oxidative stress and mitochondrial dysfunction. While the kidneys have the function of secreting erythropoietin (EPO), many CKD patients exhibit reduced EPO secretion, leading to a situation where anemia and iron deficiency coexist. Therefore, simply reducing iron exposure is not an effective method for managing CKD, and maintaining iron balance should be approached cautiously.

Urinary tract infection is one of the most common infections in the community and healthcare systems (230), with over 25% of urinary tract infections recurring, leading to the persistence of drug-resistant strains (231). Research indicates that uropathogenic E. coli (UPEC) infecting bladder epithelial cells can activate ferritinophagy when treated with ammonium ferric citrate, leading to the prolonged presence of UPEC in bladder epithelial cells. This increases the risk of recurrent infections and reinfections. In addition to activating ferritinophagy, treatment with ferric ammonium citrate also promotes host bladder epithelial cell death. Knocking out NCOA4 to inhibit ferritinophagy reduces bacterial load and decreases bladder epithelial cell death. Further research reveals that this type of cell death is neither apoptosis nor necrosis but a form of cell death induced by iron overload. However, the authors did not perform specific tests to characterize the phenotype of ferroptosis (232). In conclusion, the persistent presence of UPEC in bladder epithelial cells involves ferritinophagy, which promotes bladder epithelial cell death, exacerbating the severity of urinary tract infections. By inhibiting ferritinophagy and reducing iron content within bladder epithelial cells, it may serve as a potential therapeutic target to control UPEC proliferation. This could provide a theoretical basis for preventing recurrent urinary tract infections and reinfections.

4.5 The molecular mechanisms of ferroptosis involvement in renal cell carcinoma

Renal cell carcinoma, abbreviated as RCC, is the most common malignant tumor of the kidney, accounting for 85% to 90% of renal

malignant tumors. It can be classified into different subtypes based on pathology, including clear cell carcinoma, chromophobe carcinoma, renal papillary cell carcinoma, medullary carcinoma, and undifferentiated carcinoma (233). According to epidemiological data, the incidence of renal cell carcinoma is second only to bladder tumors and shows an increasing trend year by year (234). The primary treatment methods for renal cell carcinoma include surgical intervention, as well as radiation therapy and chemotherapy. Although there have been some advances in the treatment of renal cell carcinoma in recent years, the prognosis remains suboptimal. Hence, there is an urgent need to explore new targets for its treatment. Numerous studies indicate that inducing ferroptosis in cells may be a novel direction for the treatment of renal cell carcinoma. Yang et al. (235) tested the sensitivity of 117 cancer cell lines to Erastin-induced ferroptosis and found that diffuse large B-cell lymphoma and clear cell renal cell carcinoma (ccRCC) were particularly sensitive to GPX4-regulated ferroptosis. Zou et al. (236) found that GPX4 inhibitors exhibited strong cytotoxicity against ccRCC, with the reduction of GPX4 being a key factor in the occurrence of ferroptosis. Their further investigation revealed that in renal cancer cells, high expression of the hypoxia-inducible factor (HIF) pathway's hypoxia-inducible factor 2α enhances the sensitivity of ccRCC to ferroptosis by enriching unsaturated fatty acids through hilpda. The von Hippel-Lindau (VHL) gene acts as a major tumor suppressor in ccRCC. Miess et al. (237) discovered that exogenous overexpression of the VHL gene within cells can reduce intracellular lipid peroxides, thereby inhibiting the occurrence of ferroptosis. This study confirmed that VHL is an important gene in regulating ferroptosis sensitivity in ccRCC, demonstrating that VHL-induced ferroptosis could be a potential target for treating ccRCC. Mou et al. (238) used bioinformatics methods to discover that the expression of nuclear receptor coactivator 4 (NCOA4) is reduced in ccRCC and is associated with a poor prognosis in ccRCC patients. NCOA4 is closely linked to iron transport, and its decrease leads to a reduced sensitivity of tumor cells to ferroptosis. Therefore, promoting ferroptosis through targeting NCOA4 may be an effective method for treating ccRCC. However, this conclusion is solely based on bioinformatics analysis and may not be entirely reliable. Further experimental validation is needed to confirm its effectiveness. Artesunate (ART) is a chemical compound derived from the natural plant Artemisia annua, which has been shown to exhibit anti-tumor effects in various types of cancer (239). Markowitsch et al. found that ART can induce ferroptosis in drug-resistant kidney cancer cells, significantly inhibiting the progression of kidney cancer cells. This suggests that ART holds promise as an effective new drug for treating patients with drug-resistant renal cell carcinoma, addressing the issue of drug resistance in renal cancer (240).

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is an autosomal dominant inherited disease that originates from germline mutations in the fumarate hydratase (FH) gene. It has a low incidence rate and is often solitary, but most cases present with metastasis at the time of diagnosis, with a median survival rate of 24 months (241). Despite some progress in understanding its pathogenesis, the primary treatment remains surgery, with relatively poor treatment outcomes. Therefore, the search for better treatment methods is crucial for extending the survival of patients. Michael et al. (242) found in their research that in hereditary leiomyomatosis and renal cell cancer cells, due to the inactivation of FH, GPX4 is succinated, leading to a decrease in its activity. Additionally, the activity of nuclear factor E2-related factor 2 (NRF2) increases, thereby preventing ferroptosis. Researchers suggest that future studies could explore NRF2 activity inhibitors applicable to humans or develop inhibitors targeting succinated GPX4 to induce ferroptosis in hereditary leiomyomatosis and renal cell cancer cells. This approach could lead to better treatment options for this disease and potentially open up new avenues for the precision treatment of renal cell cancer.

4.5.1 The roles of iron metabolism and lipid metabolism in the progression of renal cell carcinoma

Iron accumulation is a main source of ROS, a crucial factor in ferroptosis. Increasing research indicates a close association between iron accumulation and the development of renal cell carcinoma, particularly ccRCC. Proteins involved in iron metabolism include ferritin light chain (FTL), ferritin heavy chain (FTH1), ferroportin (FPN), transferrin receptor 1 (TfR1) for iron uptake, and iron regulatory proteins 1 and 2 (IRP1/2) (243). Schnetz et al. found that genes related to iron metabolism are significantly upregulated in renal cell carcinoma tissues, especially in ccRCC tissues (243). In ccRCC tissues, the expression of FTL, FTH1, TfR1, and IRP1/2 proteins are upregulated, while the expression of FPN protein is downregulated, showing a phenotype of iron retention where iron accumulates within cancer cells. However, this iron accumulation does not trigger iron-induced cell death; instead, it promotes the development of renal cell carcinoma. FTH1 possesses ferroxidase activity, converting Fe^{2+} to Fe^{3+}, which, upon binding with FTL, effectively reduces the toxicity of intracellular Fe^{2+}, thus preventing cellular ferroptosis (244). Nuclear receptor coactivator 4 (NCOA4) is a component of the autophagosome involved in the process of ferritinophagy, the selective autophagy of ferritin proteins (245). Mou et al. analyzed the Cancer Genome Atlas (TCGA) database and found that NCOA4, a gene related to ferritinophagy, is closely associated with the malignancy and TNM staging of renal cell carcinoma (238). Research indicates that NCOA4 acts as a receptor for autophagy-related proteins 5 (ATG5) and 7(ATG7). The interaction between NCOA4 and ATG5/ATG7 promotes ferritinophagy, leading to a reduction in cellular ferritin levels, an increase in labile iron pools within cells, and ultimately triggering iron-dependent cell death in cancer cells. This indicates that the ATG5-ATG7-NCOA4 autophagy pathway may be a novel therapeutic target for treating renal cell carcinoma (92).

Lipid peroxidation is one of the main features of iron-dependent cell death, driving cells toward iron-induced death. However, ccRCC cells contain abundant lipid droplets but do not trigger iron-dependent cell death. The proportions of lipid components in lipid droplets vary, with PUFAs, particularly arachidonic acid and docosahexaenoic acid, playing important roles in the process of iron-dependent cell death (246). In ccRCC tissues, hypoxia-inducible factor2α (HIF-2α) can selectively enrich PUFAs through the mediation of lipid droplet-associated protein

(HILPDA). ccRCC cells are not only more sensitive to iron-dependent cell death compared to normal kidney cells but also exhibit higher levels of PUFAs as the stage of ccRCC advances. The HIF-2α-HILPDA axis holds promise as a new therapeutic pathway for advanced kidney cancer. In certain ccRCC cells (such as FR1 and FR2 cells in the 786-O series), even with an increase in PUFA content, they do not exhibit increased sensitivity to iron-dependent cell death. Zou et al. (246) discovered that polyunsaturated ether phospholipids (PUFAePLs) can promote the evasion of iron-dependent cell death and increase sensitivity. The synthesis of PUFA-ePLs is associated with enzymes such as alkylglycerone phosphate synthase (AGPS) located in peroxisomes, fatty acyl-CoA reductase 1 (FAR1), glyceronephosphate O-acyltransferase (GNPAT), 1-acyl-sn-glycerol-3-phosphate acyltransferase 3 (AGPAT3) in the endoplasmic reticulum, and plasmalogen biosynthesis enzyme phytanoyl-CoA dihydroxyacetone phosphate acyltransferase (PEDS1). FR1 and FR2 cells can reduce PUFAePL levels by spontaneously downregulating AGPS, thereby decreasing cancer cell sensitivity to iron-dependent cell death, promoting cancer cell proliferation, and metastasis. Therefore, regulating cancer cell sensitivity to iron-dependent cell death by modulating AGPS expression can serve as a therapeutic strategy for treating iron-death-insensitive cancer cells. The transcription factor Nuclear factor erythroid 2–related factor 2 (Nrf2) is closely associated with iron-dependent cell death and is a member of the solute carrier family 7 member 11 (SLC7A11). It is also an upstream regulator of GPX4 and a key regulatory factor in cellular antioxidant responses. Nrf2 can protect cells from damage caused by lipid peroxidation products such as 4-hydroxynonenal and acrolein, thereby inhibiting iron-dependent cell death. Studies have indicated that the expression of Nrf2 and its pathway is associated with the staging and grading of kidney cancer, as well as resistance to targeted therapies and poor prognoses (247, 248). Therefore, Nrf2 could serve as a potential target for the treatment of advanced kidney cancer in the future. However, research on Nrf2 inhibitors, particularly in the context of kidney cancer, is currently limited. Currently discovered Nrf2 inhibitors include chlorobutanol in lung cancer research and berberine in head and neck cancer research. Both compounds can enhance tumor cell sensitivity to ferroptosis (249, 250). Further research is needed to determine whether the application of chlorobutanol or berberine in kidney cancer yields similar effects as seen in lung cancer and head and neck cancer, respectively.

4.5.2 The role of the System X_c^- GSH-GPX4 axis in the progression of kidney cancer

The System X_c^- transports extracellular cystine into the cell and transports intracellular glutamate out of the cell to further GSH. GPX4 utilizes GSH to neutralize lipid peroxidation caused by ROS, thereby inhibiting ferroptosis in cells. Xu et al. (251) found that compared to normal kidney tissue, SLC7A11 is highly expressed in renal cancer tissue and inhibits ferroptosis by promoting GPX4 expression, thus promoting renal cancer cell proliferation, migration, and invasion. This indicates that SLC7A11 is one of the therapeutic targets for preventing the progression of renal cancer to metastatic renal cancer. GSH, as an essential intracellular antioxidant, can neutralize lipid peroxidation products. Gamma-glutamyltransferase 1 (GGT1) catalyzes the breakdown of extracellular GSH, providing cysteine for the generation of intracellular GSH, and is a component of the GSH recovery pathway. Bansal et al. (252) found that GGT1 levels are significantly increased in ccRCC cell lines (786-O and RCC10), and GGT1 can promote GSH synthesis, preventing ferroptosis caused by lipid peroxidation in tumor cells, thereby promoting tumor cell proliferation and metastasis. Studies have shown that the GGT1 inhibitor OU749 has minimal adverse effects and good efficacy, making it a promising new approach for treating ccRCC (253). Kruppel-like factor 2 (KLF2) is a member of the Kruppel-like factor family of transcription factors, characterized by a DNA-binding domain containing zinc fingers. Recent studies have found that KLF2 is involved in the development and progression of various cancers such as liver cancer, and kidney cancer, among others (254, 255). The downregulation of KLF2 expression is significantly correlated with the TNM staging of ccRCC, and ccRCC patients with low expression of KLF2 have significantly shorter overall survival and metastasis-free survival periods. In the study, a mouse model of ccRCC lung metastasis was developed, and it was found that compared to the low-expression group of KLF2, the group with KLF2 overexpression had smaller and significantly fewer lung metastatic nodules. Further research has revealed that KLF2 can bind to the promoter of GPX4 in ccRCC, leading to the downregulation of GPX4 expression, which protects ccRCC cells from ferroptosis, thus promoting tumor cell metastasis (255). Therefore, the System X_c^-GSH-GPX4 axis plays a crucial role in the progression of renal cancer and may represent a novel strategy for utilizing ferroptosis therapy in advanced renal cancer and even drug-resistant renal cancer in the future.

4.5.3 The role of ferroptosis in the treatment of renal cancer

Ferroptosis is an emerging cancer suppression strategy, and identifying renal cancer patients who are sensitive to ferroptosis inducers quickly remains a challenging issue. 18F-TRX-PET can be used to predict the sensitivity of tumors to iron-targeted therapy, but its high cost limits its clinical applicability (256). *In situ* detection technology can assess the sensitivity of tumor tissues to ferroptosis. This technology involves using a high-power laser to induce local PUFA acyl chains in cell or tissue samples, generating lipid peroxidation, and demonstrating the sensitivity of cells or tissues to ferroptosis inducers on-site (257). Therefore, *in situ* detection is a cost-effective and convenient imaging technology that holds the promise of rapidly categorizing the sensitivity of cancer patients to ferroptosis. It could accelerate the development of targeted cancer therapies focusing on ferroptosis.

(1) Ferroptosis and immunotherapy in ccRCC

As the mechanism of ferroptosis continues to be elucidated, an increasing body of research suggests a close association between ferroptosis and the tumor microenvironment. NCOA4 is an autophagic component involved in the autophagic process of iron proteins FTH1 and FTL, capable of degrading iron proteins,

releasing ferrous iron, and promoting cell ferroptosis. However, NCOA4 is generally underexpressed in ccRCC tissues. HU et al (258) discovered through the TCGA database that iron metabolism-related proteins FTH1 and FTL are upregulated in most solid tumor tissues. These proteins are associated with regulating T cells (Tregs) and tumor-associated macrophages (TAMs), especially M2 macrophages' infiltration. Iron derived from M2 macrophages in TAMs can be exported through ferroportin (FPN) and secreted in the form of the iron carrier protein lipocalin-2 (LCN-2). This allows the transfer of iron to ccRCC cells, thereby promoting ccRCC cell proliferation. This correlation is positively associated with poor prognosis in patients. Treg cells are a major factor in creating an immunosuppressive tumor microenvironment. Infiltration of Treg cells in tumors is associated with higher pathological staging and poor prognosis in patients with ccRCC (259). Therefore, NCOA4 is a key molecule linking ferroptosis and immunotherapy. Studies indicate that the ferroptosis-related gene CARS is a potential immune-infiltration-related regulator of ferroptosis. Its high expression suggests a poor prognosis for patients and is positively correlated with PD-L1 expression in ccRCC. This indicates that CARS could be a potential target for immunotherapy in ccRCC (260). The above research indicates that iron metabolism and related genes in ferroptosis could serve as a point of entry for immunotherapy in ccRCC.

(2) Targeted therapy for ferroptosis in ccRCC

Targeted therapy is a frontline treatment for ccRCC with good clinical outcomes. However, with the continuous use of targeted drugs, some patients exhibit resistance. Ferroptosis, as a newly discovered modulated form of cell death, plays a significant role in the development and progression of kidney cancer. Previous studies have often highlighted the close relationship between ferroptosis and iron metabolism disturbance. However, recent research shows that zinc plays a role similar to iron in the process of ferroptosis (261). Zinc is transported between organelles and the cytoplasm through transport proteins in the SLC39 family (ZIP) or the SLC30 family (ZNT). Among these, ZIP7 participates in cell ferroptosis along with zinc. Lowering ZIP7 expression enables RCC4 cells to resist erastin-induced ferroptosis, but this protective effect can be eliminated by supplementing ZnCl2. This suggests that ZIP7 could serve as a potential therapeutic target in ccRCC (261). ccRCC tumors contain various subcellular lineages that exhibit different sensitivities to ferroptosis. How to enhance the sensitivity of these different cell lineages to ferroptosis, thereby improving the efficacy of ferroptosis inducers, remains a question that needs to be addressed. Research indicates that the susceptibility to ferroptosis is greatly influenced by cell density and fusogenicity. Cell density affects the sensitivity of cells to ferroptosis through the Hippo pathway. Further studies have revealed two compensatory downstream molecular pathways within the Hippo pathway: the Yes-associated protein (YAP) - S-phase kinase-associated protein 2 (SKP2) pathway and the WW domain-containing transcription regulator 1 (TAZ) - ERM protein 1 (EMP1) - NADPH oxidase 4 (NOX4) pathway. In renal cancer cells, the TAZ pathway is predominant (262). NOX4 can generate and accumulate superoxide and hydrogen peroxide, thereby promoting lipid peroxidation and the onset of ferroptosis. Upregulation of SKP2 can facilitate the expression of mRNA for serine/threonine kinases and transferrin receptors, thereby contributing to ferroptosis (263). It can be seen It is evident that the Hippo pathway mainly regulates ferroptosis through the involvement of SKP2 and NOX4, providing new insights for the treatment of ccRCC.

(3) Nanotherapy for ferroptosis in ccRCC

With the advancement of materials science, an increasing number of studies have found that nanoparticles can induce ferroptosis in various tumor cells including lung cancer, breast cancer, liver cancer, and others (264). Nanoparticles exhibit advantages such as improved solubility of small molecule ferroptosis inducers, enhanced targeting specificity, lower systemic toxicity, controllable drug release, and synergistic effects with emerging therapies. Nanoparticles have broad prospects in tumor treatment (265). A recent study discovered that using a peptide-modified iron oxide (Fe3O4) nanoformulation of 1 H-perfluorobutane (1 H-PFP) called GBP@Fe3O4 can trigger thermally induced ferroptosis in 786-O cells. Under 808 nm laser irradiation, localized moderate heat (45°C) triggered the liquid-gas transition of 1H-PFP, leading to the rapid release of Fe3O4 nanoparticles. This process generates a significant amount of ROS through the Fenton reaction in the tumor microenvironment. Meanwhile, heat stress reduces GSH synthesis, inhibiting the antioxidant response of tumor cells, and further exacerbating the damage caused by ROS. Simultaneously, tumor cells undergo lipid metabolism reprogramming, producing a large amount of lipid peroxides, ultimately leading to tumor-specific ferroptosis (266). This demonstrates that nanotechnology holds great promise as a potential treatment modality for ccRCC in the future. However, it currently remains in the early stages of basic research, with limited clinical studies and a need for further safety validation. In conclusion, the main mechanisms involved in ferroptosis—iron metabolism, lipid peroxidation, and the System X_c^- GSH-GPX4 axis-are closely related to the progression of kidney cancer. They play vital roles in immunotherapy, targeted therapy, and potentially impactful nanotherapy for advanced kidney cancer. However, it remains unclear how ferroptosis regulates immune cell infiltration in the tumor microenvironment of kidney cancer and how it promotes the transition of M2 macrophages to M1 macrophages. Currently, ferroptosis inducers are still at the stage of cell and animal research due to their low solubility, weak targeting capabilities, and high systemic toxicity *in vivo*. With the advancement of nanotechnology, combining ferroptosis inducers with nanoparticles may help overcome the limitations of the inducers. However, there is limited research on their clinical application, and further investigation is required.

5 Prospects

Ferroptosis is a regulated form of cell death characterized by iron-catalyzed lipid peroxidation and can be modulated through various mechanisms. Extensive research has shown that ferroptosis plays a significant role in various cardiovascular diseases, degenerative diseases, and tumors, indicating the broad prospects for studying ferroptosis in CKD. In the regulation of ferroptosis

through epigenetic modifications, only a few microRNAs are currently known to regulate the occurrence of ferroptosis. The role of other non-coding RNAs in this regulation remains unexplored. Current research on ferroptosis and kidney disease primarily focuses on acute and chronic renal conditions, particularly in the context of how ferroptosis contributes to the pathological processes of CKD such as interstitial fibrosis, mitochondrial dysfunction, inflammation, tubular cell regeneration, and other cellular processes. Ferroptosis may serve as a driver in converting maladaptive renal responses into CKD and represents a promising therapeutic target to halt disease progression. In the future, it will still be necessary to research specific biomarkers to identify ferroptosis *in vivo*, especially in the context of kidney disease pathology and its interaction with other modes of programmed cell death. As demonstrated in a recent study (267), metformin induces ferroptosis-mediated programmed cell death, exacerbating kidney damage. Neutrophils were identified as a significant trigger for metformin-induced renal toxicity. Specifically, the protein NGAL, in conjunction with iron and metformin, forms complexes that drive neutrophil infiltration into the kidneys, ultimately leading to NETosis and worsening AKI. In the future, concerning the research of ferroptosis inhibitors, current inhibitor studies are predominantly focused on animal research. Compounds like ferrostatin-1 and liproxstatin-1 have shown promising results in animal models, but further research is essential to ensure their safety and feasibility for human use. Due to the lack of clinical studies on some drugs, the efficacy of ferroptosis inhibitors in a clinical setting remains undetermined. Although some drugs have been used in clinical settings, several issues still need to be addressed in the future. For instance, paricalcitol regulates the antioxidant function of GPX4 by activating the vitamin D receptor, thereby inhibiting cisplatin-induced AKI (268). Iron chelators deferoxamine can alleviate ferroptosis and fibrosis in CKD rats (199). Antioxidants like vitamin E and melatonin also face similar challenges. Therefore, although numerous small molecule drugs have been discovered for inducing and inhibiting ferroptosis, these drugs have not yet been translated into clinical applications to benefit patients. We believe that future research can focus on the following aspects: 1) Further elucidating the mechanism of ferroptosis and identifying more regulators targeting crucial points of ferroptosis. 2) Continuing to investigate the relationship between ferroptosis regulation and kidney-related diseases, studying the role of ferroptosis in CKD. 3) Exploring the role of epigenetic modifications in mediating ferroptosis regulation. 4) Translating currently available small molecule drugs for regulating ferroptosis into clinical use. This review elaborates on the mechanism of ferroptosis and its research progress in kidney-related diseases, providing a solid foundation for the treatment, diagnosis, and related research of kidney-related diseases.

Author contributions

ZL: Conceptualization, Validation, Writing – original draft. YL: Writing – review & editing. MY: Data curation, Formal analysis, Writing – original draft. XW: Formal analysis, Methodology, Writing – original draft. LZ: Writing – original draft. KY: Writing – review & editing.

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SLC7A11: the Achilles heel of tumor?

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The non-natriuretic-dependent glutamate/cystine inverse transporter-system Xc- is composed of two protein subunits, SLC7A11 and SLC3A2, with SLC7A11 serving as the primary functional component responsible for cystine uptake and glutathione biosynthesis. SLC7A11 is implicated in tumor development through its regulation of redox homeostasis, amino acid metabolism, modulation of immune function, and induction of programmed cell death, among other processes relevant to tumorigenesis. In this paper, we summarize the structure and biological functions of SLC7A11, and discuss its potential role in tumor therapy, which provides a new direction for precision and personalized treatment of tumors.

KEYWORDS

SLC7A11, redox homeostasis, tumor metabolism, immune, cell death

Abbreviations: ACC, Adrenocortical Carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast Invasive Carcinoma; CESC, Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon Adenocarcinoma; DLBC, Diffuse Large B-Cell Lymphoma; ESCA, Esophageal Carcinoma; GBM, Glioblastoma Multiforme; HNSC, Head and Neck Squamous Cell Carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney Renal Clear Cell Carcinoma; KIRP, Kidney Renal Papillary Cell Carcinoma; LAML, Acute Myeloid Leukemia; LGG, Low Grade Glioma; LIHC, Liver Hepatocellular Carcinoma; LUAD, Lung Adenocarcinoma; LUSC, Lung Squamous Cell Carcinoma; MESO, Malignant Mesothelioma; OV, Ovarian Serous Cystadenocarcinoma; PAAD, Pancreatic Adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate Adenocarcinoma; READ, Rectum Adenocarcinoma; SARC, Soft Tissue Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach Adenocarcinoma; TGCT, Testicular Germ Cell Tumors; THCA, Thyroid Carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma.

Introduction

Tumors, as abnormal growths in the body, often demonstrate a heightened metabolic state that fuels their relentless expansion. This amplified metabolic activity is crucial for their survival and growth, as they voraciously consume nutrients to maintain their proliferative e momentum. To achieve this, tumors enhance their uptake of essential nutrients, ensuring a steady supply for their rapid cellular division and growth. This altered metabolic behavior is a hallmark of malignancy, reflecting the tumor's adaptive response to meet its energetic and biosynthetic demands. Consequently, this heightened metabolic rate not only underscores the aggressive nature of the disease but also offers potential therapeutic targets for disrupting tumor growth (1–3). The unique amino acid transporter-Solute Carrier Family 7 Member 11 (SLC7A11) has been found to be significantly upregulated in various tumor types, with its expression levels closely associated with tumor cell proliferation, invasion, metastasis, and the tumor microenvironment (4–6). Additionally, SLC7A11 has been linked to resistance to radiation and conventional chemotherapeutic agents (7, 8).Thus, SLC7A11 may serve as a promising biomarker for the diagnosis and prognostication of clinical tumors (9).

The significant abundance of SLC7A11 suggests its potential as a promising target for tumor therapy (10). SLC7A11 plays a crucial role in facilitating the import of cystine into cells for the synthesis of glutathione, which is essential for maintaining intracellular glutathione (GSH) levels and protecting cells from oxidative stress-induced damage (5). This process is intricately linked to the initiation of ferroptosis. The SLC7A11/GSH/GPx4 axis serves as the central defense mechanism against ferroptosis, and downregulating the expression and activity of SLC7A11 has been shown to enhance the sensitivity of tumor cells to ferroptosis (11). Moreover, the elevated intracellular GSH levels induced by SLC7A11 confer inherent resistance to oxidative stress therapy in cells. A novel approach in the realm of cancer treatment involves the use of immunotherapy-activated $CD8^{+}$T cells, which release IFN-γ to enhance tumor cell ferroptosis via PD-L1 inhibition (12, 13). IFN-γ secretion decreases SLC7A11 expression, suggesting that combining SLC7A11 inhibitors with immunotherapy could improve cancer treatment (13, 14). The recent study revealed a significant reliance on NADPH and glucose in tumor cells expressing high levels of SLC7A11, thereby questioning the conventional understanding of SLC7A11 as a promoter of cancer (15). Suppression of glucose uptake in the presence of elevated SLC7A11 expression leads to intracellular disulfide stress, ultimately resulting in cell death (16).

Given the pivotal role of SLC7A11 in cancer treatment, this paper provides an overview of its structure and biological functions, as well as its involvement in oxidative stress, tumor metabolism, immune modulation, and cell death (Figure 1).

Structure and function of SLC7A11

System Xc- is composed of two subunits: the light chain functional subunit SLC7A11, also referred to as xCT, and the heavy chain structural subunit SLC3A2, also known as CD98 or 4F2hc (17, 18).

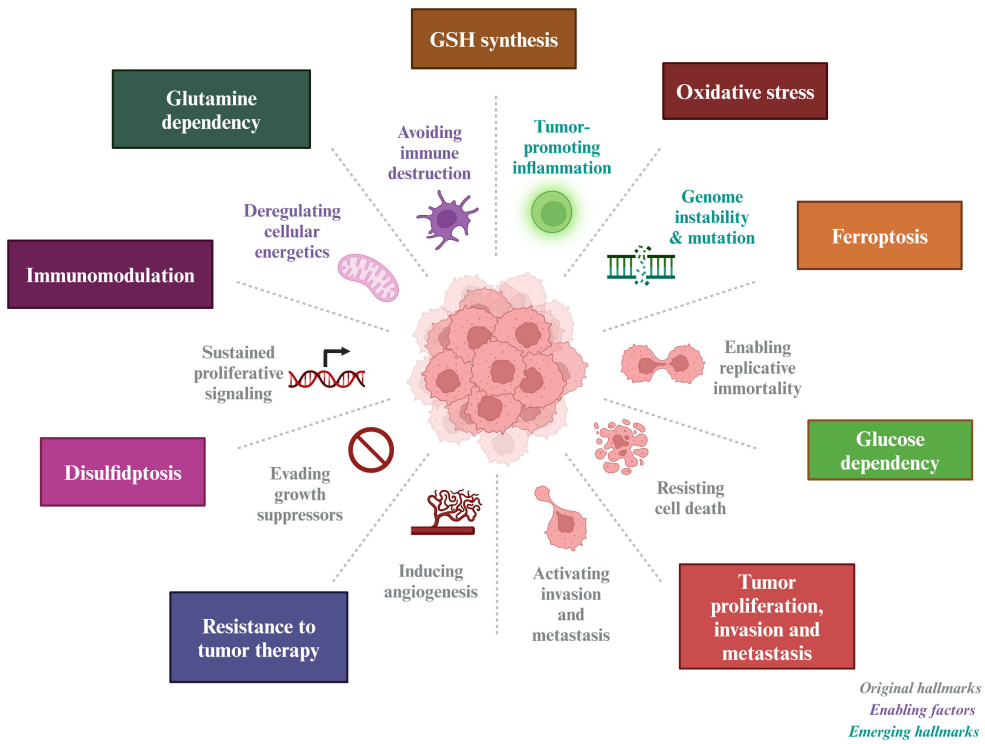

FIGURE 1
Tumor-based characterization of the interventional role of SLC7A11. SLC7A11 can improve tumor resistance to radiotherapy and inhibit tumor proliferation, invasion and metastasis by affecting GSH synthesis, inducing oxidative stress, causing glucose and glutamine dependence in tumor cells, and inducing ferroptosis and disulfidptosis in tumor cells.

In humans, the gene encoding SLC7A11 is situated on chromosome 4 and comprises 14 exons, resulting in a protein sequence of 502 amino acids. SLC7A11 is a transmembrane protein with 12 transmembrane domains, with both its N- and C-termini located intracellularly (19). This protein is abundantly expressed in various tissues and cells throughout the human body and is evolutionarily conserved among vertebrates. In contrast, SLC3A2 is a type 2 membrane glycoprotein characterized by a single transmembrane structural domain, with its N-terminal end intracellular and its C-terminal end extracellular and heavily glycosylated. SLC7A11 is linked to SLC3A2 through a disulfide bond formed between the conserved residue Cys 158 of SLC7A11 and Cys 109 of SLC3A2 (20). Notably, SLC3A2 serves as the chaperone protein for various members of the light subunits of heterodimeric amino acid transporters (LSHAT) family. Consequently, the determination of substrate specificity in system Xc-is primarily influenced by SLC7A11, while SLC3A2 plays a role in facilitating the transportation of SLC7A11 into the intracellular compartment or potentially enhancing the stability of the SLC7A11 protein (21). Furthermore, CD44 has been identified as capable of interacting with and stabilizing SLC7A11 on the membrane of cancer cells (22) (Figure 2).

A variety of amino acid transporters present on the cell surface facilitate the uptake of amino acids by cells, with system Xc- being the main known amino acid transport complex responsible for the transportation of cystine (23). This complex operates by importing cystine into the cell and exporting glutamate out of the cell in a 1:1 ratio, without the need for sodium ions. Once inside the cell, cystine is promptly converted to cysteine, which plays a crucial role as the limiting factor in the synthesis of glutathione (24). Therefore, the expression level and activity of SLC7A11 are the main factors affecting GSH content.

Expression of SLC7A11 in tumors and prognosis

Since its initial identification, research has revealed that SLC7A11 exhibits a tissue-specific distribution, with varying levels of mRNA abundance across 27 different tissues. The findings indicate that SLC7A11 is most prominently expressed in the brain, followed by the thyroid, stomach, appendix, bladder, and gallbladder, while demonstrating lower expression levels in the kidney, heart, and liver. Given the specific subcellular localization of SLC7A11 and its recognized functional significance, it is not unexpected that a multitude of studies have consistently demonstrated the involvement of SLC7A11 in various neurodegenerative, ocular, and immune disorders (25, 26).

It is noteworthy that SLC7A11 is prominently expressed in numerous tumors and exerts influence on tumor progression, invasion, metastasis, and unfavorable prognosis. Elevated levels of SLC7A11 expression have been demonstrated in a diverse array of tumor types, such as lung, liver, pancreatic, breast, ovarian, prostate, bladder, colorectal, melanoma, and leukemia, in comparison to healthy tissues (9, 27–30). Particularly in oncology patients who exhibit insensitivity to initial therapeutic agents and demonstrate resistance to radiotherapy and chemotherapy interventions (31). Table 1 lists the expression and prognosis of SLC7A11 in different tumors.

Mechanically, tumor tissues tend to enhance their own antioxidant defenses in response to high levels of oxidative stress by up-regulating SLC7A11 expression, while SLC7A11-mediated synthesis of GSH acts as a defense against the cytotoxic effects of radiotherapy or certain drugs, which further reduces the sensitivity of tumor cells to treatment.

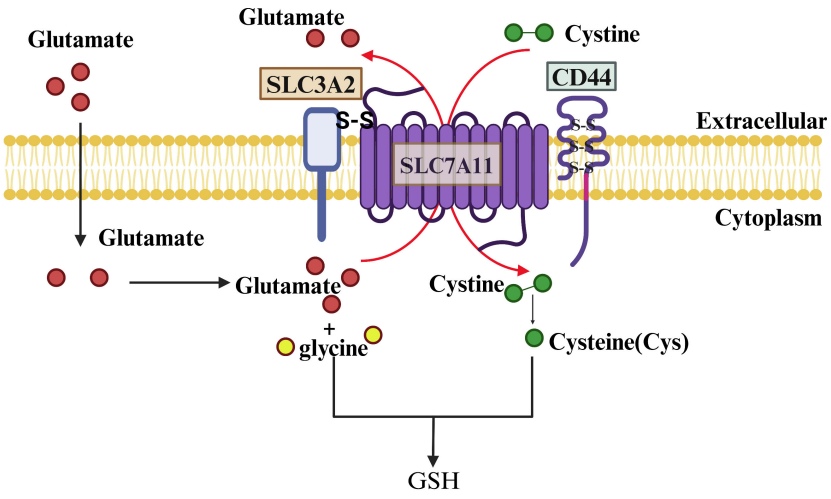

FIGURE 2
Structure and Function of SLC7A11. SLC7A11 and SLC3A2 collectively constitute the glutamate/cystine reverse transporter, with SLC7A11 being a 12-transmembrane-spanning protein featuring intracellular N- and C-termini, and SLC3A2 being a single-transmembrane-spanning protein with an extracellular N-terminus and an intracellular C-terminus. The two proteins are linked by a disulfide bond and function to facilitate the cellular uptake of cystine and the extrusion of glutamate. Cystine is rapidly reduced to cysteine inside the cell and combined with a molecule of glutamate and glycine to synthesize GSH under the action of GCL and GSS enzymes.

TABLE 1 Expression levels and prognosis of SLC7A11 in different tumors.

Tumor type	expression	Prognosis-related
ACC	UP	poor prognosis
BLCA	unchanged	No significant effects
BRCA	UP	poor prognosis
CESC	UP	No significant effects
CHOL	UP	poor prognosis
COAD	UP	poor prognosis
DLBC	UP	No significant effects
ESCA	UP	No significant effects
GBM	unchanged	No significant effects
HNSC	UP	No significant effects
KICH	UP	No significant effects
KIRC	UP	No significant effects
KIRP	UP	poor prognosis
LAML	UP	poor prognosis
LGG	UP	No significant effects
LIIC	UP	No significant effects
LUAD	UP	No significant effects
LUSC	UP	No significant effects
MESO	UP	poor prognosis
OV	UP	No significant effects
PAAD	UP	poor prognosis
PCPG	UP	No significant effects
PRAD	UP	No significant effects
READ	UP	No significant effects
SARC	UP	poor prognosis
SKCM	unchanged	No significant effects
STAD	UP	No significant effects
TGCT	UP	No significant effects
THCA	unchanged	No significant effects
THYM	unchanged	No significant effects
UCEC	UP	No significant effects
UCS	UP	No significant effects
UVM	UP	poor prognosis

Role of SLC7A11 in cancer therapy

SLC7A11 expression causes cellular ferroptosis or enhances tumor killing by immune cells by affecting oxidative status or nutrient and energy metabolism in the tumor microenvironment (TME). Here we summarize the potential role of SLC7A11 in cancer.

SLC7A11 and oxidative stress

Oxidative stress is cellular and tissue damage caused by the production of reactive oxygen species (ROS) in the organism exceeding their removal.

ROS are products of normal physiological activities and are a general term for a class of chemically active molecules and ions with high oxidative activity, which play important roles in cell signaling and tissue homeostasis (32). Essentially, ROS are partially reduced oxygen-containing molecules, including superoxide anion (O_2^-), peroxides (H_2O_2 and LOOH), and free radicals (HO· and LO·). Mitochondria are the main site of ROS production, of which more than 90% are produced by mitochondria in the normal metabolism and energy supply of the electron transport chain (33). Also, ROS can be produced by cytochrome P450, NADPH oxidase (NOX), xanthine oxidase (XO), and peroxidase in the microsomes (34). Of course, the body also exists a ROS scavenging system to maintain homeostasis, which is mainly categorized into enzymatic antioxidants and non-enzymatic antioxidants.

Non-enzymatic antioxidants scavenge free radicals by interacting directly with them. Such substances include glutathione, vitamin A, vitamin C, vitamin E, and coenzyme Q10. Enzymatic antioxidants, on the other hand, act as antioxidants by catalyzing the degradation of ROS. The main ones are Super Oxide Dismutase(SOD), Trx system and Gpx family (35).

The Gpx family of glutathione peroxidases is an evolutionarily highly conserved group of enzymes containing eight main isoenzymes, among which Glutathione Peroxidase 4(GPx4) utilizes GSH as a cofactor to convert lipid hydroperoxides into nontoxic lipids alcohols and reduce free radical accumulation (36). Inhibition of GPx4 can lead to an increase in ROS whereas increased GPx4 expression can lead to a decrease in ROS content, and GPx4 performs this function dependent on GSH (37), which is an important component of the body's endogenous antioxidant system, and is derived from glutamate, glycine, and cysteine by dehydration condensation, and its intracellular level is affected by the ability of SLC7A11 to transporter cystine (38). Studies have shown that pharmacological inhibition of SLC7A11 or knockdown of SLC7A11 reduces intracellular cysteine concentration, thereby affecting GSH concentration (39). erastin, a selective inhibitor of SLC7A11, inhibits cystine uptake, and salicylsulfonylpyrimidines and glutamate can also reduce intracellular cysteine concentration and deplete intracellular GSH by inhibiting SLC7A11 (40), depleting GSH in cells. thereby inducing cellular oxidative stress and death (Figure 3).

In conclusion, SLC7A11 has a pro-survival effect, and SLC7A11-mediated cystine uptake can help cells re-establish redox homeostasis in response to oxidative stress, whereas inhibition of SLC7A11 can lead to depletion of cellular GSH and thus make the cells more sensitive to chemotherapy or radiotherapy.

SLC7A11 and tumor metabolism

Beyond its well-documented antioxidant functions, SLC7A11 emerges as a pivotal metabolic regulator that profoundly influences intracellular nutrient processing and energy metabolism within

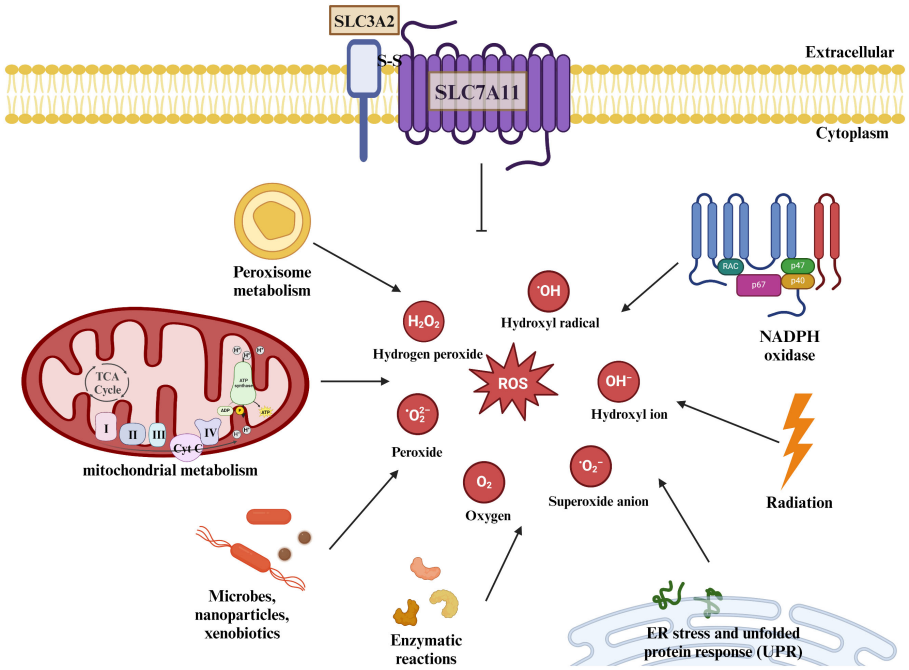

FIGURE 3
SLC7A11 regulates cellular redox homeostasis. ROS are present intracellularly in a variety of forms: including superoxide anions, peroxides, and oxygen radicals. ROS are present intracellularly in a variety of forms: including superoxide anions, peroxides, and oxygen radicals. Mitochondria produce large amounts of ROS through the electron transport chain, and the metabolism of NADPH and peroxisomes also increases intracellular ROS levels, as do some microorganisms or misfolded abnormal proteins, and studies have shown that radiation therapy also produces some ROS. The human body can scavenge excess ROS under normal physiological conditions through SLC7A11-mediated production of GSH. The body can remove excessive ROS accumulation through SLC7A11-mediated GSH production under normal physiological conditions to maintain redox homeostasis.

cancer cells. A fascinating aspect of SLC7A11's role in tumorigenesis is its ability to modulate the uptake and conversion of cystine. Cancer cells with elevated levels of SLC7A11 expression exhibit an increased affinity for cystine, which they rapidly convert into cysteine. This biochemical transformation, however, comes at an energetic cost (41).

Specifically, the conversion process requires a significant amount of NADPH, a crucial cofactor that primarily originates from the cytoplasmic glucose-pentose phosphate pathway. This pathway, in turn, plays a vital role in generating ribose-5-phosphate for nucleotide synthesis and NADPH for reductive biosynthesis and detoxification of reactive oxygen species. Consequently, cancer cells overexpressing SLC7A11 develop a heightened dependency on both the glucose and pentose phosphate pathways for their survival and proliferation (42). This enhanced reliance on glucose metabolism renders these cells particularly vulnerable to glucose deprivation. In the context of glioblastoma, for instance, glucose starvation can induce cell death more rapidly in cells with high SLC7A11 expression. This vulnerability presents a potential therapeutic window. By simultaneously targeting glucose transporter type 1 (GLUT1), a glucose transporter critical for glucose uptake, and glutathione synthesis, which is intimately linked to NADPH production and reactive oxygen species scavenging, it may be possible to deplete NADPH levels and cause a buildup of reactive oxygen species. This approach could potentially trigger synthetic lethal cell death specifically in cell lines that overexpress SLC7A11 and are thus sensitized to glucose deprivation, offering a promising avenue for targeted cancer therapies (43) (Figure 4).

SLC7A11 also affects the nutrient dependence of tumor cells through glutamine backfilling and GLS dependence (43). SLC7A11-mediated glutamate transport may deplete the intracellular glutamate/α-KG pool and activate glutamine catabolism, leading to greater glutamine uptake (44).

SLC7A11 and immune regulation

Within the tumor microenvironment, interactions involving SLC7A11 between immune cells and tumor cells play a significant role in influencing tumor survival and proliferation. Specifically, cytokines released by immune cells have the potential to impact the expression of SLC7A11 within tumors. For instance, the secretion of interferon gamma (IFN-γ) by CD8+ T cells has been shown to down-regulate the expression of SLC3A2 and SLC7A11 in tumor cells, leading to a disruption in cystine uptake (45). This disruption ultimately promotes lipid peroxidation and iron-induced cell death within the tumor cells (46). Conversely, the interplay of cysteine competition and glutamate secretion among various immune cells, as well as between immune cells and tumor cells, significantly impacts the survival of tumors. Cysteine, a crucial amino acid for T-cell activation, is integral to tumor surveillance and cytotoxicity

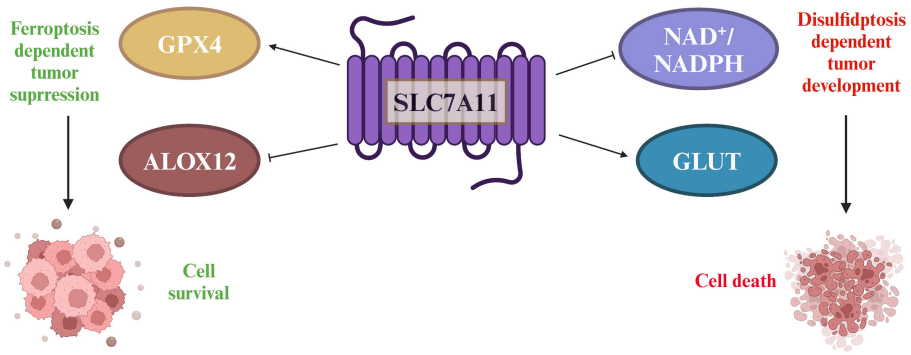

FIGURE 4
The double-edged sword played by SLC7A11 in tumors. SLC7A11 overexpression in tumors increases cysteine and GSH levels, which are important for reducing lipid peroxides through GPx4. SLC7A11 also suppresses ALOX12, decreasing lipid peroxides and promoting tumor cell survival. However, high SLC7A11 expression leads to cystine accumulation, requiring NADPH from the pentose phosphate pathway to convert it to cysteine. Inhibiting glucose transporter proteins depletes glucose, causing disulfide stress and cell death.

(12). T cells, lacking the functional SLC7A11 transporter protein and cystathionine beta-synthase enzyme, depend on neutral amino acid transporter proteins to uptake cysteine exported by antigen-presenting cells (APCs) (47).

Myeloid-derived suppressor cells (MDSCs) express the transporter SLC7A11, which selectively transports cystathionine but does not export cysteine. MDSCs compete with APCs for extracellular cystine, leading to a reduction in APC release of cysteine in the presence of MDSCs (45). This limitation of the extracellular pool of cysteine hinders T cell activation-mediated antitumor immunity. Additionally, SLC7A11-mediated glutamate release in dendritic cells inhibits metabotropic glutamate receptors, impairing T cell activation (48). The upregulation of SLC7A11 in glioblastoma (GBM) leads to heightened levels of extracellular glutamate, facilitating the proliferation, activation, and suppressive capabilities of regulatory T (Treg) cells, consequently fostering intratumoral immunosuppression (49). Metabolic alterations induced by T cells can influence the fate of GBM cells through SLC7A11, while tumor metabolism further contributes to immune evasion by impairing T cell activation via SLC7A11-mediated dysfunction (Figure 5).

SLC7A11 and cell death

In the initial investigations into cell growth conditions, researchers observed that a lack of SLC7A11-mediated cystine transport proved fatal for certain cells (50, 51). Subsequent findings revealed that cells deficient in cystine exhibited diminished levels of intracellular GSH, a key antioxidant, and that supplementation with vitamin E effectively prevented this form of cell death induced by cystine deficiency (52–54). These results imply a strong association between this mode of cell death and oxidative stress. Further research identified this specific form of cell death as ferroptosis (55). Ferroptosis is characterized by the accumulation of lipid peroxidation products resulting from iron metabolism and ROS accumulation (56). The SLC7A11/GSH/GPx4 axis plays a central role in the cell's defense against ferroptosis, with GPx4 utilizing glutathione (GSH) as a cofactor to reduce toxic lipid peroxides at the plasma membrane, thereby protecting the cell from ferroptosis. SLC7A11 is positioned at the initial stage of the ferroptosis pathway, and inhibitors targeting SLC7A11 are commonly used as inducers of ferroptosis in research studies (57)(Figure 6).

Contrary to the oncogenic implications outlined earlier, the upregulation of SLC7A11 can also induce apoptotic cell death in cancer cells under conditions. For instance, in glucose-deficient glioblastoma, heightened SLC7A11 expression leads to the generation of ROS and oxidative stress through the consumption of intracellular NADPH during the conversion of imported L-cystine to L-cysteine (58). Moreover, the addition of α-ketoglutarate (α-KG), a metabolite derived from glutamate, effectively restored the viability of cancer cells with elevated levels of SLC7A11 during glucose deprivation, indicating a potential role for exported glutamate in promoting cancer cell death (59). Subsequent research corroborated the significance of converting glutamate to α-KG for the survival of cancer cells in the absence of glucose, highlighting the regulatory function of SLC7A11 in modulating the metabolic adaptability of cancer cells (60). Cancer cells exhibiting elevated levels of SLC7A11 expression demonstrate a reliance on glucose metabolism, while cells with reduced SLC7A11 expression show heightened oxidative phosphorylation (OXPHOS) activity, as observed in lung cancer cell lines. This shift in metabolic preferences has been further validated in specific cell lines, such as A549 and H1299, where SLC7A11 knockdown led to an increase in OXPHOS and a decrease in glycolysis (5). Additionally, this phenomenon has been replicated *in vivo*, within a tumor microenvironment, where the metabolic reprogramming associated with altered SLC7A11 expression was evident (61). We underscore the credibility and reproducibility of the observed metabolic shifts in cancer cells with varying SLC7A11 expression levels (62). Given the limited scope of current experiments, additional research is necessary to explore potential underlying mechanisms or constraints contributing to this paradox. Factors such as the intensity and duration of ROS exposure may offer insight into the observed discrepancies. There appears to exist a toxicity threshold for ROS in the induction of tumor cell death (63). Cell death is only induced at

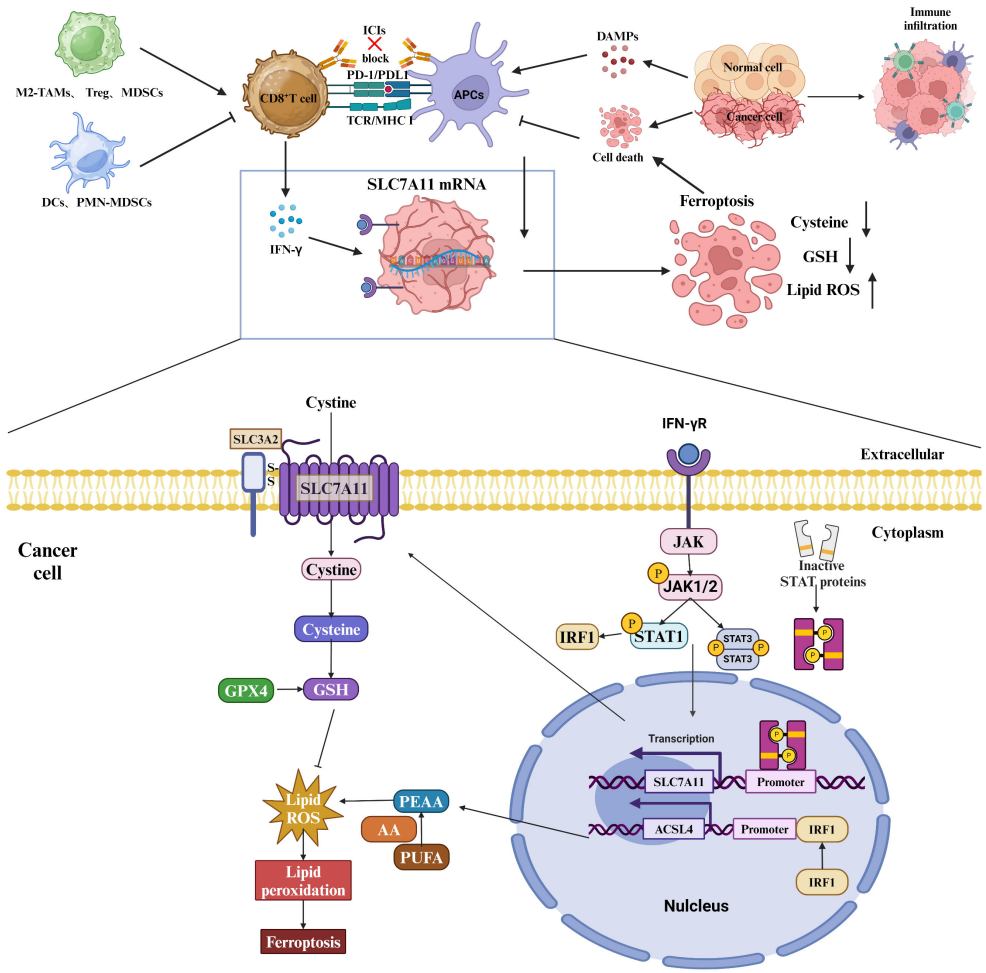

FIGURE 5
Role of SLC7A11 in regulating the tumor immune microenvironment. Myeloid-derived suppressor cells (MDSCs) express the transporter SLC7A11, which selectively transports cystathionine but does not export cysteine. MDSCs compete for extracellular cystathionine with antigen-presenting cells (APCs), resulting in a decrease in cysteine release from APCs in the presence of MDSCs. This limitation of the extracellular cysteine pool hinders T cell activation-mediated antitumor immunity. In addition, SLC7A11-mediated glutamate release in dendritic cells inhibits metabotropic glutamate receptors, thereby impairing T cell activation. Upregulation of SLC7A11 in glioblastoma (GBM) leads to elevated extracellular glutamate levels, which favors the proliferation, activation, and suppressive capacity of regulatory T (Treg) cells and thus promotes intratumorally immunosuppression. T-cell-induced metabolic alterations can affect the fate of GBM cells via SLC7A11, and tumor metabolism via SLC7A11-mediated dysfunction impairs T cell activation, which further promotes immune evasion. Specifically, IFN-γ affects the mRNA levels of SLC7A11 and ACSL4 through the JAK/STAT signaling pathway.

high levels of ROS, while levels below this threshold have been shown to enhance tumor malignancy. This phenomenon has been attributed to the inadequate redox capacity in conditions of limited glucose supply, as well as the abnormal accumulation of cystine or other disulfide molecules in cells with high expression of SLC7A11. This accumulation leads to disulfide stress, ultimately resulting in cell death through a novel form of programmed cell death known as disulfidptosis (64). Subsequent investigations revealed that elevated levels of the SLC7A11 protein unexpectedly heightened the susceptibility of tumor cells to oxidative stressors, leading to increased rates of tumor cell apoptosis. Additionally, *in vivo* experiments demonstrated that heightened SLC7A11 expression facilitated localized tumor growth while impeding tumor migration. In summary, the molecular regulatory mechanism of disulfidptosis involves multiple aspects of cystine uptake, glucose metabolic pathways, disulfide stress, and altered cytoskeletal structure (Figure 7).

These findings suggest the potential for inducing distinct forms of cell death in individuals with varying levels of SLC7A11 expression, although the interplay between ferroptosis and disulfidptosis remains an unresolved inquiry.

Tumor therapeutic strategies targeting SLC7A11

Several compounds have been identified as inhibitors of SLC7A11, including Erastin, IKE, sorafenib, DPI2, SAS, glutamate, and INF-γ. A high-throughput screening of synthetic compounds aimed at identifying substances capable of eliminating tumor cells led to the discovery of erastin, a compound exhibiting RAS-selective activity (65). Notably, erastin was observed to trigger a distinct form of cell death, non-apoptotic in nature. Subsequent

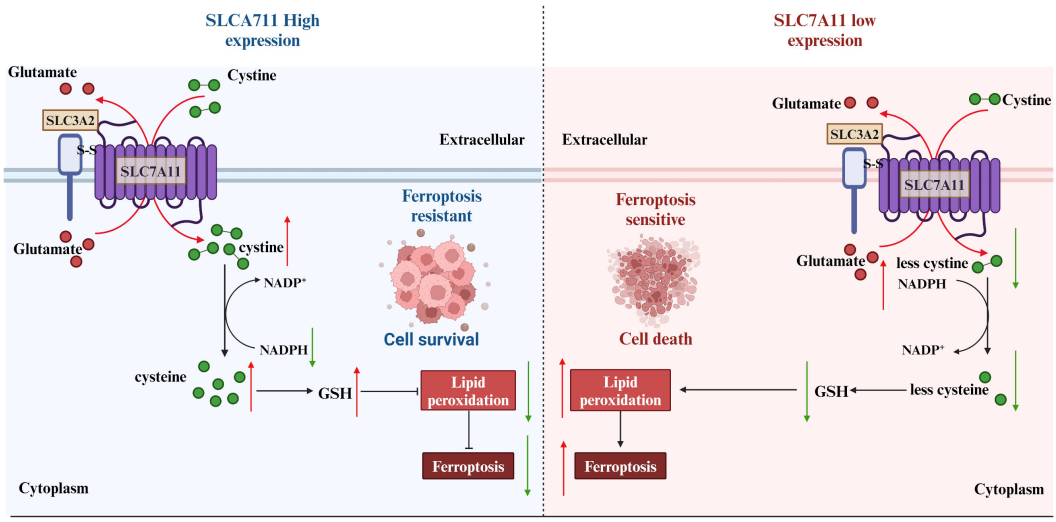

FIGURE 6
Strategies of SLC7A11 in mediating ferroptosis in the treatment of tumors. The expression levels of SLC7A11 in different cancers were characterized differently. For cells with low SLC7A11 expression, intracellular GSH deprivation leads to diminished lipid peroxide scavenging, resulting in tumor cells that are sensitive to ferroptosis. In contrast, tumor cells with high SLC7A11 expression would be naturally highly resistant to ferroptosis.

investigations delved deeper into its mechanism, revealing that erastin directly inhibits Systems Xc-, thereby depleting GSH levels. The lethality of erastin may primarily stem from its quinazolinone backbone, while other chemical moieties could potentially enhance its inhibitory effects on Systems Xc-. Additionally, erastin targets the mitochondrial voltage-dependent anion system (VDAC) (66). Beyond its direct activation of ferroptosis in the treatment of hepatocellular carcinoma, erastin also potentiates the antitumor effects of certain conventional chemotherapeutic agents in hepatocellular carcinoma cell lines. Furthermore, erastin has the potential to augment the clinical efficacy of PD1/L1 by influencing the polarization of tumor-associated macrophages (TAM) (4). In another study, aspirin was found to elicit a pronounced ferroptosis response in HepG2 and Huh7 cells, an effect that was amplified by the ferroptosis-inducing properties of erastin (67). Given its established role as a classical ferroptosis inducer, erastin has become a benchmark for researchers evaluating novel compounds or assessing the ferroptosis-inducing potential of existing drugs (Table 2).

Although erastin demonstrates a potent inhibitory effect on Systems Xc-, its practical application *in vivo* is significantly hindered by its limited water solubility and metabolic instability. To address these challenges, Imidazolidinone (IKE) was developed as a structurally improved derivative of erastin. IKE not only enhances aqueous solubility but also boosts its anticancer properties. Specifically, IKE boasts a solubility that is three times higher than erastin, and it exhibits a remarkable 50-fold reduction in the Lethal Concentration 50 (LC50) for tumor cells, indicating a significantly enhanced antitumor potency (68).

DPI2, a FIN (ferroptosis-inducing compound), which does not inhibit GPx4 activity but specifically inhibits SLC7A11 expression, consumed 90% of the GSH in BjeLR cells compared to the untreated group. The observed effect of DPI2 was comparable to that of erastin, indicating that DPI2 might trigger cellular ferroptosis via a mechanism analogous to that employed by erastin (37).

Sorafenib, a multikinase inhibitor, has gained widespread use in the treatment of clinically advanced hepatocellular carcinoma, offering patients prolonged survival. Previous research attributed sorafenib's therapeutic effect primarily to its multikinase inhibitory function, which halts cell proliferation, angiogenesis, and promotes tumor cell apoptosis (69, 70). However, recent discoveries indicate that sorafenib's toxic impact on hepatocellular carcinoma cells relies partly on ferroptosis, rather than apoptosis. This effect isn't tied to its kinase inhibitory activity but rather to its ability to trigger iron accumulation and lipid peroxidation stress in the cancer cells (71). Notably, depleting stored iron in these cells through iron chelating agents significantly diminishes sorafenib's cytotoxic effects. Further investigations have uncovered that sorafenib prompts ferroptosis by impeding the activity of the SLC7A11 transporter on the cell membrane. This reduction leads to decreased cystine levels in the cancer cells, subsequently causing insufficient GSH synthesis and a decline in GPX4 activity. Clinically, hepatocellular carcinoma patients treated with sorafenib often develop drug resistance rapidly, influencing their prognosis. Nrf2, Rb, MT-1G, and SIR are involved in regulating the sensitivity to sorafenib-induced ferroptosis through various pathways, contributing to the emergence of drug resistance (66, 72–74). Adopting a fresh perspective, targeting specific inhibitor pathways to stimulate ferroptosis represents a promising strategy to enhance sorafenib's drug resistance effectively.

Sulfasalazine (SAS) is a long-approved anti-inflammatory drug by the U.S. Food and Drug Administration, serving as a primary therapy for rheumatoid arthritis. SAS promotes ferroptosis through inhibiting the Xc-system, akin to erastin's mechanism. Nevertheless, SAS is significantly less potent in triggering ferroptosis compared to erastin. Studies have demonstrated SAS's

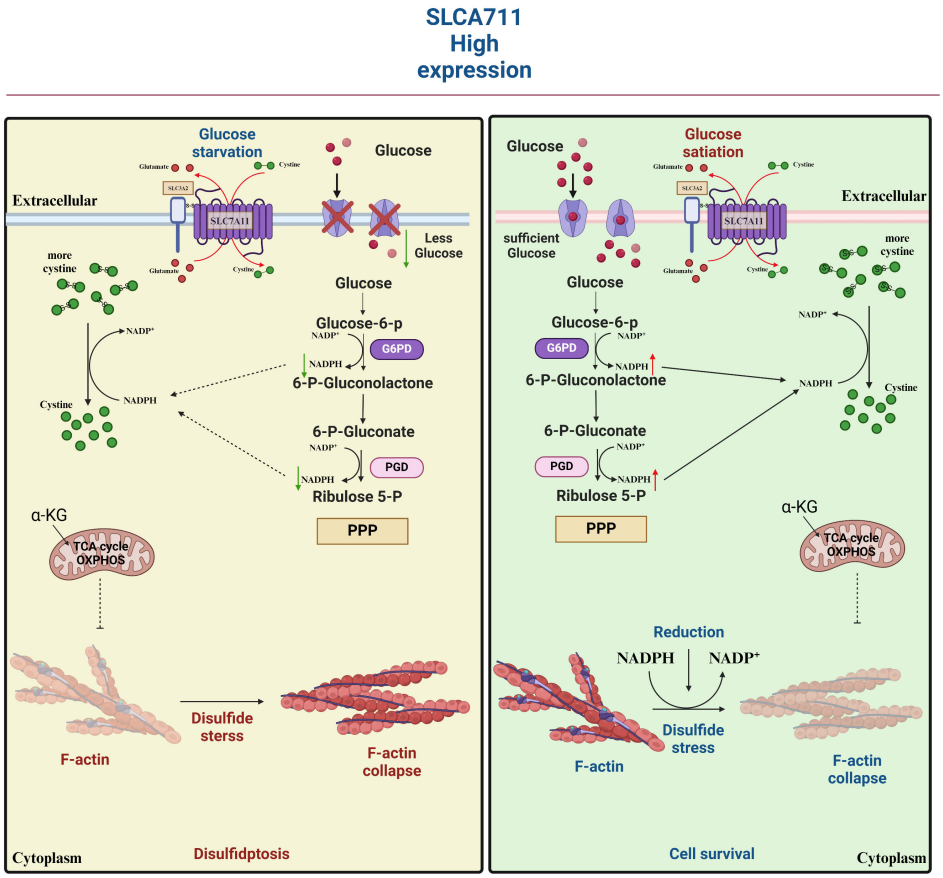

FIGURE 7
SLC7A11 is highly expressed in tumor cells in response to glucose-dependent induction of disulfidptosis. Disulfidptosis, a unique form of cell death, relies on redox reactions and disulfide bond formation. This process is controlled by cystine uptake and glucose metabolism, with SLC7A11 playing a key role in transporting cystine for glutathione synthesis. However, cystine can be toxic and must be quickly converted to cysteine to prevent harmful buildup. A shortage of NADPH during this conversion can lead to disulfide stress, causing cytoskeletal proteins to form disulfide bonds, contract, and detach from the membrane, disrupting cell function and leading to death.

ability to induce ferroptosis in diverse tumor cells, suggesting its potential as a combinatory treatment with other cancer therapies to enhance overall treatment effectiveness (75).

As an excitatory neurotransmitter that binds to both excitatory neurotransmitter binding sites and Cl^-dependent cysteine- and cystine-inhibited transporter sites, the Xc-system facilitates the transfer of glutamate out of and cystine in. Glutamate-induced toxicity of cellular ferroptosis is proportional to its ability to inhibit cystine uptake, which is in effect a negative feedback mechanism. Exposure to glutamate leads to a decrease in GSH levels and an accumulation of intracellular peroxides, resulting in oxidative stress and cell death (76).

As talked about previously, tumor-associated immunotherapy studies have shown that $CD8^+$ T cells enhance ferroptosis-specific lipid peroxidation in tumor cells. Specifically, INF-γ released by $CD8^+$T cells downregulated the expression of SLC7A11 and SLC3A2, leading to a decrease in GSH and an increase in lipid peroxidation. Inhibition of the ferroptosis pathway eliminated the synergistic effect of INF-γ on ferroptosis in tumor cells in both *in vivo* and *in vitro* mouse models (46). And retrospective analysis showed that the expression of SLC7A11 was negatively correlated with $CD8^+$ T cell signaling, INF-γ level and prognosis of tumor patients. Combining immunotherapy with SLC7A11 inhibitors produces a powerful therapeutic effect (77).

A powerful SLC7A11 inhibitor, HG106, has been discovered. This inhibitor notably decreases cystine uptake and disrupts intracellular glutathione biosynthesis. Furthermore, HG106 demonstrates targeted cytotoxicity specifically towards KRAS mutant cells. This effect is achieved by amplifying oxidative stress and triggering apoptosis mediated by endoplasmic reticulum (ER) stress. Remarkably, the administration of HG106 in KRAS mutant lung adenocarcinoma (LUAD) models significantly inhibited tumor growth and extended survival rates in multiple preclinical mouse models of lung cancer (78).

Lepadin H, a marine alkaloid, stands out as an effective inducer of ferroptosis. It demonstrates considerable cytotoxicity, stimulates p53 expression, elevates ROS generation and lipid peroxidation, while simultaneously reducing SLC7A11 and GPX4 levels. Additionally, Lepadin H upregulates ACSL4 expression. Remarkably, it exhibits minimal toxicity towards normal organs,

TABLE 2 Small molecule compounds targeting SLC7A11.

Small Molecule Compounds	Target	Cancer	Experimental Models	Reference
erastin	SLC7A11	HCC, GC, CRC	cells, animals	(66, 67)
IKE	SLC7A11	HCC	cells, animals	(68)
DPI2	SLC7A11	HCC	cells, animals	(37)
sorafenib	SLC7A11	HCC	cells, animals, clinical trial	(69–75)
SAS	SLC7A11	NSCLC	cells, animals, clinical trial	(75)
glutamate	SLC7A11	neuroblastoma	cells, animals	(76)
INF-γ	SLC7A11	NSCLC	cells, animals	(77)
HG106	SLC7A11	NSCLC	cells, animals	(78)
Lepadin E/H	SLC7A11	melanoma	cells, animals	(79)
PAB	SLC7A11	glioma	cells, animals	(80)
Ursolic acid	SLC7A11	HCC	cells, animals	(81, 82)
Butyrate sodium	SLC7A11	endometrioma, osteosarcoma	cells, animals	(83)
TPZ	SLC7A11	osteosarcoma	cells, animals	(84)
PZH	SLC7A11	HCC	cells, animals	(85)
levobupivacaine	SLC7A11	GC	cells, animals	(86)
curcumin	SLC7A11	NSCLC	cells, animals	(87)
β-Elemene	SLC7A11	CRC	cells, animals	(88)
Agrimonolide	SLC7A11	ovarian cancer	cells, animals	(89)
Ginkgetin synergized	SLC7A11	NSCLC	cells, animals	(90)
SI	SLC7A11	NSCLC	cells, animals	(91)
vitamin D	SLC7A11	CRC	cells, animals	(92)
Tanshinone IIA	SLC7A11	GC	cells, animals	(93)
Ginsenoside Rh3	SLC7A11	CRC	cells, animals	(94)
Lico A	SLC7A11	HCC	cells, animals	(95)
Talaroconvolutin A	SLC7A11	CRC	cells, animals	(56)
Saikosaponin A	SLC7A11	HCC	cells, animals	(96)

highlighting its potential as a transformative ferroptosis inducer (79).

Certain small molecule monomers possess the ability to adjust SLC7A11 activity, thereby influencing intracellular GSH levels and potentially providing therapeutic advantages for treating specific tumors. This hints at the possibility of these monomers acting as innovative ferroptosis inducers by regulating GSH content. One such example is Pseudolaric acid B (PAB), a naturally occurring diterpene acid extracted from Kaempferia roots and bark. Studies have revealed that PAB can trigger ferroptosis in glioma cells by depleting GSH through SLC7A11 inhibition. By suppressing the expression or function of the Xc-system, PAB slows tumor growth *in vivo*, while also inhibiting cancer cell invasion and metastasis (80). This cancer-suppressing effect is primarily attributed to the rapid depletion of GSH caused by SLC7A11 transporter dysfunction, leading to lipid ROS buildup and ferroptosis induction.

Ursolic acid, a pentacyclic triterpenoid derived from traditional plants, significantly boosts ROS accumulation in a hepatocellular model when paired with sorafenib (81). This enhancement is likely due to the downregulation of SLC7A11 expression, causing a drop in intracellular GSH levels and compromising ROS scavenging abilities. As a result, lipid peroxidation occurs, inhibiting ferroptosis and offering notable therapeutic benefits in hepatic cell treatment (82).

Sodium butyrate demonstrates anti-cancer properties by modulating the GSH/GSSG ratio, intracellular ROS levels, and lipid peroxide content, thereby inducing ferroptosis in endometrial cancer cells. It also shows promise in suppressing osteosarcoma growth and metastasis through ferroptosis promotion. Pre-exposure to sodium butyrate intensifies erastin-induced changes in GSH depletion, lipid peroxidation, and mitochondrial morphology in CRC cells. Mechanistically, sodium

butyrate down-regulates SLC7A11 transcription by modifying ATF3 expression (83).

Tirapazamine (TPZ), a hypoxic prodrug, is renowned for its antitumor effects in the hypoxic tumor microenvironment. TPZ effectively inhibits all three osteosarcoma cell lines tested. Additionally, TPZ enhances fluorescent staining of ferrous ions while reducing SLC7A11 and GPX4 expression, thus promoting ferroptosis and hindering the proliferation and migration of osteosarcoma cells (84).

Pientzehuang (PZH) demonstrates inhibitory effects on the diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) model in rats. The SLC7A11/GSH/GPX4 axis, associated with the ferroptosis response, is seen as a potential target for PZH in preventing the malignant transition from liver fibrosis to HCC (85).

Levobupivacaine, a renowned local anesthetic, exhibits promising anticancer capabilities. It triggers ferroptosis in gastric cancer cells by manipulating the miR-489–3p/SLC7A11 pathway, thereby hindering cancer cell proliferation (86).

Curcumin, a component derived from turmeric and used in traditional Chinese medicine, has been discovered to cause iron accumulation, GSH depletion, and lipid peroxidation in non-small cell lung cancer (NSCLC) cells. However, suppressing Fer-1 and IREB2—both inhibitors of ferroptosis—substantially diminishes curcumin's anticancer and ferroptosis-inducing effects in A549 and H1299 cells (87).

β-elemene, a naturally occurring compound, has emerged as a novel inducer of ferroptosis. When combined with cetuximab, it demonstrates enhanced sensitivity towards KRAS-mutant colorectal cancer (CRC) cells by promoting ferroptosis, offering a potential therapeutic approach for such cancers (88).

Agrimonolide, extracted from Lungwort, possesses various biomedical properties, including anticancer activity. In ovarian cancer cell lines A2780 and SKOV-3, Agrimonolide not only restricts proliferation, migration, and invasion in a dose-dependent manner but also initiates apoptosis. Its ferroptosis-inducing effects in ovarian cancer cells are evident from increased ROS, total iron, and Fe^{2+} levels, coupled with reduced expression of ferroptosis markers SLC7A11 and GPX4 (89).

Cisplatin (DDP) stands as a frontline treatment for advanced NSCLC. Ginkgetin has been found to augment DDP's cytotoxic effects in NSCLC cells by facilitating the accumulation of labile iron and lipid peroxidation. Studies confirm Ginkgetin's role in mediating ferroptosis through significant reductions in SLC7A11 and GPX4 expression, along with alterations in the GSH/GSSG ratio (90).

Mustardine (SI) has been identified as a potent anti-NSCLC agent, inducing ferroptosis by elevating subferric iron, ROS, and lipid peroxidation. Additionally, SI treatment leads to SLC7A11-dependent downregulation of P53 (91).

Colorectal cancer stem cells (CCSC) significantly impact prognosis, chemotherapy resistance, and treatment outcomes in CRC. Remarkably, Vitamin D administration substantially inhibits CCSC proliferation and reduces tumor spheroid formation *in vitro*. Further analysis reveals that Vitamin D-treated CCSC exhibit elevated ROS levels, decreased cysteine and GSH levels, and thicker mitochondrial membranes, suggesting that SLC7A11 may be a specific target of Vitamin D's action (92).

Tanshinone IIA, a bioactive compound extracted from Salvia miltiorrhiza, has been found to reduce the stem-like properties of gastric cancer cells. Its mechanism involves increasing lipid peroxidation and decreasing ferroptosis markers in these cells (93).

Ginsenoside Rh3 has proven effective in eliminating colorectal cancer (CRC) cells. It activates Gasdermin D (GSDMD)-dependent pyroptosis and inhibits SLC7A11 through the Stat3/p53/NRF2 pathway, thereby inducing ferroptosis (94).

Licorice chalcone A (Lico A), a key component of the traditional Chinese medicine Glycyrrhiza glabra, is a naturally occurring small molecule with various pharmacological effects. Both *in vivo* and *in vitro* studies have shown that Lico A promotes ferroptosis in hepatocellular carcinoma cells by suppressing SLC7A11 expression. This suppression leads to the inhibition of the GSH-GPX4 pathway and the activation of reactive oxygen species (ROS) (95).

Talaroconvolutin A (TalaA) has emerged as a new inducer of ferroptosis, exhibiting cytotoxicity against colorectal carcinoma cells in a dose- and time-dependent manner. TalaA significantly raises ROS levels to a point where ferroptosis is initiated. Additionally, it downregulates SLC7A11 channel protein expression while upregulating ALOX3, further promoting ferroptosis (56).

Multiple studies have also revealed that Saikosaponin A can induce ferroptosis in hepatocellular carcinoma (HCC) cells, both in laboratory settings and in living organisms. Through RNA sequencing analysis, it has been determined that Saikosaponin A primarily affects the glutathione metabolic pathway and suppresses the expression of the cystine transporter protein SLC7A11. Furthermore, Saikosaponin A has been observed to increase intracellular malondialdehyde (MDA) and iron levels while decreasing reduced glutathione levels in HCC cells. Interestingly, Deferoxamine (DFO), Fer-1, and GSH can reduce the cytotoxic effects of Saikosaponin A, while Z-VAD-FMK is ineffective in preventing Saikosaponin A-induced cell death in HCC (96).

Conclusions

SLC7A11, as the active subunit of the amino acid transporter on cell membranes, plays a wide range of biological functions in organisms. The regulation of SLC7A11 is affected by multiple dimensions, and also abnormal regulation of SLC7A11 leads to malignant tumors related to proliferation, invasion, metastasis and drug resistance. Given that SLC7A11 is often aberrantly expressed by tissues in tumors, it is expected to be an important biomarker for the diagnosis and prognosis of a wide range of tumors. SLC7A11 presents itself as a viable target for tumor therapy, with two primary strategies currently under consideration. The first involves the development of direct inhibitors targeting SLC7A11 to impede cystine uptake in cancer cells, thereby diminishing intracellular

GSH levels and inducing cancer cell ferroptosis. The second strategy entails the utilization of inhibitors targeting glucose transporter proteins or glutaminase in the treatment of tumors overexpressing SLC7A11. This approach capitalizes on the heightened vulnerability of SLC7A11-overexpressing tumors to glucose and glutamine deprivation, ultimately leading to tumor cell death. Nevertheless, the efficacy of the therapeutic strategy is hindered by the inadequate induction of ferroptosis caused by SLC7A11 inhibitors in the presence of intracellular cysteine. Recent studies have demonstrated that combining highly specific SLC7A11 inhibitors with immune checkpoint inhibitors PD1/PDL1 may enhance therapeutic efficacy.

Prospects and perspectives

SLC7A11 appears to represent a potential Achilles heel for tumor targeting; however, several challenges still need to be addressed. First, most of the treatments for tumors based on SLC7A11 affecting ferroptosis are preclinical studies that are not yet supported by sufficient clinical evidence. Second, the determination of how to define a baseline SLC7A11 expression level is a critical core of tumor treatment strategies based on SLC7A11 expression levels. Differences in the expression levels of SLC7A11 in different tumor tissues, and differences in the expression levels of SLC7A11 between different patients may also be closely related to the temporal, spatial, and individual heterogeneity of tumor tissues. Finally, how to achieve tissue- and cell-targeted delivery of SLC7A11 to maximize the efficacy of the drug to minimize its toxic side effects.

Author contributions

YJ: Data curation, Methodology, Writing – original draft. MS: Funding acquisition, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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GSTT1/GSTM1 deficiency aggravated cisplatin-induced acute kidney injury via ROS-triggered ferroptosis

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Introduction: Cisplatin is a widely used chemotherapeutic agent prescribed to treat solid tumors. However, its clinical application is limited because of cisplatin-induced nephrotoxicity. A known complication of cisplatin is acute kidney injury (AKI). Deletion polymorphisms of GSTM1 and GSTT1, members of the glutathione S-transferase family, are common in humans and are presumed to be associated with various kidney diseases. However, the specific roles and mechanisms of GSTM1 and GSTT1 in cisplatin induced AKI remain unclear.

Methods: To investigate the roles of GSTM1 and GSTT1 in cisplatin-induced AKI, we generated GSTM1 and GSTT1 knockout mice using CRISPR-Cas9 technology and assessed their kidney function under normal physiological conditions and cisplatin treatment. Using ELISA kits, we measured the levels of oxidative DNA and protein damage, along with MDA, SOD, GSH, and the GSH/GSSG ratio in wild-type and GSTM1/GSTT1 knockout mice following cisplatin treatment. Additionally, oxidative stress levels and the expression of ferroptosis-related proteins in kidney tissues were examined through Western blotting, qPCR, immunohistochemistry, and immunofluorescence techniques.

Results: Here, we found that GSTT1 and GSTM1 were downregulated in the renal tubular cells of AKI patients and cisplatin-treated mice. Compared with WT mice, *Gstm1/Gstt1*-DKO mice were phenotypically normal but developed more severe kidney dysfunction and exhibited increased ROS levels and severe ferroptosis after injecting cisplatin.

Discussion: Our study revealed that GSTM1 and GSTT1 can protect renal tubular cells against cisplatin-induced nephrotoxicity and ferroptosis, and genetic screening for GSTM1 and GSTT1 polymorphisms can help determine a standard cisplatin dose for cancer patients undergoing chemotherapy.

KEYWORDS

AKI, cisplatin, GSTT1, GSTM1, ROS, ferroptosis

Introduction

Acute kidney injury (AKI) is a clinical syndrome, which presents with a rapid decline in kidney function. It is pathologically characterized by tubular necrosis, which leads to high mortality (1, 2). Annually, AKI leads to approximately 1.7 million deaths worldwide (3). Moreover, AKI can progress to chronic kidney disease (CKD) and end-stage renal disease (ESRD). Currently, there is no specific treatment strategy other than supportive care and dialysis (4). Therefore, clarifying the molecular mechanism underlying AKI pathogenesis is helpful for the early diagnosis and targeted treatment of AKI.

Cisplatin is a widely used chemotherapeutic agent known for its nephrotoxic effects. It induces acute kidney injury (AKI) through several mechanisms, including the generation of reactive oxygen species (ROS), inflammation, DNA damage, and programmed necrosis of renal tubular cells (5). Cisplatin induces the production of reactive oxygen species (ROS), leading to oxidative stress and damage to cellular components, including lipids, proteins, and DNA. The imbalance between ROS production and the antioxidant defense system results in significant cellular injury (6). Additionally, cisplatin treatment triggers a robust inflammatory response in the kidneys, including the activation of various inflammatory cytokines and chemokines, recruitment of immune cells, and further exacerbation of tissue damage (7). Furthermore, cisplatin forms DNA adducts, leading to DNA damage and activation of the DNA damage response, resulting in cell cycle arrest and apoptosis, contributing to renal injury (8).

Glutathione S-transferases (GSTs) are a superfamily of phase II detoxification enzymes, which can eliminate oxygen radicals, lipid peroxidation products, and toxins by catalyzing electrophilic compounds coupled to glutathione (GSH). It is generally considered to be a detoxification reaction (9) (10). They also contribute to antioxidant defense by detoxifying ROS and other reactive intermediates, maintaining redox balance, and protecting against oxidative stress (11, 12). Human GST genes are divided into four major subfamilies, named GSTA, GSTM, GSTT, and GSTP (13). GSTs are highly expressed in most tissues, whereas GSTM1 and GSTT1 are mainly expressed in the liver and kidneys (14, 15). GSTM1 and GSTT1 are polymorphisms with null genotypes for each gene, with approximately 10% to 65% prevalence in the general population (16–19). Deletion or null genotypes of GSTM1 and GSTT1 can significantly increase the risk of ESRD (20–22). Recently, a study showed that deletion of GSTM1 exacerbates kidney injury and intensifies oxidative stress and inflammation in the model mice of CKD (23). These findings suggest that GSTM1 and GSTT1 can protect the kidneys; however, it is still unclear whether GSTM1 and GSTT1 are involved in the development and progression of AKI.

Ferroptosis is a new modality of cell death discovered by Dixon in 2012. Unlike apoptosis and necrosis, ferroptosis is characterized by an iron-dependent cell death pattern accompanied by lipid peroxidation (24, 25). The solute carrier family 7 member 11 (SLC7A11) and glutathione peroxidase 4 (GPX4) are inhibited during ferroptosis, decreasing GSH levels and increasing ROS levels (26, 27). Therefore, ROS play an integral role in the occurrence and progression of ferroptosis (28, 29). Recent studies have found that the occurrence of ferroptosis in renal tubular cells is an important pathological change in AKI, and promoting ferroptosis of renal tubular epithelial cells can expedite the progression of AKI (30). On the contrary, inhibition of ferroptosis in renal tubular epithelial cells can significantly protect against AKI (31–34). However, the specific mechanism of ferroptosis in renal tubular epithelial cells in AKI necessitates further studies.

In this study, we found that GSTT1 and GSTM1 were downregulated in the renal tubules of patients with AKI and cisplatin-treated mice. Compared with WT mice, *Gstm1/Gstt1*-DKO mice were phenotypically normal but developed more severe kidney dysfunction and higher ROS levels after receiving cisplatin, leading to ferroptosis in renal tubular cells and aggravating acute kidney injury. In conclusion, our study revealed that GSTM1 and GSTT1 can protect renal tubular cells against cisplatin-induced AKI and ferroptosis.

Results

The expression and localization of GSTM1 and GSTT1

Genetic variations in GSTM1 and GSTT1 have been linked to kidney diseases. We studied their expression in different organs of mice

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and found that they are most highly expressed in the kidneys and liver, reflecting their detoxification and antioxidant roles (Figures 1B, C). To elucidate the precise localization of GSTM1 and GSTT1 in the kidney, we utilized paraffin-embedded kidney tissues from 2-month-old mice and conducted immunofluorescence staining. Our findings indicated robust expression of GSTM1 and GSTT1 in both glomerular and tubular regions, with GSTT1 predominantly localized in the cytosol and nucleus, and GSTM1 exclusively expressed in the cytoplasm (Figure 1A).

The expression levels of both GSTM1 and GSTT1 were significantly reduced in patients with AKI

Kidney biopsy samples from patients with acute kidney injury (AKI) and kidney para-cancer tissues from patients with kidney cancer (the kidney specimens without histopathological lesions were served as normal controls) were collected for immunohistochemical staining. The results revealed a significant

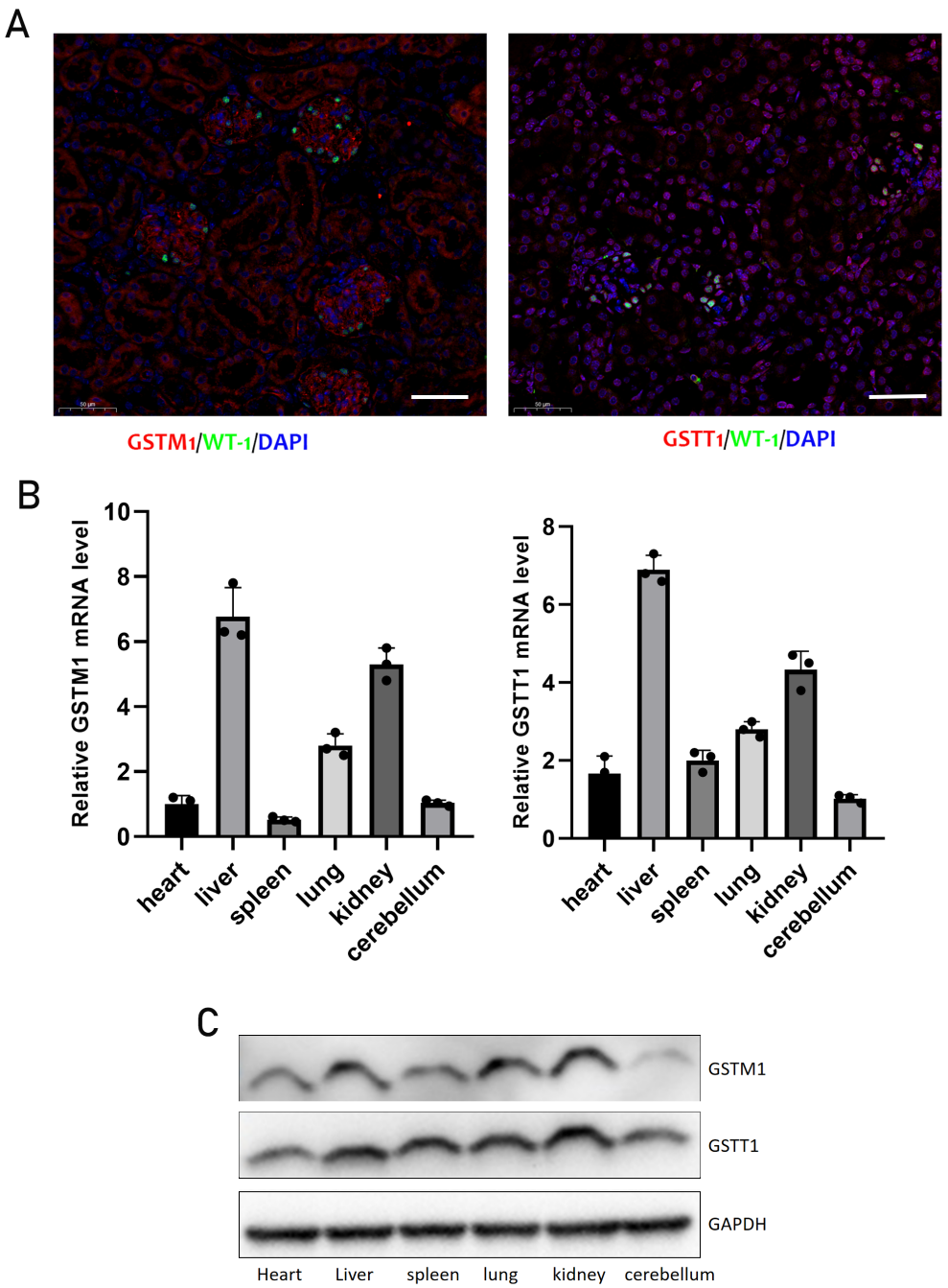

FIGURE 1
The expression and localization of GSTM1 and GSTT1 **(A)** Immunofluorescence staining of GSTT1 and GSTM1 in renal tissue from heathy mice. **(B)** qPCR analysis of Gstm1 and Gstt1 in different tissues such as heart, liver, spleen, lung, kidney and cerebellum in WT mice. **(C)** Protein expression levels of Gstm1 and Gstt1 in different tissues in WT mice. Scale bar = 50μm. n=3.

increase in the expression levels of KIM-1, a biomarker of renal tubular injury during AKI, whereas the expression levels of GSTT1 and GSTM1 were markedly decreased (Figure 2).

The expression levels of both GSTM1 and GSTT1 were significantly reduced in mice with cisplatin-induced AKI

Mice received intraperitoneal injections of saline or cisplatin, and the expression levels of GSTT1 and GSTM1 were measured. Compared to the WT + saline group, blood urea nitrogen (BUN) and creatinine (CREA) levels were significantly elevated in the WT + Cis group (Figure 3A). Immunohistochemical staining and Western blotting demonstrated a significant increase in the expression levels of KIM-1 and NGAL (Figures 3B, D). Histological staining with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) revealed marked dilation of renal tubules and protein tubule structure in mice receiving cisplatin (Figure 3C), accompanied by a significant reduction in GSTM1 and GSTT1 expression (Figure 3D).

Construction of GSTM1 and GSTT1 knockout mice

To further investigate the mechanism by which GSTM1 and GSTT1 are involved in the pathogenesis of acute kidney injury (AKI), we successfully constructed mice with GSTM1 and GSTT1 knockout (GSTM1-KO and GSTT1-KO) using CRISPR-Cas9 technology. The GSTM1 and GSTT1 genes are highly homologous between humans and mice. GSTM1 is located on chromosome 3, spanning 5724 bp and comprising 8 exons that encode 244 amino acids. GSTT1 is located on chromosome 10, spanning 14,772 bp and consisting of 5 exons encoding 190 amino acids. Exon 2 of GSTM1 (GATCCGCATGCTCCTGGAAT) and exon 3 of GSTT1 (GCAGGCTCGTGTAG) were selected as specific targets for knockout (Figure 4A). DNA was extracted from mouse tails 7 days after birth, and target bands were amplified using PCR. Due to the small size of the knockout gene fragments (5 bp and 13 bp), conventional agarose gel electrophoresis could not differentiate homozygous, heterozygous, and wild-type genotypes. Therefore, PCR amplification products were sent for gene sequencing, and comparison with wild-type sequences confirmed the successful

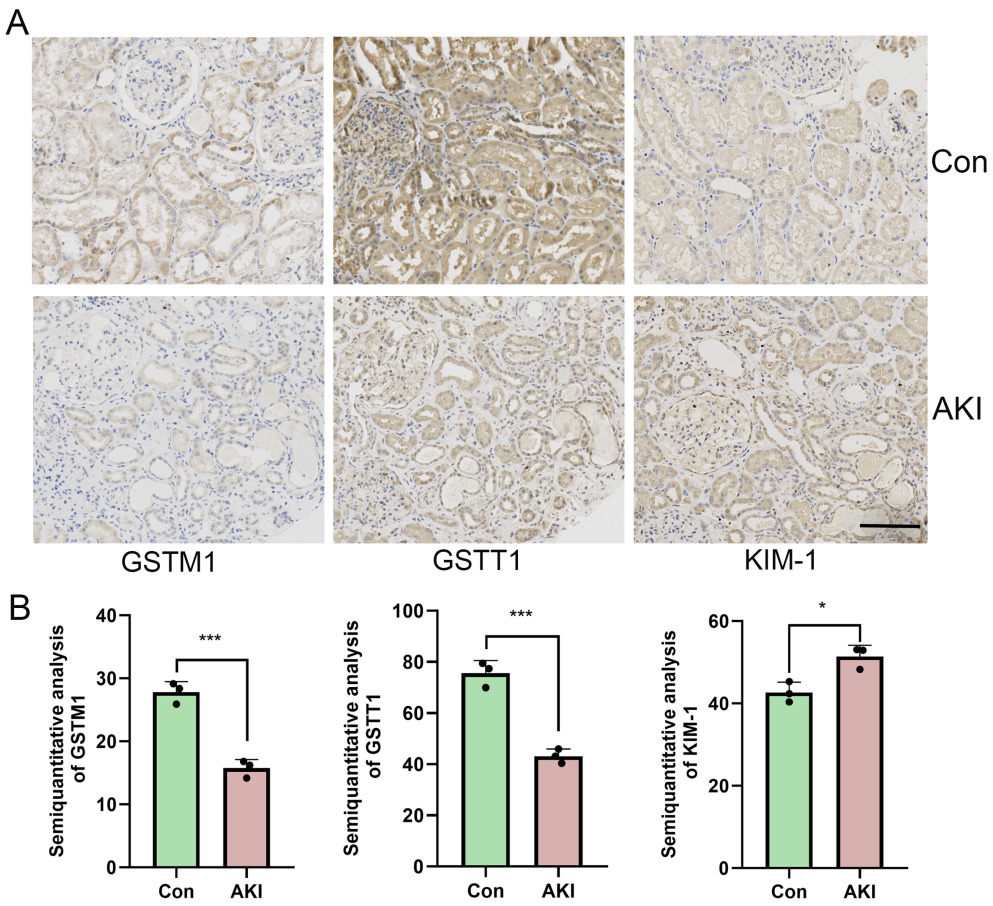

FIGURE 2
The expression of both GSTM1 and GSTT1 were significantly reduced in AKI patients **(A)** Immunohistochemical staining results of GSTT1, GSTM1 and KIM-1 in renal puncture tissue of AKI patients and para-cancerous control tissue. **(B)** Quantitative analysis of GSTM1, GSTT1 and KIM-1 immunohistochemical staining. The data are presented as the mean ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001, n=3. Scale bar =100μm.

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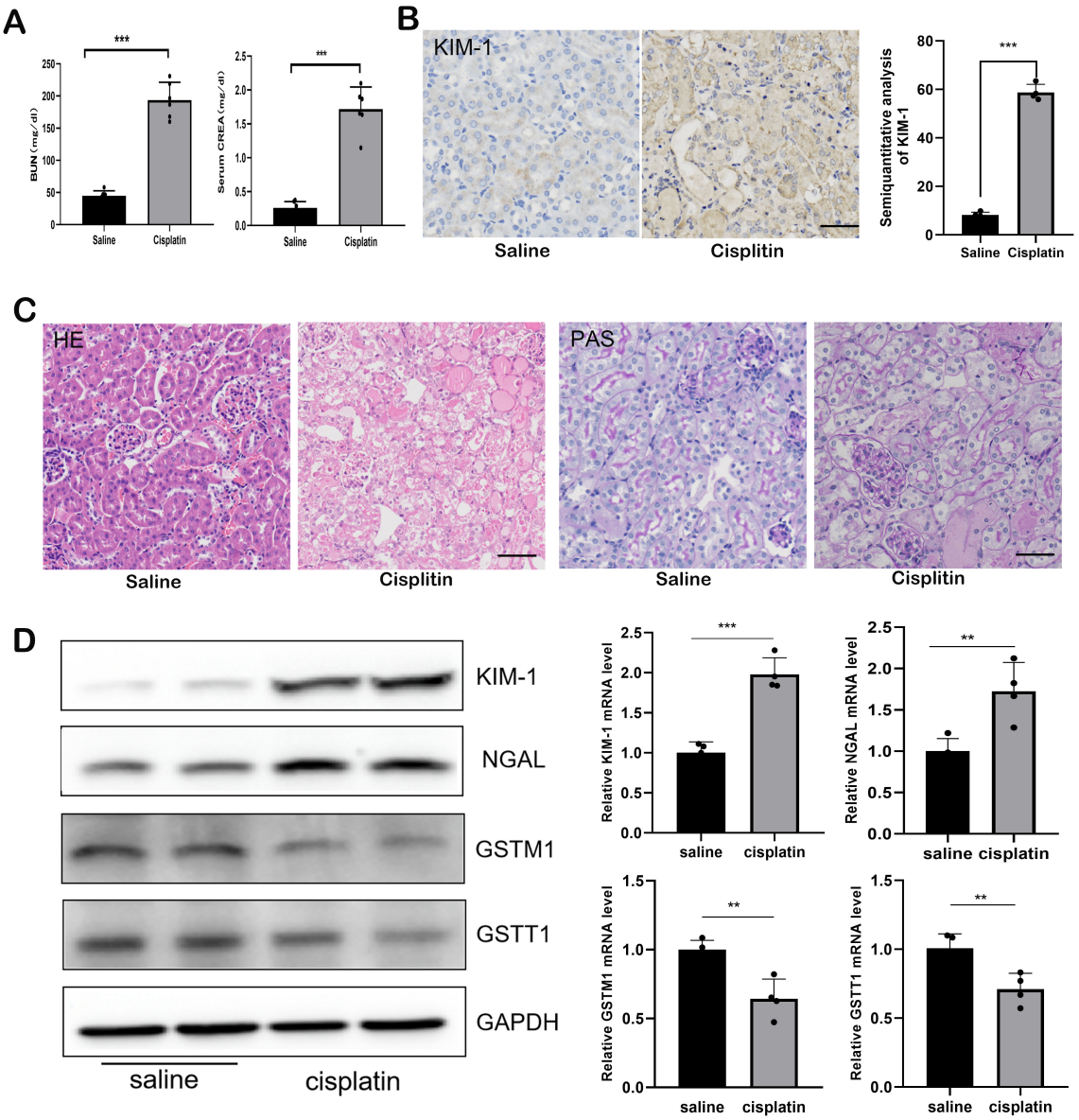

FIGURE 3
The expression of both GSTM1 and GSTT1 were significantly reduced in mice with cisplatin-induced AKI. **(A)** the BUN and CERA levels after 3 days of cisplatin and saline injection. n=6. **(B)** the expression of kim-1 was detected by immunohistochemical staining after 3days of Cisplatin and saline injection. **(C)** Kidney histology as shown by periodic acid–Schiff (PAS) and HE staining after 3 days of cisplatin and saline injection **(D)** Western blots and qPCR analysis of Kim-1, NGAL, GSTM1 and GSTT1 in renal tissue from these two groups (KIM-1 and NGAL was significantly upregulated, whereas the expression of GSTM1 and GSTT1 were significantly reduced). The data are presented as the mean ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001, n≥3. Scale bar =40μm.

deletion of the target bases in GSTM1 and GSTT1 (Figure 4B). We analyzed mRNA and protein levels to validate the knockout efficiency. Kidney tissues from 2-month-old knockout and wild-type mice were dissected for protein and RNA extraction. Western blotting and real-time PCR showed significantly reduced mRNA and protein expression levels of GSTM1 and GSTT1 in the kidneys of knockout mice compared to wild-type mice (Figures 4C, D). Immunohistochemistry and immunofluorescence staining also demonstrated significantly reduced expression levels of GSTM1 and GSTT1 (Figures 5A–C), confirming the successful construction of GSTM1-KO and GSTT1-KO mice models.

Normal kidney function in GSTM1-KO, GSTT1-KO, and Gstm1/Gstt1-DKO mice

PAS and HE staining were performed on kidney tissues from 2-month-old *GSTM1-KO*, *GSTT1-KO*, and WT mice to investigate whether the absence of Gstm1 or Gstt1 impairs kidney function and morphology. The results revealed that the kidney structure and morphology were normal in *GSTT1-KO* mice (Figure 6A), with no significant differences in kidney-to-body weight ratio (Figure 6B), BUN, and CREA levels (Figure 6C) compared to wild-type mice. Similar findings were observed in GSTM1-KO mice (Figures 6D–F).

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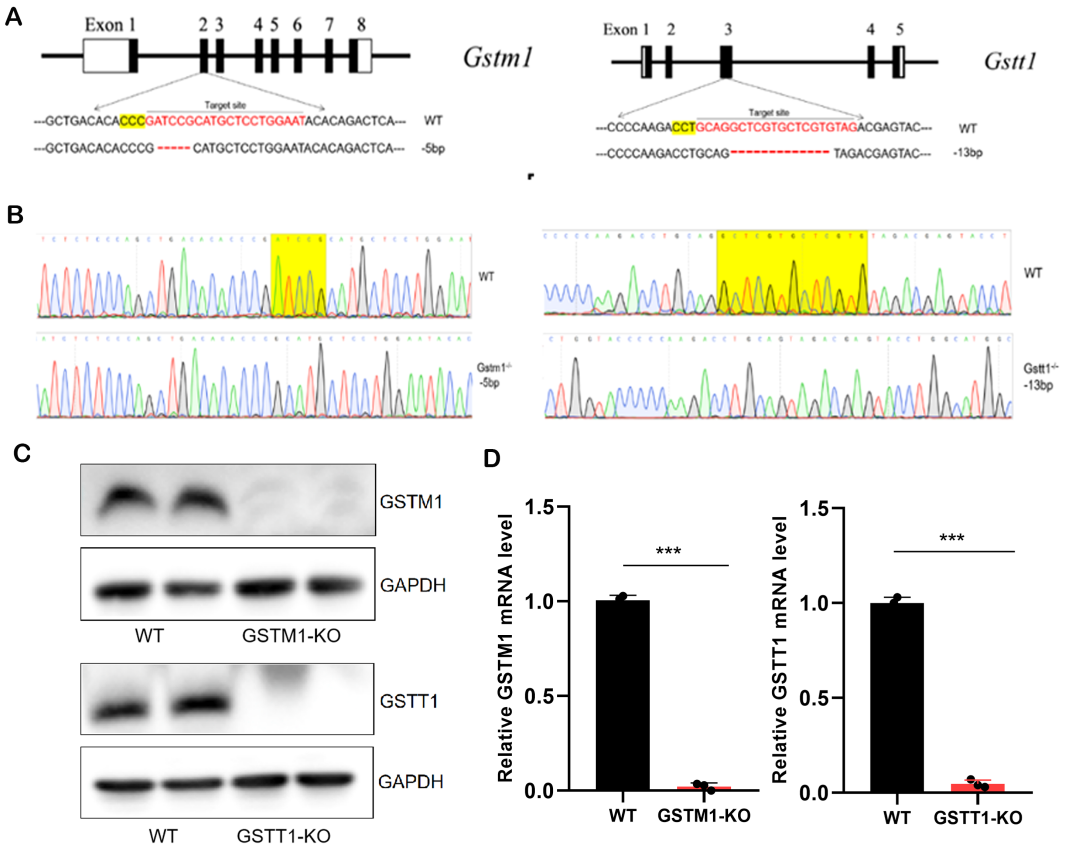

FIGURE 4
Generation of GSTM1-KO and GSTT1-KO mice **(A)** Schematic overview of generation of GSTT1 and GSTM1 knockout mice using CRISPR/Cas9 system. **(B)** Sequence of WT, GSTM1-KO, and GGSTT1-KO mice. **(C)** Protein expression levels of Gstm1 and Gstt1 in the kidney of WT, GSTM1-KO, and GSTT1-KO mice. **(D)** mRNA expression levels of Gstm1 and Gstt1 in the kidney of WT, GSTM1-KO, and GSTT1-KO mice. The data are presented as the mean ± SD, *P<0.5, **P<0.01, ***P<0.001, n≥3.

These results demonstrated that there is no significant difference in the appearance of *GSTM1-KO* or *GSTT1-KO* mice compared to WT mice.

The GSTM1/GSTT1 double-genotype deletion is frequently observed in the general population and is associated with an increased risk of various diseases, including cancer and kidney diseases (28–30). These proteins belong to the GST family, and due to their structural similarity and gene polymorphisms within the GST family, it has been hypothesized that these proteins have similar roles. Based on these considerations and previous reports, we aimed to assess this hypothesis by generating a double-knockout mice model. Therefore, we bred *GSTM1-KO* and *GSTT1-KO* mice together to obtain GSTM1/GSTT1 double-knockout mice (*Gstm1/Gstt11*-DKO). The structure and morphology of the kidneys remained intact in DKO mice (Figure 6G). In addition, there were no significant differences in kidney-to-body weight ratio (Figure 6H), BUN, and CREA (Figure 6I) between DKO mice and wild-type mice.

Deletion of GSTM1 and GSTT1 aggravated cisplatin-induced AKI in mice

Since the expression of GSTM1 and GSTT1 is downregulated in the kidney tissues of mice and patients with AKI, we hypothesized that GSTM1 and GSTT1 play an important role in the development of AKI. Mice were injected intraperitoneally with cisplatin to induce AKI, and the control mice received an equivalent amount of saline. Compared with the WT + Saline group, the levels of BUN, CREA, KIM-1, and NGAL, and the severity of renal tubular damage significantly increased in the WT + Cis group (Figures 7A, B, E), indicating the successful induction of cisplatin-induced mice model of AKI. However, the serum levels of BUN, CREA, KIM-1, and NGAL and the severity of renal tubular damage showed a greater increase in the DKO + Cis group compared to the WT + Cis group (Figures 7A, B, D, E), suggesting that co-deletion of GSTM1 and GSTT1 exacerbated cisplatin-induced AKI. Additionally, survival analysis showed a higher mortality rate in DKO mice after treatment with cisplatin (Figure 7C). These results suggest that simultaneous deletion of GSTM1 and GSTT1 aggravated cisplatin-induced AKI in mice.

Following cisplatin injection, DKO mice exhibited a significantly elevated level of oxidative stress compared to WT mice

Under normal physiological conditions, glutathione (GSH) primarily exists in its reduced form, with GSH oxidized to

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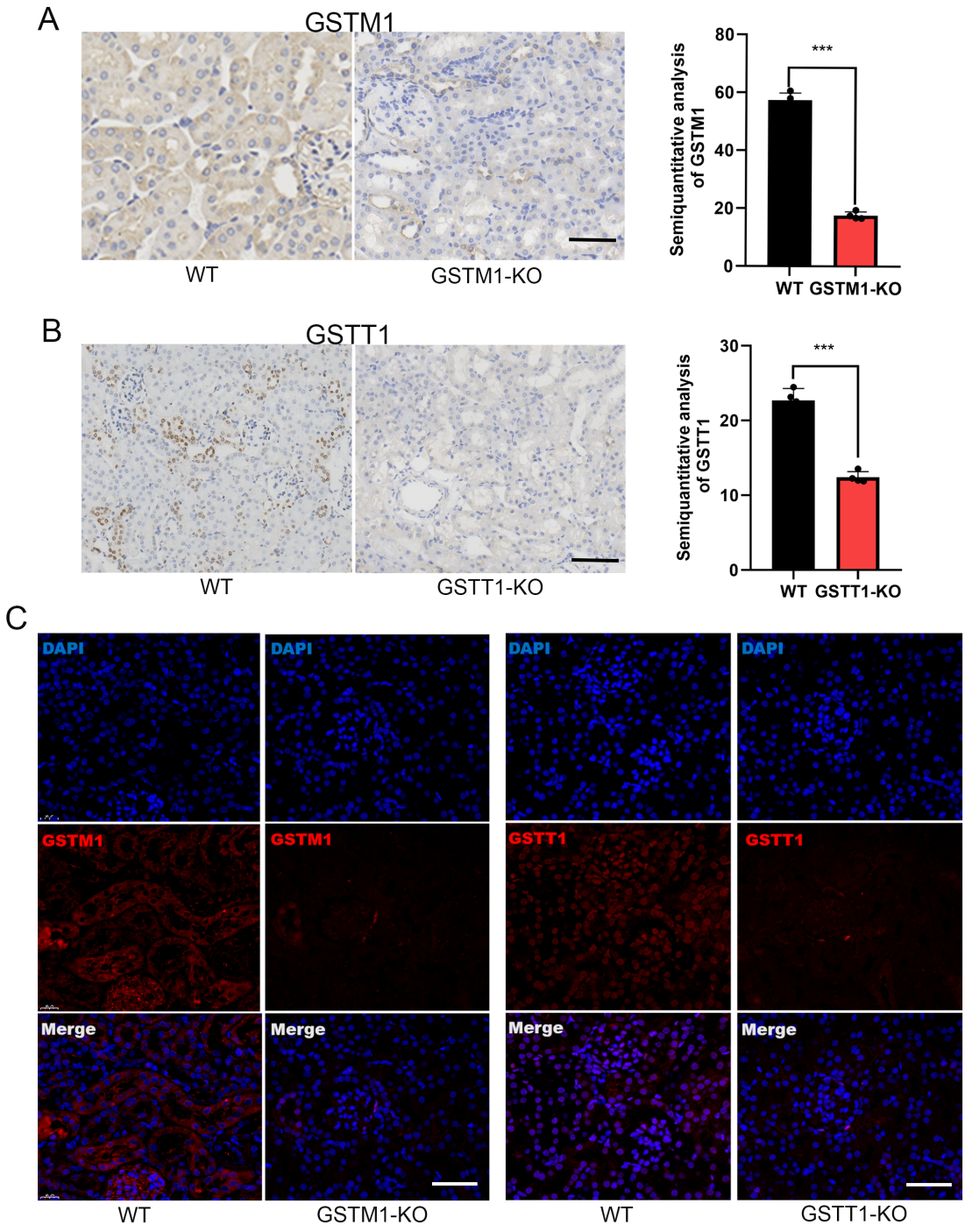

FIGURE 5
Generation of GSTM1-KO and GSTT1-KO mice. Immunohistochemistry **(A, B)** and immunofluorescence staining **(C)** also showed that the expression levels of GSTM1 and GSTT1 were significantly reduced in GSTM1-KO and GSTT1-KO mice. The data are presented as the mean ± SD, **P*<0.5, ***P*<0.01, ****P*<0.001, n=4. Scale bar =40μm.

oxidized glutathione (GSSG). GSSG is subsequently reduced back to GSH by glutathione reductase, maintaining cellular redox equilibrium. Thus, the GSH/GSSG redox system is pivotal for intracellular antioxidant capacity. ELISA revealed a significant decrease in the GSH/GSSG ratio in cisplatin-induced DKO mice (Figure 8B), indicating increased ROS production and impaired redox homeostasis after cisplatin exposure. The levels of malondialdehyde (MDA), a marker of lipid peroxidation, were notably elevated in these mice (Figure 8B). Superoxide dismutase (SOD) is an enzyme crucial for scavenging O2- and converting it into H_2O_2. H_2O_2 is further detoxified by peroxidases, catalase, and glutathione peroxidase to attenuate oxidative stress. SOD levels were significantly reduced (Figure 8B), while the mRNA and protein expression levels of the oxidative stress markers, 4-

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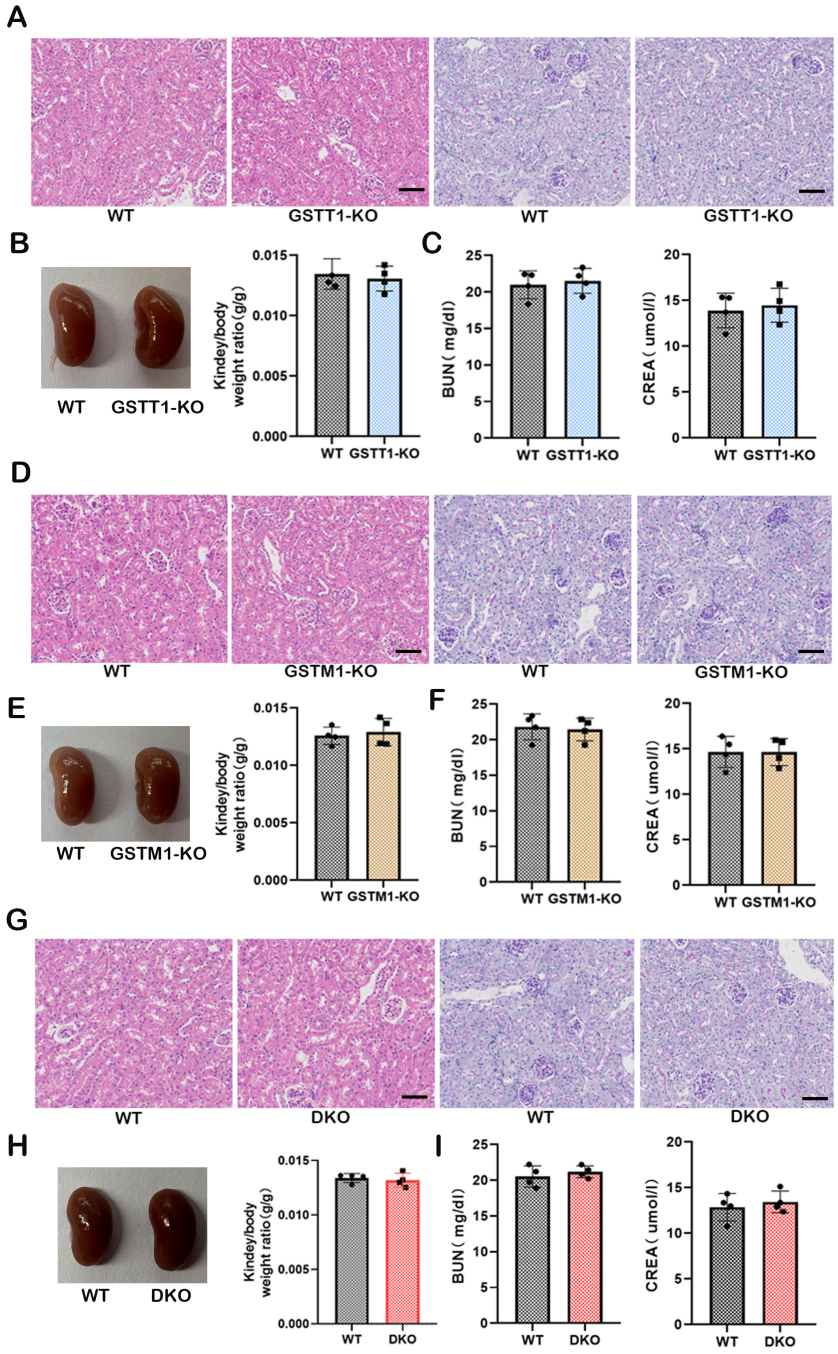

FIGURE 6
Normal kidney function in GSTM1-KO, GSTT1-KO, and Gstm1/Gstt1-DKO mice. **(A)** HE and PAS staining showed that the structure and morphology of kidney in GSTT1-KO mice was normal. **(B)** There was no difference in renal appearance and renal weight ratio between WT and GSTT1-KO mice. **(C)** BUN and CREA showed that the deletion of GSTT1 did not affect renal function in GSTT1-KO mice. **(D)** HE and PAS staining showed that the structure and morphology of kidney in GSTM1-KO mice was normal. **(E)** There was no difference in renal appearance and renal weight ratio between WT and GSTM1-KO mice. **(F)** BUN and CREA showed that the deletion of GSTM1 did not affect renal function in GSTM1-KO mice. **(G)** HE and PAS staining showed that the structure and morphology of kidney in DKO mice was normal. **(H)** There was no difference in renal appearance and renal weight ratio between WT and DKO mice. **(I)** BUN and CREA showed that the deletion of GSTT1 and GSTM1 did not affect renal function in DKO mice. Scale bar =60μm. The data are presented as the mean ± SD, **P*<0.5, ***P*<0.01, ****P*<0.001, n=4.

hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT), were markedly increased in DKO mice exposed to cisplatin (Figures 8A, C).

To illustrate the imbalance between reactive oxygen species and antioxidant systems, we evaluated oxidative damage products and detected MDA and SOD levels and GSH/GSSG ratio. 8-hydroxy-2-deoxyguanosine (8-OHdG) is an oxidative product formed from cisplatin-induced oxidative damage to nuclear DNA. It is a common clinical and experimental biomarker used to assess oxidative damage to DNA. Protein carbonylation includes

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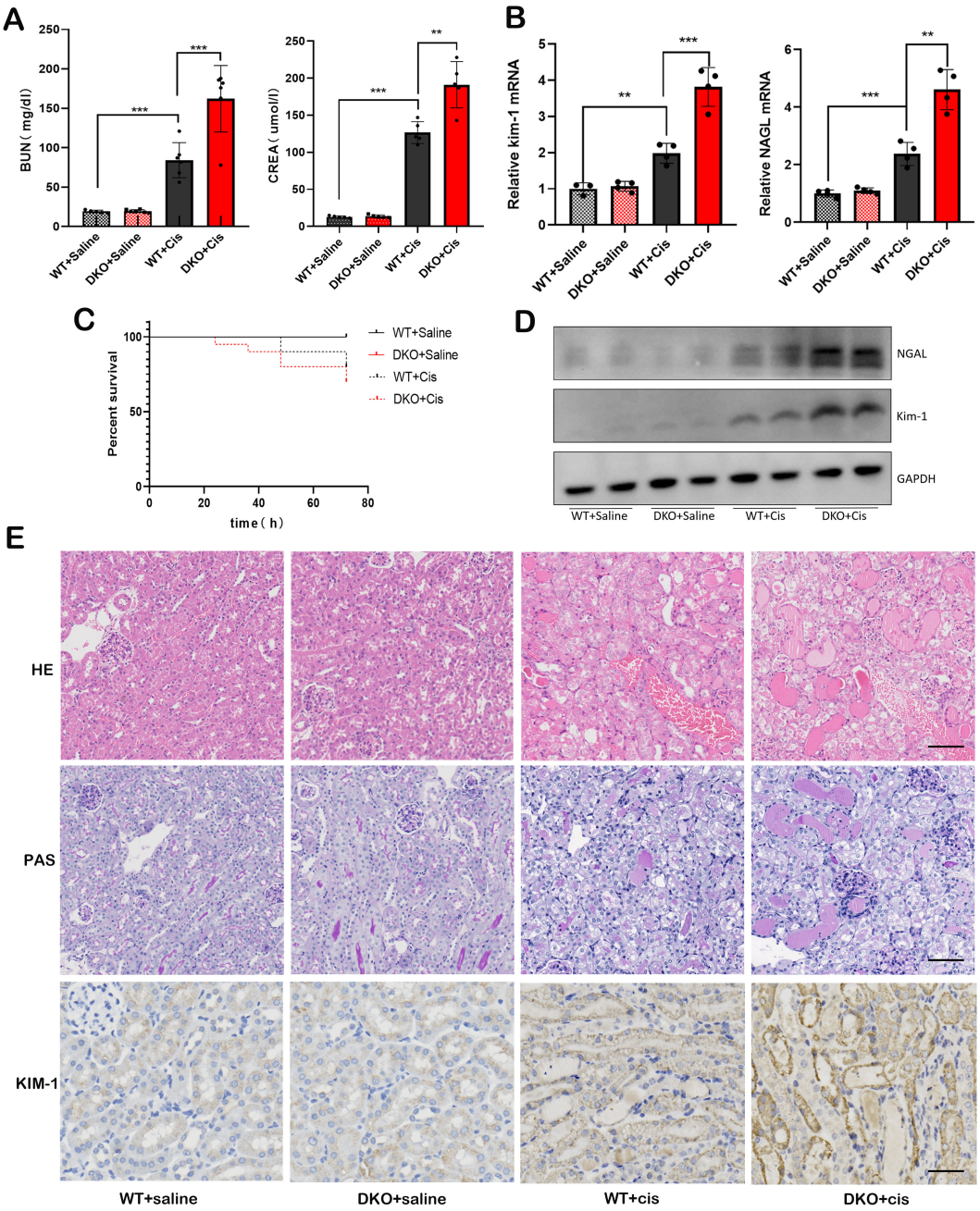

FIGURE 7
Deletion of GSTM1 and GSTT1 aggravated cisplatin-induced AKI in mice. **(A)** the BUN and CREA levels of four groups of wild type and DKO mice at 3days after cisplatin injection. **(B)** the mRNA and **(D)** protein expression levels of Kim-1 and NGAL were significantly increased in DKO mice after cisplatin injection. **(C)** the survival curve analysis of WT and DKO mice after cisplatin injection. **(E)** HE, PAS (Scale bar =50μm) and immunohistochemical (Scale bar =30μm) staining showed that after cisplatin injection, renal tubules of DKO mice were dilated and a large number of protein tubules appeared, and KIM-1 expression was significantly increased compared with WT mice. The data are presented as the mean ± SD, **P*<0.5, ***P*<0.01, ****P*<0.001, n=4.

oxidation-induced damage to amino acid side chains, altering protein structure and function. Accumulation of carbonyl products can help evaluate organismal oxidation levels. Our results demonstrated significantly elevated levels of 8-OHdG and protein carbonylation in cisplatin-induced DKO mice compared to WT mice (Figure 8B), suggesting that the deletion of GSTM1 and GSTT1 reduces the antioxidant capacity of mice.

Following cisplatin injection, DKO mice exhibited more pronounced ferroptosis compared to WT mice

Our previous study has confirmed that deletion of the GSTM1 and GSTT1 can reduce the antioxidant capacity of mice, rendering them more susceptible to cisplatin-induced oxidative damage. Specifically,

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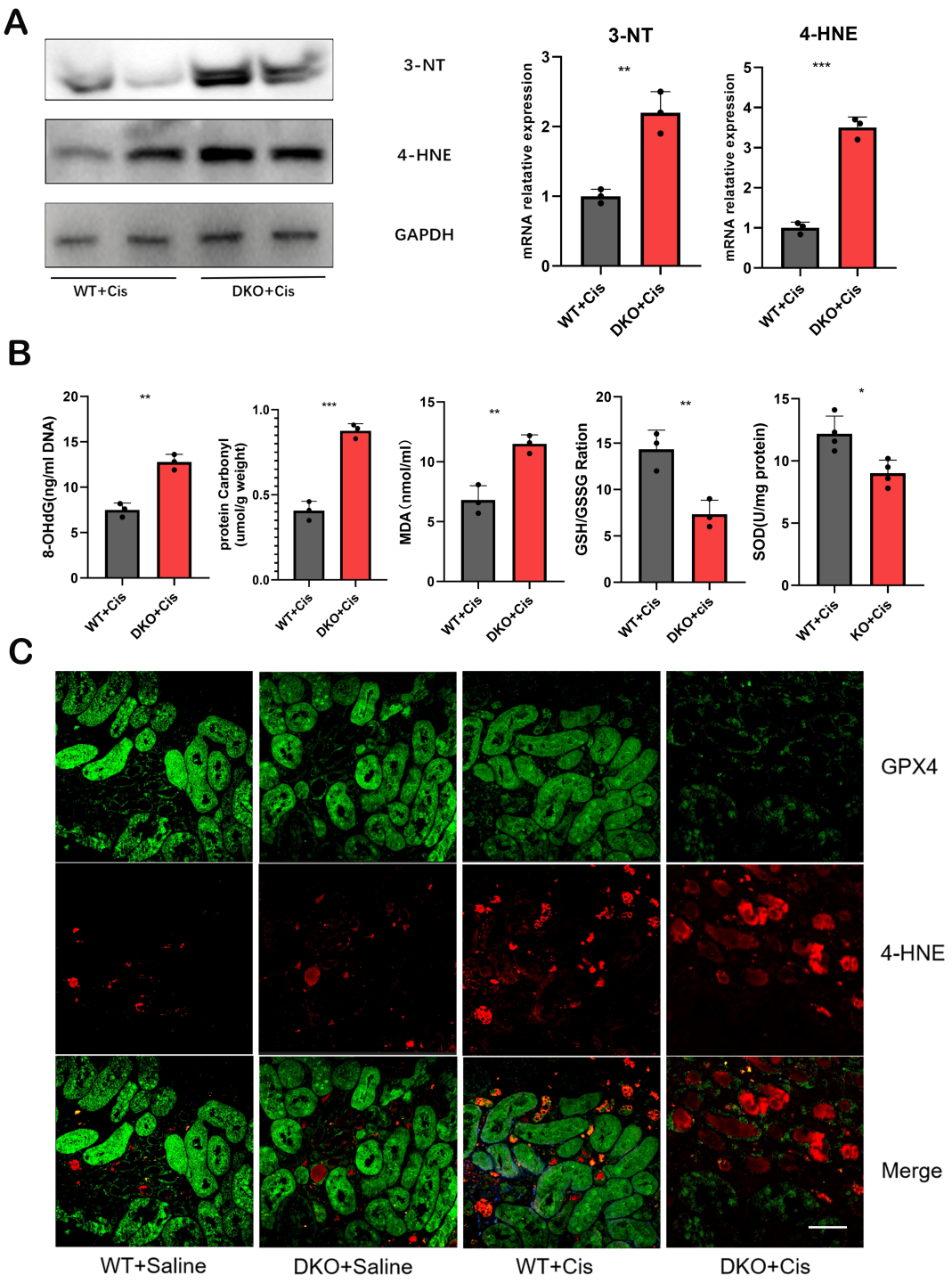

FIGURE 8
The level of oxidative stress was significantly increased in DKO mice after cisplatin injection. **(A)** the mRNA and protein expression levels of 4-HNE and 3-NT were significantly increased in DKO mice compared with WT mice after cisplatin injection. **(B)** the 8-OHdG, protein carbonyl, MDA,GSH/GSSG and SOD expression level in WT and DKO mice after cisplatin injection. **(C)** Representative immunofluorescence staining of 4-HNE and GPX4 in WT and DKO mice after cisplatin injection. Scale bar =40μm. The data are presented as the mean ± SD, **P*<0.5, ***P*<0.01, ****P*<0.001, n≥3.

the GSH/GSSG ratio significantly reduces after deleting these genes, and the expression of MDA, 3-NT, and 4-HNE markedly increases after deleting these genes. Based on these findings, we hypothesized that GSTT1 and GSTM1 knockout can primarily affect ferroptosis in renal tubular cells. To assess this hypothesis, we analyzed the expression levels of ferroptosis-related proteins in DKO mice and WT mice exposed to cisplatin. The results revealed significantly lower expression levels of SLC7A11 and GPX4 and higher expression levels of ACSL4 and COX2 in DKO mice compared to WT mice (Figure 9A). Immunohistochemical staining and immunofluorescence also validated these findings (Figures 8C, 9B, C), indicating that GSTT1 and GSTM1 knockout profoundly disrupts

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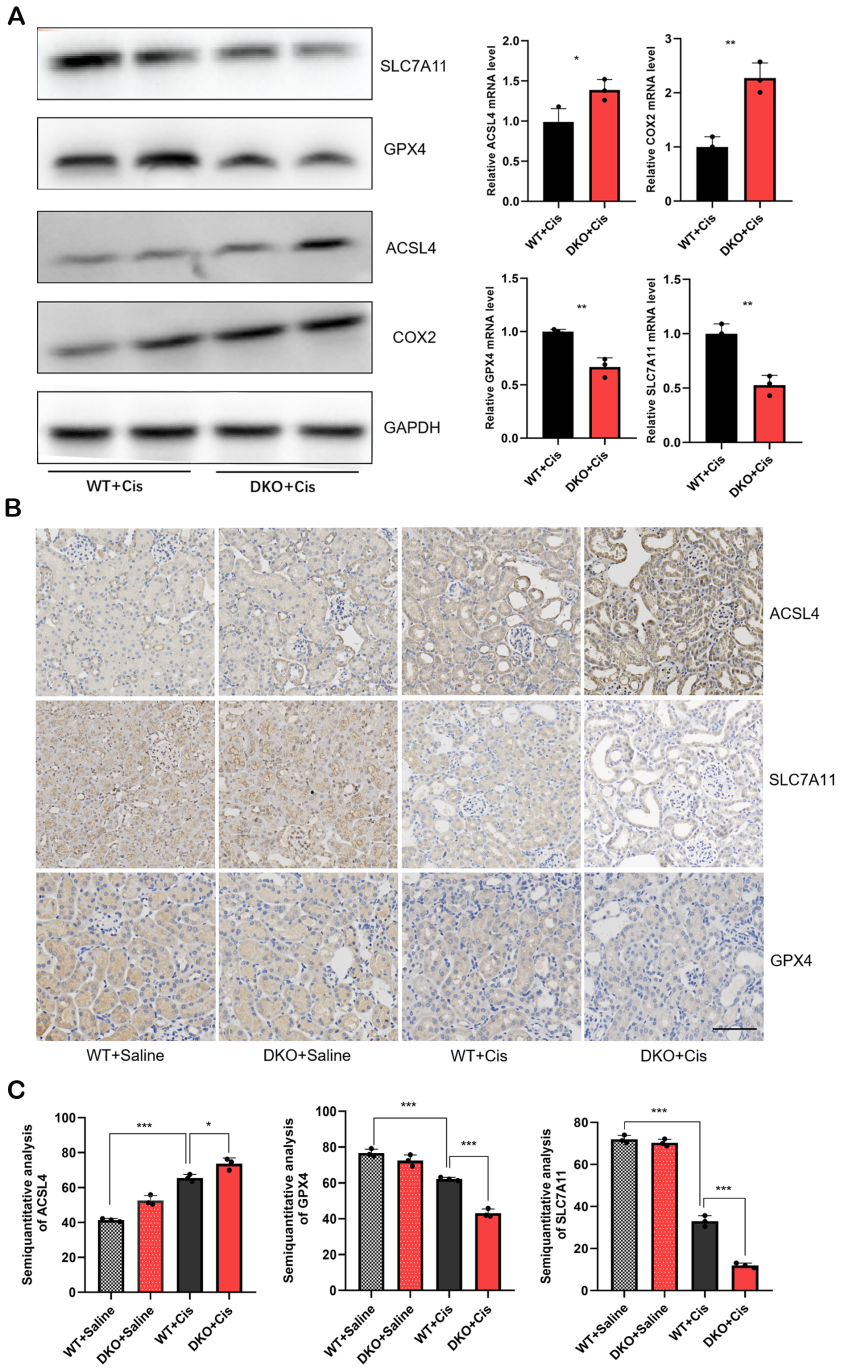

FIGURE 9
DKO mice showed more severe ferroptosis than WT mice after cisplatin injection **(A)** the mRNA and protein levels of ferroptosis related proteins in WT and DKO mice after cisplatin injection. **(B)** Immunohistochemical staining images of ferroptosis related proteins in WT and DKO mice after cisplatin injection. Scale bar =60μm. **(C)** Quantitative analysis of ACSL4, SLC7A11 and GPX4 immunohistochemical staining. The data are presented as the mean ± SD, **P*<0.5, ***P*<0.01, ****P*<0.001, n≥3.

the oxidative equilibrium in mice, aggravating lipid peroxidation and ferroptosis.

Fer-1 and NAC can alleviate cisplatin-induced AKI in *Gstm1/Gstt1*-DKO mice

We conducted a rescue experiment to validate that the primary mode of tubular cell death is ferroptosis in DKO mice exposed to cisplatin. DKO mice received 5 mg/kg of the ferroptosis inhibitor Ferrostatin-1 (Fer-1) and 325 mg/kg of the antioxidant N-Acetyl-L-cysteine (NAC) before administering cisplatin. The experimental groups included DKO, DKO + cisplatin, DKO + cisplatin + Fer-1, and DKO + cisplatin + NAC groups. Compared with cisplatin treatment group, BUN, CREA, KIM-1 and NGAL levels and the severity of renal tubule damage in Fer-1 and NAC pretreatment groups were significantly reduced. Those results demonstrated that

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Fer-1 and NAC effectively mitigated cisplatin-induced kidney damage in DKO mice (Figures 10A, B). In addition, we quantitatively analyzed the expression levels of ferroptosis related genes such as GPX4 and ACSL4, as well as kidney injury marker genes KIM-1 and NGAL to confirm the effectiveness of treatment. The result of WB showed that NAC and Fer-1 pretreatment could significantly reduce cisplatin induced ferroptosis in DKO mice (Figure 10C).

Discussion

AKI is a clinically critical condition characterized by a rapid decline in renal function in the short term. Pre-renal factors (insufficient renal blood flow caused by cardiac surgery (35, 36) and severe dehydration), renal factors (renal lesions caused by nephrotoxic drugs or sepsis) (5, 37, 38), and post-renal factors (urinary tract obstruction by urinary system tumors and stones) are

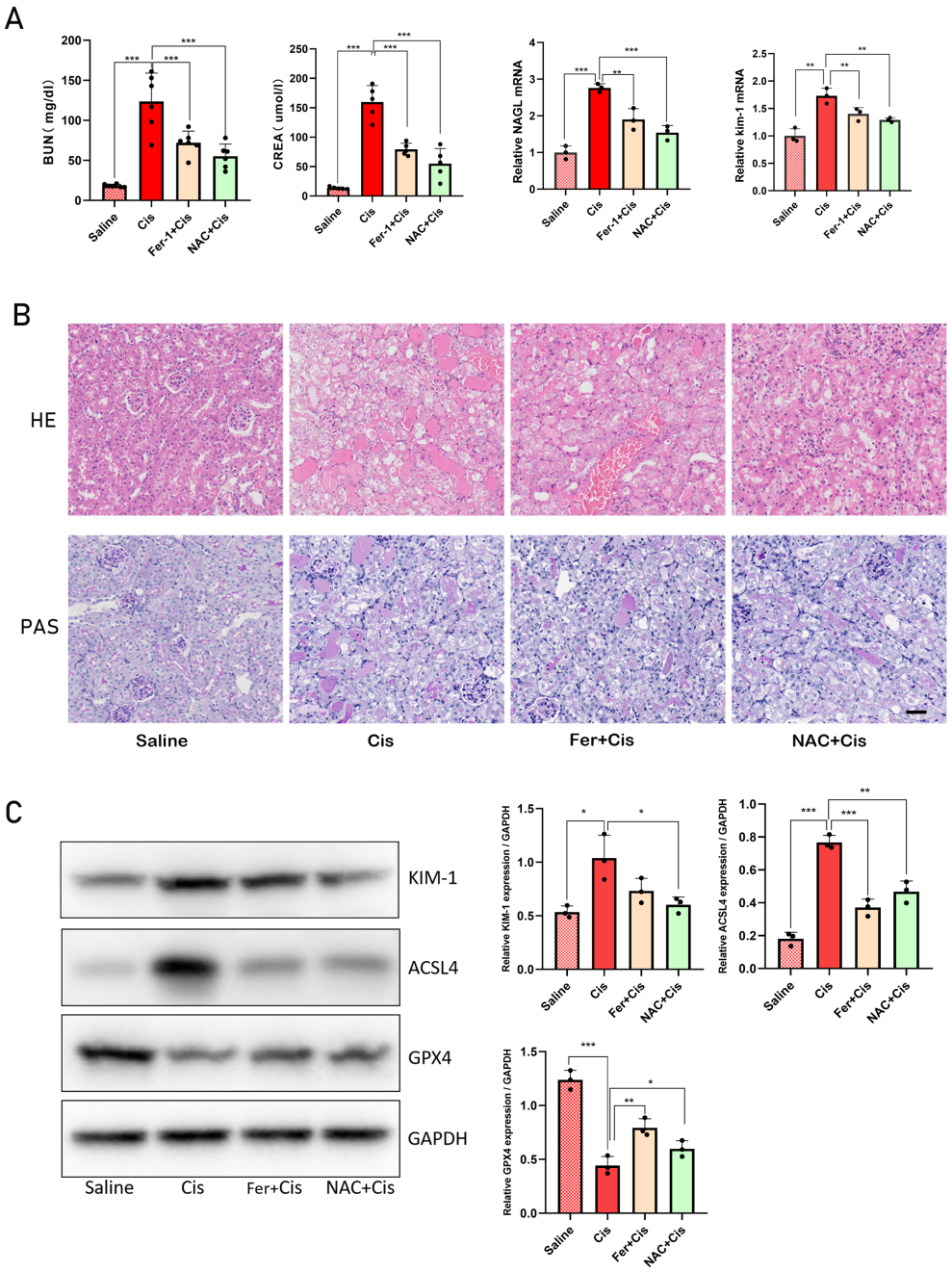

FIGURE 10
Fer-1 and NAC can alleviate cisplatin-induced AKI in Gstm1/Gstt1-DKO mice **(A)** the BUN, CREA, NGAL and kim-1 levels were measured in four groups of DKO mice treated with different treatments. **(B)** HE and PAS staining images of four groups of DKO mice treated with different treatments. **(C)** Protein expression levels of KIM-1, ACSL4 and GPX4 in different tissues in four groups of DKO mice treated with different treatments. Scale bar =50μm. The data are presented as the mean ± SD, **P*<0.5, ***P*<0.01, ****P*<0.001, n≥3.

the main causes of AKI (39). Due to the toxic effects of various drugs or foods on the kidneys, AKI caused by nephrotoxic drugs is very common. Cisplatin (CP) is currently one of the most widely used chemotherapy drugs. Cisplatin has a toxic effect on renal tubular epithelial cells; thus, the incidence of AKI in patients receiving cisplatin is as high as 30% to 40, which greatly decreases the anti-tumor use of cisplatin (40, 41). In recent years, many studies have been conducted on the pathogenesis of cisplatin-induced AKI, but it is still unclear. There is still a lack of safe and effective interventions that can adequately treat or prevent cisplatin-induced AKI. Therefore, it is important to unravel the pathogenesis of cisplatin-induced AKI and develop new therapeutic means to effectively improve AKI in patients receiving cisplatin.

Glutathione S-transferases (GSTs) are a superfamily of phase II detoxification enzymes that protect cells by catalyzing many conjugation processes of hazardous chemicals to reduce glutathione (GSH). Deletion polymorphisms of genes encoding GSTM1 and GSTT1 are widely found in humans. They are clinically of high significance as they affect the metabolism of exogenous active substances and disease susceptibility among different individuals (42–45). The relationship between these enzymes and kidney function has been a topic of considerable interest, given the kidneys' vital role in filtering and detoxifying blood. Several studies have explored the correlation between GSTT1 and GSTM1 polymorphisms and susceptibility to various renal diseases, including acute kidney injury (AKI) and chronic kidney disease (CKD) (21, 46–48). The presence or absence of these genes can influence an individual's ability to detoxify harmful substances, thereby affecting renal function. Our previous studies have also shown that the loss of GSTT1 and GSTM1 is associated with cisplatin-induced ototoxicity (49). In this study, we found that the expression of both GSTM1 and GSTT1 was significantly reduced in patients and mice with AKI. Deletion of GSTM1 and GSTT1 aggravated cisplatin-induced AKI in mice. Therefore, GSTT1 and GSTM1 play a crucial role in maintaining kidney function by reducing oxidative stress and detoxifying harmful substances.

Cisplatin induces the accumulation of lipid peroxidation products in renal tubular epithelial cells, inhibits the antioxidant system, and enhances ROS production, which are the main mechanisms leading to AKI (50). Studies have shown that glutathione consumption can increase intracellular oxidative stress. Inhibition of oxidative stress can ameliorate cisplatin-induced renal tubular epithelial cell damage. Our findings revealed that SOD and GSH/GSSG levels were significantly decreased, and MDA levels were significantly increased in cisplatin-induced DKO mice compared to WT mice. After exposure to cisplatin, 8-OHdG and protein carbonyl levels were significantly higher in DKO mice than in WT mice. In this study, we also found that after exposure to cisplatin, the oxidative stress markers, 4-HNE and 3-NT, were significantly higher in DKO mice compared to WT mice. These results indicated that the deletion of GSTM1 and GSTT1 disrupted the REDOX balance in mice.

Similar to our findings, Chang et al. showed that GSTM1 knockout *in vitro* can upregulate 4-HNE (49). Siems et al. demonstrated that GSTM1 (0) homozygous subjects had higher plasma levels of MDA than subjects with active alleles (51). Moreover, multiple studies have shown that the plasma levels of 4-HNE and MDA are significantly elevated in patients with renal failure and are directly associated with the degree of renal damage (52). Therefore, determining the relationship between GSTMI and GSTT1 genotypes and circulating 4-HNE levels in normal conditions and AKI is a future research focus.

Nrf2 can promote the transcription of certain antioxidant genes. It plays a key role in attenuating the toxicity induced by exogenous compounds. Our study showed that compared with WT mice, the mRNA and protein expression levels of Nrf2 were significantly increased in the DKO mice (data not shown). This finding suggests that the absence of GSTM1 and GSTT1 affects the expression of Nrf2, possibly because its high expression in stress conditions can slow the renal toxicity of cisplatin. In addition, our data showed that under normal physiological conditions, the expression of other GST genes, such as GSTM2 and GSTA4, was increased in GSTT1-KO mice and GSTM1-KO mice (Supplementary Figure 1). This upregulation may help explain why GSTM1 KO and GSTT1 KO mice exhibit normal renal function under normal physiological conditions despite the absence of GSTM1 or GSTT1.

Ferroptosis is a unique type of cell death triggered by intracellular accumulation of free iron, which leads to the accumulation of lipid peroxides on the cell membrane (25). In recent years, ferroptosis has been considered a key pathological form of cell death in cisplatin-induced AKI (53, 54). Our research results indicate that Gstm1 and Gstt1 double knockout (DKO) mice show increased susceptibility to cisplatin-induced acute kidney injury (AKI). We observed that cisplatin treatment led to elevated levels of oxidative stress and lipid peroxidation in DKO mice, resulting in more severe ferroptosis. The administration of NAC and Fer-1 effectively mitigated kidney damage and reduced ferroptosis levels in these mice. Gstm1 and Gstt1 are key enzymes involved in the detoxification of reactive oxygen species (ROS) through their roles in the glutathione-S-transferase (GST) pathway, which is crucial for maintaining cellular redox homeostasis and protecting cells from oxidative damage. Loss of Gstm1 and Gstt1 function can lead to increased accumulation of ROS and heightened susceptibility to oxidative stress-induced damage (55, 56). Ferroptosis is characterized by the accumulation of lipid peroxides and ROS, and is tightly regulated by cellular redox homeostasis (57). The loss of Gstm1 and Gstt1 disrupts the balance of ROS, leading to increased lipid peroxidation and triggering ferroptosis. Our findings suggest that the absence of these enzymes exacerbates ROS accumulation, thereby promoting ferroptosis and contributing to kidney injury. This disruption was evident in our study, where cisplatin treated DKO mice had significantly increased levels of oxidative stress and lipid peroxidation, leading to severe ferroptosis. Treatment with NAC and Fer-1, known for their antioxidant and ferroptosis-inhibiting properties, successfully rescued the mice from kidney damage and reduced ferroptosis levels. This suggests that Gstm1 and Gstt1 play critical roles in modulating oxidative stress and ferroptosis in cisplatin-induced AKI. Further studies are warranted to elucidate the precise molecular mechanisms by which Gstm1 and Gstt1 regulate ferroptosis, including investigating their interaction with other ferroptosis regulators such as GPX4 and SLC7A11.

At the same time, we conducted further *in vitro* experiments to evaluate the effect of Fer-1 and NAC on the antitumor activity of cisplatin. We followed the previous approach to treat cancer cell lines and HK2 with cisplatin alone (20μM) or in combination with Fer-1 (0.4μM) or NAC (10 mM) (54, 58, 59). The results showed that the addition of Fer-1 or NAC did not significantly alter the cytotoxic effects of cisplatin on these cancer cells (Supplementary Figure 2). The reason low doses of Fer-1 and NAC do not affect the anti-tumor efficacy of cisplatin but can mitigate its nephrotoxicity might be related to differences in cell types. These protective agents may exert distinct mechanisms of action in different types of cells. In renal cells of DKO mice(GSTT1/GSTM1-DKO), cisplatin-induced nephrotoxicity is primarily mediated through oxidative stress and ferroptosis pathways, with Fer-1 acting as a ferroptosis inhibitor and NAC as an antioxidant, effectively alleviating damage caused by these mechanisms (60–62). However, in cancer cells, cisplatin exerts its anti-tumor effects mainly by binding to DNA, causing DNA damage, thereby preventing DNA replication and transcription, and ultimately leading to cell death (63, 64). Therefore, low doses of Fer-1 and NAC can provide renal protection without significantly interfering with the anti-tumor efficacy of cisplatin. Additionally, different cell lines may exhibit varying sensitivities to antioxidant and anti-ferroptosis treatment.

In summary, this study indicated that GSTT1 and GSTM1 can alleviate cisplatin-induced AKI by maintaining oxidative stress balance and reducing ferroptosis in renal tubular cells. Activation of GSTT1 and GSTM1 may provide a new therapeutic strategy for AKI.

Materials and methods

Animals and ethics statement

All animal experiments (mice) were approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (Ethics Approval No. ZZU-LAC20201013[09]), and in strict accordance with the standards of the Animal Ethics Committee.

Eight-week-old male GSTT1 KO/GSTM1 KO mice and control littermates, on a CBA/J background, were obtained from Professor Gao Jiangang (Shandong University, Jinan, China) and fed under SPF conditions in the Laboratory Animal Center of Zhengzhou University (Zhengzhou, Henan, China). The mice were kept in an SPF environment with room temperature (21 ± 1°C), humidity (75 ± 5%), and light/dark cycles of 12 hours. The animals were acclimated to the environment for 2 weeks prior to the study and were given free access to standard laboratory feed and water.

Human kidney biopsy samples

Kidney biopsy samples from patients with acute kidney injury (AKI) and kidney para-cancer tissues from patients with kidney cancer (the kidney specimens without histomorphologic lesions were served as normal controls) were obtained from the First Affiliated Hospital of Zhengzhou University, China. This study was approved by the Institutional Ethics Committee of The First Affiliated Hospital. Informed consent is provided by all participants (Ethics Approval number: 2021-KY-004-02).

Animal model system

Cisplatin (Sigma Chemical Co, USA) was dissolved with 0.9% physiological saline at 2 mg/ml). AKI was induced by a single intraperitoneal (i.p.) injection of cisplatin (20 mg/kg). Control mice received 0.9% physiological saline only. For ferroptosis inhibition studies, Ferrostatin-1(Fer-1) was administered 45 minutes to 2 hours before the induction of AKI at a dose of 5 mg/kg. For ROS scavenger (Sigma-Aldrich, A9165), N-Acetyl-L-cysteine (NAC) was administered 24 hours before the induction of AKI at a dose of 200mg/kg (59, 65, 66). In this study, 8-week-old male mice were selected for each group, with at least 3 mice in each group.

Measurement of BUN and CREA

Collect the blood specimen of mice in the 1.5ml EP tube. The room temperature is static for 1-2 hours, 3000rpm 4 °C centrifuged for 15 minutes, collecting serum. The contents of BUN and CREA were detected with BUN (Urea Assay Kit) and CREA (Creatinine Assay kit (sarcosine oxidase)) test kits, which were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

MDA, SOD activity Assay and GSH/GSSG ratio

MDA, SOD levels and GSH/GSSG ratio in renal tissues were measured using the corresponding detection kits in accordance with the manufacturer's instructions. The working solution was prepared according to the instructions, and the serum was mixed with other reagents in the 96-well plate, then incubated at room temperature for 40 min, and the OD value was detected at the corresponding absorbance. Finally, the corresponding indicators were obtained according to the instructions.

Measurement of DNA and protein damage

Levels of 8-OHdG, a marker of oxidative DNA damage, were evaluated using a bioassay kit (Mlbio, ml002198) according to the instructions. The kidney tissues were homogenized with PBS and then centrifuged at 4 °C for 15 min at 3000 rpm. Transfer the supernatant diluted 4 times to the ELISA plate. Then, the conjugate reagent were added to samples mixture and incubated for an hour under 37°C. And the samples were successively treated with chromogenic agents A and B at 37°C for 15 min to initiate a color reaction. Finally, stop the color reaction with stop solution. The OD value of the samples were obtained at 450 nm.

Levels of protein carbonyl, an oxidative protein damage marker, were evaluated using the protein carbonyl content determination kit (Mlbio, ml076345) according to the manufacturer's instructions.

Kidney tissues were homogenized on ice with lysis buffer (0.1 g/mL) and centrifuged at 8000 rpm for 10 minutes at room temperature for the protein carbonyl immunoassay. Reagent I (9:1) was mixed with the supernatant and left at room temperature for 15 minutes. After centrifugation at 12000 rpm at room temperature for 10 minutes, the supernatants were transferred into clean tubes containing reagent II for 30 minutes. In order to obtain sediment, the sample mixture was added to reagent III and centrifuged for 10 minutes at 12000 rpm at 4°C. The sediment was then homogenized at 37°C for 15 minutes with reagent V after repeated additions of reagent IV. Centrifuge the sample at 4°C for 10 minutes after re-dissolving the sediment. The OD value of the samples were obtained at 370 nm.

Renal histology and immunohistochemistry staining

Dislocation of the cervical spine was used to sacrifice the mice. The kidneys of mice were quickly removed and preserved in 4% paraformaldehyde. For further analysis, the tissues were dehydrated, embedded in paraffin, and cut into 3 um-thick slices. Then a variety of staining methods were used on the kidney tissues, including H&E, PAS, and immunohistochemistry. The primary antibodies were as follows: kidney injury molecule -1 (Kim-1, 1:200, ab78494,Abcam), glutathione peroxidase 4 (GPX4, 1:100, ab125066, Abcam), ferritin heavy chain 1 (FTH-1, 1:400, 4393, CST), ACSL4 (1:200, 22401-1-AP, Proteintech), cystine/glutamate antiporter solute carrier family 7 member 11 (SLC7A11, 1:200, 26864-1-AP, Proteintech), GSTT1 (1:200, 15838-1-AP, Proteintech), GSTM1 (1:200, 12412-1-AP, Proteintech) and 4-Hydroxynonenal (4-HNE,1:100,MA5-27570,Thermo Fisher).

TABLE 1 The primer sequences data.

Gene	Forward Primer (5′ –3′)	Reverse Primer (5′ –3′)
GAPDH	ACCCAGAAGACTGTGGATGG	TTCAGCTCAGG GATGACCTT
GSTT1	TGTGGCATAAGGTGATGTTCC	CTTTGTCCTGG AGGAACTTGTCT
GSTM1	GGACTTTCCCAATCTGCCTTAC	GAACAGCCACAAAG TCAGGGT
Nrf2	GCATAGAGCAGGACATGGAGCAAG	ACTGATGGCAGCG GAGGAAGG
GPX4	CATGCCCGATATGCTGAGTGTGG	TAGCACGGCAGGTC CTTCTCTATC
SLC7A11	CCTCTGACGATGGTGATGCTCTTC	GGTGCTGAATGGG TCCGAGTAAAG
ACSL4	GCTATGACGCCCCTCTTTGT	GAATCGGTGTGTCTG AGGGG
FTH1	TGCCATCAACCGCCAGATCAAC	ATTCAGCCCGCTC TCCCAGTC
KIM-1	GGAAGTAAAGGGGGTAGTGGG	GGAAGTAAAGGG GGTAGTGGG
NGAL	GCCCAG GACTCAACTCAGAA	GACCAGGATGGA GGTGACAT
GSTA4	TGATTGCCGTGGCTCCATTTA	CAACGAGAAAAG CCTCTCCGT
GSTM2	ACACCCGCATACAGTTGGC	TGCTTGCCCAG AAACTCAGAG

Western blotting

The kidney tissues were homogenized in RIPA lysis buffer (Thermo Scientific, 89901) with PMSF and then lysed on ice for 30 min before centrifuged at 10,000rpm for 30 min at 4°C. The protein content of the supernatant is determined by the BCA method. The samples were treated with SDS loading buffer and heated to 100°C for 5 min after transferring the supernatant to an empty centrifuge tube. Using SDS-PAGE, separate each equivalent sample and then transferred to polyvinylidene fluoride(PVDF) membranes. The PVDF membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. The next day, after washing with TBST, it was incubated for 1 hour with the HRP-conjugated secondary antibody at room temperature. After washing with TBST, the bands were visualized using an ECL system (Tanon, Shanghai, China). The following primary antibodies were used: Gstm1 (1:2000, Proteintech,12412-1-AP), Gstt1 (1:2000, Proteintech,15838-1-AP). TIM-1(1:1000, Abcam, ab78494), GAPDH (1:1000, Servicebio, GB15004), NGAL (1:1000, Abcam, ab633929), Nrf2 (1:1000, Abcam, ab137550), SLC7A11 (1:1000, Proteintech, 26864-1-AP), GPX4 (1:1000, Abcam, ab125066), ACSL4 (1:1000, Proteintech, 22401-1-AP), and FTH1 (rabbit polyclonal, 1:1000, Cell Signaling Technology, 4393).

Quantitative real-time PCR

The total RNA that was derived from kidney tissues was extracted using TRIzol reagent (Invitrogen, 15596026) according to the guidance provided by the manufacturer. And then mRNA was used for reverse transcription with the PrimeScript RT Reagent Kit (Vazyme, R312-01)to produce cDNA. qPCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and Biosystems QuantStudio 5 (Thermo Fisher Scientific). Values of the housekeeping gene Gapdh were used to as an internal control, and the relative expression level of target genes were evaluated using the comparative CT formula (ΔΔCt). The primers in this study were designed and synthesized by Sangon Biotech and the sequences are listed in the Table 1.

Statistical analysis

All data were expressed as a mean ± SEM, and all tests were performed at least three times. Microsoft Excel and GraphPad Prism 8 software (GraphPad software, San Diego, CA, USA) were used to perform statistical analyses. When comparing two groups, two-tailed, unpaired Student's t tests were applied to indicate the statistical significance. A p-value less than 0.05 was considered significant difference, and the level of significance was indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

PL: Writing – original draft, Writing – review & editing. DuL: Writing – original draft, Validation. YL: Validation, Writing – original draft. SP: Data curation, Writing – review & editing. FC: Writing – original draft. SL: Data curation, Writing – original draft, Methodology. XZ: Data curation, Writing – original draft. JH: Writing – review & editing. DoL: Writing – review & editing, Resources. ZL: Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1457230/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The mRNA levels of GSTM2 and GSTA4 in WT, GSTM1-KO and GSTT1-KO mice. The data are presented as the mean ± SD, *P<0.5, **P<0.01, ***P<0.001, n=3.

SUPPLEMENTARY FIGURE 2
The HK2, colon and lung cancer cell lines were pretreated with Fer-1/NAC and then treated with cisplatin for 24 h. Cell survival rate was detected by CCK8 assay. The data are presented as the mean ± SD, *P<0.5, **P<0.01, ***P<0.001, n=4.

SUPPLEMENTARY FIGURE 3
The mRNA levels of ITGAD, NLE1 and PODN in WT and GSTT1-KO mice. The data are presented as the mean ± SD, *P<0.5, **P<0.01, ***P<0.001, n=3.

SUPPLEMENTARY FIGURE 4
The mRNA levels of GSTM6, GSTM3 and GSTM2 in WT and GSTM1-KO mice. The data are presented as the mean ± SD, *P<0.5, **P<0.01, ***P<0.001, n=3.

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Unveiling ferroptosis: a new frontier in skin disease research

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Ferroptosis, a form of regulated cell death distinct from apoptosis, necrosis, and autophagy, is increasingly recognized for its role in skin disease pathology. Characterized by iron accumulation and lipid peroxidation, ferroptosis has been implicated in the progression of various skin conditions, including psoriasis, photosensitive dermatitis, and melanoma. This review provides an in-depth analysis of the molecular mechanisms underlying ferroptosis and compares its cellular effects with other forms of cell death in the context of skin health and disease. We systematically examine the role of ferroptosis in five specific skin diseases, including ichthyosis, psoriasis, polymorphous light eruption (PMLE), vitiligo, and melanoma, detailing its influence on disease pathogenesis and progression. Moreover, we explore the current clinical landscape of ferroptosis-targeted therapies, discussing their potential in managing and treating skin diseases. Our aim is to shed light on the therapeutic potential of modulating ferroptosis in skin disease research and practice.

KEYWORDS

ferroptosis, iron metabolism, skin diseases, melanoma, psoriasis

1 Introduction

In the field of pathology, skin diseases encompass a range of cell death mechanisms, including apoptosis, necrosis, and autophagy, among others (1, 2). Among these forms of regulated cell death, ferroptosis emerges as a distinctive and noteworthy type, distinguished by its unique cellular morphology and regulatory mechanisms.

In a groundbreaking study conducted in 2012, Dixon and colleagues unveiled a remarkable pathway of cell death that is intricately linked to the metabolism of iron, particularly within cancer cells (Figure 1) (3). They coined the term "ferroptosis" to describe this unique process of cellular demise, emphasizing its significance as it extends

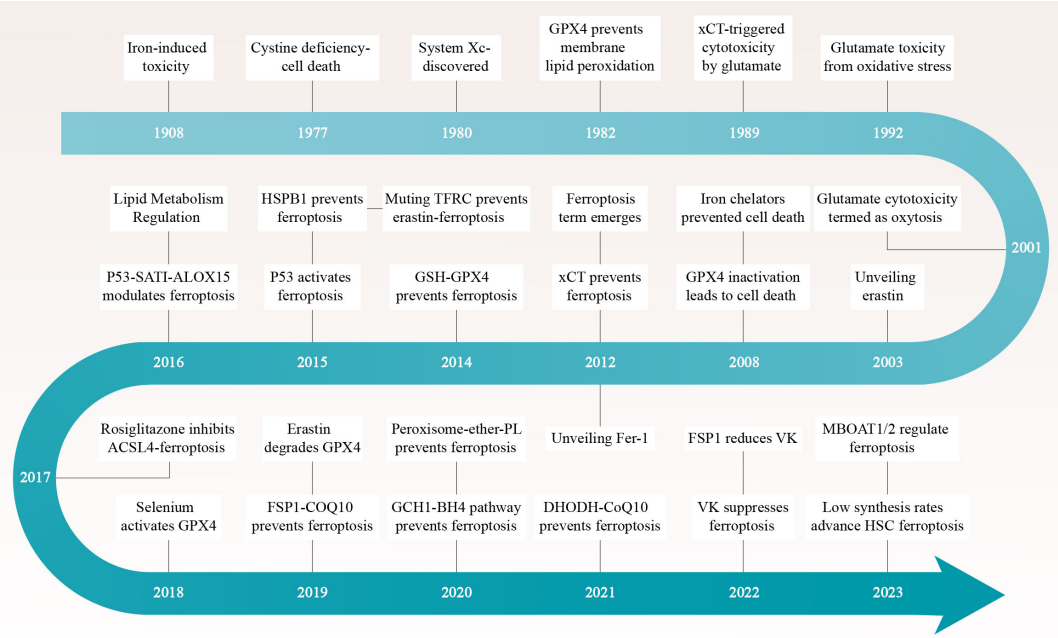

FIGURE 1
The ferric saga of ferroptosis unfolds through a compelling timeline dotted with pivotal breakthroughs. Each epoch is distinguished by seminal discoveries that have sculpted the narrative of our apprehension regarding this distinctive pathway of cellular demise. ACSL4, Acyl-CoA Synthetase Long-Chain Family Member 4; CoQ10, Ubiquinone-10; DHODH, Dihydroorotate Dehydrogenase; FSP1, Ferroptosis Suppressor Protein 1; GPX4, Glutathione Peroxidase 4; GSH, Glutathione; HSC, Hepatic Stellate Cells; MBOAT1/2, Membrane Bound O-Acylated Transferase Domain Containing 1/2; TFRC, Transferrin Receptor; VK, Vitamin K; xCT, Solute Carrier Family 7 Member 11.

beyond mammals and is prevalent across various biological kingdoms, including plants, protists, and fungi (4, 5).

In specific terms, researchers confirmed the presence of ferroptosis in *Magnaporthe oryzae*, the rice blast fungus, through characteristics such as the accumulation of lipid peroxides detected by optimized fluorescence imaging technology and dependence on iron ions, which is crucial for the developmental cell death of conidia during appressorium maturation. Ferroptosis initially occurs in the terminal cell and subsequently affects the entire conidium. Furthermore, the article indicates that specifically inducing ferroptosis in rice can restrict the invasive growth of *Magnaporthe oryzae*, thus enhancing resistance to the rice blast disease (4).

Another study discovered that with increasing age, the levels of ferrous iron (Fe^{2+}) rise and glutathione (GSH) levels decline in *Caenorhabditis elegans*, and this imbalance promotes the occurrence of ferroptosis. Treatment with ferroptosis inhibitors, such as Liproxstatin-1, can alleviate cell death caused by GSH depletion, indicating that ferroptosis plays a crucial role in the natural aging process of *Caenorhabditis elegans* (5).

This discovery not only enhances our understanding of cell death mechanisms but also offers a fresh perspective on how iron metabolism can influence the fate of cells in diverse organisms.

Ferroptosis is characterized by an overabundance of iron-dependent reactive oxygen species (ROS), a condition that is intimately associated with the diminished antioxidant defense provided by glutathione peroxidase 4 (GPX4) (3). When the cell's antioxidant defenses are unable to counteract the overwhelming presence of ROS, ferroptosis ensues within the cell (6).

In this process, the peroxidation reaction not only triggers ferroptosis but also contributes to its further progression. Hence, the molecules and signaling pathways involved in iron metabolism and peroxidation reactions assume a pivotal role in the regulation of ferroptosis. The intricate interplay of these molecules and pathways significantly influences the maintenance of the cell's antioxidant equilibrium and serves as a deterrent against the occurrence of ferroptosis.

In this review, we delve into the biological principles of ferroptosis and explore its application in various dermatological conditions. Specifically, we examine its specific impact on ichthyosis, psoriasis, polymorphous light eruption (PMLE), vitiligo, and melanoma. The review also encompasses targeted therapeutic strategies for the ferroptosis pathway and provides an evaluation of the potential and latest research trends surrounding these treatment methods in dermatological disease therapy.

2 Characteristics of ferroptosis

Iron ions are vital trace elements in the human body and are crucial for maintaining the normal functioning of living organisms (7). They play an irreplaceable role in the metabolic process of the skin, interacting with molecules such as ferritin within melanocytes to regulate cellular iron metabolism and redox balance (8). This

interaction could have a profound impact on the function and viability of melanocytes.

However, excessive accumulation of iron ions can have detrimental effects. Through the Fenton reaction, excess iron ions can generate ROS, which can result in lipid peroxidation and serve as the pathological foundation for ferroptosis (9). Ferroptosis is a condition linked to iron metabolism dysfunction, and its development is intricately connected to the buildup of ROS and the state of iron overload.

In the regulatory mechanism of ferroptosis, numerous signaling pathways and molecules play crucial roles (Figure 2). These encompass the maintenance of iron homeostasis, the metabolic process of cysteine, the activity of GPX4, the synthesis of polyunsaturated fatty acids (PUFA), the regulatory role of nuclear factor E2-related factor (NRF2), the function of the tumor suppressor protein p53, the protective mechanism of heat shock proteins (HSPs), the regulatory role of iron regulatory inhibitor-1 (FSP1), the activation state of AMP-activated protein kinase (AMPK), and the supply of nicotinamide adenine dinucleotide phosphate (NADPH). These factors collectively form a complex regulatory network that influences the equilibrium of iron ions and the cellular health status.

2.1 Iron homeostasis

Iron is crucial for preventing lipid peroxidation in the process of ferroptosis (10). The mechanisms of iron uptake, intracellular transport, and storage are significant in regulating ferroptosis (11). Organisms carefully maintain iron homeostasis to ensure proper functioning. Transferrin receptor 1 (TFR1) plays a key role in absorbing iron ions and facilitating their conversion into ferrous ions within the cell, which is a critical step in preventing ferroptosis. Divalent metal transporter 1 (DMT1) releases ferrous ions into the intracellular iron pool, while ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1) are responsible for safely storing excess iron ions in the cytoplasm, preventing their excessive accumulation (12). The proper expression and function of proteins related to iron metabolism such as hepcidin and ferroportin 1 (FPN1), are essential for controlling intracellular iron levels, as any imbalance can lead to increased iron levels, triggering metabolic issues (13, 14).

The skin serves multiple functions, acting as a protective barrier against external physical, chemical, and biological factors, while also playing roles in temperature regulation, sensory transmission, and immune responses (15). Iron homeostasis is particularly crucial for maintaining skin health, as iron ions are involved in various metabolic processes within skin cells (16). These processes include cell division, collagen synthesis, and antioxidant reactions. Optimal levels of iron ions help maintain the integrity of the skin barrier, facilitate wound healing, and support the regeneration of skin cells (17).

However, an imbalance of iron ions, whether in excess or deficiency, can have adverse effects on the skin microenvironment. Excessive iron ions can lead to oxidative stress, causing damage and triggering inflammatory reactions in skin cells (18). This, in turn, may accelerate the skin's aging process and increase the risk of skin cancer. On the other hand, a deficiency of iron ions can impact the energy metabolism and function of skin cells, potentially resulting in dryness, fragility, and abnormal pigmentation of the skin (19).

2.2 Cysteine

Cystine/glutamate antiporter (System Xc-) is recognized as a critical cystine/glutamate antiporter within cellular systems. It is composed of two integral subunits, solute carrier family 7 member 11 (SLC7A11) and solute carrier family 3 member 2 (SLC3A2), which are chemically linked together by a disulfide bridge (20). This transport system crucially defends cells by absorbing cystine and releasing equal glutamate (21).

The proper functioning of this transporter is vital for cellular health. Initially, System Xc- facilitates cystine uptake through the interaction of its subunits SLC7A11 and SLC3A2 (22). Subsequently, within the cell, cystine is converted into cysteine, a crucial precursor for GSH synthesis (23). As a potent antioxidant, GSH, catalyzed by GPX4, transforms lipid peroxides into harmless compounds, safeguarding cells from oxidative damage.

In the skin microenvironment, the level of cysteine is strictly regulated. It is taken up from outside the cell through the System Xc- and then converted into cysteine for cellular use (24). The availability of cysteine affects the levels of GSH within skin cells, thereby influencing the skin's resistance to environmental stress.

Cysteine's anti-inflammatory properties in the skin are mediated through its role in the synthesis of glutathione, a potent antioxidant. This tripeptide helps neutralize ROS, which are often elevated during inflammatory responses and can damage skin cells. By reducing oxidative stress, cysteine indirectly suppresses the activation of the NF-κB pathway, a key transcription factor that drives the expression of pro-inflammatory cytokines such as TNF-α and IL-6 (25). The inhibition of NF-κB signaling by cysteine thus leads to a decrease in the production of these cytokines, which are central to the inflammatory process, ultimately contributing to the alleviation of skin inflammation.

2.3 GPX4

GPX4, a key enzyme in the glutathione peroxidase family, is essential for managing ferroptosis by catalyzing the reduction of lipid hydroperoxides and the conversion of GSH to glutathione disulfide (GSSG), thus preventing the lipid peroxidation associated with this form of cell death (26). It neutralizes detrimental lipid hydroperoxides into benign lipid alcohols, which is vital for preserving cell membrane integrity and shielding against oxidative stress (27).

Drugs like RAS Selective Lethal 3 (RSL3) specifically target GPX4, binding covalently to inactivate its function, leading to a buildup of lipid hydroperoxides that can trigger ferroptosis (28). The level of GPX4 expression dictates the cell's vulnerability to ferroptosis; a decrease in its expression raises the risk, whereas an increase can bolster the cell's defenses against ferroptosis (29).

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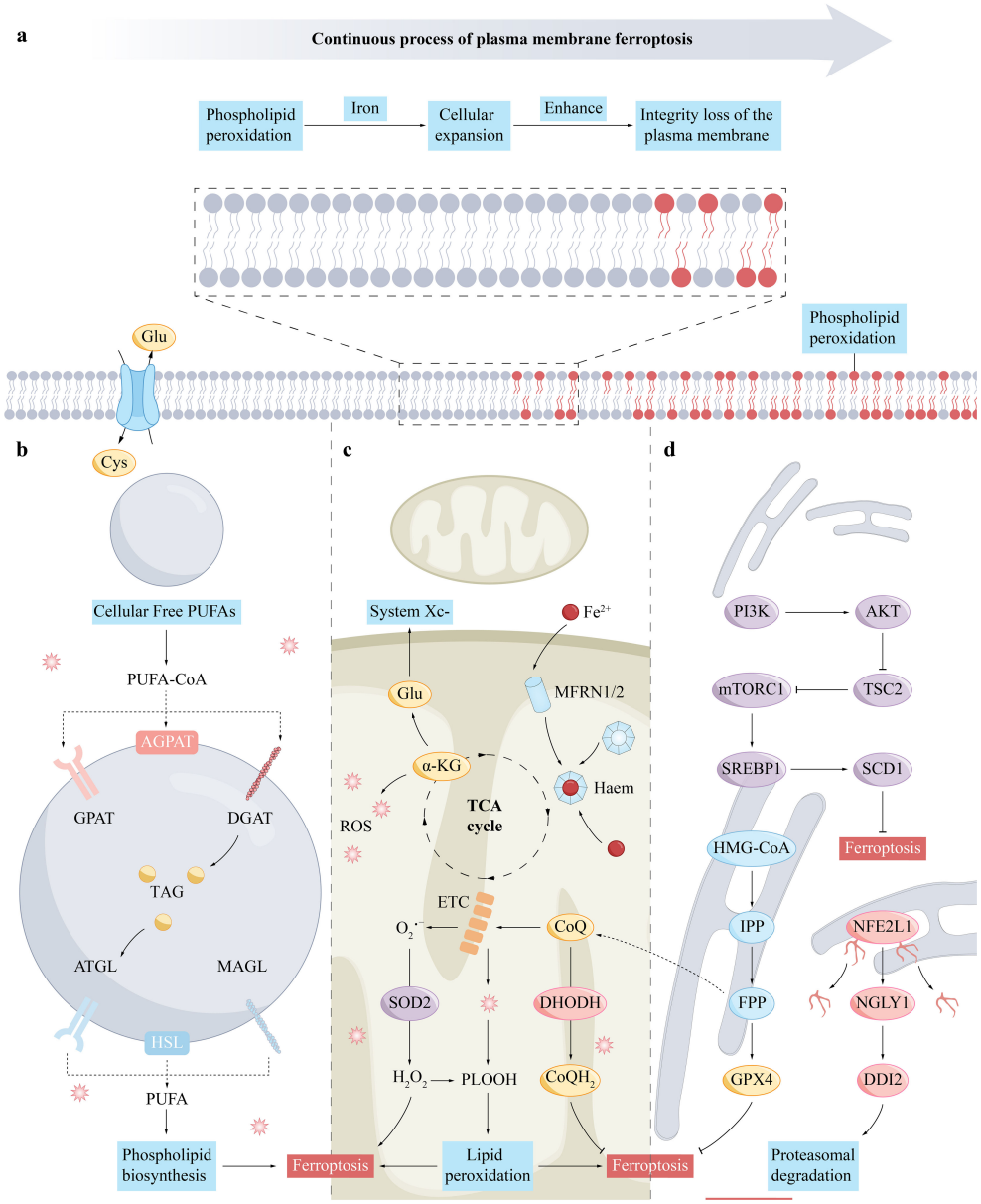

FIGURE 2
The mechanism of ferroptosis in the cell membrane **(A)**, lipid droplets **(B)**, mitochondria **(C)**, and endoplasmic reticulum **(D)**. **(A)**, Cell membrane rupture is a key event leading to cell death in the process of ferroptosis. It involves the accumulation of phospholipid hydroperoxides and the accumulation of iron ions, which can be produced through enzymatic (for example, via lipoxygenases) and non-enzymatic radical-mediated reactions. The susceptibility to ferroptosis is determined by the cell's tendency to accumulate high levels of membrane lipid peroxides. **(B)**, Free PUFAs in the cytoplasm are converted into TAG through a process of fatty acid activation and are stored in lipid droplets. PUFAs esterified within TAG can be released from lipid droplets through lipolysis or lipophagy, with lipolysis involving a series of lipid droplet-associated lipases. **(C)**, The TCA cycle supplies electrons to the mitochondrial ETC, which partially reduces oxygen to ROS, particularly superoxide at complexes I and III. Superoxide can form hydrogen peroxide through dismutation and then produce hydroxyl radicals with Fe^{2+} in the Fenton reaction. Glutamine breakdown leads to α-ketoglutarate, which may enhance mitochondrial ROS and ferroptosis. MFRN1 and MFRN2 facilitate iron transport within the mitochondria, crucial for ROS production. CoQ, an essential electron carrier in the ETC, is synthesized in mitochondria and reduced by DHODH to CoQH2, which can either transfer electrons to complex III or act as an antioxidant to mitigate lipid peroxidation and ferroptosis. **(D)**, FPP contributes to CoQ synthesis, while IPP boosts selenoprotein production, including GPX4. SREBP1 and NFE2L1, released through proteolysis, regulate genes involved in lipid metabolism and oxidative stress in the nucleus. The PI3K-mTORC1 pathway enhances ferroptosis resistance by upregulating SREBP1 and SCD1. NFE2L1 is processed by NGLY1 and DDI2 after leaving the endoplasmic reticulum. AKT, Protein Kinase B; ATGL, Adipose Triglyceride Lipase; CoQ, Coenzyme Q10; CoQH2, Ubiquinol; DDI2, DNA Damage-Inducible 1 Homolog 2; DGAT, Diacylglycerol O-acyltransferase; DHODH, Dihydroorotate Dehydrogenase; ETC, Electron Transport Chain; FPP, Farnesyl Pyrophosphate; Glu, Glutamine; GPX4, Glutathione Peroxidase 4; HMG-CoA, Hydroxymethylglutaryl-Coenzyme A; IPP, Isopentenyl Pyrophosphate; MAGL, Monoacylglycerol Lipase; MFRN1/2, Mitoferrin 1 and 2; mTORC1, Mechanistic Target of Rapamycin Complex 1; NFE2L1, Nuclear Factor (Erythroid-derived 2)-like 1; NGLY1, N-glycanase 1; PI3K, Phosphatidylinositol-3 kinase; PLOOH, Phospholipid hydroperoxide; ROS, Reactive Oxygen Species; SCD1, Stearoyl-CoA Desaturase 1; SREBP1, Sterol Regulatory Element-Binding Protein 1; SOD2, Superoxide Dismutase 2.

GPX4 serves as a protective agent in the skin environment, contributing to skin health and vitality through its antioxidant properties. As a pivotal component of the skin's antioxidant network, GPX4 can neutralize free radicals (30). These harmful agents are produced by everyday environmental factors such as ultraviolet radiation, pollutants, and stress. Furthermore, the activity of GPX4 is linked to the skin cells' capacity to repair and regenerate, thus aiding in the restoration of the skin's normal structure and function following damage (31).

2.4 PUFA

In the biological process of ferroptosis, PUFA are vital (32). They maintain cell membrane flexibility and fluidity, but their reactive double bonds make them susceptible to lipid peroxidation (33). The enzymes acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), involved in PUFA synthesis, are critical, with their activity levels determining the cell's susceptibility to ferroptosis.

Additionally, lipoxygenase (LOX), by promoting PUFA peroxidation, exacerbates ferroptosis (33, 34). Studies have found that decreasing LOX activity or expression levels can slow ferroptosis. Also, the concentration and distribution of free PUFA within the cell significantly influence the lipid peroxidation rate and ferroptosis severity (34).

PUFA are essential for skin health and functionality. They mitigate inflammation, crucial for managing skin conditions like eczema and psoriasis (35, 36). Moreover, they regulate skin cell growth and differentiation, promoting wound healing and skin regeneration (37). Since PUFA are oxidation-prone, their skin presence relates closely to the antioxidant defense mechanism (38–41). Skin's antioxidant enzymes and molecules, like glutathione peroxidase (GPX) and vitamin E, shield PUFA from free radicals, ensuring skin health.

2.5 NRF2

In the mechanism of ferroptosis, NRF2 plays a central regulatory role (42). It acts as a key transcription factor within the cell to respond to oxidative stress, maintaining cellular iron metabolism and antioxidant status through a series of complex regulatory pathways (43).

Under conditions of oxidative stress, NRF2 can dissociate from the cytosolic protein Kelch-like ECH-associated protein 1 (KEAP) to which it is bound, and rapidly translocate to the nucleus (44). This process triggers the expression of a series of antioxidant and detoxifying enzyme genes, including members of the glutathione S-transferase family (GSTs), heme oxygenase 1 (HO-1), and quinone oxidoreductase 1 (NQO1) (45). The activation of these enzymes is crucial for neutralizing ROS within the cell and mitigating the damage caused by oxidative stress (46).

Moreover, NRF2 finely regulates key genes in the iron metabolism pathway, such as ferroportin, a protein that promotes the export of iron ions out of the cell (47). This action reduces the intracellular iron concentration and the generation of ROS caused by iron overload, thus lowering the risk of ferroptosis (48). Additionally, NRF2 also participates in the regulation of iron-binding proteins, such as the TFR1, which helps the cell more effectively manage the uptake and storage of iron (49).

NRF2 plays a pivotal role in maintaining skin health. As time progresses, the skin naturally undergoes an aging process, leading to a gradual decrease in NRF2 activity (50). This reduction in NRF2 expression levels diminishes the skin's ability to defend against free radicals, thereby accelerating the external manifestations of skin aging, including wrinkles, sagging, and pigmentation (51). Consequently, this age-related decline in NRF2 activity significantly influences the skin aging process and merits attention (52).

NRF2 plays a multifaceted role in wound healing, inflammation management, and disease progression. In wound healing, NRF2 activation enhances the expression of antioxidant enzymes, which protect new cells from oxidative stress and facilitate the healing process (53). Additionally, NRF2 helps to regulate inflammation by inhibiting the production of inflammatory cytokines, thus reducing inflammation's negative impact on wound recovery (54).

In the realm of inflammatory skin diseases, the role of NRF2 is pivotal, particularly in conditions like atopic dermatitis (AD) and psoriasis. In AD, the skin's natural barrier function is compromised due to reduced NRF2 activity, which is crucial for maintaining the integrity of the skin. This weakening allows environmental irritants and allergens to penetrate more easily, leading to a heightened inflammatory response within the skin. The inflammation can manifest as redness, itching, and dryness, which are characteristic symptoms of AD (55). The lack of NRF2's protective influence results in a prolonged and often distressing experience for those affected, as the skin's ability to repair and regenerate is hindered.

In psoriasis, the situation is somewhat different but equally concerning. Here, NRF2's diminished activity is implicated not just in the exacerbation of inflammation but also in the chronicity of the disease. The reduced NRF2 levels lead to an imbalance in the skin's normal cell turnover process, causing skin cells to proliferate at an abnormally rapid rate. This over proliferation results in the formation of thick, scaly plaques that are a hallmark of Psoriasis. Moreover, the continuous cycle of rapid cell division and shedding contributes to the chronic nature of the condition, making it a long-term management challenge for patients (56). The cellular damage that accompanies this process can further disrupt the skin's ability to function properly, leading to a range of symptoms that can significantly impact a person's quality of life.

The connection between NRF2 and these inflammatory skin diseases underscores the potential for therapeutic interventions that target NRF2 activity. By enhancing NRF2's function, it may be possible to strengthen the skin barrier in AD, reducing the susceptibility to irritants and allergens, and thereby mitigating inflammation. In Psoriasis, strategies to boost NRF2 activity could help normalize skin cell turnover, reducing the formation of plaques and the associated discomfort. Such interventions could offer a more effective approach to managing these conditions, improving the prognosis and overall well-being for individuals living with AD and Psoriasis.

Furthermore, NRF2's dysregulation is implicated in the progression of certain skin cancers. In the early stages, abnormal NRF2 expression can lead to increased tumor cell proliferation and invasiveness, highlighting the importance of NRF2 in cancer development (57).

Understanding NRF2's complex role in skin health is essential for developing targeted treatments. By modulating NRF2 activity, it may be possible to enhance wound healing, alleviate inflammation in conditions like AD and psoriasis, and potentially slow the progression of skin cancers. This underscores the need for further research into NRF2's mechanisms and its potential as a therapeutic target in dermatology.

2.6 P53

The p53 protein serves a critical role in the cellular response to iron overload, maintaining a balanced state of iron by finely regulating the transcriptional activity of specific genes (58). Notably, p53 triggers the transcription of the ferroportin gene, a key membrane-bound protein that facilitates the transport of iron ions from inside the cell to the outside (59). This action effectively reduces cellular iron levels and decreases the production of ROS associated with iron overload. Additionally, p53 inhibits the expression of TFR1, thereby limiting cellular iron uptake and preventing the occurrence of iron overload (60).

Moreover, p53 is not only involved in iron homeostasis regulation but also enhances the cellular antioxidant defense system by activating the expression of a series of antioxidant enzymes, including GSTs, HO-1, and superoxide dismutase (SOD) (61). The activation of these antioxidant enzymes assists in neutralizing ROS, thereby protecting cells from oxidative stress damage.

When the skin is exposed to harmful environmental factors, such as ultraviolet rays, the p53 protein promptly activates its protective mechanism (62). It intervenes in the regulation of the cell cycle, triggering a pause in the cell cycle and providing valuable time for the cell to repair DNA damage (63). This timely cell cycle arrest not only prevents the proliferation of damaged cells but also effectively reduces the accumulation of genetic mutations, thereby significantly decreasing the incidence of skin cancer (64). Additionally, p53 promotes the synthesis of specific DNA repair enzymes, accelerating the repair of damaged DNA and maintaining the genetic stability of skin cells (65).

Moreover, p53 plays a pivotal role in combating skin aging. Over time, the activity of p53 within skin cells may gradually weaken, impacting the skin's defense against external damage and decreasing the effectiveness of the antioxidant system (66). The decline in antioxidant capacity accelerates the external manifestations of skin aging, such as the loss of skin elasticity and the appearance of fine lines and wrinkles. Furthermore, the reduction in p53 activity may affect the renewal and repair capabilities of skin cells, further contributing to the aging process (67). Understanding the multifaceted role of p53 in skin health is crucial for developing strategies to maintain skin integrity and combat aging.

2.7 HSPs

In the context of ferroptosis, HSPs play a crucial role in regulating and protecting cells through diverse mechanisms. Specifically, heat shock protein family B (small) member 1 (HSPB1), a member of the HSP family, effectively mitigates the accumulation of iron ions within cells by limiting iron absorption. This not only alleviates oxidative stress damage to cells but also helps maintain the stability of the cytoskeleton (68). Additionally, heat shock protein family B (small) member 5 (HSPB5), another member of the HSP family, is notable for its location in the endoplasmic reticulum and significantly enhances the cell's antioxidant capacity through its interaction with GPX4, providing an additional protective layer against the threat of ferroptosis (69).

The functions of HSPs extend beyond antioxidant and anti-inflammatory capacities: they also inhibit ferroptosis by safeguarding the health of mitochondria (70). Mitochondria, as the powerhouse of the cell, are vital for cell survival, and HSPs protect them from damage, thereby preventing the onset of ferroptosis (71). Furthermore, HSPs are involved in the regulation of various intracellular signaling pathways, including those closely related to cell survival and death, such as apoptosis and autophagy pathways (72). Maintaining the balance of these signaling pathways is crucial for the cell's ability to resist ferroptosis.

HSPs are instrumental in combating skin aging and photoaging. Their presence enables skin cells to combat oxidative stress induced by environmental factors, particularly ultraviolet rays, thereby mitigating the skin damage that arises from prolonged ultraviolet (UV) exposure (73). The antioxidant capacity of HSPs plays a pivotal role in eliminating harmful free radicals, which have the potential to accelerate the aging process of the skin, resulting in a loss of elasticity and luster (74). Consequently, HSPs are indispensable for maintaining the health and youthful state of the skin, offering crucial support in preserving skin integrity.

Moreover, in the context of skin diseases such as psoriasis and contact dermatitis, the expression levels of HSPs may undergo substantial changes, influencing the severity of the disease and the effectiveness of treatment (75, 76). For instance, in psoriasis, an increase in the expression of HSPs may contribute to alleviating inflammatory responses, while in contact dermatitis, changes in HSP expression may be associated with the skin's sensitivity to irritants. This underscores the significant role of HSPs in regulating the progression of skin diseases and influencing the response to treatment, thereby emphasizing their potential as targets for therapeutic interventions. Understanding the dynamic role of HSPs in skin health and disease progression is essential for developing tailored strategies to manage and treat various dermatological conditions.

2.8 FSP1

FSP1 plays a complex and multifaceted role in the process of ferroptosis, with its actions extending beyond the traditional GSH-GPX4 antioxidant pathway (77). What sets FSP1 apart is its unique ability to mitigate the effects of ferroptosis through a series of non-

traditional pathways. For instance, it can influence the balance of iron ions within cells, regulate the storage and distribution of iron, and reduce the excessive accumulation of iron ions, thereby decreasing the incidence of ferroptosis (78).

An additional crucial function of FSP1 lies in its involvement in the NADH-dependent ubiquinone-oxidoreductase reaction, wherein ubiquinone is converted to ubiquinol, effectively inhibiting lipid peroxidation, a fundamental aspect of the ferroptosis process. As part of its function, ubiquinol has the capacity to neutralize free radicals, thereby interrupting the self-oxidation chain reaction of lipids (79). Simultaneously, it promotes the regeneration of the α-tocopherol radical, indirectly inhibiting lipid auto-oxidation and further slowing down the progression of ferroptosis (80).

FSP1, a multifunctional protein, serves as a protective shield for the skin against environmental stressors, including ultraviolet rays and pollutants, owing to its antioxidant and anti-inflammatory properties (81). Its pivotal role in balancing skin iron metabolism is crucial for preventing cell damage caused by iron overload, and it may also contribute to slowing down the skin's aging process by supporting cell repair and regeneration (82). Moreover, the anti-inflammatory effects of FSP1 may aid in alleviating skin inflammatory conditions, while its involvement in ferroptosis offers promising perspectives for the prevention of skin cancer (83).

Furthermore, FSP1 may actively participate in maintaining the skin's barrier function, which is indispensable for preventing the invasion of pathogens and allergens. In the realm of skin disease treatment, the regulatory role of FSP1 could emerge as a potential target for addressing inflammatory skin conditions such as psoriasis and venous ulcers (84). These insights not only enhance our comprehension of the role of FSP1 in skin physiology and pathology but also lay a scientific foundation for the development of novel strategies for skin protection and treatment.

2.9 AMPK

AMPK plays a pivotal role in regulating ferroptosis as the primary sensor of cellular metabolic status and energy levels (85). It is activated under conditions of glucose scarcity due to the increased ratio of intracellular adenosine monophosphate (AMP) to adenosine triphosphate (ATP) (86). The activation of AMPK helps prevent the occurrence of ferroptosis, while its inhibition increases the risk of ferroptosis.

In-depth research has revealed that the activation of AMPK is regulated by its upstream kinase liver kinase B1 (LKB1), a significant tumor suppressor, particularly in malignant tumors such as lung cancer, where mutations in LKB1 are common (87). Non-small cell lung cancer cells with LKB1 mutations exhibit high sensitivity to ferroptosis, suggesting that ferroptosis inducers may hold therapeutic potential for such tumors (88).

When cells encounter ferroptosis-inducing factors, the activation of AMPK not only inhibits the activity of its downstream acetyl-CoA carboxylase 1 (ACC1) but also reduces the synthesis of fatty acids (89). This process helps decrease the accumulation of intracellular lipid peroxides and slows down the progression of ferroptosis. Further analysis of lipid metabolism has confirmed that by inhibiting the synthesis of PUFA controlled by ACC1, AMPK plays a central role in inhibiting ferroptosis (89).

During the process of skin ferroptosis, the role of AMPK is particularly important. It not only stimulates the production of collagen, which is a key component in maintaining the structure and elasticity of the skin, but also enhances the skin's firmness and elasticity through its activating effects, effectively combating signs of cell death (90).

In addition, the activation of AMPK also has anti-inflammatory effects, which can alleviate discomfort symptoms caused by skin inflammation, such as redness and itching (91). This anti-inflammatory action is particularly beneficial in alleviating the symptoms of certain skin diseases.

After the skin is damaged, the repair process is crucial. AMPK plays the role of an accelerator in this process. By stimulating cellular energy metabolism and accelerating cell proliferation, it promotes the rapid repair and regeneration of damaged skin tissue, thereby speeding up the healing of wounds (92).

2.10 Guanosine triphosphate cyclohydrolase 1

GCH1, an indispensable enzyme in the folate biosynthesis pathway, is intricately linked to ferroptosis (93). Its protective role stems from the metabolites it produces, tetrahydrobiopterin (BH4) and dihydrobiopterin (BH2), which are essential for cellular redox balance and the normal function of nitric oxide synthase (NOS) (94).

Beyond its involvement in folate generation, GCH1 also participates in the signaling of nitric oxide (NO) by converting guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and NO. NO, a molecule with diverse biological functions, profoundly influences various physiological processes within the cell, including the regulation of cell death pathways (95). The antioxidant effect of NO is evident in its ability to react with the superoxide anion to form peroxynitrite (ONOO-), a reaction that reduces levels of ROS within the cell, thereby alleviating oxidative stress (96). However, in the context of ferroptosis, the excessive accumulation of NO or its derivatives may interact with iron ions, leading to lipid peroxidation and subsequent cellular damage.

In-depth analysis has unveiled another protective mechanism of GCH1 within the cell. Cells with higher GCH1 activity exhibit characteristics indicative of containing two chains of PUFA, which demonstrate the ability to shield the phosphatidylcholine on the cell membrane from oxidative damage. This protection may be achieved by enhancing the stability of the cell membrane and limiting the spread of ROS, thereby aiding the cell in resisting oxidative stress.

Although the specific biochemical pathways and molecular interactions of this protective mechanism are not fully understood, it is evident that GCH1 plays a significant role in maintaining cellular redox balance and fortifying the cell against oxidative stress (97).

In the face of the threat of ferroptosis, the antioxidant effect of NO assumes critical importance for skin cells (98). NO's reaction with superoxide radicals to form ONOO- plays a pivotal role in neutralizing ROS, reducing oxidative stress, and providing a protective shield for cells, enabling them to resist the damage caused by ferroptosis (99).

Simultaneously, the indispensable role of GCH1 in maintaining the health of skin cells warrants attention (100). Involved in the biosynthesis of folate, a vital nutrient for cell division and DNA synthesis, GCH1 plays a key role in the renewal and repair processes of skin cells. Confronted with the challenge of ferroptosis, GCH1 ensures a stable supply of folate, promoting the rapid recovery and regeneration of damaged skin cells, thereby combating the cell damage caused by an imbalance of iron ions (101).

These multifaceted functions of GCH1 emphasize its central role in the skin's defense system. Not only does it directly participate in the battle against ferroptosis through the antioxidant mechanism of NO, but it also indirectly promotes the health and regenerative capacity of skin cells by supporting the synthesis of folate. Together, these actions underscore the crucial role of GCH1 in safeguarding the skin from the effects of ferroptosis.

3 Ferroptosis in conjunction with other cellular demise mechanisms

Ferroptosis is a unique form of regulated cell death that relies on iron and sets itself apart from other forms such as apoptosis, necrosis, and autophagy (102–104). This regulated cell demise process is characterized by the build-up of lipid peroxidation products and the collapse of cellular defenses against oxidative stress, both induced by iron accumulation (105, 106). The distinctive role of iron in ferroptosis underscores the importance of comprehending the involved molecular pathways, as it presents a novel perspective on cell death that can be therapeutically targeted.

In stark contrast to apoptosis, which is a meticulously regulated process requiring energy and involving a protease cascade, ferroptosis is marked by the loss of membrane integrity due to oxidative damage (107). It also contrasts with necrosis, typically associated with uncontrolled cell swelling and rupture in response to severe injury or infection (108, 109). Autophagy, another cellular process involving the degradation and recycling of cellular components, also differs from ferroptosis. Drawing a clear distinction between ferroptosis and these other pathways is essential for deepening our understanding of cell death in various pathological conditions and for the development of innovative therapeutic strategies.

3.1 Ferroptosis and apoptosis

Apoptosis, governed by genetic factors, is essential for maintaining tissue equilibrium and immune function. Ferroptosis and apoptosis are two key regulatory processes within cells, playing complex roles in the pathogenesis of skin diseases, especially in their interactive effects within the skin microenvironment (110). Studies indicate a possible interaction between ferroptosis and apoptosis, with cells potentially transitioning from the iron-dependent, lipid peroxidation-characterized ferroptosis to apoptosis (111, 112). This shift can escalate cell susceptibility to apoptosis, and the tumor suppressor p53 can trigger ferroptosis, influencing tumor cell death and lifespan in preclinical models (113).

The connection between ferroptosis and apoptosis in skin diseases is multifaceted. For instance, the same stimuli that induce apoptosis, such as UV radiation or certain chemotherapeutic agents, can also promote ferroptosis in skin cells (114). Moreover, the molecular pathways that regulate these two forms of cell death can intersect. For example, the tumor suppressor protein p53, which is well-known for its role in inducing apoptosis, can also activate ferroptosis under certain conditions (115).

In the context of skin cancer, the balance between ferroptosis and apoptosis can influence tumor progression and response to therapy (116). An increased understanding of how these cell death pathways interact could lead to novel therapeutic strategies that target their intersection, potentially enhancing the efficacy of treatments and providing new options for patients with skin diseases.

3.2 Ferroptosis and necrosis

Necrosis, typically an accidental cell death process, can mimic apoptotic signaling, and ferroptosis, which is driven by iron and lipid peroxidation, often occurs with necrosis under certain conditions (117). Studies have shown increased mRNA levels of markers for both ferroptosis and necrosis in cell death induced by heme chloride, suggesting an interplay between these death mechanisms (118). Research has indicated that deficiencies in ACSL4, a lipid metabolism enzyme associated with ferroptosis, can lead to increased Mixed Lineage Kinase Domain-like Protein (MLKL), a protein involved in necrosis execution, making cells more susceptible to ferroptosis. This suggests a dynamic interrelation between the two pathways (119, 120). Additionally, NADPH, vital for cellular redox balance, is thought to influence both ferroptosis and necrosis, with its consumption during necrosis potentially sensitizing neighboring cells to ferroptosis due to reduced intracellular NADPH levels (121).

In the skin microenvironment, the relationship between ferroptosis and necrosis is particularly complex and can significantly influence the pathogenesis of skin diseases. Ferroptosis, characterized by iron accumulation and lipid peroxidation, can be triggered in the skin by various stimuli, including UV radiation and oxidative stress commonly encountered in dermatological conditions (122). Necrosis, often a result of acute cellular injury, can also occur in the skin following trauma or ischemia, leading to inflammation and tissue damage (123).

The interaction between these two modes of cell death can be bidirectional. For instance, the oxidative environment that promotes ferroptosis can also damage the cell membrane, potentially leading to

necrosis (124). Conversely, the inflammatory response following necrosis can create a microenvironment that sensitizes cells to ferroptotic stimuli (125). This interplay may be particularly relevant in conditions like psoriasis, where both oxidative stress and cellular injury contribute to the disease pathology.

Moreover, the skin's immune cells, such as macrophages and neutrophils, can release ROS and pro-inflammatory cytokines that can drive both ferroptosis and necrosis (116). The resulting cellular damage and death can further exacerbate skin inflammation and impair the skin's barrier function, as seen in conditions like toxic epidermal necrolysis and bullous pemphigoid.

3.3 Ferroptosis and autophagy

Autophagy, involving lysosomal degradation of cellular components, plays a role in various diseases (126–128). It can trigger ferroptosis by degrading ferritin, leading to increased intracellular iron. This was observed in both fibroblasts and cancer cells, where autophagy enhanced ferroptosis through ferritin breakdown (129). Inhibition of autophagy-related genes like Autophagy-related protein 5 (Atg5) and Autophagy-related gene 7 (Atg7) reduced erastin-induced ferroptosis by lowering iron levels and lipid peroxidation (130, 131).

In inflammatory skin diseases such as psoriasis, the regulatory role of autophagy is particularly important, as it is responsible for clearing damaged organelles and proteins, helping to maintain cellular health and tissue homeostasis (132). Dysregulation of autophagy is associated with the abnormal cell proliferation and differentiation, as well as the exacerbation of inflammatory responses observed in psoriasis (133). Studies have found that abnormal autophagic activity in keratinocytes within the skin lesions of psoriasis may be related to the progression of the disease.

Within the skin microenvironment, the crosstalk between ferroptosis and autophagy may impact the inflammatory state and tissue repair of the disease. For instance, the activation of autophagy can alleviate inflammation, while the activation of ferroptosis may lead to the release of inflammatory mediators, intensifying the inflammatory response (134). Therefore, understanding how these two cellular processes interact is crucial for developing new strategies to treat skin diseases.

4 Ferroptosis and skin diseases

Ferroptosis, as a cell death mechanism triggered by the accumulation of intracellular iron ions and lipid peroxidation, is gradually revealing its importance in dermatological research. This mode of cell death is closely related to the pathogenesis of various skin pathological conditions, including but not limited to inflammatory skin diseases, autoimmune skin disorders, and skin tumors. In-depth research on ferroptosis has revealed its key role in the development of skin pathology, providing a new perspective for the diagnosis and treatment of skin diseases.

In the field of dermatopathology, the regulatory mechanisms of ferroptosis include the control of iron homeostasis, the production of ROS, and the balance of lipid metabolism. Disruptions in these mechanisms may lead to skin cell dysfunction or even death. For example, oxidative stress common in inflammatory skin diseases may exacerbate the accumulation of iron ions and lipid peroxidation, thereby triggering ferroptosis. In autoimmune skin diseases, the abnormal activation of the immune system may lead to iron metabolism imbalance, thus promoting the occurrence of ferroptosis. In skin tumors, ferroptosis may be related to the abnormal metabolism of tumor cells and changes in the microenvironment.

The multifaceted role of ferroptosis in dermatopathology emphasizes the necessity of in-depth research into its regulatory mechanisms. This not only helps us to better understand the pathogenesis of skin diseases but also provides the possibility for developing new treatment strategies, with the aim of improving patients' skin health and quality of life.

4.1 Ferroptosis and ichthyosis

Ichthyosis, also referred to as keratosis, is an autosomal dominant genetic disorder characterized by dry, rough, and scaly skin that resembles fish scales (135). This condition typically manifests in infancy or childhood and may become more pronounced with age. The skin lesions associated with ichthyosis usually appear on specific parts of the body, including the extensor surfaces of the limbs, the back, and the buttocks (136).

Mutations in the LOX gene family, specifically arachidonic acid 12-lipoxygenase (ALOX12B) and arachidonate lipoxygenase 3 (ALOXE3), are common causes of ichthyosis (137). In the function of the skin barrier, arachidonic acid 12-lipoxygenase (12R-LOX) is responsible for catalyzing the conversion of arachidonic acid into 12R-Hydroperoxyeicosatetraenoic acid (12R-HPETE), and epidermal-type lipoxygenase-3 (eLOX3) further transforms 12R-HPETE into biologically active compounds (138). These compounds are involved in regulating the differentiation and function of skin cells, thus maintaining the skin's barrier function. When mutations occur in the ALOXE3 or ALOX12B genes, this transformation process is disrupted, leading to abnormalities in the skin barrier function and consequently causing skin diseases such as non-bullous congenital ichthyosis (NCIE) (139).

Mixed Lineage Leukemia Protein-4 (MLL4), a pivotal histone methyltransferase, plays an essential role in biological processes such as cell differentiation and tumor suppression (140). Recent studies have revealed that MLL4 may regulate the process of ferroptosis by controlling the expression of the ALOX12B gene. In mouse models with specific knockouts of the MLL4 and ALOX12B genes, skin pathological changes similar to ichthyosis, including epidermal hyperplasia and desquamation, were observed (141). The expression of the ALOX12B gene was reduced in the epidermal tissues of these mice, suggesting a link to skin pathology (Figure 3).

Mice with functional loss mutations in the ALOX12B gene induced by ethylnitrosourea had red and shiny skin at birth, but quickly became dry and led to death, without exhibiting typical ichthyosis-like lesions (142). However, after the skin tissue of these mutant mice was transplanted into nude mice, local areas exhibited

features similar to ichthyosis. Histological examination found that the epidermis of these skin grafts was significantly thickened, with abnormal keratinization and acanthosis, related to the pathological changes of ichthyosis (143). These research findings highlight the importance of MLL4 and ALOX12B in skin health.

4.2 Ferroptosis and psoriasis

Psoriasis is a chronic and recurring skin condition characterized by raised red patches covered with silvery scales, creating a clear boundary between affected areas and normal skin (144). It's important to note that psoriasis is not contagious. Rather, it is caused by an overactive immune system leading to skin inflammation (145). Various factors, including emotional stress, infections, skin injuries, certain medications, and changes in body temperature, can trigger outbreaks or worsen psoriasis.

Ferroptosis, an iron-dependent form of regulated cell death, is closely connected to lipid peroxidation processes (146). This connection becomes especially pronounced in psoriasis patients, where skin lesions prominently display molecular markers of ferroptosis. Significantly, the keratinocytes within these lesions reveal abnormalities in lipid metabolism and an increased activation of lipid oxidation pathways, a stark contrast to healthy skin (147). These changes become even more evident at the single-cell level, where keratinocytes' lipid oxidation activity displays a positive correlation with the Th22/Th17 pathway, demonstrating a temporal and dosage-sensitive dependency on ferroptosis (148).

In psoriasis, the interplay between ferroptosis and immune pathways may occur through various mechanisms. Ferroptosis is an iron-dependent form of cell death that involves the accumulation of lipid peroxidation, which is associated with inflammation and oxidative stress observed in psoriasis. Notably, ferroptosis may interact with the Th17/Th22 pathways in psoriasis, which play a crucial role in the immune response of the disease (149).

T helper 17 cells (Th17 cells) are key immune cells in the inflammatory response of psoriasis, promoting inflammation by producing Interleukin-17 (IL-17) and other cytokines (150). IL-17 can stimulate the proliferation and activation of keratinocytes, which is one of the pathological characteristics of psoriasis (151).

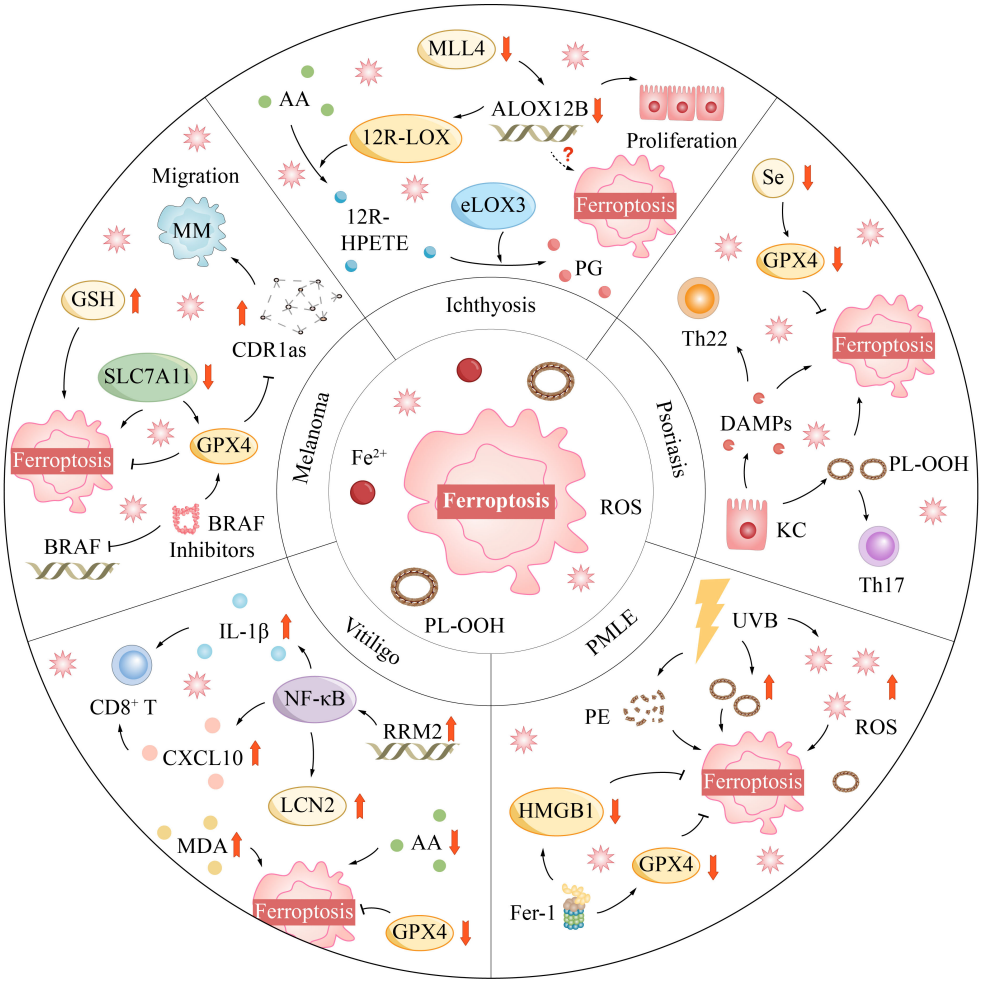

FIGURE 3
Ferroptosis mediates a variety of skin diseases.

Ferroptosis may regulate the immune response in psoriasis by affecting the function and survival of Th17 cells. For instance, inhibitors of ferroptosis, such as Ferrostatin-1, can reduce inflammatory responses in keratinocytes, which may be related to the modulation of Th17 cell functions (152).

Furthermore, in the pathogenesis of psoriasis, ferroptosis may impact the immune microenvironment by modulating the function of dendritic cells (DCs) and regulatory T cells (Tregs) (153). DCs are essential antigen-presenting cells in the immune system, playing a vital role in activating T cells and initiating immune responses (154). In psoriasis, DCs secrete cytokines like IL-23 and IL-12, promoting the differentiation and activation of Th17 cells (155). These Th17 cells then secrete IL-17 and IL-22, exacerbating inflammatory responses and the abnormal proliferation of keratinocytes (156).

The regulation of ferroptosis may affect the maturation and function of DCs. For example, the inhibition of ferroptosis may reduce the activation state of DCs, thereby diminishing their ability to activate Th17 cells (157). Additionally, ROS and lipid peroxidation products generated during ferroptosis may directly damage DCs, affecting their antigen presentation and immune regulatory functions.

Tregs are a subset of T cells with immune-suppressive functions. They suppress excessive immune responses and maintain immune tolerance by secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta (158). In psoriasis, the function of Treg cells may be suppressed, leading to an imbalance in immune regulation (159). Ferroptosis may further exacerbate this imbalance by affecting the stability and function of Treg cells. For instance, ferroptosis may induce apoptosis in Treg cells or alter their metabolic state, affecting their immune-suppressive functions (160).

Detailed investigations have highlighted alterations in expression levels of pivotal proteins like GPX4, ACSL4, Prostaglandin-endoperoxide synthase 2 (PTGS2), and Transferrin Receptor (TFRC) within psoriatic skin lesions. Of these, GPX4, an essential antioxidant enzyme, appears to have a reduced role in psoriasis pathogenesis (161). As selenium, a crucial element for GPX4 synthesis, is found deficient in psoriasis patients, this could potentially explain the decreased antioxidant capacity and increased susceptibility to ferroptosis in these individuals (162).

Furthermore, ferroptosis is not only implicated in direct cell destruction but also believed to trigger and amplify inflammatory responses by releasing damage-associated molecular patterns (DAMPs) (Figure 3) (163). This process activates immune cells and leads to an overproduction of inflammatory mediators, intensifying the chronic inflammatory environment within psoriatic lesions.

In summary, the relationship between ferroptosis and inflammation in psoriasis is complex. It involves abnormal lipid metabolism, changes in protein expression, and the escalation of inflammatory responses. Future research should strive to unravel the specific roles ferroptosis plays in psoriasis and explore the possibility of managing the disease's inflammatory aspects by targeting the molecular mechanisms linked to ferroptosis.

4.3 Ferroptosis and PMLE

PMLE is a prevalent skin disease triggered by sun exposure. It is recognized by its distinctive skin lesions, which can present in varying forms such as erythema, papules, vesicles, or plaques, often accompanied by itching (164). The manifestations of PMLE typically appear on skin areas exposed to UV radiation, including the face, neck, upper limbs, and the backs of the hands (165).

UV radiation, notably in the ultraviolet radiation B (UVB) band, poses a significant threat to skin health. Research has demonstrated that UVB radiation can induce a process known as ferroptosis in keratinocytes, the surface cells of the skin (114). This process is characterized by an accumulation of ROS and lipid peroxides within cells under the influence of UVB radiation.

UVB radiation also triggers alterations in the lipid composition of keratinocytes, notably causing an accumulation of oxidized phosphatidylethanolamine (PE), a critical factor in the onset of ferroptosis. In this context, Ferrostatin-1 has shown its importance in cell models and human skin tissue samples exposed to UVB radiation. It can markedly reduce the release of high mobility group box-1 protein (HMGB1), further highlighting the crucial role of ferroptosis in cell necrosis and inflammatory responses (166).

Further investigations have revealed that both single and repeated UV radiation exposures can lead to lipid peroxidation accumulation and iron metabolism abnormalities in keratinocytes. Despite an increase in the expression of the antioxidant enzyme GPX4 and related antioxidant components after repeated radiation exposures, the cell's defense against ferroptosis remains limited. This defense capability is notably weakened when there is an iron overload within the cells, suggesting that GPX4's protective effect may be limited by the degree of oxidative damage.

Furthermore, nicotinamide mononucleotide, a precursor of NAD^+, plays a vital role in regulating the redox imbalance caused by UVB radiation. Nicotinamide mononucleotide can stimulate the expression of GPX4, reducing the damage to mouse skin caused by UVB radiation (167). However, if GPX4 expression is inhibited, nicotinamide mononucleotide's protective effect will be significantly affected, indicating that nicotinamide mononucleotide's protective mechanism largely hinges on the normal function of GPX4 (Figure 3).

In conclusion, UV radiation significantly impacts skin health through various mechanisms, such as activating ferroptosis. These processes have implications not only on the survival of keratinocytes but also potentially influence the skin's overall immune response by triggering the release of inflammatory mediators.

4.4 Ferroptosis and vitiligo

Vitiligo manifests as white patches appearing across various body parts, a distinct characteristic of this skin condition (168). It arises when melanocytes, the cells responsible for melanin production that lends color to the skin, cease to function or are destroyed. Consequently, this leads to a loss of skin color in the

affected regions, resulting in conspicuous pale spots ranging in size from a few millimeters to several centimeters (169).

In clinical research, individuals with vitiligo have exhibited lower levels of arachidonic acid and reduced expression of the antioxidant enzyme GPX4 in their serum compared to healthy counterparts (170, 171). This decline in levels may heighten the susceptibility of melanocytes in the skin to ferroptosis and diminish the skin's antioxidant capacity.

Further investigations have revealed that in vitiligo lesions, the upregulation of the ferroptosis-related gene RRM2 activates the NF-κB signaling pathway. This activation leads to an increased release of inflammatory factors such as CXCL10 and IL-1β, consequently recruiting $CD8^+$ T immune cells to infiltrate the area and target melanocytes (172). Additionally, the activation of the NF-κB signal also results in the upregulation of LCN2, which not only accelerates the demise of melanocytes but also further amplifies the expression of RRM2.

Oxidative stress plays a pivotal role in the development of vitiligo, as it inflicts damage upon melanocytes through the accumulation of ROS, affecting the structural integrity and function of their DNA, lipids, and proteins (173). Skin lesions of vitiligo patients exhibit significantly elevated levels of hydrogen peroxide, reduced activity of antioxidant enzymes, and heightened levels of malondialdehyde, all serving as direct evidence of oxidative stress (174).

The impact of oxidative stress on melanocytes is multifaceted. It can induce the release of the protein HMGB1, disrupting the melanin synthesis process and leading to the downregulation of proteins related to melanin biosynthesis (Figure 3) (175). Furthermore, the excessive accumulation of ROS can disrupt the synthesis and stability of lipids within melanocytes, damage the mitochondrial electron transport chain, and initiate a detrimental cycle (176).

Moreover, melanocytes display a greater propensity for iron uptake than keratinocytes. Under external oxidative stress, the level of Fe^{2+} escalates, significantly heightening the sensitivity of melanocytes to ferroptosis (177). Specific triggers of ferroptosis, such as erastin, may promote this process by affecting the cystine/glutamate antiporter system or by inhibiting the activity of GSH peroxidase (178).

4.5 Ferroptosis and melanoma

Melanoma, a type of skin cancer, originates from melanocytes, the cells responsible for producing the pigment melanin that gives color to the skin (179). It stands out as one of the most aggressive forms of skin cancer and has the potential to metastasize to other parts of the body if not identified and treated promptly. Typically, melanoma is distinguished by the presence of a new or evolving mole or skin lesion, frequently exhibiting irregular borders, diverse colors, and occasionally an elevated surface (180).

Recent scientific investigations have brought to light the critical role of cerebellar degeneration-related protein 1 antisense (CDR1as) in the differentiation process of melanoma cells. Evidence has shown that the absence of CDR1as is directly associated with the heightened metastatic potential of melanoma cells, while cells expressing higher levels of CDR1as demonstrate increased sensitivity to GPX4 inhibitors (181). This revelation suggests that CDR1as could serve as a pivotal molecular target for inducing ferroptosis in melanoma cells (Figure 3).

Moreover, further research, including studies by prominent researchers like Sato, has indicated that the malfunction of System Xc- results in reduced cystine absorption by melanoma cells, leading to decreased GSH levels (182). Consequently, this hinders the cells' ability to proliferate *in vitro*, migrate, and generate tumors and metastases *in vivo*. Although these experimental findings underscore the potential involvement of the lymphatic system in melanoma metastasis, our understanding of the specific mechanisms remains incomplete (183).

Additional experiments have observed heightened levels of GSH and a higher GSH/GS-SG ratio in the bloodstream of melanoma cells, suggesting a more oxidative blood environment that could potentially promote ferroptosis. The loss of GPX4 function diminishes tumor formation via intravenous injection, a phenomenon reversible by pretreatment with ferroptosis inhibitors. Notably, lymph node metastasis remains unaffected, indicating a potential direct protective effect of lymph fluid against ferroptosis (184).

Furthermore, the mutation status of the BRAF gene and the use of BRAF inhibitors both influence the oxidative metabolism and oxidative phosphorylation levels of melanoma cells, potentially determining their sensitivity to ferroptosis inducers (185). Specifically, the downregulation of SLC7A11 and increased reliance on glutamine metabolism signify heightened sensitivity to ferroptosis in metastatic melanoma cells (186). BRAF inhibitors such as vemurafenib, by modulating lipid metabolism and enhancing dependence on GPX4, offer novel insights into addressing drug resistance in targeted melanoma therapy (187).

In the realm of immunotherapy for melanoma, PD-1 inhibitors activate $CD8^+$ T cells, inducing the secretion of IFN-γ (188). This process reduces the demand for cystine and GSH synthesis in melanoma cells, thereby augmenting the specific lipid peroxidation effect in ferroptosis. This discovery signifies that treatment strategies targeting the ferroptosis pathway can significantly enhance the efficacy of immunotherapy.

Concurrently, high expression levels of TYRO3 are associated with resistance to PD-1 inhibitors, and inhibiting TYRO3 can promote tumor cell ferroptosis (189). Additionally, reducing NRF2 levels can facilitate the infiltration of $CD8^+$ and $CD4^+$ T cells, and combining anti-PD-1 treatment can further bolster the therapeutic effect against melanoma (190).

5 Therapeutic approaches targeting ferroptosis in skin disorders

The identification of biomarkers is a critical step in the clinical diagnosis of diseases involving ferroptosis. These biomarkers can include specific lipid peroxidation products, altered iron levels, or changes in the expression of genes and proteins involved in ferroptosis. For instance, elevated levels of malondialdehyde

(MDA) or 4-hydroxynonenal (4-HNE), which are lipid peroxidation markers, could indicate ongoing ferroptosis. Additionally, the assessment of iron regulatory proteins like ferritin and transferrin can provide insights into the status of iron homeostasis.

Diagnostic tools tailored to detect ferroptosis-related biomarkers are essential for clinical practice. Advanced imaging techniques, such as MRI with iron-sensitive contrast agents, can help visualize iron accumulation in tissues. Moreover, the development of assays to quantify lipid peroxidation products in blood or tissue samples can serve as a non-invasive diagnostic method. Liquid biopsies, which involve the analysis of circulating cell-free DNA, proteins, or extracellular vesicles, may also reveal signatures of ferroptosis.

In skin diseases, histopathological examination is a valuable diagnostic tool. The presence of ferroptotic cells in skin biopsies can be identified by characteristic morphological changes, such as the accumulation of lipid droplets, the presence of iron deposits, and the absence of typical apoptotic or necrotic features. Specialized staining techniques, like the use of Prussian blue for iron detection or the detection of specific lipid peroxidation products, can further aid in the diagnosis.

The therapeutic potential of ferroptosis presents vast opportunities in the treatment of skin diseases. Through the regulation of iron metabolism, inhibition of ROS generation, induction of ferroptosis, and integration of immunotherapy, it holds promise for providing patients with more effective and safer treatment options.

For example, the utilization of iron chelators, such as deferoxamine, can effectively diminish the concentration of intracellular iron ions, thereby reducing iron-dependent ROS production (191). Additionally, antioxidants like vitamin E and selenium can play a pivotal role in curtailing the accumulation of ROS, thereby safeguarding skin cells from oxidative damage (192).

Moreover, immunotherapy has demonstrated significant potential in the treatment of skin diseases. By activating immune cells in the skin, particularly $CD8^+$ T cells, it can bolster immune surveillance and the clearance of tumor cells, thereby enhancing treatment efficacy (193).

5.1 Therapeutic approaches targeting ferroptosis in ichthyosis

Targeting ferroptosis for the treatment of ichthyosis presents an emerging therapeutic strategy. Utilizing iron chelators to reduce intracellular iron ion levels can decrease the production of hydroxyl radicals through the Fenton reaction, thereby inhibiting the accumulation of ROS (194). Additionally, modulating MLL4 and ALOX12B may prove to be an effective approach to treating ichthyosis. As a histone methyltransferase, MLL4 potentially influences the process of ferroptosis by regulating the expression of ALOX12B (141). The skin pathological changes observed in mouse models with knockouts of the MLL4 and ALOX12B genes, which resemble ichthyosis, provide experimental evidence supporting the treatment of ichthyosis through targeting the ferroptosis pathway.

To further expand on the relationship between ferroptosis and ichthyosis, it is important to consider the role of genetic factors in the development of the disease. Ichthyosis is a group of genetic disorders characterized by abnormal skin barrier function and abnormal desquamation, leading to the accumulation of scale-like skin. The identification of genes associated with ichthyosis, such as MLL4 and ALOX12B, has opened up new avenues for understanding the molecular mechanisms underlying the disease. MLL4, as a histone methyltransferase, is involved in the regulation of gene expression, including that of ALOX12B, which is implicated in skin barrier function and inflammation (195).

The modulation of these genes could potentially influence the susceptibility of skin cells to ferroptosis, thereby affecting the progression and severity of ichthyosis. This is supported by the observation of skin pathological changes in mouse models with knockouts of MLL4 and ALOX12B genes, which exhibit features similar to ichthyosis (196). These findings suggest that targeting the ferroptosis pathway may not only help in understanding the pathogenesis of ichthyosis but also in developing novel therapeutic strategies.

Moreover, the use of iron chelators and other compounds that can modulate the ferroptosis pathway could provide a novel therapeutic approach for ichthyosis. By reducing intracellular iron levels and consequently the production of ROS, it may be possible to ameliorate the symptoms of ichthyosis and improve skin health (197). This approach is in line with the current understanding of the role of oxidative stress in the pathogenesis of various skin diseases, including ichthyosis.

In conclusion, the targeting of ferroptosis in ichthyosis represents a promising area of research with potential therapeutic implications. Further studies are needed to fully understand the complex interplay between genetic factors, oxidative stress, and cell death pathways in the context of ichthyosis, which could lead to the development of more effective treatments for this group of disorders.

5.2 Therapeutic approaches targeting ferroptosis in psoriasis

Addressing psoriasis through the lens of ferroptosis presents a pioneering therapeutic strategy, concentrating on modulating cellular demise pathways intricately tied to iron homeostasis and ROS dynamics (198, 199). This strategy employs iron ion chelators to reduce the iron content within cells, thereby alleviating oxidative stress reactions and combating the pathological changes associated with psoriasis (200). Simultaneously, by targeting the activity of key enzymes such as GPX4, it can induce pathological skin cells to undergo ferroptosis, offering a novel therapeutic approach to control the progression of psoriasis (147).

To further expand on this strategy, it is important to consider the implications of ferroptosis in the clinical diagnosis and prognosis of psoriasis. The activation of ferroptosis in psoriatic lesions has been observed, with decreased expression of GPX4 and increased levels of lipid peroxidation markers, such as 4-HNE (199). This suggests that assessing the levels of these markers could potentially serve as

diagnostic indicators of psoriasis. Moreover, the modulation of ferroptosis-related genes and pathways could provide a basis for developing prognostic biomarkers and therapeutic targets.

The use of iron chelators and other compounds that target the ferroptosis pathway, such as small molecule compounds predicted to target key genes, may offer new avenues for treatment (147). These compounds could be used to modulate the expression of genes associated with ferroptosis, potentially leading to improved disease management and outcomes for patients with psoriasis.

In conclusion, the integration of ferroptosis into the therapeutic strategy for psoriasis is a promising development. By targeting the molecular mechanisms underlying ferroptosis, it may be possible to develop more effective treatments and improve the prognosis for individuals with psoriasis. Further research is needed to fully understand the role of ferroptosis in psoriasis and to translate these findings into clinical practice.

5.3 Therapeutic approaches targeting ferroptosis in PMLE

UVB radiation plays a pivotal role in inducing ferroptosis in PMLE, leading to an accumulation of oxidized PE, which is closely associated with the onset of ferroptosis (166). Notably, Ferrostatin-1 demonstrates the ability to reduce the release of HMGB1 in UVB-irradiated cells, highlighting the significance of ferroptosis in cell necrosis and inflammation. Despite an increase in GPX4 expression, repeated UV radiation can still induce lipid peroxidation and disrupt iron metabolism, particularly under conditions of iron overload (167). Furthermore, nicotinamide mononucleotide, acting as a precursor of NAD^+, regulates the redox imbalance caused by UVB and stimulates GPX4 expression, thereby reducing skin damage in UVB-exposed mice. However, the protective effect of nicotinamide mononucleotide is significantly compromised if GPX4 expression is inhibited, indicating that the protective mechanism of nicotinamide mononucleotide largely relies on the normal function of GPX4. A deeper understanding of the role of ferroptosis in skin pathology may pave the way for the development of more precise targeted therapies in the future, potentially enhancing treatment outcomes and the quality of life for PMLE patients (164, 201).

A deeper understanding of the role of ferroptosis in skin pathology may pave the way for the development of more precise targeted therapies in the future, potentially enhancing treatment outcomes and the quality of life for PMLE patients (202). The exploration of ferroptosis-related genes in the context of PMLE could provide valuable insights into the disease's pathology and identify potential therapeutic targets. For instance, the differential expression of FRGs in response to UVB radiation could serve as a biomarker for disease activity and prognosis, aiding in the development of personalized treatment strategies (203).

In conclusion, the integration of ferroptosis into the clinical management of PMLE could lead to more personalized and effective treatment approaches. Further research is needed to fully elucidate the role of ferroptosis in PMLE and to translate these findings into clinical practice.

5.4 Therapeutic approaches targeting ferroptosis in vitiligo

Targeting ferroptosis in vitiligo treatment is an emerging strategy that focuses on the modulation of GPX4 levels to reduce melanocyte susceptibility to ferroptosis (204). In vitiligo lesions, the upregulation of the RRM2 gene activates the NF-κB signaling pathway, leading to increased inflammation and melanocyte damage. The accumulation of ROS due to oxidative stress not only harms melanocytes but also elevates oxidative stress markers in skin lesions. Melanocytes, with their high iron uptake capacity, are particularly vulnerable to oxidative stress, increasing the risk of ferroptosis. Compounds such as erastin may promote ferroptosis through distinct mechanisms, further impacting melanocyte health.

The study by Wu et al. (204) provides evidence that altered expression of ferroptosis markers and dysregulated iron metabolism may play a role in vitiligo pathogenesis. The findings suggest that erastin can induce ferroptosis in melanocytes, which can be mitigated by N-acetyl-L-cysteine (NAC) treatment, indicating a potential therapeutic approach to protect melanocytes from ferroptosis.

Moreover, the research by Zhang et al. (205) identified RNA-binding protein SLC3A2 as a regulator of melanocyte ferroptosis in vitiligo through integrated analysis of single-cell and bulk RNA-sequencing. This study revealed that downregulating SLC3A2 can promote ferroptosis in melanocytes, offering new insights into the pathogenesis of vitiligo and potential therapeutic targets.

In conclusion, understanding the role of ferroptosis in vitiligo and targeting key regulators such as GPX4 and SLC3A2 may lead to the development of novel treatments that could improve outcomes for patients with vitiligo.

5.5 Therapeutic approaches targeting ferroptosis in melanoma

Recent research advancements have significantly illuminated the pivotal role of ferroptosis in the pathogenesis and treatment strategies of melanoma (206, 207). With a comprehensive understanding of this process, scientists have developed a range of new drugs for the treatment of melanoma, primarily categorized as molecularly targeted therapies and immune checkpoint inhibitors. In the realm of molecular targeted therapy, BRAFV600E inhibitors hold particular significance. Given the prevalence of BRAF gene mutations in many melanoma patients, BRAFV600E inhibitors effectively curb the growth of tumor cells by specifically targeting the activity of this mutated gene (208). Additionally, immune checkpoint inhibitors, such as anti-programmed death protein 1 (PD-1) antibodies, have demonstrated noteworthy therapeutic effects (209). These drugs bolster the body's natural defense against tumors by alleviating the suppression of the immune system by tumor cells.

Ferroptosis, an iron-dependent form of cell death, has emerged as a potential therapeutic target in melanoma. The role of ferroptosis in melanoma is multifaceted, influencing the disease's progression, metastasis, and response to treatment. Studies have

shown that certain melanoma cells are sensitive to ferroptosis inducers like erastin and RSL3, which could be exploited for therapeutic benefit (210). Furthermore, the sensitivity of melanoma cells to ferroptosis has been linked to their dedifferentiation status, with dedifferentiated cells being more susceptible to ferroptosis, suggesting a potential for combined treatment with BRAF inhibitors and ferroptosis inducers (211).

The manipulation of ferroptosis pathways could also enhance the efficacy of immunotherapies. For instance, the loss of GPX4, a key enzyme in ferroptosis, has been shown to promote an immune response against melanoma cells, indicating that ferroptosis may play a role in antitumor immunity (212). Additionally, the expression of certain microRNAs, such as miR-137 and miR-9, has been found to regulate ferroptosis in melanoma by targeting glutamate metabolism, which could have implications for therapy resistance and sensitivity.

In conclusion, the interplay between melanoma, ferroptosis, and the immune system is complex and holds promise for the development of novel therapeutic strategies. Targeting ferroptosis in melanoma may offer a new avenue for treatment, either as a standalone approach or in combination with existing therapies. As research in this field progresses, a better understanding of the mechanisms underlying ferroptosis and its role in melanoma will be crucial for the design of effective treatment strategies.

The advent of these new drugs marks substantial progress in the treatment of skin diseases. They have not only enhanced patients' survival rates but also their quality of life. With ongoing research and clinical trials, the anticipation of developing more treatment options and strategies in the future brings renewed hope to patients.

6 Conclusion

Ferroptosis, as a distinct cell death mechanism, is gaining recognition for its potential role in skin disease pathology. Our review highlights the need for further exploration into how ferroptosis intersects with conditions such as psoriasis, vitiligo, and melanoma. While the therapeutic potential of targeting ferroptosis is promising, particularly with drugs that modulate iron metabolism and lipid peroxidation, it is clear that our understanding of these interactions is still evolving.

The development of targeted therapies, possibly enhanced through nanotechnology, could offer novel treatment options for skin diseases. However, the complexity of ferroptosis and its varied implications across different pathologies underscore the necessity for cautious optimism and continued research. This includes addressing current knowledge gaps, refining drug delivery systems, and assessing the long-term safety and efficacy of ferroptosis-targeting treatments.

Looking ahead, the integration of advanced technologies such as gene editing and immunomodulation may provide additional avenues for manipulating ferroptosis in a clinical setting. As our understanding of ferroptosis deepens, so too will the potential for innovative treatments that could significantly impact the lives of those affected by skin diseases.

Author contributions

KW: Writing – original draft. YL: Writing – original draft. DZ: Writing – original draft. PL: Writing – original draft. XZ: Writing – review & editing. ZH: Writing – original draft, Writing – review & editing. HC: Writing – review & editing.

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The important role of ferroptosis in inflammatory bowel disease

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Ferroptosis is a type of regulated cell death that occurs due to the iron-dependent accumulation of lethal reactive oxygen species (ROS) from lipids. Ferroptosis is characterized by distinct morphological, biochemical, and genetic features that differentiate it from other regulated cell death (RCD) types, which include apoptosis, various necrosis types, and autophagy. Recent reports show that ferritin formation is correlated to many disorders, such as acute injury, infarction, inflammation, and cancer. Iron uptake disorders have also been associated with intestinal epithelial dysfunction, particularly inflammatory bowel disease (IBD). Studies of iron uptake disorders may provide new insights into the pathogenesis of IBD, thereby improving the efficacy of medical interventions. This review presents an overview of ferroptosis, elucidating its fundamental mechanisms and highlighting its significant involvement in IBD.

KEYWORDS

inflammatory bowel disease, intestinal epithelial cells, ferroptosis, ROS, mechanism

1 Introduction

Inflammatory bowel disease (IBD) is a chronic, incurable disorder that poses low mortality, stable course, and recurrence, and is a global health problem whose incidence and prevalence are steadily increasing (1). IBD manifests in two major forms, namely Crohn's disease (CD) and ulcerative colitis (UC). These two forms are characterized as distinct chronic and recurrent conditions and exhibit different pathological features. The CD has the potential to impact different regions of the gastrointestinal tract. However, it is commonly observed that the terminal ileal or perianal region is more susceptible to CD, resulting in a discontinuous type of transmural inflammation. Furthermore, the CD is commonly correlated to complications, including abscesses, fistulas, and strictures. In contrast, UC is characterized by persistent inflammation of the mucosal and submucosal layers of the colon, with the initial manifestation of the disease typically occurring in the rectum and subsequently extending gradually along the colon. The histopathological features of UC include the presence of a large number of neutrophils in the lamina propria and crypt, where they form microabscesses. In contrast to UC, CD is characterized by the accumulation of macrophages, which often form non-tyrosine granulomas (1, 2). Although the etiology of IBD remains unclear, studies suggest that IBD pathogenesis may be attributed to immune response, external environment, individual genetics, intestinal microflora, and type of food intake (3).

Patients with IBD were found to have an altered intestinal microenvironment, often correlated to higher reactive oxygen species (ROS) levels, leading to damage to the intestinal epithelial barrier and inflammatory response (4). ROS can cause oxidative damage to lipid

membranes by polyunsaturated fatty acids (PUFA), resulting in large amounts of lipid peroxides, which can lead to membrane damage and ferroptosis (ferroptosis) in intestinal epithelial cells (IECs) (4). IECs are a core component of the intestinal epithelial barrier. Moreover, abnormal death of IECs caused by ROS can lead to extensive epithelial erosion and disruption of the intestinal epithelial barrier, resulting in the development of UC (5, 6). In addition, ROS can act as messengers to regulate the polarization of intestinal macrophages; both NOX2-and NOX4-derived ROS can promote the polarization of primary macrophages into M1 (7) and M2 macrophages (8), respectively. The imbalance in polarization toward pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes can lead to disturbances in intestinal immune homeostasis and inflammatory responses, which have a close association with IBD severity.

Recently, cell ferroptosis was found to be involved in the development of IBD. Excessive iron intake has been reported to exacerbate the colitis model triggered by dextran sodium sulfate (DSS) in rats and increase UC risk (9, 10). A study conducted by Xu et al. revealed that ferroptosis-related genes were highly expressed in human colon biopsy samples, including ACSL4 solute carrier family 38 member 1 (SLC38A1) and glucose-6-phosphate dehydrogenase (G6PD) (11). Collectively, these results indicate a probable link between IBD and ferroptosis, such as disruption of IECs and damage to the intestinal mucosal barrier, which ultimately results in the development of IBD. Ferroptosis is a type of regulated cell death (RCD) that occurs due to the iron-dependent accumulation of lipid ROS and is characterized by distinct morphological, biochemical, and genetic features that differentiate it from other RCD types, which include apoptosis, various necrosis types, and autophagy (12, 13). The iron poisoning process is primarily mediated by the accumulation of lethal ROS and lipid peroxidation products and is significantly affected by iron metabolism (14). Excess iron resulted in ROS formation through the Fenton reaction (14, 15). Following this, a significant amount of ROS results in glutathione peroxidase 4 (GPX4) deactivation via glutathione (GSH) degradation (16). Furthermore, ROS interact with PUFAs present in cellular lipid membranes, resulting in producing numerous lipid peroxides that impair the integrity of cell membranes and ultimately result in cell death (17). Ferroptosis can occur in various disorders, including acute injury, myocardial infarction, ischemia/reperfusion (I/R), and cancer (1, 4, 7). Ferroptosis was revealed to be involved in the two major forms of IBD: CD and UC (18–21). In addition, inhibition of ferritinase by small molecule compounds alleviates experimental colitis triggered by DSS (22, 23). To conclude, ferroptosis appears to have significant implications in intestinal disorders and could potentially offer novel avenues for the management of IBD. The present review offers a comprehensive depiction of ferritin formation and its significant involvement in IBD.

2 Ferroptosis: a unique form of regulated cell death

2.1 Definition, characteristics, and development of ferroptosis

The concept of ferroptosis can be traced back to the discovery of erastin, a new agent identified in 2003 as an ferroptosis inducer. This was accomplished by screening a library of small molecule drugs that exhibited selective lethality in RAS-mutated cancer cell lines, thereby introducing a new non-apoptotic mechanism of cell death (24). Subsequently, Yagoda et al. identified the direct target of erastin to be mitochondrial voltage-dependent anion channel 2 (VDAC2, 25). Moreover, Yang et al. identified two compounds, RAS-selective lethal 3/5 (RSL3/5), with similar properties to erastin (25). They found that iron chelators have the ability to inhibit the cell death process. The nomenclature "iron poisoning" was established by Dixon et al. in 2012 to describe this newly discovered mechanism of cell death (13). This process was formally named ferroptosis, which is characterized by being iron-dependent, non-apoptotic, as well as having a large accumulation of lethal lipid ROS. This pattern is different from other types of RCD that have been previously observed. From a morphological point of view, iron-intoxicated cells exhibit certain characteristics such as small mitochondria, condensed mitochondrial membrane density, reduced or absent mitochondrial cristae, and ruptured outer mitochondrial membranes (13, 26). From a biochemical point of view, intracellular iron and ROS increase, leading to GSH depletion and impaired GPX4 activity. Consequently, this results in a breakdown of lipid peroxide metabolism, causing significant quantities of lipid ROS to accumulate and ultimately fostering ferroptosis (27). From a genetic point of view, ferroptosis is a complex biological process correlated to metabolic disturbances in several biological processes, including iron homeostasis, fatty acid synthesis, and lipid oxidation, which are regulated by an organized network of genes (28). Ferroptosis can be triggered by multiple substances, such as erastin, RSL3, p53, DPI7, FIN56, and FINO2, among others. These molecules trigger ferroptosis by different methods, GSH depletion, GPX4 inactivation, or degradation (13, 29). As the mechanism of ferroptosis has been studied, many specific inhibitors of ferroptosis have been identified, including ferrostatin-1(Fer-1), liproxstatin-1, and vitamin E, as well as iron chelators, DFO, and deoxynivalenol methanesulfonate. These substances suppress ferroptosis through the inhibition of lipid peroxide formation and iron accumulation. In addition, ferroptosis is positively or negatively regulated by several transcription factors, including nuclear factor E2-related factor 2 (NRF2), activating transcription factor 3/4 (ATF3/4), Yes-associated protein 1 (YAP1), and hypoxia-inducible factor lα (HIF1A) (28).

2.2 Mechanism of ferroptosis

The sources of lipid ROS that induce ferroptosis are multifaceted, and lipid peroxidation pathways and iron metabolism are increasingly considered to be involved in the ferroptosis process (14) (Figure 1).

2.2.1 Iron metabolism in ferroptosis

The cellular metabolism is significantly influenced by the presence of iron, owing to its ability to transition between oxidized and reduced states. This transition facilitates the generation of ROS through a Fenton-like reaction, which ultimately results in nonenzymatic lipid peroxidation (30–32). Iron serves the dual purpose of promoting nonenzymatic reactions and being conveyed to iron-containing enzymes (lipoxygenases, 15-LOX), which are implicated in lipid peroxidation (32). Consequently, iron is required as a key factor in the formation of lipid peroxides for ferroptosis to occur. Normally, heme

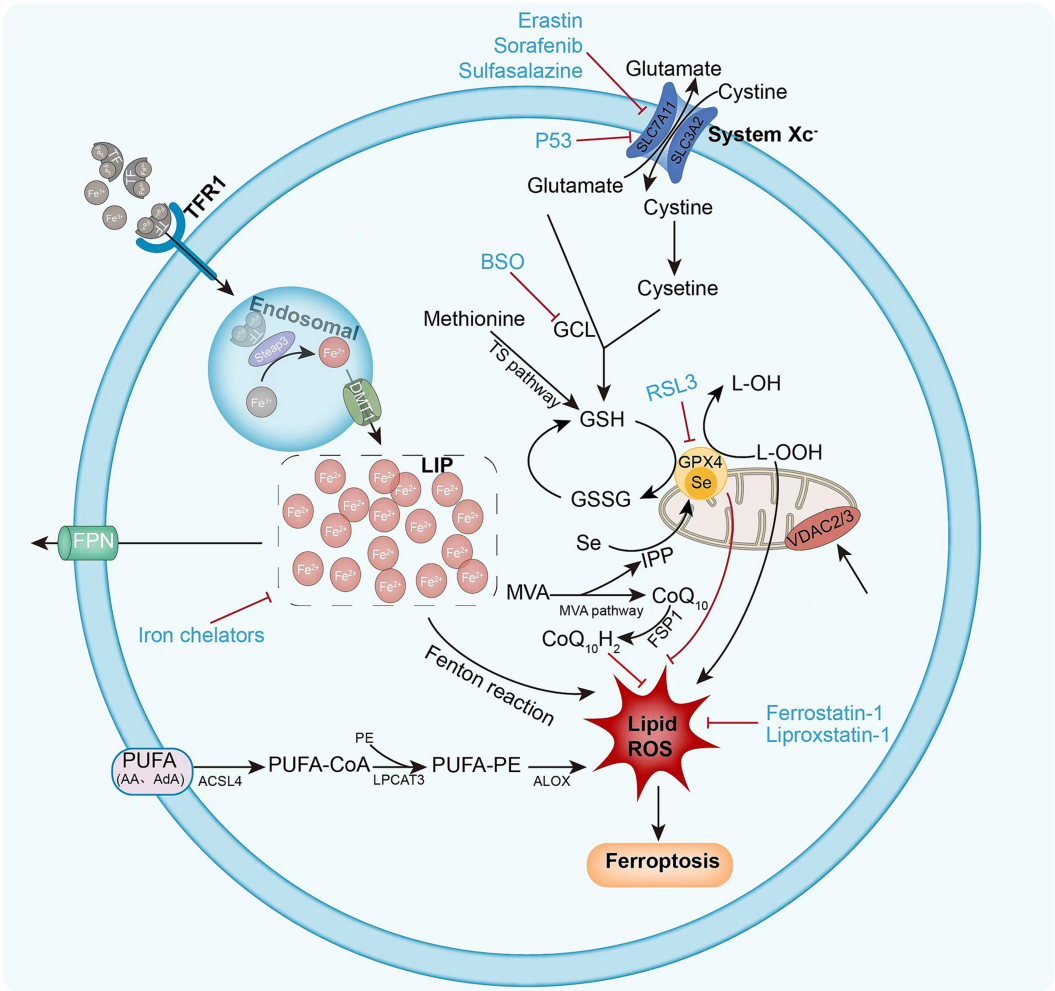

FIGURE 1
Mechanism of intracellular ferroptosis. Ferroptosis is defined by iron accumulation, excessive ROS production, and massive lipid peroxidation. Ferroptosis initiation and execution involves three major metabolic pathways: the amino acid/GSH, lipid, and iron pathways. Ferroptosis sensitivity is also controlled via additional signaling pathways and regulators. The following diagram illustrates the ferroptosis process and provides an overview of the key molecules and targets that govern iron and lipid peroxidation regulation. ACSL4 (acyl-CoA synthase long-chain family member 4), BSO (buthionine sulphoximine), DMT1 (divalent metal transporter 1), FSP1 (ferroptosis suppressor protein 1), FPN1 (ferroportin 1) GPX4 (glutathione peroxidase 4) GSH (glutathione), GSSG (oxidized glutathione), GSS (glutathione synthetase), GCL (glutamate-cysteine ligase), LOX (lipoxygenase), LPCAT3 (lysophosphatidylcholine acyltransferase 3), PUFA (polyunsaturated fatty acid), PE (phosphatidylethanolamine), ROS (reactive oxygen species), RSL3 (Ras-selective lethal 3), STEAP3 (six-transmembrane epithelial antigen of prostate 3 metalloreductase), SLC7A11 (solute carrier family 7 member 11), TF (transferrin), TFR1 (transferrin receptor 1), VDAC2/3 (voltage dependent-anion channel 2/3).

and non-heme iron are predominantly found in iron form (Fe^{3+}). Fe^{3+} is reduced to Fe^{2+} in the intestinal lumen by iron-reducing enzymes, including duodenal cytochrome b reductase (Dcytb), and transported to the enterocytes via the divalent metal 1 transporter (DMT1). Subsequently, Fe^{2+} exported by ferroportin (FPN-1) can be oxidized to Fe^{3+} by membrane-bound ceruloplasmin, which in turn binds to transferrin (TF) to form TF-Fe^{3+}, which is referred to as holotransferrin (transferrin bound to two iron atoms; Figure 2). Holotransferrin further binds to the membrane protein transferrin receptor (TFRC) to form a complex that is subsequently subjected to endocytosis (26, 31). Fe^{3+} is then reduced to Fe^{2+} by the endosomal iron reductase hexa-transmembrane prostatic antigen 3 (STEAP3) and transported across the endosomal membrane into the cytoplasm via DMT. Fe^{2+} is stored in the unstable iron pool (LIP), and ferritin is the major intracellular iron storage protein, which consists of ferritin light chain polymer (FTL) and FTH1. Excess intracellular Fe^{2+} is oxidized to Fe^{3+} and exported by FPN-1 (33). Cellular iron homeostasis is tightly regulated; in the presence of iron overload, such as reduced iron stores or increased iron uptake, excess iron can trigger ferroptosis. Knockdown of TFRC inhibits erastin-induced ferroptosis (34). Moreover, heme oxygenase 1 (HO-1) overexpression in HT-1080 fibrosarcoma cells exacerbates ergocalin-triggered cell death (35). However, silencing HO-1 increases the growth inhibition of hepatocellular carcinoma (HCC) cells after ferroptosis triggering, suggesting that HO-1 also has an anti-ferroptosis effect (36). The dual role of HO-in ferroptosis may depend on its expression and cell type. Nuclear receptor cofactor 4 (NCOA4) is a selective cargo receptor for ferritin phagocytosis, i.e., catabolism of ferritin by autophagy, and overexpression of NCOA4 increases ferritin catabolism and promotes ferroptosis (37). In addition, prominin 2 promotes ferroptosis in breast cancer through

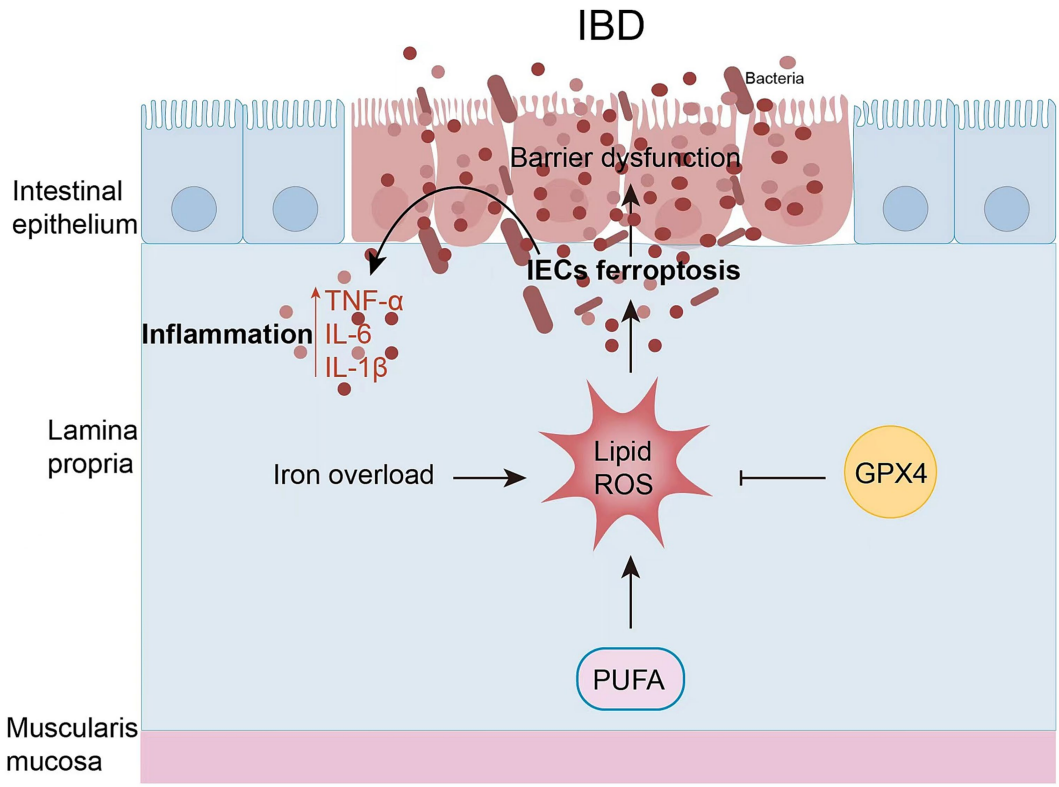

FIGURE 2
Iron metabolism in ferroptosis. Enterocytes absorb iron from the diet through the combined action of iron reductase (e.g., Dcytb) and the divalent metal transporter DMT1. The iron absorbed by the enterocytes is exported to the bloodstream via the ferroportin (FPN-1). At the same time, Fe^{2+} is oxidized to Fe^{3+} by ceruloplasmin. Subsequently, Fe^{3+} is loaded onto the circulating TF in the plasma.

iron export mediated by the Prominin2-MVB/exosomal ferritin pathway (38). Other regulators, including iron response element binding protein 2 (IREB2) and heat shock protein beta-1 (HSPB1), may also regulate ferroptosis by mediating iron uptake (39, 40). Collectively, these findings indicate that iron is an indispensable component of ferroptosis.

2.2.2 Lipid peroxidation pathway and iron reduction

Lipid peroxidation is a hallmark of ferroptosis, and PUFAs are one of the major targets of lipid peroxidation. ROS can attack PUFAs in lipid membranes, leading to lipid peroxidation. Free PUFAs (mainly arachidonic acid, AA) must be esterified to membrane phospholipids (PLs) by acyl-CoA synthase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3(LPCAT3). ACSL4 acylates AA, LPCAT3 catalyzes the acylated AA into membrane PLs, and then lipoxygenases (LOXs) catalyze PUFA PLs, producing native lipid peroxidation (41) (Figure 1). Several studies have identified ACSL4 and LPCAT3 as key determinants of susceptibility to ferroptosis, especially ACS4, which is considered a biomarker of ferroptosis (42). When GPX4 is inactive, AA and other PUFAs play an important role in ferroptosis, whereas ACSL4 and LPCAT3 promote RSL3-induced ferroptosis but not erastin-induced ferroptosis in KBM7 cells (43). ACSL4 sensitizes breast cancer cells to ferroptosis, whereas pharmacological ACSL4 inhibition prevents ferroptosis. Other researchers have reported that LPCAT3 deficiency can lead to resistance of fibroblasts to ferroptosis (42). This suggests that ACSL4 and LPCAT3 are key players in ferroptosis. It was shown that phosphatidylethanolamines (PEs) containing PUFA, especially those containing AA and epinephrine (AdA), are most susceptible to peroxidation in ferroptosis (44). Thus, overwhelming lipid peroxidation by PUFA leads to an increased susceptibility to ferroptosis.

Ferroptosis primarily results from an excessive amount of lipid peroxidation caused by the accumulation of ROS, which is reliant on the presence of iron, as previously noted. Ferroptosis can occur due to the impairment of GSH-dependent lipid peroxidation repair system, in addition to PUFA-mediated signaling. The proper functioning of GPX4, a member of the GPX family responsible for converting cytotoxic lipid hydroperoxides (L-OOH) into nontoxic lipid alcohols (L-OH), thus preventing the formation of lethal lipid ROS, is dependent on the indispensable cofactor GSH (45). GSH, an antioxidant, is a tripeptide consisting of glutamate, cysteine, and glycine. It serves as the rate-limiting enzyme for the *de novo* synthesis of GSH, which is promoted by glutamate cysteine ligase (GCL) and glutathione synthetase (GSS). Cysteine is regarded as the limiting factor in the production of GSH among these three amino acids. The system xc-antibody is a heterodimeric protein complex composed of the solute carrier family 7 member 11 (SLC7A11/xCT) and the solute carrier family 3 member 2 (SLC3A2) located on the plasma membrane. Its primary function is to facilitate the transmembrane transport of extracellular cysteine and the export of intracellular glutamate, which

is subsequently converted to cysteine and synthesized by GSH (26). The depletion of GSH results in elevated levels of oxidative stress, molecular impairment, and consequent cell ferroptosis (46). Erastin and other molecules (e.g., salazosulfapyridine, sorafenib) can induce ferroptosis by inhibiting system x and reducing GSH production (13, 30). Additionally, it was shown that P53, a significant gene in tumor suppression, has the ability to reduce SLC7A11 expression to inhibit cystine uptake by system x_c^-, thereby impairing the activity of GPX4 and leading to decreased antioxidant capacity, ROS accumulation, and ferroptosis (47). The lack of SLC7A11 in mice leads to a significant increase in plasma cysteine concentrations, and the survival of embryonic fibroblasts derived from these mice is compromised. Antioxidants such as β-mercaptoethanol (2ME), N-acetylcysteine (NAC), or vitamin E prevent cell death (48). In the absence of SLC7A11, iron treatment may lead to ferroptosis in hepatocytes (49). The deletion of SLC7A11 by CRISPR-Cas9 induces ferroptosis in cultured PDAC cells (50). The known GCI inhibitor buthionine sulfonamide (BSO) can also reduce GSH synthesis, leading to ferroptosis (17). In conclusion, GSH depletion is involved to a great extent in triggering ferroptosis.

GPX4 is a crucial factor and a master regulator in ferroptosis. The suppression of GPX4 activity may result in ferroptosis by forming lipid peroxides. The inactivation of GPX4 can be achieved through both indirect approaches, including GSH depletion, as well as direct methods involving pharmacological or genetic interventions. RSL3 is able to induce ferroptosis by directly inhibiting the enzymatic activity of GPX4 through covalent interaction with selenocysteine at the active site of GPX4 (51). In addition to RSL3, other synthetic small molecules, including DPI7, DPI, JKE1674, and JKE-1716, can inhibit GPX4 activation (26). Like RSL3, these compounds induce ferroptosis by covalently interacting with GPX4 active site without depleting cellular GSH. These compounds are categorized as "class 2" ferroptosis-inducing compounds (FINs). in contrast to "class l" FINs, which include erastin and other system x inhibitors that have an indirect effect on the blockage of GPX4 activity by blocking GSH synthesis (16). Deletion of GPX4 in mice can lead to acute renal failure and ferroptosis. Lipophilic antioxidants (e.g., liproxstatin-1) inhibit ferroptosis in GPX4-deficient mouse cells (27). GPX4 activity requires selenocysteine, the synthesis of which is mediated by selenocysteine-tRNA. The maturation of selenocysteine-tRNA can be regulated by the pentadienoic acid (MVA) pathway, which in turn can impact the synthesis of GPX4 and the development of ferroptosis (52). Thus, inhibition of the MVA pathway may lead to ferroptosis, thereby validating the crucial role of GPX4 activity in preventing ferroptosis.

2.2.3 Other mechanisms of ferroptosis

Ferroptosis can be modulated by alternative pathways as well. Mitochondrial VDACs are membrane-spanning channels that facilitate the transmembrane transport of ions and metabolites. Erastin can interact with VDAC2 protein to trigger mitochondrial malfunction and oxidative substance release, eventually leading to oxidative cell death, i.e., ferroptosis (53) (Figure 1). GSH can be synthesized via the sulfotransferase pathway, in which methionine is converted to cystine (54). Therefore, reduced methionine intake can lead to ferroptosis. Furthermore, the FSP1-CoQ10-NAD (P) H metabolic pathway (55) and p62-Keap1-NRF2 (36) exhibit proficient regulation of ROS formation of intracellular lipids and serve as a regulator of ferroptosis.

2.2.4 Box one measuring ferroptosis

Cell ferroptosis can be detected by changes in cell morphology, cell viability, ROS iron content, lipid peroxidation levels, and gene expression associated with ferroptosis (Table 1). The utilization of transmission electron microscopy is a viable approach for the identification of distinct morphological characteristics of cellular ferroptosis (56). Cell viability can be measured with the Cell Counting Kit-8 assay (57). In addition, when ferroptosis occurs, the iron level of cells increases. We can test iron levels with the Iron Assay Kit or the Phen Green SK (PGSK) fluorescent probe, a cell membrane permeable dye employed to assess intracellular iron levels by flow cytometry or confocal microscopy (14, 36). ROS and lipid peroxidation measurements are important to support the development of ferroptosis. Cellular ROS levels can be measured with ROS detection kits and dihydroethidium (DHE) kits, which indirectly reduce ROS levels by measuring superoxide anions using fluorescence microscopy (22). Oxidative lipidomics is a powerful technique to study lipid oxidation, and other methods to detect lipid peroxidation include the use of C11-BODIPY or Liperfluo probes by fluorescence microscopy (58). In addition, levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), byproducts of lipid peroxidation, may indicate cellular ferroptosis (59). Moreover, we were able to detect changes in gene/proteinexpression correlated with ferroptosis, including prostaglandin endoxylase 2 (PTGS 2), ACSL4, GPX4, and ferritin heavy chain 1 (FTH1) (4). In recent years, the detection of ferroptosis has risen from the cellular level to the genetic level with the advent of various high-throughput assays. The use of single cell RNA-sequencing technology to detect the enrichment of ferroptosis-related genes has led to more rapid and accurate detection of ferroptosis.

3 Ferroptosis in IBD

3.1 Inflammation and ferroptosis

Inflammation is a pathologic self-defense process that eliminates destructive factors, removes necrotic tissue, and repairs damage caused by the body after being stimulated by various injuries. If the inflammatory response persists, it can contribute to the development of disease through various pathways. There is a close relationship between ferroptosis and inflammation: on the one hand, ferroptosis can enhance the inflammatory response through various pathways, leading to

TABLE 1 Methods for detecting ferroptosis.

Detection object	Methods
The specific morphological features of ferroptosis	Transmission electron microscopy
Cell viability	Cell Counting Kit-8 assay (CCK-8)
Cellular iron levels	Iron assay kit; Phen Green SK (PGSK) probe
Cellular ROS content	ROS kit; DHE kit
Lipid oxidation	C11-BODIPY; Liperfluo probe
The by-products of lipid peroxidation	MDA kit; 4-HNE kit
Ferroptosis-related RNA	Single cell RNA-sequencing

increased infiltration of inflammatory cells and inflammatory mediators; on the other hand, the activation of inflammation can trigger ferroptosis. It has been shown that pathogenic bacteria or viruses in inflamed tissues promote ferroptosis in cells while activating immune cells and triggering inflammatory responses through the action of lipopolysaccharides, peptidoglycans, bacterial or viral DNA, and host cell toll-like receptors carried or released by themselves (60). The increase in AA-induced by various factors and the activation of inflammatory cells promote ferroptosis by impairing mitochondrial function and generating large amounts of free radicals. In macrophages, the upregulation of 15-LOX expression catalyzes AA dioxide, leading to lipid peroxidation, which triggers ferroptosis in macrophages (51). A significant increase in serum levels of the inflammatory factors IL-1B, TNF-a, and IL-6 was found in hemoglobin-injected mice, whereas the use of inflammatory vesicle inhibitors significantly reduced the levels of hemoglobin-induced inflammatory factors. They also reduced ferroptosis, suggesting that hemoglobin induces inflammatory vesicle formation, which in turn leads to ferroptosis (61). The inflammation-related damage factors released by cells after ferroptosis activate immune cells that further promote the progression of inflammation by impairing AA-mediated lipid metabolism, decreasing mitochondrial function, increasing oxidative stress, and reducing GPX4.

3.2 Ferroptosis and IBD in the intestinal epithelium

The intestinal epithelium is the first line of defense for the intestinal mucosa. It consists of a single layer of tightly connected columnar epithelial cells that keep out foreign antigens, microorganisms, and their toxins (62). The maintenance and continuous renewal of IEC function is maintained by cell death of IECs, including apoptosis, necrosis, necrotizing, pyrotizing, and ferrotizing. However, the imbalance in cell death of IECs leads to increased intestinal permeability and disruption of barrier function, disrupting the balance of IECs, which in turn leads to IBD. There is increasing evidence that increased intestinal permeability may be associated with ferroptosis of IECs in IBD (Figure 3). In both patients with clinical UC and experimental colitis triggered by DSS in miceferroptosis-related genes were significantly up-and down-regulated, and cellular MDA content FTH and FTL protein levels were increased in IECs, and mitochondrial atrophy and mitochondrial cristae were reduced in colonic IECs of DSS-treated mice (11). The same was observed in clinical CD patients and in trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis: increased expression of ACSL4/MDA/PTGS2/iron and inhibition of GPX4, which was ameliorated by treatment with Ferr-1 (63). Overall, these findings suggest that ferroptosis occurs in IECs with IBD and leads to increased inflammation.

3.3 Intestinal fat ferroptosis and IBD

Studies have shown that high-fat diets (HFD) are associated with an increased risk of IBD, especially diets rich in cholesterol and animal fats, such as polyunsaturated omega-6 fatty acids (PUFA) and arachidonic acid (AA) (64). The PUFAs have been observed to hinder the normal functioning of cells by creating cytotoxic protein adducts or by disrupting cell membranes through lipid peroxidation, leading to ferroptosis in IECs. GPX4 not only protects against lipid peroxidation (LPO) but also induces ferroptosis in cells either by the deletion of GPX4 allele in mice or its pharmacological inhibition. Mayr et al. found that GPX4 activity in CD epithelial cells was reduced, characteristic of LPO, and that PUFAs (especially AA) induced the release of interleukin 6 (IL-6) and chemokine (C-X-C motif) ligand 1 (CXCL1) from these lECs. Mice lacking the Gpx4 allele in IECs show signs of epithelial LPO and focal neutrophilic enteritis with a granuloma-like accumulation of inflammatory cells when fed a PUFA-rich diet (65).

Thus, a low-fat, high-fiber diet may reduce inflammatory factors and dysbiosis of intestinal flora in fetal samples and improve the life quality of patients with UC (66). Meanwhile, Zhang et al. reported that an HFD exacerbated colitis-associated carcinogenesis (CAC) by bypassing the ER stress-mediated ferroptosis pathway (67).

3.4 Dietary iron intake and IBD

Iron deficiency anemia is a prevalent complication in patients suffering from IBD, and iron replacement therapy (IRT) can improve anemia, including oral and intravenous iron supplementation, but iron overload can lead to ferroptosis (68). Iron is an indispensable element for the development of ferroptosis. Iron supplementation has been reported to alter the balance of intestinal microbiome and exacerbate CD-like intestinal inflammation in mouse models (69). Iron supplementation was found to exacerbate disease activity and oxidative stress in a rat model of colitis induced by DSS (10). In Japan, the incidence of UC has increased, and high iron intake has an effect on the development of UC compared to high zinc intake (9). Additionally, it is recognized that iron chelators have the capacity to decrease iron synthesis and ameliorate the colonic symptoms of IBD. Collectively, these results propose a potential association between IBD and the consumption of dietary iron.

3.5 Regulatory mechanisms of ferroptosis in IBD

Indeed, the molecular mechanisms of ferroptosis are diverse, and several transcription factors (e.g., TP53, NFE2L2/NRF2, ATF3, ATF4, YAPTAZTFAP2CSPHIFAEPAS/HIFA BACH TFEBJUNHIC1, and HNF4A) play multiple roles through transcription-dependent or transcription-independent mechanisms play multiple roles in shaping ferroptosis susceptibility (28). NRF2 has been identified as a potentially valuable therapeutic target for the management of IBD. The transcription factor, nuclear factor erythroid 2-like 2 (NRF2), governs the expression of antioxidant response elements, including GPX4 (70). Recently, Nrf2-Gpx4 pathway activation was found to have the ability to significantly suppress ferritin formation (71). Fer-1 has also been shown to alleviate DSS-induced colitis through the NRF2/HO-1 pathway (72). The small molecule inhibitor of Keap-Nrf2 protein–protein interaction, CPUY192018, has been found to exhibit cytoprotective properties against DSS in both NCM460 cells and mouse colon. This effect is achieved through the activation of Nrf2 signaling (73). Recent findings suggest that endoplasmic reticulum (ER) stress signaling is closely correlated to ferritin formation, which is thought to be a promoter of UC. Furthermore, phosphorylation of NF-κBp65 acts as an upstream regulator to inhibit ER stress-mediated IEC ferroptosis, thereby alleviating the disease (11).

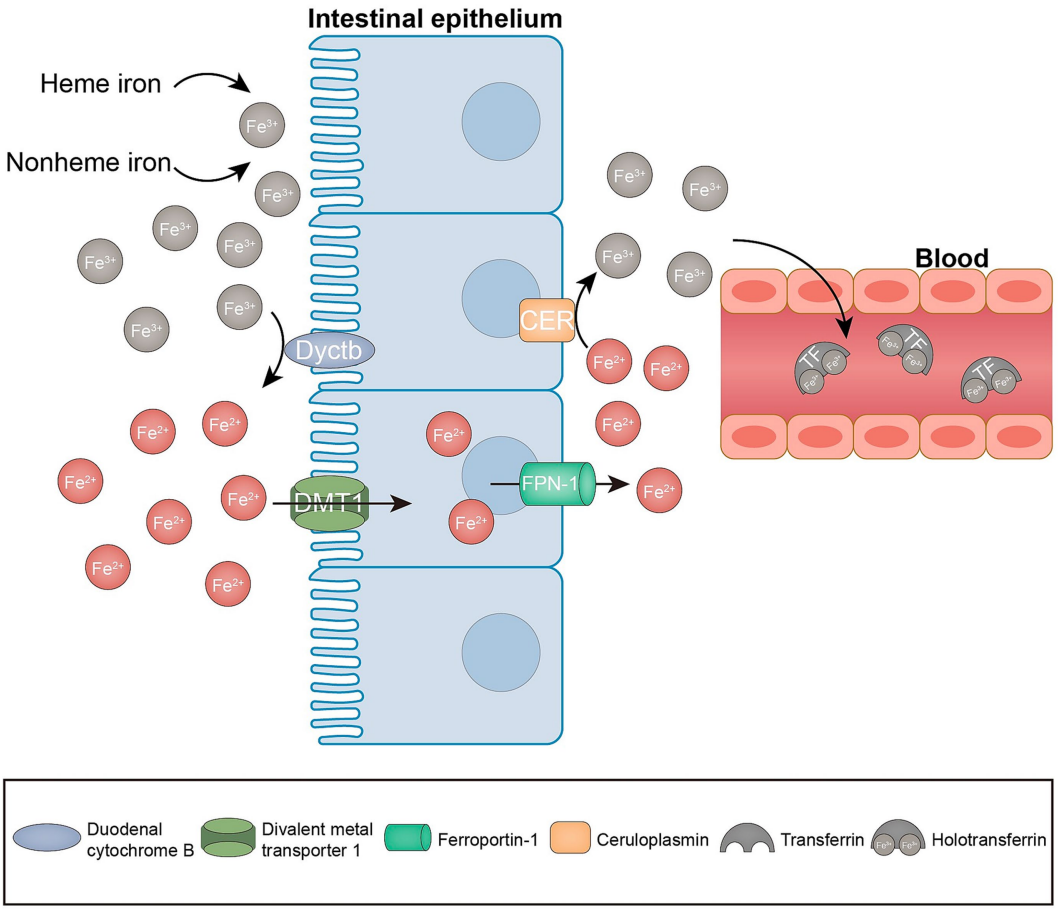

FIGURE 3
Ferritinosis in IBD. In recent studies, iron uptake disorders have been directly linked to the pathogenesis of IBD. IEC ferroptosis promotes the disruption of epithelial barrier function. As a result, immune cell and cytokine production is hyperactivated, ultimately leading to intestinal inflammation and epithelial damage.

4 Therapeutic strategies for ferroptosis

IBD has emerged as a pressing global health concern, which is anticipated to result in a substantial upsurge in healthcare expenditures due to its rapid increase in incidence and prevalence. Ferroptosis has recently been identified as a different type of RCD. As previously described, the fundamental characteristics of ferroptosis include iron overload, GSH depletion, GPX4 inactivation, and lipid peroxidation. The results of direct and indirect studies revealed a strong correlation between iron dystrophy and intestinal disorders. The clinical symptoms of IBD were revealed to include iron deficiency and anemia resulting from bleeding and malabsorption, which can significantly harm the well-being of affected individuals (74). Oral iron has been used in the clinical management of iron deficiency anemia in patients suffering from IBD (75). Nevertheless, an excessive amount of iron may cause iron overload in the gut, leading to the dysregulation of ROS production and disturbance of the gut microbiota. These effects could potentially worsen the condition of IBD (69). According to a study conducted on rats, the prevention of experimental colitis and gastric ulcers was observed through the oral administration of iron chelators deferiprone (DFP) and DFO (76). The enzymatic activity of GPX4 involves the elimination of lipid hydroperoxides and the prevention of ferroptosis. This function is attributed to its antioxidant properties. A decrease in GPX4 activity was observed in the IECs of CD patients as lipid peroxidation increased. - The consumption of diets that are high in PUFAs but low in saturated fatty acids has been found to induce focal enteritis in IEC-specific Gpx4 mice. +-More strikingly, the findings suggest that GPX4 plays a crucial role in preserving intestinal homeostasis by protecting against lipid peroxidation, as evidenced by the increased vulnerability of IEC Gpx4 mice to DSS-induced colonic inflammation relative to their wild-type counterparts (65). Curculigoside, a naturally occurring compound present in *Curculigo orchioides* Gaertn, has been discovered to possess diverse biological properties and has been shown to mitigate DSS-induced UC in mice. Curculigoside has been found to provide support for GPX4 expression, thus protecting against ferroptosis in a manner that depends on selenium (22). The efficacy of Astragalus polysaccharide (APS), a bioactive constituent of Astragalus membranaceus, in ameliorating experimental colitis has been demonstrated.

Together, these studies form the pathological concept of the involvement of ferric IECs in colitis. The presence of ferric IECs has been observed to cause a disturbance in the intestinal barrier, leading to the release of intestinal microorganisms and an overactive immune

TABLE 2 Molecules targeting ferroptosis in IBD.

Effect	Drugs	Target	Mechanisms	Model	References
Inducers	Oral iron	Iron	Exacerbates oxidative stress through the Fenton reaction	UC rats	(78)
	AA	GPX4	Trigger epithelial LPO and an inflammatory response restricted by GPX4	CD patients, $Gpx4^{+/-}$ ^{IEC} mice	(65)
Inhibitors	DDO7232	Nrf2	Reduces oxidative stress and decreases the inflammatory factors level	NCM460 cells, UC mice	(79)
	CsA, 6-TG	pro-inflammatory cytokines	Reduced mRNA expression of several pro-inflammatory cytokines in spleen and colon	UC mice	(80)
	Curculigoside	GPX4	Increases selenium sensitivity and promotes GPX4 expression	IEC-6 cells, UC mice	(22)
	DFO, DFP	Iron	Chelates excessive free iron and suppresses iron-dependent lipid peroxidation	UC mice	(21, 81)
	Lip-1	ROS	Lipophilic antioxidants	UC mice	(21)
	Fer-1	ROS	Blocks lipid peroxidation and restrains ROS overgeneration	UC mice	(21)
	Astragalus polysaccharide (APS)	Nrf2/HO-1	Antioxidant and anti-inflammatory properties	Caco-2 cells, UC mice	(82)

response within the intestines. This, in turn, has been linked to the worsening of mucosal lesions related to colitis. In addition, ferric IECs release a number of immunogenic molecules that potentially enhance the occurrence of local inflammation (77). However, it is unclear whether other intestinal immune cells besides IECs are ferroptosis in the pathogenesis of intestinal injury. Further investigation is necessary to determine whether the ferroptosis of specific types of immune cells in the intestine plays a role in the development or advancement of intestinal diseases. Given the beneficial effects of various ferroptosis inhibitors on ameliorating tissue damage associated with colitis (Table 2), there is significant therapeutic potential for targeting ferroptosis in colitis prevention and intervention. Hence, further comprehensive investigations are required to elucidate the precise function of ferroptosis in the pathogenesis of IBD and other associated intestinal disorders.

5 Conclusion and outlook

Despite significant advances, the examination of intestinal ferroptosis is still in its nascent phase, and its precise function in the context of IBD requires further exploration. Although we have summarized several approaches to studying ferroptosis from different aspects, there are no agreed criteria to directly define its occurrence. The identification of markers and other techniques for the assessment of ferroptosis *in vivo* is of the highest priority. Thus, biomarkers of ferroptosis may offer hope for indicating the severity of IBD. We found that the link between ferroptosis and iron/lipid peroxidation is a topic of debate. More evidence is required to establish a conclusive relationship between iron-induced atrophy, oxidative stress, and lipid peroxidation in the development and progression of the disease. Furthermore, it is imperative to investigate the signaling pathways and primary transcriptional regulators associated with iron uptake disorders in order to optimize their regulation and protect the gut against harm and carcinogenesis. Therefore, it is imperative to conduct further research on ferroptosis as a novel therapeutic target in the field of intestinal disorders.

Author contributions

HX: Writing – original draft, Software, Investigation, Formal analysis, Writing – review & editing, Supervision, Conceptualization. CC: Data curation, Writing – review & editing, Writing – original draft, Investigation, Conceptualization. DS: Project administration, Formal analysis, Writing – review & editing, Writing – original draft, Investigation, Conceptualization. TL: Visualization, Validation, Writing – review & editing, Conceptualization. TZ: Supervision, Resources, Writing – review & editing, Visualization, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The interplay between ferroptosis and inflammation: therapeutic implications for cerebral ischemia-reperfusion

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Stroke ranks as the second most significant contributor to mortality worldwide and is a major factor in disability. Ischemic strokes account for 71% of all stroke incidences globally. The foremost approach to treating ischemic stroke prioritizes quick reperfusion, involving methods such as intravenous thrombolysis and endovascular thrombectomy. These techniques can reduce disability but necessitate immediate intervention. After cerebral ischemia, inflammation rapidly arises in the vascular system, producing pro-inflammatory signals that activate immune cells, which in turn worsen neuronal injury. Following reperfusion, an overload of intracellular iron triggers the Fenton reaction, resulting in an excess of free radicals that cause lipid peroxidation and damage to cellular membranes, ultimately leading to ferroptosis. The relationship between inflammation and ferroptosis is increasingly recognized as vital in the process of cerebral ischemia-reperfusion (I/R). Inflammatory processes disturb iron balance and encourage lipid peroxidation (LPO) through neuroglial cells, while also reducing the activity of antioxidant systems, contributing to ferroptosis. Furthermore, the lipid peroxidation products generated during ferroptosis, along with damage-associated molecular patterns (DAMPs) released from ruptured cell membranes, can incite inflammation. Given the complex relationship between ferroptosis and inflammation, investigating their interaction in brain I/R is crucial for understanding disease development and creating innovative therapeutic options. Consequently, this article will provide a comprehensive introduction of the mechanisms linking ferroptosis and neuroinflammation, as well as evaluate potential treatment modalities, with the goal of presenting various insights for alleviating brain I/R injury and exploring new therapeutic avenues.

KEYWORDS

ferroptosis, inflammation, cerebral ischemia-reperfusion, therapeutic strategy, pharmacology

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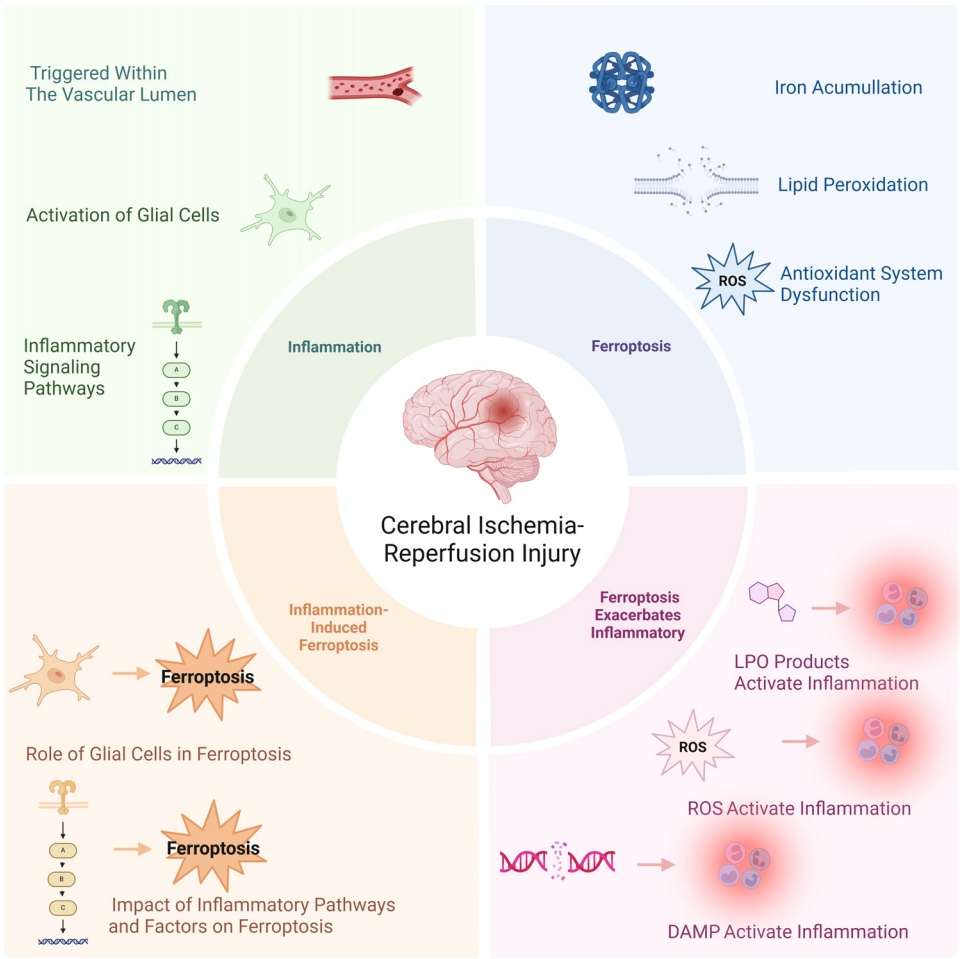

GRAPHICAL ABSTRACT

1 Introduction

Stroke remains a significant global health issue, marked by alarmingly high rates of both incidence and mortality. In the year 2019, it was responsible for approximately 6.55 million fatalities and impacted 143 million individuals, imposing a heavy burden on both society and those affected (1). Ischemic strokes comprise 71% of all stroke occurrences, typically resulting from the narrowing or blockage of cerebral arteries (2, 3). This situation leads to insufficient local blood supply, pronounced hypoxia, and disturbances in energy metabolism. As a result, these factors initiate oxidative stress and cell death, contributing to the injury of brain tissue (4). Despite the brain representing just about 2% of the total body weight, it utilizes roughly 20% of the oxygen and glucose in the body, underscoring the vital role of cerebral blood circulation in providing necessary nutrients to the brain (5). Inadequate blood flow to the brain can result in neuron death within five minutes after circulation stops. Prompt reperfusion can alleviate the harmful effects associated with cerebral ischemia, with thrombolytic therapies and mechanical thrombectomy being the most commonly utilized methods (6). Alteplase, a tissue plasminogen activator (tPA), is recognized globally as the primary thrombolytic agent (7, 8). Nevertheless, the process of reperfusion itself may inflict additional damage on ischemic brain tissues, a situation referred to as cerebral I/R injury (9).

Although our grasp of the exact mechanisms behind cerebral I/R remains incomplete, increasing evidence indicates that ferroptosis significantly contributes to this phenomenon (10). Ferroptosis represents a unique type of programmed cell death, distinguishing itself from apoptosis and necrosis. It is marked by an abnormal buildup of iron, which leads to an excessive generation of free radicals. This excessive radical production ultimately causes LPO of the cell membrane, resulting in cell death (11). Recent research has shed light on the intricate relationships between ferroptosis and cerebral I/R, encompassing LPO, damage to cell membranes, disruption of the antioxidant defense system, and inflammation—all of which are factors that contribute to cell death and worsen damage to brain tissue (12). Current strategies designed to alleviate ferroptosis induced by cerebral I/R, including the use of antioxidants and iron chelators (13, 14), show limited success.

This limitation highlights the critical need for new therapeutic approaches. Inflammation also acts as a driving force in cerebral I/R, usually appearing as a reaction to tissue damage. Cellular injury from ischemic hypoxia instigates an inflammatory response; injured neurons release pro-inflammatory cytokines and chemokines that draw immune cells, such as microglia and neutrophils, to the injury site (15, 16). These immune cells generate further inflammatory mediators, intensifying the local inflammatory response and potentially causing additional neuronal damage (17).

Although ferroptosis and inflammation are recognized as separate injury pathways, recent studies are increasingly highlighting the significant interactions that exist between them (18, 19). Exploring these mechanisms not only deepens our comprehension of cerebral I/R but also lays the groundwork for novel approaches to disease modification and the development of treatment strategies. This article succinctly outlines the crosstalk mechanisms between inflammation and ferroptosis and suggests potential therapeutic strategies, including pharmaceutical interventions, enriched environments, and the use of exosomes. On this basis, we delve deeper into potential therapeutic mechanisms for managing both inflammation and ferroptosis. Grounded in established theoretical frameworks and supported by the literature, these strategies showcase promising applications. They aim to offer insights for the treatment of cerebral I/R-related ferroptosis and inflammation.

2 Ferroptosis and cerebral I/R

In the year 2012, Stockwell and his research team made a significant breakthrough in the field of cell biology by identifying a novel form of cell death, termed ferroptosis, which is induced by erastin and is not dependent on apoptosis (20). The uncovering of ferroptosis has spurred a considerable amount of research aimed at unraveling its underlying mechanisms and exploring the broader implications of this unique cell death pathway. Ferroptosis stands apart from the more commonly understood mechanisms of cell death, such as apoptosis and necrosis, due to its distinct characteristics. The defining feature of ferroptosis is the presence of iron overload within the cellular environment, which consequently leads to the peroxidation of lipids. This process is intricately regulated by various factors that include iron metabolism, lipid metabolism, and the maintenance of redox homeostasis (21). This multifaceted interaction highlights the complexity of ferroptosis and showcases its pivotal role in cellular physiology, as illustrated in Figure 1.

2.1 Abnormal iron metabolism

Iron is crucial for numerous biological functions in the brain, playing key roles in processes like mitochondrial respiration, myelin

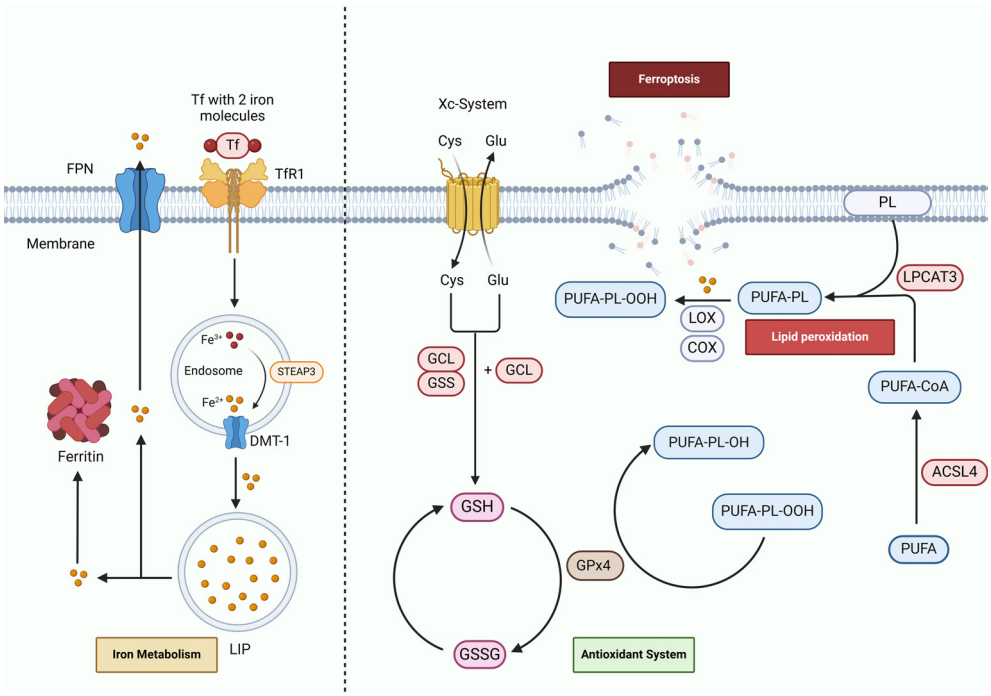

FIGURE 1
Mechanisms of ferroptosis in cerebral I/R. Tf binds to $Fe3^{+}$ and is internalized into the cell via TfR1 through endocytosis. Within the cell, $Fe3^{+}$ is reduced to Fe2+ by STEAP3 and subsequently stored in the labile iron pool (LIP) and ferritin via DMT-1. Dysregulation of iron metabolism can result in iron accumulation and ferroptosis. Iron within cells is exported extracellularly through FPN. ACSL4 and LPCAT3 incorporate free polyunsaturated fatty acids (PUFAs) into phospholipids, forming PUFA-PL. Lipoxygenases (LOX), cyclooxygenases (COX), and Fe2+ catalyze the oxidation of PUFA-PL into PUFA-PL hydroperoxides (PUFA-PL-OOH), which triggers lipid peroxidation (LPO). The antioxidant system, via the Xc-System, synthesizes GSH from amino acids. GPx4 subsequently reduces PUFA-PL-OOH to PL-OH, thereby mitigating LPO.

formation, neurotransmitter production, and other cellular functions (22). Disruptions in iron homeostasis, leading to iron accumulation, can compromise these functions. In the context of cerebral I/R, ischemia causes the release and accumulation of iron in neurons and glial cells (23). Subsequent reperfusion, which reestablishes blood flow, induces oxidative stress and inflammatory responses, thereby exacerbating iron accumulation. Excess iron can generate highly reactive hydroxyl radicals via the Fenton reaction. These radicals attack the lipid components of cell membranes, intensifying LPO and ultimately leading to ferroptosis (24, 25). In response to hypoxic injury resulting from cerebral ischemia, levels of hypoxia-inducible factor 1-alpha (HIF-1α) significantly rise, which increases iron uptake through TfR1 (26). Research has shown that patients who have suffered a stroke demonstrate markedly higher serum levels of iron and hepcidin, leading to decreased expression of FPN, reduced iron export (27), and subsequent build-up of iron, thereby creating conditions conducive to ferroptosis (28). Additionally, the compromised integrity of the blood-brain barrier (BBB) allows a greater influx of circulating iron into the brain tissue (29). In the transient middle cerebral artery occlusion (tMCAO) model, following oxygen-glucose deprivation (OGD), transferrin leaks across the BBB, and after ischemic events, DMT-1 levels in the affected brain region double, causing an accumulation in neighboring neurons (30).

2.2 LPO

Uncontrolled LPO is a significant factor leading to the destruction of cellular membrane structures and is a primary contributor to the process known as ferroptosis (31). LPO is an oxidative process targeting polyunsaturated fatty acids (PUFAs), triggered by free radicals. PUFAs are essential for maintaining the fluidity and flexibility of cell membranes, which is vital for effective signal transmission within neural pathways (32). However, due to the presence of unstable double bonds, PUFAs are highly prone to oxidation, subsequently making them major contributors to LPO (33). During this process, lipid peroxides form through a chemical interaction among lipids, oxygen, and iron. Once these lipid peroxides are generated, they interact with $Fe2^{+}$, resulting in the formation of peroxyl radicals. These radicals further extract hydrogen atoms from adjacent acyl chains in the membrane, thereby propagating the LPO cycle. This reaction not only undermines the structural integrity of cell membranes but also diminishes their functionality (31).

In the particular case of cerebral I/R, the overexpression of ACSL4 promotes the acylation of PUFAs such as arachidonic acid (AA) and adrenic acid (AdA), increasing their abundance in the cell membrane. This heightened presence makes the membrane more sensitive to oxidative stress, leading to LPO and exacerbating ferroptosis in ischemic brain injury. Conversely, knockout of ACSL4 provides protective effects for mice, significantly mitigating the associated brain damage caused by ischemic events (34). Enzymatic reactions also play a vital role in promoting LPO; for instance, the enzyme 12/15- LOX is found to be highly expressed and converts AA into 12/15-HETE, thus intensifying LPO in the pMCAO mouse model of cerebral I/R. Inhibiting this enzyme with a specific inhibitor, known as LOX Block-1 (LB-1), has proven effective in reducing LPO and significantly decreasing the infarct volume in these models (25). Moreover, studies have indicated that COX-2 and its product, prostaglandin E2 (PGE2), have the potential to exacerbate LPO in cerebral tissues, and that the inhibition of COX-2/PGE2 expression can significantly reduce the impacts of ferroptosis within the brain (35).

The antioxidant system markedly lowers levels of LPO by effectively neutralizing ROS and free radicals, thereby shielding cells from oxidative damage (36). The XC-System/GSH/GPx4 pathway is a critical component in the cellular defense against ferroptosis, a form of regulated cell death characterized by the accumulation of lipid peroxides (37). This protective mechanism centers around the XC-System, which consists of the cystine/glutamate antiporter formed by the proteins SLC7A11 and SLC3A2. These proteins regulate the balance of amino acids inside and outside the cell to synthesize GSH, thereby maintaining antioxidant status (38). In cases of cerebral I/R injury, there is a notable increase in the production of ROS, which can overwhelm the cellular defenses and hinder the functionality of the XC-System/GSH/GPx4 pathway (39). When the components of this pathway are compromised, the body's overall antioxidant capacity is notably diminished. This decline contributes to an accelerated progression of LPO, which is detrimental to cell viability (40). However, the transcription factor Nrf2 has been identified as a key regulator in this context, as it can stimulate the expression of SLC7A11. When Nrf2 activates SLC7A11, it enhances the activity of the XC-System, leading to an increase in the expression levels of both GSH and GPx4. The subsequent upregulation of these components significantly fortifies the antioxidant defense system, thereby effectively countering lipid peroxidation and inhibiting ferroptosis, illustrating the pathway's vital role in cellular resilience against oxidative stress (41, 42).

3 Inflammation and cerebral I/R

Inflammation begins rapidly during the acute phase of cerebral ischemia, occurring within minutes of the initial event. This inflammatory response can evolve into a significant mechanism of injury within just a few hours and may continue to persist for several days, affecting both intravascular and perivascular territories (43). Shortly after the occlusion of an artery, the inflammatory response is swiftly activated within the vascular lumen. Various factors contribute to this response, including hypoxia, alterations in shear stress, and the generation of ROS. These elements collectively activate the coagulation cascade and stimulate the activation of various cell types, including complement cells, platelets, and endothelial cells. As a result of this cascade, neuronal death occurs, leading to the development of an ischemic core surrounded by a region of reduced blood flow known as the penumbra (44). In the immediate aftermath of ischemia, endothelial cells begin to upregulate the expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1),

vascular cell adhesion molecule 1 (VCAM-1), P-selectin, and E-selectin. The presence of these molecules facilitates the adhesion of leukocytes circulating in the bloodstream to the endothelium. This interaction initiates a cascade of pro-inflammatory signaling that activates immune cells, thereby intensifying neuronal damage (45). The complex interplay of these processes highlights the detrimental impact of inflammation on neuronal survival during the early stages of cerebral ischemia, as depicted in Figure 2.

3.1 Activation and infiltration of glial cells

Neurons possess a remarkable susceptibility to ischemic conditions, which leads to a swift process of neuronal death. This neuronal demise prompts the release of various endogenous molecules, including ATP, UTP, HMGB1, HSPs, and others that act as DAMPs. These DAMPs are crucial because they are detected by immune cells utilizing pattern recognition receptors such as Toll-like receptors and NOD-like receptors. When these receptors recognize the DAMPs, they trigger the activation of inflammatory signaling pathways. This cascade results in the secretion of additional factors that intensify both localized and systemic inflammatory responses, thereby exacerbating the overall inflammatory condition in the affected tissues (46, 47). Microglia, which serve as the brain's intrinsic immune defenders, play a critical role in surveilling and sustaining internal equilibrium under normal resting conditions (48). Upon the onset of cerebral ischemia, these microglial cells respond swiftly, transforming their morphology to an amoeboid shape within minutes. They possess the ability to polarize into two distinct phenotypes: the pro-inflammatory M1 and the anti-inflammatory M2 (49, 50). This polarization allows microglia to effectively regulate the inflammatory response, tailoring their actions to the specific needs of the surrounding environment. Following the initial injury to neurons, the damaged cells release a chemokine known as CX3CL1, also referred to as fractalkine. This signaling molecule is recognized by the CX3CR1 receptor expressed on microglial cells, facilitating communication between the damaged neurons and the immune response (51). As a result, the activated microglia migrate to the site of injury, where they actively engulf and eliminate dead cells and cellular debris. This phagocytic activity is vital as it not only helps clear away the remnants of damage but also plays a crucial role in preventing additional cell injury and the further propagation of inflammation in the affected area (52). Astrocytes serve a crucial and multifaceted regulatory function in the context of cerebral I/R. Their roles encompass the modulation of inflammatory responses, the maintenance of the BBB, and the regulation of iron homeostasis (53). Following an episode of cerebral ischemia, the release of cytokines by both neurons and microglia occurs within hours, which triggers the activation and reactive proliferation of astrocytes (54). This rapid response is critical for mitigating brain damage. Activated astrocytes exhibit the ability to polarize into two distinct phenotypes: the pro-inflammatory "A1" and the anti-inflammatory "A2" (55). At the onset of astrocytic activation, the predominant focus is on facilitating a pro-inflammatory response. This initial response aims to swiftly clear areas of brain damage.

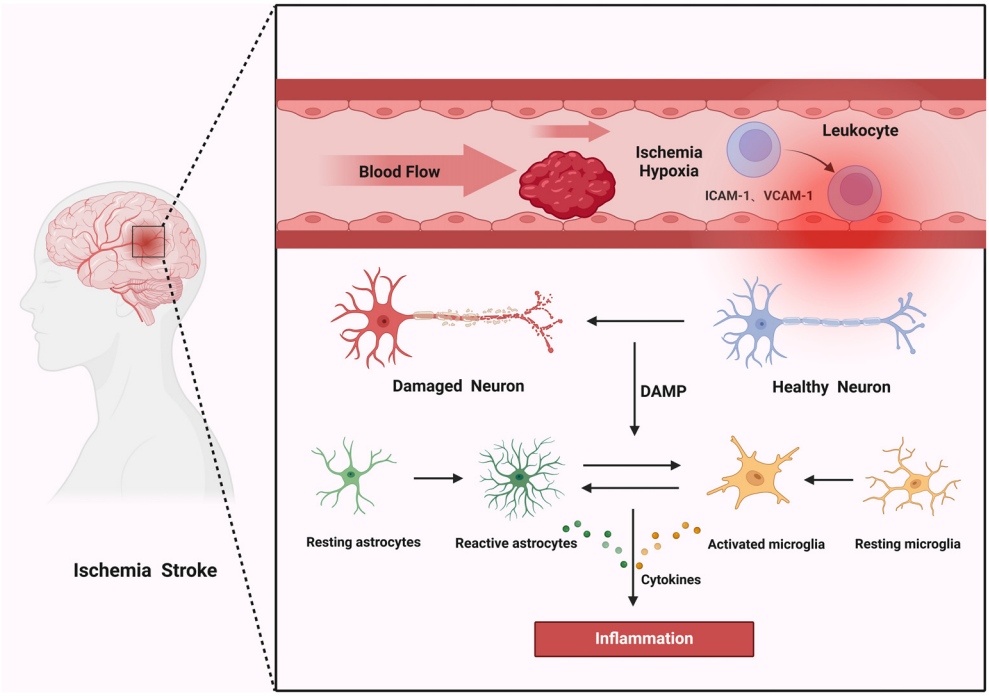

FIGURE 2
Mechanisms of inflammation in cerebral I/R. Thrombosis induces ischemic hypoxia within blood vessels, activating endothelial cells to express adhesion molecules ICAM-1 and VCAM-1. These molecules attract leukocytes, which rapidly produce pro-inflammatory signals that activate neuroglial cells. Concurrently, ischemic neurons undergo rapid cell death and release damage-associated molecular patterns (DAMPs) that further stimulate neuroglial cells to release inflammatory mediators, resulting in inflammation.

Simultaneously, it seeks to control excessive inflammation, thereby protecting neurons from sustaining further injury (47).

3.2 Inflammatory signaling pathways and factors

NF-κB functions as a crucial hub in the process of inflammation, playing a significant role in inflammatory responses, as depicted in Figure 3. In the context of cerebral I/R, NF-κB engages with pathways including Nrf2 and NLRP3 to modulate inflammation (56, 57). TLR4, which is mainly found in microglia, triggers the activation of NF-κB via signaling pathways, thereby promoting microglial activation and subsequent inflammatory responses (58, 59). Additionally, the TLR4/NF-κB signaling axis is implicated in mediating inflammatory responses within type A1 astrocytes (60). The anti-inflammatory mechanism involving Nrf2 elevates cellular HO-1 levels through gene transcription driven by antioxidant response elements (ARE) while simultaneously promoting the degradation of IκB-α by the proteasome. This action inhibits the translocation of NF-κB into the nucleus, thereby contributing to the regulation of inflammation (61).

As a key downstream target of NF-κB, the NLRP3 inflammasome functions as an essential mediator in post-ischemic inflammation, leading to a series of inflammatory responses and consequent cell death (62). Gong et al. showed that (63) the activation of the NLRP3 inflammasome occurs initially in microglia following brain I/R injury, with further expression identified in microvascular endothelial cells and, notably, in neurons. Although the primary expression of NLRP3 is found in microglia, there is growing evidence suggesting its activation in astrocytes following I/R injury, where NLRP3 activation might lead to the neurotoxic reprogramming of astrocytes (64). Hence, the modulation of NLRP3 presents considerable therapeutic promise for managing inflammation linked to cerebral I/R.

The JAK2/STAT3 signaling pathway is critical in mediating inflammatory responses, with TNF-α and IL-6 serving as major activators. These cytokines activate microglia and astrocytes in ischemic areas, affecting the expression of various inflammatory factors that contribute to brain damage caused by I/R injury (65, 66). In the setting of cerebral I/R, IL-6 functions as an initiator that fosters STAT3 phosphorylation, setting off a signaling cascade that triggers metabolic reprogramming in reactive astrocytes (67). Additionally, the interplay between the STAT3 and NF-κB pathways can amplify the activation of M1 microglia and reactive astrocytes, thus worsening neuroinflammation (68). — This revision maintains the original ideas while ensuring that phrases are restructured and synonyms are used where appropriate to avoid duplication concerns.

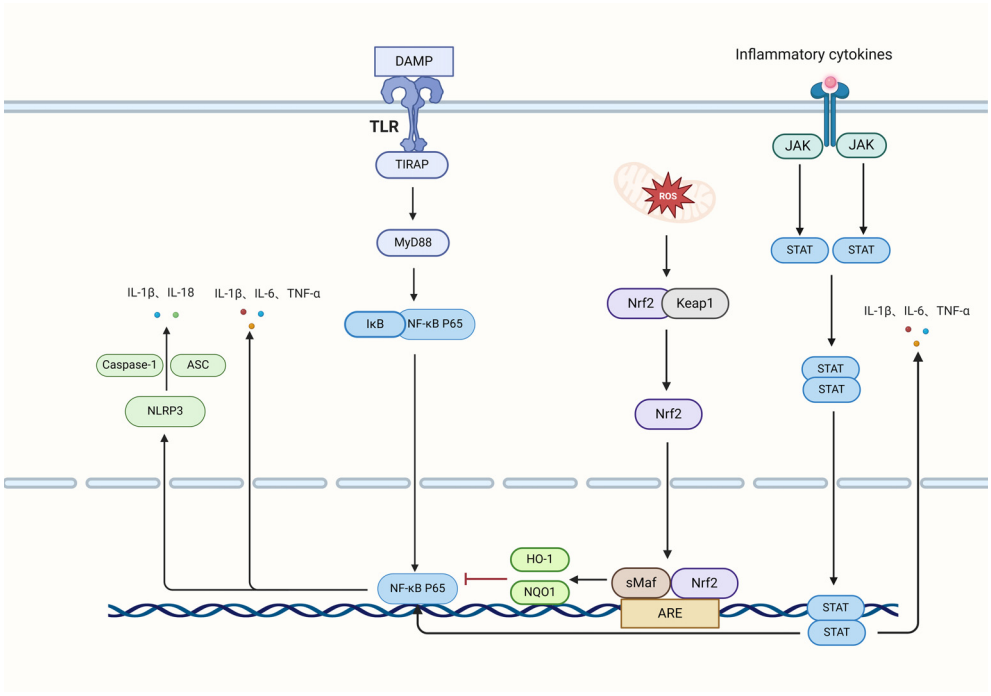

FIGURE 3
Inflammatory signaling pathways and factors. Neurons release damage-associated molecular patterns (DAMPs) that engage TLRs to transmit signals to TIRAP, subsequently activating MyD88. This activation results in the phosphorylation of IκB, which triggers the translocation of P65 into the nucleus, initiating the transcription and release of the inflammatory cytokines IL-1β, IL-6, and TNF-α. Intracellular oxidative stress activates Nrf2, which dissociates from Keap1, translocates to the nucleus, and induces the transcription of HO-1 and NQO1 via the ARE, thereby inhibiting NF-κB transcription and reducing inflammation. Additionally, NF-κB and oxidative stress activate the NLRP3 inflammasome, which, in conjunction with Caspase-1 and ASC, releases IL-1β and IL-18. The JAK pathway phosphorylates STAT, leading to its dimerization and subsequent entry into the nucleus, where it enhances the transcription of IL-1β, IL-6, and TNF-α, further activating NF-κB transcription.

4 Crosstalk between ferroptosis and inflammation in cerebral I/R

The relationship between ferroptosis and inflammation in the context of cerebral I/R is intricate and multifaceted. Although these two biological processes can operate independently, they closely interact and exert significant influences on one another. Our focus is on exploring the overlapping mechanisms between ferroptosis and inflammation to develop innovative treatment approaches for brain I/R.

(19). The connection between ferroptosis and neuroinflammation is characterized by its bidirectional nature. Neuroinflammation typically begins shortly after the onset of cerebral ischemia, setting off a cascade of biological responses. In contrast, ferroptosis is primarily observed during the reperfusion phase following ischemic injury. This chronological relationship indicates that neuroinflammation serves as a precursor to ferroptosis in the course of cerebral I/R events (25, 69). As inflammation unfolds, it engenders the activation of glial cells and various related pathways which contribute to the accumulation of iron as well as LPO. These processes compromise the antioxidant defense system's effectiveness, ultimately precipitating the occurrence of ferroptosis (70). Furthermore, products resulting from LPO that arise during ferroptosis, along with DAMPs released from disrupted cell membranes, play a significant role in activating immune cells. This activation further intensifies the inflammatory response, demonstrating how ferroptosis can exacerbate neuroinflammation (71, 72). The interplay between these two processes establishes a vicious cycle that perpetuates ongoing damage to brain tissue. Within this framework, we delve into the interactions between ferroptosis and inflammation, highlighting emerging mechanisms and potential targets for drug development. Through in-depth research into these mechanisms, we aim to offer novel insights and viable therapeutic strategies for clinical practice, thus enhancing the prognosis for patients suffering from brain I/R.

4.1 Inflammation-induced ferroptosis

In cerebral I/R, the activation of glial cells and associated signaling pathways leads to iron accumulation, LPO, and decreased activity of the antioxidant system, forming critical pathways for inflammation-induced ferroptosis (73), as depicted in Figure 4.

4.1.1 Role of glial cells in ferroptosis

Glial cells serve as facilitators for the accumulation of iron triggered by inflammation in cerebral I/R (74). They are vital for iron metabolism in the brain; while microglia store considerable quantities of iron, astrocytes have the ability to transfer this iron to adjacent neurons, effectively intertwining iron metabolism with inflammatory processes (75, 76). The presence of lipopolysaccharide (LPS) prompts microglia to generate inflammatory cytokines, including IL-6 and TNF-α, which not only activate these cells but also enhance the expression of DMT1 and downregulate FPN, thereby increasing iron uptake and retention within microglia (77). Thus, inhibiting IL-6 and TNF-α induced upregulation of DMT1 and

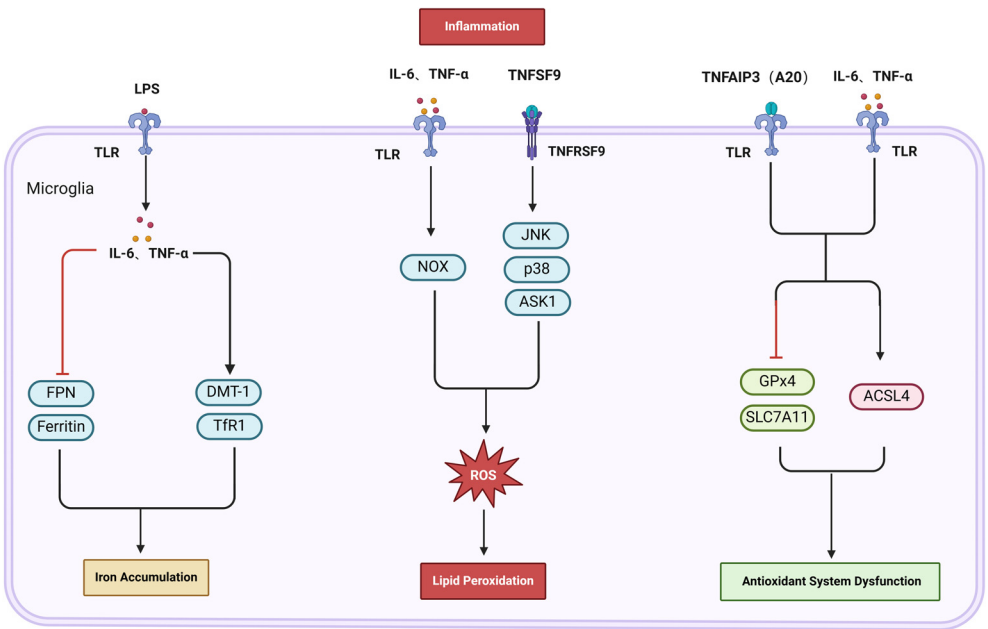

FIGURE 4
The activation of glial cells and associated signaling pathways leads to iron accumulation, LPO, and decreased activity of the antioxidant system, forming critical pathways for inflammation-induced ferroptosis. Lipopolysaccharide (LPS) activates microglia, which secrete IL-6 and TNF-α, leading to the upregulation of DMT-1 and TfR1, while downregulating ferritin and FPN. This cascade results in increased iron levels. Moreover, Tumor necrosis factor superfamily member 9 (TNFSF9) and NADPH oxidase (NOX) can induce lipid peroxidation (LPO) by generating reactive oxygen species (ROS). Furthermore, TNF-induced protein 3 (TNFAIP3, or A20) in conjunction with IL-1β and IL-6, influences the antioxidant system's activity. This is achieved by upregulating ACSL4 and downregulating the expression of GPX4 and SLC7A11, thereby intensifying LPO.

downregulation of FPN in microglia can mitigate iron accumulation. Research indicates that LPS activates M1 microglia, increasing TfR and reducing FTH, thereby elevating iron levels. Conversely, stimulation with IL-4 activates M2 microglia, which leads to an increase in both TfR and FTH, sequestering free intracellular iron and reducing iron-associated toxicity (78). Accordingly, decreasing M1 and enhancing M2 microglia expression can lower iron accumulation via adjustments in TfR and FTH. Additionally, the LPS-mediated IL-6 expression in microglia triggers STAT3 phosphorylation, signaling astrocytes to elevate hepcidin levels, which leads to a decrease in FPN1 expression in astrocytes and, subsequently, increased iron accumulation (79). Research on post-ischemic conditions by Davaanyam et al. demonstrates that (80) HMGB1 in astrocytes exacerbates inflammation and enhance hepcidin levels through TLR4 and CXCR4 signaling, resulting in an increase in iron levels and subsequent ferroptosis. Thus, by inhibiting the expression of the IL-6/STAT and HMGB1/TLR4 pathways in glial cells, FPN levels can be increased, reducing iron accumulation and consequently mitigating ferroptosis. Experimental findings indicate that (81) exosomes derived from M1-polarized BV2 microglia exhibit higher expressions of IL-1β, IL-6, and TNF-α. These cytokines promote iron release by downregulating FTH1 expression, leading to iron accumulation in neuronal cells and exacerbating neuronal injury. In contrast, exosomes from M2 microglia transport miR-124-3p to HT22 cells exposed to OGD/R, targeting NCOA4 and thereby inhibiting ferroptosis linked to iron autophagy, ultimately reducing iron accumulation in HT22 cells (82). Although the exact components of the exosomes remain unidentified, these findings indicate that exosomes can mitigate iron accumulation by inhibiting M1 microglial cells and enhancing the anti-inflammatory M2 microglial phenotype, providing a new approach to treat inflammation-driven iron accumulation.

Furthermore, the stimulation of glial cells by inflammation leads to the production of ROS and worsens LPO, ultimately resulting in ferroptosis (74). M1 microglial cells, by activating NADPH oxidase (NOX) through cytokines like TNF-α and IL-1β, produce substantial amounts of ROS, which intensify LPO. Consequently, targeting NOX to inhibit ROS generation could serve as a potential strategy to prevent ferroptosis induced by microglia-enhanced LPO (83, 84). Subsequently, Li et al. (85) found that tumor necrosis factor ligand superfamily member 9 (TNFSF9) engages with TRAF1 to activate mouse BV2 microglia, promoting ROS generation via the JNK, ASK1, and p38 signaling pathways. This activation reduces SLC7A11 and GPx4 activities, thus promoting ferroptosis; however, this process can be reversed by inhibiting the TNFSF9/TRAF1 signaling cascade. Therefore, investigating TNFSF9 to curb inflammation-driven microglial ferroptosis shows considerable potential. Reducing the activity of the antioxidant system represents another pathway by which inflammation exacerbates ferroptosis. Liao et al. revealed that (86) the M1 microglial phenotype encourages the discharge of IL-1β and IL-6, while simultaneously inhibiting IL-10. This imbalance causes reduced levels of GSH and GPx4, which aggravates iron-mediated cellular death and neurological harm after OGD/R. The TNF-induced protein 3 (TNFAIP3, or A20), a significant regulator of inflammatory responses, negatively regulates microglial polarization via the TLR/NF-κB pathway (87). Liu et al. highlighted that (88) in microglia with A20 knockdown induced by RSL3, the heightened levels of IκBα/NFκB/iNOS resulted in increased inflammation, whereas upregulation of GPx4 and SLC7A11 along with downregulation of ACSL4 decreased the microglial susceptibility to ferroptosis. This observation may appear contradictory to the belief that inflammation intensifies ferroptosis; however, it suggests that A20 plays a dual role in governing both inflammatory responses and ferroptosis in microglia. Microglia that without A20 knockdown showed more pronounced ferroptosis, indicating that knocking down A20 expression could help mitigate ferroptosis triggered by microglial activation. This indicates the need to delve further into the specific molecular mechanisms at play between A20 knockdown and the inflammatory and ferroptosis responses in microglial cells, evaluating A20's therapeutic target potential. Additionally, IL-6 can activate astrocytes through the STAT3 pathway, leading to a downregulation of GPx4 and FSP1 and promoting ferroptosis, a process significantly countered by the anti-inflammatory agent IL-10 (89).

Neuroglial cells play a crucial role in inflammation-induced ferroptosis. By modulating the activation state of these cells and targeting iron accumulation mechanisms mediated by TNF-α, IL-6/STAT, and HMGB1/TLR4, strategies to inhibit ROS production by M1-type microglial cells and restore the activities of GPX4, SLC7A11, and ACSL4 can effectively prevent ferroptosis. Furthermore, these pathways may represent new approaches to treating inflammation-induced ferroptosis. For example, suppressing TNFSF9 expression can downregulate the ROS produced by inflammation, thereby mitigating ferroptosis. Additionally, knocking down A20 reduces both inflammation and ferroptosis in microglia. Moreover, inflammation-related pathways and factors are also significant in governing the interplay between inflammation and ferroptosis.

4.1.2 Impact of inflammatory pathways and factors on ferroptosis

Inflammatory pathways are essential in facilitating ferroptosis during cerebral I/R events (70). NF-κB serves as a key regulator of inflammation and triggers ferroptosis upon its activation. For example, within the HT22 cell model, activation of the STAT3/NF-κB pathway leads to elevated $Fe2^{+}$ levels and ACSL4 expression, while concurrently inhibiting SLC7A11 and GPX4 activities, ultimately inducing ferroptosis (90). Conversely, the Nrf2 signaling pathway plays a protective role by blocking the transcription of NF-κB p65, which helps reduce inflammation and decrease the incidence of ferroptosis (91). These results highlight the protective function of Nrf2 against ferroptosis; however, there is also evidence indicating that (92, 93) the Nrf2/HO-1 pathway might inadvertently contribute to iron accumulation as it metabolizes heme into CO, biliverdin, and iron (94). Research by Wang et al. has demonstrated that (95) inhibiting HO-1 with minocycline during cerebral I/R can mitigate iron accumulation in microglia and subsequent ferroptosis. While the Nrf2/HO-1 pathway is generally viewed as advantageous for preventing ferroptosis, it may also have negative repercussions; therefore,

additional research is necessary to develop strategies aimed at preventing iron buildup mediated by this pathway.

As a pathway downstream of NF-κB, the NLRP3 inflammasome plays a role in inducing ferroptosis. The activation and assembly of NLRP3 lead to the release of IL-1β and IL-18, which subsequently affect the activities of the Nrf2/HO-1 pathway and the xc-GSH-GPX4 system, contributing to iron accumulation (96). Research conducted by Wang et al. revealed that (97) the knockout of NLRP3 in mice significantly diminished brain infarct volume, along with a reduction in the expression levels of IL-18 and IL-1β when compared to wild-type MCAO mice. Furthermore, the absence of NLRP3 resulted in increased levels of GSH and GPX4, while decreasing $Fe2^{+}$ and MDA concentrations, likely associated with the Nrf2/HO-1 pathway.

HMGB1, recognized as a damage-associated molecular pattern, is released in response to cellular damage and can intensify inflammatory reactions (98). In the setting of cerebral I/R, the increased levels of HMGB1 inhibit the expression of both Nrf2 and GPx4, thus affecting the efficacy of the antioxidant system and promoting ferroptosis. Therefore, blocking HMGB1 expression could potentially elevate the activities of both Nrf2 and GPx4, lessening the occurrence of ferroptosis (99, 100).

These results highlight that during cerebral I/R, inflammation mediated by NF-κB, NLRP3, and HMGB1 can trigger ferroptosis. Although the upregulation of HO-1 might result in iron accumulation, interventions targeting Nrf2 generally suppress inflammation and diminish ferroptosis. Thus, promoting Nrf2 while inhibiting NF-κB, NLRP3, and HMGB1 could be beneficial in addressing inflammation-induced ferroptosis. The relationship between inflammation and ferroptosis is reciprocal, as ferroptosis can also provoke inflammatory responses, thereby worsening ischemic damage in cerebral I/R.

4.2 Ferroptosis exacerbates inflammatory responses

The buildup of iron ions enhances oxidative stress, leading to a significant increase in the generation of lipid peroxides and ROS. These oxidative byproducts not only inflict direct harm to cellular components but also trigger and escalate inflammatory responses via multiple pathways, which in turn inflicts additional damage on brain tissue (71), as demonstrated in Figure 5.

4.2.1 LPO products activate inflammation

Products of LPO, catalyzed by both COX and LOX, display properties that promote inflammation (35, 101). The COX-2/PGE2 pathway is acknowledged as a key factor in ferroptosis triggered by

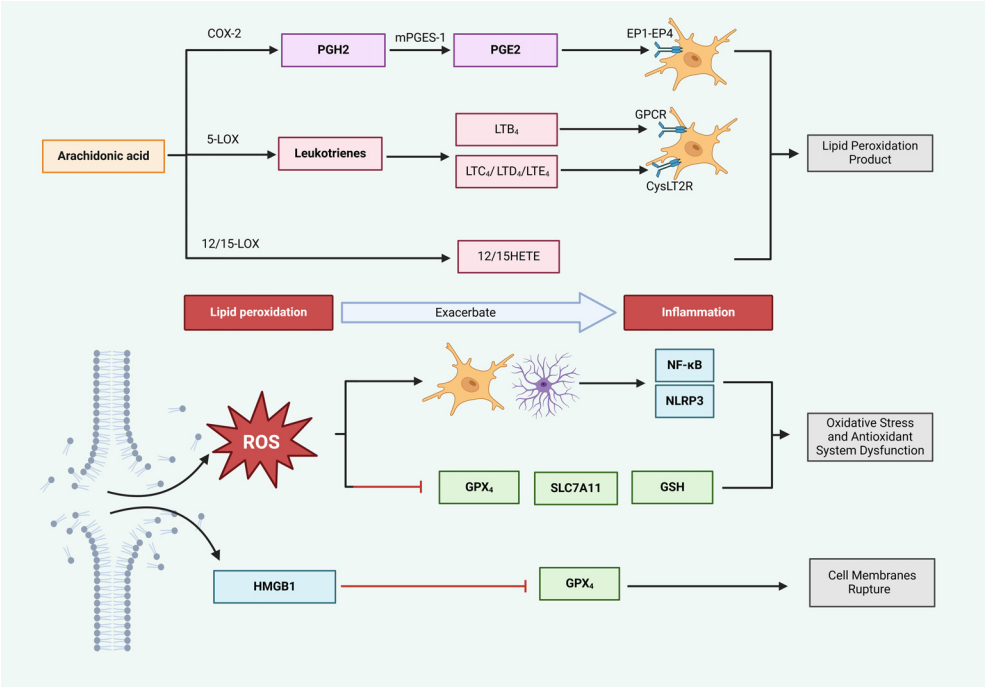

FIGURE 5
Ferroptosis exacerbates inflammatory responses. Cyclooxygenase (COX) and lipoxygenase (LOX) enzymes catalyze arachidonic acid (AA) to produce lipid peroxides, which trigger inflammation. Specifically, COX-2 converts AA into prostaglandin H2 (PGH2), which is subsequently transformed into prostaglandin E2 (PGE2) by microsomal prostaglandin E synthase-1 (mPGES-1). PGE2 interacts with EP1-EP4 receptors, stimulating glial cells and provoking inflammatory responses. Concurrently, 5-LOX metabolizes AA into leukotrienes; leukotriene B4 (LTB4) interacts with G protein-coupled receptors (GPCR), while LTC4, LTD4, and LTE4 engage with CysLT2 receptors, collectively stimulating glial cells and promoting inflammation. In the lipid peroxidation (LPO) process, ROS are generated, activating glial cells and inducing inflammation via the NF-κB and NLRP3 pathways. Additionally, ROS suppress the activity of GPx4, SLC7A11, and GSH, thereby accelerating the LPO-mediated generation of ROS and indirectly intensifying inflammation. Furthermore, HMGB1, released from ruptured cell membranes, further suppresses GPx4 activity, elevates ROS levels, and exacerbates inflammation.

cerebral I/R (102). In this process, COX-2 facilitates the transformation of AA, which results in heightened levels of PGE2. The resulting PGE2 binds to various prostaglandin receptors (EP1-EP4), leading to the increased migration of microglia and neutrophils, thus intensifying the inflammatory response. Furthermore, the generation of ROS may activate COX-2, resulting in a self-reinforcing cycle (103). Therefore, COX-2/PGE2 signifies a promising target for addressing inflammation caused by ferroptosis-related products.

Moreover, 5-LOX, a specific variant of LOX, has the capability to trigger ferroptosis and generate harmful LPO byproducts, positioning it as a crucial target for inhibiting this pathway (101). This enzyme facilitates the peroxidation of AA, resulting in the creation of inflammatory substances, including leukotrienes (LTB4, LTC4, LTD4, and LTE4). LTB4 subsequently engages their G-protein-coupled receptors. The engagement of these receptors initiates the migration and activation of immune cells, thus enhancing local inflammation (104). Additionally, LTC4, LTD4, and LTE4 provoke inflammatory reactions through the activation of cysteinyl leukotriene receptors (CysLTR), which may activate the NF-κB signaling cascade, further promoting neuronal damage and inflammation, as well as encouraging M1 polarization within microglia (105, 106). By targeting the lipid peroxidation products linked to 5-LOX, leukotrienes, and their corresponding receptors, it may be possible to mitigate the infiltration of immune cells (such as microglia and neutrophils) and downregulate the expression of NF-κB, thereby lessening inflammatory responses.

The heightened activity of 12/15-lipoxygenase (12/15-LOX) is significantly linked to the process of ferroptosis, with its lipid peroxidation derivatives, specifically 12-HETE and 15-HETE, being associated with ischemic stroke (107). Research into the inflammatory mechanisms related to ischemic stroke has shown that (108) 12/15-LOX can trigger the activation of NLRP1 and NLRP3, which subsequently leads to the secretion of IL-1β. This release promotes inflammation and contributes to brain injury in pMCAO mice. Through these LPO processes, PUFAs are ultimately converted into the peroxidation product 4-hydroxynonenal (4-HNE) (109). Due to its pro-inflammatory nature, 4-HNE activates microglia through the TLR4/NF-κB signaling pathway, leading to an increased expression of the inflammatory factors IL-1β and TNF-α, thus intensifying neuroinflammation. Therefore, the LPO product 4-HNE represents a potential therapeutic target for inflammation caused by ferroptosis (110). Furthermore, ACSL4 plays a role in both ferroptosis and inflammation by converting free arachidonic acid (AA) into arachidonyl-CoA, thereby enhancing ferroptosis (111). Simultaneously, ACSL4 is capable of activating microglial NF-κB pathways, resulting in the production of cytokines such as TNF-α, IL-6, and IL-1β. Importantly, the silencing of ACSL4 reverses the expression levels of these inflammatory markers (34, 112). Although these processes seem to be interconnected, treatment with RSL3 did not lead to an increase in inflammatory cytokines in LPS-stimulated microglia that were silenced for ACSL4 (112). This observation implies that while the inhibition of ACSL4 may alleviate ferroptosis and associated neuroinflammation, the link between reduced neuroinflammation and ferroptosis may be limited, suggesting that further investigations are necessary to determine whether ACSL4 can be effectively targeted to treat inflammation induced by ferroptosis and to clarify the relationship between these processes.

LPO can produce substantial quantities of ROS, which activate reactive astrocytes and microglia. This activation triggers the transcription of inflammatory cytokines such as IL-6, TNF-α, IL-1β, and IL-18 through various pathways like NF-κB and NLRP3, thereby initiating an inflammatory response (113). The NOXfamily is considered a key contributor to cellular ROS generation (114). Research conducted by Yauger et al. revealed that (115) iron exposure results in ROS production within microglia; however, the use of NOX2 and NOX4 inhibitors diminishes this iron-induced ROS production, highlighting NOX as a potential target for the suppression of microglial activation triggered by ferroptosis.

Excessive ROS can surpass the capabilities of the antioxidant system, resulting in swift dysregulation and promoting the unrestrained advancement of LPO (116). As a result, bolstering the antioxidant system can significantly alleviate ferroptosis and inflammation induced by LPO. However, during the process of ferroptosis, the antioxidant system tends to become rapidly exhausted, indicating the need for its reinforcement to control ROS produced and to mitigate inflammation, particularly through the xc−/GSH/GPX4 pathway (117). The Nrf2 signaling pathway has the ability to enhance the expression of GPX4, SLC7A11, and GSH, subsequently improving the functionality of the antioxidant system and lowering inflammation brought about by ROS (118). In addition, the RNA-binding protein Pumilio 2 (PUM2) binds to PUM-binding elements (PBEs) found in the 3' untranslated regions (UTRs) of particular mRNAs, and it has been demonstrated to worsen neuroinflammation caused by cerebral I/R by facilitating ferroptosis (119). In their study, Liu et al. established that (120) PUM2 reduces the expression of SIRT1, which in turn restrains the activity of the downstream target SLC7A11, leading to increased levels of ACSL4 and TFR1, while decreasing FTH1 and GPX4 levels. This sequence of events contributes to ferroptosis and worsens neuroinflammation as well as brain injury linked to cerebral I/R. Thus, targeting PUM2 expression may produce positive outcomes, as the suppression of the SIRT1/SLC7A11 pathway by PUM2 is associated with ferroptosis and increased neuroinflammation.

4.2.2 Activation of inflammatory signaling following cellular injury

Excessive LPO leads to the disruption of neuronal cell membranes, which facilitates the release of DAMPs like HMGB1 into the extracellular environment. These DAMPs subsequently stimulate immune cells through the TLR/NF-κB signaling pathway, resulting in neuroinflammation (121). Research by Wen et al. established that (122) HMGB1 released during the process of ferroptosis encourages the production of inflammatory cytokines, suggesting that targeting the regulation of HMGB1 could be an effective strategy for managing inflammation linked to ferroptosis.

The pathways through which ferroptosis triggers neuro inflammation involve lipid peroxidation-derived products such as PGE2 and leukotrienes, along with ROS and HMGB1 released as a result of cell membrane damage. These mediators can activate neuroglial cells, worsening inflammation via pathways like TLR/

NF-κB, NLRP3, and COX-2/PGE2. Consequently, targeting pathways such as COX-2/PGE2, 5-LOX/leukotrienes, 12/15-LOX, NOX/ROS, and HMGB1 may alleviate neuroinflammation associated with ferroptosis. Furthermore, inhibiting the PUM2-induced downregulation of SIRT1 could present a novel mechanism for addressing inflammation resulting from ferroptosis, although the specific mechanisms by which PUM2 facilitates inflammation via ferroptosis necessitate additional exploration. Additionally, targeting the ferroptosis byproduct 4-HNE represents another therapeutic strategy for inflammation; however, no drugs have yet been confirmed effective in this context. Therefore, ongoing research is essential to elucidate specific pathways for targeting 4-HNE in treating brain ischemia/reperfusion (I/R) associated with ferroptosis and inflammation.

5 Therapeutic mechanisms

As illustrated in Table 1.

5.1 Modulating inflammation-induced ferroptosis

Glycyrrhizin, a key ingredient found in the traditional herb licorice, acts as a blocker of HMGB1 and is utilized in treating cerebral I/R (123). Research by Zhu et al. demonstrated that (124) glycyrrhizin lowers the levels of TNF-α, IL-6, and IL-1β through the HMGB1/GPX4 pathway, effectively preventing neuroinflammation-related neuronal ferroptosis and alleviating damage from cerebral ischemic hypoxia. Icariin II, which is the main active component of the traditional herb horny goat weed, possesses anti-inflammatory, antioxidant, anti-ferroptotic, and anti-apoptotic attributes in the context of cerebral I/R (125). According to a study by Gao et al. (91), prior treatment with icariin II in astrocytes notably enhances Nrf2 expression, which subsequently reduces the secretion of IL-1β, IL-6, and TNF-α via the OXPHOS/NF-κB pathway. This mechanism leads to diminished production of mitochondrial ROS, MDA, and iron following ischemic stroke, while also increasing the ratios of NADPH/NADP, enhancing SOD2 activity, elevating GPX4 levels, and boosting SIRT5 activity, thereby averting LPO-triggered ferroptosis. These results suggest that icariin lessens inflammation-related ferroptosis through the Nrf2/OXPHOS/NF-κB pathway, offering significant neuroprotection after ischemic stroke. Findings from Zhang et al. indicate that (126) dimethyl fumarate (DMF), an activator of Nrf2, promotes the upregulation of IκBα while inhibiting the NF-κB pathway, resulting in increased expression of crucial ferroptosis mediators, including HO-1, NQO1, and GPx4, thereby safeguarding cells from oxidative stress and ferroptosis. Additionally, recent studies on cerebral I/R have demonstrated that (127) the Nrf2 activator sulforaphane (SFN) enhances the levels of Occludin and ZO1, which helps maintain the integrity of the blood-brain barrier (BBB). SFN also leads to decreased levels of NF-κB, IL-18, and IL-1β, diminishes inflammation, alleviates $Fe2^+$ concentrations, and boosts GSH and GPX4 levels, ultimately mitigating ferroptosis and reducing iron buildup through the enhancement of the antioxidant defense system. Oxysophoridine (OSR), a bioactive alkaloid sourced from sophora flowers, displays antioxidant and anti-inflammatory characteristics while providing protection against cerebral I/R damage in mice (128, 129). The TLR/p38 MAPK pathway is acknowledged for its crucial role in modulating inflammation during cerebral I/R (130). Recent research indicates that (131) OSR restrains the activity of the TLR4/p38 MAPK signaling pathway in rats experiencing MCAO, resulting in a decrease in $Fe2^+$ accumulation and a marked downregulation of ACSL4 and TFR1 protein levels. Simultaneously, OSR upregulates levels of FTH1 and GPx4, thereby countering ferroptosis. Salvia miltiorrhiza, commonly known as Danshen, is extensively employed in the treatment of cerebrovascular disorders owing to its properties that include anticoagulant, antioxidant, anti-inflammatory, and anti-apoptotic effects (132). It comprises active compounds like salvianolic acid B and tanshinone IIA, although their precise constituents remain to be thoroughly elucidated (133, 134). In a mouse model of tMCAO, the administration of Danshen markedly suppresses the activation of microglia and astrocytes, leads to a downregulation of TNF-α and IL-6 expressions, as well as the phosphorylation of STAT3. This sequence of events mitigates neuroinflammation-induced upsurge in ROS and ACSL4 levels, while also decreasing GPX4 and FPN1, thus providing an inhibition of ferroptosis, sustaining synaptic integrity, and lessening neuronal death. These actions illustrate lasting neuroprotection in the aftermath of ischemic brain damage (135). Nao Tai Fang (NTF), which is made up of traditional Chinese medicinal components like Astragalus, Chuanxiong, Dilong, and Jiangcan, has been found to safeguard rats against neuronal ferroptosis triggered by acute cerebral ischemia (136, 137). Research by Liao et al. revealed that (86) key active ingredients in Nao Tai Fang, such as ferulic acid, Ligustilide, astragaloside IV, and tetramethylpyrazine, impede M1 microglia polarization while encouraging M2 polarization. This adjustment results in a reduction of IL-1β and IL-6/STAT3 expression alongside an increase in IL-10 secretion. Additionally, NTF attenuates $Fe2^+$ buildup and the excessive production of ROS induced by LPO through the BMP6/SMADs signaling pathway associated with iron metabolism, all while boosting the levels of GSH and GPx4, thereby inhibiting ferroptosis.

5.2 Modulating inflammation caused by ferroptosis

Caffeic Acid (CA), a naturally occurring phenolic compound generally found in medicinal plants, serves as an inhibitor of COX-2/PGE2 (138). In studies involving the pMCAO rat model, CA has been shown to hinder the activation of COX-2 induced by ferroptosis and reduce the associated increase in ROS, thus preventing the activation of both microglia and astrocytes. Moreover, CA diminishes the release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, effectively reversing the damage resulting from cerebral I/R (139). Furthermore, it has been shown that CA acts as a 5-LOX inhibitor, mitigating inflammation related to brain I/R (140). In rat models, CA administration leads to a reduction in 5-LOX expression, downregulating NF-κB p65 activation, and decreasing the release of iNOS (141). Researchers, including Keuters (142), have

TABLE 1 Therapeutic mechanisms for ferroptosis and inflammatory crosstalk in cerebral ischemia-reperfusion.

Therapeutic Mechanisms	Name	Pathway	Effects on cytokine levels	Reference
Inhibiting inflammation-induced ferroptosis	Glycyrrhizin	HMGB1/GPX4↓	TNF-α, IL-6, IL-1β↓	(123, 124)
	Icariin II	Nrf2/OXPHOS/NF-κB↓	TNF-α, IL-6, IL-1β, ROS, MDA↓ SIRT5, NADPH/NADP, SOD2, GPX_4↑	(91, 125)
	Dimethyl fumarate (DMF)	NRF2/NF-κB↓	IL-1β, TNF-α, IL-6↓ GSH, SOD, GPX_4↑	(126)
	sulforaphane (SFN)	NRF2/NF-κB↓	Occludin, ZO1↑ IL-18, IL-1β, $Fe2^+$↓, GSH, GPX4↑	(127)
	Oxysophoridine (OSR)	TLR/p38 MAPK↓	Fe^{2+}, ACSL4, TFR1↓ FTH1, GPX4↑	(128–131)
	Salvia miltiorrhiza (Active ingredients are Salvianolic Acid B and Tanshinone IIA)	IL-6/STAT3↓	TNF-α, ROS, ACSL4↓ FPN1, GPX4↑	(132–135)
	Nao Tai Fang (Active ingredients are Ferulic Acid, Ligustilide, Astragaloside IV, and Tetramethylpyrazine)	M1 microglia, IL-6/STAT3↓ M2 microglia, BMP6/SMADs↑	IL-1β, IL-6, Fe^{2+}, ROS↓ IL-10, GSH, GPX_4↑	(86, 136, 137)
Inhibiting ferroptosis-induced inflammation	Caffeic Acid (CA)	COX-2/PGE2, 5-LOX NF-κB p65↓ Nrf2↑	ROS, TNF-α, IL-6, IL-1β iNOS↓	(138–141)
	ADA-409-052	COX-2/PGE2, Microglial activation, Macrophage↓	TNF-α, IL-6, IL-1β↓ GSH, GPx4↑	(142, 143)
	Edaravone	5-LOX↓ Nrf2/FPN↑	Fe^{2+}, MDA, IL-6, IL-1β, TNF-α, leukotriene↓ GSH↑	(144–147)
	Panax notoginseng saponins (PNS)	Nrf2↑	$Fe2^+$, ACSL4, SLC7A11, IL-6, IL-1β, TNF-α↓ GSH, GPX_4 ↑	(148, 149)
	Gastrodia elata polysaccharides	NRF2/HO-1↑ HMGB1, NLRP3↓	Fe^{2+}, ROS, IL-1β, IL-6 TNF-α↓	(150, 151)
	Srs11-92 (AA9)	NRF2/NF-κB↓ NRF2/HMGB1↓	GSH, GPX_4↑	(152)
Other Therapeutic Strategy	Enriched environments (EE)	IL-6/STAT3, Astrocytic activation↓	ROS, $Fe2^+$ ↓, GPX4↑	(153–156)
Exosomes	ADSC-Exo	Fxr2/Atf3/Slc7a11, M2 microglia↑ M1 microglia↓	Fe^{2+}, hepcidin, TfR1, DMT1, TNF-α, IL-6, IL-1β, ACSL4↓ FPN1, GPX4, HIF-1α↑ IL-1β, IL-2, IL-6, IL-12 p70, IL-23, TNF-α, MDA ROS, Fe^{2+}↓ IL-10, TGFβ1, IL-4, IL-13↑	(159)
	SRC-3-exo	Ferroptosis, Microglial activation, Astrocytic activation↓	MDA, IL-1β, IL-6, TNF-α↓ GSH/GSSG↑	(160)
	MSCs-EVs	MSCs-EVs/Fer-1↑ GPX4/COX-2/PGE2↓	TNF-α, IL-1β↓	(161)

↑ is upregulating ↓ is downregulating.

discovered a novel small molecule, ADA-409-052, which features an arylthiazine framework and inhibits LPO, thereby preventing neuronal cell death caused by GSH depletion or GPx4 inhibition. In addition, ADA-409-052 curtails the pro-inflammatory activation of BV2 microglia, lessens the release of TNF-α, IL-6, and IL-1β, and provides protection to N2a neuronal cells from inflammation-induced cell death mediated by RAW 264.7 macrophages. Similar to well-established ferroptosis inhibitors like Fer-1, which can alleviate neuroinflammation (143), ADA-409-052 interacts with the COX-2/PGE2 pathway to target ferroptosis, helping to reduce

both severe neuronal injury and inflammation, making it a potential therapeutic approach for neurological disorders (142). Edaravone represents a highly effective neuroprotective agent that functions as a potent scavenger of free radicals, thereby inhibiting both ferroptosis and inflammation associated with ischemic stroke (144, 145). Recent studies have shown that following cerebral I/R, there is a notable decrease in the expression levels of Nrf2 and FPN; however, these alterations can be reversed with edaravone treatment (146). This therapeutic intervention not only diminishes the concentrations of Fe2+ and MDA within the brain tissue of MCAO/R rats but also elevates GSH levels, suppresses ferroptosis, and reduces the concentrations of inflammatory markers such as IL-6, IL-1β, and TNF-α. These observations suggest that edaravone mitigates cerebral I/R injury through the activation of the Nrf2/FPN signaling pathway, which leads to the inhibition of both ferroptosis and inflammation. Nonetheless, additional research is necessary to clearly define the relationship between reduced inflammation and the Nrf2/FPN pathway. Moreover, edaravone lowers extracellular levels of leukotrienes by blocking the 5-LOX pathway, thus safeguarding SD rats and PC12 cells from inflammatory damage induced by ischemia (147). Panax notoginseng saponins (PNS), which are extracted from the Chinese medicinal herb Panax notoginseng, display various beneficial effects including antioxidation, anti-inflammation, anti-apoptosis, and the promotion of angiogenesis (148). In MCAO/R rat models, the administration of PNS activates the Nrf2 signaling pathway, suppresses $Fe2^+$ and ACSL4 expression, decreases levels of LPO, and enhances the concentrations of GSH, GPX4, and SLC7A11, thereby strengthening the antioxidant defense system, inhibiting ferroptosis, and subsequently reducing the levels of IL-6, IL-1β, and TNF-α to mitigate neuroinflammation and address cerebral I/R (149). Moreover, polysaccharides from Gastrodia elata, a traditional Chinese herb, exhibit properties such as antioxidant, anti-inflammatory, and anti-ferroptotic effects (150). In models of MCAO mice, treatment with these polysaccharides leads to a marked activation of the NRF2/HO-1 signaling pathway, a decrease in the harmful buildup of $Fe2^+$, and an enhancement of GSH and GPx4 activities. By obstructing LPO, the treatment helps avert damage to cell membranes and the ensuing release of HMGB1 and ROS, thus mitigating neuronal ferroptosis. This mechanism is associated with the stimulation of the NLRP3 pathway, which subsequently triggers the release of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, ultimately reducing neuroinflammation (151). These findings highlight the protective effects of Gastrodia elata polysaccharides against ferroptosis and inflammation induced by cerebral ischemia/reperfusion. The Ferrostatin-1 derivative, Srs11-92 (AA9), has been shown to impede iron accumulation and ROS generation, boost GPX4 and Nrf2 expression, and inhibit the levels of HMGB1 and NF-κB p65 within the hippocampal region. This intervention led to a reduction in infarct size, lessened neuronal injury, and improved neurological outcomes in the MCAO/R mouse model. Conversely, the Nrf2 inhibitor ML385 negated the protective effects of AA9, suggesting that AA9 alleviates neuronal ferroptosis and neuroinflammation induced by OGD/R through the Nrf2 pathway (152).

5.3 Other therapeutic strategies

Enriched environments (EE) serve as a rehabilitation approach for stroke patients, aimed at offering greater space, innovative game tools, and more social interactions. This method fosters improved sensory, cognitive, motor, and social engagement, which supports neuronal reorganization and functional recovery after injury, while also enhancing the central immune response (153, 154). Research indicates that EE can reduce the activation of IL-6-induced STAT3 phosphorylation in MCAO rats, leading to lower hepcidin levels in astrocytes, reduced TfR1 and DMT1 expression, increased FPN1 activity, and a decrease in iron accumulation due to neuroinflammation (155). Moreover, EE effectively reduces iron accumulation and mitigates the downregulation of GPX4 induced by inflammatory cytokines like TNF-α, IL-6, and IL-1β in the brain tissues of MCAO rats. This intervention also boosts HIF-1α expression while inhibiting the upregulation of ACSL4, thereby reducing ferroptosis and aiding in the recovery of function following brain I/R injury (156).

Exosomes are tiny membrane-bound vesicles that are expelled from cells and are being researched for their potential in treating a range of neurological conditions (157). In cases of cerebral ischemia, exosomes have demonstrated their ability to lessen neuronal injury and enhance the brain's microenvironment by modulating inflammation, preventing ferroptosis, mediating apoptosis in cells, fostering axonal development, and facilitating vascular remodeling (158). Research by Wang et al. revealed that (159) exosomes derived from adipose stem cells (ADSC-Exo) act as inhibitors of ferroptosis, significantly decreasing M2 microglial sensitivity to this process and suppressing M1 microglial marker expression through the Fxr2/Atf3/Slc7a11 signaling pathway in MCAO mice. Furthermore, these exosomes diminish the levels of MDA, ROS, and $Fe2^+$, leading to a decrease in pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-12 p70, IL-23, TNF-α, while simultaneously increasing the levels of anti-inflammatory cytokines like IL-10, TGF-β1, IL-4, and IL-13, which further mitigates inflammation and supports neuronal survival. Additionally, exosomes that overexpress steroid receptor coactivator-3 (SRC-3-exo) significantly elevate the GSH/GSSG ratio while decreasing the activity of catalase, MDA, and LPO, thereby preventing neuronal ferroptosis induced by OGD/R. This action also leads to the inhibition of microglial and astrocytic activation, resulting in a lowered production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the brains of MCAO mice. SRC3-exo treatment additionally reduces brain edema and infarction size in mice, promoting neuronal recovery and enhancing neurological performance (160). These findings suggest that exosomes may bolster the antioxidant defense mechanisms to counteract LPO-induced ferroptosis during cerebral I/R, further inhibiting the overactivation of neuroglial cells that plays a role in neuroinflammation. Additionally, extracellular vesicles derived from bone marrow mesenchymal stem cells (MSCs-EVs) are capable of transporting Fer-1 to neurons in the hippocampus. The combination of MSCs-EVs and Fer-1 enhances the levels of GPX4, suppresses COX-2 expression, and decreases the expression

of TNF-α and IL-1β via the COX-2/PGE2 signaling pathway, thereby reducing neuroinflammation caused by ferroptosis and offering protection during cerebral I/R events (161).

5.4 Clinical feasibility and potential risks of treatment

The treatment strategies derived from basic research provide promising avenues for addressing the interplay between ferroptosis and inflammation in brain I/R. Anti-inflammatory drugs and environmental enrichment (EE) reduce inflammation by regulating pathways like HMGB1, Nrf2/NF-κB, TLR/p38 MAPK, and IL-6/STAT3, thereby mitigating ferroptosis (91, 124, 131, 155). Ferroptosis inhibitors and exosomes, on the other hand, target COX-2/PGE2, 5-LOX/leukotrienes, and Nrf2-mediated pathways, reducing the inflammatory impact of ferroptosis on inflammation (147, 152, 161). Innovations such as the regulation of M1/M2 microglial polarization by NTF, which influences the BMP6/SMADs pathway, demonstrate novel mechanisms to alleviate inflammation-induced ferroptosis (86). Bone morphogenetic protein 6 (BMP6), a regulator of hepcidin and iron metabolism, is upregulated in response to increased iron levels, triggering a SMAD signaling cascade that activates the hepcidin gene—a potential new approach to manage iron level alterations caused by microglia (162). Additionally, the ferroptosis inhibitor ADSC-Exo protects M2 microglia through the Fxr2/Atf3/Slc7a11 pathway, managing the inflammatory microenvironment (163, 164). Under ADSC-Exo's influence, Fxr2 is upregulated, indirectly suppressing Atf3 while boosting SLC7A11 under oxidative stress, thus enhancing antioxidant defenses and inhibiting ferroptosis (159). New pharmacological developments like ADA-409-052 target the COX-2/PGE2 pathway to improve outcomes in severe neuronal death and neuroinflammation (142), while AA9 reduces hippocampal damage, inhibits iron deposition and ROS accumulation to alleviate ferroptosis, and subsequently suppresses neuroinflammation via the Nrf2 pathways (152).

Despite their innovative mechanisms offering new therapeutic possibilities, the potential risks and side effects of these strategies cannot be ignored. When evaluating their large-scale clinical application, initial small-scale clinical trials are crucial. These trials should thoroughly assess patient-specific conditions, drug safety, and potential interactions with other treatments. Possible side effects include immune suppression and elevated blood pressure from anti-inflammatory drugs. For instance, glycyrrhizin might increase blood pressure and lead to hypokalemia and hypernatremia—risks that necessitate cautious use, especially in hypertensive patients (165). Dimethyl fumarate may cause lymphocytopenia, a risk manageable with preemptive blood tests (166). Furthermore, using high doses of ferroptosis inhibitors, like caffeic acid, might impair iron absorption, leading to deficiency if not carefully managed (167).

Additionally, treatment plans should be tailored based on patient age, gender, and the specific brain regions affected. For example, elderly patients, who typically have diminished neuroplasticity and are more susceptible to iron accumulation, might benefit from combinations of iron chelators and stem cell exosomes (168). Female patients, potentially responding better to treatments due to the neuroprotective effects of estrogen, may have better outcomes (169, 170). Regional differences in brain sensitivity also dictate tailored approaches; for instance, the hippocampal region's high demand for oxygen and glucose and its active iron metabolism make it particularly vulnerable to ischemic damage and ferroptosis. Therefore, treatment of the hippocampal region may primarily involve ferroptosis inhibitors, coupled with appropriate anti-inflammatory agents, and improving mitochondrial energy supply to neurons to support their survival and functional recovery (171, 172). The basal ganglia, critical for controlling muscle strength and tone, are frequently affected by strokes (173). Cerebral ischemia/reperfusion can increase the permeability of the blood-brain barrier (BBB) in this region (174). Therefore, we should increase the use of anti-inflammatory drugs and employ drug delivery systems to more effectively target the basal ganglia, protecting the integrity of the BBB. Additionally, combining ferroptosis inhibitors and motor neuron nutrients should be considered to mitigate the effects of ferroptosis and inflammation in this area. By fully understanding these variables, clinicians can develop more effective, personalized treatment strategies, ultimately improving patient outcomes in brain I/R scenarios.

6 Conclusion and prospects

Ferroptosis, an iron-dependent form of cell death, has garnered increasing attention for its role in ischemic brain diseases in recent years. Current research indicates that during ischemia-reperfusion injury, the accumulation of iron ions promotes lipid peroxidation by catalyzing ROS, a critical mechanism underlying ferroptosis. Furthermore, the inflammatory response significantly contributes to cerebral I/R injury. The activation of microglial cells leads to the release of numerous inflammatory mediators, which further exacerbate brain tissue damage. Ferroptosis not only induces direct neuronal death but may also worsen cerebral I/R injury by activating neuroglial cells and associated pathways that release inflammatory factors. While inflammation serves as a self-defense mechanism, excessive inflammation can be detrimental, resulting in iron accumulation and LPO-induced ferroptosis.

From a therapeutic standpoint, novel iron chelators and anti-inflammatory drugs have shown promise in experimental studies. For instance, the Ferrostatin-1 analog Srs11-92 has been demonstrated to reduce iron accumulation and activate the Nrf2 pathway, thereby alleviating both ferroptosis and inflammatory responses (152). Additionally, caffeic acid has been found to mitigate inflammation by inhibiting COX-2 and 5-LOX-induced inflammatory LPO (139, 141). Targeted treatment of inflammatory pathways, such as the administration of Salvia miltiorrhiza, significantly inhibits the activation of neuroglial cells, downregulates the expression of TNF-α, IL-6, and the phosphorylation of STAT3, thereby suppressing neuroinflammation-induced ferroptosis (135). Similarly, glycyrrhizin inhibits neuroinflammation-induced neuronal ferroptosis by downregulating TNF-α, IL-6, and IL-1β expression through the HMGB1/GPX4 pathway, demonstrating protective effects against ischemic brain damage (124).

Despite recent advances, the crosstalk mechanisms between ferroptosis and inflammation in cerebral I/R injury remain incompletely understood. Future research must delve deeper into the interaction mechanisms between these two processes and identify more effective strategies for their regulation to optimize therapeutic outcomes. For instance, while ferroptosis inhibitors have been shown to alleviate both ferroptosis and inflammation, the specific interaction mechanisms between them are not fully elucidated, and the potential health risks associated with their long-term use remain uncertain. Similarly, although inhibiting ACSL4 can mitigate both ferroptosis and inflammation, the upstream and downstream factors governing ACSL4 expression and activity, as well as their interactions under varying physiological and pathological conditions, are not yet fully understood, highlighting the need for further foundational research. Additionally, clinical studies are essential to validate the translatability and safety of findings from laboratory research. Future research directions should include the development of novel iron chelators and anti-inflammatory agents, alongside the exploration of combination therapy strategies. Moreover, advancements in biomarkers and imaging techniques will facilitate the early identification and quantitative assessment of the extent of ferroptosis and inflammation, thereby providing more precise timing and targets for clinical interventions. Through these comprehensive strategies, we aspire to achieve more effective treatment and prevention of cerebral I/R in the future.

In summary, the interplay between ferroptosis and inflammation in cerebral I/R injury represents a complex yet promising field of investigation. By thoroughly examining the interaction and regulatory mechanisms of these two processes, we can enhance our understanding of cerebral I/R development and establish a theoretical foundation for the development of novel therapeutic approaches.

Author contributions

YH: Software, Writing – original draft. JW: Visualization, Writing – review & editing. CY: Conceptualization, Writing – review & editing. KX: Formal analysis, Writing – review & editing. JL: Methodology, Writing – review & editing. BW: Project administration, Writing – review & editing. JG: Investigation, Writing – review & editing. ZY: Validation, Writing – review & editing. YZ: Data curation, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Immune imbalance in Lupus Nephritis: The intersection of T-Cell and ferroptosis

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Ferroptosis is a novel form of cell death characterized by unlimited accumulation of iron-dependent lipid peroxides. It is often accompanied by disease, and the relationship between ferroptosis of immune cells and immune regulation has been attracting increasing attention. Initially, it was found in cancer research that the inhibition of regulatory T cell (Treg) ferroptosis and the promotion of CD8+ T cell ferroptosis jointly promoted the formation of an immune-tolerant environment in tumors. T-cell ferroptosis has subsequently been found to have immunoregulatory effects in other diseases. As an autoimmune disease characterized by immune imbalance, T-cell ferroptosis has attracted attention for its potential in regulating immune balance in lupus nephritis. This article reviews the metabolic processes within different T-cell subsets in lupus nephritis (LN), including T follicular helper (TFH) cells, T helper (Th)17 cells, Th1 cells, Th2 cells, and Treg cells, and reveals that these cellular metabolisms not only facilitate the formation of a T-cell immune imbalance but are also closely associated with the occurrence of ferroptosis. Consequently, we hypothesize that targeting the metabolic pathways of ferroptosis could become a novel research direction for effectively treating the immune imbalance in lupus nephritis by altering T-cell differentiation and the incidence of ferroptosis.

KEYWORDS

ferroptosis, T cells, Lupus Nephritis, immune imbalance, cellular metabolism

1 Introduction

The key mechanism of ferroptosis involves the upregulation of lipid peroxidation pathways driven by iron, reactive oxygen species (ROS), and polyunsaturated fatty acids (PUFAs), coupled with the downregulation of antioxidant mechanisms, primarily selenium-dependent glutathione peroxidase 4 (GPX4) (1). Increasing evidence suggests

that modulating metabolic targets involved in T-cell ferroptosis can alter immune imbalances. Initially, this regulatory mechanism attracted attention in the context of tumor immunomodulation. Ferroptosis in Treg cells is often suppressed by upregulated antioxidant mechanisms, which contribute to tumor immune evasion (2). However, ferroptosis in CD8+ T cells is frequently promoted by increased lipid synthesis, thereby reducing their capacity for immune recognition and killing of tumor cells (3). Notably, altering T-cell ferroptosis-related metabolic targets, such as promoting ferroptosis in Treg cells or inhibiting ferroptosis in CD8+ T cells, has been shown to improve cancer prognosis. Therefore, given that T-cell ferroptosis is a promising disease immunoregulatory mechanism, its potential therapeutic role in other diseases has also garnered significant interest (4).

Systemic lupus erythematosus (SLE) is characterized by immune tolerance disorders and hyperactivity of immunological reactivity, leading to immune imbalance (5). LN is a common complication and cause of death in SLE patients (6). Recent studies have shown that T-cell immune imbalance is the key pathogenesis of LN (7, 8). Specifically, upregulated effector T (Teff) cells contribute to kidney injury through the formation of a proinflammatory environment and the promotion of fibrosis (9). By assisting in humoral immunity, TFH cells produce more pathogenic antibodies, exacerbating autoimmune inflammation (10). Additionally, the downregulation of Treg cells in LN fails to maintain immune tolerance, allowing the adaptive immune system to no longer protect self-antigens while recognizing and eliminating pathogens and accelerating kidney injury (11). Although T-cell immune imbalance is crucial in LN, little is known regarding its regulation. Therefore, research on the mechanisms by which T-cell ferroptosis regulates T-cell immune imbalance in the LN has attracted significant attention.

In this review, we first described the unique manifestations of immune imbalances among various T-cell subsets in LNs. We highlighted that metabolic alterations in T cells within the LN not only promote the aberrant differentiation of T cells, leading to immune dysregulation but also variably augment the occurrence of ferroptosis across different T-cell subsets. Similarly, we revisited the current understanding of how the regulation of metabolic targets related to ferroptosis in these T-cell subsets could modulate immune imbalances in LN. We emphasized the potential of metabolic enzymes and molecules associated with ferroptosis as future therapeutic targets for treating immune imbalances in LN.

2 The mechanism of Ferroptosis

The continuous accumulation of unstable iron, increase of ROS, and supply of PUFAs in cells, coupled with weakened antioxidant mechanisms, lead to an unlimited increase in lipid ROS in cells, which is the key factor that induces ferroptosis (1). Among them, weakened antioxidant mechanisms are closely related to glutathione metabolism and the GPX4 enzyme. Therefore, abnormal metabolism in cells is often the key to identifying ferroptosis. In addition, many studies have shown that mitochondria usually exhibit atrophic and dense morphology during ferroptosis, which is also key evidence for verifying the occurrence of ferroptosis (12). A deeper understanding of metabolic changes in ferroptosis is highly important for identifying the occurrence of T-cell ferroptosis in diseases (Figure 1).

2.1 Accumulation of cellular iron

Once iron enters the bloodstream from different sources in the human body, it must undergo absorption, use, storage, and excretion by cells. Cellular iron absorption involves the uptake of transferrin-bound iron (TBI) into the cell via traditional transferrin receptor 1 (TfR-1, also known as CD71) (13), as well as the uptake of nontransferrin-bound free iron (NTBI), mostly by liver cells (14). Upon entering the cytoplasm, TBI and NTBI are transiently held in the cytoplasm's "labile iron pool" (LIP) in an unstable ferrous state. Iron in the LIP is then weakly attached and carried to the mitochondria for use. Iron inside mitochondria is a crucial component for the production of heme and Fe-S clusters, which are involved in a variety of cellular metabolic processes (15, 16). The majority of unutilized iron is stored inside the cell as ferritin (17). Excess iron is specifically linked to ferritin by the iron chaperones poly r(C)-binding protein (PCBP) 1 and PCBP 2 (18, 19) and then transported out of the cell exclusively by ferroportin (FPN)-1 (20). Furthermore, the iron content in the unstable iron pool inside the cell is carefully regulated by iron regulators and iron regulatory proteins. These proteins control the release and storage of iron in the cell to prevent it from exceeding 5% of the total iron content and maintain a balanced iron level within the cell (17, 21, 22). Disruption of iron metabolism may result in the aberrant buildup of iron inside the cell, which can lead to ferroptosis. Studies have shown that the process of autophagy, which involves the breakdown of ferritin (a protein that stores iron), may lead to the build-up of unstable iron in cells and facilitate ferroptosis (23–25). Disruption of the main receptor TfR1, which is responsible for cellular iron absorption, has been shown to successfully prevent ferroptosis (26). Facilitating the elimination of iron from inside cells has been scientifically shown to successfully prevent iron-induced cell death (27, 28).

2.2 ROS accumulation

ROS represent a collective term for oxygen-containing radicals and peroxides associated with oxygen metabolism in living organisms. ROS are characterized by unpaired electrons, rendering them highly chemically reactive. When they encounter nonradical species abundantly present in the body, they initiate electron-snatching chain reactions (29).

The ROS generated by the Fenton reaction are considered the primary source of ROS in ferroptosis. In LIP, divalent iron reacts with cytoplasmic hydrogen peroxide (H2O2) via the Fenton reaction, producing trivalent iron, OH−, and highly reactive hydroxyl radicals (OH•) (30, 31). OH• subsequently engages in

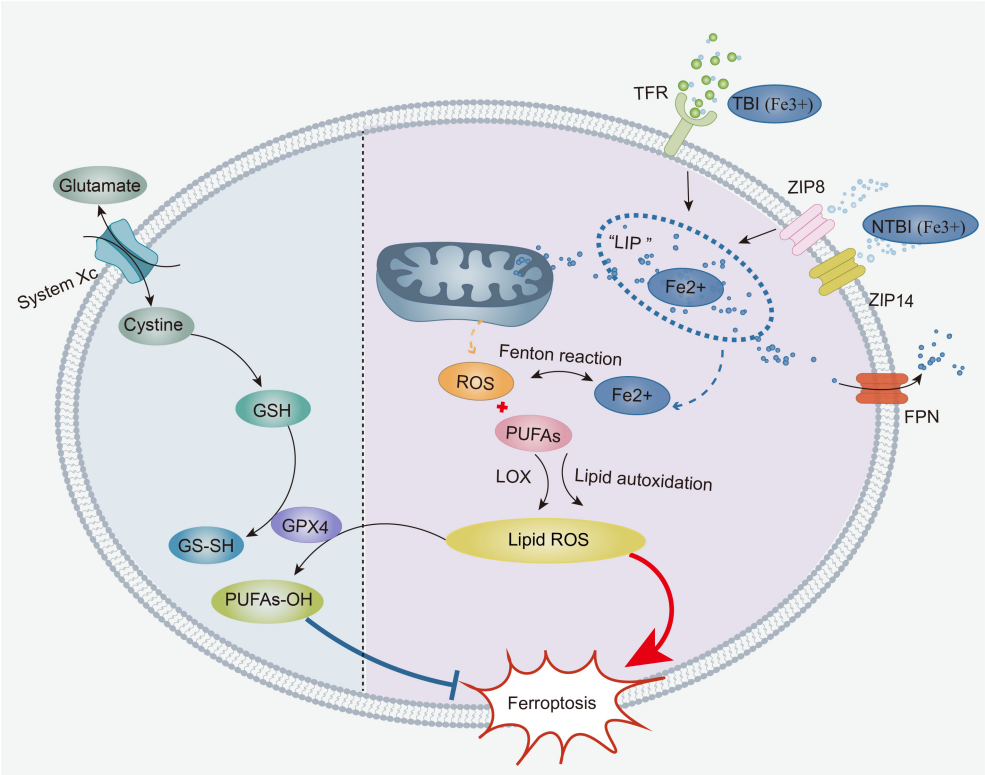

FIGURE 1
The core mechanisms of ferroptosis: The accumulation of labile iron, ROS, and PUFAs in cells contributes to the onset of ferroptosis. TBI and NTBI enter the cell and are temporarily stored in an "iron pool". Subsequently, labile iron from the "iron pool" initiates a Fenton reaction that produces an abundance of ROS, leading to an increase in intracellular ROS. Under the effect of LOXs, intracellular ROS first undergoes enzymatic lipid peroxidation with PUFAs, generating lipid ROS. Once a critical threshold is reached, intracellular ROS directly triggers non-enzymatic lipid autoxidation with membrane-bound PUFAs, resulting in a large amount of lipid ROS. Furthermore, the weakening of the xCT-GPX4 antioxidant system promotes ferroptosis. Cystine is transported into the cell via System xCT, participating in the synthesis of reduced GSH. Then, reduced GSH acts on GPX4 to reduce lipid ROS to lipid alcohols, thereby inhibiting the production of lipid ROS. ROS, Reactive Oxygen Species; PUFAs, Polyunsaturated Fatty Acids; TBI, Transferrin-bound Iron; NTBI, Non-Transferrin-Bound Iron; TFR, Transferrin Receptors; Non-Transferrin Receptors, ZIP8, ZIP14; LOXs, Lipoxygenases; GSH, glutathione.

electron-snatching chain reactions with cellular lipids, leading to the generation of toxic lipid ROS [also called lipid peroxides, LPOs or phospholipid hydroperoxide (PLOOH)].

Furthermore, numerous studies have shown that mitochondrial ROS (mtROS) (32) and nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase (NOX)-derived ROS are significant electron predators involved in the generation of lipid ROS (33–35). Therefore, in addition to intracellular iron accumulation, the accumulation of ROS from multiple sources within cells, which are critical triggers for lipid ROS production, represents the second major factor in ferroptosis.

2.3 The occurrence of lipid peroxidation

Many studies have shown that PUFAs in the phospholipids of endoplasmic reticulum (ER) membranes, primarily arachidonic acid (AA) and adrenic acid (AdA), are key lipid substrates for the occurrence of lipid peroxidation (36–39). Acyl-CoA synthetase long-chain family member 4 (ACSL4) promotes the synthesis of PUFAs (40), whereas lysophosphatidylcholine acyltransferase 3 (LPCAT3) facilitates the esterification of PUFAs and their integration into membrane phospholipids (41).

PUFAs and ROS undergo a process of lipid peroxidation that involves both enzymatic and nonenzymatic stages. The enzymatic stage refers to the process by which PUFAs, under the action of lipoxygenases (LOXs), react with ROS to form lipid ROS (36, 42). Arachidonate lipoxygenase 15 (ALOX15) has been identified as the key enzyme in the enzymatic peroxidation of PUFAs (42–44). In addition, arachidonate lipoxygenase 12 (ALOX12) (45) and cytochrome P450 enzymes (CYPs) (46) have also been found to induce lipid peroxidation. The nonenzymatic stage occurs when intracellular iron accumulates, leading to Fenton reactions between intracellular ROS and membrane-bound PUFAs, thereby initiating lipid autoxidation (31, 47). This nonenzymatic lipid peroxidation plays a dominant role in the accumulation of lipid ROS during ferroptosis (42). Current research still debates the sequence of the enzymatic and nonenzymatic stages, but many studies support that once the enzymatically produced lipid ROS reach a critical threshold, they can trigger nonenzymatic lipid peroxidation (31,

47). Ultimately, lipid ROS first accumulate in the ER membrane and then in the cell membrane (38). Ferroptosis, a form of regulated cell death, occurs when the cell's capacity to repair membrane damage is overwhelmed, leading to cell demise (42).

2.4 Antioxidant mechanisms

The strengthening of iron-dependent lipid peroxidation and the weakening of the antioxidant system are the third significant triggers for ferroptosis. Recent studies in cancer cells have demonstrated that altering the intracellular redox state can induce ferroptosis (48). Therefore, when an oxidative–reductive imbalance occurs within a cell, vigilant monitoring of the potential induction of ferroptosis is essential.

2.4.1 The xCT- GPX4 antioxidant system

In general, the xCT-GPX4 system is considered the primary antioxidant mechanism against ferroptosis. Its key components include cystine/glutamate antiporter solute carrier family 7 member 11 (SLC7A11), which is also known as xCT, and GPX4. Most cells initially take up cysteine (Cys) via xCT. Subsequently, cysteine is converted to cystine through the action of the reducing agent glutathione (GSH) or thioredoxin reductase 1 (TXNRD1), and it participates in GSH synthesis (47, 49). In mammals, TXNRD1 belongs to the thioredoxin reductase (TrxR) family and is also a selenoprotein. The Trx reductive system to which TXNRD1 belongs, along with the GSH reduction system, collaboratively eliminates ROS *in vivo*, maintaining the cellular redox balance (50). Through the action of GPX4, synthesized GSH subsequently reduces lipid ROS to lipid alcohols, thereby inhibiting ferroptosis (51–53). Therefore, the xCT-GPX4 system is a necessary antioxidant mechanism that directly targets the inhibition of lipid ROS generation. Studies have shown that GPX4 knockout cells exhibit high accumulation of ROS and lipid peroxidation products, providing further evidence for this mechanism (54). Notably, Gpx4, as a selenoprotein, requires the presence of selenium for its antioxidative function (51, 55).

Conversely, inhibition of the xCT-GPX4 system can induce ferroptosis. On the basis of this mechanism, various ferroptosis inducers (FINs), such as buthionine sulfoximine (BSO) and erastin, have been developed and have garnered widespread attention in cancer therapy research (56–58). Notably, recent studies have suggested that ras-selective lethal 3 (RSL3), as a type of FINs, may act as an inhibitor of TXNRD1, not only GPX4. First, RSL3 increases ROS levels by inhibiting the TrX system, promoting the generation of lipid ROS. Second, it enhances the accumulation of lipid ROS by inhibiting GPX4, thereby increasing susceptibility to ferroptosis through a dual mechanism (53, 59). Furthermore, recent research has revealed a significant association between the anticancer mechanism of the tumor suppressor gene P53 and the inhibition of xCT-GPX4-induced ferroptosis. In contrast to FINs, P53 achieves its anticancer effects through the dual regulation of peroxidation and weakening antiperoxidation mechanisms. P53 downregulates SLC7A11 expression, directly inhibiting the xCT-GPX4 antioxidant system. However, P53 downregulation of SLC7A11 leads to the release of ALOX12 bound to it, promoting membrane lipid peroxidation and accelerating ferroptosis (45, 60). In summary, weakening of the xCT-GPX4 system, a critical intracellular antioxidant mechanism, is essential for inducing ferroptosis, and this mechanism holds promise for targeting various pathogenic cells in the treatment of various diseases.

In LN, there is a significant increase in oxidative stress due to the presence of autoantibodies, immune complexes, and cytokines such as interferon-α (61, 62). This oxidative stress leads to the depletion of antioxidants, including GPX4, making kidney cells more vulnerable to damage and death (61, 63). Lipidomic analyses of kidneys confirm excessive lipid peroxidation consistent with ferroptosis in LN (64). Ferroptosis, driven by GPX4 inhibition or dysfunction, is increasingly recognized as a mechanism of kidney cell death in LN (65, 66). This process exacerbates inflammation and tissue injury in the renal microenvironment (65, 67). The low expression of GPX4 in these kidneys correlates with tubular damage, highlighting its protective role against ferroptosis-induced injury (68).

Beyond its role in ferroptosis, GPX4 also influences immune cell function, particularly in regulating B cells and neutrophil, which are central to LN pathogenesis. Research indicates that LN is associated with attenuated expression of SLC7A11, this disrupts the antioxidant system, further reducing GPX4 activity and significantly enhances ferroptosis in B cells and reduces their proliferation (69). Research has found that in LN, GPX4 mechanisms involve autoantibodies and interferon-α in serum, which promote neutrophil ferroptosis by enhancing cAMP response element modulator (CREM)α binding to the GPX4 promoter (70). This binding reduces GPX4 expression and leads to an increase in autoantigens produced by neutrophil ferroptosis (70). Given the critical role of GPX4 in ferroptosis, further investigation into GPX4-mediated T cell ferroptosis and its regulation would be of great significance for understanding and potentially treating LN.

2.4.2 The non xCT- GPX4 antioxidant systems

In addition to GPX4, ferroptosis suppressor protein 1 (FSP1, also known as AIFM2) and coenzyme Q10 (CoQ10, also referred to as ubiquinone) constitute the second major antioxidant system in ferroptosis. Unlike the xCT-GPX4 system, which combats oxidation by reducing already generated lipid ROS, the FSP1-CoQ10 system functions as an antioxidant by reducing the generation of lipid ROS, as described in the following mechanism. FSP1 utilizes NAD(P)H to reduce CoQ10 to its reduced form, Coenzyme Q10H2 (CoQ10H2, also known as ubiquinol), thereby consuming ROS and reducing the generation of lipid ROS (71, 72).

Dihydroorotate dehydrogenase (DHODH) shares a similar function with FSP1, as it can also reduce ubiquinone to ubiquinol, forming an antiferroptotic mechanism similar to that of the FSP1-CoQ10-CoQ10H2 system, known as the DHODH-CoQ10H2 system, to inhibit ferroptosis (73). The distinction between these two systems lies in their subcellular localization; FSP1 is primarily distributed throughout the plasma membrane and

other nonmitochondrial membranes, whereas DHODH is mainly localized within the mitochondria (73). However, both systems work in coordination with their respective xCT-GPX4 systems to counteract the occurrence of lipid peroxides (74).

In the case of the GTP cyclohydrolase-1 (GCH1) -tetrahydrobiopterin (BH4) pathway, GCH1 is a critical enzyme in BH4 biosynthesis, and both play essential roles in ferroptotic antioxidant mechanisms. BH4 can promote the synthesis of CoQ10, indirectly counteracting oxidative stress through the FSP1-CoQ10 system (75). Cells overexpressing GCH1 were found to possess two polyunsaturated fatty acyl chains, a structural feature that significantly protects against the depletion of PUFAs in ferroptosis, serving as a physical defense mechanism against ferroptosis (76).

NAD(P)H:quinone oxidoreductase 1 (NQO1), an NAD(P)H-dependent quinone reductase similar to FSP1, can synergistically promote the conversion of ubiquinone to ubiquinol, inhibiting oxidation (77, 78). However, NQO1 is substrate dependent, and depending on the substrate, it may either promote or reduce ROS generation, suggesting that the inhibitory effect of NQO1 on oxidation may be unstable (79).

Therefore, as mentioned above, the FSP1-CoQ10 system clearly plays a crucial role in the antioxidant mechanism of ferroptosis by collaborating with multiple antioxidant systems, and this system holds significant research prospects.

2.4.3 Other antioxidant systems

In the crucial xCT-GPX4 antioxidant system, Cys not only participates in the classic xCT-GPX4 pathway of antioxidation but also promotes the synthesis of sulfane sulfur (S°), thereby enhancing the antioxidative mechanism (80). Cys serves as the primary source of intracellular elemental sulfur (S (0)) and contributes to the biosynthesis of hydrogen sulfide, hydrogen polysulfides, and polysulfides, among other S° species (81). Among these, hydrogen sulfide, in particular, is a potent ROS scavenger capable of reducing lipid ROS generation to inhibit ferroptosis (80).

Furthermore, some mechanisms counteract ferroptosis by inhibiting the synthesis of lipid ROS. A previous study revealed that the gene encoding the phospholipid transporter SLC47A1 can be activated by the transcription factor peroxisome proliferator-activated receptor α (PPARA). And the activated PPARA-SLC47A1 pathway inhibits the production of esterified PUFAs, namely, cholesterol esters (CEs), to prevent lipid peroxidation in ferroptosis (82). Additionally, LOXs, a critical driver of lipid ROS formation, can be targeted to prevent ferroptosis. For example, research has shown that inhibiting ALOX15 in cancer-associated fibroblasts (CAFs) can block lipid ROS production, thereby suppressing ferroptosis (83).

2.5 Mammalian target of rapamycin

The mTOR pathway is a central regulator of cell growth, autophagy, and metabolism, comprising two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (84). While mTORC2 is not well understood, mTORC1 has become a central regulator of cell metabolism, proliferation, differentiation, autophagy, and immune responses (85). The compositional differences between mTORC1 and mTORC2 influence their sensitivity to rapamycin, rapamycin have shown therapeutic potential, though combination therapies may be required to overcome resistanc (86). The phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, also called Akt)/mTOR (PAM) signaling pathway is activated by immune stimulation and is tightly regulated at multiple levels to prevent uncontrolled cellular proliferation (87). phosphatase and tensin homolog (PTEN), as a lipid phosphatase, dephosphorylates phosphatidylinositol-3,4,5–trisphosphate (PIP3) back to phosphatidylinositol-4,5-bisphosphate (PIP2), negatively regulating AKT signaling (87). Additionally, the tuberous sclerosis complex (TSC) acts as a negative regulator of mTORC1 by inhibiting ras homolog enriched in the brain (Rheb), a GTPase that activates mTORC1 (88, 89). adenosine 5'-monophosphate-activated protein kinase (AMPK) is activated due to low ATP levels, it inhibits mTORC1 both directly through phosphorylation and indirectly by activating the TSC (90, 91). Feedback loops within the pathway also ensure that mTORC1 activation suppresses upstream signaling to prevent excessive cell growth and maintain metabolic balance (87).

In LN, hyperactivation of mTOR signaling has been linked to glomerular damage, mesangial proliferation, and immune cell dysregulation (92). Recent studies show that cordyceps proteins (CP) modulate the mTOR pathway in LN, significantly reducing interleukin-6 (IL-6) and interleukin-1β (IL-1β) levels (93). Pharmacological inhibition of mTOR, such as with rapamycin, mycophenolate, etc., has demonstrated renoprotective effects in LN, underscoring its therapeutic potential (92, 94, 95). Research has also found that magniferin (MG) and Astragali Radix downregulate the mTOR pathway, thereby restoring T cell imbalance in LN (67, 96). These compounds may offer therapeutic potential by modulating the immune response and reducing inflammation in LN through their effects on mTOR signaling.

In LN, the activated T-cell receptor (TCR) regulates multiple metabolic pathways through mTOR, enabling T cells to undergo metabolic reprogramming from fatty acid oxidation and pyruvate oxidation metabolic patterns to glycolysis and glutaminolysis (97–99). During this process, changes associated with ferroptosis metabolism, such as increased lipid synthesis and ROS production (100), accumulation of labile iron (101), and weakened glutathione metabolism (102), occur within T cells (103, 104). Therefore, targeting the mTOR pathway in LN may not only help restore T cell balance but also reduce ferroptosis in T cells, providing a potential therapeutic strategy for managing LN (102).

2.6 NAD(P)H

NAD(P)H can serve as a biomarker for determining sensitivity to ferroptosis (105). The pentose phosphate pathway is the primary source of NAD(P)H, NAD(P)H plays a crucial role in ferroptosis regulation by providing reducing equivalents for antioxidant

defense mechanisms (106). For instance, NAD(P)H provides hydrogen ions to convert cystine into cysteine, potentially influencing GSH production and promoting the xCT-GPX4 antioxidant system, which inhibits ferroptosis (107, 108). NAD(P)H functions through the FSP1-CoQ10-NAD(P)H pathway, alongside GPX4 and glutathione, to prevent phospholipid peroxidation (72). The study reveals that the mechanism of NAD(P)H in ferroptosis involves the membrane-associated RING-CH-type finger 6 (MARCHF6) E3 ubiquitin ligase in the transmembrane endoplasmic reticulum interacts with NAD(P)H through its C-terminal region, enhancing its activity and reducing ferroptosis (109). However, NAD(P)H can also induce ferroptosis. Electrons from NAD(P)H are transferred to oxygen by oxidoreductases, generating hydrogen peroxide, which then reacts with iron in the Fenton reaction, promoting ferroptosis (110). Therefore, NAD(P)H, as a key double-edged regulator of ferroptosis, plays a crucial role by supporting antioxidant defense mechanisms to suppress ferroptosis while also contributing to its occurrence through the generation of reactive oxygen species.

NAD(P)H-mediated ROS contributes to the immune imbalance observed in LN. Superoxide production, driven by NOX, is elevated in LN, contributing to the occurrence of ferroptosis (111). However, ROS from NOX are also involved in efferocytosis, enhancing the removal of dead cells and decreasing antigen production by influencing pH levels and proteolysis in efferosomes (112). Additionally, recent studies have demonstrated that NOX plays a role in SLE immunomodulation through its activity in the myeloid compartment and its selective inhibition of TLR7 signaling in B cells (113).

3 The role of T-cell ferroptosis in immune regulation

3.1 T-cell ferroptosis and immune regulation in cancer

The immune regulatory mechanism associated with metabolic goals related to T-cell ferroptosis has attracted early interest in the area of tumor research (Figure 2). Inducing ferroptosis in CD8+ T cells is a vital tactic used by tumor cells to evade immune surveillance since it is the primary mechanism by which these cells are eliminated (114). CD8+ T cytotoxic (Tc) cells can be classified into many subtypes, such as Tc1, Tc2, Tc9, Tc17, and Tc22 cells. The Tc1 fraction is recognized as the conventional cytotoxic T lymphocyte (CTL) fraction and functions as the main effector subtype of CD8+ T cells (115). Furthermore, studies have shown that activated CD8+ T lymphocytes are highly susceptible to ferroptosis when they are present in the tumor microenvironment (TME) (116). Research has shown that upregulation of the fatty acid receptor CD36 on CTL results in increased production of PUFAs inside the cells. This, in turn, facilitates the ferroptosis of CTL

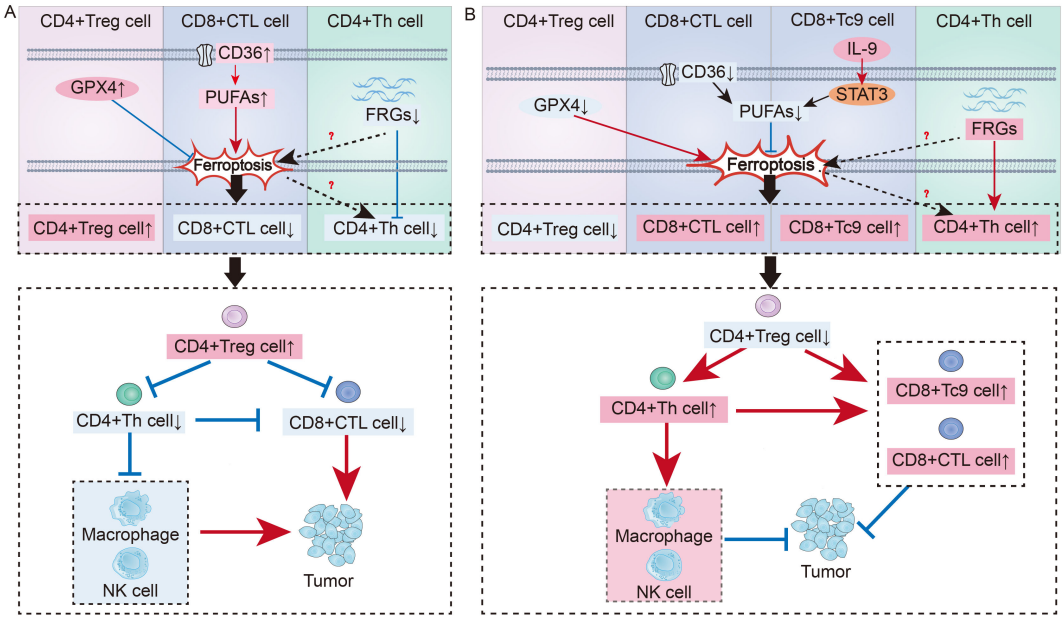

FIGURE 2
Immune regulation of T cell ferroptosis in cancer: **(A)** Mechanisms of T Cell Ferroptosis in Promoting Tumor Growth: In the tumor microenvironment, upregulation of Gpx4 in CD4+ Treg cells inhibits ferroptosis, leading to an increase in CD4+ Treg cells. Upregulation of the CD36 receptor on the surface of CD8+ CTL enhance intracellular PUFAs synthesis, promoting ferroptosis and resulting in a decrease in CD8+ CTL. Suppression of FRGs in CD4+ Th cells can promote ferroptosis, leading to a reduction in CD4+ Th cells. Thus, T cell immune imbalance regulated by ferroptosis contributes to tumor development. **(B)** Mechanisms by Which T Cell Ferroptosis Suppresses Tumor Growth: Downregulating Gpx4 in Treg cells can promote ferroptosis, increasing the number of CD4+ Treg cells. Blocking the CD36 receptor on CTL can inhibit ferroptosis, leading to an increase in CTL. Tc9 can suppress PUFAs synthesis through the IL-9-STAT3-fatty acid oxidation pathway, avoiding ferroptosis. After the suppression of FRGs in Th cells is lifted, it can promote an increase in Th cells. By reversing the T cell immune imbalance in the tumor environment, tumor development can be inhibited.

(3, 117). By blocking CD36 or suppressing CD8+ T cell ferroptosis, it is possible to effectively restore their antitumor function.

Additionally, recent research has revealed the role of ferroptosis in other CD8+ T cell subsets in tumor immune regulation. Among the CD8+ effector T-cell subsets, Tc9 cells are characterized by IL-9 secretion (118). Although significantly fewer in number than CTL, they possess a tumor-killing capability per cell comparable to that of individual CTL (118). Notably, unlike CTL, Tc9 cells in the TME can upregulate fatty acid oxidation through the IL-9-STAT3-fatty acid oxidation pathway, reducing the accumulation of PUFAs and preventing ferroptosis (119). Given that Tc9 cells are less prone to ferroptosis in the TME than are CTL and have a higher survival rate, adoptive replenishment of Tc9 cells is expected to be a new therapeutic target for treating tumors.

In addition to enhancing the cytotoxic impact of CD8+ effector T cells, CD4+ Th cells also stimulate B cells and other effector cells to exhibit antitumor effects (120–122). Research has shown that in the gastric cancer TME, inhibiting the expression of ferroptosis-related genes (FRGs) in CD4+ Th cells weaken their activation, which is associated with poor tumor prognosis (123). Conversely, relieving the suppression of FRGs enhances the activation of CD4+ T cells in GC patients, improving the outcomes of immunotherapy. Therefore, ferroptosis in CD4+ Th cells is closely linked to immune imbalance, but the specific regulatory mechanisms require further investigation for clarification.

Furthermore, the high infiltration of Treg cells in cancer patients often indicates a low survival rate, as Treg cells play a key role in tumor immune evasion by helping tumor cells withstand antitumor immune responses (124). Several studies have shown that Treg cells in tumors exhibit increased GPX4, which is positively correlated with Treg cell survival (2, 125). Moreover, Treg cells lacking Gpx4 can not only induce ferroptosis in Tregs but also enhance the antitumor effect of Th17 cells through increased IL-1β, both of which are mechanisms that inhibit tumor progression (2). However, if lipid peroxides are further neutralized or iron chelators are used, Treg cells can regain their protumor survival state of high infiltration (2). Therefore, targeting Treg cell GPX4 or promoting ferroptosis in Treg cells could be key to improving tumor immunotolerance (Figure 2).

3.2 T-cell ferroptosis and immune regulation in other diseases

In recent years, the immune regulation of T-cell ferroptosis has also garnered attention in other diseases. Studies have shown that in autoimmune encephalomyelitis, inhibiting GPX4 promotes an excess of pathogenic T cells (126). Therefore, enhancing the xCT-GPX4 axis or inhibiting other metabolic pathways that cause lipid peroxidation in pathogenic T cells could be a new therapeutic direction to improve the prognosis of patients with autoimmune diseases. Research has shown that during external pathogen infection, the xCT-GPX4 antioxidant system helps to suppress the occurrence of ferroptosis in effector CD8+ and CD4+ T cells, promoting their expansion and enabling them to mount an immune response against pathogen invasion (4). Studies have also shown that by inhibiting mitochondrial ROS accumulation and promoting GPX4 effects, ferroptosis in memory CD4+ T cells can be suppressed, thereby contributing to long-term viral immune protection (127). In summary, metabolism related to T-cell ferroptosis and its key enzymes are critical targets for regulating immune balance in diseases and warrant further study.

4 Ferroptosis and T-cell immune imbalance in LN

LN is characterized by immune imbalance as an autoimmune disease. In LN, there is often an immune imbalance characterized by the upregulation of TFH cells, Teff cells and CD8+ effector T cells, along with the downregulation of Treg cells (128, 129). Inducing ferroptosis in B cells has been shown to reduce plasma cell formation and antibody production, greatly improving the prognosis of SLE (130). The cell debris produced by neutrophil ferroptosis is a stable source of autoantigens in SLE, and inhibiting neutrophil ferroptosis helps to alleviate SLE (70). However, recent studies provide a direct challenge to the concept that NETs promote autoimmunity and target organ injury in SLE (131).

T-cell ferroptosis also plays an immunoregulatory role has garnered significant attention. Research in LN patients has shown that iron accumulation within T cells promotes gene transcription by driving DNA hydroxymethylation and demethylation, thereby facilitating CD4+ T cell activation, which exacerbates lupus manifestations (132). Targeting excessive iron uptake in T cells could improve outcomes in SLE patients (133). Moreover, an *in vitro* study revealed that the maturation of peripheral T cells is closely related to T-cell ferroptosis (134). Research has shown that T-cell GPX4 deficiency can induce ferroptosis and inhibit T-cell proliferation (4). In the LN, to fulfill their corresponding effector functions, various differentiated T-cell subsets need to utilize all reprogrammed metabolic pathways to varying degrees (11, 135, 136). And the distinct metabolic pathway preferences of different T-cell subsets lead to changes in ferroptosis-related metabolism within their respective cells (137). Therefore, ferroptosis-related metabolism regulate abnormalities in the activation, development, maturation and differentiation of T cells in the LN (Figure 3).

Below, we discuss the connection between immune imbalance and ferroptosis-related metabolism in different T-cell subsets in the LN, including TFH, Treg, CD8+ T and Teff cells, and summarize the potential immunoregulatory mechanisms of T-cell ferroptosis in the LN.

4.1 TFH cell

Upon activation, naïve T cells differentiate into Teff in the LN, which are promptly dispatched to sites of inflammation to mount an immune response, whereas differentiated TFH cells remain within the lymph nodes or lymphoid follicles, specifically the germinal centers (GCs) (138). The characteristic phenotype of TFH cells includes the expression of C-X-C chemokine receptor type 5 (CXCR5), induced T cell costimulator (ICOS), programmed

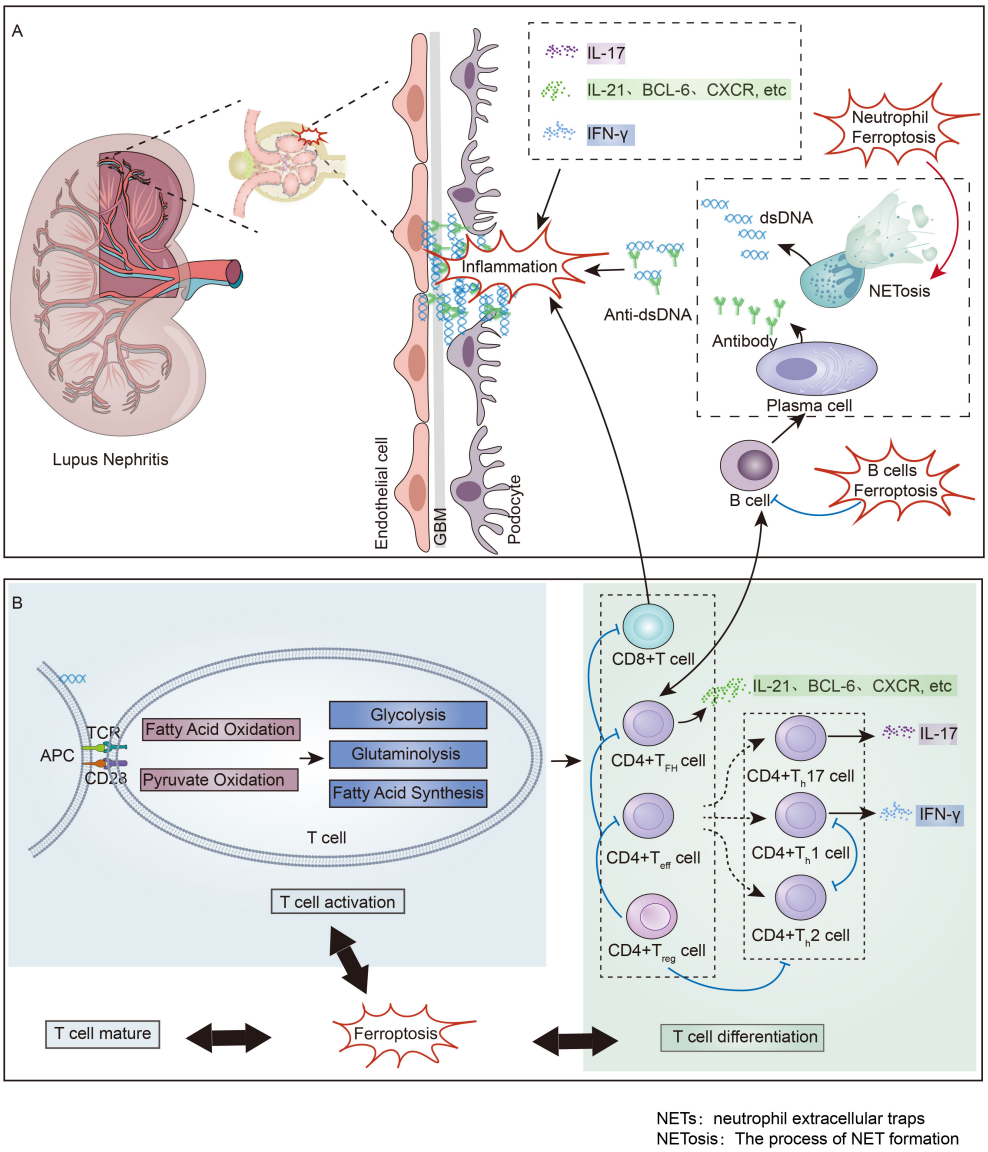

FIGURE 3
Immune Imbalance and Ferroptosis in Lupus Nephritis: **(A)** Non-T cell Immune Imbalance and Ferroptosis in Lupus Nephritis. Neutrophil ferroptosis induces the formation of NETs, which promotes the production of a large number of pathogenic antigens. These antigens stimulate B cells to activate into plasma cells and secrete antibodies, and the deposition of antibody-antigen complexes in the kidney leads to the onset of lupus nephritis. B cell ferroptosis has been found to suppress B cells, thereby inhibiting the occurrence of lupus nephritis. **(B)** T cell Immune Imbalance and Ferroptosis in Lupus Nephritis. Following the deposition of antibody-antigen complexes in the kidney, T cells in the kidney are promoted to become activated and differentiated, ultimately secreting various inflammatory factors that advance renal inflammation. Ferroptosis regulates the state of T cell immune imbalance by participating in the maturation, activation, and differentiation processes of T cells. NETs, neutrophil extracellular traps; NETosis, The process of NET formation.

cell death protein 1 (PD-1), B-cell lymphoma 6 (Bcl-6), and interleukin-21 (IL-21), which are integral to regulating the differentiation of TFH cells and the formation of GCs (139). The interaction between CXCR5 and its ligand C-X-C chemokine ligand 13 (CXCL13) facilitates the migration of TFH cells to GCs and promotes sustained activation of B cells within GCs (140). BCL-6 is the master transcription factor for TFH cells, leading to the differentiation of naïve T cells into TFH cells. Moreover, BCL-6 plays a crucial role in activating and differentiating B cells and forming GCs by regulating various target genes involved in antigen-triggered calcium signaling within TFH cells (141). Upon binding to its receptor on TFH cells, the cytokine IL-21 upregulates the expression of BCL-6, CXCR5, and ICOS through the janus kinase (JAK)- signal transducer and activator of transcription (STAT) axis, indirectly promoting the migration of TFH cells and the production of pathogenic antibodies (139). Studies have shown that targeting the autocrine cytokine IL-21 in the TFH cells of mouse models of SLE can help suppress the proliferation and development of TFH and Th17 cells, providing a treatment for SLE (142). Therefore, the upregulation of TFH cells in the LN, through high expression of the aforementioned phenotypes, can promote the migration of TFH cells to GCs and foster sustained reciprocal stimulation between

TFH cells and B cells within GCs (142–144). And the upregulation of TFH cells is a significant factor in the production of pathogenic antibodies in LN and accelerates the progression to end-stage renal disease in patients with LN (145).

Iron is an important element in promoting the normal differentiation of T cells and maintaining normal metabolism (146). Recent studies have shown that miR-21 overexpression in CD4+T cells promote iron accumulation by inhibiting 3-hydroxybutyrate dehydrogenase 2 (BDH2) in lupus-susceptible mice. Cellular iron accumulation can promote BCL6 gene hydroxymethylation by enhancing Fe2+-dependent TET enzyme activity in TFH cells, thereby promoting TFH cell differentiation (147). Given that the accumulation of intracellular iron not only is crucial for TFH cell differentiation but can also induce ferroptosis, whether the balance between these two factors can be modulated to improve TFH cell immune imbalance has attracted increasing attention (148).

Upon TCR activation, there is a marked increase in intracellular ROS within T cells, which is primarily mediated by the mTOR pathway, which represents the predominant source of ROS (149). Studies have revealed that activated TFH cells contribute to elevated ROS levels not only through intrinsic TCR signaling but also through sustained interaction with B cells in GCs, thereby increasing ROS production (99, 150). Furthermore, these activated TFH cells accumulate lipid peroxides, and mitochondrial alterations are consistent with a ferroptosis phenotype (150). Advanced investigations have shown that specific deletion of GPX4 in mouse T cells accelerates the depletion of TFH cells and attenuates the TFH-B-cell interaction within GCs, leading to reduced production of pathogenic antibodies. Conversely, selenium supplementation has been found to reverse this trend (150). Thus, the selenium-GPX4-ferroptosis axis is a central regulator of TFH cell immune homeostasis. However, whether this axis can modulate immune imbalances caused by increased TFH cells in the LN remains to be confirmed by further research (Figure 4).

So far, clinical studies highlight the potential of targeting TFH cells for LN treatment. Recent clinical studies have shown that a CXCR5-directed antibody promotes the depletion of TFH cells in SLE, demonstrating its clinical potential for treating autoimmune diseases (151). Furthermore, follicular regulatory T (TFR) cells localize to the GC where TFH cells reside by expressing CXCR5 and FoxP3 and regulate their function (152). A clinical study found that PD-1 on TFR cells promotes hypermethylation in the CNS2 region of the FoxP3 gene, leading to reduced FoxP3 expression and impaired suppressive function (153). However, IL-2 supplementation therapy can restore this lost regulatory function *in vitro* (153). Notably, recent clinical studies have demonstrated the potential of low-dose IL-2 therapy in restoring TFR-TFH regulation *in vivo* (154).

4.2 Treg cell

The primary function of Treg cells is to inhibit hyperactive pathogenic immune responses to maintain immune homeostasis. They exert their immunosuppressive effects by directly or indirectly targeting T cells and B cells, with the expression of Forkhead Box Protein 3 (FOXP3) being essential for sustaining immune tolerance (136). Studies in LN have identified deficiencies in both the number and function of Treg cells (155, 156). Treg cells highly express CD25, and IL-2 can bind to the IL-2 receptor (IL-2R) subunit CD25, which is also known as IL-2Rα (157). IL-2 stimulation is crucial for the maintenance of Treg cells and for the differentiation of CD4+ T cells into specific effector T cell subsets following antigen-mediated activation (158).

The metabolic environment in LN following Naïve T cell activation has been found to be detrimental to Treg cell survival, resulting in the downregulation of Treg cells (156). These changes are closely associated with ferroptosis-related pathways, suggesting that modulating ferroptosis could potentially enhance Treg cell survival and help restore immune balance (128). For instance, the survival and differentiation of Treg cells rely more on fatty acid oxidation and oxidative phosphorylation for energy production, necessitating a metabolic environment in Tregs that generates less lipid ROS (159). Furthermore, research has found that the high expression of thioredoxin-1 in human Treg cells counteracts the oxidizing effects of ROS, resulting in a low ROS environment that favors Treg cell survival (160). Therefore, inhibiting ferroptosis in Tregs appears to be conducive to the metabolic environment required for their proliferation and differentiation. Recent studies in SLE have shown that blocking the cell surface protein CD71 to reduce intracellular iron accumulation and inhibit ferroptosis promotes Treg proliferation (133). In cancer-related research, upregulation of GPX4 has been identified as a key mechanism to inhibit ferroptosis in Treg cells and promote their survival (2, 161). Hence, in LN, enhancing GPX4 activity or suppressing the metabolic processes that lead to lipid ROS production in Treg cells could likely be a critical mechanism to promote Treg proliferation and differentiation, representing an important target for improving LN prognosis.

A prospective study found that vitamin D treatment, currently used in LN therapy, is beneficial for increasing Treg cells in SLE patients (162). In SLE patients, Treg cell dysfunction has been found to result from a persistent decrease in Foxp3 expression in Tregs, mediated by the involvement of the OX40L/OX40 axis (163). In a recent Phase I trial, infusion of umbilical cord-derived mesenchymal stromal cells (MSCs) led to an increase in Treg cells in SLE patients, showing effectiveness in treating lupus (164, 165). Since CD25 is the high-affinity subunit of IL-2R, low-dose IL-2 preferentially expands Treg cells, while higher doses further stimulate effector T cells and NK cells (158). Phase II clinical trials have confirmed that low-dose IL-2 is beneficial for active SLE by upregulating Treg cells (166, 167). These researchs demonstrate the potential of Treg cell therapies for treating LN.

4.3 CD4+ Teff cell

In LN, an increased immune imbalance involving Teff cells, including Th17, Th1, and Th2 cells, is an important factor that promotes inflammatory immune responses (168). Among these factors, the significant increase in Th17 cells is a crucial pathogenic mechanism in the renal damage experienced by LN patients (169).

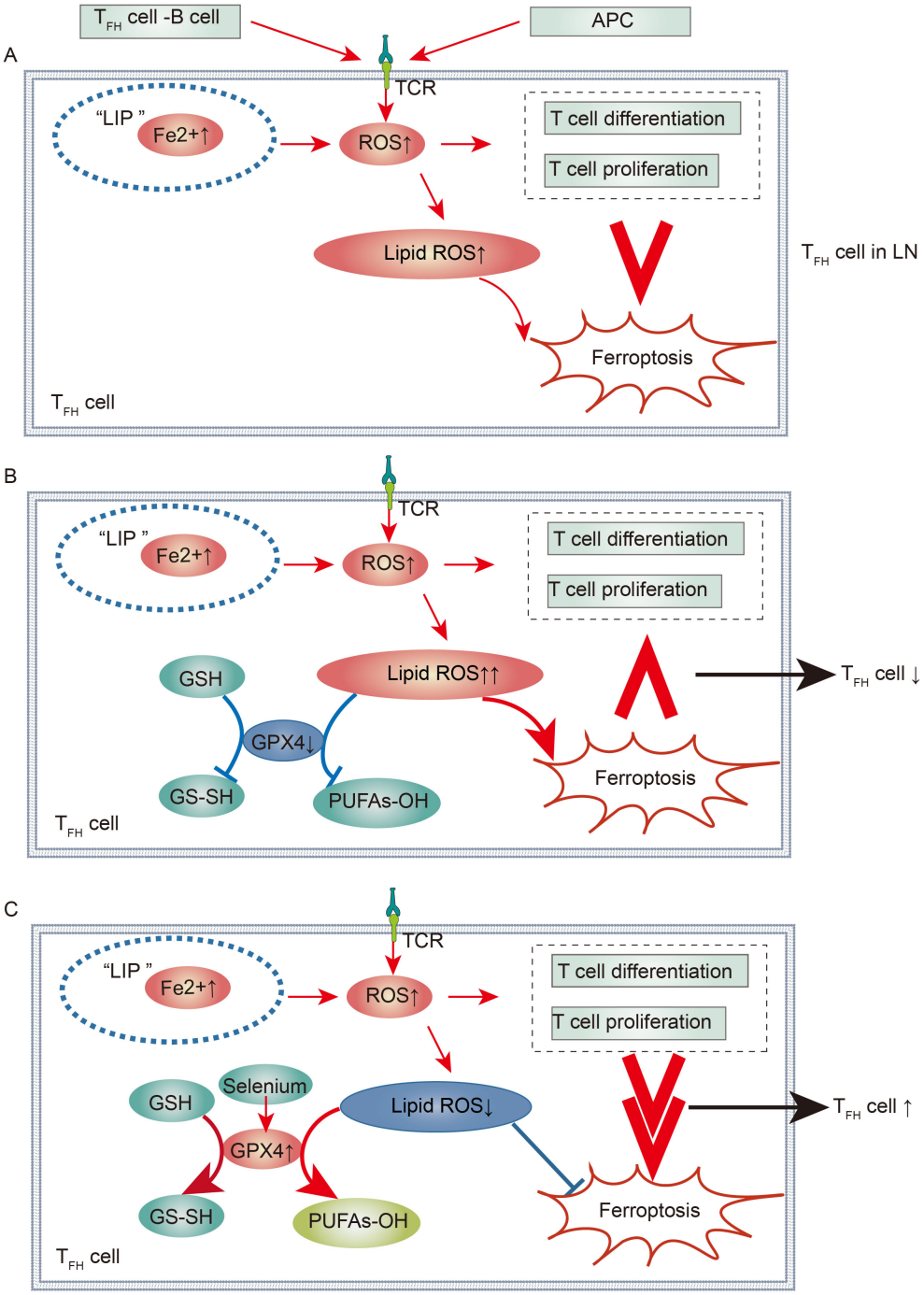

FIGURE 4
Immunological Regulation of Ferroptotic TFH Cells in Lupus Nephritis: **(A)** TFH Cells in Lupus Nephritis. In the context of lupus nephritis, the interaction between TFH and B cells, along with TCR stimulation activation, leads to a significant increase in ROS within TFH cells. Concurrently, there is an increase in Fe uptake within TFH cells. This not only promotes TFH cell differentiation and proliferation but also ferroptosis. However, the overall effect of ferroptosis is less than that of promoting differentiation and proliferation, resulting in a large number of TFH cells contributing to the progression of lupus nephritis. **(B, C)** Regulation of TFH Cell Immune Imbalance in Lupus Nephritis via the Selenium-GPX4-Ferroptosis Axis. By downregulating GPX4, ferroptosis can be promoted, making its effect greater than that of promoting differentiation and proliferation, leading to a reduction in TFH cells. Conversely, by increasing selenium to upregulate GPX4 activity, ferroptosis can be inhibited, thus diminishing its effect compared to differentiation and proliferation, resulting in an increase in TFH cells.

The differentiation of Th17 cells in the LN requires the cytokines IL-6 and transforming growth factor β (TGFβ), which activate the lineage-defining transcription factor receptor–related orphan nuclear receptor γt (RORγt) through the JAK-STAT pathway (170–172). Furthermore, the cytokine interleukin-23 (IL-23) is vital for the expansion and survival of pathogenic Th17 cells in LN by activating the STAT-3 pathway (173). Inflammatory cytokines secreted by Th17 cells, such as interleukin-17A (IL-17A),

interleukin-17F (IL-17F), and interleukin-22 (IL-22), are key factors that drive the progression of autoimmune kidney diseases (174). Several studies have shown that the Th17/IL-17 axis significantly contributes to structural and functional renal damage in lupus nephritis by fostering proinflammatory environments and activating profibrotic pathways through cytokine secretion (175, 176).

In healthy organisms, Th1 cells secrete interleukin-12 (IL-12) and interferon-gamma (IFN-γ) to exert cellular immunity to prevent bacterial and viral invasion (177). Th2 cells secrete interleukin-4 (IL-4), IL-6, and interleukin-4 (IL-10) to perform humoral immunity against helminths and other extracellular microbes (177). Research has shown that IFN-γ secreted by Th1 cells inhibits Th2-related functions, whereas IL-4 and IL-10 secreted by Th2 cells suppress Th1-related functions (178, 179). In LN, an increasing body of research has demonstrated that a Th1 differentiation advantage, which enhances cellular immune attack on healthy tissue cells, is involved in the progression of LN (180, 181). Moreover, inflammatory cytokines secreted by Th1 cells promote the formation of an inflammatory environment in the LN (182–184). Specifically, autocrine interleukin-12 (IL-12) from Th1 cells can promote self-differentiation of Th1 cells and, together with T-bet and the transcription factors STAT4 and STAT1, drive the production of the major inflammatory cytokine IFN-γ in lupus nephritis (185).

Research has revealed that in mammals, there are two types of mTOR protein complexes: mTORC1 and mTORC2 (186). mTORC1 promotes the differentiation of Th1 cells and Th17 cells, whereas mTORC2 mediates the differentiation of Th2 cells (187). In lupus patients, studies have shown that inhibiting the mTORC1 pathway contributes to disease outcome (92, 188). Therefore, the mTORC1 pathway is crucial for the pathogenesis of LN, and it promotes the formation of an immune imbalance characterized by the upregulation of Th17 cells and a Th1 cell differentiation advantage in LN. In LN, since the mTOR protein complex pathway is closely related to cellular metabolism, it affects various Teff cells, including the synthesis of PUFAs, ROS production, and other ferroptosis-related metabolic changes (189, 190). Thus, whether ferroptosis can regulate the Teff immune imbalance through ferroptosis metabolic targets is worth investigating.

In LN, the hyperactivated PI3K/Akt/mTOR pathway mediates high glycolytic metabolism and an elevated glutaminolysis pattern, which promotes the hyperactivity of synthetic metabolic processes such as protein, lipid, and carbohydrate metabolism within cells, fostering the differentiation and proliferation of Teff cells (128, 133). Studies have shown that the glucose transporter 1 (Glut1) is expressed on Teff cells, which can further increase the glycolytic metabolism of Teff cells (191). Further research revealed that calcium/calmodulin-dependent protein kinase IV (CaMK4) promotes the glycolytic process in Th17 cells by stimulating the Akt/mTORC1 pathway and upregulating Glut1 (192). Additionally, studies have shown that the mTOR pathway promotes the expression of hypoxia-inducible Factor 1α (HIF1α) in Th17 cells, enhancing glycolysis (193). Compared with other Teff cells, Th17 cells present high glycolytic metabolism, which significantly promotes iron death-related metabolic changes, such as increased ROS production and increased synthesis of PUFAs, which are positively correlated with the differentiation trends of each Teff cell type.

An *in vitro* study revealed that glutaminolysis is crucial for Th17 inflammatory diseases such as LN. Owing to the different dependencies of Teff cells on the glutaminolysis pathway, glutaminolysis promotes the differentiation of Th17 cells but constrains the differentiation of Th1 cells and CTLs (194). The glutaminolysis induced by mTOR leads to changes in the accumulation of ROS in Teff cells (191). Initially, the breakdown of glutamine in Teff cells yields glutamate. Glutamate can subsequently increase the synthesis of GSH to inhibit the production of ROS (195). Moreover, glutamate can be further metabolized to produce α-ketoglutarate (α-KG). α-KG can promote ROS generation through the tricarboxylic acid cycle to enhance the mTOR-mediated metabolic pathway, and it can also alter chromatin accessibility by affecting histone methylation, thereby promoting cell differentiation (196). Therefore, the differentiation of various Teff cells promoted by glutaminolysis is positively correlated with the accumulation of ROS within these cells.

In summary, the differentiation trends of Teff cells are positively correlated with the accumulation of ROS and PUFAs within these cells. The antioxidant gene nuclear factor erythroid 2-related factor 2 (NRF2) can significantly inhibit the differentiation of Th17 cells in LN by suppressing ROS, thereby improving prognosis, which also confirms this point (197). Notably, these metabolic processes not only promote the differentiation and proliferation of Teff cells but also facilitate the occurrence of ferroptosis. Moreover, recent studies have shown that the expression of the transferrin receptor CD71 on the surface of Teff cells in SLE is significantly increased, with noticeable accumulation of intracellular iron (133). Past research has indicated that different Teff cells have varying levels of labile iron stores, which may lead to inconsistent regulation of ferroptosis among these cells (198). Notably, recent studies have shown that activated CD4+ Teff cells do not exhibit the characteristic changes in lipid ROS deposition associated with ferroptosis as much as TFH cells do, which may be related to TFH cells having more sources of ROS (148, 150). Similarly, since Th17 cells accumulate more ROS and PUFAs than other Teff cells do, promoting an increase in lipid ROS or inhibiting antioxidant mechanisms seems to be more conducive to the occurrence of ferroptosis in Th17 cells. Promoting Th17 cell ferroptosis is a promising therapeutic direction for LN and warrants further investigation.

Studies have found that in SLE patients, CaMK4 inhibits the transcription of IL-17A and IL-17F through dual mechanisms, by suppressing the activation of CREMα and the AKT/mTOR pathway, thereby indirectly inhibiting Th17 differentiation (199). After 6 months of vitamin D supplementation in SLE patients, it not only has favorable clinical effects on SLE, but also produces beneficial immunological effects by promoting a decrease in Th1 and Th17 cells in these patients (162, 200). Ustekinumab is a monoclonal antibody targeting the shared p40 subunit of IL-12 and IL-23 (201). IL-12 promotes the differentiation of Th1 cells and the secretion of IFN-γ, while the IL-23/Th17 axis plays a key role in the development of lupus (202). After ustekinumab treatment in

SLE patients, a significant reduction in the IFN-γ response was observed, but no modulation of Th17-related genes was detected (202). Currently, the potential of Teff cell-driven treatment strategies for LN is significant, but many aspects remain unexplored and require further investigation.

4.4 CD8+ T cell

In LN, an increase in CD8+ T cells suggest a poor prognosis (203, 204). Studies have shown that in patients with juvenile-onset SLE, there is a significant increase in total CD8+ T cells and naïve CD8+ T cells, whereas effector memory CD8+ T cells are decreased (205). A clinical study indicated that the expansion of CD8+ memory T cells was associated with a poor prognosis for patients with LN (206). In LN, classic CD8+ effector T cells, namely, CTL, have been found to have defects in their cytotoxic function. This not only promotes autoimmune hyperactivity but also facilitates the invasion of pathogens (207). Moreover, the potential effects of other nonclassical CD8+ effector T cell subpopulations in the LN are beginning to receive attention (208–210). For example, subpopulations with effects similar to those of Treg cells have been identified, and increasing their numbers may become a new treatment method for LN (208, 211). Research has also revealed that effector CD8+ T cells characterized by high expression of granzyme K (GzmK) and low expression of granzyme B (GzmB) and perforin have relatively weak cytotoxic effects, driving the development of LN inflammation through the secretion of cytokines (207, 212, 213). Therefore, molecules that regulate the immune balance of CD8+ T cell subpopulations and related signaling pathways are potential therapeutic targets for LN.

The mTORC1 pathway affects the response of CD8+ effector T cells, whereas mTORC2 activity regulates memory CD8+ T cells (214). Therefore, metabolic processes within CD8+ T cells in the LN promote ferroptosis caused by the accumulation of lipid ROS and enhance cell differentiation. Studies have shown that Gpx4 is a major factor for the survival of peripheral CD8+ T cells in the TME and that ferroptosis induced by GPX4 deficiency can limit the expansion of CD8+ T cells (4). Research has also demonstrated different sensitivities to ferroptosis among CD8+ T cell subpopulations (119). Thus, metabolic targets of ferroptosis, such as GPX4, are likely key in regulating the proportions of different CD8+ T cell subpopulations and suppressing the expansion of pathogenic CD8+ T cells in the LN. However, the regulatory mechanisms of ferroptosis in various subpopulations of CD8+ T cells in the LN are still under exploration and hold great promise.

In clinical studies of SLE patients, IFN-γ produced by CD8+ T cells is a key factor in enhancing indoleamine 2,3-dioxygenase (IDO) activity, which promotes the therapeutic effect of allogeneic MSCs in lupus (215). CD8+ T cells are the primary producers of IFN-γ in LN (216). Notably, the monoclonal antibody AMG 811, which targets IFN-γ, has demonstrated limited and transient effects in LN patients (217). Currently, the effects of mTOR inhibitors on CD8+ T cells have been identified in other diseases. For instance, Everolimus, an mTOR inhibitor, has been shown to significantly reduce the abundance and proliferation of CD8+ CD28- effector memory T (TEM) cell in post-kidney transplant patients, thereby decreasing the progression of inflammation (218, 219). Given the importance of mTOR in CD8+ T cells, clinical research on its role in CD8+ T cells remain lacking.

5 Targeted ferroptosis in the treatment of LN

5.1 Feasibility of targeting ferroptosis in LN patients

Targeting ferroptosis in LN could offer a novel and more precise approach compared to traditional therapies. One potential strategy involves the use of ferroptosis inhibitors, such as ferrostatin-1 and liproxstatin-1, which are known to prevent lipid peroxidation by inhibiting the enzyme system responsible for ferroptosis (220, 221). These inhibitors have been shown to attenuate kidney damage and experimental models of SLE, suggesting their potential for clinical application (70).

Another promising approach is the modulation of iron metabolism. Iron chelators, such as deferasirox, have been used in various diseases to reduce iron overload and prevent ferroptosis (222, 223). In the context of LN, iron chelation may help decrease iron overload, thereby reducing ferroptosis-associated kidney injury (68, 224, 225). Additionally, agents such as Erastin, sulfasalazine (SSZ), and BSO inhibit the xCT-GPX4 system (226), while allosteric GPX4 activators promote the xCT-GPX4 system (227). Extensive research in diseases like cancer has demonstrated the effectiveness of these approaches (226). Regulating the GPX4 pathways could provide another avenue for therapeutic intervention LN (228, 229).

5.2 Potential side effects and safety considerations

While targeting ferroptosis offers exciting therapeutic potential, there are several important considerations regarding safety and potential side effects. Ferroptosis inhibitors and iron chelators, although effective in preclinical studies, may have off-target effects that need to be carefully evaluated in clinical trials (230). For instance, ferroptosis inhibitors may interfere with the normal functioning of oxidative stress pathways, which play a critical role in cellular defense against pathogens and cancer (231). Inhibition of ferroptosis may impair the ability of immune cells to respond to infections or tumorigenic cells, potentially increasing susceptibility to infections or promoting tumorigenesis (232).

In addition, iron chelation may lead to iron deficiency, which can impair cellular functions, particularly in rapidly dividing cells such as those involved in immune responses and erythropoiesis (233–235). Chronic iron depletion may also result in adverse effects on other organs, including the heart and liver, leading to organ dysfunction (236, 237).

Therefore, a balanced approach to targeting ferroptosis in LN is essential. Therapeutic strategies should aim to specifically modulate ferroptosis in the kidney and immune cells without affecting other

critical physiological processes. Careful monitoring of iron levels, ROS generation, and immune function will be necessary to avoid unwanted side effects (238).

5.3 Current state of therapeutic development

Currently, the development of ferroptosis-targeted therapies for LN is in its early stages, with most studies being conducted in preclinical models. However, several promising strategies are being explored, and early-phase clinical trials are underway. For instance, liproxstatin-1 have shown efficacy in treating autoimmune diseases models, including LN (70). Iron chelators are being evaluated for their ability to reduce albuminuria in LN, but their effects and potential side effects related to ferroptosis and iron metabolism have not yet been fully explored (225).

Furthermore, understanding the immune regulatory role of ferroptosis in LN is critical for optimizing therapeutic strategies. Ferroptosis has been shown to influence the activation and differentiation of immune cells, such as T cells and B cell, which are central to the pathogenesis of LN (130, 147). By modulating ferroptosis in these immune cells, it may be possible to not only mitigate kidney injury but also restore immune tolerance and reduce autoimmunity (133).

6 Conclusions

Lupus nephritis often manifests as an immune imbalance characterized by the upregulation of Teff cells and CD8+ effector T cells, alongside the downregulation of Treg cells, which is related to the abnormal differentiation of various T cells. We further discovered that the cellular metabolism that induces T-cell differentiation in LNs also leads to the accumulation of lipid ROS within each T-cell subset. We found that Treg cell differentiation in the LN is restricted, whereas the intracellular accumulation of lipid ROS promotes ferroptosis. The differentiation of Teff cells in LN and the accumulation of intracellular lipid ROS both exhibit varying degrees of promotion, with a positive correlation observed between these enhancements. Among these, TFH cells have a greater source of ROS, leading to greater accumulation of lipid ROS in activated TFH cells, followed by Th17 cells, with other Teff cells accumulating even less.

Given the close relationship between T-cell ferroptosis metabolic targets and the generation of lipid ROS, we have summarized the potential mechanisms of immunoregulation by ferroptosis metabolic targets in LN. We found that reducing the accumulation of lipid ROS in Treg cells can promote Teff differentiation and inhibit Teff ferroptosis, whereas enhancing the accumulation of lipid ROS in Teff cells can significantly promote Teff ferroptosis, an effect greater than its ability to promote Teff differentiation. Notably, we emphasize the central regulatory role of the selenium-GPX4-ferroptosis axis in the immune dysregulation of TFH cells.

Ferroptosis also plays a role in other autoimmune diseases, including rheumatoid arthritis (RA) and multiple sclerosis (MS), though the mechanisms differ from those in LN. In RA, iron accumulation in synovial fibroblasts and macrophages contributes to the inflammatory environment within the joints (239). Excess iron promotes ROS production, leading to oxidative stress, which damages joint tissues and accelerates disease progression (240). However, RA primarily involves joint inflammation and does not feature the same degree of systemic immune cell dysfunction as in LN (241). In MS, oligodendrocytes is the cells responsible for the formation of myelin sheaths in the central nervous system (242). Iron accumulation in oligodendrocytes contributes to cell death through ferroptosis, impairing myelin regeneration and promoting neurodegeneration (243). While ferroptosis is implicated in the pathogenesis of MS, the disease is more focused on central nervous system damage rather than systemic immune dysfunction. In diseases such as autoimmune thyroiditis, inflammatory bowel disease (IBD), and myasthenia gravis (MG), ferroptosis-related metabolism influences immune cell activation and inflammation (244). Compared to LN, where ferroptosis directly contributes to immune dysfunction and kidney damage, the role of ferroptosis in these conditions is less directly reported.

Ferroptosis in LN has already garnered significant attention from researchers. On one hand, ferroptosis in renal cells during inflammation promotes tissue damage and triggers regional inflammatory responses (68, 245). On the other hand, immune cells, particularly T cells, contribute to persistent inflammation by continuously releasing inflammatory cytokines and phagocytosing healthy renal cells, underscoring their pivotal role in sustaining inflammatory processes in LN (246, 247). Ferroptosis in immune cells has been found to contribute throughout the process (248, 249). Neutrophils ferroptosis contributing to the generation of autoantigens (70). And inducing ferroptosis in B cells has been identified as an optimal strategy to reduce sustained antibody production (130). Although there is no direct evidence linking ferroptosis in T cells to LN, changes in ferroptosis-related metabolic pathways in T cells—including ROS, PUFAs, iron, GPX4, and GSH (100–102)—mediated through the mTOR pathway indirectly suggest a critical role for T-cell ferroptosis in regulating T-cell imbalance in LN (250).

Currently, various novel ferroptosis modulators, such as mitochondrial-targeted nanodrug systems (251), have been used to induce or block ferroptosis. However, the treatment of ferroptosis in LN is still mainly at the animal experiment or limited case study stage. More clinical trials are needed in the future to verify their safety and efficacy. Based on the current understanding of ferroptosis-related metabolic changes in T cells, we propose that ferroptosis-targeted drugs, by modulating ROS, PUFAs, iron, GPX4, GSH, etc., could be more beneficial for targeting T cells. Additionally, since ferroptosis manifests differently in each T cell type, determining the appropriate dose and adjusting drug combinations for inhibiting or promoting ferroptosis in appropriate T cell subset will be a key focus for future research. Currently, there is still much to explore regarding T cell ferroptosis treatment in LN, which warrants further attention.

In summary, research on the role of T-cell ferroptosis in immune regulation within the LN is still in its early stages. A

better understanding of this potential immune regulatory mechanism from the perspective of ferroptosis metabolism will undoubtedly lead to novel therapeutic concepts. Not only could this involve mitigating LN-induced tissue damage by inhibiting the differentiation and survival of Teff cells, but it could also enhance immune tolerance by increasing the number of Treg cells, allowing for a more targeted approach to treating LN. Targeting additional metabolic aspects of T-cell ferroptosis may represent a promising future direction for the treatment of immune imbalance in LN.

Author contributions

YF: Writing – original draft. KM: Writing – original draft. YL: Writing – original draft. JR: Writing – original draft. HP: Writing – original draft. LY: Writing – review & editing. MN: Writing – review & editing. XJ: Writing – review & editing. KW: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Deciphering ferroptosis in critical care: mechanisms, consequences, and therapeutic opportunities

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Ischemia-reperfusion injuries (IRI) across various organs and tissues, along with sepsis, significantly contribute to the progression of critical illnesses. These conditions disrupt the balance of inflammatory mediators and signaling pathways, resulting in impaired physiological functions in human tissues and organs. Ferroptosis, a distinct form of programmed cell death, plays a pivotal role in regulating tissue damage and modulating inflammatory responses, thereby influencing the onset and progression of severe illnesses. Recent studies highlight that pharmacological agents targeting ferroptosis-related proteins can effectively mitigate oxidative stress caused by IRI in multiple organs, alleviating associated symptoms. This manuscript delves into the mechanisms and signaling pathways underlying ferroptosis, its role in critical illnesses, and its therapeutic potential in mitigating disease progression. We aim to offer a novel perspective for advancing clinical treatments for critical illnesses.

KEYWORDS

ferroptosis, critical illness, iron overload, lipid metabolism, mitochondrial dysfunction

1 Introduction

Critical illness, marked by organ-level pathophysiological disruptions, remains a significant global health challenge (1, 2). Despite its impact, intensive care unit (ICU) outcomes are often underrepresented in global disease burden studies. For instance, a 2017 report revealed that sepsis-related deaths accounted for 47% of all fatalities worldwide, underscoring an urgent need for focused research (2, 3). While advancements in critical care have improved symptomatic management—such as organ support and fluid resuscitation—progress in targeted therapies has been limited. Identifying novel mechanisms and therapeutic strategies is, therefore, paramount. Mechanisms linked to metabolic dysregulation, including ferroptosis, autophagy, and oxidative stress—processes

driven by lipid peroxidation and cellular ion imbalances—present promising avenues for innovation in critical care.

Cell death is a fundamental process critical for growth, homeostasis, and the progression of various diseases (2, 4). Programmed cell death pathways, such as necrosis, autophagy, pyroptosis, and apoptosis, operate through well-defined signaling regulatory mechanisms and are closely tied to disease pathophysiology (2, 5, 6). Among these, ferroptosis—an iron-dependent form of regulated cell death triggered by lipid peroxidation—has emerged as a key contributor to critical illnesses, including sepsis, acute respiratory distress syndrome (ARDS), acute kidney injury (AKI), and Ischemia-reperfusion injuries (IRI) (2, 7–9). Growing evidence underscores the importance of targeting ferroptosis to better understand and manage these life-threatening conditions. Iron, an essential trace element in the human body, is involved in numerous biological processes, including energy metabolism and nucleotide synthesis and repair (2, 10). While the concept of ferroptosis dates back to the 1980s, it was formally named by Dixon in 2012 (2, 7, 11). Ferroptosis development is driven by iron-induced reactive oxygen species (ROS), making it susceptible to inhibition by lipophilic antioxidants and agents such as ferritin and iron chelators (2, 12).

Current evidence highlights ferroptosis as a key player in the onset and progression of various diseases, positioning it as a potential target for clinical therapies (Figure 1). This review summarizes the occurrence, characteristics, regulatory mechanisms, and critical molecular pathways of ferroptosis, emphasizing its direct and indirect roles in the etiology of serious disorders. Additionally, we explore potential therapeutic targets linked to ferroptosis, offering new insights for clinical applications in treating critical conditions.

2 Discovery of ferroptosis

In 1980, System Xc^- was identified as a transporter that exchanges glutamate for cystine, enabling cystine entry into cells (2, 13). In 2003, Dolma et al. discovered that NSC146109 (later known as erastin) selectively killed BJeLR cells with mutated Ras oncogenes during high-throughput screening (2, 14). In 2008, compounds like RAS-selective lethal small molecules 3 (RSL3) and RSL5 were identified as non-apoptotic agents that induce cell death (2, 15). This form of cell death is distinct from previously recognized categories, as apoptosis, necrosis, and autophagy inhibitors did not prevent cell death induced by these compounds. However, significant inhibition could be achieved

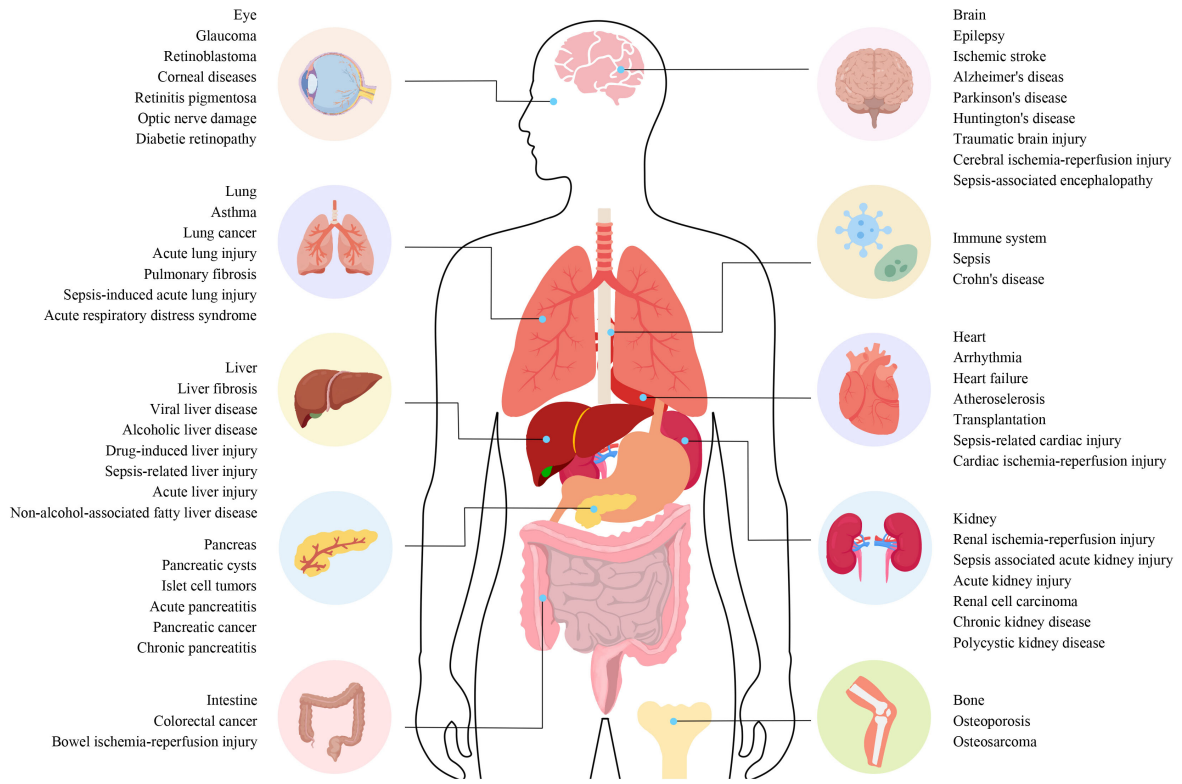

FIGURE 1
The connection between different diseases and ferroptosis. The connection between different diseases and ferroptosis. Ferroptosis plays a role in the regulation of various systemic diseases, such as diseases of the nervous system, cardiovascular system, digestive system, musculoskeletal system, autoimmune system, visual system, lung, liver, and kidney. Ferroptosis often involves systemic interactions, where iron metabolism and oxidative stress affect multiple organ systems. For example, diseases like sepsis can trigger widespread inflammation and organ failure involving ferroptotic pathways in the liver, lungs, kidneys, and cardiovascular system. Understanding these pathways is essential for developing targeted therapies that can mitigate ferroptosis-induced damage across various diseases.

using iron chelators and lipid peroxide inhibitors (2, 16). In 2012, Dixon et al. demonstrated that erastin, a product linked to the Ras oncogene, induced cell death in tumor cells and coined the term ferroptosis for this iron-dependent mode of cell death (2, 11). The concept of ferroptosis gained formal recognition in 2018 when the Cell Death Nomenclature Committee defined it as a potentially regulatory mode of cell death (2, 17).

3 Ferroptosis and other types of cell death

Ferroptosis differs from other forms of programmed cell death by exhibiting distinct morphological features (2, 18). These include a reduction or absence of mitochondrial cristae, rupture of the outer mitochondrial membrane, mitochondrial shrinkage, and an increase in membrane density. While nuclear size remains normal, chromatin condensation is absent, and membrane blebbing occurs without full rupture (2, 19, 20). Biochemically, ferroptosis is marked by elevated iron and ROS levels, decreased glutathione (GSH), and reduced glutathione peroxidase 4 (GPX4) activity. It also involves disruption of the cysteine uptake system, changes in mitochondrial membrane potential, and arachidonic acid-mediated release of functional factors, all leading to lipid peroxidation and mitochondrial dysfunction. Additionally, specific gene expression changes have been observed, and ongoing studies are exploring the relationship between ferroptosis and other forms of cell death (21).

3.1 Ferroptosis and apoptosis

Both ferroptosis and apoptosis are programmed cell death mechanisms, but they differ in their biological characteristics, triggers, and signaling pathways, though there are notable intersections (22). For instance, B-cell lymphoma-2 (Bcl-2) family proteins, key regulators in the mitochondrial pathway of apoptosis, also influence ferroptosis. These proteins interact with Beclin1 (BECN1) to inhibit autophagy and ferroptosis, prevent cytochrome c release from mitochondria in apoptosis to block cell death, and regulate lipid metabolism and redox status in ferroptosis to prevent its occurrence (23, 24). Additionally, in cancer therapy, inducing ferroptosis is considered a strategy to target apoptotic cancer cells, particularly those resistant to conventional apoptosis-inducing treatments.

3.2 Ferroptosis and necroptosis

In some cases, the signaling pathways of ferroptosis and necroptosis converge. For example, under certain stress conditions, receptor-interacting protein kinase 1 (RIPK1) not only mediates necroptosis but can also influence ferroptosis by modulating cellular redox status (25). RIPK1 regulates the activity of antioxidant enzymes, indirectly affecting lipid peroxide levels and either promoting or inhibiting ferroptosis (26). During ferroptosis, cell membrane rupture releases several damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1), which activate immune cells and trigger inflammation. Necroptosis, an inflammatory mode of cell death, similarly leads to membrane disruption through mixed lineage kinase domain-like protein (MLKL), causing the release of numerous inflammatory factors (27). Inflammation-related diseases may involve both ferroptosis and necroptosis, acting synergistically to exacerbate tissue damage and inflammation.

3.3 Ferroptosis and necrotic death

The transition from necrotic death to ferroptosis can be facilitated by phosphatidylethanolamine binding protein 1 (PEBP-1) and 15-lipoxygenase (28). Belavgeni et al. suggest that necrotic death may represent an initial phase in the ferroptosis-mediated spread of cell death (29). A small-molecule inhibitor, necrostatin-1f, was developed to investigate this relationship. Although it effectively inhibits necrotic death, it weakly inhibits ferroptosis, highlighting the distinct yet interconnected nature of these cell death pathways (30).

3.4 Ferroptosis and pyroptosis

Pyroptosis can be induced by ROS-generating drugs and iron ions through the ROS-Tom20-Caspase3-GSDME signaling pathway (31). During ferroptosis, lipid peroxides break down into 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which interact with proteins, nucleophiles, and DNA bases, causing significant cytotoxicity (32). This process amplifies ROS signaling, activating mitochondrial caspase pathways typically associated with pyroptosis. These findings suggest a potential link between ferroptosis and pyroptosis, indicating an overlap between these cell death mechanisms.

3.5 Ferroptosis and autophagy

Ferroptosis regulation and progression are tightly linked to selective autophagy, which facilitates the degradation of key ferroptosis-related molecules and organelles. Recent studies highlight autophagy's role in controlling ferritin levels, the primary iron storage protein (7). Nuclear receptor coactivator 4 (NCOA4), a transport receptor that selectively degrades ferritin, is a key player in regulating intracellular iron homeostasis. Overexpression of NCOA4 enhances ferritin degradation, increasing labile iron levels, which promotes ferroptosis (33).

3.6 Ferroptosis and oxidative death

First described in 2001, oxidative death is a non-apoptotic form of cell death characterized by glutathione depletion and oxidative stress (34). This process shares similarities with ferroptosis, as both

activate the expression of eIF2α (35). Moreover, studies show that CRISPR/Cas9-mediated gene knockdown can protect synapses from both ferroptosis and oxidative death (36).

3.7 Ferroptosis and cuproptosis

Cuproptosis, a recently identified form of metal ion-dependent cell death, is driven by intracellular copper levels. Excess copper directly binds to fatty acylated components of the tricarboxylic acid cycle (TCA), disrupting protein homeostasis and causing the accumulation of fatty acylation-related proteins, ultimately triggering cell death (37). Recent studies suggest that ferroptosis inducers can initiate and accelerate cuproptosis in liver cancer. The concurrent use of ferroptosis and cuproptosis inducers leads to enhanced cell death (38).

3.8 Ferroptosis and disulfidptosis

Cancer cells with high levels of solute carrier family 7 member 11 (SLC7A11) undergo disulfidptosis, a thiol-dependent cell death induced by disulfide stress. This process is marked by the formation of multiple disulfide bonds in the cytoplasm, particularly under glucose starvation. This emerging phenomenon suggests a potential link between disulfidptosis and ferroptosis (39). However, evidence on the interaction between these two processes remains limited, and future research is needed to explore and clarify their relationship.

4 Regulatory mechanisms of ferroptosis

4.1 Regulation of iron homeostasis

Iron is essential for various physiological functions, and its homeostasis is crucial to preventing ferroptosis (40). Iron metabolism, which includes absorption, activation, storage, and recycling, is tightly regulated to ensure cellular health. Iron primarily enters the bloodstream through dietary intake and macrophage-mediated erythrocyte breakdown. It is absorbed in the Fe^{3+} form, binds to transferrin (TF), and is recognized by transferrin receptor 1 (TfR1) on cell membranes, after which the complex is internalized through endocytosis (41). TfR1 has been suggested as a potential marker for ferroptosis (42).

Inside cells, ferric reductase six-transmembrane epithelial antigen of prostate 3 (STEAP3) reduces Fe^{3+} to Fe^{2+}, which binds to ferritin or is transported by ferroportin (Fpn) out of the cell. Divalent metal transporter 1 (DMT1) imports free Fe^{2+} from nucleosomes into the cytoplasm, contributing to the labile iron pool (LIP), where it plays a role in metabolic processes (43–45). Ferroportin and enzymes such as ceruloplasmin (CP) export Fe^{2+} from cells, converting it back to Fe^{3+} to maintain the iron cycle (46).

Iron homeostasis is tightly regulated by iron response elements (IREs) and iron regulatory proteins (IRPs), which control the translation of genes such as DMT1 and TfR1. Iron-regulated protein 2 (IRP2) binds to IREs under low-iron conditions to regulate iron levels, while IRP1 acts as a coenzyme when bound to an Fe-S cluster. When iron levels are high, IRP2 is degraded, and IRP1 remains bound to the Fe-S cluster, inhibiting further regulation and stabilizing iron homeostasis (47, 48).

The hepcidin-ferroportin-1 (Fpn1) axis is pivotal for iron regulation (49). Ferroportin exports iron, while ferritin stores excess iron (41, 50). In ferroportin-deficient mice, iron homeostasis is severely disrupted, leading to iron overload (51). Excessive iron, primarily stored in ferritin or as free Fe^{2+}, can induce ferroptosis. Iron can overwhelm TF binding capacity, resulting in non-transferrin bound iron (NTBI), which catalyzes harmful reactions, such as Fenton and Haber-Weiss, producing ROS (52). These ROS damage lipids, driving ferroptosis. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), a leucine zipper protein, plays a crucial role in reducing excess iron and preventing ferroptosis by regulating iron intake, storage, and recycling.

In IRI, tissue damage occurs due to hypoxia during ischemia, and subsequent reperfusion further exacerbates ROS generation. Iron participates in the Fenton reaction, catalyzing the conversion of hydrogen peroxide into free radicals, thus intensifying cellular oxidative damage and potentially triggering ferroptosis. In sepsis, the inflammatory response significantly alters iron metabolism, redistributing iron from storage sites and decreasing serum iron levels. Excessive iron accumulation in cells and the reticuloendothelial system promotes ROS generation, induces ferroptosis, and exacerbates organ dysfunction. Inflammatory mediators in septic patients also regulate iron metabolism-related proteins, such as increasing hepcidin expression to drive iron accumulation. This retention and overload activate oxidative stress responses, damaging cellular structures and leading to tissue damage and organ failure.

4.2 Lipid metabolism

Ferroptosis is strongly influenced by lipid peroxidation, especially through polyunsaturated fatty acids (PUFAs), which undergo peroxidation in the presence of lipoxygenases and ROS. As integral components of cell membranes, PUFAs play key roles in immune response, inflammation, and cell proliferation (50). Free PUFAs, acting as precursors for lipid signals, must first esterify into membrane phospholipids and undergo oxidation to transmit ferroptosis signals. This process is facilitated by lysophosphatidylcholine acyltransferase 3 (LPCAT3) and acyl-CoA synthetase long-chain family member 4 (ACSL4) (53, 54).

Adrenoic acid (AdA) and arachidonic acid (AA) are primary PUFAs involved in inducing ferroptosis (54). ACSL4, in association with Coenzyme A (CoA), forms AA-CoA or AdA-CoA intermediates, which LPCAT3 esterifies into phosphatidylethanolamines, such as PE-AA or PE-AdA. These phospholipids are oxidized by lipid oxidases (LOXs) or via autoxidation, leading to cell death (53). ACSL4 also upregulates ferroptosis in a feedback loop, particularly when the neurofibromin 2-yes-associated protein 1 (NF2-YAP) pathway is inhibited (55).

Phosphorylation of ACSL4 enhances lipid peroxidation and facilitates the incorporation of PUFAs into plasmalogens, a process

mediated by protein kinase C beta type isoform 2 (PKCβII) (55). In contrast, integrin α6β4 inhibits ferroptosis by downregulating ACSL4 expression. Moreover, thiazolidinedione hypoglycemic agents and safranin effectively suppress ferroptosis through ACSL4 targeting (56, 57). Knockdown of LPCAT3 confers protection against ferroptosis, underscoring its critical role (54, 57).

LOXs catalyze the oxidation of PUFAs both directly and within biofilms (53, 58). Among them, 15-LOX is particularly involved in lipid peroxidation, with its interaction with PUFAs, such as sn2-15-hydroperoxy-eicosatetraenoyl-phosphatidylethanolamines (sn2-15-HpETE-PE), playing a pivotal role in signaling ferroptosis (59). The involvement of 12-LOX in p53-mediated ferroptosis remains debated, despite its acknowledged importance (60).

Lipophagy, the autophagic degradation of lipid droplets (LDs), regulates ferroptosis by modulating lipid peroxidation (61). Inhibiting RAB7A or promoting tumor protein D52 (TPD52)-mediated lipid storage can prevent ferroptosis (61, 62). A deeper understanding of lipid peroxidation and its regulatory enzymes opens new therapeutic avenues for diseases such as cancer.

During the ischemic phase of IRI, oxygen and nutrient supply to cells is insufficient, disrupting energy metabolism and lipid homeostasis. Reperfusion leads to the influx of oxygen and iron ions, triggering oxidative reactions. Unsaturated fatty acids, particularly those in phospholipids containing polyunsaturated fatty acids, are prone to oxidation and serve as primary substrates for lipid peroxidation (62). Additionally, IRI affects the expression and activity of enzymes involved in lipid metabolism, altering intracellular lipid composition and further disrupting cellular homeostasis.

Sepsis accelerates the mobilization and oxidation of fatty acids, leading to an accumulation of lipid peroxidation byproducts such as 4-HNE and MDA. These products are toxic to cells, compromising membrane integrity and inducing cell death through ferroptosis (63). The inflammatory response during sepsis promotes fatty acid mobilization and increases the proportion of polyunsaturated fatty acids in cell membranes, heightening susceptibility to lipid peroxidation. This is particularly detrimental in endothelial cells and leukocytes, where lipid peroxidation and iron overload synergistically exacerbate cell damage and contribute to multi-organ failure. Sepsis-induced inflammation also boosts ROS production, further enhancing lipid peroxidation and establishing a vicious cycle that amplifies cellular damage.

4.3 Mitochondrial dysfunction

Mitochondria, essential for lipid and energy metabolism in eukaryotic cells, are pivotal in regulating ferroptosis (64, 65). They synthesize heme and Fe-S clusters, which are critical for ferroptosis regulation (66, 67). Iron enters mitochondria through mitoferrin 1 (SLC25A37) in erythrocytes and mitoferrin 2 (SLC25A28) in non-erythrocytes (68). Overexpression of SLC25A28 can lead to redox-active iron accumulation, increasing ferroptosis susceptibility (69). Conversely, reducing SLC25A28 activity can prevent erastin-induced ferroptosis.

Heme acts as a cofactor in metabolism and electron transfer (70). Excess heme is either exported by feline leukemia virus subtype C receptor-related protein 1B (FLVCR1B) or metabolized by heme oxygenase 1 (HO-1) into Fe^{2+}, carbon monoxide (CO), and biliverdin (71). While HO-1 activation can induce ferroptosis by causing iron overload, modest upregulation of HO-1 confers protection against ferroptosis (72, 73).

Cysteine desulfurase (NFS1) is essential for Fe-S cluster synthesis, and its inhibition sensitizes cancer cells to ferroptosis (74). Increased expression of mitoNEET (CISD1) inhibits erastin-induced ferroptosis, while overproduction of NAF1 (CISD2) prevents sulfadiazine-induced ferroptosis (75, 76). The ABCB7 transporter, critical for mitochondrial iron homeostasis, also plays a role in ferroptosis regulation (77).

Overexpression of mitochondrial ferritin (FtMt) reduces erastin-induced ferroptosis by enhancing iron storage and lowering the LIP, indicating a protective role (78, 79). Voltage-dependent anion channels (VDACs), located in the outer mitochondrial membrane, facilitate chemical and energy transport and are involved in programmed cell death (80). Yagoda et al. demonstrated that knockdown of VDAC2/3 reduces erastin sensitivity in tumor cells (81). Erastin targets VDAC2/3, causing mitochondrial dysfunction and increased ROS production, suggesting that mitochondria are potential therapeutic targets for mitigating ferroptosis.

In IRI, the ischemic phase disrupts mitochondrial energy supply, decreases adenosine triphosphate (ATP) production, and causes metabolic disturbances. Although oxygen re-supply during reperfusion restores mitochondrial activity, it also dramatically increases ROS generation due to the sudden activation of the electron transport chain (82). Iron overload within mitochondria exacerbates ROS accumulation and lipid peroxidation, triggering ferroptosis. Additionally, mitochondrial membrane damage releases signaling molecules that activate downstream cell death pathways, further exacerbating tissue damage.

Sepsis, a complex systemic inflammatory response, often results in severe mitochondrial dysfunction. The release of inflammatory mediators, oxidative stress, and increased metabolic load contribute to mitochondrial damage. Disrupted iron metabolism and mitochondrial iron overload are particularly prominent in sepsis, making mitochondria a major source of ROS (83). Overproduction of mitochondrial ROS worsens lipid peroxidation, triggering ferroptosis and promoting organ dysfunction and failure. Furthermore, mitochondrial dysfunction in sepsis impairs antioxidant defenses, notably by reducing GPX4 activity. This impairs lipid peroxide reduction, fostering ferroptosis. Loss of mitochondrial function also disrupts cellular energy metabolism, diminishes the ability to manage oxidative and metabolic stress, and increases susceptibility to ferroptosis.

4.4 System Xc^--GSH-GPX4 axis

The System Xc^-–GSH–GPX4 axis, a crucial component of the antioxidant system, plays a pivotal role in inhibiting ferroptosis. System Xc^- facilitates cystine uptake, enabling GSH synthesis, which, in turn, supports GPX4 in reducing peroxides, maintaining cellular stability, and preventing ferroptosis (84). Dysregulation of

this axis influences ferroptosis and contributes to various pathologies (Figure 2).

System Xc^-, a sodium-independent cystine/glutamate antiporter, consists of two subunits: SLC7A11 and solute carrier family 3 member 2 (SLC3A2) (85, 86). It exports glutamate while importing cystine, initiating intracellular GSH production (85). Inhibition of SLC7A11 triggers ferroptosis, and its expression is modulated by factors such as ubiquitin aldehyde-binding protein 1 (OTUB1), BECN1, p53, and activating transcription factor 3 (ATF3) (85).

The ferroptosis inducer erastin inhibits System Xc^-, depleting GSH and promoting lipid peroxidation (11). Mutant p53 suppresses SLC7A11 expression, increasing lipid peroxide accumulation and inducing ferroptosis, although p53 can also upregulate GSH levels through p21 activation (87, 88). ATF3 and ATF4 regulate System Xc^- activity, either promoting or mitigating ferroptosis depending on context (89, 90). Nrf2 enhances SLC7A11 transcription, boosting GSH synthesis, while its loss amplifies ferroptosis susceptibility (91).

Phosphorylated BECN1 inhibits SLC7A11, inducing ferroptosis (92). In liver cancer, the ferroptosis-inducing drug sorafenib is effective at low doses but often encounters resistance (93, 94). DJ-1 suppression can enhance sorafenib efficacy (94). Additionally, dipeptidase-1 (DPEP1) exacerbates oxidative stress, increasing ferroptosis susceptibility (95).

GPX4, essential for lipid peroxide detoxification, relies on selenium and GSH. Selenium not only supports GPX4 expression but also plays a role in immune responses; its deficiency results in embryonic lethality (96, 97). The mevalonate pathway further regulates GPX4 expression and ferroptosis sensitivity (98, 99). Ferroptosis inducers such as Ferroptosis-Inducer-56 (FIN56) and

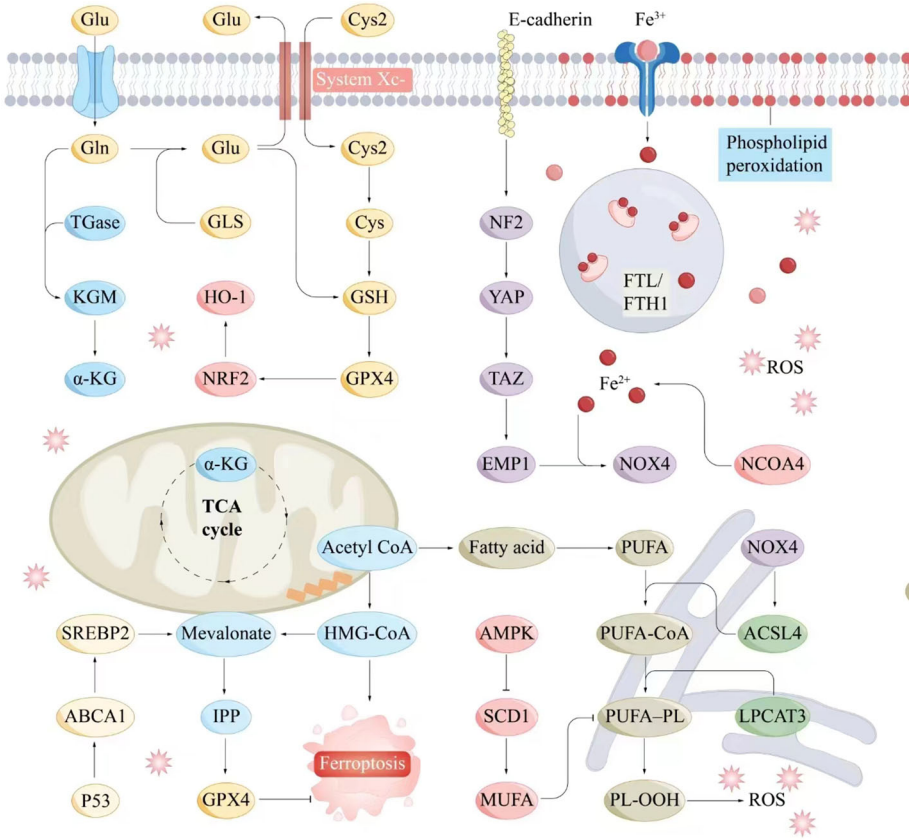

FIGURE 2
Molecular interaction diagram related to ferroptosis mechanisms. Key molecules and pathways involved in the process of ferroptosis include iron metabolism, antioxidant responses, lipid peroxidation, and related signaling pathways. Iron metabolism plays a central role in ferroptosis, as the accumulation of iron within cells can catalyze the formation of ROS through the Fenton reaction. Antioxidant Responses include GSH, GPX4, NADPH and FAD. Serve as electron donors in the regeneration of GSH and other antioxidants, supporting the cellular antioxidant response to counteract ferroptosis. Lipid peroxidation is a hallmark of ferroptosis, involving the oxidative degradation of PUFAs in cellular membranes. ABCA1, ATP-binding cassette transporter A1; Acetyl CoA, Acetyl coenzyme A; ACSL4, Acyl-CoA synthetase long-chain family member 4; AMPK, AMP-activated protein kinase; α-KG, Alpha-Ketoglutarate; Cys, Cysteine; E-cadherin, Epithelial cadherin; EMP1, Epithelial membrane protein-1; Fe^{3+}, Iron ion (trivalent); Fe^{2+}, Iron ion (divalent); FTL/KGM, Ferritin light chain/keratinocyte growth factor; FTH1, Ferritin heavy chain 1; Gln, Glutamine; Glu, Glutamate; GLS, Glutaminase; GPX4, Glutathione peroxidase 4; GSH, Glutathione; HMG-CoA, 3-Hydroxy-3-methylglutaryl coenzyme A; HO-1, Heme oxygenase 1; IPP, Isoprenoid pyrophosphate; LPCAT3, Lysophosphatidylcholine acyltransferase 3; Mevalonate, Mevalonic acid; MUFA, Monounsaturated fatty acid; NCOA4, Nuclear receptor coactivator 4; NF2, Neurofibromin 2; NOX4, NADPH oxidase 4; NRF2, Nuclear factor erythroid 2-related factor 2; P53, Tumor protein P53; PL-OOH, Phospholipid hydroperoxide; PUFA, Polyunsaturated fatty acid; PUFA-CoA, Polyunsaturated fatty acid coenzyme A; ROS, Reactive oxygen species; SCD1, Stearoyl-CoA desaturase-1; SREBP2, Sterol regulatory element-binding protein 2; System Xc^-, Cystine/glutamate antiporter; TAZ, Transcription coactivator with PDZ-binding motif; TGase, Transglutaminase; TCA cycle, Tricarboxylic acid cycle; YAP, Yes1-associated transcriptional regulator.

RSL3 target GPX4 (87, 100). Additionally, ferroptosis suppressor protein-1 (FSP1), identified through CRISPR screening, inhibits ferroptosis independently of GPX4, highlighting the importance of the Coenzyme Q10 (CoQ10)–FSP1–NAD(P)H axis (101, 102).

In IRI, dysfunction of the System Xc^-–GSH–GPX4 axis exacerbates oxidative stress and cellular damage. Reperfusion generates large amounts of ROS, while impaired System Xc^- function limits GSH replenishment, rendering GPX4 inactive. The resulting lipid peroxidation intensifies cell injury and ferroptosis. Similarly, in sepsis, extensive inflammation, oxidative stress, and immune activation disrupt this axis. System Xc^- inhibition reduces GSH availability, weakening cellular defenses against oxidative damage. Additionally, reduced GPX4 activity prevents lipid peroxidation resolution, leading to membrane instability and ferroptosis, which accelerates the progression of multiple organ failure.

4.5 CoQ10-FSP1-NADH axis

CoQ10, also known as ubiquinone, is a lipophilic metabolite synthesized through the mevalonate pathway. Comprising an isoprenoid polymer and a benzoquinone ring, CoQ10 is indispensable for mitochondrial energy production (103). In its reduced form, it functions as a potent antioxidant, neutralizing free radicals generated by lipid peroxidation and thereby preventing ferroptosis (104).

CoQ10 is synthesized from acetyl-coenzyme A through the MVA pathway and is regulated at both transcriptional and translational levels (105). FSP1, initially identified as p53-responsive gene 3 (PRG3) and associated with p53-mediated apoptosis, shares sequence similarities with apoptosis-inducing factor (AIF) (106, 107).

FSP1, a flavoprotein oxidoreductase, interacts with DNA and uses NADPH to reduce ubiquinone to ubiquinol outside mitochondria, thereby inhibiting lipid peroxidation independently of GPX4 and GSH (101, 102). Elevated FSP1 expression enhances resistance to ferroptosis, a process regulated by murine double minute 2 (MDM2) and murine double minute X (MDMX) through peroxisome proliferator-activated receptor α (PPARα) (108). Plasma-activating mediators (PAM) reduce FSP1 expression, triggering ferroptosis in lung cancer, while the compound NPD4928 inhibits FSP1 and promotes ferroptosis (106, 107). Additionally, FSP1 reduces vitamin K to vitamin K hydroquinone (VKH2), which scavenges free radicals and prevents ferroptosis (109). The CoQ10-FSP1-NADH axis underscores FSP1 as a distinct ferroptosis inhibitor, separate from GPX4.

In IRI, the ischemic phase is marked by inadequate oxygen supply, reduced cellular metabolism, and diminished ATP and NADH production. Upon reperfusion, oxygen is reintroduced, rapidly generating ROS and inducing severe oxidative stress. FSP1 mitigates the expansion of lipid free radicals by reducing CoQ10 to panthenol using NADH, thus decreasing oxidative stress, limiting cell damage, and preventing ferroptosis. In sepsis, persistent inflammation and immune activation lead to excessive ROS production and enhanced lipid peroxidation. FSP1 function or expression may be impaired, exacerbating cellular antioxidant demands. The CoQ10-FSP1-NADH axis provides a crucial antioxidant pathway, removing lipid peroxides, maintaining membrane integrity, and reducing ferroptosis, thereby preventing multi-organ dysfunction to some degree.

4.6 DHODH-CoQH2 axis

The inner mitochondrial membrane hosts dihydroorotate dehydrogenase (DHODH), a key enzyme in pyrimidine ribonucleotide synthesis. Recent studies suggest that DHODH also functions as an antioxidant, similar to FSP1, by reducing CoQ10 and modulating ferroptosis independently of mitochondrial GPX4, highlighting its role in oxidative stress management and cell survival (110). DHODH catalyzes the oxidation of dihydroorotate (DHO) to orotate (OA) and facilitates the conversion of ubiquinone (CoQ) to ubiquinol (CoQH2), integral to respiratory complexes and electron transfer in oxidative phosphorylation (111). High DHODH expression confers ferroptosis resistance, while low expression increases susceptibility. DHODH functions independently of FSP1, working alongside GPX4 to prevent mitochondria-associated ferroptosis (110). Wang et al. demonstrated that DHODH provides an independent antioxidant pathway, complementing GPX4 in ferroptosis regulation (112). DHODH inhibitors, such as SA771726 (a leflunomide metabolite) and Brequinar sodium (BQR), are important in cancer therapy (113).

In IRI, the initial ischemic phase disrupts mitochondrial function and blocks the electron transport chain. The subsequent restoration of oxygen during reperfusion triggers the rapid generation of ROS. The DHODH-CoQH2 axis mitigates this damage by maintaining mitochondrial redox balance, reducing ROS-induced cellular damage, preventing lipid peroxidation, and inhibiting ferroptosis. In sepsis, sustained oxidative stress from systemic inflammation results in mitochondrial dysfunction and enhanced lipid peroxidation. The DHODH-CoQH2 axis alleviates oxidative stress, supports mitochondrial and cellular membrane integrity through continuous CoQH2 supply, and inhibits ferroptosis, thereby protecting cells and reducing organ dysfunction.

4.7 GCH1-BH4 axis

Tetrahydrobiopterin (BH4), synthesized by GTP cyclohydrolase 1 (GCH1) from guanosine triphosphate, plays a crucial role in limiting intracellular CoQ and ROS accumulation, preventing ferroptosis. It specifically inhibits the consumption of phospholipids containing polyunsaturated fatty acyl tails (114). While BH4 is essential for various enzymes, its role in ferroptosis inhibition is newly discovered. As an antioxidant, BH4 reduces lipid peroxidation, traps free radicals, and safeguards cells, particularly under conditions where GPX4 is inhibited. BH4's protective effects are enhanced through regeneration by dihydrofolate reductase (DHFR) (115).

Kraft et al. demonstrated that GCH1 increases BH4 synthesis, facilitates lipid remodeling, and reduces lipid peroxidation, thereby

inhibiting ferroptosis (114). Additionally, BH4 aids in converting phenylalanine to tyrosine, which is subsequently transformed into 4-OH-benzoate, a precursor for CoQ10 synthesis, further promoting ferroptosis inhibition. Studies underscore the GCH1-BH4 axis as critical in regulating tumor cell susceptibility to ferroptosis by modulating iron metabolism and mitigating ferritin toxicity through inhibition of NCOA4-mediated ferritin autophagy in colorectal cancer (115, 116). Moreover, Nrf2 has been shown to activate GCH1, promoting BH4 synthesis and protecting cells from oxidative stress, particularly in radiation-induced damage (117). The GCH1-BH4 axis thus represents a major regulatory pathway for ferroptosis, offering a strategy independent of the GSH/GPX4 system.

In IRI, the GCH1-BH4 axis enhances antioxidant defense by boosting BH4 synthesis, reducing ROS damage to cellular membranes, and preventing ferroptosis-induced cell death. In sepsis, this axis helps minimize cellular damage, stabilizes the intracellular environment, and reduces lipid peroxidation, thereby lowering the risk of organ dysfunction.

4.8 p53

The tumor suppressor gene p53 plays a central role in managing cellular stress, including ribosomal stress, hypoxia, and DNA damage. Mutations in p53 and associated signaling pathways are key drivers of tumorigenesis (118). Ferroptosis, a process regulated by p53, is modulated through both transcription-dependent and independent mechanisms (119). p53 promotes ferroptosis by downregulating SLC7A11 expression through Ubiquitin-specific proteinase 7 (USP7), reducing histone H2B monoubiquitination (2). It binds to the SLC7A11 promoter, thereby affecting GPX4 activity and inducing lipid peroxidation (60). However, p53 does not significantly impact GSH levels or GPX4 function, likely due to the activation of Glutaminase 2 (GLS2), TIGAR (p53-induced glycolysis-regulated phosphatase), and CDKN1A (60). p53 activation increases expression of TfR1 and SLC25A28, leading to iron accumulation and heightened sensitivity to ferroptosis (69, 120).

Additionally, p53 targets Spermidine/spermine N1-acetyltransferase 1 (SAT1), enhancing ALOX15 activity and promoting lipid peroxidation under oxidative stress (121). In contrast, p53 can inhibit ferroptosis by enhancing GSH levels through the p53-p21 pathway and regulating ROS through Parkin (122). The p53-DPP4 interaction within the nucleus suppresses Dipeptidyl peptidase-4 (DPP4) and inhibits NADPH oxidase (NOX1), offering protection against ferroptosis (108). DPP4 inhibitors, such as vildagliptin, may reduce the anticancer efficacy of ferroptosis activators (88). MDM2 ubiquitinates p53, but MDM2 inhibitors like nutlin-3 can delay ferroptosis (122). p53 also inhibits erastin-induced ferroptosis by inducing CDKN1A in specific cell types (122). Overall, p53's regulation of ferroptosis is complex, influencing lipid, energy, and iron metabolism in a cell-type and context-dependent manner.

In IRI, p53 enhances cellular susceptibility to ferroptosis by inhibiting antioxidant defenses and promoting lipid peroxidation. This process may be protective by clearing damaged cells in ischemia-reperfusion, but it can also exacerbate tissue damage and dysfunction. In sepsis, p53 similarly mediates oxidative stress-induced ferroptosis. Inflammation-driven persistent oxidative stress activates p53, which suppresses antioxidant systems and drives lipid peroxidation, thus triggering ferroptosis. While this cell death aids in clearing damaged cells and pathogens, excessive ferroptosis can damage tissues and organs, exacerbating sepsis-related organ failure.

4.9 Nrf2

Cloned in 1994, Nrf2 is a member of the basic leucine zipper (bZIP) transcription factor family, including cap "n" collar (CNC) proteins. It is crucial for maintaining redox homeostasis and preventing oxidative stress by regulating genes associated with oxidative stress (123). During ferroptosis, Nrf2 activates cytoprotective genes involved in iron metabolism and redox signaling.

Nrf2 promotes the transcription of antioxidant-related genes by binding to antioxidant response elements (AREs) (124). Upon oxidative stress, Nrf2 dissociates from Kelch-like ECH-associated protein 1 (KEAP1) and translocates to the nucleus, triggering the expression of genes linked to downstream AREs. These include HO-1, quinone oxidoreductase 1 (NQO1), Fpn1, ferritin heavy chain 1 (FTH1), ferritin light chain (FTL), and Fpn1, which collectively promote GSH synthesis and limit ROS production (125, 126).

The Nrf2/KEAP1 pathway enhances the expression of System Xc^-, which targets SLC7A11, indicating that SLC7A11 may mitigate Nrf2-mediated ferroptosis (127). Nrf2 has been shown to inhibit ferroptosis in mice with folate-induced kidney injury through the regulation of GPX4. It also facilitates NADPH production in the pentose phosphate pathway (99). Metallothionein 1G (MT1G), an Nrf2 target gene, helps hepatocellular carcinoma (HCC) cells resist ferroptosis (128). Additionally, Nrf2-driven ATP-binding cassette subfamily C member 1 (ABCC1/MRP1) promotes GSH efflux, potentially influencing ferroptosis regulation (129).

Nrf2 serves as a critical negative regulator of ferroptosis within complex molecular signaling networks. However, further preclinical and clinical research is essential to fully elucidate Nrf2's role in ferroptosis resistance and the therapeutic potential of nuclear factor erythroid 2-like 2 (NFE2L2) inhibitors.

In IRI, Nrf2 mitigates ferroptosis-induced cell damage by upregulating antioxidant enzymes and related protective proteins, thereby reducing ROS accumulation and lipid peroxidation. This protective effect is crucial for minimizing tissue damage and facilitating repair after reperfusion. In sepsis, Nrf2 effectively reduces excessive ROS generation and lipid peroxidation, regulating antioxidant gene expression to preserve cell survival and function. Nrf2 activation stabilizes the intracellular environment, preventing multiple organ injuries induced by ferroptosis and improving sepsis prognosis.

4.10 YAP1/WWTR1

The Hippo signaling pathway depends on two key effector proteins: WW domain-containing transcriptional regulator 1

(WWTR1/TAZ) and Yes1-associated transcriptional regulator (YAP1, also known as YAP), which control organ size, tissue homeostasis, and cancer growth (130, 131). Targeting YAP1/WWTR1 has therapeutic potential in cancer treatment and regenerative medicine (130, 131). These proteins act as biomechanical and morphological sensors, responsive to the cellular environment.

Cadherin 1 (CDH1/E-cadherin)-dependent cell adhesion enhances ferroptosis resistance through the Hippo pathway (55). E-cadherin activates the tumor suppressor neurofibromin 2 (NF2) in epithelial cells, reducing YAP1 activity and ferroptosis (55). Conversely, inhibiting Hippo pathway effectors such as NF2/merlin, large tumor suppressor kinases 1 (LATS1), and 2 (LATS2) reinstates cancer cell susceptibility to ferroptosis, particularly at high cell densities (55).

WWTR1 modulates angiopoietin-like 4 (ANGPTL4) and epithelial membrane protein 1 (EMP1) levels, while YAP1 influences transferrin receptor complexes (TFRC) and ACSL4 translation, promoting lipid peroxidation and iron accumulation, thereby inducing ferroptosis (55, 132). In conclusion, YAP1 and WWTR1 are key transcriptional regulators of ferroptosis, though further *in vivo* studies are necessary.

During IRI, YAP1/WWTR1 activation helps cells manage oxidative stress and attenuate ferroptosis by promoting antioxidant gene expression and defense mechanisms. Modulating YAP1/WWTR1 activity may alleviate tissue injury following ischemia-reperfusion and protect cells from lipid peroxidation and ferroptosis. In sepsis, YAP1/WWTR1 acts as a regulator, helping cells cope with excessive ROS generation and oxidative damage (133). Its activation promotes antioxidant gene expression, metabolic regulation, and reduces ferroptosis-associated apoptosis and histological damage, thereby preserving organ function and minimizing multiple organ damage caused by sepsis.

4.11 Activating transcription factor

ATF, part of the cAMP response element-binding protein 2 family, which includes ATF3 and ATF4, is critical for pathways involving autophagy, oxidative stress, and inflammation (134). Endoplasmic reticulum (ER) stress stimulates ATF expression, influencing metabolism and oxidative balance (135).

In human fibrosarcoma cells, ATF3 directly represses SLC7A11 during erastin-induced ferroptosis (89). Conversely, ATF4 activation enhances SLC7A11 expression, modulating ferroptosis (90). Overexpression of DNA damage-inducible transcript 3 (DDIT3/CHOP) in human glioblastoma, pancreatic, and Burkitt's lymphoma cells links ATF4 to ferroptosis-associated diseases, such as Burkitt's lymphoma and diabetic myocardial IRI (136, 137).

ATF4's target gene, ChaC glutathione-specific γ-glutamyl cyclotransferase 1 (CHAC1), catalyzes GSH degradation, increasing the susceptibility of triple-negative breast cancer cells to ferroptosis (138). In contrast, heat shock 70 kDa protein 5 (HSPA5), an ER chaperone, mitigates ferroptosis by stabilizing GPX4 (139). These findings underscore ATF4's involvement in ferroptotic cell death through complex signaling pathways, highlighting the need for further research on its precise role in ferroptosis (90).

In IRI, ATF4 is activated under ER stress and regulates cellular responses to protein misfolding, crucial for maintaining cellular function and preventing ferroptosis. Enhancing ATF4 activity has been shown to improve cellular tolerance to oxidative stress induced by ischemia-reperfusion (I/R), reducing cell death associated with ferroptosis (140). ATF4 also protects against oxidative damage and modulates immune responses in sepsis by regulating cellular redox balance. Its activation increases the expression of antioxidant proteins, alleviates lipid peroxidation, and prevents ferroptosis caused by excessive ROS accumulation, thereby safeguarding organs and tissues.

4.12 Hypoxia-inducible factor

HIF plays a pivotal role in cellular responses to fluctuating oxygen levels. It is composed of a stable β component, aryl hydrocarbon receptor nuclear translocator (ARNT1/HIF1B), and a labile α subunit, endothelial PAS domain protein 1 (EPAS1/HIF2A), which together drive the expression of hypoxia-inducible factor 1α (HIF1A) and HIF3α, forming a heterodimeric transcription factor (141).

Under normoxic conditions, HIF1α and EPAS1 are hydroxylated by EGLN enzymes, which target them for degradation via the Von Hippel-Lindau (VHL) E3 ubiquitin ligase complex (142). HIF modulates ferroptosis in tumor cells through two mechanisms: EGLN serves as a key target for iron chelators like deferoxamine, which stabilize HIF by inhibiting EGLN (143).

Hypoxia induces HIF1α expression in HT-1080 fibrosarcoma cells, leading to increased transcription of fatty acid binding proteins 3 (FABP3) and 7 (FABP7), enhancing lipid uptake and storage, which inhibits ferroptosis (144). In contrast, EPAS1 upregulates hypoxia-induced lipid droplet-associated proteins (HILPDA/HIG2) in clear-cell carcinoma cells, promoting ferroptosis through lipid peroxidation and increased PUFAs synthesis (145).

These findings suggest that tumor type influences HIF's regulation of ferroptosis. Understanding HIF's role across different cancers could inform innovative therapeutic strategies for cancer treatment and prevention.

HIF is activated during ischemia in IRI, promoting cellular adaptation to hypoxic conditions and mitigating ROS damage by regulating antioxidant genes and metabolic pathways during reperfusion. This regulation inhibits ferroptosis. By controlling glycolysis and other metabolic pathways, HIF enhances cellular energy production and antioxidant capacity, which is critical for responding to hypoxia and oxidative stress. In sepsis, HIF modulates metabolic and inflammatory responses by regulating hepcidin and ferroportin expression, thereby maintaining intracellular iron homeostasis. This reduces free iron accumulation, limits excessive ROS generation through the Fenton reaction, and promotes antioxidant enzyme expression, aiding in ROS scavenging and preventing oxidative stress-induced ferroptosis.

5 Role of ferroptosis in critical illness

5.1 IRI

IRI is a common, life-threatening clinical complication with significant local and systemic effects. Recent studies suggest that ferroptosis plays a role in the pathophysiology of IRI across various organs, including the brain, intestines, liver, heart, and kidneys (20, 72, 146, 147). Modulating lipid peroxidation and iron levels influences susceptibility to ferroptosis, thereby affecting IRI outcomes (Figure 3).

5.1.1 Cardiac IRI

Cardiac IRI and myocardial infarction are prevalent cardiovascular conditions associated with poor prognosis and limited early intervention options. Before the identification of ferroptosis, studies indicated that mitochondrial overexpression of GPX4 had cardioprotective effects against IRI (148). Ferroptosis predominantly occurs during the cardiac reperfusion phase, rather than during ischemia. Prolonged reperfusion results in a decline in GPX4 levels, while ferroptosis markers, including ACSL4, iron, and MDA, increase (149).

In 2015, Gao et al. first highlighted the role of ferroptosis in cardiac I/R (150). Iron concentrations increase near coronary blood flow after prolonged myocardial ischemia (151). Following cardiac IRI, cardiomyocytes become more vulnerable to ferroptosis as excess iron is absorbed by lipid peroxides through the Fenton reaction and enters the cells. Moreover, cardiomyocytes exposed to high iron levels become more susceptible to oxidative stress due to increased ROS, which, in turn, triggers ferroptosis.

Iron overload significantly contributes to myocardial cell injury, with lipid peroxidation playing a pivotal role in oxidative IRI (152, 153). In response to I/R, the heart exhibits increased production of ferritin heavy chain (FTH) and FTL, indicating elevated non-heme iron levels in ischemic myocardial tissue (72). Cardiomyocytes treated with erastin or RSL3 show decreased Fe^{2+}, ROS, MDA, and cell death levels when ATF3 is overexpressed, preventing ferroptosis in these cells (152).

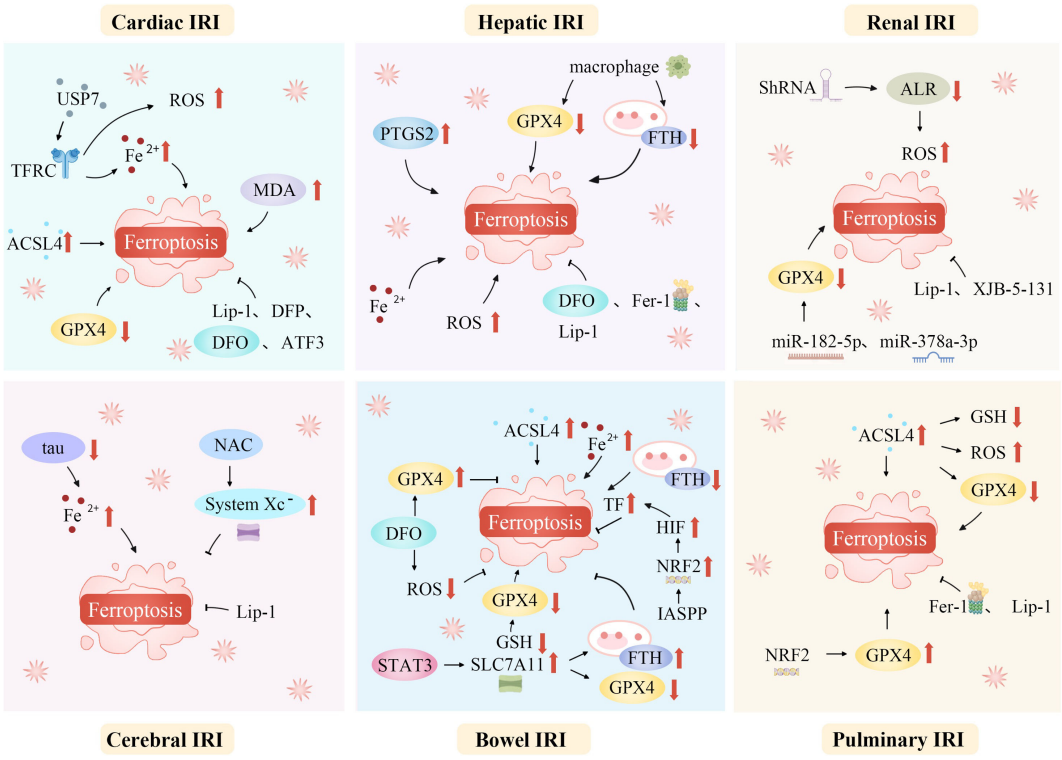

FIGURE 3
Ferroptosis mediates a variety of ischemia-reperfusion injuries. Ferroptosis, a unique regulated cell death form, mediates various IRIs. During ischemia, restricted blood supply causes hypoxia, disrupting metabolism and leading to intracellular changes like ROS generation in mitochondria's electron transport chain. Reperfusion restores blood flow but worsens oxidative stress. Increased ROS overwhelms antioxidant systems like GPX4, triggering ferroptosis. Abundant tissue iron ions in the ROS-influenced Fenton reaction generate hydroxyl radicals, initiating lipid peroxidation. This is key in ferroptosis. In organs like the heart, brain, kidneys, and liver, ferroptosis-caused lipid metabolism disruption and peroxide accumulation damage cell membranes, causing cell death. Thus, ferroptosis-mediated damage in ischemia-reperfusion contributes to tissue dysfunction and organ failure. ACSL4, Acyl-CoA synthetase long-chain family member 4; ALR, Augmenter of liver regeneration; ATF3, Activating transcription factor 3; DFO, Deferoxamine; DFP, Deferiprone; Fe^{2+}, Iron ion; Fer-1, Ferrostatin-1; FTH, Ferritin heavy chain; GPX4, Glutathione peroxidase 4; GSH, Glutathione; HIF, Hypoxia-inducible factor; IASPP, Inhibitor of apoptosis stimulating protein of p53; Lip-1, Liproxstatin-1; IRI, Ischemia-reperfusion injury; NAC, N-acetylcysteine; NRF2, Nuclear factor erythroid 2-related factor 2; PTGS2, Prostaglandin-endoperoxide synthase 2; ROS, Reactive oxygen species; ShRNA, short hairpin RNA lentivirals; SLC7A11, Solute carrier family 7 member 11; STAT3, Signal transducer and activator of transcription 3; System Xc^-, Cystine/glutamate antiporter; TF, Transferrin; TFRC, Transferrin receptor complexes; USP7, Ubiquitin-specific proteinase 7.

An *in vitro* study revealed that phospholipid oxidation products negatively affect cardiomyocyte viability during I/R (154). Oxidized lipidomic analysis identified oxidized phosphatidylethanolamine as a specific indicator of ferroptosis, providing direct evidence for cardiac ferroptosis (147). Moreover, I/R-treated hearts demonstrated the formation of oxidized phosphatidylethanolamine in their mitochondria, underscoring the role of cardiac mitochondria in lipid peroxide generation and ferroptosis signaling (155). Additionally, inhibiting or knocking down USP7 suppresses TFRC expression, reducing myocardial IRI primarily by decreasing iron levels, lipid peroxidation, and ferroptosis (120).

Li et al. investigated the *in vivo* inflammatory responses triggered by IRI following heart transplantation. Their results demonstrated that ferroptosis facilitated endothelial cell adhesion in cardiac tissues via the type I interferon/TLR4/Trif signaling pathway, which in turn promoted neutrophil recruitment to the injured myocardium (147). Moreover, using a coronary artery ligation model to induce myocardial IRI, they found that suppression of ferroptosis improved heart function and reduced infarct size.

5.1.2 Hepatic IRI

Hepatic IRI poses a significant challenge in liver transplantation, as it induces an immune response and inflammatory cascade that impairs donor liver function and worsens recipient prognosis (154). In pediatric living donor liver transplants (LTx), donor iron burden has been identified as an independent risk factor for liver IRI (156). Iron overload, lipid peroxidation, and the upregulation of Prostaglandin-endoperoxide synthase 2 (PTGS2)—a marker of ferroptosis—are key features of IRI progression in the liver.

In vitro studies have shown that hepatic macrophages play a role in ferroptosis during IRI, with macrophage co-culture leading to ferroptosis through downregulation of GPX4 and FTH1 (157). Additionally, mice subjected to a high-iron diet exhibited aggravated IRI, which was mitigated by treatment with DFO104.

5.1.3 Renal IRI

Ferroptosis is firmly linked to renal IRI, where it significantly contributes to the pathophysiological processes associated with the condition. The tubular manifestation of ferroptosis plays a crucial role in the progression of renal IRI (158).

In the context of renal injury and repair, augmenter of liver regeneration (ALR) has been shown to influence ferroptosis (159). Blocking ALR with short hairpin RNA (shRNA) exacerbates the condition, increasing ROS production, mitochondrial damage, and ferroptosis (159). Using a rat model of renal IRI, Ding et al. demonstrated that miR-182-5p and miR-378a-3p directly bind to the 3′-untranslated region of GPX4 mRNA, suppressing GPX4 expression and promoting ferroptosis (160).

Reducing iron-related antioxidants and administering iron chelators can improve renal function in patients with renal IRI (161). These findings underscore the close association between ferroptosis and renal IRI, involving multiple regulatory mechanisms.

5.1.4 Cerebral IRI

The pathophysiological mechanisms underlying cerebral IRI are multifactorial, involving factors such as heightened inflammatory responses, calcium overload, oxidative damage, excessive release of excitatory amino acids, and processes leading to apoptosis or necrosis (162). Most research on ferroptosis in cerebral IRI focuses on oxygen-glucose deprivation/reoxygenation models and animal models of focal or global brain injury.

In focal cerebral IRI, a middle cerebral artery occlusion (MCAO) model, based on the traditional MCAO technique, revealed reduced ferritin expression. In this model, ferritin overexpression modulated p53/SLC7A11-mediated ferroptosis, improving memory and cognitive function (163). Significantly suppressed tau expression, characteristic of cerebral I/R, is linked to Alzheimer's disease and promotes iron efflux. This impairs iron transport to the extracellular space, increases intracellular iron levels, and induces neurotoxicity and ferroptosis, thereby exacerbating neuronal injury (164, 165).

Due to the complex nature of cerebral IRI and the incomplete understanding of ferroptosis mechanisms, it is critical to consider the interplay between multiple factors when studying the effects of ferroptosis on cerebral IRI. Results from single-perspective studies often fail to yield conclusive outcomes in broader animal models or clinical settings.

5.1.5 Bowel IRI

The intestine, particularly the small intestine, is one of the most vulnerable organs to IRI, which typically results from a sudden decrease in blood flow followed by reoxygenation upon restoration of circulation (165). This condition is associated with various forms of regulated cell death (RCD), including ferroptosis, apoptosis, necroptosis, and autophagy (166, 167). Lipid peroxidation and ROS generation play a pivotal role in initiating and completing ferroptosis in intestinal IRI (168).

A crucial enzyme, ACSL4, regulates lipid content and is vital for initiating ferroptosis (57). Li et al. demonstrated that intestinal IRI leads to decreased levels of GPX4, ferritin heavy chain, and GSH, while increasing ACSL4 and iron levels. This process results in ferroptosis of intestinal histiocytes (146). Inhibiting ACSL4, a key ferroptosis controller, through medicinal and biological approaches successfully prevented lipid peroxidation and ferroptosis. This intervention also alleviated cell injury and dysfunction of the intestinal barrier caused by IRI (57, 146).

Ferroptosis in intestinal IRI has been further investigated through modulation of SLC7A11, which influences intestinal IRI-induced acute lung injury (ALI) (169). The inhibitor of apoptosis-stimulating protein of p53 (IASPP) mitigates ferroptosis in intestinal IRI by activating the Nrf2-HIF1-TF signaling pathway, thereby reducing ALI (170).

Additionally, intestinal microbiota and its metabolites can inhibit ferroptosis in intestinal IRI. A potential mechanism involves capsaicin ester, a metabolite of the microbiota, which activates transient receptor potential cation channel subfamily V

member 1 (TRPV1) to enhance GPX4 expression, thus suppressing ferroptosis (169).

5.1.6 Pulmonary IRI

Pulmonary IRI remains a complex and not fully understood condition. Despite significant recent research, our understanding of the mechanisms underlying pulmonary IRI and ferroptosis is still in its early stages, warranting further comprehensive investigation.

Inhibition or knockdown of ACSL4 prevents I/R-induced lung injury by reducing lipid peroxidation and elevating GSH and GPX4 levels, thereby mitigating ferroptosis (171). These findings suggest that increased Nrf2 expression protects against lipid peroxidation-induced injury in I/R by upregulating GPX4 and SLC7A11 (172). Future research should focus on elucidating the mechanisms of pulmonary IRI to identify more precise therapeutic targets and develop effective clinical treatments.

5.2 Sepsis

Sepsis is a syndrome in which the host's response to infection becomes dysregulated, potentially leading to multiple organ failure and life-threatening dysfunction (173). It involves complex processes such as activation of the innate immune system, inflammatory cascades, procoagulant and antifibrinolytic pathways, altered cellular metabolism, and modifications to signaling pathways. However, the central pathophysiological change in sepsis is acquired immunological dysfunction (174, 175). Ferroptosis, strongly linked to immune cell dysfunction, oxidative stress, and metabolic disturbances, significantly impacts the immune response, influencing the onset and progression of sepsis (Figure 4) (176).

5.2.1 Sepsis-induced cardiac injury

Sepsis-induced heart failure, known as septic cardiomyopathy, significantly raises mortality rates (177). Previous reviews have highlighted ferroptosis as a key component of the pathogenesis of septic cardiomyopathy (178). Lin et al. identified lipid peroxidation products, including malondialdehyde, 4-hydroxynonenoic acid, and lipid ROS, in H9c2 myofibroblasts and a mouse model of lipopolysaccharide (LPS)-induced septic cardiomyopathy. The authors observed morphological signs of LPS-induced mitochondrial damage, consistent with microstructural alterations characteristic of ferroptosis in mitochondria (179).

In a mouse model of CLP-induced septic heart injury, Wang et al. found that CLP elevated the cardiac injury marker troponin I (TNI). Moreover, CLP significantly reduced levels of GSH and

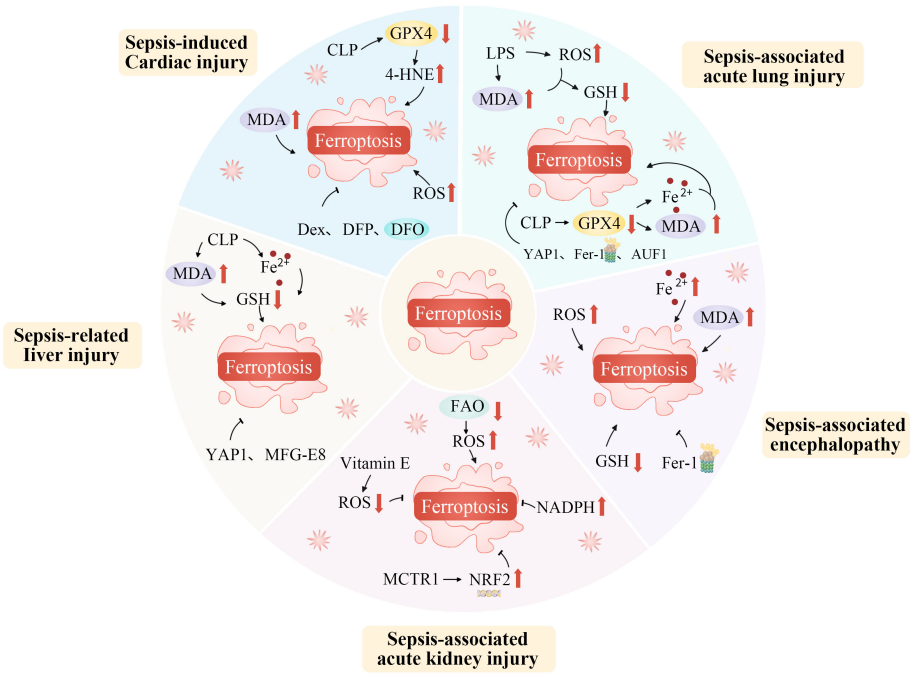

FIGURE 4
Ferroptosis plays an important role in the occurrence and progression of sepsis. Ferroptosis, as a specific form of regulated cell death, plays an indispensably important role in the occurrence and progression of sepsis. During the development of sepsis, the complex pathophysiological environment, including excessive inflammation, oxidative stress, and disrupted iron metabolism, creates favorable conditions for ferroptosis. The dysregulation of iron homeostasis within cells leads to an accumulation of iron ions, which act as catalysts for lipid peroxidation. Inflammatory cytokines and reactive oxygen species generated during sepsis can further disrupt the antioxidant defense system within cells, thereby exacerbating lipid peroxidation. This process of ferroptosis in turn aggravates tissue damage, impairs organ function, and promotes the continuous progression of sepsis, ultimately having a profound impact on the prognosis of patients with this severe condition. AUF1, AU-rich element RNA binding factor 1; CLP, Cecal ligation and puncture; Dex, Dexmedetomidine; DFO, Deferoxamine; DFP, Deferiprone; FAO, Fatty acid oxidation; Fe^{2+}, Iron ion; Fer-1, Ferrostatin-1; GSH, Glutathione; GPX4, Glutathione peroxidase 4; LPS, Lipopolysaccharide; MCTR1, Maresin conjugates in tissue regeneration-1; MDA, Malondialdehyde; MFG-E8, Epidermal growth factor 8; NADPH, Nicotinamide adenine dinucleotide phosphate; NRF2, Nuclear factor erythroid 2-related factor 2; ROS, Reactive oxygen species; YAP1, Yes1 associated transcriptional regulator; 4-HNE, 4-hydroxynonenal.

GPX4, while increasing concentrations of HO-1, TfR1, caspase-3, inducible nitric oxide synthase, and Fe^{2+} (180).

Ferroptosis was also found to heighten atrial vulnerability in the LPS-induced sepsis model, which contributed to the onset of new atrial fibrillation. Conversely, knocking down Fpn or administering ferroptosis inhibitors mitigated atrial fibrillation development (181). These findings suggest that ferroptosis plays a crucial role in sepsis-related heart damage and that targeting ferroptosis may offer therapeutic potential.

5.2.2 Sepsis-associated lung injury/ARDS

Acute respiratory distress syndrome (ARDS), a common consequence of sepsis, is also linked to ferroptosis (182, 183). Xu et al. emphasized the role of ferroptosis in the pathophysiology of SALI (184). In a study of LPS-induced septic lung injury in A549 cells, LPS was shown to elevate ROS, iron, and MDA levels, while reducing GSH in alveolar cells (185). Additionally, in a murine model of ALI caused by sepsis via cecal ligation, ferroptosis marker GPX4 expression was downregulated, while MDA and iron ion levels increased.

YAP1 mitigates SALI by inhibiting ferroptosis via ferritin phagocytosis. In a CLP-induced murine model, YAP1 knockout exacerbated SALI, as indicated by increased expression of mitochondrial membrane proteins SFXN1 and NCOA4, alongside decreased levels of GPX4, FTH1, and SLC7A11 (186).

The KEAP1-Nrf2/HO-1 pathway plays a protective role in the pathogenesis of SALI. Activation of Nrf2 by American ginseng phenol has been shown to ameliorate septic ALI by upregulating HO-1 expression (187). Furthermore, mucin and itraconazole protect against macrophage ferroptosis and alleviate SALI by enhancing GPX4 expression via Nrf2-mediated pathways (188, 189). Overall, ferroptosis is a critical factor in SALI development, representing a promising target for both preventive and therapeutic interventions.

5.2.3 Sepsis-related liver injury

Emerging evidence highlights ferroptosis as a key mechanism in hepatocellular injury during sepsis. In a CLP-induced sepsis model, mice exhibited hepatic ferroptosis, characterized by elevated iron levels, increased MDA, and reduced GSH.

YAP1 prevents ferroptosis driven by ferritin phagocytosis, offering protection against septic liver injury, while its deficiency worsens liver damage during sepsis (190). Irisin has also been shown to reduce ferroptosis in LPS-treated hepatocytes and CLP-induced sepsis models by modulating GPX4 expression (191). Therefore, targeting ferroptosis may offer a promising approach for treating septic liver injury.

5.2.4 Sepsis-associated acute kidney injury

Sepsis often leads to AKI, which is marked by rapid deterioration in renal function. The incidence of SA-AKI has been increasing (192). Recent studies suggest that ferroptosis contributes significantly to the pathophysiology of AKI (193).

Fatty acid oxidation (FAO) is a primary energy source for proximal tubular epithelial cells. During ischemia, an imbalance between fatty acid intake and utilization impairs FAO due to the accumulation of free fatty acids. This disruption promotes lipid accumulation, enhances lipid peroxidation, and accelerates ROS production, thereby amplifying the inflammatory response and aggravating kidney injury.

Inhibition of NADPH oxidase, an antioxidant produced by mitochondria that increases NADPH concentrations, effectively reduces ferroptosis in renal cells (194). Ye et al. demonstrated that LOX inhibitors improve renal function, reduce proteinuria, and enhance creatinine clearance in LPS-induced AKI mice (195). These findings strongly suggest that targeting ferroptosis could be a viable strategy for treating SA-AKI.

5.2.5 Sepsis-associated encephalopathy

SAE affects up to 70% of patients, with symptoms ranging from delusions and disorientation to coma (196, 197). The full pathophysiology of SAE remains unclear, but sepsis exacerbates SAE by promoting ferroptosis (198). Xie et al. showed that glutamate contributes to neuronal injury during SAE through ferroptosis (199). Thus, ferroptosis plays a crucial role in SAE development.

The hippocampus, critical for memory and learning, is particularly vulnerable to damage from inflammation and hypoxia (200). SAE-induced ferroptosis in the hippocampus is characterized by increased ROS, iron content, and MDA levels, alongside reduced GSH levels and altered expression of ferroptosis-related proteins (GPX4, ACSL4, and SLC7A11).

In CLP-induced mouse models, survival rates were reduced, and cognitive dysfunction, hippocampal damage, and mitochondrial injury were observed. However, in SAE mice, irisin alleviated neurological impairment, reduced hippocampal ferroptosis and microglial activation, and mitigated CLP-induced learning and memory deficits (201, 202).

Research in animal models of sepsis suggests that cytokines such as TNF-α and IL-6 cross the blood-brain barrier, disrupting cerebral microcirculation and leading to oxidative stress, mitochondrial damage, and cell death (199). Therefore, ferroptosis is a key contributor to SAE pathogenesis.

5.2.6 Sepsis-associated immunosuppression

The role of ferroptosis in sepsis-related immunosuppression is gaining recognition, with immune cell depletion and immune dysfunction being prominent effects. However, further research is needed to clarify the specific pathways and mechanisms through which ferroptosis regulates immune suppression.

5.3 Other critical illness

5.3.1 ARDS/ALI

ARDS and ALI are characterized by diffuse interstitial and alveolar edema, which can lead to hypoxic respiratory insufficiency or failure. These conditions can be triggered by both direct (intrapulmonary) and indirect (extrapulmonary) factors.

Ferroptosis significantly impacts the onset and progression of ALI (203).

Mice with ALI induced by oleic acid (OA) or LPS exhibited iron overload, reduced GSH, MDA, and 4HNE levels, and downregulated ferritin, SLC7A11, and GPX4 expression (204, 205). Downregulation of CircEXOC5 mitigated lung injury by reducing ROS levels, downregulating GPX4, and upregulating ACSL4, thus suppressing ferroptosis (206).

Purearin has been shown to reduce epithelial damage by lowering iron concentrations in lung epithelial cells and suppressing GPX4 and GSH expression, thereby inhibiting ferroptosis (207). Additionally, electroacupuncture (EA) stimulation at Zusanli (ST36) prevents ferroptosis in alveolar epithelial cells by activating the α7 nicotinic acetylcholine receptor (α7nAchR), mitigating LPS-induced ARDS/ALI (208). These findings suggest that targeted suppression of ferroptosis in the lungs may benefit ARDS/ALI patients.

5.3.2 AKI

AKI is a prevalent clinical condition, affecting 11.6% of individuals in China (209). Approximately 700,000 adult hospitalizations annually result in death from AKI (210). Ferroptosis plays a pivotal role in the development of AKI (211). Among the various causes of AKI, tubular cells, particularly the metabolically active and energy-dense proximal tubular cells, are highly susceptible to ferroptosis (212). Therefore, ferroptosis-induced proximal tubular injury may be a key mechanism in the pathogenesis of AKI.

In the context of rhabdomyolysis, myocyte rupture releases significant amounts of myoglobin, which is subsequently broken down into iron and heme. These byproducts damage tubular epithelial cells through lipid peroxidation, eventually leading to ferroptosis. The transcriptional regulator YAP mediates the upregulation of ACSL4 in skeletal muscle cells, contributing to ferroptosis and promoting the progression of rhabdomyolysis following exercise-induced heatstroke. Targeted suppression of ACSL4 and ferroptosis may, therefore, offer a potential therapeutic strategy to mitigate rhabdomyolysis-induced AKI (213). Furthermore, Wang et al. demonstrated that ACSL4 deficiency protects against AKI mediated by ferroptosis (214).

Cisplatin, a first-generation platinum chemotherapy drug, is associated with nephrotoxicity, which remains its primary side effect. Both a single high dose and repeated low doses can lead to AKI. To prevent AKI progression, Zhao et al. administered iron sucrose to a cisplatin-induced AKI mouse model (215). Their findings indicated that iron supplementation upregulated ferritin and iron transporters. Additionally, activation of vitamin D receptors partially inhibited ferroptosis through GPX4 trans-regulation, thus alleviating cisplatin-induced AKI (216).

In GPX4 knockout mice, there is a notable increase in both the incidence and mortality of spontaneous AKI (20). Legumain, a conserved asparagine endopeptidase, stabilizes GPX4 in its absence, thereby reducing tubular cell ferroptosis (217). Moreover, HO-1 significantly inhibits ferroptosis in renal proximal tubular cells (218). As such, targeting ferroptosis holds promise for improving therapeutic outcomes in AKI.

5.3.3 Acute liver injury

Acute liver injury is characterized by a sudden decline in hepatic function, typically without prior signs of liver disease. It is caused by hepatotoxic agents such as alcohol, narcotics, IRIs, or viral hepatitis (219). Numerous studies have explored the mechanism of ferroptosis induced by acetaminophen (also known as N-acetyl-p-aminophenol, or APAP), a widely used nonsteroidal anti-inflammatory drug (220). Intracellular GSH levels are critical for the activation of ferroptosis, with APAP overdose leading to a significant depletion of GSH (221).

The mechanisms underlying APAP-induced liver injury have been linked to iron accumulation and lipid peroxidation. Yamada et al. identified ferroptosis, marked by increased lipid peroxides derived from n-6 PUFAs, as a key factor in APAP-induced acute liver injury in a mouse model (222). Moreover, mice deficient in GPX4 showed that α-tocopherol could inhibit excessive lipid peroxidation (223).

Recent research underscores the pivotal role of mitochondria in ferroptosis development (224). In animal models of APAP-induced acute liver injury, ferroptosis inhibitors, such as UAMC-3203 and VDAC1 oligomerization inhibitor VBIT-12, were shown to preserve mitochondrial function and reduce ferroptosis (225). These findings suggest that ferroptosis may serve as a promising therapeutic target for treating acute liver injury.

5.3.4 Stroke

Stroke, encompassing both hemorrhagic and ischemic subtypes, has been linked to ferroptosis (226). Following severe hypoxic-ischemic brain injury, regions such as the thalamus, subcortical white matter, periventricular areas, and basal ganglia exhibit significant iron accumulation (227).

In ischemic stroke models, a reduction in GSH levels and GPX4 activity is observed, along with increased lipid peroxidation in neuronal cells (228). Lu et al. demonstrated that lncRNA PVT1, through miR-214-mediated modulation of TFR1 and p53 expression, regulates ferroptosis in acute ischemic stroke (229).

Ferroptosis also contributes to neuronal death after hemorrhagic stroke (230). In a hemorrhagic stroke model, GPX4 levels significantly decreased within 24 hours post-intracerebral hemorrhage. However, a single dose of selenium enhanced GPX4 expression, mitigating inflammation, oxidative stress, blood-brain barrier disruption, cerebral edema, and neuronal dysfunction (231). Therefore, ferroptosis modulators represent potential pharmacological targets for stroke therapy.

5.3.5 Traumatic brain injury

TBI is a major cause of head injuries, fatalities, and disabilities due to accidents such as falls and crashes (232). Ferroptosis contributes to neurological impairment and neuronal death following TBI (233). Pathophysiological mechanisms include iron accumulation, disrupted iron metabolism, overexpression of ferroptosis-related genes, reduced GPX activity, and increased ROS levels, all of which are linked to TBI progression (234).

Ruxolitinib has demonstrated neuroprotective effects in animal models of TBI by inhibiting the JAK-STAT pathway, thereby

preventing iron accumulation, neurotoxicity, brain edema, and lesion expansion. This treatment also improves motor deficits, cognitive dysfunction, and anxiety-like behaviors (235). Moreover, ruxolitinib reverses the decrease in GPX4 levels and the increase in TfR1, cyclooxygenase-2 (COX2), and 4-HNE observed in the acute phase of TBI.

Liang et al. reported that peroxisome proliferator-activated receptor γ (PPARγ) expression was reduced in neurons undergoing ferroptosis following TBI (236). However, pioglitazone, a PPARγ agonist, reversed this reduction, alleviating neuronal ferroptosis by reducing MDA and COX2 levels both *in vitro* and *in vivo*. Furthermore, pioglitazone reduced neuronal loss, lesion size, and neurological severity scores (NSS) in TBI mice.

In summary, inhibiting ferroptosis offers a promising therapeutic strategy for neuroprotection in TBI. A deeper understanding of the mechanisms underlying ferroptosis-targeting treatments will pave the way for novel therapeutic approaches for TBI patients. This could significantly improve clinical outcomes and establish a new theoretical framework for TBI management.

6 Therapeutic approaches targeting ferroptosis in critical care diseases

Ferroptosis is a key target for therapeutic interventions aimed at both treating and preventing severe illnesses, as it plays a role in the progression of various critical conditions. This paper reviews several drugs and compounds known to inhibit ferroptosis, exploring their potential applications in a range of serious diseases (see Supplementary Table 1).

6.1 IRI

6.1.1 Cardiac IRI

Feng et al. utilized a mouse cardiac I/R model to demonstrate that early administration of Liproxstatin-1 (Lip-1) during reperfusion increased GPX4 levels in mitochondria, thereby reducing ischemic infarct size and improving mitochondrial function and morphology (237). Both deferiprone (DFP) and deferoxamine (DFO) mitigate myocardial IRI by inhibiting ferroptosis (149, 150). Additionally, 2,2-Bipyridine and MitoFerroGreen have been shown to target mitochondrial iron reduction pharmacologically, offering protection against cardiac IRI in mice (238, 239).

Compound 968 has been shown to reduce myocardial IRI in ex vivo studies by limiting glutamine availability (150). Moreover, by inhibiting p53, USP22-mediated deubiquitination prevents ferroptosis, leading to the overexpression of SLC7A11 and a decrease in ROS generation, which ultimately reduces myocardial IRI (240). Baicalin exerts its protective effects by inactivating ACSL4, thus resisting ferroptosis and preventing myocardial IRI (56). Other compounds, such as xanthohumol, naringenin, and resveratrol, have been shown to protect against I/R-induced heart ferroptosis by modulating GPX4 levels (241–243).

6.1.2 Hepatic IRI

The first-generation ferroptosis inhibitor, ferrostatin-1 (Fer-1), functions as a synthetic antioxidant, preventing membrane lipid damage and inhibiting cell death (11). In animal models, Fer-1 and α-tocopherol have been demonstrated to reduce inflammatory responses and ameliorate hepatic IRI (20, 156). Li et al. showed that inhibiting ferroptosis with Lip-1 significantly reduced myeloperoxidase (MPO) activity and alleviated liver histopathological damage (146).

6.1.3 Renal IRI

In a renal IRI model, administration of Lip-1 improved ferroptosis inhibition (241). Zhao et al. found that renal tubular epithelial cells undergoing IRI-induced AKI exhibited ferroptosis, while XJB-5-131 ameliorated IRI-induced AKI by inhibiting ferroptosis (244).

6.1.4 Cerebral IRI

Lip-1 also protected mice from brain IRI by modulating iron-related gene and protein activity (234). The antioxidant N-acetylcysteine (NAC) prevented ferroptosis induced by cysteine depletion and System Xc^--inhibition by enhancing cysteine bioavailability (245, 246). Furthermore, NAC inhibits heme-induced ferroptosis in brain cells by opposing the toxic lipids produced through arachidonate 5-lipoxygenase (ALOX5) activity and cooperating with prostaglandin E2 (247).

6.1.5 Bowel IRI

DFO has been shown to prevent lipid peroxidation induced by intestinal I/R and restores reduced GPX4 activity (248). Nrf2 agonists, such as dimethyl fumarate and signal transducer and activator of transcription 3 (STAT3), mitigate ferroptosis in intestinal IRI-ALI by increasing the mRNA expression of FTH1 and GPX4, reducing lipid peroxidation, and preserving GSH levels through upregulation of SLC7A11. This decreases lipid ROS and intracellular ROS levels (249, 250).

6.1.6 Pulmonary IRI

Fer-1 and Lip-1 alleviate pulmonary edema, atelectasis, necrosis, inflammation, and fibrosis in I/R mice, exacerbated by iron or Erastin treatment through tail vein injection (170, 249). However, while ferroptosis inhibitors have shown lung protection in animal models, they have not yet been translated into clinical practice, requiring further investigation into their regulatory mechanisms.

6.2 Sepsis

6.2.1 Sepsis-induced cardiac injury

The α2 adrenergic agonist dexmedetomidine (Dex) reduces ferroptosis by activating the SLC7A11-GPX4 signaling pathway. Clinical studies indicate that Dex mitigates myocardial injury in sepsis, although its precise mechanism remains unclear (251). Similarly, DFP and DFO attenuate sepsis-induced cardiac injury by inhibiting ferroptosis (252).

6.2.2 SALI/ARDS

In patients with SALI, hydrogen sulfide reduces ferroptosis and blocks mTOR signaling (253). Additionally, the RNA-binding protein AUF1 contributes to improved SALI outcomes by preventing ferroptosis via the Nrf2 pathway (254). AUF1 suppression exacerbates lung injury by inducing ferroptosis and significantly reduces survival in a CLP-induced SALI model (207).

Intraperitoneal injection of recombinant cold-induced RNA-binding protein (CIRP) downregulates GPX4 expression and elevates lipid ROS levels in lung tissue. This effect is mitigated by Fer-1. Ferroptosis and lung damage are less prevalent in CIRP mutant cells in CLP-induced SALI models, and the CIRP inhibitor C23 further reduces ferroptosis and SALI severity *in vivo* (255).

6.2.3 Sepsis-related liver injury

Serum levels of the specialized protein MFG-E8, which inhibits ferroptosis, are lower in septic patients compared to healthy individuals (256). Intraperitoneal administration of recombinant MFG-E8 in mice improves liver recovery, reduces oxidative stress and ferroptosis in liver tissue, and enhances survival rates in septic mice.

6.2.4 SA-AKI

Antioxidants that neutralize free radicals, such as vitamin E and phenolic compounds, have been shown to enhance ferroptosis and improve renal function (257). Maresin conjugates in tissue regeneration-1 (MCTR1) mitigate SA-AKI by modulating Nrf2 expression, thereby further preventing renal ferroptosis (258).

6.2.5 SAE

In SAE, the ferroptosis inhibitor Fer-1 prevents neuronal death and reduces glutamate toxicity (259). Animal studies have also shown that Fer-1 significantly improves survival rates in SAE mice and alleviates cognitive dysfunction by inhibiting ferroptosis (260). In parallel, the mTOR inhibitor rapamycin reduces inflammatory responses, mitigates SAE, and improves cognitive deficits in septic mice by activating autophagy and suppressing pyroptosis (261, 262).

6.3 Other critical illness

6.3.1 ARDS/ALI

In vitro and *in vivo* studies have demonstrated that panaxydol (PX), a polyacetylene compound from ginseng root, dose-dependently increases GSH and GPX4 expression while significantly reducing Fe^{2+} accumulation. These effects lead to substantial improvements in lung histopathology in lipopolysaccharide-induced injury (183).

Artesunate (AS) enhances the anti-apoptotic pathways of Nrf2 and HO-1 while activating the mTOR/AKT signaling pathway, reducing lung tissue damage and neutrophil infiltration (263, 264). Certain metabolites, including oxgonolone and itaconic acid, also protect against LPS-induced ALI by activating the Nrf2 pathway (204, 265).

6.3.2 AKI

Skouta et al. initially confirmed the ferroptosis-inhibiting effects of hepostatin-1 in a rhabdomyolysis-induced AKI model (266). Fer-1 significantly reduces creatinine and blood urea nitrogen levels, mitigating kidney damage in cisplatin-induced AKI and nephrotoxic folate-induced AKI models (216, 267). FG-4592 prevents ferroptosis by activating Nrf2, protecting against folate-induced kidney damage (268). Paricalcitol inhibits cisplatin-induced AKI by modulating GPX4's antioxidant properties through vitamin D receptor activation (216).

Quercetin inhibits ferroptosis by increasing GSH levels and reducing both MDA and lipid ROS levels (269). Nuciferine directly reduces ferroptosis by limiting iron accumulation, reducing oxidative stress, and minimizing lipid peroxidation, all in a GPX4-dependent manner (270). Tocilizumab and small-molecule compounds from traditional Chinese medicine (e.g., nobiletin and tectorigenin) also inhibit fibrotic progression and ferroptosis (271–273).

6.3.3 Acute liver injury

Lőrincz et al. demonstrated that Fer-1 offers limited protection against APAP-induced ferroptosis in primary murine hepatocytes (274). According to Schnellmann et al., DFO's intracellular iron chelation reduces liver injury caused by APAP (275).

The antioxidant protein sestrin2 (Sesn2) plays a crucial role in responding to various stressors and maintaining cellular homeostasis (276). Cells expressing Sesn2 exhibit resistance to ferroptosis, ROS generation, and GSH depletion induced by erastin. *In vivo*, adenovirus-mediated Sesn2 expression completely mitigated the elevated serum ALT/AST levels and histological changes in animals pretreated with phenylhydrazine (277).

Glycyrrhizic acid, an inhibitor of HMGB1, can suppress ferroptosis by mitigating oxidative stress, thus reducing liver injury (278). Similarly, promethazine alleviates acute liver failure induced by LPS and d-galactosamine (GalN) by inhibiting lipid peroxidation and reducing cell death (279).

6.3.4 Stroke

In the context of stroke, Tuo et al. demonstrated that tau-mediated iron export alleviated ferroptosis-induced damage in gerbils following ischemic stroke. Carvacrol further inhibited ferroptosis by enhancing GPX4 levels (280, 281). DFO effectively blocked brain injury induced by MCAO, although its short half-life limits its efficacy, requiring sustained administration for cerebral protection (282). Compound 2-(1-(4-(4-methylpiperazin-1-yl) phenyl)ethyl)-10H-phenothiazine (51) also demonstrated efficacy in preventing ferroptosis in an MCAO ischemic stroke model (283).

Furthermore, Fer-1 significantly mitigated secondary brain injury following intracerebral hemorrhage (284). When administered via lateral ventricle injection, Fer-1 reduced lesion volume, neurodegeneration, and iron deposition, ultimately improving the long-term cognitive outcomes of affected animals (234).

6.3.5 TBI

In TBI, overexpression of Lip-1 in a mouse model improved cognitive function by reducing brain lesion size and neurodegeneration (285). The neuroprotective effects of Lip-1 are attributed to its ability to lower iron and lipid peroxidation levels and restore glutathione in the injured brain tissue. Polydatin has been shown to reverse the deposition of free iron ions/Fe^{2+}, elevate MDA levels, decrease GPX4 activity in the injured area, and protect neurons in TBI models (286).

Tetrandrine (Tet) treatment improved neurological scores in TBI mice, reduced brain contusion and edema, and increased expression of GPX4, GSH, SLC7A11, and FTH, while decreasing MDA levels (287). Melatonin also mitigates lipid peroxidation via the circPtpn14/miR-351-5p/5-LOX signaling cascade, reducing iron deposition and neurodegeneration, ultimately improving neurological function after TBI (288, 289).

7 Clinical application of ferroptosis

Fer-1 has shown protective effects against doxorubicin-induced cardiac injury without altering iron levels, preventing cell death in cultured cardiomyocytes (72, 290), and reducing mitochondrial damage. Additionally, Fer-1 significantly alleviates cardiac injury, inflammation, and survival time in LPS-treated mice (252). Fer-1 has also been demonstrated to inhibit iron deposition in various acute kidney injury models, exerting renoprotective effects (193). Lip-1, a spiroquinoxaline amine derivative identified through high-throughput screening in $Gpx4^{-/-}$ cells (20), has been shown to significantly reduce palmitate-induced cardiac injury (291). In both unilateral ureteral obstruction (UUO) and radiation-induced lung fibrosis models, Lip-1 administration improved ferroptosis and fibrosis (292, 293) outcomes. MitoTEMPO, a mitochondria-targeted antioxidant, prevented ferroptosis-induced cardiac injury by inhibiting lipid peroxidation in murine hearts (72).

Ferroptosis, an iron-dependent form of programmed cell death, can be inhibited by iron chelators (11). Dexrazoxane, a cyclic ethylenediaminetetraacetic acid derivative, easily crosses cell membranes and accumulates in the mitochondria of cardiomyocytes, where it chelates intracellular free iron (294, 295). Similarly, DFO reduces ferroptosis and fibrosis in CKD rat models (296). While iron chelators protect various tissues and organs from injury, their protective effects may not solely result from ferroptosis inhibition.

Rapamycin reduces TfR1 expression, lowering intracellular iron levels, and activates the IRP1/2 system to compensate for the downregulation of iron transporters, thereby shielding cardiomyocytes from iron overload and ferroptosis (297, 298). Zinc protoporphyrin IX, a competitive inhibitor of HO-1, prevents doxorubicin-induced iron accumulation and ferroptosis in the murine heart by blocking heme degradation and limiting free iron release (72). Additionally, atorvastatin alleviates isoproterenol-induced cardiac dysfunction and remodeling in mice by inhibiting ferritin autophagy-mediated ferroptosis (299). Puerarin protects cultured cardiomyocytes from ferroptosis induced by both erastin and isoproterenol (300). Cyanidin-3 glucoside (CG3), an anthocyanin, mitigates ferroptosis by reducing Fe^{2+} content through TFR1 and FTH1 modulation, which in turn reduces myocardial IRI (301).

8 Discussion and prospect

As research progresses, ferroptosis is increasingly recognized for its critical role in various diseases, offering new perspectives for the treatment of related disorders. The involvement of ferroptosis in IRI and sepsis-induced tissue damage varies across organs, influenced by differences in metabolic activity, iron homeostasis, antioxidant defenses, and cellular sensitivity to oxidative stress. Understanding these variations clarifies why certain tissues are more vulnerable under pathological conditions.

Organs such as the heart, brain, and kidneys, which have high metabolic rates and oxygen demands, are particularly susceptible to severe oxidative stress during IRI and sepsis. Reperfusion after ischemia triggers increased ROS production. For example, the brain's high PUFA content makes it especially vulnerable to lipid peroxidation, a key step in ferroptosis (162). In contrast, tissues with lower metabolic rates generate fewer ROS and are less prone to ferroptosis.

Iron metabolism varies across tissues, influencing susceptibility to ferroptosis. The liver, a primary iron storage site, has elevated iron levels that promote ROS production, increasing ferroptosis risk during IRI or sepsis (277). Tissues with lower iron content, like skeletal muscle, exhibit reduced ferroptotic activity. Additionally, an organ's antioxidant capacity is pivotal. The liver, equipped with robust antioxidants such as glutathione and superoxide dismutase (SOD), counters oxidative stress but becomes vulnerable to ferroptosis if these defenses are compromised during sepsis or IRI (277). Similarly, the kidney's high susceptibility to ferroptosis arises when its protective mechanisms are overwhelmed, resulting in acute injury.

Lipid composition also plays a key role in ferroptosis susceptibility. The brain's high PUFA content predisposes it to lipid peroxidation under stress, exacerbating damage during IRI and sepsis (227). Conversely, tissues like the pancreas, with distinct lipid profiles, show varied susceptibility. Organ-specific inflammatory responses during sepsis further influence ferroptosis development. The liver and kidneys, with significant immune cell infiltration, are particularly prone to ROS-induced damage and ferroptosis due to the pro-inflammatory environment (212).

Ferroptosis represents a unique form of cell death that interacts with other cell death pathways, offering potential for combination therapies in the future. Despite extensive research into the molecular mechanisms of ferroptosis, significant challenges remain, as the field is still emerging. For example, the molecular signaling pathways that regulate ferroptosis are not fully elucidated. The mechanisms behind p53's bidirectional regulation in ferroptosis remain unclear. Furthermore, while iron is crucial for ferroptosis, it is unknown whether other metal ions can replace it. What are the downstream genes regulated by iron metabolism, and what are the specific molecular mechanisms involved? Moreover, current techniques cannot detect ferroptosis *in vivo* through specific biomarkers. Identifying biomarkers that reflect ferroptosis and disease severity would significantly enhance clinical diagnostics. Future research

could focus on discovering ferroptosis biomarkers through transcriptional or metabolomic approaches. Additionally, the clinical efficacy of ferroptosis inhibitors remains uncertain, presenting a major research gap. Although some studies have explored clinically used drugs in animal models or specific cell types, research on clinical safety and efficacy is sparse. Comprehensive studies are needed to define the therapeutic potential of ferroptosis inducers and inhibitors, including optimal timing, dosages, administration routes, and treatment durations. Further preclinical and clinical research is crucial to assess ferroptosis's impact in humans and facilitate the development of targeted therapies for human diseases.

In conclusion, investigating ferroptosis, a recently identified cell death pathway, may lead to groundbreaking advancements in critical care and the treatment of severe diseases. Key questions remain, such as how ferroptosis is regulated in various critical conditions and how best to modulate it. Answers to these questions will provide a foundation for halting disease progression and identifying effective therapeutic targets.

Author contributions

RT: Conceptualization, Writing – original draft, Writing – review & editing. CG: Writing – original draft. YY: Conceptualization, Writing – original draft. HG: Resources, Writing – original draft. XH: Resources, Writing – original draft. QZ: Supervision, Writing – original draft. QD: Funding acquisition, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1511015/full#supplementary-material

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Identification and validation of five ferroptosis-related molecular signatures in keloids based on multiple transcriptome data analysis

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Introduction: Keloids are a common skin disorder characterized by excessive fibrous tissue proliferation, which can significantly impact patients' health. Ferroptosis, a form of regulated cell death, plays a crucial role in the development of fibrosis; however, its role in the mechanisms of keloid formation remains poorly understood.

Methods: This study aimed to identify key genes associated with ferroptosis in keloid formation. Data from the NCBI GEO database, including GSE145725, GSE7890, and GSE44270, were analyzed, comprising a total of 24 keloid and 17 normal skin samples. Additionally, single-cell data from GSE181316, which included 8 samples with complete expression profiles, were also evaluated. Differentially expressed genes were identified, and ferroptosis-related genes were extracted from the GeneCards database. LASSO regression was used to select key genes associated with keloids. Validation was performed using qRT-PCR and Western blot (WB) analysis on tissue samples from five keloid and five normal skin biopsies.

Results: A total of 471 differentially expressed genes were identified in the GSE145725 dataset, including 225 upregulated and 246 downregulated genes. Five ferroptosis-related genes were selected through gene intersection and LASSO regression. Two of these genes were upregulated, while three were downregulated in keloid tissue. Further analysis through GSEA pathway enrichment, GSVA gene set variation, immune cell infiltration analysis, and single-cell sequencing revealed that these genes were primarily involved in the

Abbreviations: DEGs, Differentially expressed genes; GEO, Gene Expression Omnibus; LASSO, Least absolute shrinkage and selection operator; AUC, Area under the receiver operating characteristic curve; GO, Gene ontology; ROC, Receiver operating characteristic curve; GSVA, Gene Set Variation Analysis; GSEA, Gene set enrichment analysis; BCA, Bicinchoninic Acid; RIPA, Radioimmunoprecipitation Assay.

fibrotic process. The qRT-PCR and WB results confirmed the expression patterns of these genes.

Discussion: This study provides novel insights into the molecular mechanisms of ferroptosis in keloid formation. The identified ferroptosis-related genes could serve as potential biomarkers or therapeutic targets for treating keloids.

KEYWORDS

keloid, ferroptosis, fibrosis, immune infiltration, oxidative stress

1 Introduction

Keloid scarring, a complex fibroproliferative dermatosis, remains poorly understood in terms of its pathogenesis. It is characterized by excessive collagen deposition and abnormal fibroblast proliferation (Chike-Obi et al., 2009), predominantly affecting individuals aged 10–30 years and is especially prevalent among African, Hispanic, and Asian populations (Robles and Berg, 2007). Common sites of occurrence include the chest, earlobes, shoulders, and back. Beyond cosmetic disfigurement, keloids can significantly impact mental health and restrict local limb functionality. Despite the availability of various treatments, none have emerged as definitively effective (Ojeh et al., 2020). This underscores the urgent need for deeper insights into the molecular mechanisms driving keloid pathogenesis to improve therapeutic strategies.

Ferroptosis, according to Dixon, is a type of iron-dependent cell death characterized by lipid peroxidation and iron buildup (Dixon et al., 2012). Numerous diseases have been linked to pathophysiology of ferroptosis, including neoplasms, neurological diseases, ischemia-reperfusion injuries, cardiovascular diseases, and organ fibrosis, according to recent investigations (Hu et al., 2021; Mou et al., 2019). Fibrosis is an abnormal tissue repair process often associated with chronic inflammation. Inflammatory cells, such as macrophages and lymphocytes, infiltrate the affected area and release various pro-inflammatory factors, including IL-1, IL-6, and IFN. This cascade of events further promotes the activation of fibroblasts and the deposition of extracellular matrix components. Characteristics of ferroptosis include lipid peroxidation, GPX4 inhibition, and iron accumulation, which harm parenchymal cells and lead to fibrotic lesions that abnormally collect in tissues, thereby promoting fibrosis of tissues and organs (Weiskirchen et al., 2019). Currently, the role of ferroptosis in fibrosis has been validated by multiple studies, including those focusing on renal, cardiac, hepatic, and pulmonary fibrosis. El-Horany found that in a mouse model, bleomycin-induced pulmonary fibrosis was associated with increased ferroptosis markers (El-Horany et al., 2023). Chen J et al. observed that TRIM23 inhibits hepatic stellate cell (HSC) ferroptosis by regulating p53 ubiquitination, which in turn promotes cell activation and liver fibrosis (Chen et al., 2024). Wang et al. demonstrated that modulation of the JNK/p53 pathway by the MLK3 pathway which promotes ferroptosis attenuates myocardial fibrosis (Wang et al., 2020). These studies indicate that ferroptosis plays a crucial role in regulating fibrosis, potentially promoting fibrosis in various organs and tissues.

Keloids are characterized by persistent inflammation, progressive fibrosis, and irregular patterns of cell proliferation and apoptosis (Li et al., 2022). Inflammation, excessive oxidation, and iron accumulation are all linked to ferroptosis, suggesting that this cell death process influences fibrosis via multiple mechanisms, and may play a significant role in the development of keloids. The expression and function of ferroptosis-related genes in keloids remain poorly understood. This study focuses on screening ferroptosis-related genes to investigate how ferroptosis may influence keloid formation.

The flowchart of the study is shown in Figure 1.

2 Materials and methods

2.1 Downloading data

The NCBI GEO public database included the Series Matrix File for GSE145725 (Kang et al., 2020), annotated as GPL16043, which contained expression profile data for 19 individuals, 10 in the normal group and 9 in the illness group. Comparably, the expression profile data for ten patients—equally divided between the normal and illness groups—were found in the Series Matrix File for GSE7890 (Smith et al., 2008), annotated as GPL570, which was also acquired from the same database. In addition, expression profile data for 12 patients—three in the normal group and nine in the illness group—were obtained from the Series Matrix File for GSE44270 (Hahn et al., 2013), labeled as GPL6244. The NCBI GEO public database included the single-cell data file for GSE181316, which contained complete expression profiles for 8 samples.

2.2 Analysis of differential expressions

The R package Limma is employed for differential expression analysis of expression profiles to identify genes with significantly different expression levels between groups (Ritchie et al., 2015). The "Limma" R program was used to create heat maps and differential gene volcano plots, identify genes with differential expression between disease and control samples, and analyze keloid data for variations in molecular processes. This facilitated the identification of genes with differential expression between disease and control samples. Heat maps and differential gene volcano plots were generated, with thresholds set at P Value <0.05 and |logFC| > 1 for differential gene screening.

2.3 Analysis of functional enrichment

The Metascape database (www.metascape.org) was used to perform functional annotation of intersecting genes to

TABLE 1 The sequences of the primers for key genes.

Genes		Sequence	Size (bp)
HOMO_GAPDH	Forward	5′-TCAAGAAGGTGGTGAAGCAGG-3′	115 bp
	Reverse	5′-TCAAAGGTGGAGGAGTGGGT-3′	
HOMO_SOCS2	Forward	5′-GAGCCGGAGAGTCTGGTTTC-3′	239 bp
	Reverse	5′-ATCCTGGAGGACGGATGACA-3′	
HOMO_SLC38A1	Forward	5′-GCTTTGGTTAAAGAGCGGGC-3′	295 bp
	Reverse	5′-AGCTTGACACCCCTGTTAGC-3′	
HOMO_SNCA	Forward	5′-ATTCGACGACAGTGTGGTGT-3′	102 bp
	Reverse	5′-GTTTTCTCAGCAGCAGCCAC-3′	
HOMO_PLIN2	Forward	5′-TGATGGCAGGCGACATCTAC-3′	239 bp
	Reverse	5′-CTGGCTGCTCTTGTCCATCT-3′	
HOMO_AKR1C3	Forward	5′-GTCACTTCATGCCTGTCCTG-3′	249 bp
	Reverse	5′-GGACCAACTCTGGTCGATGAA-3′	

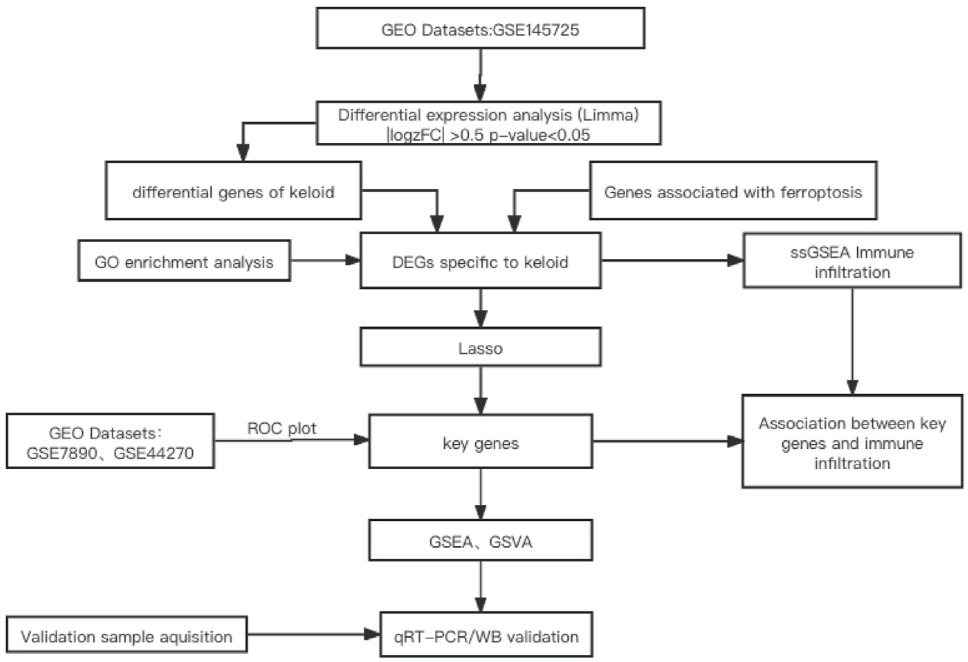

FIGURE 1
Flowchart of the research methodology.

investigate their functional significance comprehensively. These genes underwent Gene Ontology (GO) pathway analysis, with statistical significance determined by a minimum overlap of ≥3 and $p \leq 0.01$.

2.4 Predictive modeling

After selecting the potential genes, a predictive correlation model was developed using lasso regression (Friedman et al.,

2010). A risk score formula was constructed for each patient based on the expression values of each gene. Weights were applied according to the predicted regression coefficients from the lasso regression analysis. This algorithm was used to compute each patient's risk score. ROC curves assessed the model's prediction accuracy (Robin et al., 2011).

2.5 Analysis of single-cell sequencing

First, the data was processed using the Seurat software, followed by the application of the t-SNE method to evaluate the data and ascertain the spatial relationships among clusters. Next, the celldex package was used to annotate the clusters, associating them with specific cells relevant to the disease's occurrence. Finally, the FindAllMarkers function's parameter was set to 1 to identify marker genes from single-cell expression data for each cell subtype. Marker genes were selected based on criteria of adjusted p-value <0.05 and |avg log2FC| > 1 (Tang et al., 2009; Ziegenhain et al., 2017).

2.6 Analysis of immune cell infiltration

Single-sample Gene Set Enrichment Analysis (ssGSEA) is a commonly used method to evaluate various types of immune cells inside the microenvironment. B cells, NK cells, and T cells are among the 29 human immune cell phenotypes that may be identified with this approach. In this study, the ssGSEA algorithm was employed to quantify immune cells within expression profiles, facilitating the estimation of relative proportions among 29 types of immune-infiltrating cells. Furthermore, a Spearman correlation analysis was performed to explore the relationship between gene expression levels and the content of immune cells.

2.7 Gene set enrichment analysis (GSEA)

Gene Set Enrichment Analysis (GSEA) utilizes predefined gene sets and ranks genes based on their differential expression levels between two sample classes. The analysis then determines whether these predefined gene sets are significantly enriched at either the top or bottom of the ranking list. This study employs GSEA to compare the differences in signaling pathways between high and low expression groups, thereby exploring the molecular mechanisms of key genes in both groups of patients. For the analysis, the number of permutations is set to 1,000, with phenotype as the permutation type.

2.8 Gene set variation analysis (GSVA)

Gene Set Variation Analysis (GSVA) is a non-parametric, unsupervised method for assessing the enrichment of gene sets in transcriptomic data (Hanzelmann et al., 2013). GSVA evaluates biological functions of samples by converting gene-level variations into pathway-level changes through an integrated scoring of the gene sets of interest. In this study, gene sets will be downloaded from the Molecular Signatures Database and the GSVA algorithm will be applied to each set to generate comprehensive scores, assessing potential biological function changes in different samples.

2.9 Sources of tissue samples

Ethical approval for this study was granted by the Research Ethics Committee of the Second Hospital of Lanzhou University (the project approval number is 2023A-796), and informed consent was obtained from all participants. Samples were collected from both the Plastic and Reconstructive Surgery Ward and the Outpatient Clinic at the Second Hospital of Lanzhou University. A total of five samples each of normal skin tissue and keloid tissue were collected. The exclusion criteria for keloid tissue samples included: 1) previous radiotherapy or injection therapy within the past 2 years, and 2) presence of diabetes, autoimmune diseases, or malignant tumors.

2.10 Quantitative real-time polymerase chain reaction and RNA extraction (RT-qPCR)

Total RNA was extracted from 5 normal tissue samples and 5 keloid tissue samples using the Trizol method. Subsequently, the extracted total RNA was reverse transcribed into complementary DNA (cDNA) using HiScript II Q RT SuperMix for qPCR (+gDNA wiper). Quantitative PCR (qPCR) was then performed using Taq Pro Universal SYBR qPCR Master Mix. The thermal cycling conditions for qPCR were set as follows: an initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 60°C for 30 s. Primer sequences for each gene are listed in the attached table. The sequences of the primers for key genes are listed in (Table 1). Relative gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method, with GAPDH as the endogenous control.

2.11 Western blot analysis and protein extraction

Total protein was extracted from five normal samples and five keloid samples using radioimmunoprecipitation assay (RIPA) buffer, and quantified using the bicinchoninic acid (BCA) assay. Equal amounts of protein were loaded onto SDS-PAGE. The gel was then transferred, and the membrane was blocked with 5% bovine serum albumin (BSA). The membrane was subsequently incubated with primary and secondary antibodies. The following antibodies were used: AKR1C3 rabbit polyclonal antibody, SLC38A1 rabbit polyclonal antibody, SNCA rabbit polyclonal antibody, SOCS2 rabbit polyclonal antibody (Abcam, Shanghai, China), PLIN2 rabbit polyclonal antibody, β-actin mouse monoclonal antibody, HRP-conjugated anti-rabbit secondary antibody, and HRP-conjugated anti-mouse secondary antibody (all from ProteinTech, Wuhan, China). After washing the membrane with TBST solution, the results were detected using chemiluminescence. Relative quantification was analyzed using ImageJ software for densitometry.

2.12 Statistical analysis

Data collection and statistical analyses were performed using R (version 4.2.2), and all tests were two-sided. GraphPad Prism

(version 9.0) was used for qPCR and Western blot analyses. Data for keloid and normal skin tissues were presented as mean ± SEM. Differences between the groups were compared using Student's t-test and Welch's correction. A p-value of <0.05 was considered statistically significant.

3 Results

3.1 Keloid-specific DEG collection

We downloaded the GSE145725 dataset from the NCBI GEO public database, which included samples from 19 patients, with 10 in the control group and 9 in the disease group. Differential gene expression analysis was conducted using the limma package. Genes were selected based on the criteria of $p < 0.05$ and $|logFC| > 1$. A total of 471 differentially expressed genes were identified, comprising 225 upregulated and 246 downregulated genes (Figures 2A, B). Subsequently, we utilized the GeneCards database (https://www.genecards.org/) to extract genes related to ferroptosis with a Relevance score >1. An intersection of ferroptosis-related genes with the differentially expressed genes yielded 14 genes associated with ferroptosis (Figure 2C). Further pathway analysis on this intersected set revealed significant enrichment in pathways such as monocarboxylic acid metabolic process, epithelial cell differentiation, and molecular adaptor activity (Figure 3).

3.2 Construction of a predictive model for genes associated with ferroptosis in keloids

To perform feature screening by Lasso regression, we used the GSE145725 dataset as the training set and the GSE7890 and GSE44270 datasets as the validation sets. Lasso regression identified five key genes associated with keloids, which were then utilized to construct the predictive model (Figures 4A, B). RiskScore = SNCA × (−0.226932318008185) + SLC38A1 × (−0.144538726326697) + SOCS2 × (−0.033573222490279) + AKR1C3 × 0.0671905003157174 + PLIN2 × 0.102513500537292 was the model formula (Figure 4C). The prediction model (Figure 4D) showed an area under the curve (AUC) of 1, indicating high diagnostic effectiveness. AUC (Area Under the Receiver Operating Characteristic Curve) is an important performance evaluation metric, particularly suitable for imbalanced datasets, and it can comprehensively assess classification performance, ensuring the selection of the most appropriate metric to reflect the accuracy of predictive results (Parodi et al., 2022). Therefore, we employed the GSE7890 and GSE44270 datasets as external validation sets to further substantiate the model. The model's remarkable stability was validated by the validation findings, which showed an AUC of 0.7407 (Figure 4E) for the GSE44270 dataset and 0.72 (Figure 4F) for the GSE7890 dataset.

3.3 Single-cell sequencing analysis

Using the Seurat software, we conducted a single-cell analysis after downloading the single-cell data from the GSE181316 dataset. The tSNE technique was used to cluster the cells, producing 36 different isoforms (Figure 5A). The 36 clusters were divided into six cell types using the SingleR R package to annotate every subtype: fibroblasts, endothelial cells, epithelial cells, monocytes, adipocytes, and $CD8^+$ T-cells (Figure 5B). Figures 5C, D shows the expression levels of important genes within each of these six cell types. According to our investigation, endothelial cells, epithelial cells, monocytes, and adipocytes are the main cell types that exhibit high expression of SNCA. Similarly, SLC38A1 exhibited high expression in endothelial cells, monocytes, adipocytes, epithelial cells, and $CD8^+$ T-cells. SOCS2 was predominantly expressed in endothelial cells and adipocytes. AKR1C3 showed high expression in fibroblasts, endothelial cells, and adipocytes, while PLIN2 was highly expressed across fibroblasts, monocytes, endothelial cells, $CD8^+$ T-cells, adipocytes, and epithelial cells.

3.4 Analysis of key gene infiltration by immune cells

Clinical therapeutic response and disease diagnosis are significantly influenced by the intricate interplay of immune cells, growth factors, extracellular matrix components, inflammatory mediators, and specific physicochemical features that comprise the microenvironment. There is a notable association between fibrosis and immune infiltration, particularly during inflammatory diseases and tissue repair processes (Brandt et al., 2020; Distler et al., 2019; Rosenbloom et al., 2017). In order to get further insight into the connection between important genes and immune infiltration, we examined the keloid dataset to determine the possible biochemical pathways by which these genes affect the development of keloid formation. The figure (Figures 6A, B) shows the percentage of immune cells present in each patient and the connections between different immune cells. Notably, we found that the groups differed significantly in the expression of APC co-inhibition, B cells, molecules that promote inflammation, T cell co-inhibition, Th1 cells, and Type II IFN response (Figure 6C). We also looked at the connections between immune cells and key genes. According to our research, SNCA has a substantial negative correlation with Th1 cells but a significant positive correlation with T cell and APC co-inhibition (Figure 7A). SLC38A1 exhibited a positive association with Type II IFN response, a substantial negative correlation with MHC class I, and significant positive correlations with B cells and T cell co-inhibition (Figure 7B). Significant positive relationships were found between SOCS2 and the Type II IFN response, T cell co-inhibition, and APC co-inhibition (Figure 7C). Furthermore, AKR1C3 was considerably negatively connected with B cells and significantly positively correlated with neutrophils and the Type I IFN response (Figure 7D). PLIN2 also showed a substantial negative correlation with Type II IFN response and B cells, but a significant positive correlation with neutrophils and CCR (Figure 7E).

3.5 Key genes and various immune factor correlation

Using information from the TISIDB database, We looked at the connections between five key genes and additional immunological elements, including immunomodulators (Figures 8A,

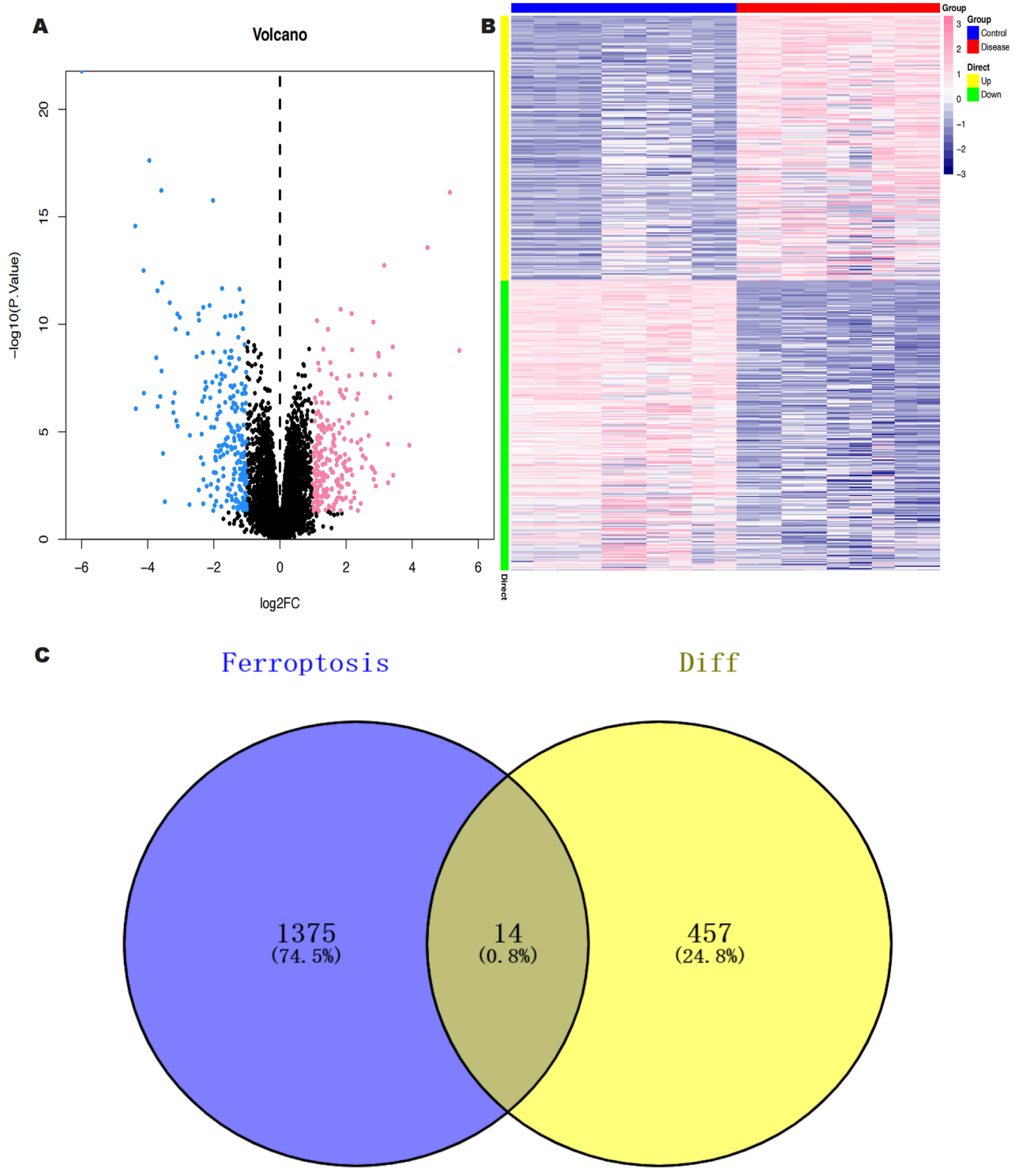

FIGURE 2
(A) Volcano plot of differentially expressed genes (DEGS) between keloid and normal skin samples; red color represents up-regulated genes, black color represents non-significantly different genes, and blue color represents down-regulated genes. **(B)** Heat map of DEGs between keloid and normal skin samples. **(C)** Screening of keloid DEGS intersecting with Ferroptosis genes.

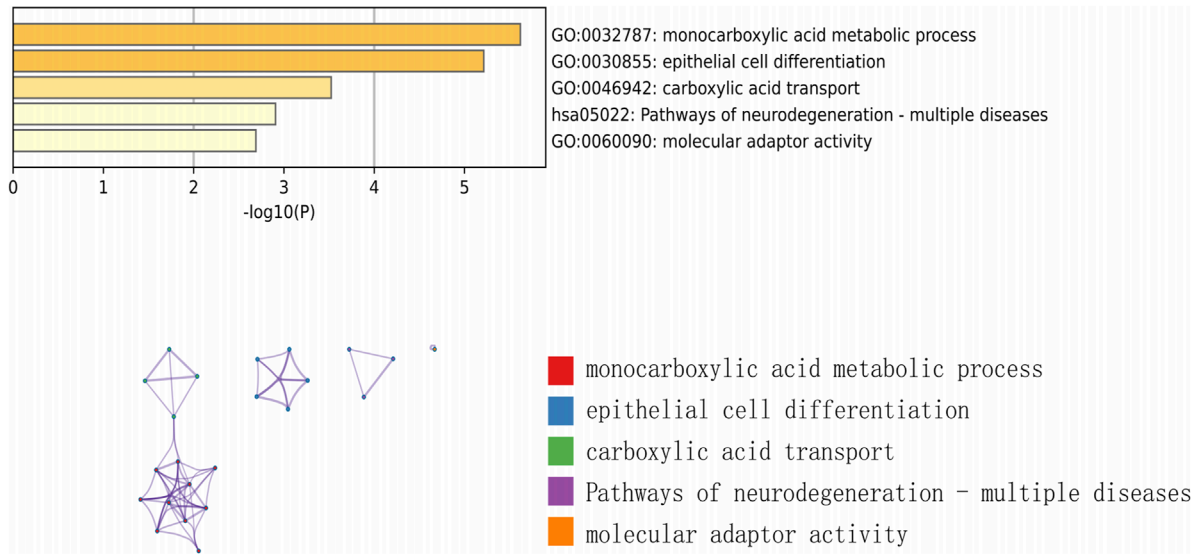

FIGURE 3
GO functional enrichment analysis of keloid ferroptosis intersecting DEGs.

B), MHC (Figure 8C), chemokines (Figure 8D), and cellular receptors (Figure 8E). Our results indicate that the key genes are essential to the immunological milieu and that the level of immune cell infiltration is highly linked with them.

3.6 Gene set enrichment analysis

We used GSEA to investigate the possible molecular pathways via which these important genes affect keloid growth. The results revealed that SLC38A1 is rich in pathways like the presentation and processing of antigens, Notch signaling, and protein export (Figure 9A). AKR1C3 is enriched in peroxisome, drug metabolism via cytochrome P450, and beta-alanine metabolism pathways (Figure 9B). SOCS2 exhibits enrichment in the ErbB, Hedgehog, and Wnt signalling pathways (Figure 9C). PLIN2 is enriched in galactose metabolism, the pentose phosphate pathway, and gap junctions (Figure 9D). SNCA is enriched in adherens junctions, beta-alanine metabolism, and cell adhesion molecules (CAMs) pathways (Figure 9E).

3.7 Gene set variation analysis

The GSVA results indicated that high expression of PLIN2 is enriched in several signaling pathways including IL-6 JAK-STAT3 signaling, Wnt/β-catenin signaling, p53 pathway, and TGF-β signaling (Figure 10A). Similarly, high expression of SOCS2 is predominantly associated with downregulated KRAS signaling, inflammatory response, hedgehog signaling, and peroxisome pathways (Figure 10B). High expression of SNCA was found to enrich the reactive oxygen species pathway, E2F targets, and mTORC1 signaling (Figure 10C). Furthermore, high expression of SLC38A1 is correlated with downregulated KRAS signaling, MYC targets v2, and peroxisome pathways (Figure 10D). High expression of AKR1C3 is involved in bile acid metabolism, IL-2 STAT5 signaling, notch signaling, and TGF-β signaling (Figure 10E). These findings suggest that key genes may influence the progression of keloids through these pathways.

3.8 Validation of key genes

We further validated the expression of five key genes in scar keloid and normal skin samples using RT-qPCR and Western blot analyses. We examined mRNA levels of these genes in five normal and five keloid samples. As shown in (Figures 11A–F), at the protein level, the expression of PLIN2 and AKR1C3 was significantly increased in keloid samples compared to normal samples, whereas the expression of SOCS2, SLC38A1, and SNCA was significantly decreased. Additionally, as depicted in (Figures 11G–K), at the mRNA level, there was also a significant increase in the expression of PLIN2 and AKR1C3 in keloid samples, and a significant decrease in the expression of SOCS2, SLC38A1, and SNCA. These findings are consistent with the expression patterns of key genes identified during the screening phase.

4 Discussion

Keloids represent an abnormal formation of fibrous tissue, typically developing on injured skin. Excessive tissue formation often results from overgrowth of granulation tissue or increased Type III collagen during the healing process. To date, the pathogenesis of keloids remains elusive. These scars are notoriously difficult to treat due to their tendency to recur after excision and significantly affect patients' quality of life, stemming from both aesthetic concerns

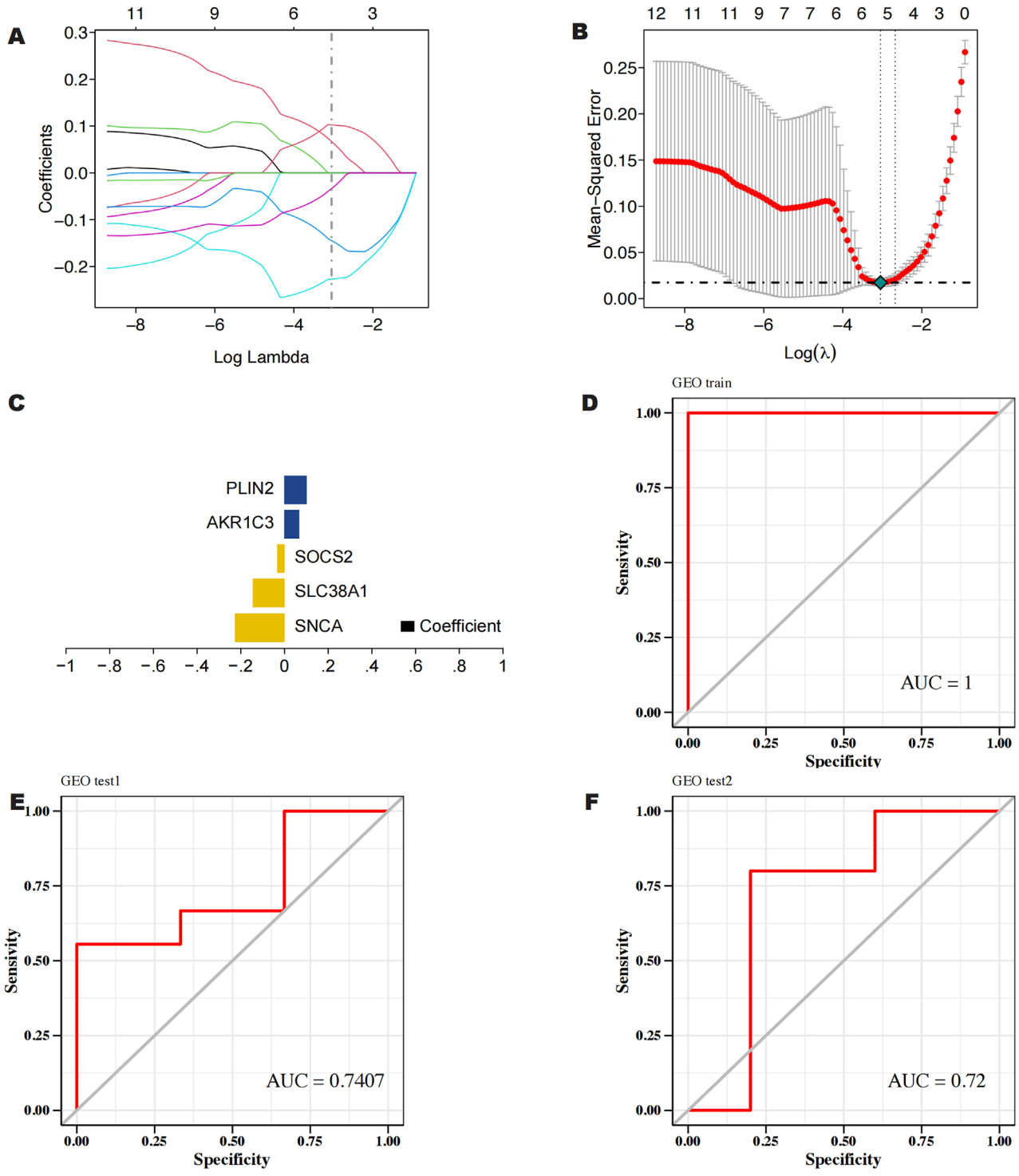

FIGURE 4
(A, B) Screening of characterized genes by LASSO regression analysis. **(C)** Construction of prediction models for 5 characterized genes **(D)** ROC curves of LASSO regression models for 5 characterized genes in GSE145725. **(E)** ROC curves of 5 characterized genes in GSE44270. **(F)** ROC curves of 5 characterized genes in GSE7890.

and functional impairments that can lead to psychological distress. Therefore, identifying molecular characteristics that can effectively prevent progression and enhance the prognosis of keloids is crucial.

Ferroptosis is a distinct mode of cell death, regulated by various cellular metabolic processes. The accumulation of intracellular iron and lipid peroxidation are two critical signals for membrane oxidative damage during ferroptosis (Rochette et al., 2022).

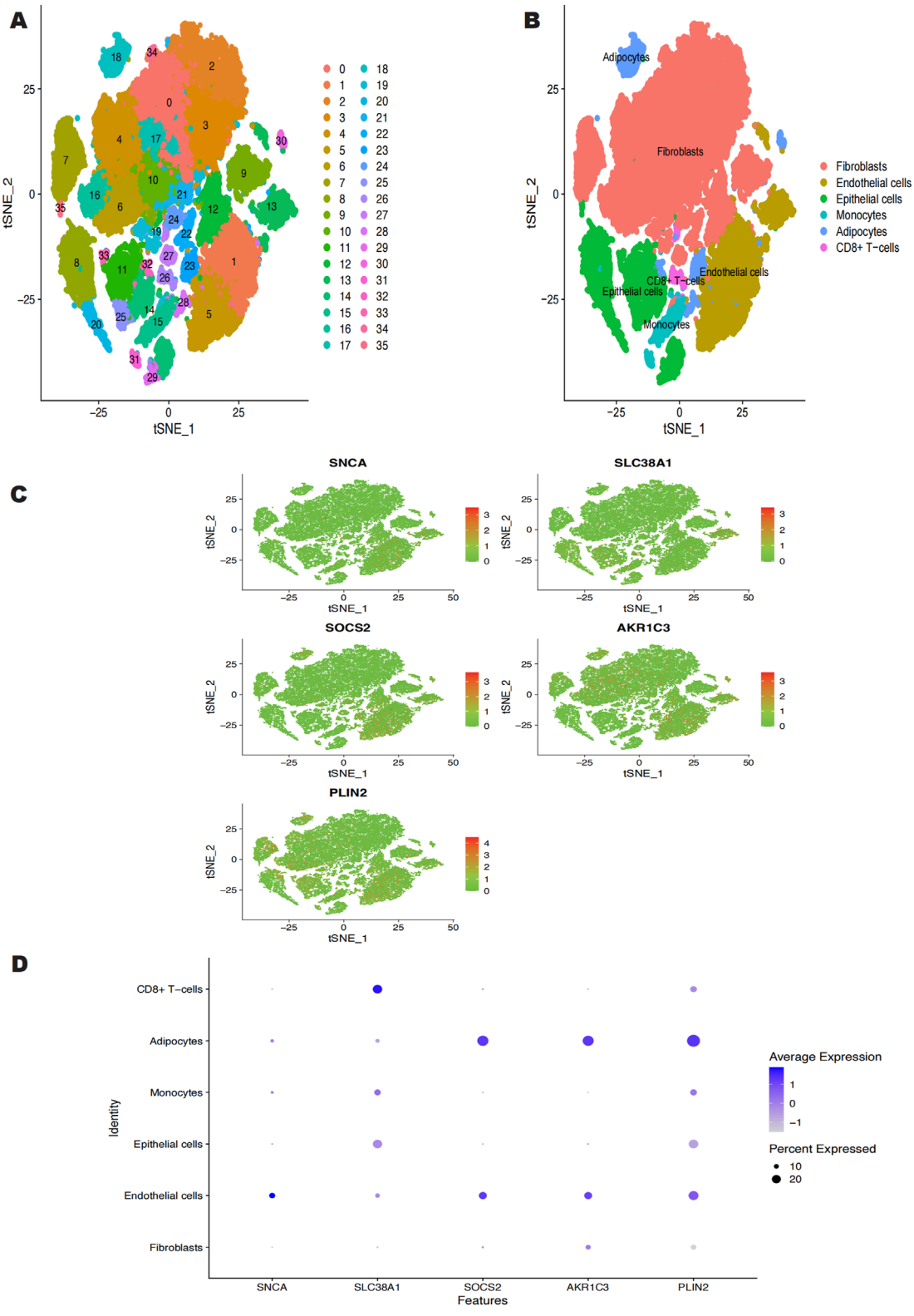

FIGURE 5
(A) GSE181316 single cell sequencing analysis, 36 isoforms were obtained. **(B)** 36 clusters were annotated into 6 cell categories, Fibroblasts, Endothelial cells, Epithelial cells, Monocytes, Adipocytes, CD8+ T-cells. **(C, D)** Expression of the 5 characterized genes in the 6 cell categories.

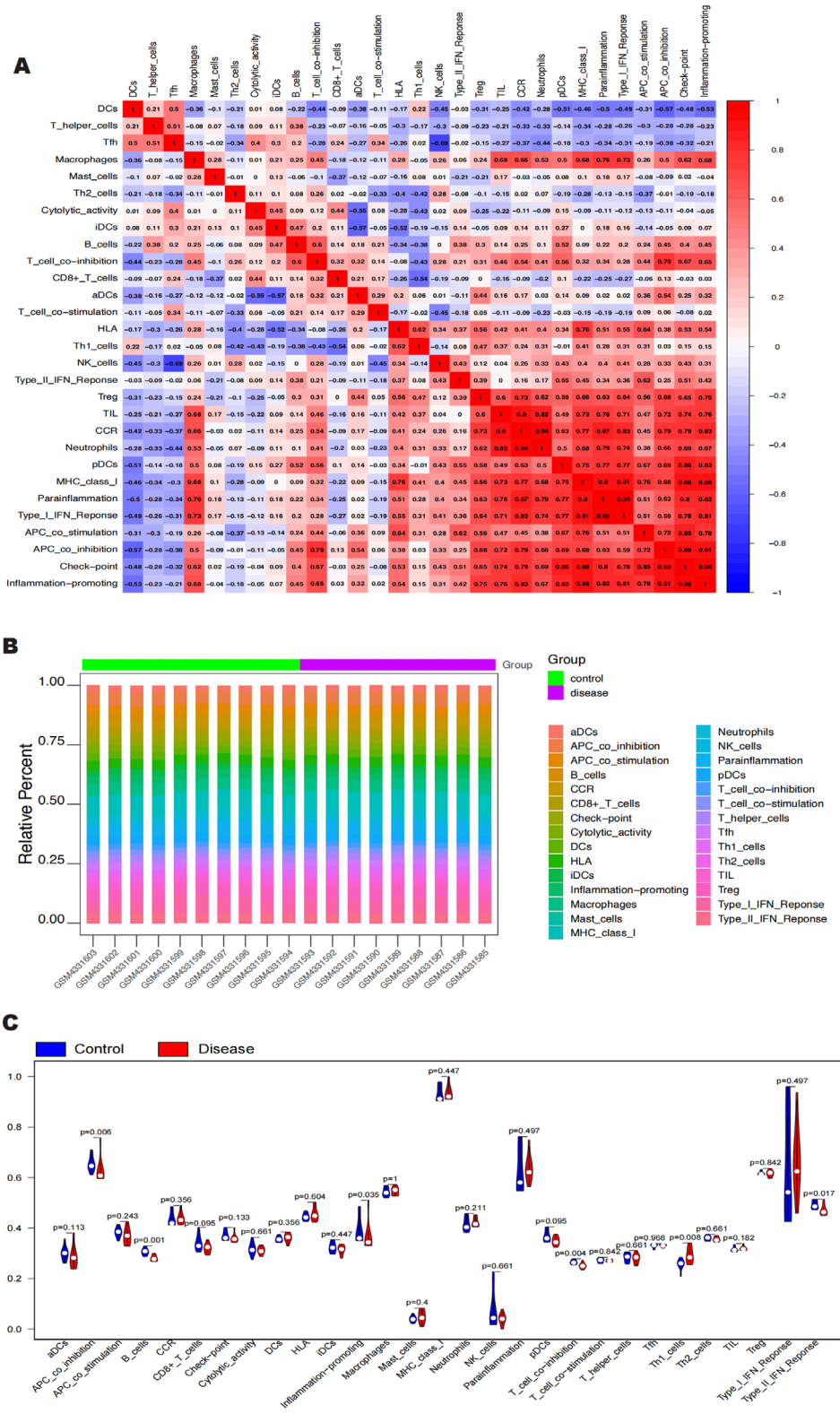

FIGURE 6
(A, B) Immune cell content as a percentage of each patient and correlation between immune cells. **(C)** APC_co_inhibition, B cells, Inflammation-promoting. T_cell_co_inhibition, Thl_cells, and Type_II_IFN_Reponse were significant in intergroup expression.

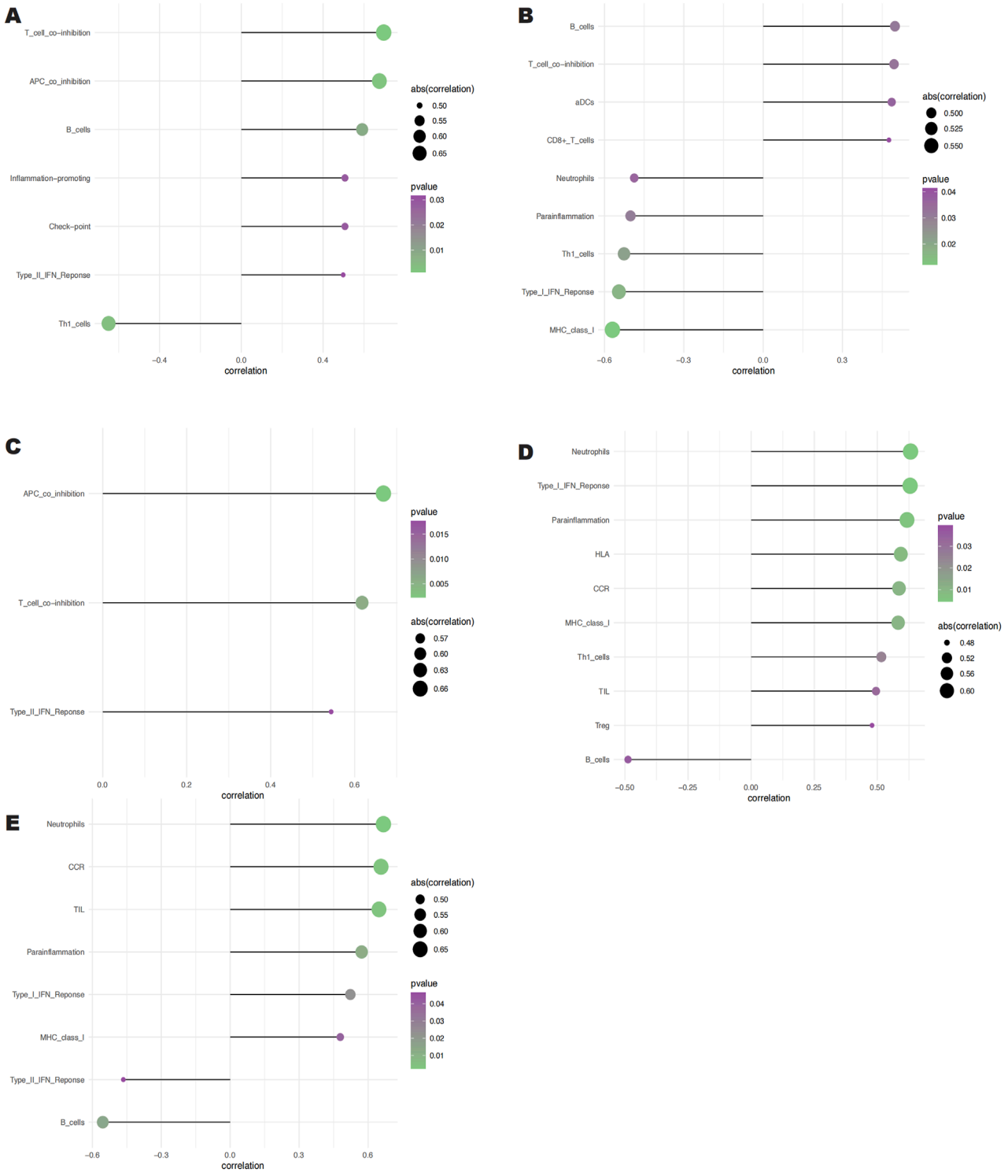

FIGURE 7
Relationships between the 5 key genes and immune cells. **(A)** Relationship between SNCA and immune cells. **(B)** Relationship between SLC38A1 and immune cells. **(C)** Relationship between SOCS2 and immune cells. **(D)** Rela- tionship between AKR1C3 and immune cells. **(E)** Relationship between PLIN2 and immune cells.

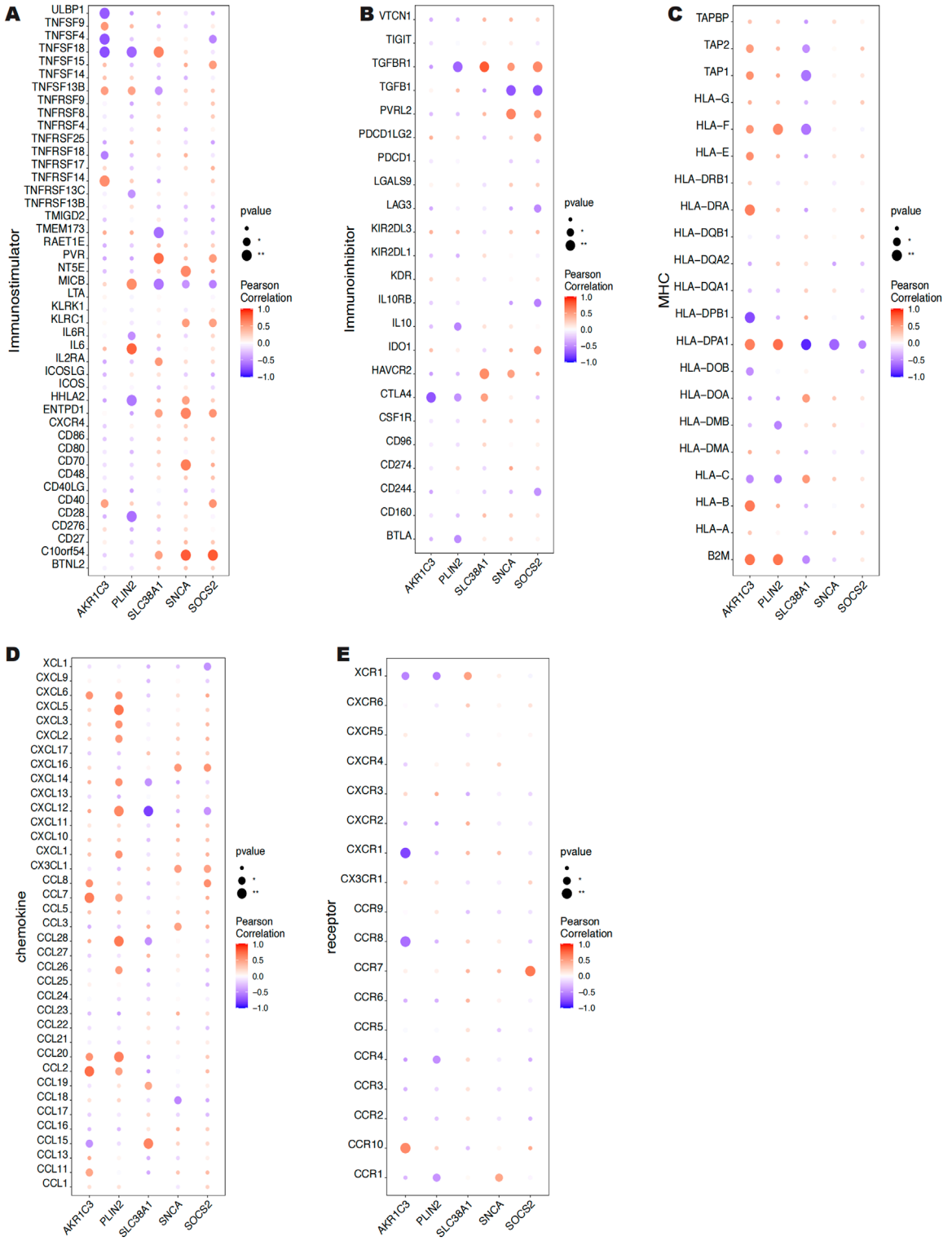

FIGURE 8
Correlation between 5 key genes and immunomodulatory factors, MHC, chemokines and cellular receptors.

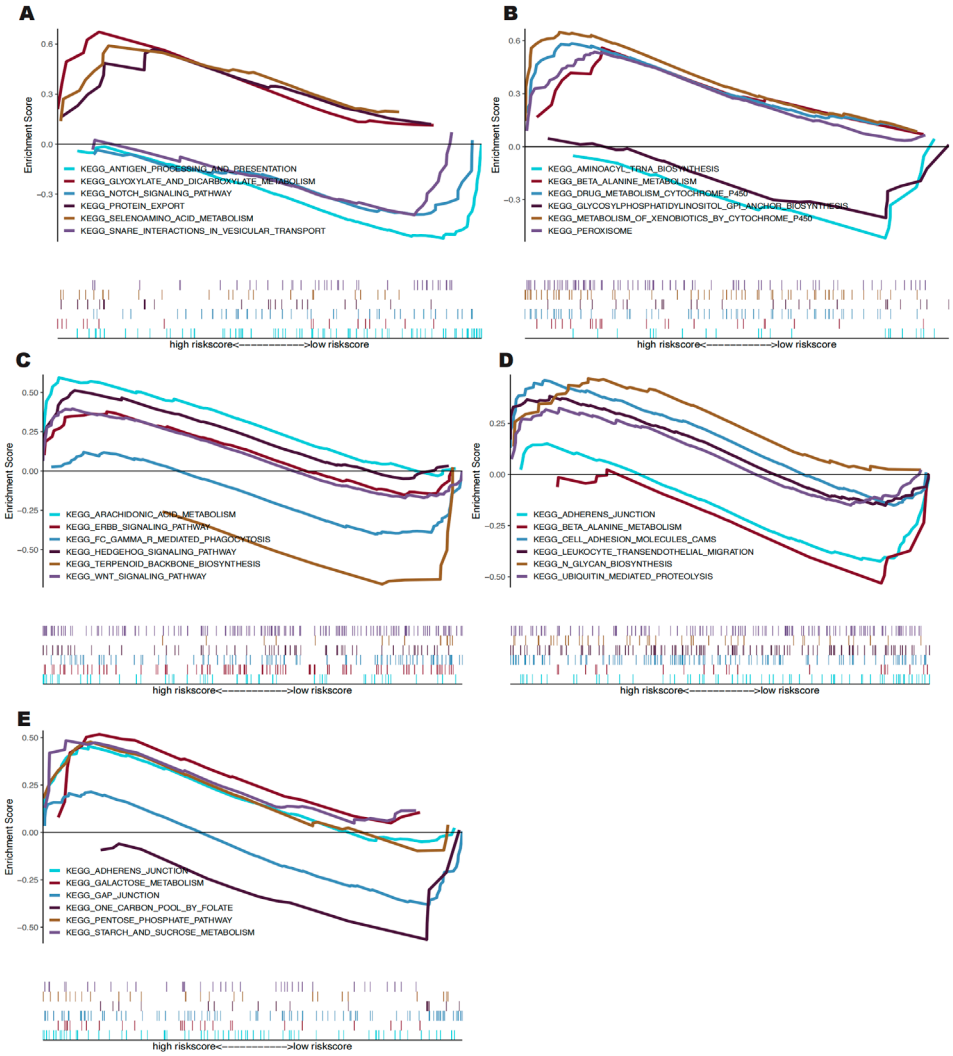

Figure 9. (A-E) Gene set enrichment analysis (GSEA).

FIGURE 9
Gene pathway enrichment analysis (GSEA).

Excessive production of reactive oxygen species (ROS) within cells, beyond their scavenging capacity, leads to oxidative stress (Sies et al., 2022). The formation of keloid scars largely manifests oxidative stress, appearing to result from dysregulated wound healing. Excessive inflammation or local hypoxia can distort wound repair, generating an abundance of free radicals and ROS. Tissue mediators such as transforming growth factor-beta (TGF-β) can induce angiogenesis and lead to wound fibrosis (Distler et al., 2019; Lichtman et al., 2016; Muppala et al., 2017). Ferroptosis is linked to the pathobiology of organ fibrosis and may impact nearly all organ systems, including the skin, lungs, heart, liver, and kidneys (Liu and Wang, 2022; Wang et al., 2023). Therefore, we have investigated the potential mechanisms by which ferroptosis influences the development of keloids.

Firstly, we used GEO database data mining to get transcriptome data from both normal and keloid skin tissue. By creating difference sets from the control and disease-related DEGs and crossing them with ferroptosis genes, we were able to identify 14 hub genes linked to keloid formation. According to the GO functional enrichment study, the functional module's genes were connected to monocarboxylic acid metabolic processes, carboxylic acid transport, pathways of neurodegeneration in multiple diseases, epithelial cell differentiation, and molecular adaptor activity. According to reports, energy metabolism and cell signaling are involved in the metabolism of monocarboxylic acid, and these procedures could be essential for the development, spread, and penetration of tumor cells (Halestrap and Wilson, 2012; Payen et al., 2020). It has been demonstrated that the pathway of neurodegeneration in multiple diseases is involved in oxidative stress and inflammatory responses (Michael et al., 2019). The epithelial cell differentiation pathway directly impacts the reconstruction of the skin epidermis and repair processes (Caldon et al., 2010). These mechanisms may occur in keloids. Research has shown that oxidative stress is linked to keloids, and that apoptosis, cell proliferation,

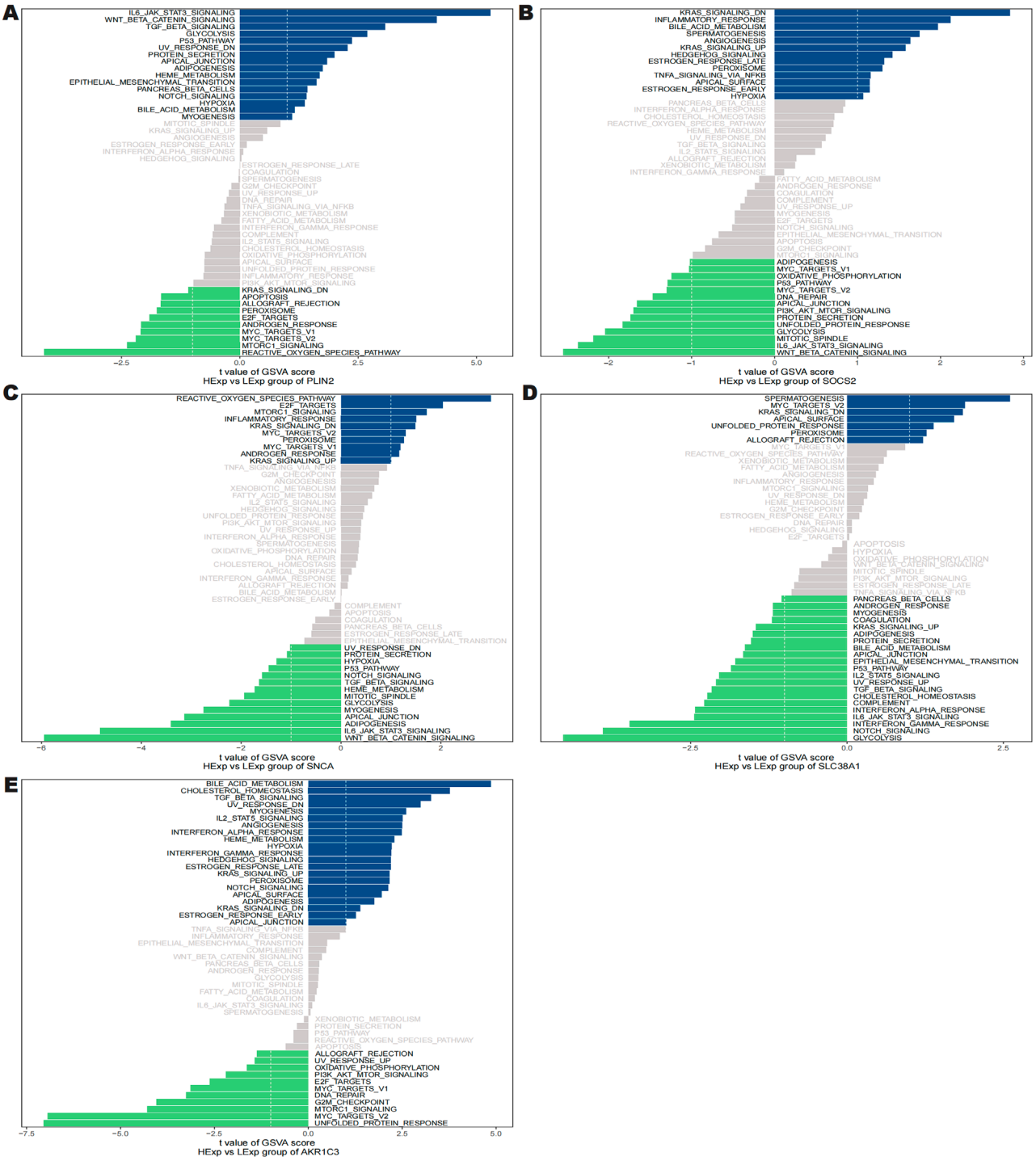

Figure 10. (A-E) Gene set variation analysis (GSVA).

FIGURE 10
Gene set variance analysis (GSVA).

and inflammatory responses may have an impact on keloids' development.

The LASSO regression reduced the dimensionality of the feature module from 14 genes to five key genes, revealing upregulation of AKR1C3 and PLIN2, and downregulation of SOCS2, SLC38A1, and SNCA in the initial sequencing results. Subsequently, the accuracy of the model's predictions was investigated using ROC curves, and the diagnostic efficacy of AKR1C3, PLIN2, SOCS2, SLC38A1, and SNCA was validated through an external dataset. Single-cell sequencing analysis of the five genes annotated them to certain

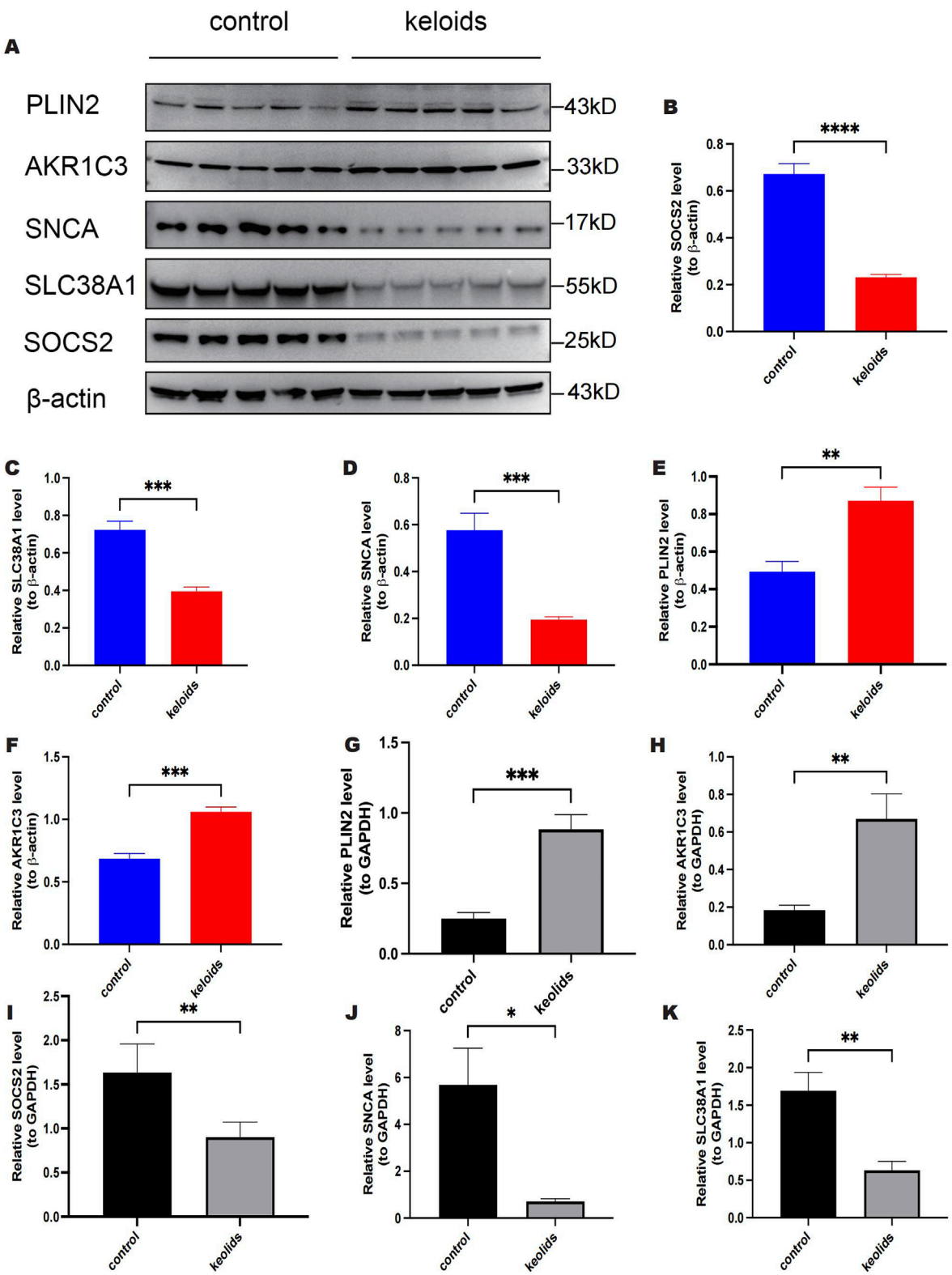

FIGURE 11
Expression of PLIN2, AKRIC3, SOCS2, SLC38A1 and SNCA in validation samples at protein and mRNA levels. **(A)** Protein blot analysis of PLIN2, AKRIC3, SOCS2, SLC38A1 and SNCA. **(B)** Relative quantitative results of Western Blot detection of SOCS2 expression. **(C)** Relative quantitative results of Western blot detection of SLC38A1 expression. **(D)** Relative quantitative results of Western blot detection of SNCA expression. **(E)** Relative quantitative results of PLIN2 expression detected by Western blot. **(F)** Relative quantitative results of AKRIC3 expression detected by Western blot. **(G)** RT-qPCR results of PLIN2. **(H)** RT-qPCR results of AKR1C3. **(I)** RT-qPCR results of SOCS2. **(J)** RT-qPCR results of SNCA. **(K)** RT-qPCR results of SLC38A1.

cell types significantly associated with disease pathogenesis. We employed ssGSEA to calculate and compare the proportions of different immune cells within scar keloid samples. This approach allowed us to assess the relationships between key genes and various immune cells, as well as to explore the correlations between these genes and different immune factors. Additionally, results from GSEA and GSVA provided evidence of the potential pathways and functions involving AKR1C3, PLIN2, SOCS2, SLC38A1, and SNCA. Finally, the expression of these genes was validated using qRT-PCR and Western blot analyses.

We conducted further research on the five key genes identified through model selection. These five genes are predominantly expressed in six cell types: fibroblasts, endothelial cells, epithelial cells, monocytes, adipocytes, and $CD8^+$ T-cells. Fibroblasts play a crucial role in scar formation by synthesizing and secreting extracellular matrix components, such as collagen, thereby facilitating tissue remodeling during wound healing. Studies have shown that excessive accumulation of iron leads to increased lipid peroxidation, which damages cell membranes and subsequently induces apoptosis in fibroblasts (Hu et al., 2024). Ferroptosis may indirectly influence scar formation through multiple mechanisms, including the induction of fibroblast apoptosis, modulation of their function, regulation of inflammatory responses, and alterations in iron metabolism (Yin et al., 2024). Endothelial cells are vital for angiogenesis and the maintenance of vascular permeability. During the scar healing process, they support the repair of surrounding tissues by providing oxygen and nutrients. Research indicates that ferroptosis induces endothelial cell damage and dysfunction, increases oxidative stress and inflammatory responses, and inhibits angiogenesis (Yu et al., 2021). Damage to endothelial cells results in localized hypoxia, which further impacts the function of fibroblasts, promoting their transition towards fibrosis, a process that contributes to the formation and progression of scars (Bai et al., 2020; Raslan et al., 2024). Epithelial cells are essential for the barrier function of the skin and other organs and primarily participate in tissue regeneration and repair. Luo et al. found that ferroptosis in epithelial cells may affect their proliferation and migration capabilities, thereby influencing wound healing and scar formation (Luo et al., 2024). Another study confirmed that ferroptosis induces oxidative stress and lipid peroxidation, leading to the death and dysfunction of keratinocytes (Vats et al., 2021). Consequently, this epithelial cell damage increases inflammatory responses and promotes fibroblast activation, which further enhances scar formation (Deng et al., 2023). Monocytes play a significant role in inflammatory responses and immune responses, differentiating into macrophages that participate in tissue repair. Research has demonstrated that ferroptosis contributes to lung fibrosis by driving macrophage polarization, fibroblast proliferation, and extracellular matrix deposition (Hu et al., 2024). These processes may also relate to scar formation. Ferroptosis induces M1 macrophage polarization, enhancing their role in inflammatory responses and resulting in the release of additional pro-inflammatory factors (Handa et al., 2019; Zhou et al., 2018), thereby promoting fibroblast proliferation and collagen synthesis, which exacerbates scar formation. Adipocytes are crucial for energy storage and metabolic regulation and are closely associated with inflammatory responses (Lindhorst et al., 2021). Iron acts as an important regulator in the energy metabolism of adipose tissue (Ma et al., 2021). The occurrence of ferroptosis is often associated with oxidative stress. Under oxidative stress conditions, adipocytes may release inflammatory factors, leading to inflammation in surrounding tissues. The formation of keloids is closely linked to chronic inflammation; thus, the regulation of ferroptosis in adipocytes may indirectly influence the occurrence of keloids. $CD8^+$ T cells play a vital role in defending against viral and bacterial infections and in tumor immunity (Pritykin et al., 2021). However, their role in scar formation remains unclear. Studies have found that ferroptosis in $CD8^+$ T cells can weaken their anti-tumor and anti-infection capabilities (Li et al., 2024). Imbalances in the immune microenvironment promote inflammatory responses, which may further correlate with scar formation. Single-cell sequencing results indicate that ferroptosis-related genes expressed in the aforementioned cell types primarily participate in processes such as lipid metabolism, oxidative stress, and inflammatory responses, all of which may significantly influence fibrosis and scar formation.

The results of immune infiltration analysis reveal that these five genes exhibit significant correlations with various immune cell types, including antigen-presenting cell (APC) co-inhibition, B cells, inflammation-promoting cells, T cell co-inhibition, Th1 cells, and the Type II interferon response. These immune cells not only effectively respond to infections and tumors but also maintain the immune balance of the organism by regulating immune responses (Chen and Flies, 2013; Krieg et al., 2007; Wynn, 2015; Zeng et al., 2018; Zhang et al., 2015). Specifically, SNCA shows a significant positive correlation with T cell co-inhibition and APC co-inhibition, suggesting its potential role in regulating immune responses. In contrast, its significant negative correlation with Th1 cells may indicate that SNCA plays a role in suppressing certain types of immune responses. SLC38A1 is significantly positively correlated with both B cell and T cell co-inhibition, indicating its involvement in modulating the activity of these cells. Additionally, its significant negative correlation with the Type II interferon response and MHC Class I molecules suggests that SLC38A1 may exert an inhibitory effect on antigen presentation and cellular immune responses. Furthermore, SOCS2 is significantly positively correlated with APC co-inhibition, T cell co-inhibition, and the Type II interferon response, highlighting its crucial role in regulating immune cell activity and inflammatory responses (Yoshimura et al., 2007). AKR1C3 shows a significant positive correlation with neutrophils and the Type I interferon response, which may indicate its role in promoting inflammatory responses. Its significant negative correlation with B cells suggests that AKR1C3 may inhibit B cell activity. Moreover, PLIN2 is significantly positively correlated with neutrophils and CCR, indicating its potential role in regulating immune cell infiltration and inflammatory responses (Reiss et al., 2001). The significant negative correlation with B cells and the Type II interferon response suggests that PLIN2 may have an inhibitory effect on B cell activity and immune responses. Additionally, the five genes exhibit significant correlations with various immune factors, including immunostimulators, MHC molecules, receptors, immunoinhibitors, and chemokines. These molecules interact through intercellular signaling and regulation, determining the intensity and type of immune system responses (Alberto et al., 2004; Dhatchinamoorthy et al., 2021; Deng et al., 2022). The aforementioned findings further confirm the close relationship between the expression of these five ferroptosis-related

genes and the level of immune cell infiltration. It is well-established that activated immune cells, such as macrophages and neutrophils, generate substantial amounts of ROS. Continuous oxidative stress may activate various inflammatory signaling pathways, such as NF-κB and JAK-STAT, which typically exacerbate inflammatory responses (Banerjee et al., 2017; Lawrence, 2009). Prolonged inflammation can stimulate the activation and proliferation of fibroblasts, promoting the occurrence of fibrosis, and may play a critical role in the formation of keloids.

Previous studies have found that PLIN2 is associated with the perilipin protein family and plays a major role in regulating intracellular lipid metabolism and the formation of lipid droplets (Kimmel and Sztalryd, 2016). In hepatocellular carcinoma (HCC), overexpression of PLIN2 promotes cell proliferation, potentially by inhibiting the degradation of HIF1α, which facilitates HCC cell proliferation (Liu et al., 2022). Another study indicates that PLIN2 deficiency reduces triglyceride (TG) levels in wild-type mouse embryonic fibroblasts (MEFs) by enhancing autophagy and can prevent fatty liver disease (Tsai et al., 2017). The upregulation of PLIN2 in keloids may influence the scar healing process by promoting cell proliferation and reducing autophagy. AKR1C3 plays a crucial role in androgen and steroid metabolism and is highly expressed in metastatic prostate cancer specimens and circulating tumor cells (Penning, 2019). AKR1C3 is significantly upregulated in HCC and promotes tumor proliferation and metastasis through the activation of NF-κB signaling (Wu et al., 2022; Zhou et al., 2021). The upregulation of AKR1C3 in keloids may contribute to scar formation by affecting cell proliferation and extracellular matrix component deposition. SOCS2, a negative regulatory factor, primarily modulates cell growth and differentiation by inhibiting cytokine signaling pathways, and its abnormal regulation is associated with various inflammatory and cancerous diseases (Rico-Bautista et al., 2006). Research shows that SOCS2 can enhance IL-2 and IL-3-induced STAT phosphorylation and promote cytokine-induced cell proliferation (Tannahill et al., 2005). High SOCS2 expression facilitates the ubiquitination and degradation of SLC7A11, promoting ferroptosis in HCC. Targeting SOCS2 may improve the efficiency of radiotherapy and patient prognosis in HCC (Chen et al., 2023). In keloids, the expression of SOCS2 is downregulated, which may affect the scar healing process by modulating inflammation, cell proliferation, and other pathways. SLC38A1 encodes an amino acid transporter involved in regulating amino acid metabolism both intracellularly and extracellularly (Kandasamy et al., 2018; Schiöth et al., 2013). Studies have shown that knockout of SLC38A1 significantly inhibits HCC cell growth and migration, and that SLC38A1 regulates the occurrence and progression of HCC through glutamine-mediated energy metabolism by modulating the PI3K/AKT/mTOR signaling pathway (Hg et al., 2023). In research on the pathogenesis of selective intrauterine growth restriction (sIUGR), overexpression of hypoxia-inducible factor (HIF-1α) induces upregulation of SLC38A1, which reduces cell growth and migration (Chen et al., 2022). In bleomycin (BLM)-induced pulmonary fibrosis rat lung tissues and transforming growth factor-β1-treated HFL1 cell models, downregulation of miR-150-5p targets SLC38A1 expression, leading to increased expression of inflammatory cytokines, fibroblast activation, and elevated ROS levels (Yang et al., 2020). Our research finds that SLC38A1 is downregulated in keloids, suggesting that it may have a significant impact on processes such as inflammation and cell proliferation. SNCA encodes α-synuclein, which is widely expressed in the nervous system and is associated with neuron survival, synaptic function, and cellular stress responses (Burré et al., 2018). Numerous studies indicate that SNCA is linked to tumorigenesis; its deficiency disrupts iron metabolism, leading to ferritin-iron accumulation and cell apoptosis (Shekoohi et al., 2021). Overexpression of SNCA in medulloblastoma can inhibit tumor invasion and induce apoptosis (Li et al., 2018). And SNCA overexpression may inhibit the proliferation of lung adenocarcinoma cells via the PI3K/AKT pathway (Zhang et al., 2022). The differential expression of SNCA in keloids may influence scar formation through processes such as cytokine regulation, cytokine signaling, iron metabolism, and neuroregulation. In summary, the upregulation of AKR1C3 and PLIN2 in keloids may inhibit ferroptosis by affecting oxidative stress, as well as lipid metabolism and storage, thereby promoting cell proliferation and fibrosis, which further contributes to scar tissue formation. Additionally, changes in the expression of SOCS2, SLC38A1, and SNCA are closely related to the regulation of ferroptosis by oxidative stress, inflammatory responses, and imbalances in iron metabolism. The interactions among these processes may significantly impact cell proliferation, apoptosis, and fibrosis, collectively promoting the formation of keloids.

GSEA and GSVA results indicate that high expression of SNCA is enriched in the reactive oxygen species pathway, E2F targets, and MTORC1 signaling, which play roles in cell growth regulation, redox balance, and cellular metabolic stress responses (Kent and Leone, 2019; Napolitano et al., 2022; Thannickal and Fanburg, 2000). High expression of SLC38A1 is enriched in KRAS signaling dn, MYC targets v2, and Peroxisome pathways, primarily regulating cell proliferation, growth, metabolic energy balance, and redox status (Bryant et al., 2014; Okumoto et al., 2020; Schulze et al., 2020). High expression of AKR1C3 is enriched in bile acid metabolism, IL2 STAT5 signaling, NOTCH signaling, and TGF-β signaling, which are involved in cell proliferation and growth regulation, metabolic and energy balance, immune regulation, and inflammatory responses (Chiang, 2015; Distler et al., 2019; Meurette and Mehlen, 2018; Ross and Cantrell, 2018). Similarly, high SOCS2 expression is enriched in KRAS signaling dn, Hedgehog signaling, inflammatory response, and Peroxisome pathways, participating in cell metabolism regulation, proliferation, growth control, and redox balance (Bryant et al., 2014; Ingham, 2022; Okumoto et al., 2020). Moreover, high PLIN2 expression is enriched in IL6 JAK STAT3 signaling, WNT/β-catenin signaling, P53 Pathway, and TGF-β signaling, which interact in cell proliferation, growth regulation, inflammatory response, immune modulation, and tissue regeneration (Clevers and Nusse, 2012; Joerger and Fersht, 2016; Zegeye et al., 2018). The results indicate that the pathways enriched by these five genes are not only involved in cell proliferation and growth but also interact with processes of oxidative stress and inflammatory responses. Ferroptosis is widely recognized for promoting lipid peroxidation and oxidative stress, leading to cell death and inflammation, thereby accelerating the fibrotic process. These processes play a crucial role in the regulation of ferroptosis and may have a significant impact on the formation of keloids.

In summary, we identified AKR1C3, PLIN2, SOCS2, SLC38A1, and SNCA as characteristic genes associated with ferroptosis in keloids through comprehensive transcriptomic data analysis. Additionally, we collected and validated samples, confirming these genes as molecular markers of ferroptosis in keloids and further exploring the role of ferroptosis in the fibrotic process. However, this study has certain limitations, primarily regarding the reliance on transcriptomic data and the urgent need for functional validation. While transcriptomic analysis can reveal gene expression changes in specific samples, it may not fully reflect cellular behaviors across different individuals or pathological states. Although some genes exhibit significant differences in transcriptomic analysis, their functions in cellular or animal models have not been adequately validated. To address these limitations, future research will focus on systematic functional validation of the key genes identified in the transcriptomic analysis. This will include experiments such as gene knockout, overexpression, and small molecule inhibition to observe the effects of these genes on fibroblast survival, proliferation, and collagen synthesis. Through these studies, we aim to achieve a more comprehensive understanding of the specific mechanisms linking ferroptosis to keloid formation. This approach provides new insights for regulating cellular death mechanisms by targeting ferroptosis-related genes, potentially paving the way for developing novel therapeutic strategies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The studies involving humans were approved by Lanzhou University Second Hospital Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

ZS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing–original draft. YQ: Project administration, Supervision, Writing–review and editing. XZ: Project administration, Supervision, Writing–review and editing, Methodology.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The role of ferroptosis in colorectal cancer and its potential synergy with immunotherapy

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Colorectal cancer (CRC) is one of the most prevalent and deadly malignancies worldwide. Recently, ferroptosis, a novel form of regulated cell death characterized by iron dependency and lipid peroxidation, has garnered significant attention from researchers. The mechanisms underlying ferroptosis, including intracellular iron levels, lipid peroxidation, and antioxidant system regulation, offer new insights into cancer treatment strategies. This study aims to explore the emerging role of ferroptosis in the context of immunotherapy for CRC, highlighting its potential mechanisms and clinical applications. We employed a comprehensive review of current literature to elucidate the biological mechanisms of ferroptosis, its relationship with CRC, and the interplay between ferroptosis and immunotherapy. Ferroptosis reshapes the tumor microenvironment (TME) by regulating intracellular iron levels, lipid metabolism, and antioxidant systems, significantly enhancing the efficacy of immune checkpoint inhibitors (ICIs). Meanwhile, traditional Chinese medicine therapies promote antitumor immunity by modulating the TME and inducing ferroptosis. Additionally, advances in nanotechnology have facilitated precise therapy by enabling targeted delivery of ferroptosis inducers or immunomodulators, transforming "cold" tumors into "hot" tumors and further boosting ICI efficacy. This study comprehensively reviews the latest developments in ferroptosis, immunotherapy, traditional Chinese medicine, and nanotechnology in CRC, highlighting the importance of ferroptosis-related biomarkers and novel inducers for personalized treatment. In summary, ferroptosis offers a promising strategy to overcome CRC therapy resistance and enhance immunotherapy efficacy, warranting further investigation and translational application.

KEYWORDS

ferroptosis, colorectal cancer, immunotherapy, immune checkpoint inhibitors (ICBs), tumor microenvironment

Introduction

Globally, colorectal cancer is the third most common malignant tumor in terms of incidence, with a mortality rate of 9.3% of the total cancer prevalence worldwide (1). Although early screening has been shown to be effective in reducing morbidity and mortality in the general population, approximately 25% to 50% of patients with early-stage colorectal cancer are still at risk for tumor metastasis, and approximately 25% of patients are in advanced stages at the time of diagnosis (2, 3). These factors seriously affect treatment outcome and survival prognosis (4–6). In recent years, the age of onset of colorectal cancer has shown a tendency to be younger, and studies have shown that the incidence of colorectal cancer in young and middle-aged people under the age of 50 has continued to rise since the mid-1980s (7). The phenomenon may be closely related to factors such as the widespread use of antibiotics,intestinal flora dysbiosis increased obesity rates, and other factors (7–9). Currently, significant progress has been made in the treatment of colorectal cancer, including surgical resection, radiation therapy, targeted therapy, chemotherapy and immunotherapy. For early-stage colorectal cancer patients, radical surgery combined with adjuvant radiotherapy and chemotherapy can usually achieve a better prognosis and a higher five-year survival rate (10). However, for patients with metastatic, malignant or invasive colorectal cancer, the effectiveness of existing treatments remains limited and the prognosis is poor (11). Therefore, colorectal cancer has become a major public health challenge that needs to be addressed globally. Improving early detection rates and optimizing treatment strategies, especially in advanced metastatic cases, remains a major challenge in the current treatment of colorectal cancer. There is an urgent need to develop new and more effective treatments to improve the survival prognosis of patients.

Cell death plays a pivotal role in development and physiological homeostasis, being essential in embryogenesis and mechanisms like immune surveillance and tissue repair in adults. According to the Nomenclature Committee on Cell Death (NCCD), cell death is classified into accidental cell death (ACD) and regulated cell death (RCD) (12). ACD arises from unpredictable damaging stimuli exceeding repair capacity, while RCD is a gene-regulated process mediated by signal amplification complexes critical for development and homeostasis. Programmed cell death (PCD), a subtype of RCD, removes aberrant, aging, or damaged cells under physiological conditions, maintaining immune and tissue balance (13). Dysregulation of cell death mechanisms can lead to pathological conditions, such as excessive neuronal apoptosis in neurodegenerative diseases or failure to eliminate mutated cells in tumorigenesis, highlighting its dual role in disease progression (14–16).

In recent years, the classification of cell death has been further refined, leading to the emergence of new types of cell death such as autophagic death, pyroptosis, and cupric death, which have unique physiological and pathological characteristics. Among these novel modes of cell death, ferroptosis, a non-apoptotic programmed cell death mode dependent on iron, has gradually attracted widespread attention. Ferroptosis, first proposed in 2012, is a novel programmed cell death mechanism that is mainly triggered by iron-dependent lipid peroxidation and the accumulation of reactive oxygen species (ROS) (17). This mechanism differs from conventional apoptosis in that ferroptosis is characterized by distinctive cellular morphological changes, such as a marked reduction in the size of mitochondria, a decrease in the number of mitochondrial cristae, and an increase in the density of membranes (17, 18).

Research on ferroptosis has revealed its potential role in various pathological states, particularly in diseases such as intestinal disorders, neurodegenerative diseases, and cardiovascular diseases, where ferroptosis is believed to be a key factor in pathological progression (15, 19–22). Therefore, regulating iron metabolic pathways, especially by inducing ferroptosis, has become a new direction for treating tumors. By modulating the ferroptosis pathway, researchers hope to develop innovative therapeutic approaches for targeting tumor cells, especially when conventional therapies fail or develop resistance. As an emerging mechanism of cell death, ferroptosis could serve as an alternative therapeutic strategy for tumor cells that are insensitive to conventional chemotherapy, including how to improve the selectivity of ferroptosis inducers and avoid side effects on normal tissues (15, 19–23).

Immunotherapy has become an important direction in the treatment of colorectal cancer in recent years. Immune checkpoint inhibitors (ICIs), such as PD-1/PD-L1 and CTLA-4, restore and enhance the body's immune surveillance and attack ability against tumors by relieving the inhibition of tumor cells on the immune system (24, 25). However, the efficacy of existing immunotherapy is still limited for patients with microsatellite stable (MSS) or other immunotherapy-resistant colorectal cancer, so there is an urgent need to find new combination treatment strategies to improve the efficacy.

Some studies have shown a strong link between ferroptosis and immunotherapy. Ferroptosis-related oxidative stress and cell death mechanisms may enhance the efficacy of immunotherapy by influencing immune escape mechanisms in the tumour microenvironment (17, 18, 26). In particular, immune checkpoint inhibitors have shown better efficacy in microsatellite unstable (MSI-H) colorectal cancer, especially in microsatellite stable (MSS) colorectal cancer, where efficacy is still unsatisfactory. However, in patients with microsatellite-stable (MSS) or other immunotherapy-resistant colorectal cancers, the efficacy of existing immunotherapies is still limited (27, 28). Therefore, researchers are actively exploring new therapeutic strategies, such as combination chemotherapy, radiotherapy, targeted therapies, and novel ferroptosis-based therapies, with the aim of improving the response rate and efficacy of immunotherapy, and bringing more effective treatment options to colorectal cancer patients.

Novel mechanism to regulate cell death: ferroptosis

The goal of most cancer treatment strategies is to selectively eliminate cancer cells without damaging non-malignant cells.

Different sub-programs of programmed cell death (RCD) have different effects on tumor progression and treatment response (29). In contrast to accidental cell death, RCD is controlled by specific signaling pathways that can be intervened by pharmacological or genetic means. One such ferroptosis is a type of iron-dependent RCD.

In 2012 Dixon et al. named the discovery of a unique form of cell death by the first induction of the small molecule compound erastin as ferroptosis (17). Morphologically, ferroptosis cells show typical necrotic features, including shrinkage of mitochondria, reduction of cristae, increase in membrane density, and rupture of the outer membrane, but are not accompanied by the typical hallmarks of apoptosis (12). Further studies revealed that ferroptosis is mediated by mitochondrial voltage-dependent anion channels (VDACs); specifically, erastin promotes mitochondrial uptake of ferric ions, reactive oxygen species (ROS) generation, elevated mitochondrial potential, and oxidative stress, thereby triggering ferroptosis. Studies over the years have demonstrated that mitochondria play a key role in ferroptosis, affecting the onset of ferroptosis through the modulation of mitochondrial lipid metabolism, energy metabolism, and iron metabolism, among others (18). In addition to the mitochondrion, other organelles such as the endoplasmic reticulum, the Golgi, and lysosomes are also participate in the regulation of ferroptosis, which they promote through oxidative stress, lipid peroxidation, and dysfunction, respectively (21, 23). In the presence of the antioxidant glutathione (GSH), glutathione peroxidase 4 (GPX4) exhibits a unique ability to inhibit lipid peroxidation and protect the cells from ferroptosis. This finding reveals a complex interaction between iron, cysteine, and lipid metabolism as an important regulatory mechanism for ferroptosis and a series of related regulators have been identified. Because cancer cells require large amounts of iron to meet their metabolic demands and promote growth, they are more susceptible to ferroptosis (30, 31). Since the discovery of ferroptosis, a large number of studies have targeted ferroptosis for its anticancer potential, and these effects may be mediated through immune-mediated mechanisms (Figure 1).

Mechanisms and signaling pathways of ferroptosis

Mechanisms of lipid peroxidation

Peroxidation of lipid components is the central driving mechanism of ferroptosis. In this process, accumulation of lipid bilayer peroxides (PLOOHs) irreversibly triggers ferroptosis when it exceeds the antioxidant capacity of the cell (32). Acyl coenzyme A synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) play key roles in this process and are responsible for the synthesis, activation, and incorporation of polyunsaturated fatty acid phospholipids (PUFA-PLs), whereas arachidonic acid (AA) and adrenoic acid (AdA), which are the most susceptible lipids to peroxidation, are present in PUFA. ACSL4 forms

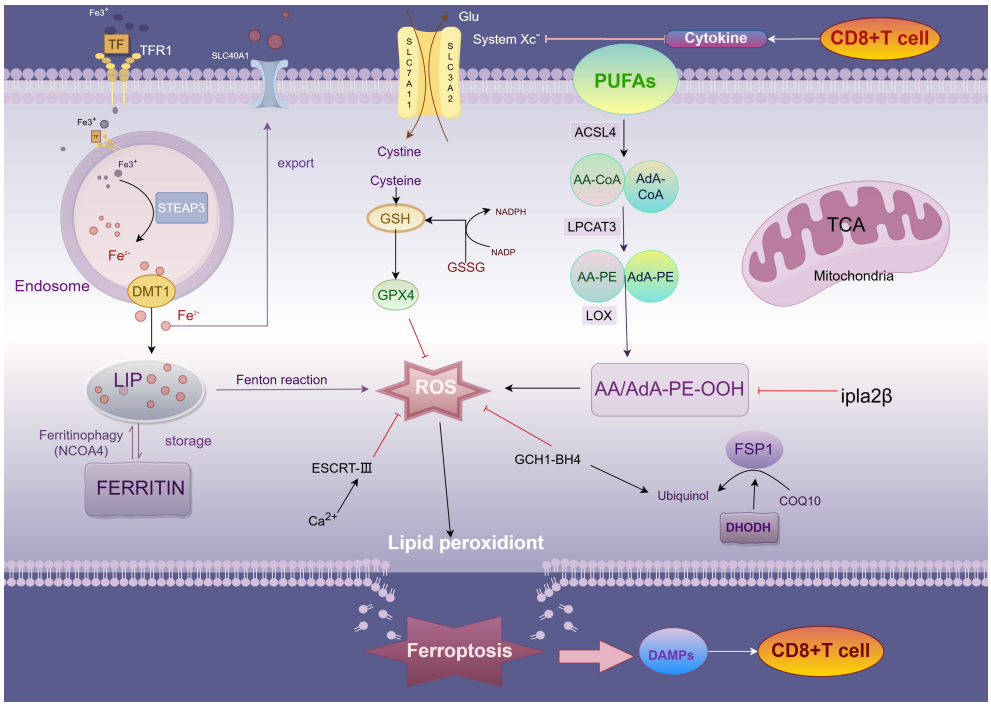

FIGURE 1
Mechanism of ferroptosis. TFR1, transferrin receptor 1; DMT1, divalent metal transporter 1; peptidase-4 STEAP3, six-transmembrane epithelial antigen of prostate; LIP, labile iron pool; PUFA, polyunsaturated fatty acids; ACSL4, acyl-CoA synthetase long-chain family member 4; AA, arachidonic acid; AdA, adrenic acid; LPCAT3, lysophosphatidylcholine acyltransferase 3; LOX, lipoxygenases; System Xc–, cystine-glutamate exchange system; FSP1, ferroptosis suppressor protein 1.

AA -CoA or AdA-CoA by catalyzing the attachment of free PUFA to coenzyme A (CoA), which is subsequently esterified to membrane phosphatidylethanolamine (PE) by LPCAT3 and integrated into the cell membrane structure (26). As the multiple double-bond structure of PUFA is susceptible to attack by reactive oxygen species (ROS) generated by the Fenton reaction, lipid peroxides (LPO) are formed. With the accumulation of LPO, the cell membrane structure is impaired, ultimately inducing ferroptosis (23). In addition, deletion of ACSL4 or, more recently, P53 has been shown to activate the expression of calcium-independent phosphatase A2β (Ipla2β).

Ipla2β is an enzyme capable of hydrolyzing lipid peroxidation, which hydrolyzes oxidized PUFA-PLs from the phospholipid molecules of the cell membrane and reduces the accumulation of lipid peroxidation, thus inhibiting ferroptosis to a certain extent (33). Not only that, as lipid peroxidation continued to accumulate, protein kinase CβII (PKCβII) was activated and interacted with the Thr328 site of phosphorylated ACSL4, increasing the production of PUFA-PLs and further inducing ferroptosis (33, 34). Notably, accumulation of lipid peroxides sustains activation of PKCβII, creating a positive feedback loop that in turn enhances the sensitivity of tumor cells to immune-mediated killing.

Antioxidant defenses

The Xc-system consists of two subunits, SLC7A11 and SLC3A2. Under normal homeostatic conditions of the internal environment, lipid peroxides (PLOOH) in cells are reduced to nontoxic lipid alcohols through the glutathione (GSH)-glutathione peroxidase 4 (GPX4) antioxidant system (i.e., the Xc-system) (26, 34). This reduction process effectively prevents further accumulation of lipid peroxides and avoids overproduction of reactive oxygen species (ROS), thereby inhibiting oxidative stress and ferroptosis.GPX4, an important regulator of ferroptosis, maintains the integrity and function of cell membranes through its own antioxidant action by reducing peroxisomal phospholipids to hydroxyphospholipids form (31, 35, 36). However, when intracellular GSH is depleted or GPX4 is inactivated due to certain factors (e.g., ubiquitination, etc.), lipid peroxides are no longer efficiently scavenged and begin to accumulate in large amounts in the cell membrane (30). Since the structure of PUFA-PL contains multiple double bonds, it is susceptible to ROS attack, leading to the peroxidation chain reaction. This autocatalytic process exacerbates lipid oxidation, which ultimately destroys the structure and function of the cell membrane, resulting in an increase in membrane permeability, loss of membrane potential, and a complete breakdown of intracellular homeostasis. When lipid peroxidation reaches a certain critical value, the whole lipid peroxidation system enters an uncontrolled state, and the cell is exposed to intense oxidative stress pressure, which ultimately triggers ferroptosis. The oxidative damage in this process not only severely destabilizes the cell membrane, but also promotes irreversible cell death through the activation of various downstream signaling pathways, such as apoptosis-related signals, inflammatory response pathways, and autophagy pathways. Erastin, an inhibitor of ferroptosis, induces ferroptosis by inhibiting the function of the Xc- system (17, 23). Not only that, cisplatin also induced ferroptosis in colorectal cancer A549 and HCT116 cells, and the combination of cisplatin and Erastin increased the antitumor activity even more (37).

High expression of GPX4 in different tumor cells has different survival outcomes. For example, in pancreatic cancer cells, high expression of GPX4 was positively correlated with survival, whereas high expression in colorectal cancer cells had a poor survival outcome (36, 38, 39). Thus, GSH depletion as well as functional imbalance of GPX4 may be a potential strategy to improve cancer treatment outcomes. In addition to the GSH-GPX4 system, there are other antioxidant pathways in cells that counteract ferroptosis. For example, tetrahydrobiopterin (BH4), an endogenous antioxidant, inhibits ferroptosis by selectively preventing the depletion of phospholipids containing two PUFA tails through the expression of its GTP cyclase hydratase 1 (GCH1), and GCH1 is also associated with an increase in CoQ10 synthesis through the BH4-mediated phenylalanine to tyrosine conversion (40). Whereas Ferroptosis suppressor protein 1 (FSP1, also known as apoptosis-inducing factor mitochondrial 2 AIFM2) acts as an oxidoreductase in the cell membrane, reducing ubiquinone 10 to ubiquinol (CoQ10H2) with NADPH as the electron donor, ubiquinol can act as a lipid-soluble RTA, thus inhibiting lipid peroxidation and preventing ferroptosis (41, 42). In CRC, FSP1 is inhibited by. Not only that, the sorting complex (ESCRT-III) in endosomes, as well as by promoting cell membrane repair, was also able to partially alleviate the membrane damage triggered by ferroptosis. And in colorectal cancer cells, especially in tumors, these antioxidant systems act synergistically through different mechanisms to help cancer cells resist excessive oxidative stress and slow down ferroptosis (23). Thus, dysregulation of the antioxidant system is closely related to the induction of ferroptosis, especially in tumor therapy, and modulation of these pathways promises to be a new therapeutic strategy.

Iron metabolism pathway

Iron metabolism is closely related to the process of ferroptosis, which is characterized by iron-dependent lipid peroxidation. Under normal physiological conditions, iron is transported in the blood as Fe^{3+} bound to transferrin. Transferrin receptor 1 (TFR1) mediates the uptake of Fe^{3+}, which is followed by the reduction of ferrous iron in acidic endosomes in response to prostate six transmembrane epithelial antigen 3 (STEAP3), which in turn passes through the divalent metal transporter protein 1 (DMT1) or enters the cytoplasmic pool of unstable iron that stores reactive iron (LIP) (43, 44). Intracellular iron is mainly stored in ferritin, and nuclear receptor coactivator 4 (NCOA4) binds directly to ferritin heavy chain (FTH1), mediating ferritin degradation through the autophagy pathway (also known as Ferritinophagy) and releasing iron into the LIP (45, 46). Inhibition of NCOA4 reduces iron content in LIP and prevents ferroptosis, whereas enhancement of ferritin autophagy increases iron content in LIP and promotes ferroptosis in CRC cells. The expression level of NCOA4 is positively correlated with sensitivity to ferroptosis inducers (45).

Specific small molecule inhibitor 9a significantly improves the clinical prognosis of acute myeloid leukemia (AML) by targeting the interaction between NCOA4 and FTH1, leading to depletion of iron content in LIP, thereby inhibiting ferritin autophagy, blocking lipid peroxidation and ferroptosis, and exerting an inhibitory effect on the self-renewal and viability of leukemic stem cells (47, 48). Not only that, compound 9a demonstrated a significant protective effect against ischemia-reperfusion injury (47). Once intracellular free iron accumulates, excess Fe^{2+} reacts with hydrogen peroxide (H_2O_2) via the Fenton reaction to produce ROS (mainly highly reactive hydroxyl radicals), which triggers lipid peroxidation and membrane damage, inducing ferroptosis. The regulation of hepcidin (iron regulator) affects iron release and reutilization. FINO2 and other novel ferroptosis-inducing agents that trigger the generation of lipid peroxides by oxidizing Fe^{2+} to Fe^{3+} represent a fourth class of ferroptosis-inducing compounds.

Association between colorectal cancer and ferroptosis

Ferroptosis, an iron-dependent form of programmed cell death, has garnered significant attention in recent years. Its regulation plays a critical role in tumorigenesis. Dysregulation of ferroptosis during tumor progression leads to increased oxidative stress within the tumor microenvironment, promoting invasion, metastasis, and immune evasion (49–51). In colorectal cancer, ferroptosis is a key factor influencing tumor progression and clinical prognosis (52–55). It regulates cell growth, migration, invasion, and chemosensitivity, thereby shaping clinical outcomes. For instance, the expression of ferroptosis-related genes such as TFAP2C, ACACA, and NOS2 correlates significantly with patient survival, as demonstrated by receiver operating characteristic (ROC) and Kaplan-Meier (K-M) analyses, even surpassing traditional TNM staging in prognostic assessment (56–58). Further studies indicate that ferroptosis sensitivity is closely linked to metabolic reprogramming in tumor cells. Recent findings reveal a connection between mTORC1 and ferroptosis, with mTORC1 acting as a critical regulator (59, 60). Notably, the combination of aspirin and RSL3 effectively induces ferroptosis in PIK3CA-mutant CRC cells by inhibiting the mTOR/SREBP-1/SCD1 signaling pathway (61). Thus, ferroptosis emerges as a multifaceted mechanism in CRC pathogenesis and progression. Investigating its molecular mechanisms, particularly identifying ferroptosis-related biomarkers, could pave the way for novel approaches in early diagnosis and personalized treatment strategies.

The role and application of ferroptosis in CRC immunotherapy

Mechanisms of ferroptosis in CRC immunotherapy

The resistance to tumor immunotherapy is primarily associated with the immunosuppressive characteristics of the tumor microenvironment (TME). In CRC, the TME is enriched with immunosuppressive cells such as tumor-associated macrophages (TAMs) and regulatory T cells (Tregs), which secrete inhibitory cytokines and express immune checkpoint molecules like PD-L1, suppressing T-cell activity and evading immune surveillance (62, 63). Ferroptosis significantly influences the dynamic equilibrium of the TME by regulating the function of immune cells (23). TAMs, dendritic cells (DCs), and Tregs in CRC are particularly sensitive to ferroptosis, highlighting its pivotal role in both tumor metabolism and the modulation of immune responses.

$CD8^+$ T cells

As core effector cells in antitumor immunity, CD8+ T cells enhance tumor cell lysis by secreting interferon-γ (IFN-γ), which inhibits the expression of SLC7A11 and SLC3A2—subunits of the system Xc^- in tumor cells. This suppression reduces cystine uptake, depletes glutathione levels, and induces lipid peroxidation, ultimately triggering ferroptosis (64–66). The resulting tumor cell lysis releases damage-associated molecular patterns (DAMPs) such as ATP, HMGB1, and CRT, which activate innate immunity and amplify antitumor responses (67, 68). Additionally, studies have identified apolipoprotein L3 (APOL3) as a key player in CRC, enhancing CD8+ T-cell antitumor activity by promoting ubiquitin-mediated degradation of lactate dehydrogenase A (LDHA), making it a potential biomarker for ferroptosis-based immunotherapy (69).

Conversely, ferroptosis can contribute to CD8+ T-cell exhaustion. In the TME, lipid uptake and intracellular lipid peroxidation accumulate in infiltrating CD8+ T cells, leading to ferroptosis-mediated dysfunction (70, 71). Oxidized low-density lipoproteins (OxLDL) in the TME, taken up by CD36-expressing CD8+ T cells, increase lipid peroxidation and activate stress response protein p38, triggering ferroptosis. This process reduces the expression of IFN-γ and TNF-α, impairing CD8+ T-cell function and weakening antitumor efficacy. Additionally, CD36 expression correlates positively with immune checkpoint proteins PD-1 and TIM-3. Overexpression of antioxidant enzyme GPX4 can inhibit OxLDL-induced lipid peroxidation, preserving T-cell function and restoring antitumor immunity (72, 73). Furthermore, APOL3 overexpression enhances the synergistic effects of ferroptosis inducers like RSL3 and PD-1 inhibitors in CRC (69). Therefore, ferroptosis in the CRC microenvironment exerts multifaceted regulatory effects on CD8+ T cells, facilitating tumor immune clearance through diverse mechanisms.

Treg cells

Regulatory T cells (Tregs) play a crucial role in maintaining immune tolerance and facilitating tumor immune evasion. The expression of GPX4 in Tregs is essential for resisting oxidative stress within the TME (31). Studies have demonstrated that in MC38 colorectal cancer and B16F10 melanoma models, specific deletion of GPX4 in Tregs leads to significant accumulation of lipid peroxides (LPOs), inducing ferroptosis upon TCR/CD28 co-

stimulation and impairing Treg function. GPX4-deficient Tregs are highly susceptible to ferroptosis, which alters immune homeostasis in the TME by enhancing pro-inflammatory cytokine interleukin-1β (IL-1β) secretion. IL-1β promotes the differentiation and activation of Th17 cells, creating a pro-inflammatory environment. This inflammation further activates dendritic cells (DCs), improving antigen presentation, and enhances the cytotoxic activity of CD8+ T cells. These effector cells are central to antitumor immunity, significantly boosting immune clearance of tumor cells (32). Therefore, GPX4 is vital in regulating oxidative stress resistance and immunosuppressive functions of Tregs, maintaining immune tolerance within the TME.

TAMs

TAMs are the most abundant innate immune cells in the TME, comprising pro-tumorigenic M2 macrophages and pro-inflammatory, antitumorigenic M1 macrophages (74). While M1 and M2 macrophages exhibit similar LPO levels and expression of key metabolic regulators (e.g., ACSL4, LPCAT3, GPX4), M1 macrophages display greater ferroptosis resistance due to high inducible nitric oxide synthase (iNOS) expression. Conversely, M2 macrophages are more susceptible to ferroptosis. Ferroptosis not only induces direct tumor cell death through lipid peroxidation but also reshapes macrophage phenotypes within the TME. This process drives the transition of M2 macrophages toward the antitumor M1 phenotype, enhancing immune response efficacy (75). For instance, the natural compound dictamnine inhibits ERK phosphorylation and suppresses M2 TAMs, thereby inducing ferroptosis in CRC cells (76).

Additionally, increased intracellular iron and ROS levels elevate TNF-α expression, promoting macrophage polarization toward the M1 phenotype and further enhancing ferroptosis-mediated cytotoxicity (77). Ferroptosis-induced macrophages secrete pro-inflammatory cytokines such as TNF-α and IL-6, activating other immune cells in the TME and amplifying antitumor immune responses (74). These dual effects of TAMs in ferroptosis regulation highlight novel avenues for tumor immunotherapy (Figure 2).

DCs

Ferroptosis impacts tumor immunity not only by inducing tumor cell death but also by modulating immune cell activity and cytokine secretion, shaping the TME (78). In the early stages of ferroptosis, tumor cells release damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1) and ATP. These molecules significantly enhance dendritic cell (DC) maturation and antigen-presenting capability, activating specific T-cell responses. DAMP release also promotes DC migration to tumor sites, increasing antigen presentation efficiency and reducing immune evasion. Within the TME, DAMPs facilitate antitumor immunity and contribute to the transformation of "cold" tumors into "hot" tumors.

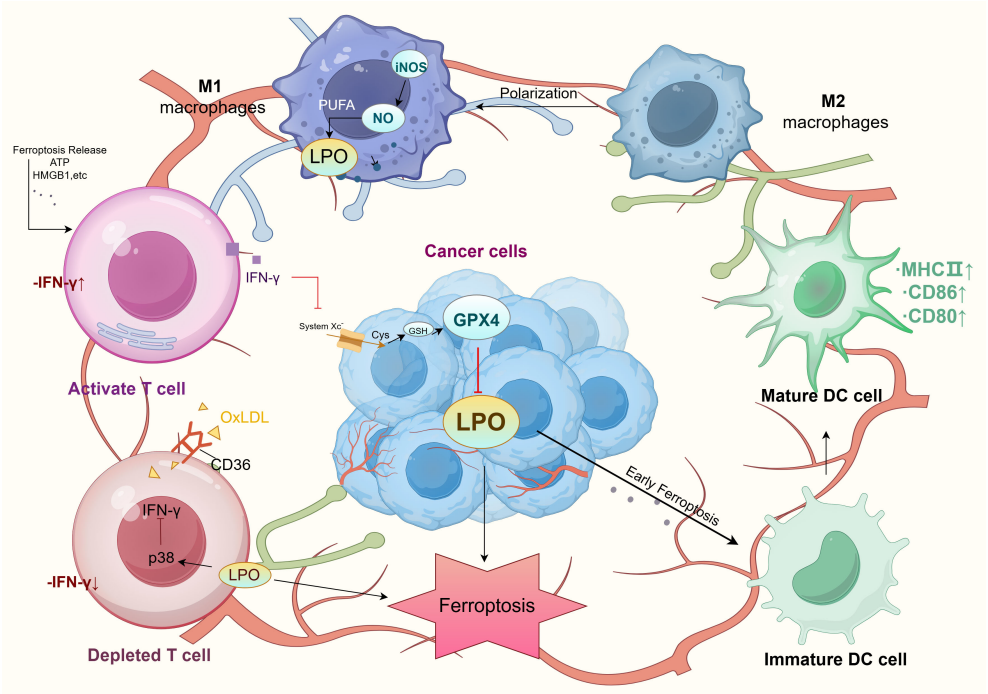

FIGURE 2
Tumor microenvironment and ferroptosis mechanism. LPO, Lipid Peroxidation; PUFA, Polyunsaturated Fatty Acids; iNOS, Inducible Nitric Oxide Synthase; NO, Nitric Oxide; GPX4, Glutathione Peroxidase 4; GSH, Glutathione; Cys, Cysteine; System Xc-, Amino acid transport system; OxLDL, Oxidized Low-Density Lipoprotein; MHC II, Major Histocompatibility Complex Class II; DC cell, Dendritic Cell.

However, in the late stages of ferroptosis, although dying cells are effectively phagocytosed, DC antigen-presentation capacity declines, limiting sustained immune responses. This highlights the dual effects of ferroptosis on the TME, presenting both stimulatory and inhibitory influences. Regulation targeting specific stages of ferroptosis may offer novel strategies to enhance immunotherapy efficacy, especially in colorectal cancer, by optimizing ferroptosis-associated immune modulation to inhibit tumor progression and immune escape (79, 80) (Figure 2).

MDSCs

Myeloid-derived suppressor cells (MDSCs), originating from bone marrow mesenchymal stem cells, are key immunosuppressive cells in the TME (81). They facilitate tumor immune evasion and suppression through metabolic reprogramming and immune regulation (82). Research indicates that N-acylsphingosine amidohydrolase 2 (ASAH2) is upregulated in MDSCs in colorectal cancer, enhancing heme oxygenase-1 (Hmox1) expression via p53 destabilization. This reduces lipid reactive oxygen species (ROS) production and inhibits ferroptosis, maintaining MDSC activity and immunosuppressive function in the TME. The specific ASAH2 inhibitor, NC06, suppresses ceramidase activity, increases lipid ROS, and induces ferroptosis in MDSCs. In tumor-bearing mouse models, NC06 treatment reduced MDSC infiltration and activated tumor-infiltrating cytotoxic T lymphocytes (CTLs), leading to tumor growth inhibition (83). Thus, NC06-mediated MDSC ferroptosis offers a novel therapeutic approach to reduce MDSC infiltration and enhance antitumor immunity within the TME.

The combination of ferroptosis inducers and immunotherapy

Immunotherapy represents a revolutionary breakthrough in cancer treatment, particularly in disrupting tumor immune evasion mechanisms. The immune system combats cancer by recognizing and eliminating tumor cells, but tumors often evade this through immune checkpoint molecules such as PD-1, PD-L1, and CTLA-4 (84). Immune checkpoint inhibitors (ICIs), as an emerging strategy for colorectal cancer treatment, have shown promising clinical applications in recent years (85, 86). ICIs enhance the anti-tumor immune response by inhibiting the immunosuppressive pathway between tumor cells and TME, and relieving the immune escape of tumor cells from T cells. Combining ferroptosis inducers with ICIs demonstrates synergistic effects by enhancing tumor immunogenicity and reversing immune suppression within the TME. This approach transforms immune-suppressive TME into an inflammatory environment rich in antitumor immune cells, particularly improving the response to "cold" tumors (87, 88). In microsatellite-stable (MSS) colorectal cancer patients, ICIs are less effective due to low mutational burden and reduced neoantigen expression, resulting in limited immunogenicity. Exploring ICIs in combination with other therapies, including ferroptosis inducers, is a critical avenue for improving CRC immunotherapy outcomes.

In recent years, the role of ferroptosis in CRC immunotherapy has garnered significant attention. Ferroptosis inducers such as RSL3 and Erastin have been shown to enhance tumor immunogenicity by directly or indirectly activating ferroptosis pathways (39, 89). Erastin induces ferroptosis by inhibiting the system Xc^- and depleting glutathione, while sorafenib, a multi-target tyrosine kinase inhibitor, triggers ferroptosis by suppressing GPX4 activity, synergizing with ICIs like pembrolizumab and nivolumab (90). These combinations not only recruit effector T cells but also convert "cold" tumors to "hot" tumors, improving therapeutic efficacy. For instance, RSL3 enhances PD-1 blockade efficacy by targeting GPX4 to induce lipid peroxidation in CRC cells. Similarly, combining vitamin C (VitC) with cetuximab, an EGFR-targeting antibody, suppresses drug-resistant cell emergence, restricts CRC organoid growth, and delays acquired resistance development in CRC xenografts. Cetuximab impairs glucose metabolism while VitC disrupts iron homeostasis and elevates ROS levels to potentiate ferroptosis (91). Ferroptosis also enhances the sensitivity of CRC to ICIs. For example, Chen c et al. showed that in CRC cells, the expression of CYP1B1 was negatively correlated with that of ACSL4. CYP1B1 activated the PKC signaling pathway by degrading ACSL4, increased the expression of FBXO10, and promoted the ubiquitination and degradation of ACSL4, thereby making tumor cells resistant to ferroptosis. In addition, inhibition of CYP1B1 increased the sensitivity of CRC to anti-PD-1 antibodies and enhanced the response of CRC patients to anti-PD-1 therapy (92). A novel ferroptosis inducer, N6F11, specifically degrades GPX4 via TRIM25-mediated ubiquitin-proteasome pathway activation, triggering ferroptosis in CRC cells. HMGB1 released from ferroptotic cells acts as a DAMP, activating antitumor CD8+ T-cell responses. Additionally, N6F11 suppresses the mTOR/SREBP-1/SCD1 pathway to boost checkpoint blockade therapy (93).

Notably, Traditional Chinese Medicine (TCM) demonstrates significant potential in oncology due to its multi-target, multi-pathway mechanisms, structural stability, and safety profile (94). Active molecules in TCM, such as artemisinin, baicalein, and tanshinone, have been shown to exert antitumor effects by modulating ferroptosis. TCM enhances the immune system and TME immune signaling pathways, showing promise as an adjuvant to ICIs, particularly in CRC.Unlike conventional therapies focusing on direct tumor cell cytotoxicity, TCM leverages TME immune signaling modulation to stimulate immune responses, suppress tumor progression, and improve immunity (95, 96). For instance, ginsenoside Rh3 (GRh3) induces ferroptosis and pyroptosis in CRC cells via the Stat3/p53/NRF2 axis, reducing SLC7A11 expression, depleting intracellular GSH, and increasing ROS and MDA accumulation, thus enhancing immunotherapy efficacy (97). BXD (Banxia Xiexin Decoction) targets the PI3K/AKT/mTOR axis, inducing CRC ferroptosis by increasing intracellular iron and ROS. Similarly, Wei-Tong-Xin (WTX) promotes ferroptosis via the PI3K/AKT pathway, while SSG (Shenqi Sanye Granules) inhibits CRC proliferation through Hmox1-mediated ferroptosis pathways (98, 99). These studies highlight TCM's potential to

combine direct tumor inhibition with immune modulation and ferroptosis, offering innovative strategies for cancer immunotherapy. Its holistic, multi-target approach, particularly in inducing ferroptosis and modulating immune responses, represents a promising avenue for advancing CRC treatment.

In addition, nanomaterials are increasingly being used in ferroptosis-mediated immunotherapy. Nanotechnology enhances the targeting, controlled release, and stability of drugs while minimizing side effects in CRC treatment (79). Smart nanocarriers, designed to respond to TME-specific signals, enable precise drug delivery and improved therapeutic outcomes. For example, Li Q et al. investigated leukocyte membrane-encapsulated polylactic acid-hydroxyacetic acid-encapsulated glycyrrhizinopropyls (GCMNPs), which, when combined with ferumoxyto, an iron supplement, decreased GPX4 expression, led to increased levels of lipid peroxidation, and induced ferroptosis (100). In contrast, Xin Y et al. designed a fish oil-based microemulsion system, which can effectively orally deliver the inhibitory peptide OPBP-1, which blocks PD-1/PD-L1, to induce ferroptosis in tumor cells and also synergistically interact with ICIs to further enhance the antitumor effect (101). Furthermore, advanced dual-targeting systems, such as GOx@FeNPs, leverage photothermal therapy (PTT) and ferroptosis synergy to boost CRC cell immunogenicity, enhancing dendritic cell (DC) maturation and CD8+ T-cell activation. Combined with αPD-L1, this system showed remarkable efficacy in CRC growth suppression (102). Li et al. further developed a pH- and glutathione-responsive nanodrug delivery system (PMDC NPs) targeting dihydroartemisinin (DHA) and CORM-401, inducing oxidative stress-related apoptosis and ferroptosis while triggering immunogenic cell death (ICD). These nanoparticles activate immune effector cells, including CD4+ and CD8+ T cells, mature DCs, and M1 tumor-associated macrophages (TAMs), while inhibiting Tregs and M2 TAMs (103). Overall, these innovations highlight nanotechnology's pivotal role in ferroptosis-based CRC immunotherapy, offering potential for overcoming resistance, enhancing efficacy, and enabling personalized treatment strategies. This progress establishes a foundation for future precise and effective CRC therapies. In conclusion, recent research underscores the diversity of colorectal cancer (CRC), highlighting its potential as a natural tumor-suppressing process and a targetable pathway to overcome therapeutic resistance. These advancements offer critical insights into CRC pathogenesis and reveal promising strategies to leverage ferroptosis for enhanced treatment outcomes. Comprehensive understanding—from identifying genetic traits to deploying novel drug interventions—could support the development of precise and effective therapeutic strategies. Such approaches may aid in preventing tumor progression and overcoming CRC resistance. Table 1 summarizes the application of ferroptosis inducers with ICIs in CRC.

Future prospects and conclusions

Ferroptosis as an emerging mechanism of programmed cell death shows promise in CRC therapy (112). It triggers cancer cell death through accumulation of ferroptosis, depletion of GSH and increased lipid peroxidation, and in the process triggers an anti-tumor immune response. In particular, DAMPs released during ferroptosis can activate DCs and CD8+ T cells, thus enhancing immune surveillance of tumors and making it an effective adjunct to immunotherapy. Thus, ferroptosis provides an effective immune adjunct to CRC therapy.

Nevertheless. The physiological roles and regulatory mechanisms of ferroptosis remain many unanswered questions, and in-depth exploration of its regulatory network, dynamic changes in lipid metabolism, and synergistic effects with other cell death modalities, such as apoptosis and autophagy, will reveal its dynamics in the tumour microenvironment (TME), especially in the regulation of

TABLE 1 Application of ferroptosis in the treatment of CRC.

Drugs Name	Target	Mechanism of ferroptosis	Ref.
ACADSB	GPX4	ACADSB induces ferroptosis of CRC cells by negatively regulating GPX4	(104)
Andrographis	β-catenin/Wnt-signaling pathways	Activation of ferroptosis and suppression of β-catenin/Wnt-signaling pathways were the key mediators for the anticancer and chemosensitizing effects of andrographis	(105)
Lipocalin 2	GPX, system Xc^-	Overexpression of Lipocalin 2 inhibits ferroptosis and promotes CRC progression	(106)
Cisplatin	GPX4	Depletion of reduced glutathione caused by cisplatin and the inactivation of GPX4 plays the vital role in CRC cells to induce ferroptosis	(90)
Cetuximab	NRF2, ROS	Cetuximab inhibits Nrf2/HO-1 pathway to promote ferroptosis in CRC	(107)
Tagitinin C	NRF2/HO-1, lipid peroxidation	Tagitin C activates NRF2/HO-1 pathway to induce ferroptosis	(108)
Sulfasalazine (SSZ)	System Xc^-GSH	Inhibiting plasma membrane Cys transporter Xc (-) and effectively depleting cellular GSH	(109)
IMCA	SLC7A11	IMCA induces ferroptosis mediates by SLC7A11 through the AMPK/mTOR pathway	(110)
Hydroxy camptothecincinnamaldehyde loaded nanoparticles(PCH)	ROS	Producing ROS	(111)

lipid metabolism and immune cell activity. It is a promising strategy to develop specific ferroptosis inducers targeting these molecular mechanisms, which should not only have high selectivity and low toxicity, but also good tissue penetration to effectively reach the tumor site. In addition, the optimal combination in the combination therapy strategy deserves further exploration. Existing studies have shown that ferroptosis inducers in combination with ICIs (e.g., PD-1/PD-L1 inhibitors) can activate both innate and adaptive immunity, thereby substantially enhancing the anti-tumor effect. However, the optimal drug combinations, dosages, and timing of administration need to be formulated through in-depth studies for the wide application of such combination therapy regimens in the clinic. Especially in terms of individualized treatment, the tumor microenvironment and immune status of different CRC patients vary greatly, and how to combine these features to design personalized treatment plans will be an important step toward achieving precision therapy. Conducting clinical trials to validate the safety and efficacy of these treatment strategies is essential. In addition to the need to focus on the toxic side effects of ferroptosis inducers, their efficacy in patients with different stages of CRC should be evaluated. Through these multilevel studies, ferroptosis is expected to become an important component of CRC treatment, providing patients with more therapeutic options and significantly improving their prognosis. In this context, traditional Chinese medicine (TCM) demonstrates significant potential in CRC immunotherapy due to its multi-target mechanisms and low side effects. Active compounds like ginsenosides can regulate ferroptosis-related pathways and improve the immune state of the tumor microenvironment (TME). However, TCM faces challenges such as low bioavailability, complex compound structures, and unclear mechanisms. Future research should focus on elucidating TCM molecular mechanisms and optimizing its synergy with immunotherapy and ferroptosis inducers to enhance therapeutic design and overcome existing limitations. Moreover, nanotechnology offers innovative approaches to CRC immunotherapy. Nano-delivery systems improve drug stability and targeting, addressing issues like uneven biodistribution and reducing side effects. Functionalized nanoparticles enable controlled release and TME-specific targeting, enhancing the efficacy of combined therapies. In summary, ferroptosis introduces a novel perspective and strategy for CRC immunotherapy. Future advancements require interdisciplinary collaboration, integrating immunotherapy, ferroptosis inducers, TCM, and nanotechnology to refine treatments and accelerate clinical translation. Multicenter clinical trials are essential to validate efficacy and safety, while personalized strategies based on patient-specific factors can achieve precision treatment and better outcomes. These efforts promise to offer more therapeutic options, significantly improving survival rates and quality of life for CRC patients.

Author contributions

WX: Writing – original draft, Writing – review & editing. YL: Conceptualization, Writing – review & editing. YZ: Writing – review & editing. ZK: Writing – review & editing. ZL: Writing – review & editing. JT: Writing – review & editing. HZ: Writing – review & editing. WS: Writing – review & editing. JZ: Writing – original draft, Writing – review & editing.

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Conflict of interest

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New insights into the ferroptosis and immune infiltration in endometriosis: a bioinformatics-based analysis

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Background: Ferroptosis, a recently discovered iron-dependent cell death, is linked to various diseases but its role in endometriosis is still not fully understood.

Methods: In this study, we integrated microarray data of endometriosis from the GEO database and ferroptosis-related genes (FRGs) from the FerrDb database to further investigate the regulation of ferroptosis in endometriosis and its impact on the immune microenvironment. WGCNA identified ferroptosis-related modules, annotated by GO & KEGG. MNC algorithm pinpointed hub FRGs. Cytoscape construct a ceRNA network, and ROC curves evaluated diagnostic efficacy of hub FRGs. Consensus cluster analysis identified ferroptosis subclusters, and CIBERSORT assessed immune infiltration of these subclusters. Finally, RT-qPCR validated hub FRG expression in clinical tissues.

Results: We identified two ferroptosis modules of endometriosis, and by enrichment analysis, they are closely linked with autophagy, mTOR, oxidative stress, and FOXO pathways. Furthermore, we identified 10 hub FRGs, and the ROC curve showed better predictive ability for diagnosing. RT-qPCR confirmed that the tissue expression of 10 hub FRGs was mostly consistent with the database results. Subsequently, we developed a ceRNA network based on 4 FRGs (BECN1, OSBPL9, TGFBR1, GSK3B). Next, we identified two ferroptosis subclusters of endometriosis and discovered that they are closely linked with endometriosis stage. Importantly, immune enrichment analysis illustrated that the expression levels of immune cells and immune checkpoint genes were significantly different in the two ferroptosis subclusters. Specifically, the ferroptosis subcluster with stage III-IV of endometriosis is more inclined to the immunosuppressive microenvironment.

Conclusions: Our study showed that ferroptosis may jointly promote endometriosis progression by remodeling the immune microenvironment, offering new insights into pathogenesis and therapeutics.

KEYWORDS

ferroptosis, endometriosis, WGCNA, immune infiltration, immune checkpoint genes

1 Introduction

Endometriosis (EMs), an estrogen-dependent inflammatory disease, is characterized by abnormal growth of endometrial-like tissues outside the uterus, affecting approximately 10% of women of reproductive age globally (1). Common symptoms include pelvic pain and infertility, which severely jeopardize the physical and mental health of patients (2). Hormone drugs and surgery are the primary treatments for EMs; however, these treatments are closely linked to various adverse effects, such as contraception and menopause-related symptoms (1). Currently, the etiology of EMs remains elusive. Previous studies have shown that iron overload and excessive accumulation of reactive oxygen species (ROS) are pathological characteristics of EMs (3, 4), which lays the foundation for the occurrence of cellular ferroptosis.

Ferroptosis, a novel form of iron-dependent programmed cell death, is characterized by the accumulation of ROS and lipid peroxidation-mediated membrane damage (5). Mechanistically, ferroptosis stands apart from other forms of cell death, such as apoptosis, autophagy, and necrosis, primarily due to distinct differences observed in cell morphology, metabolism, and protein expression patterns (5). Ferroptotic cell death manifests as damage of the mitochondrial membrane, increased membrane density, reduced mitochondrial cristae, and intact nuclear membranes (5). The critical mechanisms underlying ferroptosis are intricately linked to iron metabolism, lipid metabolism, and glutathione metabolism (6). Several lines of study showed that ferroptosis played a crucial role in the pathogenesis of various diseases, including EMs (7–10). Prior investigations have elucidated the complex function of ferroptosis in EMs, highlighting its significance in disease development and progression (11–13). On the one hand, endometriotic lesions present resistance to ferroptosis, impeding the clearance of ectopic endometrium and facilitating its proliferation and migration (11, 14), while ferroptotic cell death also release inflammatory cytokines and trigger downstream regulatory pathways, thereby promoting proliferation and angiogenesis in adjacent tissues (15). However, the exact mechanisms of ferroptosis in EMs remains poorly understood and needs further investigation.

As we all know, immune dysfunction, characterized by abnormalities in the number and function of immune cells, stands as a pivotal feature of EMs (16). Recent studies have highlighted the close relationship between ferroptosis and the immune microenvironment as well as immunotherapy for diseases (5). For instance, IL-6 from M1 macrophages can induce lipid peroxidation and disrupt iron homeostasis in bronchial epithelial cells, leading to ferroptosis (17). Ferroptotic cell death could also activate the innate immune system and promote the development of inflammatory diseases by releasing the damage-associated molecular patterns (DAMPs). Additionally, ferroptotic cell death exposes tumor antigens, boosting tumor cell immunogenicity and enhancing the effectiveness of immunotherapy (18). Therefore, the exploration of the interaction between ferroptosis and immune cell infiltration in EMs is crucial for understanding the pathogenesis and developing effective treatment strategies of the disease.

In this study, we initially employed the weighted gene co-expression network analysis (WGCNA) to identify ferroptosis-related modules in EMs samples and annotated their potential functions. Subsequently, we constructed subclusters of EMs based on ferroptosis-related genes (FRGs), and evaluated the immune infiltration of subclusters. Following this, we identified hub FRGs and constructed a ceRNA network for hub FRGs, and further validated their expression levels in tissue samples through RT-qPCR. Taken together, our study reveals new insights into the mechanism of ferroptosis in the development of EMs.

2 Methods

2.1 Data acquisition

The microarray data of EMs, including mRNA (GSE51981), miRNA (GSE105765), and lncRNA (GSE105764), were obtained from GEO databases (19). FRGs were acquired from the FerrDb database (20).

2.2 Weighted gene coexpression network analysis

We used the 'WGCNA' package to reconstruct coexpression gene networks associated with disease clinical characteristics in GSE51981. Subsequently, we determined an appropriate soft threshold power and employed a dynamic tree-cut strategy to hierarchically cluster and merge similar modules. Next, we calculated the module eigengene, module membership, and gene significance to identify clinical trait modules.

2.3 Functional annotation of key modules and identification of hub FRGs

Gene Ontology (GO) biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the clusterProfiler package to further interpret and visualize diverse biological functions of those key modules. Then, WGCNA was firstly utilized to identify gene–gene interactions in two important modules, and then the hub FRGs were obtained using the MNC genetic algorithm. To further confirm the accuracy of the results, we validated the expression of these hub FRGs in clinical tissue samples. Moreover, the receiver operating characteristic (ROC) analysis was performed to assess the specificity and sensitivity of these hub FRGs for diagnosing EMs.

Identification of new ferroptosis subclusters of endometriosis and immune infiltration analysis

We analyzed the expression of ferroptosis genes in endometriosis samples of GSE51981 using consensus cluster analysis to identify novel ferroptosis molecular subclusters. Subsequently, the CIBERSORT package was utilized to estimate

the relative expression percentages of 22 infiltrated immune cell types for each subcluster. Meanwhile, the expression levels of immune checkpoint genes in the subclusters were assessed using Wilcoxon tests.

2.4 Construction of a ceRNA network

TargetScan (21), miRDB (22), and miRTarBase (23) were utilized for miRNA prediction, while Mircode was employed for predicting miRNA–lncRNA interactions with a stringent approach (24). Subsequently, a lncRNA–miRNA interaction network was established (Additional File 1). DESeq2 R package was then applied to analyze the differential expression of lncRNAs (Additional File 2), and lncRNAs with a significance level of $P<10^{-6}$ were chosen to construct the ceRNA network (Additional File 3). The interaction networks were visualized using Cytoscape software.

2.5 Collection of tissue samples

In this study, based on the use of specimens in the GSE51981 dataset, we also used eutopic endometrium from ovarian endometriosis (OEM) patients (n=22) as case group, which allowed us to investigate the molecular changes in the endometrium associated with the disease process. Furthermore, we used normal endometrium from cervical intraepithelial neoplasia grade III (CIN III) patients (n=10) as a control group. The comparison between these two types of samples highlights the differences in gene expression between control and diseased endometrium. All samples were obtained from the Department of Gynecology at the Fourth Hospital of Hebei Medical University in China. Patients who underwent hormone therapy within six months prior to surgery or exhibited concurrent tumor conditions were excluded from the study. This research was approved by the Ethics Commission of the Fourth Affiliated Hospital of Hebei Medical University (2021KY016). Written informed consent was obtained from all patients.

2.6 Total RNA extraction and RT–qPCR

TRIzol reagent (Thermo Fisher Scientific, Waltham, USA) was utilized for total RNA extraction from the tissues, with RNA quality assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Subsequently, 1μg of total RNA was reverse transcribed into cDNA employing SweScript All-in-One First-Strand cDNA Synthesis SuperMix (Servicebio, Wuhan, China), followed by qPCR using Hieff® qPCR SYBR Green Master Mix (YEASEN, Shanghai, China). The PCR procedure as follows: 95°C for 5 min followed by 40 cycles at 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s. Primer sequences of genes can be found in Additional File 4. Each sample underwent triplicate analysis, with gene expression levels normalized to GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method.

2.7 Statistical analysis

GraphPad software version 8.0 was utilized for statistical analyses. An unpaired t-test was conducted to ascertain the statistical significance of pairwise differences between groups, with a significance level set at $P<0.05$. The diagnostic efficacy of the hub FRGs was assessed through ROC curve analysis.

4 Results

4.1 Identification of key modules

Firstly, we integrated genes from GSE51981 dataset combined with ferroptosis genes from FerrDb dataset, and the four ferroptosis modules (Turquoise, Blue, Brown, and Grey) related to EMs and controls were identified through hierarchical clustering analysis and a dynamic cutting algorithm using a cut-off R^2 value of 0.93 and an appropriate soft threshold β value (Figures 1A–C). Among these modules, the turquoise module exhibited a significant positive correlation with EMs, having a correlation coefficient of 0.25, while the blue module showed a significant negative correlation with EMs, with a correlation coefficient of -0.33 (Figure 1D). Consequently, we focused on analyzing the turquoise and blue modules in subsequent investigations.

4.2 Enrichment analysis of key modules

Subsequent to this, we conducted GO and KEGG pathway analyses to elucidate the potential molecular functions and biological processes of the turquoise and blue module. The results depicted in Figure 2 illustrated that the turquoise module predominantly correlated with oxidative stress, autophagy, and mTOR signalling pathways (Figures 2A–D). The blue module was primarily linked to shigellosis, ferroptosis, FOXO, and the NOD-like receptor signalling pathway (Figures 3A–D). These findings suggest a potential role of these pathways in modulating the ferroptosis process in EMs.

4.3 Identification of the hub FRGs

It's well known that the hub genes represent the biological significance of key modules, thus, we identified 10 hub FRGs in the aforementioned modules by the MNC algorithm (Figure 4A). Subsequently, we evaluated the expression level of hub FRGs in GSE51981 (Figure 4B). As shown in Figures 4C–L, CFL1, CHMP6, and CISD3 exhibited higher expression levels in the eutopic endometrium of patients with EMs compared to the normal endometrium of controls. Conversely, BECN1, EIF2AK4, GSK3B, IREB2, OSBPL9, RICTOR, and TGFBR1 were found to be upregulated in control patients when compared to those with EMs. Furthermore, we also validated the expression of hub FRGs in the GSE25628 database. The expression trends of most genes are consistent with those in GSE51981, which indicates that the genes we have screened are representative and reliable (Supplementary Figure S1).

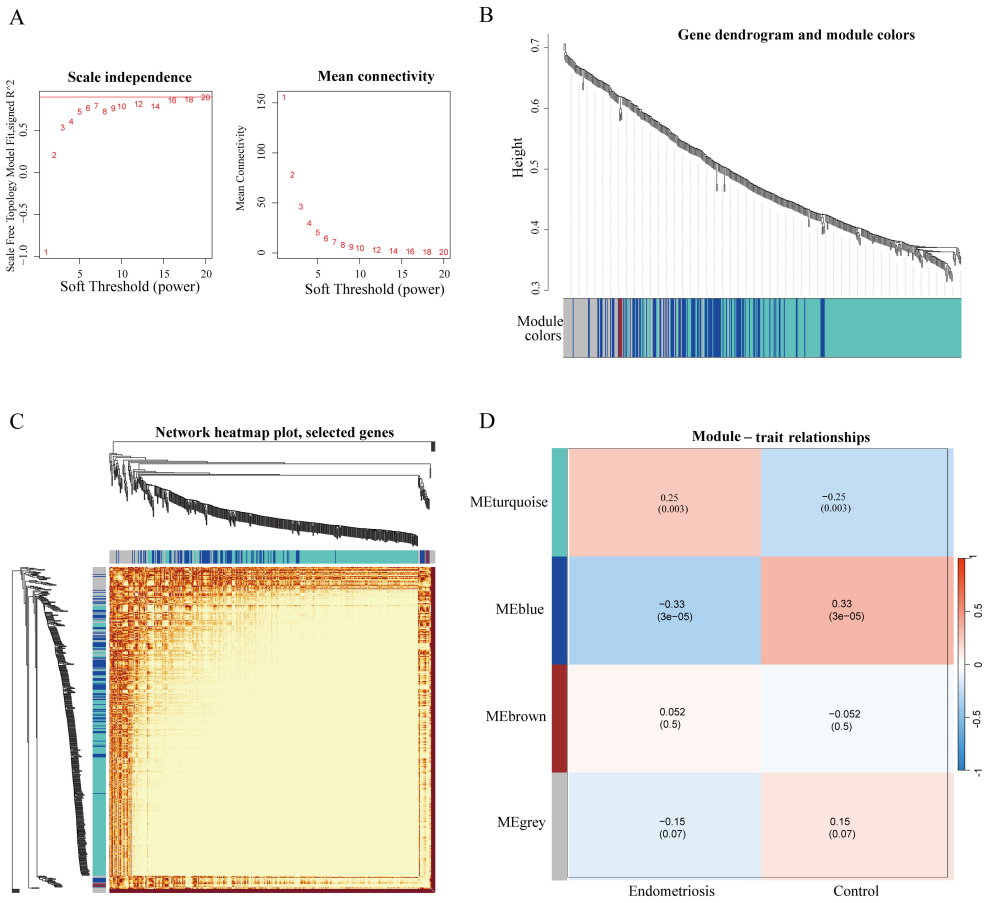

FIGURE 1
Identification of ferroptosis-related modules of endometriosis by WGCNA analysis. **(A)** Network topology analysis for various soft threshold powers (The association between the scale-free fit index and diverse soft-thresholding exponents (former); The connection between average connectivity and diverse soft-thresholding exponents (latter). **(B)** Hierarchical cluster tree diagram of co-expression modules based on a topological overlap matrix (1-TOM). Each branch in the clustering tree represents a gene, and co-expression modules are constructed and represented by different colors. **(C)** The heatmap of the co-expression network. **(D)** Correlation of the key modules and control and endometriosis group. Each row corresponds to a module eigengene, column to a clinical trait. Each module includes the respective correlation coefficient and corresponding *p*-value. And we identified turquoise and blue modules are correlated with the diseases.

4.4 Diagnostic values of hub FRGs

To evaluate the diagnostic efficacy of hub FRGs, we conducted ROC curve analysis. The results depicted in Figure 5A showed that the area under the curve (AUC) of ROC for all 10 hub FRGs exceeded 0.6 in GSE51981. Specifically, the AUC values were 0.637 for BECN1, 0.629 for CFL1, 0.646 for CHMP6, 0.639 for CISD3, 0.695 for EIF2AK4, 0.697 for GSK3B, 0.679 for IREB2, 0.652 for OSBPL9, 0.639 for RICTOR, and 0.67 for TGFBR1. These results suggest their potential as diagnostic markers.

4.5 Construction of ceRNA network

Numerous evidence has been suggested that the ceRNA regulatory network plays a vital role in the pathological processes of diseases. Therefore, to further understand the regulatory mechanism of hub FRGs in EMs, we also constructed the ceRNA network using GSE105765 and GSE105764 combined with several bioinformatics database, which involves 4 hub FRGs (BECN1, TGFBR1, GSK3B, and OSBPL9), 7 miRNAs, and 27 lncRNAs (Figure 5B). These results provide new insights for further understanding the regulatory mechanisms of hub FRGs in EMs.

4.6 Identification of new ferroptosis subclusters in endometriosis

Although molecular subtypes have become crucial for predicting disease prognosis and guiding targeted therapy, there is currently no molecular subgroup identified for EMs. To address this gap, a cluster analysis was conducted to identify ferroptosis subclusters based on the FRGs in EMs samples from GSE51981. The analysis revealed that the most stable number of subclusters was achieved when k=2 (Figure 5C), which was further validated in GSE25628 databases (Supplementary Figure S1). Subsequent evaluation of the distribution of EMs stages in the two ferroptosis subclusters showed that cluster 1 was predominantly associated with moderate/severe stages of EMs,

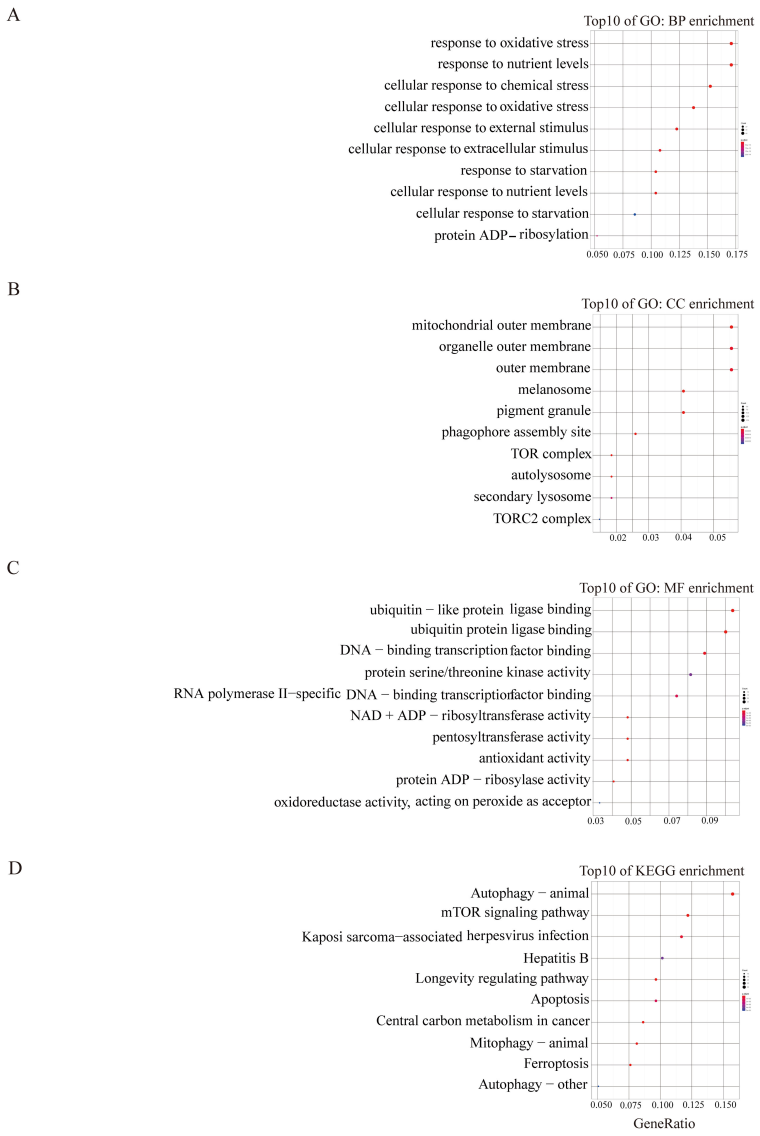

FIGURE 2
GO and KEGG enrichment analysis of turquoise module **(A–D)**. The color and size of each circles represent p-value and the number of the enriched genes, respectively.

while cluster 2 had a higher abundance of minimal/mild stages compared to moderate/severe stages (Figure 5D). This suggests that the two ferroptosis subclusters could reflect to some extent the severity of conditions in EMs and are worth further investigation.

4.7 Immune landscapes of ferroptosis subclusters

Emerging evidence suggests a close relationship between ferroptosis and immunity (25, 26). Therefore, we analyzed the immune landscapes of two subclusters using CIBERSORT (Figure 6). In cluster 1, there was a higher proportion of B cells naïve, T cells CD4 memory resting, activated NK cells, M2 macrophages, activated dendritic cells, and resting mast cells, while cluster 2 showed a higher proportion of T cells CD8, regulatory T cells, resting NK cells, monocytes, and resting dendritic cells. Furthermore, we also examined the expression of immune checkpoint genes in these subclusters and found statistically significant differences in most immune checkpoint genes. Our results depict the landscape of ferroptosis and immune microenvironment of EMs interacting closely, providing a theoretical basis for further research on the specific mechanisms of the two in the disease.

4.8 Tissue expression of hub FRGs and its relationship with clinicopathological features

To further verify our results, we assessed the expression levels of 10 hub FRGs in the eutopic endometrium of endometriosis patients

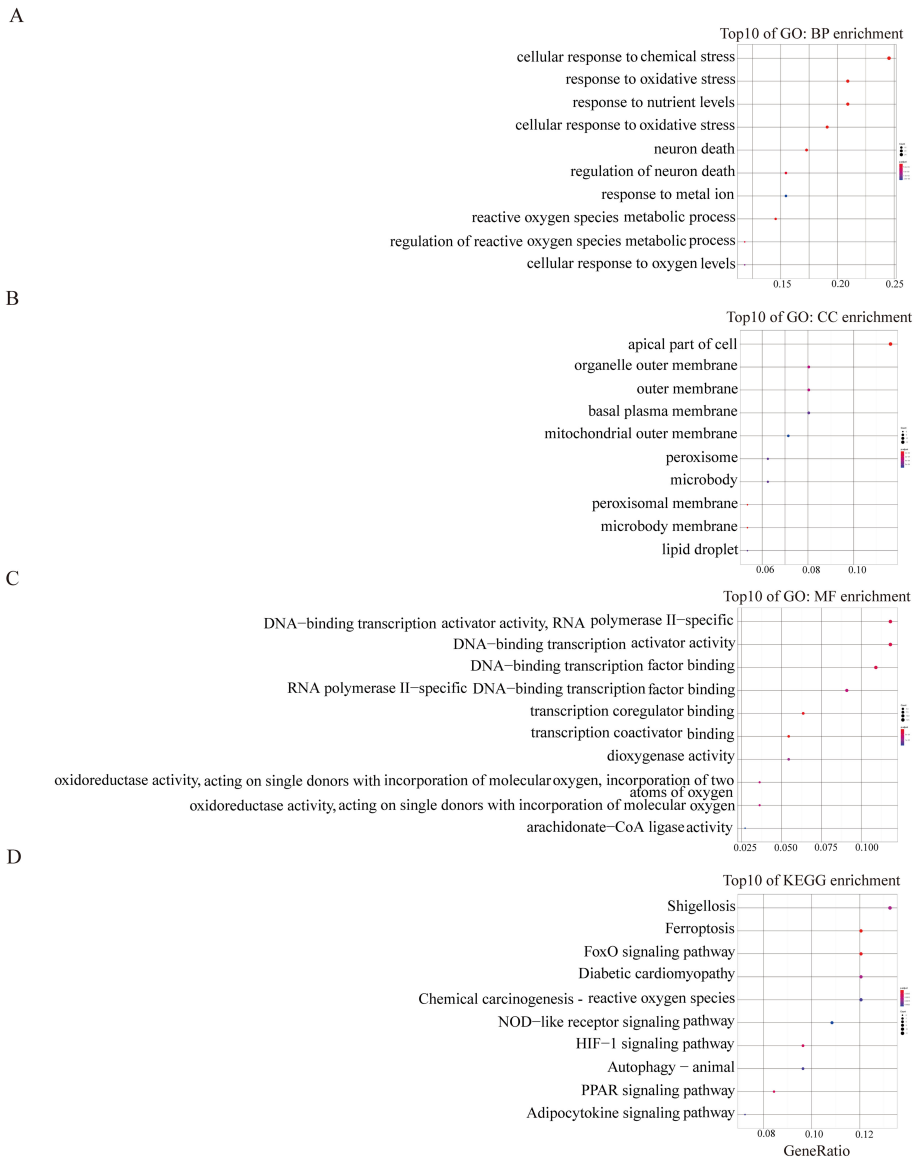

FIGURE 3
GO and KEGG enrichment analysis of blue module **(A–D)**. The color and size of each circles represent p-value and the number of the enriched genes, respectively.

(Endometriosis group) and in the normal endometrium of patients with CIN III (Control group) (Figure 7). As we can see, the expression trend of hub FRGs in clinical samples was roughly consistent with the results of GSE51981, which confirms the reliability of our results. Furthermore, based on the median value of each hub FRGs expression, we analyzed the correlation between the expression of these hub FRGs and the clinicopathological features of endometriosis patients (Supplementary Tables S1-S6). Specifically, the expression of BECN1 positively correlates with disease staging and negatively correlates with CA125 levels. The expression of CFL1 is associated with a history of infertility. The expressions of CISD3 and CHMP6 negatively correlate with disease staging. The expression of IREB2 positively correlates with CA125 levels and the diameter of ovarian cysts, and negatively correlates with disease staging.

5 Discussion

The pathogenesis of EMs remains unclear. Although some studies suggest a potential involvement of ferroptosis in the development of EMs, its precise role remains incompletely understood. In this study, we utilized the latest ferroptosis-related genes retrieved from the FerrDb database and employed the WGCNA to identify ferroptosis modules that are most relevant to EMs. Subsequently, functional enrichment analysis demonstrated that the turquoise module, positively correlated with EMs, was primarily associated with oxidative stress, autophagy, and the mTOR signaling pathway. Conversely, the blue module, which demonstrates a negative correlation with EMs, was predominantly implicated in shigellosis and ferroptosis, while also being associated with the FOXO and NOD-like receptor signaling pathways.

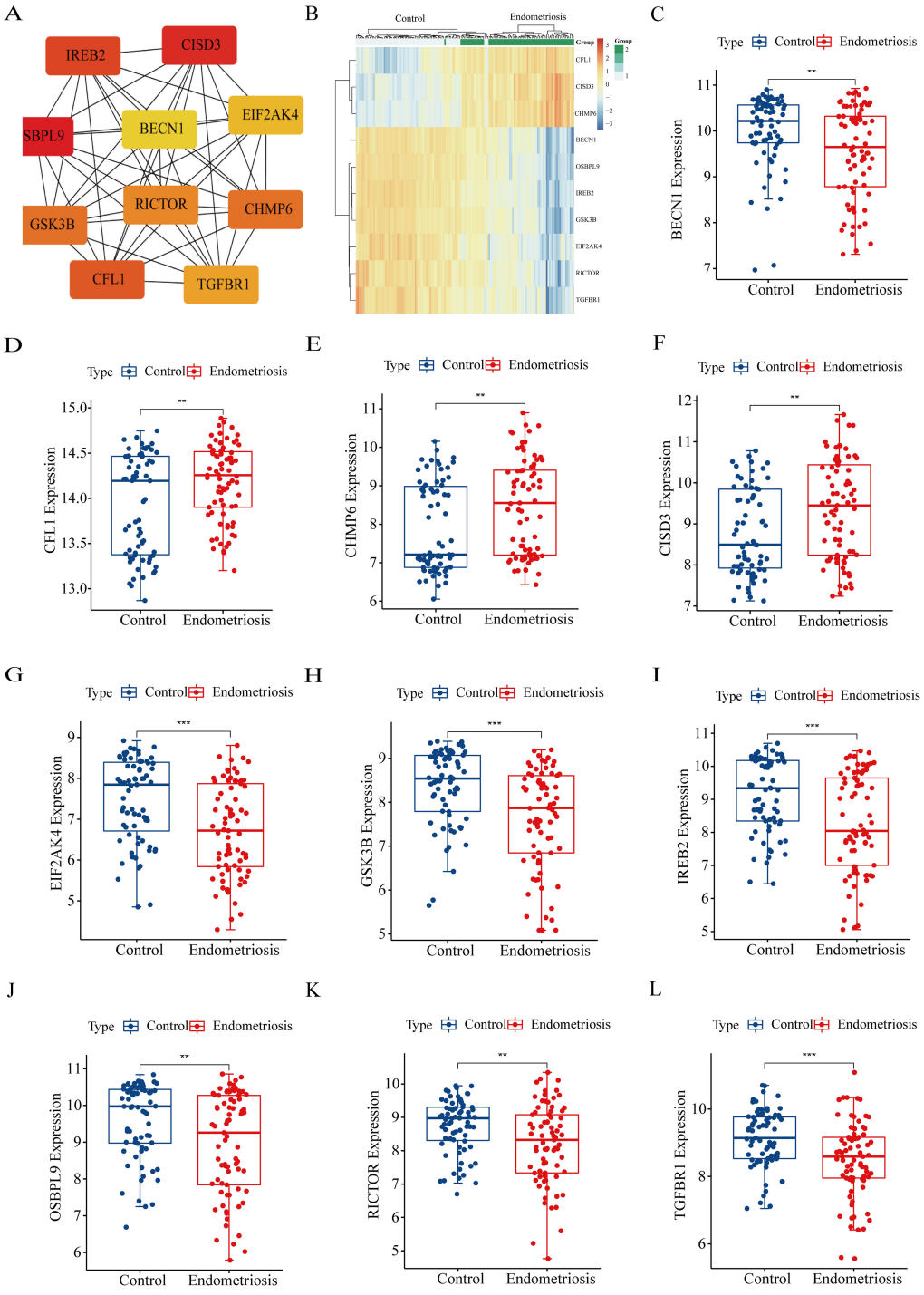

FIGURE 4
Identification and the expression levels of hub FRGs. **(A)** Identification of hub FRGs using MNC algorithm. Three indicators (degree, closeness and betweenness) were, respectively, calculated to evaluate the importance of each node and the top 10 nodes were selected. The six hub genes were their common nodes. **(B)** The heatmap of hub FRGs in GSE51981. **(C–L)** The expression box plots of hub FRGs in GSE51981. *P<0.05, **P<0.01, ***P<0.001.

Furthermore, existing evidence supports the association of these pathways with the pathological process of EMs. Autophagy is a crucial cellular degradation pathway responsible for eliminating damaged or aged organelles (27). Research has shown that in normal endometrial cells, the induction of autophagy exhibits a pro-apoptotic effect, whereas the autophagy pathway is suppressed in ectopic endometrial cells of EMs patients (28). A recent study has revealed that, in EMs, Açai Berries promotes autophagy by reducing mTOR expression to regulate the PI3K/AKT/ERK1/2 pathway. Meanwhile, it also activates mitochondrial autophagy, which enhances the clearance of damaged mitochondria, reduces ROS production, and restores oxidative balance (29). Furthermore, it has

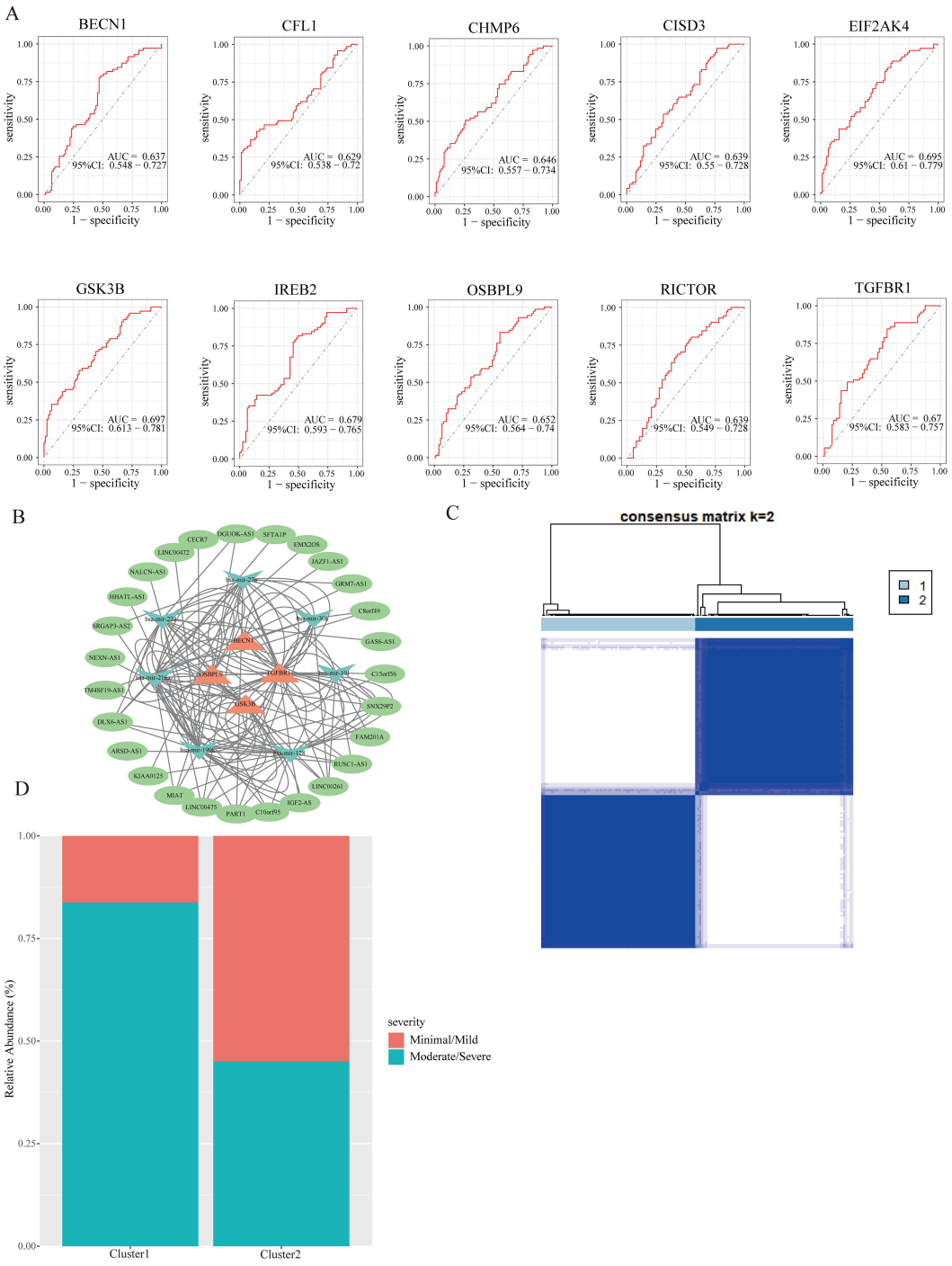

FIGURE 5
The ROC curve and ceRNA network of hub FRGs, and identification of subclusters. **(A)** The ROC curves of hub FRGs. **(B)** CeRNA network of hub FRGs. **(C)** Two ferroptosis subclusters were identified by consensus clustering analysis of EMs patients in GSE51981. **(D)** The relationship between EMs staging and subclusters.

been reported that mTOR, an important negative regulator of autophagy, is abnormally activated in the endometriotic lesions (30). The FOXO and NOD-like receptor signaling pathway participate in the inflammation process and cytokine signal transduction of EMs (31, 32). Consequently, these pathways might play a pivotal role in the involvement of ferroptosis in the development of EMs.

Next, we identified 10 hub FRGs using the MNC algorithm, including BECN1, GSK3B, IREB2, OSBPL9, EIF2AK4, RICTOR, TGFBR1, CFL1, CHMP6, and CISD3, which are potentially involved in the development of EMs through bioinformatics analysis. The AUC-ROC of all hub FRGs exceeded 0.6, indicating the high sensitivity and specificity of these hub FRGs in distinguishing EMs patients from healthy controls. Among these

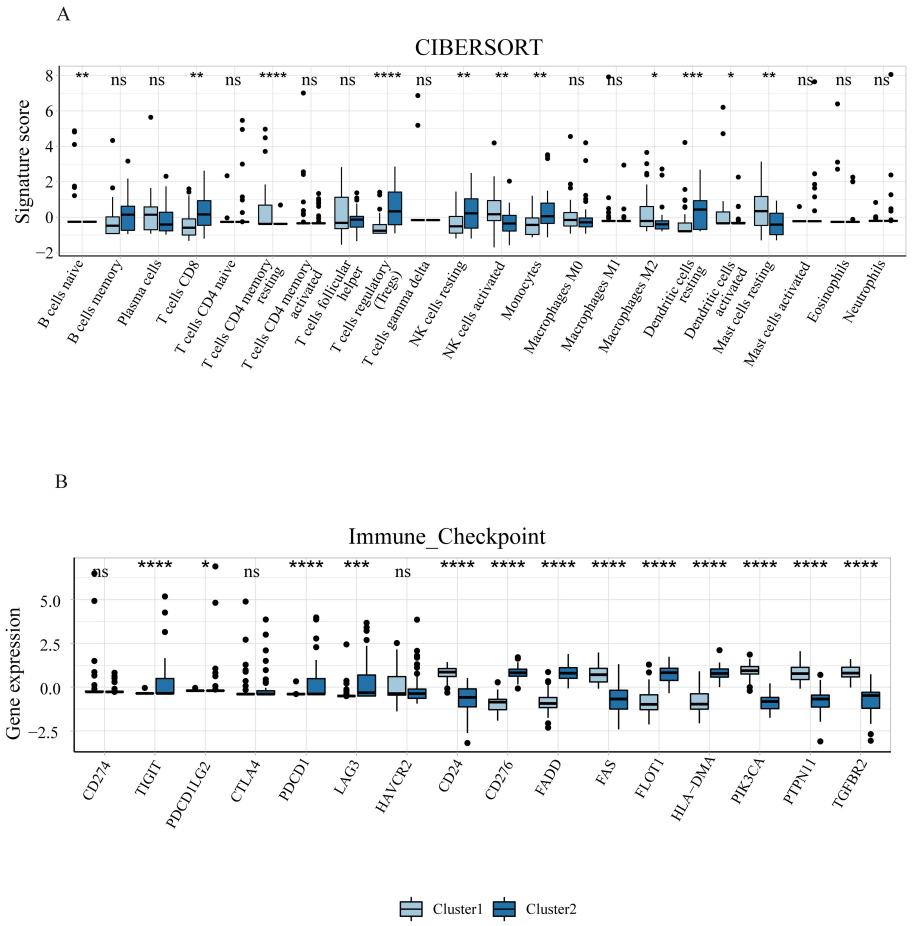

FIGURE 6
Immune infiltration of ferroptosis subclusters in endometriosis. **(A)** The difference of 22 types of immune cells' infiltration between the two subclusters. **(B)** The expression levels of immune checkpoint genes in the two subclusters. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

genes, BECN1, GSK3B, IREB2, OSBPL9, EIF2AK4, RICTOR, and TGFBR1 were downregulated in the eutopic endometria of EMs patients compared to the endometria of normal controls. In contrast, CFL1, CHMP6, and CISD3 were upregulated in EMs patients. Beclin1 (BECN1), a key component of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, is crucial in regulating macroautophagy/autophagy (33, 34). It inhibits system Xc− by interacting with SLC7A11, consequently promoting ferroptosis (35, 36). Meanwhile, hucMSC-exosome-derived BECN1 enhances HSC ferroptosis by inhibiting the expression of cystine/glutamate transporter (xCT)-driven GPX4 (37). Previous studies have shown that BECN1 expression was decreased in both eutopic and ectopic endometrium of patients with EMs compared to normal endometrium (38, 39), which is consistent with our bioinformatics analysis and PCR results. BECN1 plays a role in processes such as progesterone synthesis and apoptosis in EMs through its involvement in autophagy (40, 41). However, its role in ferroptosis in EMs remains unclear, and we will investigate this in subsequent experiments. Iron-responsive element binding protein 2 (IREB2) is a pivotal posttranscriptional regulator of cellular and systemic iron metabolism. A recent study found that miR-19a targets IREB2 to inhibit ferroptosis in colorectal cancer (42). Additionally, the upregulation of IREB2 disrupts iron homeostasis, leading to the accumulation of a labile iron pool and lipid peroxidation, which in turn triggers ferroptosis (43, 44). Previous research has indicated that iron overload and lipid peroxidation are implicated in the pathogenesis of EMs. Our results indicated that IREB2 was downregulated in the eutopic endometrium of EMs patients. Thus, based on the existing evidence, we hypothesize that IREB2 may play a crucial role in regulating ferroptosis through its influence on iron balance in endometrial cells, warranting additional study. GSK3B, a widely expressed serine/threonine protein kinase, is a key regulator of redox homeostasis by modulating nuclear factor erythroid 2-related factor (Nrf2) (45, 46). Wu et al. demonstrated that GSK3B inhibited Nrf2 expression, leading to the accumulation of ROS and malondialdehyde, thereby promoting erastin-induced ferroptosis in breast cancer cells (47). Our study indicated that GSK3B was downregulated in eutopic endometrium compared to normal endometrium, suggesting that GSK3B may play a role in suppressing ferroptosis in eutopic endometrium through similar mechanisms mentioned above. Transforming growth factor beta receptor 1 (TGFBR1, also known as ALK4/5) plays a crucial role in transmitting extracellular stimuli to the downstream TGF-β

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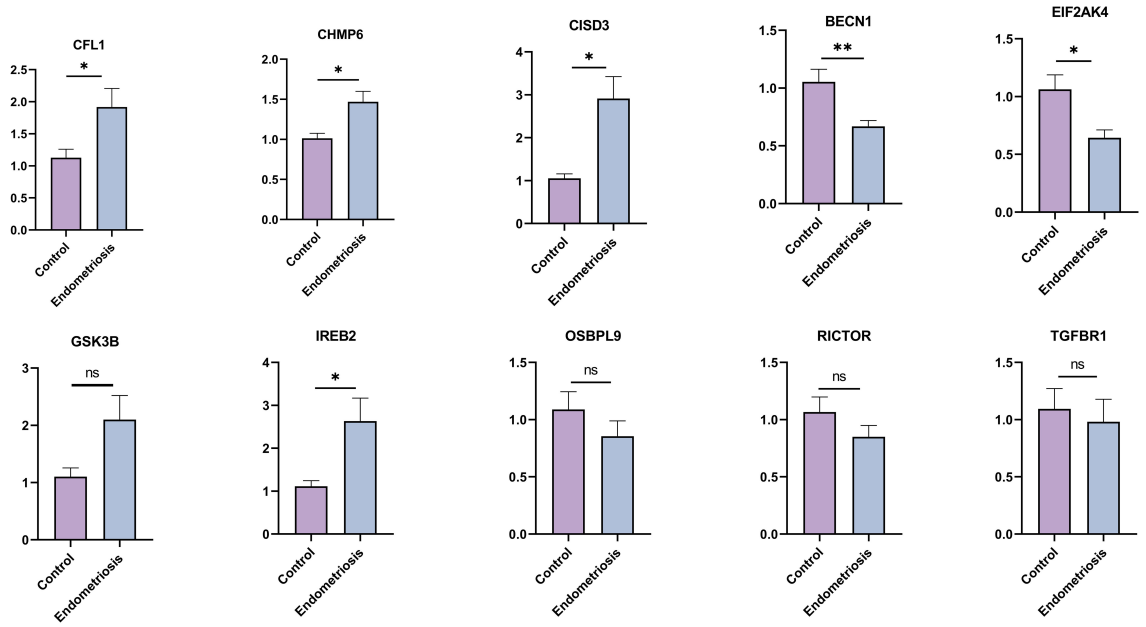

FIGURE 7
The expression levels of hub FRGs in tissue samples by RT-qPCR. Control group: normal endometrium of CINIII patients (n=10); Endometriosis group: eutopic endometrium of endometriosis patients (n=22). The data are presented as the Mean ± SD. *P<0.05, **P<0.01.

signalling pathway (48). Research has demonstrated that the inhibition of ALK4/5 effectively attenuates erastin-induced ferroptosis in HK-2 cells by potentiating Nrf2 signalling pathways (49) Previous studies have indicated that TGFBR1 expression was elevated in ectopic endometrium compared to eutopic endometrium in EMs patients (50). However, our research demonstrated that TGFBR1 was upregulated in normal endometrium compared to eutopic endometrium. This inconsistency could be attributed to variations in the tissue samples utilized in the two studies. Although TGFBR1 is involved in the migration and invasion processes of EMs, its role in ferroptosis within this condition remains elusive and deserves further exploration. Eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4, also known as GCN2) primarily participates in the cellular amino acid starvation response and regulates the immune response (51). The activation of the GCN2/ATF4 axis is triggered under cystine-deprived or xCT-KO conditions, thus contributing to ferroptosis (52, 53). Oxysterol-binding protein-like 9 (OSBPL9), a member of the oxysterol-binding protein family, is localized at the cellular membrane and exchanges molecules or signals between organelles, functioning as an essential transporter (54). Although there are few studies on the relationship between OSBPL9 and ferroptosis, OSBPL9 may be involved in ferroptosis by regulating the integration of sterol and sphingomyelin metabolism, sterol transport, and neutral lipid metabolism (55). Rapamycin-insensitive companion of mTOR (RICTOR) is a core component and functional element of mTORC2 that participates in PI3K/AKT pathway activation, cell proliferation, migration, autophagy, and metabolism (56). Research demonstrated that mTORC2 inhibited xCT activity through serine 26 phosphorylation at the cytosolic N-terminus (57), which may trigger ferroptosis. However, further investigations are warranted to elucidate the relationship between RICTOR and ferroptosis. Charged multivesicular body protein 6 (CHMP6) is an ESCRT-III subunit that is involved in membrane repair to inhibit necroptosis and pyroptosis (58). Dai et al. discovered that the silencing of CHMP6 expression inhibits the repair of the ferroptotic plasma membrane and enhances ferroptosis by modulating lipid peroxidation and DAMP release (59). CDGSH iron sulfur domain 3 (CISD3), also known as Miner2 or MiNT, is a mitochondrial protein belonging to the NEET protein family. Members of the NEET family are key regulators of iron and ROS homeostasis (60). CISD3 downregulation leads to a reduction in the mitochondrial membrane potential (MMP) and an increase in mitochondrial labile iron and ROS accumulation, thereby triggering ferroptosis (61). Cofilin-1 (CFL1) is a member of the ADF/cofilin family and plays a crucial role in actin depolymerization (62). Existing evidence suggests that the upregulation of CFL1 increases glutamate- and elastin-induced ferroptosis (63). Moreover, CFL1 triggers ferroptosis by modulating NF-κB signalling or the ER stress pathway to induce acute kidney injury (AKI) (64). Prior study suggested that CFL1 was significantly upregulated in eutopic endometrium from EMs patients compared to the normal endometrium (65), which is consistent with our results. The high expression of CFL1 in eutopic endometrium can promote the proliferation, adhesion, and invasion of endometrial stromal cells (ESC), while inhibiting apoptosis. However, there is no research on the role of CFL1 in ferroptosis in EMs. Taken together, known evidence from prior research, along with our findings, demonstrates that further elucidating the role of these hub FRGs in ferroptosis in EMs may provide potential therapeutic targets for this disorder. Currently, targeting ferroptosis seem to a promising therapy for

treating diseases (66). However, given the dual role of ferroptosis in EMs and its complex interaction with the immune system, it is uncertain which targeted ferroptosis treatment methods, such as inhibiting ferroptosis or promoting ferroptosis, have a definitive therapeutic effect on EMs. This needs further research in the future to confirm.

It is reported that a large number of miRNAs and lncRNAs are involved in the occurrence and development of EMs. However, the current research focusing on the correlation between non-coding RNA and ferroptosis has primarily concentrated on cancer, with comparatively fewer reports in the context of endometriosis. Herein, we have constructed a ceRNA network of hub FRGs, which includes 4 hub FRGs, 7 miRNAs, and 27 lncRNAs. These miRNAs are closely related to EMs or ferroptosis, such as miR-119a, miR-128, miR-101, miR-30a, miR-27a, miR-223, and miR-216a. For example, miR-119a is downregulated in the ectopic stromal cells (ESCs) of patients with ovarian EMs which inhibits the migration and invasion of ESCs by targeting PAK4 (67). miR-223 inhibits the proliferation, migration and invasion and promotes apoptosis of ESCs in EMs. In hepatitis B-related viral nephritis, exosomal miR-223-3p derived from bone marrow mesenchymal stem cells downregulates STAT3 phosphorylation by targeting HDAC2, thereby alleviating hbx-induced ferroptosis in renal podocytes (68). Meanwhile, we have also identified 27 lncRNAs, which can participate in the progression of multiple diseases through mediating various mechanisms. In summary, the ceRNA network of hub FRGs constructed in our study provides new insights for further understanding the regulatory mechanisms of ferroptosis in EMs.

Various diseases have recently been classified into molecular subtypes to guide prognosis and targeted treatment. In this study, we identified two ferroptosis subclusters related to EMs based on FRGs. Among them, Cluster 1 was mainly associated with moderate/severe stages, while Cluster 2 was predominantly linked to minimal/mild stages. Previous research has shown a strong connection between ferroptosis and immunity, yet a comprehensive analysis of the relationship between ferroptosis and immune infiltration in EMs remains unexplored. Using CIBERSORT, we determined the proportions of different immune cell types in the two clusters. Interestingly, Cluster 1 showed higher proportions of immune cells like activated dendritic cells (DCs), activated natural killer (NK) cells, and M2 macrophages, while Cluster 2 had elevated levels of immunosuppressive cells such as regulatory T cells (Tregs). Macrophages facilitate the progression of EMs by attenuating their phagocytic capacity and promoting the synthesis of inflammatory mediators, while M2 macrophages are involved in anti-inflammatory processes, immunosuppression, and neuroangiogenesis (67). Dendritic cells (DCs) act as a bridge between innate and adaptive immune responses. Prior studies revealed a greater proportion of immature DCs (iDCs) in the peritoneal DCs of EMs patients than in those of control patients, while iDCs facilitate angiogenesis, immune tolerance and the establishment of endometriotic foci (69). NK cells, cytotoxic effector lymphocytes, can regulate innate and adaptive immune responses. Wu et al. demonstrated that activated NK cells were more abundant in the eutopic endometria of patients with EMs than in those of controls (70), which is in accordance with our results. Tregs are a specialized subset of T cells primarily generated in the thymus that exert suppressive effects on immune responses. They are more concentrated within the peritoneal fluid of EMs and facilitate local immunosuppression and induce angiogenesis by interacting with proinflammatory cytokines (71). These results seemingly suggest that stage III-IV exhibits greater immunosuppressive and angiogenic capacity than stage I-II thus promoting the growth of endometriotic lesions, which is consistent with the distribution of immune cells in disease severity in our results. Moreover, immune checkpoint genes were notably differentially expressed between the two clusters. Specifically, TIGIT, PDCD1LG2, PDCD1, LAG3, CD276, FADD, FLOT1 and HLA-DMA are notably high expressed in cluster 2. While the CD24, FAS, PIK3CA, PTPN11 and TGFBR2 are significantly upregulated in cluster 1. These genes play an immunosuppression role in the progression of diseases. Such as, TIGIT, identified in 2009, is expressed on Tregs, memory T cell subsets, and NK cells which induces the exhaustion of immune cells and mediates immune suppression (72). PDCD1, also known as PD-1, is a vital immune checkpoint gene for cancer immunotherapy, which interactions with PD-L1 negatively regulating adaptive immune response mainly by inhibiting the activity of effector T cells and enhancing the function of immunosuppressive Tregs (73). Previous studies demonstrated a notable upregulation of PD-1 and PD-L1 expression in EMs (74, 75), suggesting that targeting PD-1 maybe a promising immunotherapy for EMs. LAG3, often expresses on the lymphocytes, including $CD4^+$ T cells, NK cells, $CD8^+$ T cells, and Treg cells, impeding the tumor immune microenvironment through accelerating T cell exhaustion and blocking T cell proliferation. As a result, high LAG3 expression promotes tumor growth by inhibiting the immune microenvironment (76). CD276 (B7-H3), a novel immune checkpoint of the B7 family, is widely overexpressed in tumor tissues associated with a worse prognosis and provides an immunosuppression environment for diseases progression (77). CD24 is predominantly expressed on the surface of the T and B lymphocytes and is overexpressed in multiple cancer cell types. During the interaction between immune cells and cancer cells, it inhibits phagocytosis leading to tumor-mediated immune escape (78). Fas participates in the regulation of cell death, plays a key role in immune homeostasis and immune surveillance (79). PIK3CA, a most frequently mutated gene, could activate multiple genetic programs and influence intratumoral heterogeneity which its wild types may have favorable immunotherapy outcomes for patients with breast cancer (80). In a word, our findings suggest that these immune checkpoint genes may provide a favorable immune environment for the progression of EMs. However, we must admit that, although our study indicates that these genes may play a role in the development of different stages of EMs, there are currently limited reports on their specific functions in EMs thus whether these genes are indeed associated with the stage of the disease still requires further confirmation through future research. These findings suggest that ferroptosis is closely related to immune microenvironment during the progression of EMs and further provides the new insights for understanding the interaction between the both.

There are several limitations that must be noted. First, further functional experiments are warranted to verify the role and mechanisms of the hub FRGs in EMs. Second, *in vitro* and *in vivo* experiments are needed to confirm the interaction between ferroptosis and immune cells in EMs. Subsequently, we will undertake additional experiments to confirm the findings of this study.

6 Conclusion

In summary, our study has revealed that autophagy, mTOR, oxidative stress, and FOXO signaling pathway may play a crucial role in the process of ferroptosis in EMs and provided the evidence that ferroptosis could be involved in reshaping the immune microenvironment in EMs. Additionally, we have also identified 10 key FRGs and constructed their regulatory network, which will enhance our understanding of the role of ferroptosis in EMs. Taken together, our study provides valuable information for understanding ferroptosis and immunity of EMs, and lays a theoretical foundation for subsequent research.

Data availability statement

The original contributions presented in the study are included in the article Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Fourth Affiliated Hospital of Hebei Medical University (No:2021KY016). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LL: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. FH: Data curation, Software, Writing – original draft. ND: Formal analysis, Writing – original draft. YL: Methodology, Writing – review & editing. AD: Software, Visualization, Writing – original draft. SK: Conceptualization, Writing – review & editing. BL: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1507083/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The expression levels of hub FRGs in the GSE25628 databases. **(A–G)** The expression box plots of hub FRGs in GSE25628. **(F)** Two ferroptosis subclusters were identified by consensus clustering analysis of EMs patients in GSE25628.

SUPPLEMENTARY TABLE 1
The correlation of the expression of BECN1 and clinicopathological features in endometriosis patients.

SUPPLEMENTARY TABLE 2
The correlation of the expression of EIF2AK4 and clinicopathological features in endometriosis patients.

SUPPLEMENTARY TABLE 3
The correlation of the expression of CFL1 and clinicopathological features in endometriosis patients.

SUPPLEMENTARY TABLE 4
The correlation of the expression of CISD3 and clinicopathological features in endometriosis patients.

SUPPLEMENTARY TABLE 5
The correlation of the expression of CHMP6 and clinicopathological features in endometriosis patients.

SUPPLEMENTARY TABLE 6
The correlation of the expression of IREB2 and clinicopathological features in endometriosis patients.

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Broadening horizons: research on ferroptosis in lung cancer and its potential therapeutic targets

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Ferroptosis is a novel form of cell death distinct from traditional mechanisms, characterized by the accumulation of iron ions and the production of lipid peroxides. It not only affects the survival of tumor cells but is also closely linked to changes in the tumor microenvironment. Lung cancer is one of the leading malignancies worldwide in terms of incidence and mortality, and its complex biological mechanisms and resistance make treatment challenging. Recent studies have shown that ferroptosis plays a key role in the onset and progression of lung cancer, with its intricate regulatory mechanisms influencing tumor development and response to therapy. As research into ferroptosis deepens, related molecular pathways, such as glutamate metabolism, iron metabolism, and antioxidant defense, have been gradually revealed. However, in clinical practice, ferroptosis-based therapeutic strategies for lung cancer are still in their early stages. Challenges remain, including the incomplete understanding of the specific mechanisms of ferroptosis, insufficient research on related regulatory factors, and limited insight into the interactions within the tumor microenvironment. Therefore, effective modulation of ferroptosis to enhance lung cancer treatment remains an urgent issue. This review summarizes the biological mechanisms of ferroptosis, analyzes the regulatory factors of ferroptosis in lung cancer cells and their interaction with the tumor microenvironment, and further explores potential therapeutic strategies targeting ferroptosis. By synthesizing the latest research, this paper aims to provide new perspectives and directions for lung cancer treatment, with the goal of advancing clinical applications.

KEYWORDS

lung cancer, ferroptosis, cell death mechanisms, tumor microenvironment, therapeutic strategies, molecular pathways

1 Introduction

Lung cancer is one of the most common cancers worldwide and has an extremely high mortality rate (1). According to global cancer statistics, the annual death toll from lung cancer has surpassed 2 million, making it one of the leading causes of cancer-related deaths (2). In some regions, the incidence and mortality rates of lung cancer continue to rise year

by year, posing a significant public health challenge (3–6). The high mortality rate of lung cancer is primarily attributed to factors such as the lack of obvious early symptoms, late diagnosis, and treatment difficulties. Additionally, smoking, air pollution, occupational exposure, and other factors are considered major risk factors for lung cancer, with their impact varying significantly across different regions (7).

Traditional treatment methods for lung cancer mainly include surgery, radiotherapy, and chemotherapy. However, these approaches have significant limitations when treating advanced-stage lung cancer, especially small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (8). Surgical treatment is effective for early-stage cases, but in patients with locally advanced or metastatic lung cancer, chemotherapy and radiotherapy often show limited effectiveness and are accompanied by high toxic side effects (9, 10). Moreover, due to tumor heterogeneity and drug resistance, many patients still face the risk of recurrence and metastasis after receiving conventional treatments (11). Despite advancements in early screening and treatment methods for lung cancer in recent years, the prognosis remains poor, with a five-year survival rate of less than 20% (12). Therefore, there is an urgent need to explore new treatment strategies to improve the survival rate and quality of life for lung cancer patients. A comparison of the advantages and disadvantages of various lung cancer treatment methods is shown in Table 1:

Iron plays a crucial role in cellular metabolism, as it is involved not only in oxygen transport and energy metabolism, but also in DNA synthesis and repair (13). However, excessive iron accumulation can lead to an increase in reactive oxygen species (ROS) within cells, triggering oxidative stress that damages cellular DNA and other macromolecules, thereby promoting tumor initiation and progression (14, 15). In recent years, increasing evidence has shown that dysregulation of iron metabolism is closely associated with the development of various cancers, including lung cancer (16). Iron is not only a key element required for tumor cell growth but also a critical regulator of cell death. In particular, in lung cancer, abnormal iron metabolism may represent an important feature of the tumor microenvironment, influencing tumor cell growth and metastasis.

In this context, ferroptosis, a newly discovered form of programmed cell death, has gradually gained attention from researchers. Ferroptosis is a form of cell death driven by the accumulation of iron-dependent lipid peroxides, and it differs from traditional forms of cell death such as apoptosis and necrosis (17, 18). For instance, cells undergoing ferroptosis typically exhibit membrane rupture and organelle swelling, changes not typically observed in apoptosis. Ferroptosis was initially identified in tumor research, but more recent studies have indicated that it also plays a significant role in various neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, as well as other pathological conditions. The mechanism of ferroptosis involves multiple biological processes, including iron metabolism, lipid metabolism, and antioxidant responses (19, 20). Importantly, ferroptosis plays a critical role in the tumor microenvironment, and inducing ferroptosis may offer novel therapeutic strategies for cancer treatment (21). By modulating iron metabolism and lipid peroxidation, ferroptosis can be induced in tumor cells, thereby inhibiting tumor growth and metastasis, and overcoming resistance to traditional therapies (22, 23).

In lung cancer, the expression profiles of GPX4 (glutathione peroxidase 4) and SLC7A11 (solute carrier family 7 member 11) are closely associated with patient prognosis. Studies have shown that high expression of GPX4 in lung adenocarcinoma (LUAD) is linked to tumor aggressiveness and drug resistance, with its expression level negatively correlated with patient survival. Similarly, high

TABLE 1 The advantages and disadvantages of current treatments for lung cancer.

Therapies	Advantages	Disadvantages
Surgical excision	1. Directly removing the tumor may lead to a cure. 2. Significant efficacy for early-stage lung cancer.	1. The trauma is significant, and the recovery time is long. 2. It may cause complications, such as infection and bleeding. 3. The range of resection is limited and is suitable for some early-stage lung cancers. 4. Some patients are not suitable for surgery due to their physical condition.
Radiation therapy	1. Good control effect on local tumors. 2. Suitable for some patients who cannot undergo surgery. 3. Combined with chemotherapy can improve efficacy.	1. May cause side effects such as radioactive pneumonia and esophagitis. 2. Long-term radiotherapy may lead to skin damage, fatigue, etc. 3. Radiotherapy equipment and technology have high requirements.
Chemotherapy	1. Inhibitory effect on tumor cells throughout the body. 2. Suitable for advanced lung cancer and adjuvant therapy after surgery. 3. Combined with radiotherapy can enhance efficacy.	1. The side effects are significant, such as nausea, vomiting, and hair loss. 2. The issue of drug resistance may affect efficacy. 3. Chemotherapy drugs are expensive, placing a heavy financial burden on patients.
Targeted therapy	1. Highly targeted with relatively minor side effects. 2. Long-lasting efficacy and higher quality of life for patients. 3. Combining with chemotherapy can enhance efficacy.	1. Only applicable to lung cancer patients with specific gene mutations. 2. The drug is expensive, placing a heavy financial burden on patients. 3. There may be issues with drug resistance.
Immunotherapy	1. Significant efficacy for patients with advanced lung cancer. 2. Significantly improved patients' survival rates and quality of life. 3. Combined with chemotherapy and radiotherapy, it can enhance efficacy.	1. Possible side effects such as autoimmune reactions may occur. 2. Treatment costs are high, placing a heavy financial burden on patients. 3. Efficacy and safety vary due to individual differences.

expression of SLC7A11 is considered a marker of poor prognosis in lung cancer patients. Specifically, overexpression of SLC7A11 is associated with tumor stage, lymph node metastasis, and chemotherapy resistance. Furthermore, the expression patterns of ferroptosis-related genes can serve as biomarkers to predict survival and therapeutic response in lung cancer patients. For example, one study developed a prognostic model based on ferroptosis-related genes using RNA-seq data from NSCLC patients, revealing that patients in the high-risk group had significantly lower overall survival compared to those in the low-risk group (24).

These findings indicate that ferroptosis-related genes not only play a pivotal role in the initiation and progression of lung cancer but also have potential clinical applications, providing a foundation for the development of personalized treatment strategies. Therefore, exploring the role of ferroptosis in lung cancer not only aids in understanding the biological characteristics of the disease but may also provide important insights for the development of novel therapeutic approaches.

2 The definition and mechanism of ferroptosis

2.1 Biological characteristics of ferroptosis

Ferroptosis is a novel form of programmed cell death that differs significantly from traditional mechanisms of cell death in its biological characteristics. The hallmark of ferroptosis is the accumulation of iron-dependent lipid peroxides, leading to cell membrane rupture and cell death. Additionally, the morphological features of ferroptosis cells differ from other forms of cell death, typically exhibiting cell shrinkage, disruption of cell membrane integrity, and the formation of intracellular lipid vesicles (25, 26). Ferroptosis is closely associated with excessive intracellular iron accumulation and the generation of ROS, which together lead to lipid peroxidation and cellular damage. Research has shown that ferroptosis not only plays a crucial role in cancer cells but is also closely linked to the pathogenesis of various diseases, including neurodegenerative diseases, cardiovascular diseases, and kidney disorders (27–32).

The regulation of ferroptosis involves multiple molecular pathways and signaling cascades. Key molecules, such as GPX4 and SLC7A11, are central to ferroptosis regulation (Figure 1). GPX4 plays a critical protective role during ferroptosis, and its loss of function increases the cell's susceptibility to ferroptosis. By catalyzing the reduction of lipid peroxides, GPX4 protects cells from oxidative damage and is therefore considered an inhibitor of ferroptosis. On the other hand, SLC7A11 is responsible for the uptake of cysteine, which is essential for glutathione synthesis, thereby enhancing the cell's resistance to oxidative stress and suppressing ferroptosis.

In lung cancer, abnormal expression of ferroptosis-related molecules is considered closely linked to tumor initiation and progression. The expression levels of GPX4 and SLC7A11 are significantly upregulated in lung cancer cells, and this upregulation correlates with the malignancy and prognosis of the tumor, making them potential therapeutic targets (33, 34). Studies have shown that high expression of SLC7A11 is closely associated with the proliferation and survival of lung cancer cells, while the expression of GPX4 is linked to chemo resistance (35). Additionally, other ferroptosis-related molecules, such as NCOA4 (nuclear receptor coactivator 4) and TXNIP (thioredoxin-interacting protein), also play important roles in ferroptosis in lung cancer (36). Therefore, targeted therapeutic strategies aimed at these molecules may offer new treatment options for lung cancer patients, particularly in the management of drug-resistant tumors (37). Furthermore, ferroptosis inducers and inhibitors affect its occurrence through different signaling pathways, further highlighting its complex biological characteristics (38).

The study of ferroptosis provides new insights into the mechanisms of cell death and its role in disease, especially in the development of therapeutic strategies for cancer and neurodegenerative disorders. Thus, a deeper understanding of the biological features and underlying mechanisms of ferroptosis is crucial for developing targeted treatments for related diseases.

2.2 Molecular mechanisms of ferroptosis

The molecular mechanisms of ferroptosis primarily involve iron metabolism, lipid peroxidation, and related signaling pathways (Figure 1). Excessive iron accumulation in cells can generate ROS through the Fenton reaction, leading to lipid peroxidation (39). Lipid peroxidation is one of the core events in ferroptosis, as excessive ROS generation oxidizes polyunsaturated fatty acids (PUFAs) in cell membranes, forming lipid peroxides that further trigger cell death (40–42). Studies have shown that ferroptosis is closely related to the depletion of glutathione (GSH), an important antioxidant. A lack of GSH reduces the cell's ability to resist oxidative stress, thereby promoting ferroptosis (43, 44). Furthermore, GSH depletion is often associated with iron overload, a state that predisposes cells to ferroptosis, which has been validated in various diseases, such as acute kidney injury and neurodegenerative diseases (45).

GPX4 is a critical regulator in preventing ferroptosis, as it inhibits ferroptosis by catalyzing the reduction of lipid peroxides (Figure 1). Therefore, dysfunction of GPX4 leads to increased ferroptosis, while iron deficiency or failure of related antioxidant systems can exacerbate this process (46, 47). Additionally, ferroptosis is also associated with the activation of several signaling pathways, with the Nrf2 pathway playing a key role in regulating the cellular antioxidant capacity (48). Recent research has increasingly highlighted the role of non-coding RNAs (such as miRNAs and lncRNAs) in the regulation of ferroptosis. These molecules can influence the process of ferroptosis by modulating the expression of related genes (49–52). In summary, the molecular mechanisms of ferroptosis are complex and involve interactions among multiple biological pathways (Figure 1). By modulating lipid metabolism and glutathione levels, new strategies for treating related diseases may emerge. Research in this field is continuously advancing, providing a theoretical foundation for clinical applications.

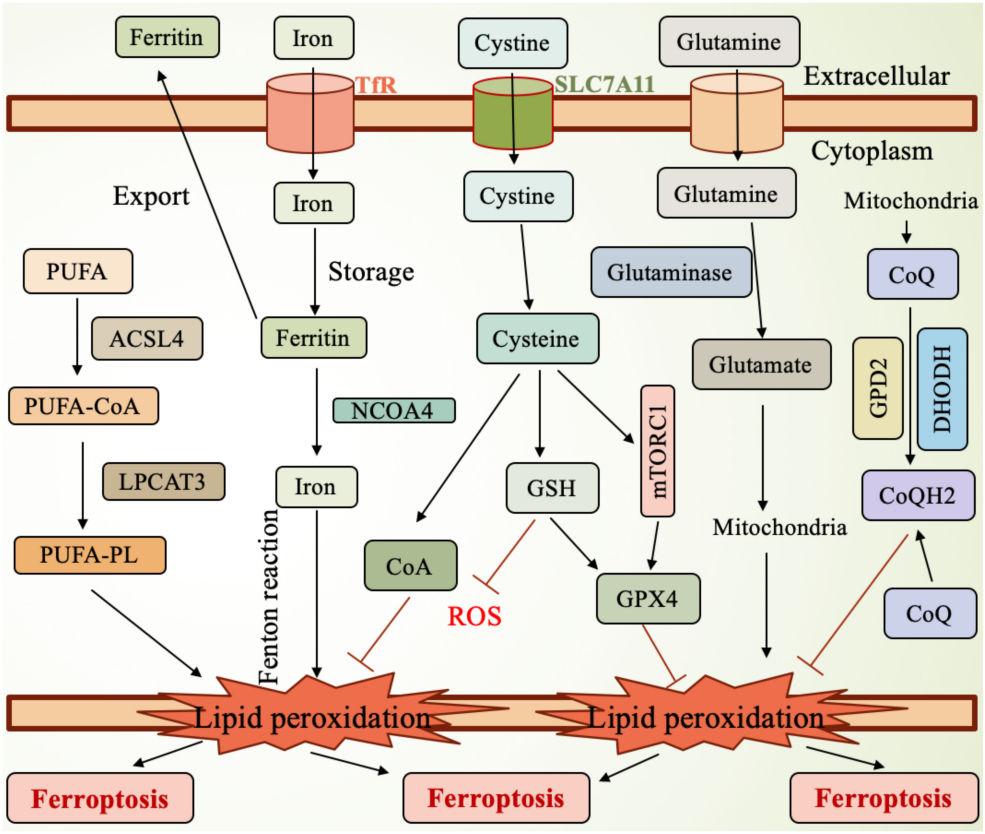

FIGURE 1
Mechanism of ferroptosis. SLC7A11, the cystine transporter solute carrier family member 11; GSH, glutathione; ROS, reactive oxygen species; GPX4, glutathione peroxidase 4; mTORC1, mammalian target of rapamycin complex 1; GPD2, glycerol-3-phosphate dehydrogenase 2; DHODH, dihydroorotate dehydrogenase; CoQH2, ubiquinol; TfR, transferrin receptor; NCOA4, nuclear receptor coactivator 4; PUFA, polyunsaturated fatty acid; ACSL4, Acyl-CoA synthetase long-chain family member 4; LPCAT3, lysophosphatidylcholine acyltransferase 3; PUFA-PL, PUFA-containing phospholipids.

2.3 The relationship between iron metabolism and oxidative stress

Oxidative stress refers to the imbalance in the cellular environment caused by excessive ROS production and insufficient antioxidant capacity, leading to cell damage and death (Figure 1). There is a close interplay between iron metabolism and oxidative stress. Iron, an essential trace element, participates in various biochemical reactions within cells, but excess iron can promote ROS generation, triggering oxidative stress (53, 54). Oxidative stress not only directly damages cell membranes and organelles but also activates several signaling pathways that further exacerbate iron accumulation and cellular damage. In diseases affecting organs such as the kidneys and liver, abnormal iron metabolism is closely related to oxidative stress, and together they promote cellular damage and dysfunction (27, 55). For instance, in conditions of iron overload, increased ROS within cells can further amplify iron accumulation, creating a vicious cycle that ultimately leads to ferroptosis (56, 57). Moreover, oxidative stress can also influence the expression of proteins involved in iron metabolism, creating a feedback loop that accelerates cell death under the combined pressures of oxidative stress and iron metabolism imbalance (58). Understanding the relationship between iron metabolism and oxidative stress is crucial for developing new therapeutic strategies to address related diseases (Figure 1).

2.4 The role of ferroptosis in tumor immunity

Ferroptosis plays a complex role in tumor immunity. Recent studies have shown that ferroptosis not only serves as a form of tumor cell death but also influences immune cell functions within the tumor microenvironment. Ferroptosis can induce immunogenic cell death, and the released cellular contents (such as damage-associated molecular patterns, or DAMPs) can activate dendritic cells, thereby enhancing anti-tumor immune responses (59). However, inhibition of ferroptosis may allow tumor cells to escape immune surveillance, reducing the effectiveness of immunotherapy (60). Additionally, the combination of ferroptosis and immune checkpoint inhibition (ICI) therapy has shown potential synergistic effects, possibly improving the efficacy of cancer treatments (61). Studies suggest that ferroptosis can enhance the effects of immunotherapy by inducing immunogenic death of tumor cells and activating anti-tumor immune responses.

However, the oxidative byproducts released during ferroptosis may also inhibit immune cells in the tumor microenvironment, leading to immune tolerance, which in turn affects the outcome of immunotherapy (62). For example, tumor-associated macrophages can secrete certain metabolites that promote tumor cell resistance to ferroptosis, further influencing tumor progression and the effectiveness of immunotherapy (63). Therefore, in-depth research into the role of ferroptosis in tumor immunity not only helps us understand the mechanisms of immune escape in tumors but may also provide a theoretical foundation for developing new immunotherapeutic strategies.

3 The role of ferroptosis in lung cancer

3.1 Abnormal iron metabolism in lung cancer cells

Iron metabolism in lung cancer cells is notably dysregulated, and this dysregulation is closely linked to the occurrence of ferroptosis. Studies show that lung cancer cells typically exhibit excessive iron accumulation, which leads to an increase in ROS, triggering lipid peroxidation and ultimately resulting in ferroptosis. In lung cancer cells, the disruption of iron metabolism is characterized by upregulation of iron transport proteins such as transferrin (Tf) and hepcidin, while the expression of ferroportin, an iron export protein, is reduced (Figure 1). These alterations affect the cell's ability to uptake and store iron, thus promoting tumorigenesis and progression (64–66). Additionally, some studies have found abnormal expression of ferroptosis-related genes in lung cancer patients, suggesting that the alteration of iron metabolism could serve as a new therapeutic target for lung cancer treatment (67, 68). Therefore, regulating iron metabolism and enhancing ferroptosis to inhibit tumor growth may represent a novel therapeutic strategy for lung cancer.

Iron metabolism plays a crucial role in the onset and progression of lung cancer. Research indicates that dysregulated iron metabolism not only promotes tumor growth but is also closely linked to metastasis and drug resistance. The cellular state of iron (such as ferrous iron Fe^{2+} and ferric iron Fe^{3+}) exerts different effects on the growth and survival of lung cancer cells. For instance, studies have shown that the accumulation of ferrous iron inhibits the proliferation of lung cancer cells, whereas ferric iron has little impact on cell growth (69). Moreover, cancer-associated fibroblasts (CAFs) regulate the iron metabolism of lung cancer cells by secreting exosomes, which in turn affect the occurrence of ferroptosis. This process involves the interaction of long non-coding RNAs (such as ROR1-AS1) with genes related to iron metabolism. Disruption of iron metabolism not only accelerates lung cancer progression but may also contribute to treatment resistance. Therefore, further investigation into the interplay between iron metabolism and lung cancer progression could provide new strategies and targets for therapy. Overall, the interaction between ferroptosis and iron metabolism offers new insights into the onset and development of lung cancer, highlighting the importance of iron as a potential therapeutic target.

3.2 The relationship between ferroptosis and lung cancer cell proliferation

Ferroptosis plays a dual role in lung cancer cell proliferation. On one hand, the induction of ferroptosis can suppress lung cancer cell proliferation. Research suggests that by regulating iron metabolism and inducing ferroptosis, the growth and spread of lung cancer cells can be effectively inhibited. For example, ubiquitin-specific protease 35 (USP35), a deubiquitinase, when overexpressed in lung cancer cells, stabilizes ferroportin and prevents ferroptosis, thus promoting cell proliferation (70). In contrast, inhibiting USP35 expression leads to an increase in ferroptosis and a suppression of lung cancer cell proliferation. On the other hand, inhibition of ferroptosis may accelerate the proliferation of lung cancer cells, which is closely associated with the malignancy and chemotherapy resistance of the tumor (71). Additionally, long non-coding RNAs (lncRNAs) also play a crucial role in regulating ferroptosis. For example, the upregulation of LUCAT1 is associated with increased lung cancer cell proliferation, while its inhibition promotes ferroptosis. Therefore, modulating ferroptosis pathways may offer new therapeutic approaches for lung cancer, particularly in overcoming chemotherapy resistance, by inducing ferroptosis to enhance tumor cell sensitivity to treatment (71, 72).

3.3 The role of ferroptosis in lung cancer metastasis

Ferroptosis also plays an important role in the metastasis of lung cancer. Studies have found that ferroptosis can promote lung cancer metastasis by modulating the tumor microenvironment and affecting immune cell function. For example, the occurrence of ferroptosis may lead to the release of pro-inflammatory cytokines from tumor cells, which alters the tumor microenvironment and influences the metastatic capacity of the cancer (19). Some studies suggest that after ferroptosis, immune cells such as macrophages and neutrophils in the tumor microenvironment become activated and release pro-inflammatory factors, further promoting tumor growth and metastasis (71, 73). Moreover, ferroptosis may influence the epithelial-mesenchymal transition (EMT) process in tumor cells, thus enhancing the invasiveness and metastatic potential of lung cancer cells (74). Research indicates that inhibiting ferroptosis could aid in suppressing lung cancer cell metastasis. For instance, CAFs secrete lncRNAs (such as ROR1-AS1) in exosomes to inhibit ferroptosis in lung cancer cells, thereby promoting metastasis (75). Furthermore, the dysregulation of iron metabolism is closely associated with the metastatic ability of tumors. Iron accumulation not only promotes tumor cell proliferation but also enhances oxidative stress and induces inflammation, which can further promote metastasis (76). Therefore, therapeutic strategies targeting ferroptosis may help inhibit lung cancer metastasis and improve

patient prognosis. By modulating iron metabolism and inducing ferroptosis, new anti-metastasis therapies could be developed, offering better treatment options for lung cancer patients.

4 Regulatory factors of ferroptosis

4.1 Lipid peroxides and their metabolic enzymes

Ferroptosis is an iron-dependent form of cell death, characterized by the accumulation of lipid peroxides in the cell membrane. The generation of lipid peroxides is closely linked to intracellular iron levels, with excessive iron accumulation driving the production of ROS through the Fenton reaction, which, in turn, exacerbates lipid peroxidation (Figure 1). Studies have shown that the sensitivity to ferroptosis is regulated by various lipid metabolic enzymes, such as long-chain acyl-CoA synthetase 4 (ACSL4) and lipoxygenases (LOX) (Figure 1). These enzymes play critical roles at different stages of lipid peroxidation, influencing the cell's response to ferroptosis. For example, an increase in ACSL4 expression promotes the accumulation of polyunsaturated fatty acids (PUFAs), which enhances the sensitivity of cells to ferroptosis (Figure 1). The regulation of these metabolic enzymes not only affects cell survival but is also closely associated with the development of various diseases, including cancer and neurodegenerative disorders (77, 78). Furthermore, the presence of PUFAs in the cell membrane significantly impacts the occurrence of ferroptosis, as these PUFAs are highly susceptible to peroxidation, leading to membrane damage and cell death (39). Therefore, regulating the activity of lipid metabolic enzymes and maintaining the balance of lipid components are crucial strategies for modulating ferroptosis, providing new insights and targets for the treatment of related diseases (79–81).

4.2 The role of antioxidants in ferroptosis

Antioxidants play a crucial role in the regulation of ferroptosis. The occurrence of ferroptosis is often accompanied by the dysregulation of the intracellular antioxidant system, particularly the loss of function of GPX4, which significantly reduces the cell's ability to defend against lipid peroxidation (Figure 1). For example, in the tumor microenvironment, the imbalance of antioxidants can lead to the accumulation of iron and the increase of lipid peroxides, thereby inducing ferroptosis. Therefore, the role of antioxidants is highly dependent on the cellular environment and specific physiological and pathological conditions (82, 83). Research has shown that antioxidants such as vitamin E and other natural compounds can effectively inhibit the occurrence of ferroptosis by enhancing the cell's antioxidant capacity and mitigating the impact of lipid peroxidation. Additionally, nuclear factor erythroid 2-related factor 2 (Nrf2), a key transcription factor, can regulate the expression of antioxidant genes within the cell, thereby influencing the sensitivity to ferroptosis. Thus, utilizing antioxidants to boost the cell's antioxidant capacity may become a novel strategy for treating diseases associated with ferroptosis (84–86).

4.3 The impact of tumor microenvironment on ferroptosis

The tumor microenvironment is one of the key regulators of ferroptosis. The metabolic characteristics of tumor cells in this environment, such as hypoxia, acidity, and nutrient deprivation, can influence iron metabolism and the oxidative state of lipids, thereby modulating the sensitivity to ferroptosis (87). Studies have shown that the interactions between immune cells and tumor cells in the tumor microenvironment may affect tumor progression and metastasis by regulating ferroptosis pathways. For example, certain immune cells in the tumor microenvironment might promote ferroptosis in tumor cells or help them resist ferroptosis through the release of cytokines and metabolic byproducts. Moreover, tumor cells' ability to adapt to ferroptosis may contribute to resistance to anticancer therapies. Additionally, the high iron concentration and oxidative stress present in the tumor microenvironment can also promote the occurrence of ferroptosis, a mechanism that plays a crucial role in tumor progression and metastasis. Therefore, further research into the influence of the tumor microenvironment on ferroptosis will not only help to understand tumor biology but also provide a basis for the development of new therapeutic strategies (81, 88, 89).

5 Interaction between ferroptosis and other forms of cell death

5.1 Interaction between ferroptosis and apoptosis

Ferroptosis is a newly identified form of programmed cell death, primarily driven by iron-dependent lipid peroxidation, leading to cellular death. In contrast, apoptosis is executed through intracellular signaling pathways, particularly via the activation of caspases. Therefore, ferroptosis differs significantly from classical apoptosis. Studies have shown a complex interplay between ferroptosis and apoptosis. On one hand, in certain contexts, ferroptosis can induce apoptosis by promoting the generation of ROS within the cell. For instance, lncRNA LINC00618 has been found to promote both apoptosis and ferroptosis in leukemia cells, suggesting an interaction between these two cell death mechanisms (90). On the other hand, ferroptosis may inhibit apoptosis. For example, in some cancer cells, the activation of ferroptosis may suppress apoptotic pathways, thereby affecting tumor growth and metastasis (91). Furthermore, in some cases, ferroptosis may occur prior to apoptosis, resulting in cell death before apoptotic signals emerge (92). Thus, a deeper understanding of the interaction between ferroptosis and apoptosis not only helps unravel the complexity of cell death mechanisms but may also provide new avenues for cancer therapy (93).

5.2 Relationship between ferroptosis and necroptosis

Necroptosis is a regulated form of necrosis, typically triggered by intracellular death receptor signaling, and shares morphological and biochemical features with ferroptosis. Both processes involve rupture of the cell membrane and the release of cellular contents, triggering inflammatory responses (94). Research has shown that ferroptosis and necroptosis interact in certain contexts. For instance, iron overload can promote necroptosis, and the activation of necroptosis can exacerbate ferroptosis by promoting iron release (95). Excessive iron accumulation can not only induce ferroptosis but may also promote necroptotic cell death by affecting mitochondrial function and the integrity of the cell membrane (96). Additionally, certain inhibitors, such as KW-2449 and Necrostatin-1, have been found to suppress both cell death pathways simultaneously, suggesting that they may share common regulatory mechanisms. In pathological conditions like ischemia-reperfusion injury, ferroptosis and necroptosis work together in the cellular damage process, regulating the type and extent of cell death (97). Therefore, combined intervention targeting these two forms of cell death may offer new strategies for treating related diseases.

5.3 Interaction between ferroptosis and autophagy

Autophagy is a cellular self-degradation process that clears damaged organelles and proteins, maintaining intracellular homeostasis (Figure 2). Recent studies have revealed that autophagy plays a crucial role in promoting ferroptosis. By regulating intracellular iron levels and lipid metabolism, autophagy facilitates the occurrence of ferroptosis. For example, autophagy has been shown to increase the levels of free iron in cells by degrading ferritin, the iron storage protein, thereby accelerating lipid peroxidation and leading to ferroptosis (98). Additionally, oxidative stress is considered a key factor in autophagy-induced ferroptosis. Research indicates that ROS can activate autophagy, further promoting the onset of ferroptosis (99). Therefore, autophagy is not only an important regulatory factor in ferroptosis but also a protective mechanism in cells responding to iron overload and oxidative stress. Inhibition of autophagy significantly impacts ferroptosis. Studies have shown that when autophagy is suppressed, cells become less sensitive to ferroptosis. For example, in cells lacking autophagy-related genes, iron accumulation and lipid peroxidation levels are significantly reduced, thus protecting the cells from ferroptosis (98). Moreover, autophagy inhibitors like 3-methyladenine (3-MA) reduce cell sensitivity to ferroptosis inducers, suggesting that autophagy plays a critical role in regulating ferroptosis (100). In certain pathological conditions, such as atherosclerosis, there is a close interplay between autophagy inhibition and ferroptosis activation, where autophagy suppression alleviates ferroptosis-related cellular damage (101). Therefore, modulating autophagic activity may provide new therapeutic strategies for diseases associated with ferroptosis.

5.4 Interaction between ferroptosis and cuproptosis

Cuproptosis is a form of cell death induced by excessive copper, involving mitochondrial dysfunction and protein aggregation (102).

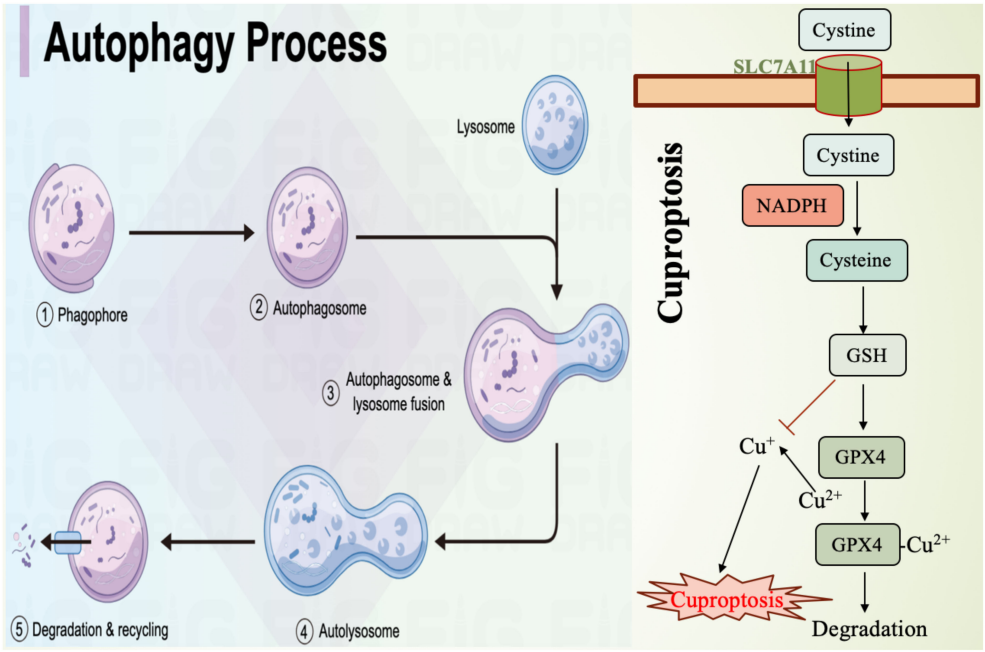

FIGURE 2
Mechanism diagram of autophagy and cuproptosis. SLC7A11, the cystine transporter solute carrier family member 11; GSH, glutathione; GPX4, glutathione peroxidase 4.

Studies have shown that there is a complex interaction between these two cell death mechanisms (Figure 2). For example, copper can enhance ferroptosis by promoting the degradation of GPX4 (103). Additionally, copper deficiency increases cellular sensitivity to ferroptosis, suggesting a metabolic crossover between iron and copper. This may occur through the regulation of antioxidant mechanisms and lipid metabolism, thereby influencing each other (104). Understanding this metabolic intersection is crucial for uncovering the regulatory mechanisms of cell death and its role in pathological conditions such as cancer and neurodegenerative diseases. The crosstalk between ferroptosis and cuproptosis involves multiple signaling pathways (Figure 2). Research has found that copper accumulation can influence the occurrence of ferroptosis by modulating iron metabolism pathways within mitochondria. This mechanism includes increasing intracellular iron levels by inhibiting enzymes associated with iron metabolism (105). Meanwhile, ferroptosis inducers such as erastin can enhance copper-dependent lipid acylation and protein aggregation, thereby promoting the occurrence of cuproptosis (102). Furthermore, the interaction between iron and copper may also affect cell death fate by regulating intracellular ROS levels. Excessive ROS accumulation causes cellular damage and may promote cell death through the activation of various signaling pathways (106). Therefore, in-depth exploration of the interactions between these signaling pathways could help develop combined therapeutic strategies targeting both ferroptosis and cuproptosis.

As emerging mechanisms of cell death, ferroptosis and cuproptosis show great potential in cancer therapy (Figure 2). By targeting and inducing either ferroptosis or cuproptosis, researchers hope to overcome tumor resistance to conventional treatments. For instance, combining ferroptosis inducers with chemotherapy or immunotherapy drugs could significantly enhance anti-tumor efficacy (107). Additionally, copper ion carriers and copper compounds are also being explored as new therapeutic strategies, especially for tumor types that are resistant to standard treatments (108). Moreover, the interaction between ferroptosis and cuproptosis provides new insights for cancer therapy. Studies suggest that targeting both cell death mechanisms simultaneously may yield a synergistic effect, improving treatment outcomes (109). Therefore, a deeper understanding of the mechanisms underlying ferroptosis and cuproptosis and their applications in cancer therapy may offer new treatment options for cancer patients.

The comparison table of ferroptosis and other cell death modes is shown in Table 2.

6 Therapeutic strategies targeting ferroptosis

6.1 Development of ferroptosis inducers

In recent years, the development of ferroptosis inducers has emerged as a novel strategy for cancer treatment. Numerous studies have shown that ferroptosis inducers can effectively induce ferroptosis in tumor cells by targeting iron metabolism and lipid peroxidation, thereby inhibiting tumor growth. In clinical applications, ferroptosis inducers have demonstrated the potential to overcome chemotherapy resistance in certain cancers (111, 112). For example, small molecules such as Erastin, RSL3, and FIN56 have been proven to induce ferroptosis by inhibiting GPX4, offering a new approach for cancer treatment (113, 114).

Furthermore, advances in nanotechnology have made it possible to target the delivery of ferroptosis inducers, enhancing the bioavailability and therapeutic efficacy of these drugs while reducing toxicity to normal cells (115, 116). Researchers are exploring the use of nanocarriers to improve the specificity and effectiveness of ferroptosis inducers, thus achieving precise targeting of tumor cells (117). Additionally, strategies targeting iron-sulfur clusters (ISCs) have been proposed to induce ferroptosis in cancer cells. By modulating intracellular iron metabolism and lipid peroxidation, ferroptosis inducers are expected to address the issue of resistance in cancer therapies (118, 119). These advances offer new perspectives and possibilities for the clinical application of ferroptosis inducers. A comparison of various ferroptosis inducers is shown in Figure 3.

6.2 The potential of combined therapy

Combination therapy strategies have shown promising prospects in cancer treatment, particularly when ferroptosis inducers are used in conjunction with other therapeutic modalities. This is because inducing ferroptosis can enhance the sensitivity of tumor cells to other treatments while reducing immune suppression in the tumor microenvironment (120). For example, combining ferroptosis inducers with immunotherapy or targeted therapy has been shown to significantly improve treatment outcomes. Ferroptosis can overcome the resistance of cancer cells to conventional therapies, especially in highly heterogeneous tumors such as glioblastoma and melanoma (121, 122). Studies have found that ferroptosis inducers can enhance the antitumor activity of BRAF inhibitors and immune checkpoint inhibitors, thereby improving the prognosis of patients with malignant melanoma (123).

Radiotherapy combined with ferroptosis inducers has also demonstrated enhanced efficacy, as radiotherapy promotes ferroptosis by increasing intracellular iron accumulation (124). Specifically, radiotherapy induces ROS and activates lipid metabolism enzymes (such as ACSL4), which in turn promote ferroptosis. Moreover, the induction of ferroptosis can enhance the effects of radiotherapy, increasing tumor cell sensitivity and improving treatment outcomes. For instance, certain ferroptosis inducers, when used alongside radiotherapy, significantly enhance the antitumor effects of radiotherapy by increasing the accumulation of lipid peroxides inside cells (125, 126). Clinical studies have shown that combining ferroptosis inducers with radiotherapy can significantly inhibit tumor growth and overcome resistance to radiotherapy in some cancers (127–129).

In the context of chemotherapy, ferroptosis is also considered a novel strategy for overcoming chemotherapy resistance by enhancing the cytotoxic effects of chemotherapeutic agents. Research has shown that combining chemotherapy drugs like cisplatin with ferroptosis inducers can reverse tumor cell resistance, thereby improving the efficacy of chemotherapy (130, 131). This combination strategy not

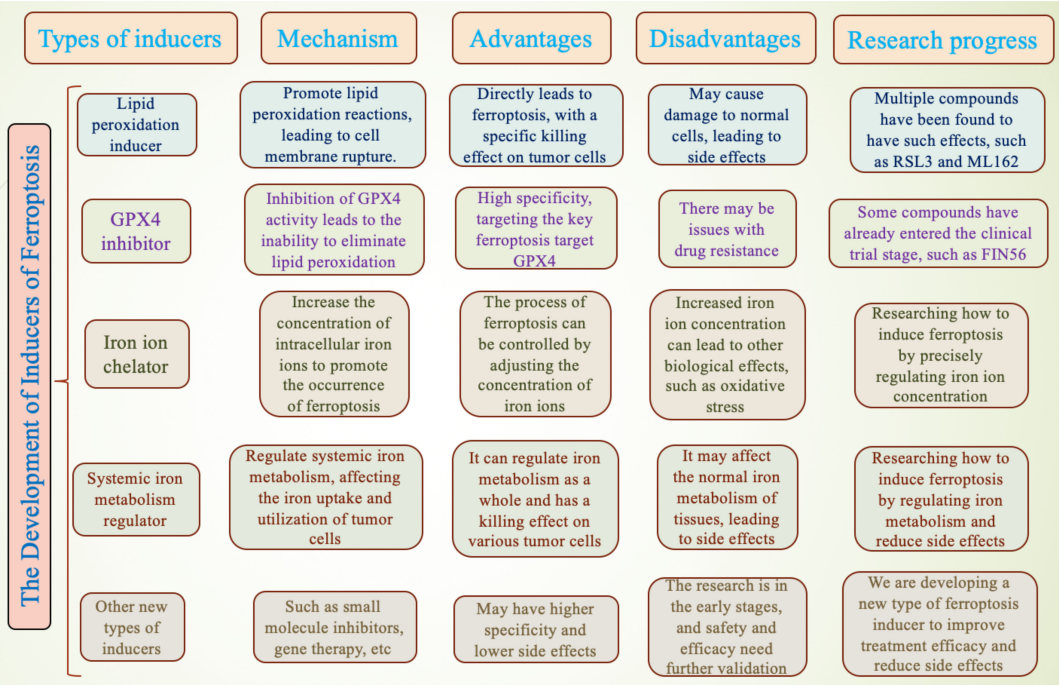

FIGURE 3
Comparison of various ferroptosis inducers.

TABLE 2 Comparison of ferroptosis and other cell death modes.

Forms of cell death	Description	Morphological characteristics	Biological significance	Regulatory mechanism	References
Ferroptosis	A type of programmed cell death caused by lipid peroxidation dependent on iron.	Cell membrane rupture, cell shrinkage, mitochondria shrinkage but membrane intact.	Eliminate damaged or abnormal cells to maintain tissue homeostasis.	Regulation of molecules such as GPX4, ACSL4, and FSP1.	(39)
Apoptosis	The process of cell self-destruction controlled by genes is divided into exogenous (death receptor-mediated) and endogenous (mitochondria-mediated) pathways.	The cell membrane remains intact, the cell shrinks, the nucleus fragments, forming apoptotic bodies.	Eliminate excess, damaged, or hazardous cells to maintain tissue homeostasis.	Regulation involving Caspase and the BCL-2 family.	(110)
Autophagy	The process by which cells degrade their own organelles and proteins through the formation of autophagosomes is divided into macroautophagy and microautophagy.	Autophagosome formation encapsulates and degrades organelles and proteins, releasing amino acids and other nutrients for cellular reuse.	Maintain cellular homeostasis to cope with conditions such as hunger and stress.	Regulation involving the ATG family.	(100)
Necroptosis	The way cells suddenly die due to physical, chemical, or biological damage.	Cell membrane rupture, cell swelling, release of cellular contents, leading to an inflammatory response.	The passive process after cell damage may lead to tissue injury and inflammatory response.	Regulation involving mechanisms such as cellular energy depletion and oxidative stress.	(96)
Cuproptosis	The process of programmed cell death caused by lipid peroxidation due to copper ions overload, resulting in the production of a large amount of reactive oxygen species.	Cell volume shrinkage, membrane rupture, organelle swelling, mitochondrial shape changes, while the nucleus remains relatively intact.	It can serve as a potential therapeutic target for anti-tumor treatment by inducing copper death in tumor cells to achieve therapeutic effects.	Regulation by antioxidant enzymes and lipid metabolism enzymes.	(39)

only increases the tumor cell death rate but may also improve patient prognosis, especially in the case of refractory cancers (132, 133). This approach not only overcomes the resistance associated with single therapies but also targets tumor cells through multiple mechanisms, thus enhancing overall treatment efficacy (134).

Studies have also shown that combining therapies with different mechanisms can effectively improve patient response rates and reduce the risk of tumor recurrence (135). However, the specific mechanisms, optimal dosages, and treatment regimens for combination therapy still require further clinical trials and basic research to determine, in order to achieve better therapeutic outcomes and minimize side effects. Therefore, research into the combination of ferroptosis inducers with traditional therapies offers new treatment options and improved prognosis for cancer patients (136).

6.3 Preclinical and clinical research status

Currently, therapeutic strategies targeting ferroptosis have made significant progress in preclinical and clinical research. Several studies have validated the antitumor effects of ferroptosis inducers in animal models, demonstrating their potential applications across different cancer types. For example, research on colorectal cancer has shown that ferroptosis inducers can effectively overcome chemotherapy resistance and significantly increase tumor cell death rates (137, 138). In terms of clinical research, although still in its early stages, some small-scale clinical trials are assessing the safety and efficacy of ferroptosis inducers. These studies lay the foundation for future clinical applications and highlight the potential of ferroptosis as a novel therapeutic target (139). Furthermore, these studies not only focus on the direct role of ferroptosis in cancer therapy but also explore its synergistic effects in radiotherapy and immunotherapy (140). While the current findings are promising, more clinical trials are needed to validate the practical effects and indications of ferroptosis-targeted therapy in different cancer types. As our understanding of ferroptosis mechanisms deepens and related drugs continue to be developed, ferroptosis-targeted therapies are expected to become a new option for cancer treatment in the near future.

Research has also explored the impact of modulating ferroptosis-related genes on the prognosis of lung cancer patients, with results indicating that specific ferroptosis-related genes could serve as prognostic biomarkers to predict patient survival. Additionally, clinical trials are exploring strategies to combine ferroptosis inducers with traditional chemotherapy drugs to overcome resistance in lung cancer. Although clinical applications targeting ferroptosis are still in their infancy, the available research results provide a foundation for future clinical trials, suggesting that modulation of ferroptosis could become a new direction for lung cancer treatment.

Ferroptosis, as a novel targeted therapy strategy, demonstrates potential in the treatment of lung cancer. Studies indicate that lung cancer cells are particularly sensitive to ferroptosis, especially in cases of chemotherapy resistance, making ferroptosis induction a promising therapeutic approach (141). For example, certain natural compounds have been found to enhance the effectiveness of chemotherapy for lung cancer by inducing ferroptosis, offering a new solution to chemotherapy resistance. Additionally, researchers are developing new ferroptosis inducers that not only exhibit good selectivity and bioavailability but can also be combined with other treatments such as radiotherapy and immunotherapy to improve therapeutic outcomes. In conclusion, ferroptosis, as a new direction in targeted therapy, holds great promise in the future treatment of lung cancer, particularly in overcoming resistance and enhancing treatment efficacy.

7 Conclusion and outlook

In this review, we explored the significance and mechanisms of ferroptosis in lung cancer, emphasizing its role in the tumor microenvironment and its impact on the survival and proliferation of lung cancer cells. Ferroptosis, an iron-dependent form of programmed cell death, has been shown to play a crucial role in various cancer types, particularly in lung cancer. Its importance is highlighted by the complex relationship between ferroptosis and tumor cell survival, proliferation, and drug resistance. Research suggests that ferroptosis not only influences tumor cell survival and proliferation by regulating intracellular iron homeostasis, lipid peroxidation, and antioxidant responses, but it may also alter the sensitivity of tumors to conventional therapies. These findings shed light on the complexity of ferroptosis and its feasibility as a therapeutic target in lung cancer.

Targeting ferroptosis offers a novel perspective for lung cancer treatment. Studies have shown that promoting ferroptosis can effectively suppress tumor cell growth while enhancing responses to chemotherapy and immunotherapy. The potential of this new therapeutic strategy is particularly evident in lung cancer cases resistant to existing treatments. Therefore, the development of drugs or therapies targeting iron metabolism could become a significant direction in lung cancer treatment, warranting further attention from clinical researchers and drug developers. Future research should focus on further elucidating the molecular mechanisms of ferroptosis and its role in the heterogeneity of lung cancer. Additionally, exploring the differences in ferroptosis characteristics between different types of lung cancer (e.g., small cell lung cancer and non-small cell lung cancer) and combining it with other therapeutic approaches will be an important part of future studies. Moreover, understanding the complex interactions between ferroptosis and the tumor microenvironment is crucial for identifying new biomarkers and therapeutic targets.

However, despite the clear potential of ferroptosis in lung cancer treatment, we must be cautious in interpreting existing research results. The factors influencing ferroptosis and the associated signaling pathways vary across different experimental conditions, cell models, and animal studies. There are also some discrepancies in the mechanisms of ferroptosis and its role in lung cancer across different studies. Therefore, it is essential to adopt more standardized experimental designs and conduct multi-center clinical trials to validate the true potential of ferroptosis in lung cancer therapy. Future research should focus on clarifying the molecular mechanisms of ferroptosis and how to effectively leverage this mechanism in clinical practice. Exploring the regulatory factors of

ferroptosis in different tumor microenvironments and assessing its combined use with other therapeutic strategies will be key areas of future research. Additionally, developing new ferroptosis inducers and screening biomarkers to predict patients' responses to ferroptosis-targeted therapies will provide important insights for personalized treatment. Future studies need to be more systematic and in-depth to unravel the complex mechanisms of ferroptosis in the occurrence, progression, and outcome of lung cancer. This will not only enhance our understanding of tumor biology but also lay the foundation for the development of new targeted therapeutic strategies.

Ferroptosis-related therapies hold great potential for clinical application but also face several challenges. Balancing the promotion and inhibition of ferroptosis, ensuring the selectivity and safety of these therapies, is a critical issue that researchers must address. Furthermore, individual differences and the complexity of the tumor microenvironment will affect the clinical efficacy of ferroptosis-based treatments. Therefore, future research should emphasize interdisciplinary collaboration, combining basic research with clinical trials, to facilitate the translation of ferroptosis-related therapies into clinical practice.

In conclusion, ferroptosis, as a novel strategy for lung cancer treatment, shows broad developmental prospects. By integrating multidisciplinary research efforts and delving deeper into the mechanisms of ferroptosis and its potential for clinical application, we hope to provide more effective treatment options for lung cancer patients.

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Intricating connections: the role of ferroptosis in systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a chronic inflammatory and autoimmune disease with multiple tissue damage. However, the pathology remains elusive, and effective treatments are lacking. Multiple types of programmed cell death (PCD) implicated in SLE progression have recently been identified. Although ferroptosis, an iron-dependent form of cell death, has numerous pathophysiological features similar to those of SLE, such as intracellular iron accumulation, mitochondrial dysfunction, lipid metabolism disorders and concentration of damage associated-molecular patterns (DAMPs), only a few reports have demonstrated that ferroptosis is involved in SLE progression and that the role of ferroptosis in SLE pathogenesis continues to be neglected. Therefore, this review elucidates the potential intricate relationship between SLE and ferroptosis to provide a reliable theoretical basis for further research on ferroptosis in the pathogenesis of SLE.

KEYWORDS

systemic lupus erythematosus (SLE), ferroptosis, interferons (IFNs), mitochondrial, damage-associated molecular patterns (DAMPs)

1 Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease characterized by inflammation and immune-mediated injury to various organs and systems. Approximately 3.4 million people are affected by SLE, with approximately 400,000 people diagnosed with SLE each year worldwide (1, 2). Despite the diversity of clinical manifestations

and the extremely complex pathogenesis in SLE, high levels of interferons (IFNs) and autoantibodies are common features (3), which also are primarily the result of cell death. In SLE, excessive cell death and impaired clearance machinery contribute to the continuous release of intracellular nucleic acids and their complexes into the extracellular space, thereby promoting responses by autoreactive B cells and IFN-responsive mechanisms (4). Interestingly, some prevalent features of SLE, including iron overload, mitochondrial dysfunction, lipid metabolism disorders and damage associated molecular pattern (DAMP) concentrations, are complexly associated with cell death, especially ferroptosis (5, 6).

Ferroptosis is an iron-dependent immunogenic form of cell death that is distinct from cell death mediated by perforin rupture (7). The occurrence of ferroptosis is attributed mainly to an imbalance in intracellular oxidation and antioxidant mechanisms, which triggers a cascade of hydrogen peroxide-lipid reactions and mediates the rupture of the cell membrane. Its mechanism mainly includes lipid peroxidation (mainly involving iron homeostasis and mitochondrial dysfunction) and antioxidant system disorders [mainly involving the cystine/glutamate transport system (xCT), glutathione (GSH)-glutathione peroxidase 4 (GPX4)] (5). Ferroptosis is induced by many factors, such as excessive DAMPs [IFN-α, IFN-γ, high-mobility group box 1 (HMGB1)] (8), ionizing radiation (9), the accumulation of iron, and increases in free radicals and oxidized lipids (10). Although the physiological role of ferroptosis is still unclear, its pathological role in a variety of acute and chronic diseases has been widely reported (11, 12), and it is potentially closely related to the pathogenesis of SLE (13, 14).

To date, the pathogenesis of SLE has not been classified. PCD has been shown outstanding role in SLE pathogenesis. Ferroptosis is a novel form of PCD, which exhibits several pathophysiological features reminiscent of those seen in SLE, including intracellular iron accumulation, mitochondrial dysfunction, lipid metabolism disorders, and regulation by DAMPs (Table 1). These similarities warrant further exploration of the potential interplay between ferroptosis and SLE pathogenesis. Although ferroptosis has been reported to play a role in SLE, the relationship between ferroptosis and SLE remains unclear. Therefore, this review discusses those parallel features between ferroptosis and SLE promote further research on ferroptosis in the pathogenesis of SLE.

2 Primary regulation in ferroptosis

Current evidence demonstrated intracellular oxidation primary related to some intracellular alterations, encompassing iron accumulation, mitochondrial dysfunction and lipid metabolism disorder (5). And antioxidant systems related to defend ferroptosis mainly including the xCT-GPX4, ferroptosis suppressor protein 1 (FSP1)-coenzyme Q10 (CoQ10), Guanosine triphosphate cyclohydrolase 1 (GCH1)-tetrahydrobiopterin (BH4) and dihydroorotate dehydrogenase (DHODH)-dihydroubiquione (CoQH2) systems (5, 25). Conceivably, biological processes related to the homeostasis of oxidation-reduction are peculiarly prone to regulate ferroptosis, such as lipogenesis (26, 27), autophagy (mainly including ferritinophagy, lipophagy and mitophagy) (28, 29), and the tricarboxylic acid (TCA) cycle (17). Moreover, plenty of and metabolic molecules are crucial for primary ferroptostic suppressors, such as: isopentenylation is required for the synthesis of selenoenzymes including GPX4 and ubiquinone (also known as coenzyme Q) is a major substrate of FSP1, both them is metabolites of mevalonate pathway (30). Additionally, multiple DAMPs can regulate ferroptosis, such as: IFNs can regulate solute carrier family 7a member 11 (SLC7A11, subunit of xCT) and GPX4 expression by activating JAK-STAT1 signaling (8); HMGB1 can induce ferroptosis by promoting autophagy and iron accumulation (31, 32); Mitochondrial DNA (mtDNA) performs ability to trigger ferroptosis by activating the cyclic GMP-AMP receptor stimulator of interferon genes (STING) (33).

3 Similar features in SLE and ferroptosis

3.1 Iron accumulation

Iron is one of the essential trace elements in the human body and is crucial for maintaining various biological functions, such as transporting oxygen, participating in the production of energy in the respiratory chain, and being involved in key biological reactions as a key component of various enzymes (34). However, iron accumulation can also mediate a series of pathophysiological

TABLE 1 Parallel features in SLE and ferroptosis.

Parallel features	Key points	
	SLE	Ferroptosis
Iron accumulation	Iron accumulation is pervasive in SLE kidney and pathogenic immune cells (15)	Iron overload is a vital trigger for ferroptosis (12)
Mitochondrial dysfunction	impaired mitochondria are central mediators of injury in different tissues and organs in SLE (16)	Mitochondria serves as key organelles for triggering ferroptosis (17)
Lipid metabolism disorders	Dyslipidemia and amplification of lipid peroxides are common features in SLE (18)	Lipid peroxides is executor of ferroptosis and some enzymes and intermediates of cholesterol metabolism are regulators for ferroptosis (19)
HMGB1 concentration	HMGB1 serve as a biomarker for SLE and potential aggravates the disease progression (20)	HMGB1 serves as the main DAMP of ferroptosis and can trigger ferroptosis in neighboring cells (21–23)
Regulation by IFNs	SLE is regarded as "IFNs signature" disease (3, 24)	IFN-I/II are key regulators of ferroptosis (8)

processes, such as reactive oxygen species (ROS) production, lipid peroxide production and mitochondrial dysfunction, thereby promoting disease progression, such as SLE (35). Iron accumulation has been detected in the kidneys of both SLE patients and lupus mice (36, 37), and multiple molecules related to iron metabolism [such as ferritin, transferrin, ceruloplasmin, and neutrophil gelatinase-associated lipid carrier protein (NGAL)] have been identified as biomarkers of SLE and/or LN, which are associated with disease activity and/or renal involvement (38–43). Additionally, the excessive intake of iron exacerbates SLE progression (44–46), whereas iron chelation can alleviate the disease (37, 47).

Kidney is major damaged organ in SLE and main organ attacked by iron overload because it is site of iron filtration and reabsorption. Over transferrin bound (TBI) and non-transferrin bound iron (NTBI) can overwhelm the heavy chain ferritin (FtH) capacity, which lead to release of labile iron and render proximal tubular epithelial cells (PTEC) susceptible to iron oxidant damage, especial in distal tubular epithelial cells (DTEC) with lack iron storage (light and heavy chain ferritin) and export protein (ferroportin) (15). Moreover, this damage can be worse under some pathological condition like LN with glomerular injury resulting in an increased leakage of TBI and NTBI (48). Additionally, SLE is a typical autoimmune disease, and the overactivation of $CD4^+$ T lymphocytes is a feature of SLE (28). In SLE, $CD4^+$ T cells are over differentiated into pathogenic Th1 and Th17 cells, whereas Treg cells are suppressed (49, 50). Interestingly, iron is crucial for regulating the activation, proliferation and differentiation of $CD4^+$ T lymphocytes (51). Some reports have demonstrated that the iron concentration in $CD4^+$ T cells is significantly greater in SLE patients than in healthy controls (52, 53). CD71 (also referred to as ferritin receptor TfR1, which mediates iron endocytosis) on the surface of $CD4^+$ T cells is essential for their activation and proliferation and promotes their differentiation into Th17 cells (54–56). Moreover, Th17 cells in SLE patients express high levels of CD71, which is positively correlated with disease activity (56). Similarly, follicular helper T (Tfh) cells, as a specific subset of $CD4^+$ T cells, contribute to the pathogenesis of SLE by promoting the maturation of germinal center B cells and the production of antibodies (57, 58). However, the accumulation of iron in $CD4^+$ T cells in SLE can promote their differentiation into Tfh cells and aggravate the progression of SLE (53). Moreover, Treg cells can restrict $CD4^+$ T cell overactivation and its dysfunction is involved in SLE pathogenesis (59–61). Gao XF et al. demonstrated iron deficiency could alleviate pristane-induced lupus progression by promoting Treg cell expansion (62). And Feng P et al. verified iron overload leaded to systemic autoimmune disorders by aggravating Treg cell death (63). These findings verify that iron overload is involved in the pathogenesis of SLE, but whether it occurs through ferroptosis is not clear.

Iron overload is also conducive to ferroptosis. Too much iron intake, too little iron excretion, or impaired iron metabolism can contribute to iron overload. Physiologically, there are several ways to reduce the level of intracellular iron when the level of intracellular iron is excessive: i. iron-responsive proteins/iron-responsive elements (IRPs/IREs) hamper the transfer of iron by disrupting the stability of transferrin receptor mRNA while promoting the transcription and synthesis of ferritin to enhance the binding of free iron (64–66); ii. increased expression of hepcidin promotes the expression of iron transfer protein (ferroportin), which promotes iron outward transport (67–69); and iii. ferritin is targeted to lysosomes by nuclear receptor coactivator 4 (NCOA4)-mediated autophagosomes for degradation (often referred to as ferritinophagy) (70, 71). Iron overload is a vital trigger for ferroptosis. i.Iron-mediated Fenton reactions are necessary for ferroptosis and can facilitate the production of phospholipid hydroperoxides (PLOOH, a biomarker of ferroptosis). ii.Key enzymes that trigger lipid peroxidation [lipoxygenases (LOXs) and cytochrome P450 oxidoreductases (PORs)] require iron catalysis. iii.Iron is required for numerous redox-based metabolic processes and is a major source of intracellular ROS (11, 34). iv.Iron chelating agents not only prevent ferroptosis but also reduce the production of lipid peroxides (72–75). Feng P et al. reported that iron overload in Treg cells can promote iron-dependent cell death (63). Liu Y et al. shown iron overload in the joint cavity of rheumatoid arthritis patients and *in vitro* can promote ferroptosis in macrophages (76). Additionally, Alli, A. A. et al. demonstrated iron sequestration within the proximal tubules promoted LN progression by exacerbating ferroptosis (77). This evidence demonstrated that iron accumulation can trigger ferroptosis not only in the extracellular space but also in the intracellular space.

Thus, iron overload is a shared feature of SLE and ferroptosis and multiple evidences showed iron overload was involved in disease progression by triggering ferroptosis (Figure 1). Although, numerous reports demonstrated iron overload can aggravate the progression of SLE, whether the effect is ascribed to promoting ferroptosis. Anyhow, preventing iron accumulation in tissues and cells by mediating the key molecules of iron metabolism (transferrin receptor, ferroportin, hepcidin, etc.) may be a novel direction for the treatment of SLE.

3.2 Mitochondrial dysfunction

Mitochondria are essential organelles for maintaining the normal physiological function of the cell and play a crucial role in physiological processes such as energy production, calcium homeostasis, and iron metabolism. It is also a key site for ROS elevation, self-nucleic acid antigen production, and the promotion of various forms of cell death (such as ferroptosis), which are closely related to a variety of diseases, such as SLE (26, 78, 79). Overactivation and immune intolerance of immune cells is the most pivotal link in the pathogenesis of SLE (28). The rapid proliferation and differentiation of immune cells require mitochondria to supply a large amount of energy. Oxidative phosphorylation (OXPHOS) of the electron transport chain (ETC) on the inner mitochondrial membrane is the process of ATP production, which is also accompanied by ROS production (80). Dysfunction of mitochondria limits ATP production but aggravates the release of ROS, which are closely related to immune disorders, autoantibody production, excessive cell death and ineffective clearance mechanisms (81). Previous studies have shown that mitochondria in $CD4^+$ T cells from SLE patients exhibit numerous abnormalities, such as large size (mitochondrial fusion), membrane

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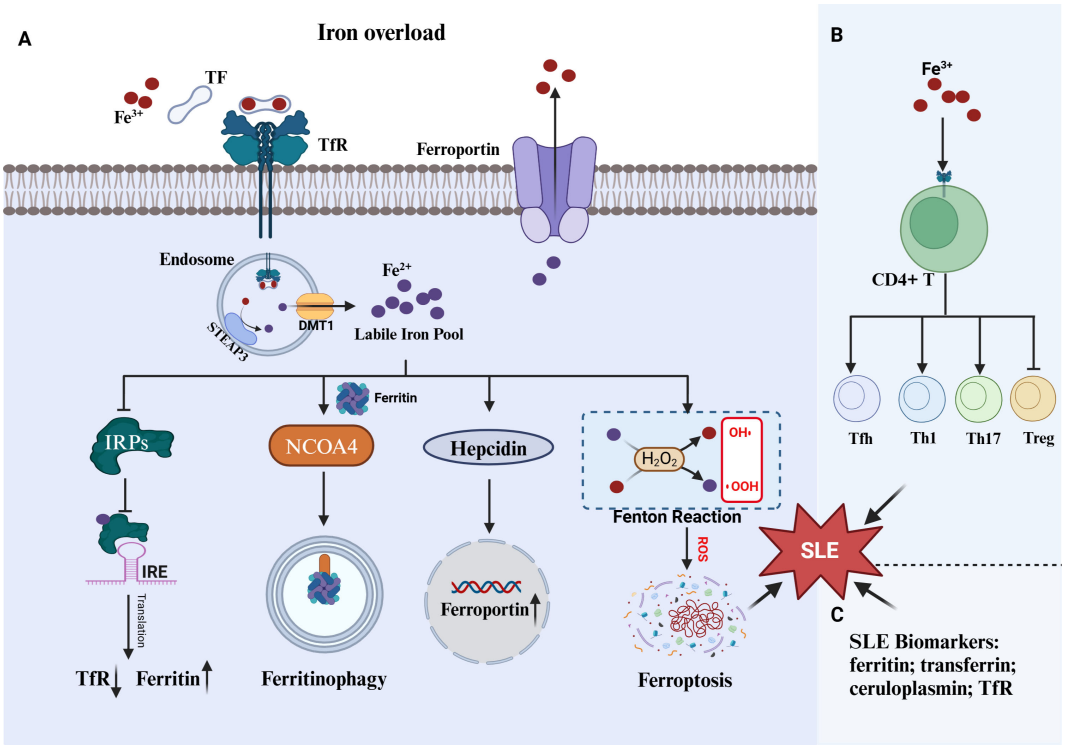

FIGURE 1
Iron overload in ferroptosis and SLE. **(A)** Three mechanism including IRPs/IRE system, ferritinophagy and hepcidin-ferroportin roadways against iron overload. The cell undergoes ferroptosis when those mechanism are impaired. **(B)** Iron overload promote $CD4^+T$ abnormal proliferation and differentiation, which can promote SLE progress. **(C)** Numerous serum protein closed to iron regulation were identified as SLE biomarkers.
TF, transferrin; TfR, transferrin receptor; IRPs, iron-responsive proteins; IREs, iron-responsive elements; STEAP3, six-transmembrane epithelial antigen of the prostate 3; DMT1, Divalent metal transporter 1; NOCA4, nuclear receptor coactivator 4.

hyperpolarization (elevated mitochondrial transmembrane potential), ATP depletion, fragility, increased mitochondrial ROS (mtROS) and decreased antioxidant (GSH) levels (82–84). Moreover, massive ROS production in SLE patients is the main contributor to mitochondrial dysfunction (85). Second, mitochondrial DNA (mtDNA) oxidized by ROS (ox-mtDNA) has been identified as an important DAMP. Under normal physiological conditions, it can be dissociated from mitochondrial transcription factor A (TFAM) and degraded in lysosomes. However, in SLE, the restriction of TFAM function promotes the continuous accumulation of Ox-mtDNA. In addition, mtDNA that is released into the cytoplasm can not only activate cyclic GMP-AMP synthase (cGAS) or Toll-like receptors 9 (TLR9) to promote the production of IFNs (86, 87) but also serve as an antigen to directly activate the immune system to exacerbate autoantibody production (81). Since the kidney is one of the most involved organs in lupus and iron consumption is prevalent in it, mitochondrial are particularly susceptible to oxidative attack in glomerular and tubular cells. Tian Y at al. Demonstrated the molecular mechanism of iron-induced injury in these cells was contributed to mtROS overproduction (88). Remarkably, recent evidence demonstrated impairment of mitochondrial degradation in glomerular and tubular cells was implicated in proteinuria and renal failure in LN (16, 89, 90). Moreover, both mtROS and impair mitochondrial degradation promote Ox-mtDNA accumulation (91). On the other side, Ox-mtDNA can exacerbate mtROS production in a vicious cycle, which further aggravate the disease progression.

Moreover, mitochondria serve as central hubs for multiple forms of cell death, such as apoptosis, necroptosis, and pyroptosis, and are also key organelles that trigger ferroptosis (5, 78, 92). It is still debated whether mitochondria are necessary for ferroptosis. For example, some cells become insensitive to ferroptosis-inducing agents after the removal of mitochondria, whereas others do not (93, 94). Moreover, it has also been shown that mitochondria are required for erastin-induced ferroptosis but not for RSL3-induced ferroptosis (17). However, deficiency of optic atrophy protein 1 (OPA1), which maintains mitochondrial homeostasis and function, leads to resistance to both erastin- and RSL3-induced ferroptosis (95). In ferroptosis, significant changes in mitochondrial function and morphology are observed, including a decrease in volume, increase in membrane density, and decrease in ridge and ATP production (7). The role of mitochondria in triggering ferroptosis is explained as follows: First, mitochondria are one of the main sources of ROS and the key site of energy production and iron metabolism (65, 96). Mitochondrial dysfunction not only reduces ATP production but also contributes ETC to leaking large amounts of ROS (such as $O_2^{\cdot-}$ and H_2O_2) and disturb iron homeostasis. H_2O_2 combines with the Fe^{2+}-mediated Fenton cascade and further exacerbates the massive release of ROS, which promotes lipid peroxidation (97). Depletion of mtROS can alleviate mitochondrial ferroptosis (98). Second, the tricarboxylic acid cycle (TCA) cycle is located in the mitochondria. The mitochondrial TCA cycle can increase ferroptosis sensitivity

(17). Glutamate metabolism is an important complementary part of the TCA cycle. In mitochondria, glutamine can be converted to glutamate and TCA intermediate metabolites (α-ketoglutarate, αKG), both of which can promote ferroptosis (99–101). Moreover, the inactive αKG dehydrogenase complex can prevent ferroptosis induced by cystine depletion (102). Additionally, ferroptosis has been identified as a form of autophagy-dependent cell death (103). And interesting, some reports demonstrated mtDNA could trigger autophagy-dependent ferroptosis by activating STING (33) and peroxisome proliferator activated receptor alpha (PPARα) (104). Moreover, mtDNA is prone to transform B- to Z-DNA structures under mtROS stress (105), which could promote ferroptosis by activating Z-DNA binding protein 1 (ZBP1) (106). These results suggest that mitochondrial dysfunction can promote ferroptosis.

In summary, in SLE patients, mtROS accumulation and mtDNA release may promote ferroptosis and subsequent release of intracellular antigens and DAMPs, thereby mediating the progression of SLE (Figure 2). Therefore, limiting mtROS production and mtDNA release to maintain mitochondrial function may be a promising treatment strategy for SLE.

3.3 Lipid metabolism disorder

Lipid metabolism is a major physiological process that includes catabolism [fatty acid oxidation (FAO)], anabolism (*de novo* lipogenesis) and storage [lipid droplets (LDs)], and lipid metabolism disorders are closely related to multiple pathological processes and diseases, including immune dysfunction and SLE (107). Dyslipidemia, a major type of lipid metabolic disruption, is most common in SLE patients (68%-100%) and is characterized primarily by increased plasma levels of very low-density lipoprotein (VLDL), triglyceride (TG) and total cholesterol (TC) but decreased high-density lipoprotein (HDL) levels (108). This disorder not only is closely related to SLE activity and organ involvement (including the cardiovascular system and kidney) but is also related to high mortality (109, 110). Among them, low HDL-cholesterol (HDL-c) levels are the most common sign of dyslipidemia in SLE patients (18, 111, 112). Under normal conditions, HDL performs anti-inflammatory and antioxidative functions despite its cholesterol efflux capacity. However, in SLE, it functions in an opposite manner with an uncertain mechanism (113, 114). Interestingly, a previous study demonstrated that limiting cholesterol flux could aggravate the IFN and ISG response in a STING-dependent manner in macrophages (115), and its precursor concentration, 7-dehydrocholesterol (7-DHC), could also significantly increase I-IFN production via the phosphatidylinositol 3-kinase (PI3K)-protein kinase B 3 (AKT3)-interferon regulatory factor 3 (IRF3) pathway (116). However, 7-DHC, as a precursor to form Vitamin D3 in the skin by solar ultraviolet B radiation, is the main source of Vitamin D. The deficiency Vitamin D is prevailed in SLE population and its supplementation seems to ameliorated the diseases (117), which may attribute that adequate level of Vitamin D prevents the synthesis of 7-DHC. Additionally, the metabolite of cholesterol, 7α, 25-dihydroxycholesterol (7α, 25-OHC), is dramatically elevated in the plasma of SLE patients and binds to the G protein-coupled receptor (GPCR) Epstein–Barr virus-induced gene 2 (EBI2) in macrophages to alleviate the IFN and ISG response for protecting against SLE progress (118).

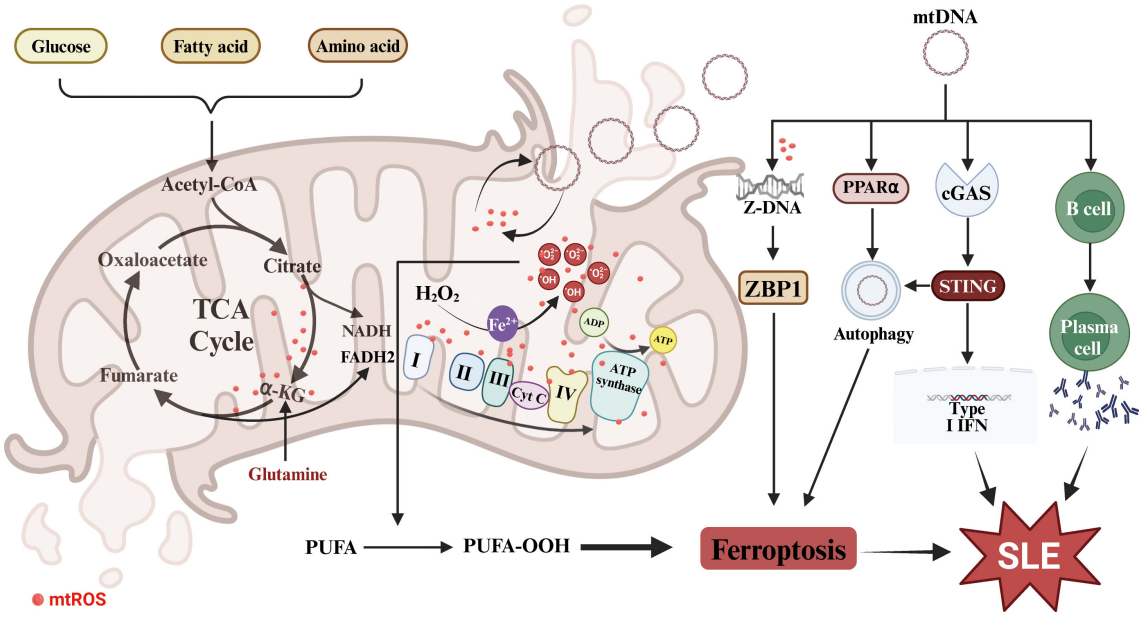

FIGURE 2
Mitochondrial dysfunction in SLE and ferroptosis. The dysfunction promotes mtROS accumulation and mtDNA release, which triggers cell ferroptosis and enhances the production of autoantibodies and IFNs. PUFA, polyunsaturated fatty acids; TCA, the tricarboxylic acid cycle; α-KG, α-ketoglutarate; mtROS, mitochondrial reactive oxygen species; mtDNA, mitochondrial DNA; ZBP1, Z-DNA binding protein 1; PPARα, peroxisome proliferator activated receptor alpha.

Consequently, homeostasis lipidemia, especially cholesterol metabolism, is crucial for SLE pathogenesis.

Additionally, lipids are highly vulnerable to oxidation, and many lipid peroxides, including oxidized HDL (ox-HDL), ox-LDL, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), have been identified as potential biomarkers for SLE and are related to disease activity (111, 118–121). Because oxidative stress is involved in the pathogenesis of SLE, aberrant oxylipins have been revealed in SLE patients by lipidomic analysis (122, 123). Oxylipin is a series of oxidative metabolites produced by polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), linoleic acid (LA), and alpha-linolenic acid (ALA), that undergo non-enzymatic or specific enzymatic [lipoxygenase (LOX) and cytochrome P450 (CYP450)] oxidations. Multiple oxylipins, such as 12-hydroxy-heptecotrienoic acid (12-HHTrE) and prostaglandin E1 (PGE1), are critical for SLE pathogenesis and have been identified as biomarkers for SLE pathogenesis (124, 125). Interestingly, the concentration of serum PUFAs is significantly decreased in SLE patients, which demonstrates that the antioxidant system is impaired and that lipid peroxidation is prevalent (107, 124). Moreover, lysophospholipids containing PUFA chains and lipid peroxidation substances (i.e., 4-hydroxyalkenals) strongly accumulate in the peripheral blood mononuclear cells of SLE patients (126). In summary, lipid metabolic disorder is one of the outstanding features in SLE, especially pertaining to oxidized-lipid metabolism.

Moreover, lipid metabolic disorder is essential for ferroptosis, because the executor of this cell death is lipid peroxidation, which triggers ion flux disturbances and ultimately plasma membrane permeabilization and fragmentation (127–129). Lipid metabolism is crucial for the initiation, propagation and termination of lipid peroxidation (130). PUFAs [especially AA (C20:4) and LA (C18:2)] are regarded as primary inducers of ferroptosis because whose bis-allylic hydrogens have relatively low bond dissociation energies and are more sensitive to lipid peroxidation (19, 131). In brief, the contributions of PUFAs to ferroptosis can be divided into two main categories: extension into the membrane and peroxidation. First, PUFAs are taken into the cell or converted by other lipids inside the cell. Then, extension can occur into membrane phospholipids (PUFA-PLs) via acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), which are very vulnerable to peroxidation. Second, PUFA-PLs are oxidated by lipoxygenases or via nonenzymatic autoxidation reactions [such as the Fenton reaction). The lipid peroxidation chain reaction is subsequently maintained by lipid radicals (such as the phospholipid peroxyl radical (PLOO·)] derived from lipid peroxidation. When antioxidative agents, including GPX4 and coenzyme Q10 (CoQ10), are depleted, the cell ultimately undergoes ferroptosis (132).

Although the direct relationship between SLE and ferroptosis related to lipid metabolism has not been reported, there are numerous potential connections as follows: i.The dyslipidemia feature of SLE further promotes cholesterol metabolic dysfunction, which can affect ferroptosis. The intermediate, isopentenyl pyrophosphate (IPP), is required for the synthesis of GPX4 (133, 134) and CoQ10 (135–137), which are known to prevent ferroptosis. Similarly, squalene (138, 139) and 7-DHC (140, 141) effectively prevent lipids from autoxidation and subsequent fragmentation (142). Furthermore, some enzymes related to cholesterol metabolism serve as potential suppressors of ferroptosis, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) in mitochondria (143) and sterol C5-desaturase (SCD5), whereas 7-dehydrocholesterol reductase (DHCR7) (140) and squalene monooxygenase (SQLE) (138) function as pro-ferroptostic molecules (3). Elevation of the cellular cholesterol content specifically restrains elevated lipid peroxidation and reduces susceptibility to ferroptosis by decreasing membrane fluidity (138). ii.PUFA, an inducer of ferroptosis, is significantly decreased in the extracellular space but increased in the intracellular space in SLE patients (Figure 3). Moreover, the levels of lipid peroxidation substances, especially MDA and 4-HNE (ferroptostic biomarkers), are markedly increased in SLE patients. Moreover, elevated MDA was related with multiple lupus manifestations (such as vasculitis, musculoskeletal, cutaneous and nephritis) (144–146). And also, the serum levels of anti-MDA IgG antibody positively were related with disease activity, active nephritis, inflammatory indictors and the consumption of complement factors (147). This phenomenon may be explained by the fact that PUFAs are taken into cells to be involved in ferroptosis in SLE patients.

3.4 Function of DAMPs

3.4.1 HMGB1

DAMPs are endogenous molecules that are released into the extracellular space under some pathologic conditions, mainly during cell death. Although numerous DAMPs released from cell death or necrosis, including TNF-α (148, 149), IL-1β, IL-18 (150, 151) and IL-33 (152), have been identified as biomarkers for SLE, their potential role in SLE has not been well defined.

However, extracellular HMGB1, a ubiquitous DAMP, belongs to the HMG family and performs well in SLE pathogenesis. HMGB1 was originally thought to be located only in the nucleus as a DNA chaperone and participated in regulating chromatin structure and function; however, recently, it was found that it can also be located in the cytoplasm and cell membrane. In addition, HMGB1 can be secreted actively or passively into the extracellular space to perform various biological functions (153, 154). In recent years, much more attention has been given to its extracellular function. A growing number of reports have suggested that extracellular (both urine and plasma) HMGB1 can serve as a biomarker for SLE, which could predict disease activity and renal involvement (152–157). Moreover, anti-HMGB1 antibodies in plasma have also been identified as biomarkers of SLE (158, 159). Extracellular HMGB1 can bind to numerous receptors, such as toll-like receptors (TLRs), and advanced glycosylation end-product-specific receptor (AGER), triggering receptor expression on myeloid cells-1 (TREM1), which promotes the release of pro-inflammatory factors. In addition, when released outside the cell, HMGB1 usually binds to

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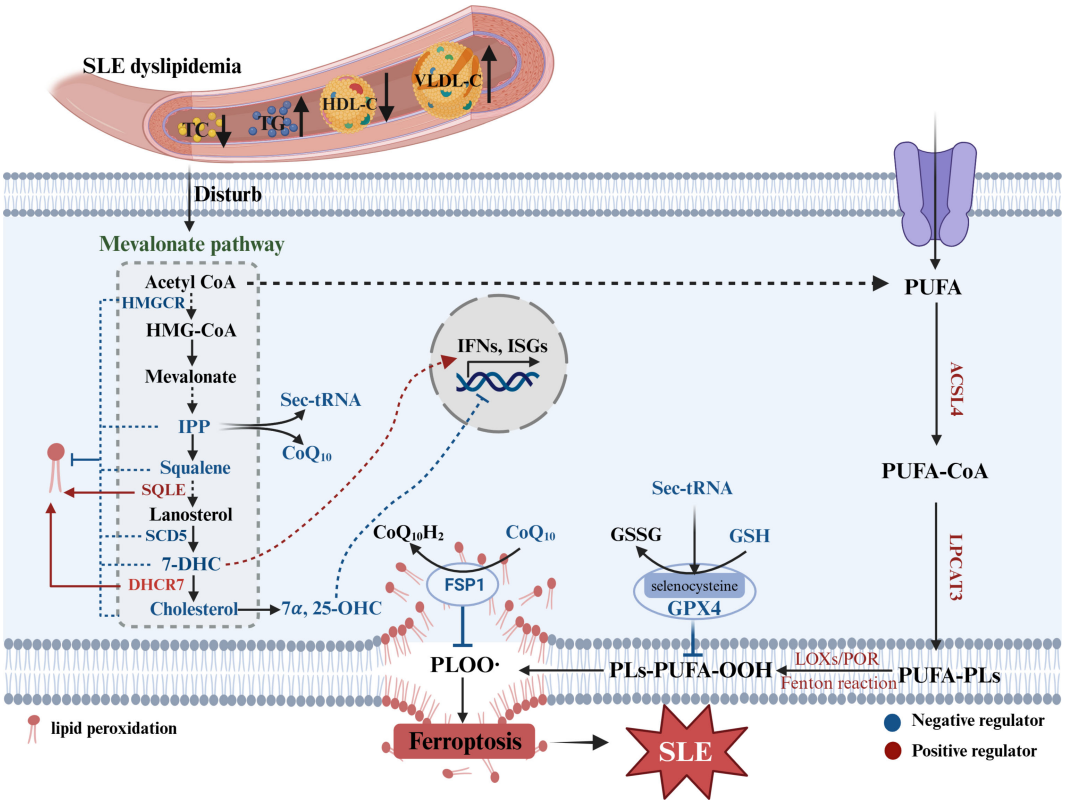

FIGURE 3
Lipid metabolism disorder in SLE and ferroptosis. SLE dyslipidemia can disturb cholesterol metabolism, which intermediates or enzymes not only affect IFNs response but mediate ferroptosis. Sec-tRNA, selenocysteine tRNA required for GPX4 synthesis; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low density lipoprotein-cholesterol; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IPP, isopentenyl pyrophosphate; SQLE, squalene monooxygenase; CoQ10, coenzyme Q10; 7-DHC, 7-dehydrocholesterol; DHCR7, 7-dehydrocholesterol reductase; SCD5, sterol C5-desaturase; 7α,25-OHC, 7α, 25-dihydroxycholesterol; FSP1, ferroptosis suppressor protein 1; ISGs, interferon-stimulated genes; LOXs, lipoxygenases; POR, cytochrome P450 reductase; PLs, phospholipids; ACSL4, acyl-CoA synthetase long-chain family member 4; LPCAT3, lysophosphatidylcholine acyltransferase 3.

intranuclear material (e.g., RNA, DNA), which can activate multiple intracellular nucleic acid sensors, including TLR7, TLR9 and cGAS (160, 161). These sensors are tightly correlated with increased IFN production and SLE pathogenesis (162, 163). In addition, both I-IFN and II-IFN can promote HMGB1 release, resulting in a vicious cycle (24, 164), which may aggravate the progression of SLE. Overall, numerous results suggest that extracellular HMGB1 is involved in the pathogenesis of SLE. Similarly, treatment with an HMGB1 monoclonal neutralizing antibody can alleviate the phenotype of MRL/*lpr* and BXSB Lupus mice, but it has also been shown to be ineffective in MRL/*lpr* mice (20).

Extracellular HMGB1 is derived mainly from cell death, including apoptosis, necroptosis and ferroptosis (165). Various ferroptostic agonists (including erastin and RSL3) can increase HMGB1 release, and this process is blocked by ferroptostic antagonists (such as ferrostatin-1 and liproxstatin-1) or pro-ferroptostic genetic deficiency (e.g., Acsl4 shRNA) in cancer and noncancer cells (21). Wiernicki B et al. recently demonstrated that ferroptosis can be clearly distinguished into three stages: (1) At the beginning, there is intracellular lipid peroxide accumulation; (2) In the intermediate stage, cell membrane permeability increases, resulting in ATP release; and (3) In the final stage, there is complete disintegration of the cell membrane, and HMGB1, LDH and inflammatory factors are released (166). HMGB1 serves as the main DAMP of ferroptosis and mediates the pathological effects of ferroptosis. It is released during ferroptosis of M2 macrophages and can promote the inflammatory response of M1 cells through the TLR4/STAT3 pathway. Moreover, the inhibition of ferroptosis rescues this effect (167). Wang CB et al. revealed that the hepatotoxic effect of methotrexate (MTX) was mainly mediated by HMGB1 released from ferroptostic cells (31). In addition, Zhang DF et al. reported that renal dysfunction related to imidacloprid was primarily attributed to HMGB1 release from ferroptosis, which promoted the activation of the nucleotide-binding domain (NBD), leucine-rich repeat (LRR), and pyrin domain (PYD)-containing protein 3 (NLRP3) inflammasome in neighboring cells through the RAGE/TLR4-NF-κB signaling pathway, further aggravating pyroptosis (22). Interestingly, numerous recent reports have shown that HMGB1 released from ferroptosis can also trigger ferroptosis in neighboring cells, thus forming a vicious cycle and aggravating the progression of the disease. Wei Q et al. revealed that ultraviolet B radiation induces autophagy-dependent ferroptosis and that subsequently released HMGB1 can promote ferroptosis in neighboring cells through TfR1 (32). Davaanyam D et al.

demonstrated that ferroptosis occurs in neurons at the early stage of cerebral ischemia and that HMGB1 can upregulate the expression of hepcidin through TLR4/C-X-C chemokine receptor type 4 (CXCR4) to promote ferroptosis and aggravate cerebral ischemia. Moreover, inhibition of HMGB1 can intercept ferroptosis and alleviate cerebral ischemia (23). In addition, Deng YL et al. reported that the overexpression of casepase-6 in THP-1 cells promoted ferroptosis in HTR8/SVneo cells. However, HMGB1-neutralizing antibodies can prevent this process (168). Taken together, these findings indicate that HMGB1 is an important effector of ferroptosis that can mediate the involvement of ferroptosis in various pathological mechanisms and disease progression.

In summary, ferroptosis can increase the release of HMGB1, which can intensify the release of inflammatory factors through its sensors. Additionally, binding to nuclear substances can activate related nucleic acid receptors (TLR7/9, cGAS, etc.) to mediate the mass production of IFNs, and subsequently, IFNs can promote ferroptosis and accelerate the release of HMGB1. Moreover, HMGB1 can further promote ferroptosis in neighboring cells. Overall, these events form a vicious cycle, which may exacerbate the progression of SLE (Figure 4).

3.4.2 IFNs

SLE is known as an "interferon signature" disease and the overactivation of the interferon system is closely related to SLE pathogenesis, especially I-IFN, which is elevated before the onset of SLE and closely associated with disease activity and organ involvement (169–171). Nucleic acid substances bind to the endosomal or intracellular nucleic acid receptors (TLRs, cGAS, Nucleotide-binding and leucine-rich repeat receptors (NLRs), etc.) and promote the massive production of interferon-stimulated genes (ISGs) and Type I IFNs (I-IFNs), subsequently contributing to SLE progression. In the past, type I IFNs (II-IFNs) were considered to play the most crucial role in the pathogenesis of SLE (169). Recently, other types of IFNs, especially II IFNs, have also been confirmed to play an important role in the pathogenesis of SLE (170, 171). Moreover, in recent years, IFNs have been shown to be crucial for the regulation of ferroptosis (8). Li PC et al. reported that, in SLE, IFN-α or IgG can restrain GPX4 transcription through

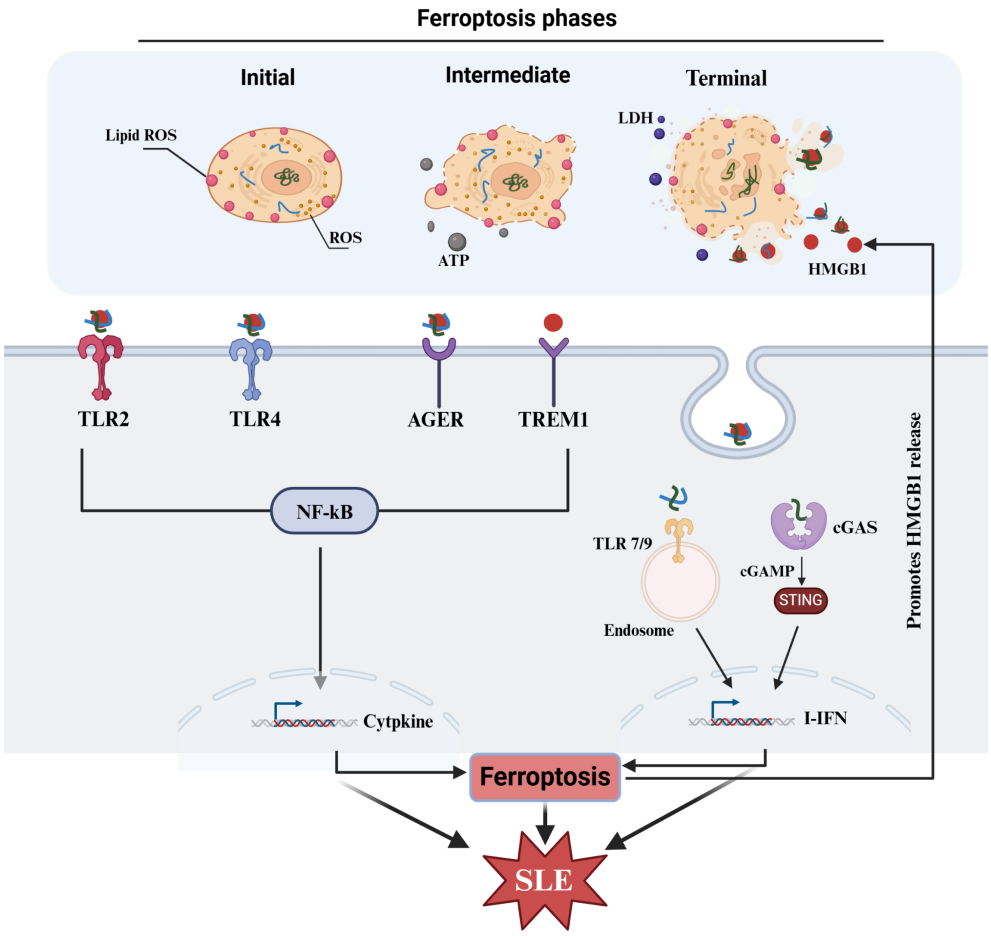

FIGURE 4
HMGB1 in SLE and ferroptosis. HMGB1 as a major DAMPs released from ferroptosis can intensify the production of IFNs and other cytokines, which subsequently contribute ferroptosis and SLE progress. HMGB1, high-mobility group box 1; TLR, toll-like receptors; AGER, advanced glycosylation end-product-specific receptor; TREM1, triggering receptor expressed on myeloid cells-1; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; cGAMP, cyclic GMP-AMP; ISGs, interferon-stimulated genes.

CaMKIV/CREMα, which promotes an increase in the level of intracellular lipid ROS and ultimately facilitates neutrophil ferroptosis. Moreover, they confirmed that neutrophil ferroptosis is a key form of cell death involved in the pathogenesis of SLE (172). In addition, evidence that IFN-α can induce ferroptosis was confirmed by Zhang SL et al. (173). They reported that manganese could disturb the expression of DHODH by activating cGAS-STING pathway, promoting an increase in mitochondrial lipid peroxides and ROS, ultimately leading to ferroptosis of tumor cells. Zhang SL et al. also found that blocking IFNAR could rescue this effect of manganese. As the downstream pathway of IFNs, the JAK-STAT pathway is considered one of the most important pathways involved in the pathogenesis of SLE (mainly JAK-STAT1) (174, 175). In addition, many JAK inhibitors (such as tofacitinib, baricitinib, upadacitinib and filgotinib) are being investigated in clinical trials (176). Recent studies have confirmed that spermine can effectively alleviate the progression of SLE by inhibiting JAK1 (177).

Moreover, a close correlation between the IFNs-JAK-STAT pathway and ferroptosis has been demonstrated in recent years. In another autoimmune disease, Sjogren's syndrome, high expression of IFN-γ could decrease the expression of solute carrier family 3 member 2 (SLC3A2, a subunit of xCT), glutathione, and GPX4 in salivary gland epithelial cells and then trigger ferroptosis through activation of the JAK/STAT1 pathway, which can be inhibited by JAK1/2 or STAT1 antagonists (178). In addition, in retinal pigment epithelial cells, IFN-γ can downregulate the expression of solute carrier family 40 member 1 (SLC40A1, iron export protein) and SLC7A11 through the JAK1-2/STAT1 pathway, which promotes the accumulation of intracellular Fe^{2+} and the exhaustion of glutathione and ultimately induces ferroptosis (179). In tumor cells, IFN-γ can also prevent the transcription of both SLC3A2 and SLC7A11 and then accelerate ferroptosis via JAK1/2-STAT1-IRF1 (180). Furthermore, a chromatin immunoprecipitation assay confirmed that IFN-γ promoted the binding of STAT1 to the SLC7A11 promoter to intercept its transcription (181). However, it has also been reported that inhibition of STAT1 can downregulate GPX4 and SLC7A11 expression to promote ferroptosis (182). Fan Li also confirmed that STAT1 can bind to the promoter region of SLC7A11 and promote its transcription using a dual luciferase reporter assay (183). These results suggest that STAT1 can regulate ferroptosis, but the regulatory mechanism is contradictory and may be related to differences in cells and their environment.

In conclusion, JAK-STAT1 not only play important roles in the pathogenesis of SLE but also regulate ferroptosis. Since the regulation of IFNs-JAK-STAT signaling is inconsistent in ferroptosis, whether this signaling is implicated in SLE pathogenesis by regulating ferroptosis needs more evidences (Figure 5). Although many inhibitors targeting the JAK-STAT pathway have been investigated in clinical trials, their

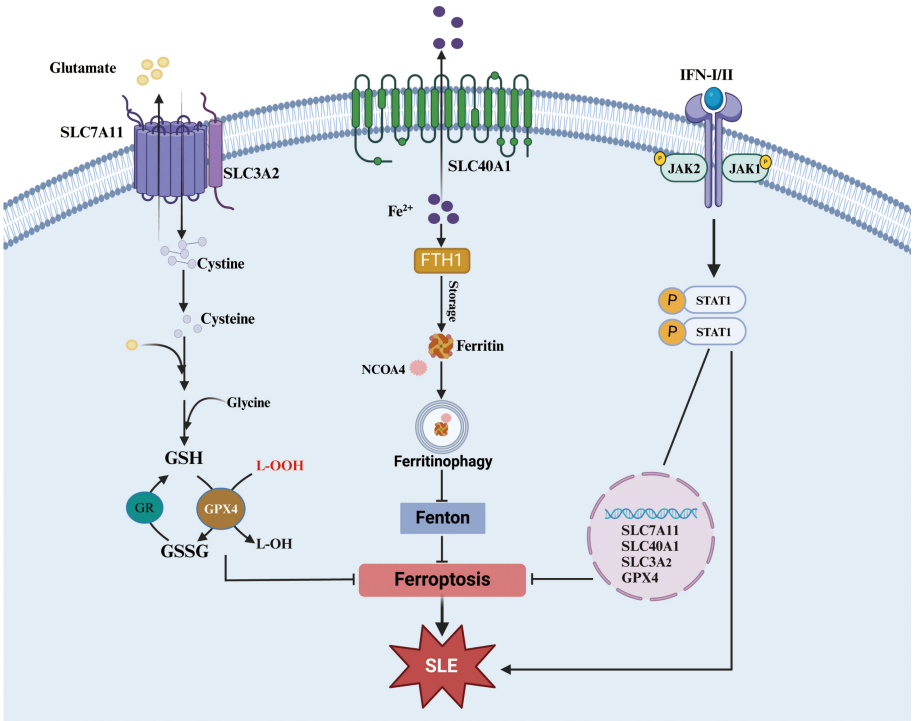

FIGURE 5
JAK-STAT1 pathway in ferroptosis. Both xCT-GPX4 and FTH1-Ferritinophagy are important roadways to against ferroptosis. And the accumulation of some cytokines (especial I/II-IFN) could significantly affect pivotal molecules of the two roadways although JAK-STAT1 pathway. xCT, cystine/glutamate transport system; SLC7A11, solute carrier family 7a member 11, SLC3A2, solute carrier family 3 member 2; SLC40A1, solute carrier family 40 member 1; GPX4, glutathione peroxidase 4; GSH, glutathione; GSSG, glutathione disulfide; FTH1, ferritin heavy chain 1; NCOA4, nuclear receptor coactivator 4.

actual effects are not satisfactory (176). Notably, whether the inhibition of the JAK-STAT pathway potentially aggravates ferroptosis and promotes the progression of SLE deserves further explore.

4 Promising therapeutical reagents for SLE by inhibiting ferroptosis

Given that several pathophysiological features of ferroptosis, including iron accumulation, mitochondrial dysfunction, overproduction of lipid peroxidation and suppression xCT-GPX4 pathway are potentially implicated in SLE progression, reversing those features may be beneficial to SLE disease. Below, we summarize some the bioactive pharmacological agents that can against those ferroptostic process (Table 2).

4.1 Inhibiting iron accumulation

Intracellular iron accumulation is the chief culprit of ferroptosis and can promote SLE progression, the therapeutical reagents to reduce iron concentration may be beneficial to treat SLE. Iron chelators, including deferiprone (DFP), dexrazoxane (DXZ) and deferoxamine (DFO), are commonly used in clinical. Although only few reports demonstrated DFO could alleviated SLE progression, multiple evidences confirmed those chelators performed an effectivity on some chronical diseases by preventing ferroptosis, such as osteoarthritis (184), I/R-induced injury (185), non-alcoholic steatohepatitis (204), neurodegeneration (205) and so on. Additionally, hepcidin, the iron-regulatory hormone, has been verified it could reduce progression and severity of LN by decreasing renal iron accumulation (47).

4.2 Recovering mitochondrial dysfunction

Mitochondrial dysfunction not only drivers ferroptostic process but promotes SLE progression. Recovering mitochondrial dysfunction to prevent ferroptosis may improve prognosis of SLE disease. Recent evidences demonstrated mTOR inhibition suppress ferroptosis by mediating mitochondrial dysfunction (186). Sirolimus is an mTOR inhibitor that server as an antifungal or immunosuppressive agent in clinical, which also effectively alleviates lupus mice manifestation (187, 188). Moreover, Lai ZW et al. demonstrated sirolimus improve SLE patient activity by recovering mitochondrial dysfunction (189). Metformin, commonly used in clinical to treat type II diabetes, can reduces oxidants by normalizing mitochondrial dysfunction (190, 191). Remarkably, it has been verified to improve SLE patients by recovering mitochondrial dysfunction (206–208). Additionally, MitoQ, as an analogue of CoQ10, special for preventing mtROS production, was shown to alleviate lupus mice (86, 192).

4.3 Reducing lipid peroxidation

Lipid peroxidation is essential to ferroptosis and prevent the process was shown to improve numerous ferroptosis-related diseases. Thiazolidinediones (TZDs), including rosiglitazone, pioglitazone and troglitazone, are applied to treat type II diabetes. Remarkably, TZDs performed an ability to suppress ferroptosis

TABLE 2 The promising therapeutical reagents for SLE by inhibiting ferroptosis.

Targeted pathway	Reagent	Proposed mechanism	Reference
Iron homeostasis	Deferiprone (DFP)	Iron chelator	(47, 121)
	Dexrazoxane (DXZ)	Iron chelator	(123)
	Deferoxamine (DFO)	Iron chelator	(184, 185)
	Hepcidin	Reducing iron accumulation	(47)
Mitochondria dysfunction	Sirolimus	an mTOR inhibitor and recovering mitochondrial dysfunction	(186–189)
	Metformin	normalizing mitochondrial dysfunction	(190, 191)
	MitoQ	Analogue of CoQ10, special for preventing mtROS	(86, 192)
Lipid peroxidation	Thiazolidinediones (TZDs)	Selectively inhibiting ACSL4	(193, 194)
	Liproxstatin-1 (Lip-1)	Special ferroptostic inhibitor	(172)
	CoQ10	A potent radical-trapping antioxidant and inhibiting lipid peroxidation	(195)
	Idebenone	Analogue of CoQ10	(196)
	Vitamin K	A potent radical-trapping antioxidant and inhibiting lipid peroxidation	(197–199)
xCT-GPX4 pathway	N-acetylcysteine (NAC)	Increasing cysteine levels and facilitating GSH synthesis	(200–202)
	Selenium (Se)	Essential for maintaining GPX4 activity	(79)
	Glycyrrhizin	Increasing GSH and GPX4, decreasing MDA, Fe2+, ROS	(203)

though selectively inhibiting ACSL4 and reduce mortality in kidney-specific *Gpx4* knockout mice (193, 194). Liproxstain-1 (Lip-1), a spiroquinoxalinamine derivative, was identified as a special ferroptostic inhibitor by high-throughput screening. Recent evidence shown it could alleviate MRL/*lpr* lupus mice symptom (172). Additionally, some lipid antioxidants also display potential therapeutical prospect as follow: i. CoQ10 is a major substrate of FSP1 that known as ferroptostic suppressor. The dietary supplement of it as a potential candidate for the treatment of various noncommunicable diseases (195). Moreover, its analogues, idebenone with better bioavailability and efficacy, was shown to attenuate murine lupus (196). ii. Vitamin K, resembled CoQ10 structure, was used to overcome warfarin poisoning in clinical practice and its supplementation could positively affect multiple chronical diseases (197). Interesting, it also was identified as a lipid radical-trapping antioxidant to prevent ferroptosis via FSP1-mediated pathway (198, 199).

4.4 Activating xCT-GPX4 pathway

As described above, the xCT and GSH-GPX4 pathway are identified as primary defense mechanism to ferroptosis. N-acetylcysteine (NAC), a precursor to cysteine, has been shown to prevent ferroptosis by increasing cysteine levels and facilitating the synthesis of γ-glutamyl-cysteine and GSH (200). And its supplement is potentially beneficial for comorbid disorders associated with heavy alcohol consumption (201) and Parkinson's disease symptoms (202). Micronutrient selenium (Se) is essential for maintaining GPX4 activity. Optimal Se supplement could improve hemorrhagic and ischemic stroke prognosis via inhibiting ferroptosis in mice (79). Additionally, glycyrrhizin, extracted from the glycyrrhiza and commonly used in clinical, could alleviate acute liver failure by increasing GSH and GPX4, decreasing MDA, Fe^{2+}, ROS to inhibit ferroptosis (203).

5 Conclusion

In summary, SLE is a complex chronic disease for which its pathophysiology and effective treatments remain speculative. Ferroptosis is a prevalent type of pathogenic cell death in chronic disease. Although the studies on ferroptosis in SLE pathogenesis are still in early stages, there are intricate and close connections among them, which provides opportunities for understanding SLE pathophysiology and development of novel therapeutics.

Author contributions

GZ: Writing – original draft, Formal analysis, Writing – review & editing. XL: Writing – review & editing, Conceptualization, Writing – original draft. YZ: Writing – original draft, Conceptualization, Writing – review & editing. XW: Visualization, Writing – review & editing. LD: Visualization, Writing – review & editing. JX: Visualization, Writing – review & editing. SJ: Resources, Writing – review & editing. ZZ: Resources, Writing – review & editing. LX: Resources, Writing – review & editing. ML: Resources, Writing – review & editing. FY: Supervision, Writing – review & editing. JQ: Funding acquisition, Writing – review & editing. PF: Funding acquisition, Writing – review & editing.

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Conflict of interest

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Iron and ferroptosis in kidney disease: molecular and metabolic mechanisms

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Maintaining iron homeostasis is necessary for kidney functioning. There is more and more research indicating that kidney disease is often caused by iron imbalance. Over the past decade, ferroptosis' role in mediating the development and progression of renal disorders, such as acute kidney injury (renal ischemia-reperfusion injury, drug-induced acute kidney injury, severe acute pancreatitis induced acute kidney injury and sepsis-associated acute kidney injury), chronic kidney disease (diabetic nephropathy, renal fibrosis, autosomal dominant polycystic kidney disease) and renal cell carcinoma, has come into focus. Thus, knowing kidney iron metabolism and ferroptosis regulation may enhance disease therapy. In this review, we discuss the metabolic and molecular mechanisms of iron signaling and ferroptosis in kidney disease. We also explore the possible targets of ferroptosis in the therapy of renal illness, as well as their existing limitations and future strategies.

KEYWORDS

kidney disease, ferroptosis, mechanism, treatment, metabolic

1 Introduction

Various forms of controlled cell death, including apoptosis, necroptosis, pyroptosis, and autophagy, have been linked to the development of kidney diseases (1). In recent years, extensive research has indicated the pivotal role of ferroptosis, a non-apoptotic form of cell death that relies on iron and results in the accumulation of lipid hydroperoxides, in the pathophysiology of the progression of kidney diseases, including Acute Kidney Injury (AKI) and Chronic Kidney Disease (CKD) (1, 2).

Iron is a crucial trace element ubiquitous in almost all living organisms. It is crucial in various biological functions, such as energy metabolism, nucleotide synthesis, and repair processes (3). Iron imbalance is frequent in patients (4). Prevalence rates as high as

24%-85% have been reported for chronic inflammatory conditions, such as CKD (5). Iron deficiency increases mortality in CKD patients (6). Conversely, iron accumulation leads to oxidative damage through Fenton reactions, which may lead to renal injury (7), indicating that iron negatively impacts CKD development and that iron accumulation plays a role in initiating ferroptosis. Therefore, it is essential to regulate proteins involved in iron metabolism to restore kidney iron metabolism and reduce the occurrence of ferroptosis.

Renal injury is distinguished by increased generation of mitochondrial reactive oxygen species (ROS) and disrupted iron homeostasis (8). Imbalances in the redox systems can result in the oxidation of specific biomolecules, thereby inducing structural and anatomical changes in these molecules. The mitochondrial cytochrome oxidase enzymes, such as cytochrome P450, regulate this process, which takes place in the mitochondria (9). Research conducted on mice models by Su et al. (10) and Zhu et al. (11) revealed that mitochondrial dysfunction precedes the onset of proteinuria and podocyte effacement. The production of ROS, metabolic byproducts that occur during this process, contributes to the development of atherosclerosis in CKD and accelerates renal damage progression.

The available evidence strongly suggests that ferroptosis has a vital function in advancing different kidney disorders. Numerous studies have identified ferroptosis as a primary cause of impaired kidney repair and the development of inflammatory responses in proximal tubular cells (12). Moreover, excessive ferroptosis, regulated by various signaling and metabolic pathways, has demonstrated its involvement in developing AKI, CKD, and renal cell cancer (RCC). This review elucidates the complex mechanisms of regulating iron homeostasis, glutathione (GSH) synthesis and lipid metabolism in the kidney. Additionally, we explore the latest indicators of ferroptosis in renal diseases and offer invaluable insights into the potential for developing novel therapeutic interventions to inhibit ferroptosis in the kidney.

2 Development of ferroptosis

Ferroptosis was initially reported in 2012 as a new type of cell death that can be inhibited by the iron-chelating compound deferoxamine (13). However, numerous types of cell death associated with iron and oxidative stress have been recognized for years (14). In 2003, researchers made a significant discovery related to a unique form of non-apoptotic cell death, later termed “ferroptosis” (15). Subsequent studies determined that this cell death is regulated iron-dependent (16) and is highly modulatable with molecular perturbations (17, 18). Subsequently, Marcus Conrad and his colleagues demonstrated that genetic modulation of crucial genes regulating redox state induced non-apoptotic cell death (19–21). Ferroptosis may have emerged due to understanding cysteine depletion-induced cancer cell death and glutamate-induced cytotoxicity (22, 23). Since its discovery, ferroptosis has been identified as an iron-dependent form of programmed cell death where the formation of lethal lipid peroxides is catalyzed by iron (24) (Figure 1).

3 Molecular and metabolic drivers of ferroptosis

Ferroptosis cells exhibit distinct genetic, biochemical, morphological, and metabolic characteristics compared to other recognized forms of cell death, including apoptosis, necroptosis and pyroptosis (25). In contrast to other forms of cell death, ferroptosis can rapidly propagate within cell populations in a wave-like manner (26). Regarding morphological alterations, electron microscopy can be used to observe mitochondrial abnormalities in cells undergoing ferroptosis. These changes include swelling, density variations and outer membrane rupture (27). Finally, the pathways involving iron, glutathione and lipid metabolism intersect to regulate the onset and progression of ferroptosis, particularly in renal tubular epithelial cells. The following section discusses the impact of these metabolic pathways on ferroptosis and kidney disease.

3.1 Iron metabolism and ferroptosis in the kidney

3.1.1 modulation of iron homeostasis in the renal system

Cellular iron homeostasis is closely connected to iron absorption, storage, circulation and utilization (28). The human digestive system typically absorbs 1-2mg of iron daily from the diet (29). With the aid of cytochrome b on the brush boundary, dietary ferric iron is converted to catalytically active ferrous iron (30), which is then absorbed by intestinal epithelial cells through the assistance of the divalent metal transporter 1 (DMT1, SLC11A2) (31).

The kidney receives 20% to 25% of the overall cardiac output and is primarily a metabolic organ (32). Iron is both filtered and reabsorbed in the kidney (33). Renal cells can uptake transferrin-bound iron (TBI) and non-transferrin-bound iron (NTBI). The transferrin receptor 1 (TfR1) is expressed on both proximal and distal tubule epithelial cells, enabling the absorption of TBI (34). Transferrin is endocytosed as an additional method for TBI uptake (35). The endosome undergoes acidification, a process mediated by vacuolar ATPase, which results in the conversion of ferric iron to ferrous iron by the six transmembrane epithelial antigens of prostate 3 (STEAP3) (36, 37). Natural resistance-associated macrophage protein 2 (NRAMP2) (also called DMT1) aids in the transfer of ferrous iron from the endosome to the cytoplasm (38, 39).

The uptake of NTBI in the kidneys occurs independently of transferrin. The reabsorption of NTBI is achieved by utilizing other iron transporters, such as DMT1 (40). Zinc transporters ZIP8 and ZIP14, which were initially identified as zinc transporters, are expressed in the proximal and distal tubules and potentially contribute to the uptake of NTBI (41). These transporters can directly transport divalent iron (Fe^{2+}) in the form of NTBI through the apical plasma membrane. In addition to these iron transporters, there are several additional receptors and transporters that facilitate the renal iron reabsorption process (42). Regarding the absorption of hemoglobin (Hb) and TBI, proximal tubule epithelial cells employ the megalin-cubilin complex, while distal tubule epithelial

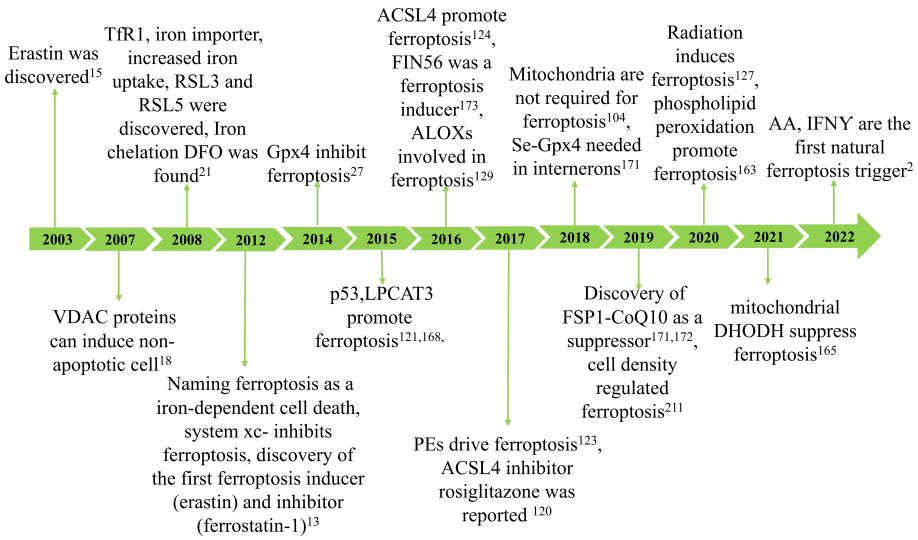

FIGURE 1
The development of ferroptosis. The concept of ferroptosis originated in 2003 with the discovery that erastin can induce a unique form of cell death characterized by the absence of traditional caspase-3 activation, DNA fragmentation, and preservation of nuclear morphology. Due to its iron dependency, this phenomenon was officially termed ferroptosis in 2012. Key hallmarks of ferroptosis include lipid peroxidation and iron accumulation, which result in diminished glutathione peroxidase (GPX4) activity and subsequent cell death. Ferroptosis arises from reduced GPX4 activity, leading to decreased cellular antioxidant capacity and the buildup of lipid reactive oxygen species (ROS). Research has demonstrated that ferroptosis is closely associated with the pathogenesis and progression of various diseases, including ischemia-reperfusion injury, kidney damage, neurological disorders, cancer, and hematological conditions.

cells utilize the neutrophil gelatinase-associated lipocalin receptor (NGALR, also known as SLC22A17) (33). Both megalin and NGALR have been connected to the tubular absorption of filtered Hb during hemolysis (43).

In general, imported ferrous iron can be preserved in the cytosol within the ferritin complex, comprised of the heavy chain (Fth) and ferritin light chain (Ftl) (44). This mechanism contributes to stabilizing volatile iron concentrations and mitigates the production of ROS (45). Ferritinophagy also called the degradation of iron-saturated ferritin, is controlled by Nuclear receptor coactivator 4 (NCOA4) (46). Ferritinophagy triggers the degradation of intracellular ferritin within lysosomes, leading to the subsequent release of iron (47), This liberated iron is transported from lysosomes to the cytosol through lysosomal NRAMP2 (38). Ferritin may play a role in protecting renal cells against cisplatin-induced toxicity by regulating iron levels. Increased ferritin levels can store iron and mitigate the harmful effects of cisplatin on the kidneys. Conversely, the silence of Fth may cause cell death (48). Subsequent studies have identified that ferritin degradation is linked to the activation of NCOA4-mediated ferritinophagy. Inhibiting NCOA4-mediated ferritinophagy can effectively hinder ferritin degradation and potentially prevent kidney injury (49). Ferritin and NCOA4 play a vital role in regulating iron levels in the kidney. Moreover, a limited amount of ferrous iron is preserved in the labile iron pool (LIP), which supports cellular metabolism under normal physiological conditions (50).

Ferroportin (Fpn), the identified iron export protein in mammals, is responsible for transporting iron into the bloodstream to fulfill the needs of the organism (51–53). However, Fpn is only expressed in the epithelial cells of the proximal tubule, which plays a vital role in iron export (54). Given that Fpn is not expressed in distal tubules and these cells lack additional iron exporters, it appears that distal tubules do not have a notable function in the reabsorption and recycling of iron under normal physiological conditions (33).

Fpn significantly impacts the maintenance of plasma iron levels. When the ferroportin1 (FPN1) gene, which encodes Fpn, was knocked out in AKI mice, it improved renal function by regulating iron levels (55). Hepcidin, a factor that protects against AKI, plays a central role in regulating iron homeostasis through Fpn. Hepcidin exerts its anti-kidney damage properties by promoting the ubiquitination of ferroportin through the E3 ubiquitin-protein ligase RNF21 (56), which is associated with the restoration of iron homeostasis and the suppression of inflammation (57). Hepcidin pretreatment reduces inflammation in AKI animal models and inhibits renal ischemia-reperfusion injury (IRI)-induced dysregulation of systemic iron homeostasis (58). The hepcidin-ferroportin pathway may be a promising novel therapeutic indicator for the treatment of AKI (56, 57). (Figure 2).

While the glomerulus can filter iron and haem proteins, there is currently limited knowledge about how glomerular cells process iron. A research study demonstrated that podocytes can uptake Hb via megalin-cubilin endocytosis, which can be metabolized by heme oxygenase 1 (HMOX1/HO-1) (59). Podocytes also contain ferritin and can take up transferrin via endocytic processes, indicating that these cells can transport and process iron (58–60). Cultured human glomerular endothelial cells have been shown to express TfR1, Fpn and DMT1 (61). Additionally, both rat and mouse mesangial cells express ferritin, TfR1 and iron regulatory protein 1 (IRP1). The induction of HO1 has been observed in these cells, indicating the involvement of iron transport and regulation (62).

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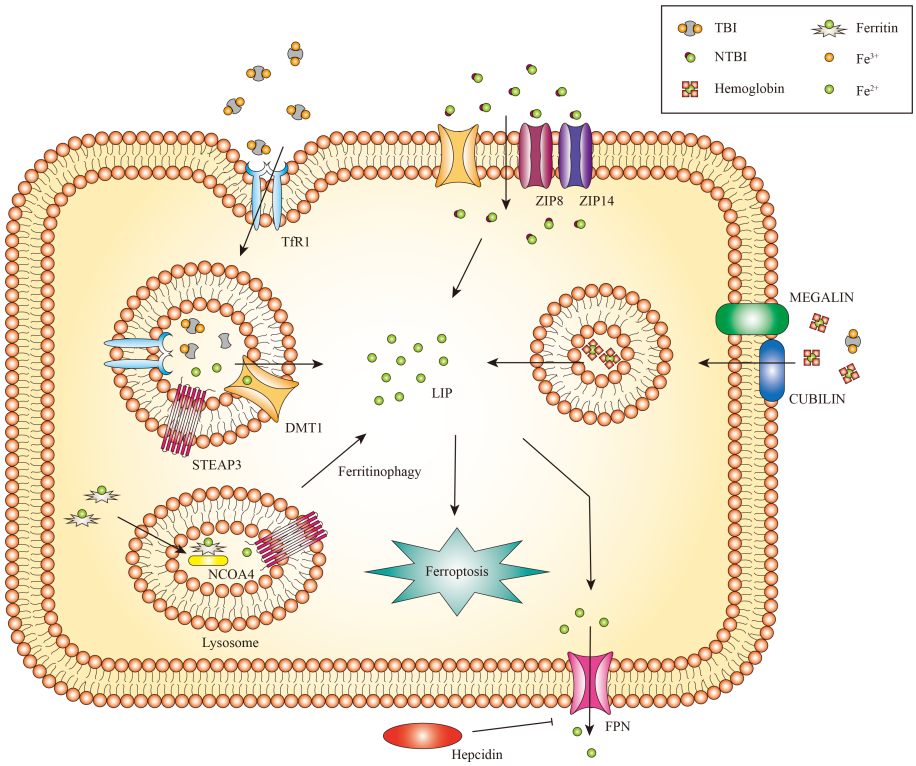

FIGURE 2
The metabolism of renal iron. In glomerular endothelial cells, endocytic uptake of TBI has been demonstrated, likely involving DMT1. Proximal and distal tubule epithelial cells reabsorb TBI from the tubular lumen through TfR1 mediated endocytosis. Divalent iron [Fe(II)] is transported into the cytosol by DMT1, zinc transporter ZIP8 and/or ZIP14. Proximal tubule epithelial cells also take up Hb and TBI through the megalin-cubilin complex. Iron can be exported to the cytoplasm by NRAMP2 after a metalloreductase STEAP3-mediated reduction. In addition, iron can be released from FTH via NCOA4-mediated autophagic degradation of ferritin, a process known as ferritinophagy. Iron export might occur via FPN. Only the proximal tubule epithelial cells express FPN for iron export, which can be inhibited by circulating hepcidin.

The kidneys are iron-sensitive (57). During the iron cycle, NTBI and unstable ferrous irons can undergo oxidation and reduction through Fenton and Haber-Weiss reactions, resulting in oxidative damage to the kidney.

3.1.2 Iron metabolism in ferroptosis

Iron is an essential trace metal element that plays a crucial role in various physiological activities, including oxygen transport, cellular respiration, DNA synthesis, and neurotransmitter biosynthesis in the nervous system (63, 64). Maintaining iron homeostasis is essential for normal cellular function, as iron deficiency and accumulation can lead to detrimental effects. Iron deficiency often leads to anemia, while an excess of iron can increase metabolic enzyme activity, such as Lipoxygenases (LOXs), which in turn enhances the generation of ROS, this process can cause tissue damage and elevate the risk of cancer (65). Therefore, iron homeostasis regulation is vital for maintaining cellular health and preventing the onset of various diseases.

The kidney has two prominent iron regulatory mechanisms: the iron-responsive element (IRE)-IRP system and the Hypoxia-inducible factor (HIF) regulatory system. The IRE-IRP1 system strictly maintains the body's iron concentrations. The kidney expresses IRP1 and IRP2, but IRP1 is especially abundant in the proximal tubule and is vital in controlling this organ's iron metabolism (34). In iron-depleted cells, IRP1 and IRP2 bind to IREs in the 3′ untranslated region (UTR) of target mRNAs, such as TfR1 and DMT1, leading to their stabilization and transcription of these mRNAs. On the other hand, binding of IRPs to IREs present at the 5'UTR of target mRNAs, such as Fth1 (encoding ferritin heavy chain), FPN1 and HIF-2α, prevents their translation (66, 67). This mechanism helps maintain the balance of LIPs.

HIF proteins also regulate gene transcription in cellular iron regulation (68). HIF-1α is expressed in tubular cells, whereas HIF2α is restricted to renal endothelial and interstitial cells (69). HIF-2 regulates the production of erythropoietin (EPO) in hypoxic renal interstitial fibroblast-like cells, and this process is susceptible to inhibition by IRP1 (67, 70). The regulatory role of HIF on proteins involved in iron regulation in kidney cells remains unclear. Furthermore, different proteins controlling iron balance within cells can impact the cell's vulnerability to ferroptosis (71) (Table 1).

Impairment of the uptake, transportation, storage, and utilization of iron within cells can result in an excessive accumulation of free divalent iron. This accumulation can trigger the Fenton reaction, generating hydroxyl radicals (53) and ROS (85). Subsequently, a cascade of peroxidation processes occurs on the cell membrane, involving polyunsaturated fatty acids (PUFAs), resulting in the generation of lipid peroxides, which in turn degrade the structure of the cell membrane, ultimately triggering cell ferroptosis (86).

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TABLE 1 Main proteins modulating iron metabolism in ferroptosis.

Gene	Protein	Location	Function	Effects of genetic deletion or overexpression	Refs.
CISD1	CDGSH iron–sulfur domain-containing protein 1 (also known as mitoNEET)	Outer mitochondrial membrane and cytoplasm	Inhibiting mitochondrial iron uptake	Deletion promotes erastin-induced ferroptosis	(72)
CISD2	CDGSH iron–sulfur domain-containing protein 2 (also known as NAF1)	ER and mitochondria associated ER membranes	Regulating mitochondrial iron homeostasis	Deletion promotes sulfasalazine-induced ferroptosis	(73)
CP	Ceruloplasmin	Cellular membranes, extracellular	Oxidizing Fe2+ to Fe3+ to mediate FPN effect	deletion promotes erastin-induced ferroptosis	(24)
SLC11A2	Natural resistance-associated macrophage protein 2	Cellular membranes, endosomal membranes	Iron importer	Deletion suppresses ferroptosis	(31)
FTH1	Ferritin heavy chain 1	Cytoplasm	Storing Iron	Deletion promotes ferroptosis in AKI	(33)
FTMT	Ferritin mitochondrial	Mitochondria	Mitochondrial iron storage	overexpression of FTMT inhibits ferroptosis	(74)
HMOX1	Heme oxygenase 1	Cytoplasm, the Golgi apparatus	Catabolizes heme	overexpression inhibits ferroptosis	(75, 76)
IREB2	Iron-responsive element binding protein 2	Cytoplasm	Regulates the translation of mRNAs that affect iron homeostasis	Deletion suppresses erastin-induced ferroptosis	(13)
NCOA4	Nuclear receptor coactivator 4	Cytoplasm	Regulates ferritinophagy	Deletion suppresses erastin-induced ferroptosis	(77, 78)
PCBP1	Poly(rC)- binding protein 1	Cytoplasm	Iron chaperones	Deletion increases ferroptosis	(79)
PROM2	Prominin 2	Multivesicular bodies	Regulates ferritin export	Deletion promotes ferroptosis	(80)
SLC25A28	Mitoferrin 2	Outer mitochondrial membrane	Regulates mitochondrial iron uptake	Deletion suppresses erastin-induced ferroptosis	(81)
SLC39A8	Solute carrier family 39 member 8 (also known as ZIP8)	Cellular membranes	Mediating the cellular uptake of Fe2+	Deletion suppresses ferroptosis	(41)
SLC39A14	Solute carrier family 39 member 14 (also known as ZIP14)	Cellular membranes	Mediating the cellular uptake of Fe2+	Deletion suppresses ferroptosis in diabetic nephropathy	(82)
SLC40A1	Solute carrier family 40 member 1(also known as ferroportin/FPN)	Cellular membranes	Mediates iron export	Deletion promotes siramesine and lapatinib-induced ferroptosis	(83)
TF	transferrin	Extracellular, endosome	Transporting iron	Deletion promotes ferroptosis	(34)
TFR1	Transferrin receptor protein 1	Extracellular, endosome	Transports iron	Deletion suppresses ferroptosis	(84)
Steap3	six transmembrane epithelial antigen of prostate 3	Cellular membranes, endosome	Mediating iron uptake	Deletion promotes ferroptosis	(36, 37)

Iron dysregulation is primarily caused by the abnormal barrier function of iron transporters Fpn (87), TfR1 and DMT1 (88). Thus, the proteins responsible for regulating the transportation of cellular iron could influence a cell's vulnerability to ferroptosis (89).

Firstly, the deletion of TfR1 inhibits the development of renal fibrosis induced by unilateral ureteral obstruction (UUO) in a mouse model of diabetic kidney disease (DKD) by regulating iron import and suppressing ferroptosis (84). Furthermore, the induction of ferroptosis in breast cancer cells by siramesine and lapatinib is hindered by the overexpression of Fpn, which regulates iron efflux. Conversely, silencing Fpn has the opposite effect (83). Alternatively, prominin 2 (PROM2), a membrane glycoprotein, produces ferroptosis resistance in epithelial and breast cancer cells by promoting exosome-dependent iron export (80). Furthermore, CDGSH iron-sulfur domain-containing protein 1 (CISD1), also known as mitoNEET, is involved in transporting iron within mitochondria and maintaining iron and ROS balance in the outer mitochondrial membrane (90, 91). Limiting the uptake of mitochondrial iron, the upregulation of CISD1 expression protects human hepatocellular carcinoma cells against elastin-induced ferroptosis (72). CISD2 has been linked to developing resistance to sulfasalazine-induced ferroptosis in head and neck cancer. Reducing CISD2 enhances the accumulation of mitochondrial iron and restores the sensitivity of ferroptosis-

resistant cells to sulfasalazine-induced cell death (73). Therefore, modulation iron transport pathway on cell membranes can regulate ferroptosis.

Alternatively, NCOA4-mediated ferritin degradation increases iron storage and accumulation in the active LIP. This triggers the Fenton reaction, resulting in mitochondrial damage, activation of LOXs function, and a rise in ROS levels (92), ultimately leading to ferroptosis. Inhibition of NCOA4-mediated ferritinophagy reduces iron storage and prevents ferroptosis in cancer cells (77, 78). Furthermore, Poly(rC)-binding protein 1 (PCBP1), an iron chaperone, interacted with NCOA4 in regulating iron flux into and out of ferritin (93). The levels of labile iron and lipid peroxidation increased in the PCBP1 knockout mice, indicating that PCBP1 may have a crucial function in preventing diseases associated with ferroptosis (79). Moreover, the overexpression of mitochondrial ferritin (FTMT), a mitochondria iron-storage protein, inhibits ferroptosis in neuroblastoma cells (74), indicating an essential antiferroptotic role in mitochondria.

Genes related to iron metabolism also can regulate ferroptosis. TfR1, Fth1 and Ftl expression during ferroptosis is influenced by the iron metabolism regulator iron-responsive element binding protein 2 (IREB2). The silencing of IREB2 affects the expression of iron-regulated genes TfR1, Fth1 and Ftl during ferroptosis (13). Furthermore, Nuclear factor erythroid 2-related factor 2 (NFE2L2) is a regulatory protein that influences heme and iron metabolism by increasing the transcription of various genes, including HO-1. HO-1 contributes to iron homeostasis (94). However, HO-1 has a dual function in ferroptosis (95, 96). The dual mechanism of protection versus cytotoxicity of HO-1 has already been confirmed by Suttner DM and Dennery PA (97). Non-canonical ferroptosis is caused by excessive HO-1 activity (98). Additionally, the serine/threonine kinase ATM enhances ferroptosis by blocking the movement of metal regulatory transcription factor 1 (MTF1) into the nucleus, which triggers the synthesis of Fpn, Fth1 and Ftl, ultimately resulting in a reduction in iron toxicity (87).

These data demonstrate that various variables influence iron metabolism during ferroptosis (4). Therefore, further investigation is required.

3.1.3 Iron associated kidney disorders

Disturbances in iron homeostasis have significant impacts on the function of various organs. Iron overload, iron deficiency and imbalances in iron distribution impact iron-utilizing and recycling. As advancements in treatments for iron disorders have improved, reducing major complications and increasing patient survival, new complications are emerging, such as renal damage (99, 100).

3.1.3.1 Systemic iron overload

3.1.3.1.1 Hereditary haemochromatosis

Hereditary hemochromatosis (HH) is a prevalent condition characterized by excessive iron accumulation throughout the body, resulting from either hepcidin deficiency or hepcidin resistance. This condition is caused by inherited defects in genes encoding proteins involved in hepcidin production (HAMP), function (Fpn), or regulation [hereditary hemochromatosis protein (HFE), TfR2 and haemojuvelin (HJV)] (9), resulting in systemic iron overload and ferroptosis. White individuals primarily develop hereditary hemochromatosis due to a homozygous alteration in the HFE gene, which is situated on chromosome 6. Iron deposition in the kidneys and iron leakage into the urine was observed in mouse models of hemochromatosis, suggesting impaired kidney function (101). Therapeutic phlebotomy is the standard treatment for HH, involving the removal of a significant quantity of iron from erythrocytes (75, 102). This intervention leads to a progressive depletion of iron reserves in the organism.

3.1.3.1.2 Halassaemia syndromes

Reduced production of α-globin or β-globin chains leads to α-thalassaemia or β-thalassaemia, respectively, which disrupts hemoglobin synthesis and subsequently impairs erythropoiesis (103). Patients with β-thalassaemia may exhibit diverse mutations and have different treatment needs (104). In both β-thalassaemia intermedia and major, ineffective and increased erythropoiesis results in reduced hepatic hepcidin production (105). Frequent red blood cell (RBC) transfusions for the treatment of anemia and the inherited defect exacerbate systemic iron overload (104). Iron removal therapy in patients with β-thalassaemia involves the treatment of iron chelation medication. β-thalassaemia has been associated with increased renal iron exposure, as reflected by increased urinary iron excretion, deposition, and kidney injury. Renal injury and iron accumulation are frequently observed in patients with β-thalassaemia. These patients may exhibit elevated levels of urinary markers for kidney injury, including cystatin C, β2-microglobulin and N-acetyl-β-d glucosaminidase, in the absence of kidney failure (106–109).

3.1.3.2 Systemic iron deficiency

Iron deficiency disorders can be classified into two types: absolute and functional iron deficiency (FID), both of which can result in anemia. Iron deficiency anemia (IDA) is most common in women of childbearing age, pregnant women and children (5). In these individuals, insufficient body iron stores result from inadequate dietary iron intake, increased iron need, or iron loss, which hampers the support for erythropoietic needs. Anemia due to FID can be inherited or acquired. Mutations in TMPRSS6, which encodes matriptase-2, have been found to result in the iron-refractory IDA (IRIDA) (110). The mechanisms of matriptase-2 mediated inhibition of hepcidin synthesis have yet to be resolved entirely. Matriptase-2 cleaves various components in hepcidin-activating pathways, including HJV, Activin receptor-like kinase 2 (ALK2) and HFE. However, in patients with IRIDA, matriptase-2 is non-functional, leading to elevated hepcidin levels. These high levels of hepcidin impede the absorption of dietary iron and the release of iron from macrophages (111, 112). Acquired FID frequently occurs in patients with elevated levels of inflammation, including individuals with systemic inflammatory disorders such as CKD or cancer. This condition is also known as anemia of inflammation or anemia of chronic disease (110). Iron levels can be restored through oral or IV iron supplementation for patients with IDA or FID. However, individuals with FID may require additional therapy to stimulate erythropoiesis and decrease hepcidin levels (113, 114).

3.2 GSH metabolism and ferroptosis in the kidney

Glutamate and glutamine are also important regulators of ferroptosis (115). System xc- (also known as xCT) facilitates the exchange of glutamate and cystine in equal proportions. It is a cystine-glutamate antiporter system, with SLC7A11 as its active subunit (116). The function of system xc -is affected by glutamate levels. Indeed, elevated levels of extracellular glutamate concentrations inhibit system xc- and induce ferroptosis (13). Knockout mice lacking system xc- knockout mice exhibit protection against brain damage due to the reduction of extracellular brain glutamate levels (117). Thus, the concentration of extracellular glutamate may induce ferroptosis.

System xc^- regulated cystine uptake is critical in enhancing GSH biosynthesis (118). The entry of cystine into the cell affects the production of cysteine and GSH (13). GSH activates glutathione peroxidase 4 (GPX4), significantly influencing intracellular redox homeostasis maintenance. By inhibiting system xc- activity, sulfasalazine and sorafenib can disrupt cystine absorption and GSH synthesis, leading to a reduction in GPX4 activity and cell antioxidant capacities, an increase in lipid ROS accumulation, and the gradual onset of oxidative damage and ferroptosis. By utilizing mice with inducible GPX4 knockout, we have uncovered a crucial function of the GSH/Gpx4 axis in preventing sudden kidney malfunction and the related occurrence of ferroptosis (27).

Human tissues and plasma contain glutamine in its natural state. The glutaminolysis of glutamine can promote the tricarboxylic acid (TCA) cycle and lipid biosynthesis processes. The absence of glutamine or glutaminolysis inhibition hampers the accumulation of ROS, thereby preventing lipid peroxidation and ferroptosis. This finding can be explained by the requirement of α-ketoglutarate (αKG), a product of glutaminolysis, for ferroptosis (115).

Since ferroptosis has been recognized as a significant mechanism of cell death in tissue injury (119, 120), targeting glutaminolysis may emerge as a practical therapeutic approach for managing organ damage mediated by ferroptosis. Indeed, studies have demonstrated that inhibiting glutaminolysis can reduce kidney damage induced by ischemia/reperfusion (I/R) in experimental models (120).

3.3 Lipid metabolism and ferroptosis in the kidney

Ferroptosis is characterized by uncontrolled oxidation of lipids (121). The oxidation of PUFAs in the membranes of mammalian cells is a crucial process in ferroptosis (122). Genome-wide and CRISPR/Cas9-based screens have revealed that Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) (123–125), both membrane-remodeling enzymes, are significant contributors to the induction of ferroptosis. Upregulation of ACSL4 increases the content of PUFAs in phospholipids, thereby rendering the cell more susceptible to ferroptosis (123, 126). Although the role of ACSL4 in the kidney is unclear, increased expression and activity of ACSL4 might promote ferroptosis under various pathophysiological conditions (127). Identifying this enzyme as a potential therapeutic target shows promise in reducing cell death in skeletal muscle and preventing rhabdomyolysis (128). Another study demonstrated a close association between ferroptosis and intestinal IRI, highlighting the critical role of ACSL4 in this lethal process (129). Furthermore, ionizing radiation can trigger the activation of ACSL4 and facilitate ferroptosis in malignant cells. Deleting ACSL4 significantly eliminates ionizing radiation-induced ferroptosis and promotes radioresistance, thus highlighting the crucial role of ACSL4 in cancer treatment (130). By contrast, ACSL3 catalyzes the conversion of exogenous monounsaturated fatty acids (MUFAs) to fatty acyl-CoAs and inhibits ferroptosis (131, 132).

LOXs, a group of iron-containing enzymes, can directly oxygenate PUFAs in biological membranes (133), indicating that LOXs may mediate lipid peroxidation in ferroptosis. This hypothesis is supported by the evidence that certain pharmacological inhibitors of LOX can inhibit ferroptosis (134, 135). 12/15-LOX knockout protected mice against ischemic brain injury (136, 137). However, the genetic removal of 12/15-LOX on the GPX4 knockout background failed to prevent ferroptosis in mouse AKI (27). The results indicate that alternative mechanisms compensate for LOX loss (138). Furthermore, it has been demonstrated that the scaffold protein PEBP1 can bind and guide 15-LOX toward PUFAs in the membrane, thus facilitating the process of ferroptosis (139).

The kidney is extremely vulnerable to oxidative harm, and lipid peroxidation is vital in kidney injury caused by ROS. Malondialdehyde (MDA), 4-hydroxyhexenal (4-HHE) and 4-hydroxynonenal (4-HNE) are significant end products of PUFAs oxidation and are frequently utilized as indicators of lipid peroxidation (140). The average serum MDA levels were significantly increased in chronic renal failure (CRF) patients. Additionally, a high plasma level of MDA was identified as a robust and independent predictor of mortality in CRF patients (141, 142). These findings support the hypothesis that lipid peroxidation plays a role in the severity of kidney disease. Lipid peroxidation drives ferroptosis by damaging cellular membranes (143). *In vivo*, removing lipid peroxides could increase the lifespan of mice with deficiencies by around 35% (27). A certain compound has been demonstrated to inhibit the characteristic morphological alterations of ferroptosis cells by decreasing MDA and lipid ROS levels in proximal tubular epithelial cells (144). Quercetin treatment abolished lipid ROS, protecting against functional acute renal failure (144). Overall, both direct and indirect evidence from various studies strongly supports the crucial involvement of lipid peroxidation in ferroptosis.

3.4 Mitochondria and kidney ferroptosis

The density of mitochondria in kidneys is among the highest. Mitochondria coordinate essential metabolic processes. Dysfunction in the mitochondria may result in an energy crisis, impact the redox status of cells, and control ferroptosis. Mitochondrial defects in renal tubules contribute to the

development of kidney disease by causing epithelial atrophy, inflammation and cell death (9).

Iron exerts its functions mainly by forming iron-sulphur (Fe-S) clusters and haem. Fe-S clusters are assembled from ferric iron in the mitochondria and incorporated into Fe-S proteins primarily in the mitochondria and cytosol (145, 146). Mitochondria acquire iron via mitoferrin 1 and mitoferrin 2 (81). Subsequently, iron binds to mitochondrial ferritin (gene named FTMT) to inhibit the production of ROS. Moreover, mutations in FTMT are associated with mitochondrial iron overload and cytoplasmic iron deficiency (147). Currently, the mechanism responsible for the export of iron from the mitochondria remains unknown (146). Moreover, the export of Fe-S clusters into the cytoplasm may depend on the presence of iron-sulfur clusters transporter ABCB7 and mitochondrial ABCB7 and ABCB8 (148). Additionally, it has been shown that mitochondrial ABCB10 plays a role in regulating the initial stages of haem synthesis within mitochondria (149).

Haem, acting as a cofactor in facilitating catalysis and electron transfer (150), is synthesized in the mitochondria through a complex biogenic pathway involving aminolevulinic acid synthase (ALAS, also known as ALAS-H), which plays a crucial role in the rate-limiting initial step (151). Excess haem can be transported to the cytoplasm through feline leukemia virus subgroup C receptor-related protein 1B (FLVCR1B) or catabolized via the HO-1 pathway into Fe^{2+} (152). The expression of HO-1 is ubiquitously induced in response to oxidative stress (153). In mammals, haem efflux is primarily mediated by the plasma membrane exporter FLVCR1A and membrane importer FLVCR2 (154, 155). There are other haem transporters, such as haem carrier protein 1 (HCP1) and haem responsive gene protein 1 (HRG1), but their functions need to be better understood (156).

Mitochondria are the primary source of ROS in most cells (157). Since 2012, it has been recognized that mitochondria play a crucial regulatory role in ferroptosis. Scientists observe changes in mitochondrial morphology during ferroptosis, such as decreased mitochondrial volume and elevated mitochondrial membrane density (13). Certain compounds have been specifically engineered to target mitochondria and exhibit protective effects against ferroptosis (158). In contrast, the evidence contradicts the relationship between mitochondria and ferroptosis. Previous research has indicated that a cell line with mitochondrial defects is equally susceptible to ferroptosis compared to its parental cell line. Moreover, this sensitivity can be mitigated by administering iron chelators (13, 159). However, there is significant controversy surrounding these findings. The involvement of mitochondria in ferroptosis could vary depending on the context (27, 160).

Kidney tubule cell death is the predominant phenotype observed in global GPX4 knockout mice, highlighting the crucial involvement of GPX4 and ferroptosis in kidney disease (27). Conversely, conditional GPX4 deletion promotes multiple organ dysfunction (161). The GPX4 gene in mammalian cells encodes cytosolic and mitochondrial isoforms. The mitochondrial isoform of GPX4 localizes in mitochondria due to a mitochondrial targeting signal at its amino terminus (162, 163). The cytosolic GPX4 isoform can accumulate in the mitochondrial intermembrane, playing a role in suppressing mitochondrial lipid peroxidation (162). Interestingly, the overexpression of cytosolic GPX4, rather than the mitochondrial isoform, prevented the death of GPX4 knockout mouse embryonic fibroblasts (164). Overall, these findings reveal that the expression of cytosolic GPX4 is adequate to prevent ferroptosis.

In vivo, experiments have also shown that mitochondria play a significant role in ferroptosis. The mitochondria ROS scavenger (MitoTEMPO) has been demonstrated to protect against ferroptosis-induced nephropathy (165). Furthermore, mice that lack FTMT encounter heightened brain injury and neurological impairments, along with characteristic molecular characteristics of ferroptosis. Conversely, FTMT overexpression reverses these changes. This research proves that FTMT is critical in protecting against cerebral I/R-induced ferroptosis and subsequent brain damage (166).

Multiple studies have demonstrated that mitochondria can act as initiators or amplifiers of cystine starvation and GSH depletion induced by ferroptosis. The ability of mitochondrial dihydroorotate dehydrogenase (DHODH) and mitochondrial coenzyme Q10 (CoQ10) to suppress ferroptosis triggered in this manner (167), highlights this function of mitochondria. The localization of DHODH occurs within the inner membrane of mitochondria, facilitating dihydroorotate transformation into orotate (168). Interestingly, it has been demonstrated that DHODH inactivation coupled with low expression of GPX4 leads to a significant increase in mitochondrial lipid peroxidation and ferroptosis (167). Furthermore, the transportation of GPX4 to the mitochondria restores RSL3-induced ferroptosis in cells lacking DHODH (167). The mitochondrial GSH importer, SLC25A39, has been discovered, indicating its potential role in controlling mitochondrial lipid peroxidation and ferroptotic cell death (169). However, further investigations are necessary to elucidate the *in vivo* role of DHODH and discover other mitochondrial enzymes that regulate ferroptosis (Figure 3).

3.5 Other pathways that regulate ferroptosis

Other pathways can also regulate Ferroptosis. First, the p53 pathway is essential, which performs dual regulatory functions in ferroptosis. On the one hand, p53 can enhance ferroptosis by inhibiting the expression of SLC7A11 (170). In contrast, p53 inhibits ferroptosis by diminishing the activity of dipeptidyl peptidase-4 (DPP4) and enhancing the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A) (171). Research has shown that p53 can promote ferroptosis in glomerular podocyte injury induced by high fructose (172). However, the exact role of p53 in various kidney diseases still needs to be better understood. Second, ferroptosis suppressor protein 1 (FSP1) (173, 174). The FSP1-coenzyme Q10-nicotinamide adenine dinucleotide phosphate (FSP1-CoQ10-NADPH) axis functions as a parallel system that collaborates with GPX4 and GSH to inhibit phospholipid peroxidation and suppress ferroptosis in cancer (174, 175). The absence of FSP1 makes kidneys more susceptible to tubular ferroptosis in a manner that relies on GSH, leading to

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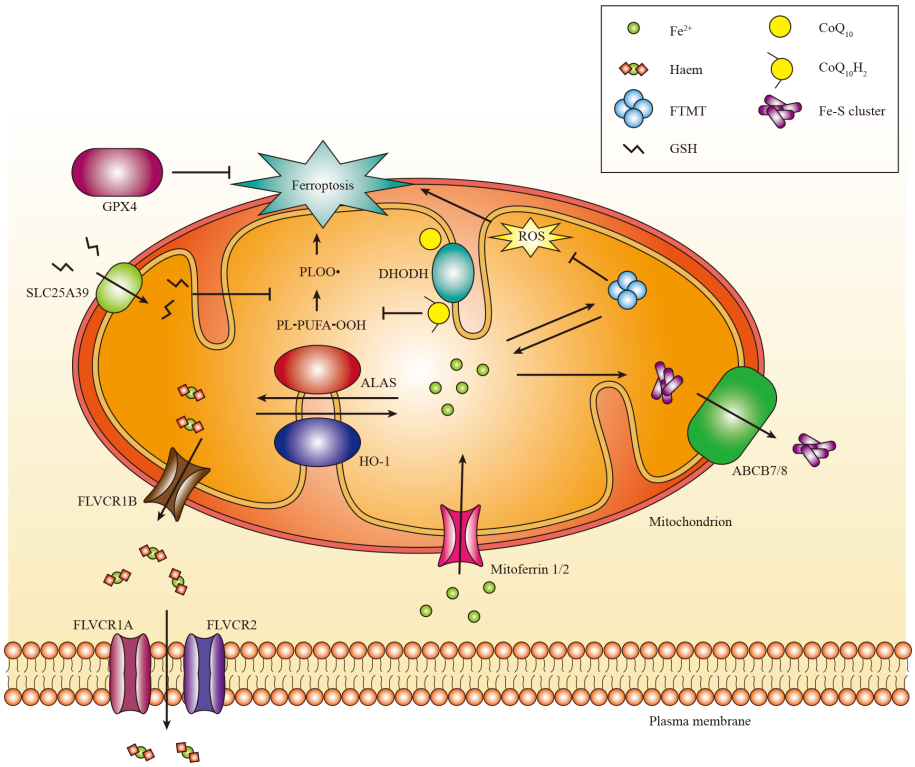

FIGURE 3
The role of mitochondria in ferroptosis. Mitochondria host a wide range of key metabolic processes of ferroptosis, such as lipid peroxidation. Separate mitochondria-localized defense systems have evolved to prevent mitochondrial lipid peroxidation and ferroptosis. For example, either the mitochondrial version of phospholipid hydroperoxide GPX4 or mitochondrial (DHODH) can specifically detoxify mitochondrial lipid peroxides. Moreover, FTMT protects mitochondria from iron overload induced oxidative injury. Iron is primarily used to Fe-S clusters and haem in the mitochondria. Excess iron can be stored in the FTMT. Mitoferrin 1 and mitoferrin 2 mediate the transport of iron across the mitochondrial membrane. FLVCR1B promotes haem efflux into the cytoplasm, whereas the export of Fe-S clusters into the cytoplasm might require Fe-S clusters transporter ABCB7 and ABCB8. PL-PUFA-OOH, polyunsaturated fatty acid-containing phospholipid hydroperoxides; PLOO·, phospholipid peroxyl radical.

a distinct morphological pattern of tubular necrosis (176). The precise role of FSP1 in the kidney still needs to be better comprehended.

4 Ferroptosis in kidney disease

4.1 AKI

AKI is a clinical syndrome marked by a sudden decrease in renal excretory function, such as the accumulation of creatinine (Cr), urea nitrogen (BUN) and other metabolic waste products. The pathogenesis of AKI is complex, involving multiple factors, including renal IRI, drug-induced AKI, severe acute pancreatitis-induced AKI, and sepsis-associated AKI. These factors are closely associated with the occurrence of ferroptosis.

4.1.1 Renal IRI

Renal IRI is a severe condition contributing to AKI and substantially impacting mortality rates. Ferroptosis is responsible for the death of tubular cells, a crucial element in I/R renal injury (177). In the mouse IRI model, ferroptosis inhibitors demonstrated protective effects against AKI and other organ damage (176). Renal IRI is the primary factor responsible for AKI following cardiac surgical procedures in clinical settings. The development of postoperative AKI in the heart is intricate, with the liberation of unbound iron playing a crucial part (178). Elevated free iron levels can trigger cellular ferroptosis, resulting in tubular necrosis (179). This finding highlights the significance of maintaining iron homeostasis in AKI induced by I/R (179). In addition, I/R-induced AKI is linked to the GSH-glutathione peroxidase (GSH-GPx) system. A rat I/R kidney injury model revealed that miR-182-5p and miR-378a-3p could bind GPX4 and SLC7A11 mRNAs to inhibit their expression, which in turn activated ferroptosis to reduce I/R kidney injury (180). Another research verified that the inhibition of Legumain, which is involved in the lysosomal degradation of GPX4 during ferroptosis, mitigated both ferroptosis and tubular damage in a model of ischemia/reperfusion kidney injury by elevating intracellular GPX4 levels (181).

4.1.2 Drug-induced AKI

Drugs frequently contribute to the occurrence of AKI. Folic acid (FA) can induce AKI in mice. Diego et al. found that FA-induced AKI in mice was associated with lipid peroxidation and suppression of GSH metabolism, consistent with the typical profile of ferroptosis. Moreover, administering ferrostatin-1, a ferroptosis

inhibitor, decreased kidney damage and oxidative stress levels in mice with FAI. These findings suggest that ferroptosis is closely linked to the process of FA-induced AKI (182). In addition, cisplatin is a commonly used antitumor drug; however, it is nephrotoxic and can cause AKI in patients. Cisplatin induces acute tubular necrosis through DNA damage and disruption of mitochondrial function. Research indicated that iron was detectable in the medium after exposure to cisplatin. The increase in iron levels can catalyze free radical reactions while using iron chelators significantly reduces cisplatin-induced cytotoxicity. These findings suggest a crucial involvement of iron, although the precise mechanism remains elusive (183). Ferritin plays an essential role in iron metabolism. Fth1 knockout mice are more susceptible to developing AKI after cisplatin treatment, which emphasizes the protective role of Fth1 in AKI (33). Myo-inositol oxygenase (MIOX) is an enzyme to promote ferroptosis, which can inhibit GPX4 activity and increase ferritin phagocytosis. The renal pathological damage and ferroptosis levels were elevated in cisplatin-treated mice overexpressing MIOX. These findings suggest a strong association between the cisplatin-induced AKI process and ferroptosis (184).

4.1.3 Severe acute pancreatitis-induced AKI

AKI is a common complication of severe acute pancreatitis (SAP) (185). The exact pathophysiology of AKI in SAP remains unclear. Previous studies have indicated that AKI induced by SAP is primarily caused by the loss and dysfunction of renal tubular epithelial cell barriers induced by oxidative stress (186). Deliang et al. conducted a rat model of SAP-induced AKI and observed an elevation in the expression of ACSL4, a positive ferroptosis regulator. However, negative regulators expressions, such as GPX4 and Fth1, were suppressed. Moreover, morphological features of ferroptosis were observed, suggesting an association between ferroptosis and SAP-induced AKI. In addition, using Liproxstatin-1, a ferroptosis inhibitor, effectively reduced histopathological damage in the pancreas and kidneys of SAP rats (187). Other studies have shown that AKI may result from various forms of cell death in SAP (188). However, further investigation is required to better understand the interconnections among these cell death processes.

4.1.4 Sepsis-associated AKI (SA-AKI)

Sepsis is a major cause of AKI in intensive care units. Patients with SA-AKI experience prolonged hospital stays and have a greater mortality risk than others (189). Previous studies have demonstrated that renal tubular cells undergo necrosis, apoptosis, and autophagy in the SA-AKI model (190). Ji et al. initially confirmed the presence of ferroptosis in SA-AKI, and further experiments indicated that Nrf2, an essential protein regulating iron metabolism, was involved in inhibiting ferroptosis in the SA-AKI model (191). Similarly, Qiu et al. used Ferrostatin-1 to inhibit ferroptosis and thereby reduce SA-AKI and found that melatonin also inhibited this process. Subsequent studies have confirmed that melatonin inhibits ferroptosis by activating the Nrf2/HO-1 pathway, which is involved in the inflammatory response and antioxidant processes in SA-AKI, reducing ROS accumulation (192).

4.2 CKD

Due to its high incidence and mortality, CKD is a significant public health problem. The intricacy of molecular mechanisms in CKD has sparked a growing interest in this particular disease area. The pathology of CKD is significantly influenced by ferroptosis (193).

4.2.1 Diabetic nephropathy (DN)

DN, one of the significant complications of diabetes, is a severe microangiopathy. The main features are the progressive decline in renal function and the increase in proteinuria. Recent findings indicate that ferroptosis plays a role in developing DN (194). The decreased expression of GPX4 and elevated lipid peroxidation were confirmed in the renal tissue of DN mice, and the administration of ferrostatin-1 reversed these changes (195). In addition, researchers have observed inhibition of Nrf2 expression in DN models. Reducing Nrf2 levels enhances the susceptibility of DN cells to ferroptosis and fenofibrate by upregulating Nrf2 expression and inhibiting ferroptosis, which can decelerate DN progression. This finding suggests a promising avenue for developing future therapeutic interventions (196). It has been verified that HMGB1 relocates to the cell's nucleus and diminishes the Nrf2 expression along with its subsequent objectives. The potential for innovative therapeutic approaches in DN lies in targeting HMGB1 and ferroptosis (197). Furthermore, DN mice exhibited elevated lipid peroxidation levels and iron accumulation, resulting in the upregulation of ferroptosis indicator ACSL4 and the downregulation of GPX4 in renal tubular cells (76). In particular, the upregulation of HO-1 expression has been demonstrated to inhibit oxidative stress and improve redox balance, potentially offering benefits for DN (198).

4.2.2 Renal fibrosis

Renal fibrosis is crucial in advancing CKD to end-stage renal disease. Its main pathological features include glomerulosclerosis, tubulointerstitial fibrosis, and excessive accumulation of extracellular matrix (1). Previous studies have demonstrated an increased expression of prostaglandin-endoperoxide synthase (PTGS2), a potential marker of ferroptosis, in animal models of renal fibrosis (199). Other ferroptosis-related molecules, such as GPX4, iron and lipid peroxides, have also been shown to be involved in ferroptosis in the pathogenesis of renal fibrosis (200). TTGF-β1 is widely recognized as a pivotal factor in developing renal fibrosis. TGF-β1 inhibits the expression of SLC7A11 and GPX4, demonstrating synergistic effects with ferrostatin-1 in inhibiting ferroptosis (195). Other studies have demonstrated that Deferasirox (DFX) may reduce renal fibrosis by inhibiting TGF-β1/Smad3, inflammation and oxidative stress pathways (201). It has also been suggested that roxadustat may impede renal fibrosis by inhibiting ferroptosis; however, further investigation is required to ascertain the specific mechanism (202).

4.2.3 Autosomal dominant polycystic kidney disease (ADPKD)

ADPKD is the prevailing inherited renal disorder resulting from mutations in PKD1 (encoding polycystin-1) or PKD2 (encoding

polycystin-2), which mainly presents with cysts of varying sizes in the kidneys bilaterally. The gradual enlargement of the renal cysts ultimately destroys the kidneys' structure and function, leading to end-stage renal failure (203). The researchers observed an increase in intracellular ferroptosis in the PKD1 mutant mouse model, characterized by elevated expression of HO-1 and iron importers (TfR1, DMT1), enhanced lipid peroxidation levels, and decreased expression of GSH and GPX4. Ferroptosis inducer erastin accelerates ADPKD progression, and ferroptosis inhibitors, such as ferrostatin-1, can reduce ADPKD progression (204). Schreiber et al. used ferroptosis inducer RSL to promote ADPKD cysts growth by activating TMEN16A. Conversely, ferrostatin-1 inhibited TMEN16A expression, preventing cysts progression (205). The results reveal that ferroptosis plays a vital role in regulating ADPKD progression.

4.3 RCC

RCC is a malignant neoplastic disease that originates from the renal tubular epithelium. Oxidative phosphorylation and metabolic reprogramming are important features of RCC (206). Numerous studies have substantiated the essential significance of ferroptosis in RCC (207). Evidence indicates that the inhibition of ferroptosis is a common occurrence in cancer cells, enabling their survival and progression. The resistance to RCC therapy remains a significant challenge. However, it has been observed that cancer cells, resistant to conventional chemotherapeutic drugs, exhibit high susceptibility to ferroptosis inducers. Inducing ferroptosis can effectively overcome the resistance of cancer to standard therapies (208). For example, RCC can be susceptible to erastin-induced ferroptosis in mouse tumor xenograft models (209). In addition, RCC is strongly linked to mutations in the von Hippel Lindau (VHL) gene. Previous studies have found that exogenous expression of pVHL can enhance oxidative metabolism in RCC cells, thereby leading to the inhibition of ferroptosis (210). Additionally, Xu et al. found that Acyl-CoA thioesterase 8 (ACOT8) expression was significantly reduced in RCC tissues, and its high expression suggested a poor prognosis. Functional analysis revealed that ACOT8 correlated with Oxidative phosphorylation and fatty acid metabolism. Moreover, the analysis revealed a positive association between ACOT8 and GPX4 while demonstrating a negative correlation with factors that inhibit ferroptosis. These findings imply that ACOT8 potentially regulates RCC development by suppressing ferropsosis (211). Moreover, Yang et al. found that cell density affects the sensitivity of RCC cells to ferroptosis, and the Hippo-Yes-associated protein (YAP)/Transcriptional coactivator with PDZ-binding motif (TAZ) pathway is involved in this process. Meanwhile, suppression of TAZ expression inhibited ferroptosis and overexpression of TAZS89A promoted ferroptosis. TAZ regulates NADPH Oxidase 4 (NOX4) levels by regulating epithelial membrane protein 1 (EMP1) expression, essential for ROS processes in ferroptosis. The findings reveal that cell density-regulated ferroptosis is mediated by TAZ through the regulation of EMP1-NOX4, suggesting its therapeutic potential for RCC (212). Emerging evidence suggests that ncRNAs act as pivotal regulators of various cellular processes in RCC. RNA-based therapeutics offer an alternative approach to modulating ferroptosis in cancer therapy, fostering ferroptosis in cancer treatment. These findings emphasize the need for further investigation into the therapeutic potential of ncRNAs in RCC (213).

4.4 Other kidney-related diseases

SARS-CoV-2 virus leads to COVID-19, resulting in a range of symptoms from mild to severe in humans (214). While SARS-CoV-2 primarily induces lung damage, it is also capable of causing extrapulmonary manifestations, such as renal involvement (215). Several studies have confirmed that AKI induced by SARS-CoV-2 is associated with up to a five-fold increase in in-hospital mortality compared to patients without AKI (216, 217). Ninety percent of hospitalized patients have abnormal serum iron levels, which correlate with the severity of the disease (218). Iron metabolism dysfunction has been widely documented in many COVID-19 patients. Consequently, this dysregulation could induce ferroptosis in renal tubule cells (219, 220). Ferroptosis has been indicated as a potential therapeutic target for the treatment of COVID-19 (221). Renal dysfunction and AKI frequently manifest in patients with initially healthy kidneys during severe SARS-CoV-2 infection (222). Moreover, through the use of RNA sequencing (RNA-seq), it has been observed that cilastatin, an inhibitor of dipeptidase-1 (DPEP1), is capable of preventing SARS-CoV-2 infection and the subsequent ferroptosis (219).

Cardiorenal syndrome (CRS) encompasses a spectrum of disorders involving the heart and kidneys, where acute or chronic dysfunction in one organ can precipitate acute or chronic dysfunction in the other. This syndrome can be classified into five subtypes: Type 1 (acute CRS), Type 2 (chronic CRS), Type 3 (acute renocardiac syndrome), Type 4 (chronic renocardiac syndrome), and Type 5 (secondary CRS) (223). Empagliflozin (EMPA) is a selective inhibitor of sodium-glucose cotransporter 2 (SGLT2). It has been shown to significantly enhance cardiac and renal function by mitigating doxorubicin-induced ferroptosis, fibrosis, apoptosis, and inflammation in mice through mechanisms involving NLRP3 and MyD88-related pathways (82).

5 Ferroptosis as a promising treatment target

Increasing research on ferroptosis has provided mounting evidence suggesting its ability to inhibit tumor growth and enhance the efficacy of chemotherapeutic drugs (116). However, ferroptosis exerts distinct effects on different types of renal diseases. This therapeutic target shows great promise for treating and preventing kidney disease. We have compiled a summary of ferroptosis targeting agents with therapeutic potential in kidney diseases in Table 2.

TABLE 2 Summary of ferroptosis targeting agents with therapeutic potential in kidney diseases.

Drugs	NCT	Mechanism	Targets	Refs
Ferrostatin 1		Inhibits lipid peroxidation	I/R-induced AKI,folic acid-induced AKI	(27, 120, 181)
			lipid-oxidation-induced acute renal failure	(222)
			primary kidney renal tubule damage model	(224)
			ADPKD mice, cisplatin induced renal injury	(203, 223)
			DN	(82, 194)
UAMC-3203		Inhibits lipid peroxidation	multiorgan injury, including kidney	(225)
			renal I-R injury	(226)
liproxstatin 1		Inhibits lipid peroxidation	lipid-oxidation-induced acute renal failure	(21)
			Fe(II)-dependent renal tubular injury model	(227)
			SAP-induced AKI	(186)
Tectorigenin		Inhibits lipid peroxidation	UUO mouse model	(228)
Nec-1f		Inhibits necroptosis and ferroptosis	renal I-R injury	(175)
Deferoxamine	00870883	Inhibits iron overload	CIN	(229)
	04633889		renal tubulointerstitial fibrosis	(230)
			renal injury	(231)
Deferasirox		Inhibits iron overload	CKD rat model	(200)
			iron-induced renal injury	(232)
Deferiprone	01146925	Inhibits iron overload	CIN	(233)
	01770652		aluminum chloride-induced AKI	(234)
N-acetylcysteine	01218178		CIN	(235)
	00780962		AKI	(236–238)
SRS 16-86		Ferroptosis inhibition	renal I-R injury	(120)
rosiglitazone		ACSL4 inhibitor	I/R-induced AKI	(128)
			DN	(239)
ciclopirox olamine (CPX-O)		Inhibits iron overload	ADPKD	(240)
L-buthionine (S,R)-sulfoximine		Inhibits lipid peroxidation	RCC	(241)

I–R, ischaemia–reperfusion; DN, diabetic nephropathy; ADPKD, autosomal dominant polycystic kidney disease; SAP, severe acute pancreatitis; UUO, unilateral ureter obstruction; CIN, Cisplatin-induced nephrotoxicity.

5.1 Ferrostatin-1, liproxstatin-1 and antioxidants

Ferrostatin-1 is a first-generation inhibitor of ferroptosis (13). Since the discovery of the anti-ferroptotic effects of this small-molecule compound, ferrostatin-1 has undergone extensive testing in various diseases, including kidney disease. Ferrostatin-1 attenuates cell death in cellular models of kidney dysfunction by inhibiting lipid peroxidation (224). Ferrostatin-1, the first known inhibitor of ferroptosis, also protected Gpx4-/- cells from I/R-induced renal damage (27). Furthermore, a study demonstrated that ferrostatin-1 could effectively prevent ferroptosis cell death and inhibit the proliferation of Pkd1-deficient cells in the kidneys of Pkd1 mutant ADPKD mice. Moreover, it was observed that the increased levels of lipid peroxidation product in Pkd1-deficient mice enhanced the proliferation of the surviving Pkd1 mutant cells by activating Akt, S6, Stat3, and Rb signaling pathways during ferroptosis (204). Pretreatment of ferrostatin-1 has significantly alleviated cisplatin-induced renal injury via trans-regulation of vitamin D receptor (VDR)/GPX4 (204). Furthermore, studies have demonstrated that ferrostatin-1 can reduce renal fibrosis in DN and lessen the injury to renal tubular basement membranes in diabetic db/db mice models by modulating the HIF-1α/HO-1 pathway (242). Another study showed that ferrostatin-1 could also prevent ferroptosis by inhibiting the ZIP14 initiation of ferroptosis in DN, suggesting a promising novel therapeutic strategy for the treatment of DN (225). Furthermore, treatment with ferrostatin-1 has been shown to protect renal function and reduce histologic injury, oxidative stress, and tubular cell ferroptosis in a mouse model of FA-induced AKI. Ferrostatin-1 also inhibited the upregulation of interleukin-33 (IL-33) and the infiltration of macrophages (182). A subsequent study demonstrated the effectiveness ferrostatin-1 in mitigating oxidative stress-induced

ferroptosis in renal tubular epithelial cells through the modulation of the PGE2 pathway (226). Furthermore, hemopexin and hemopexin accumulate in the proximal tubular cells and exacerbate kidney injury in both the cisplatin-induced and the unilateral kidney IRI models of AKI. Ferrostatin-1 inhibits this harmful effect of Hb and hemopexin in proximal tubular cells, suggesting the involvement of iron toxicity in the mechanism underlying hemopexin-mediated injury (243).

Several studies have revealed that the *in vivo* efficacy ferrostatin-1 is comparatively lower than its *in vitro* activity due to its limited stability in plasma (227). UAMC-3203, a novel inhibitor derived from ferrostatin-1, has been developed to function as a stronger and more stable ferroptosis inhibitor. These analogues exhibit superior properties to ferrostatin-1, demonstrating *in vivo* efficacy and representing novel compounds with therapeutic potential in ferroptosis-driven disease models, including kidney disease (227, 228).

Liproxstatin-1 was initially identified as a specific inhibitor of ferroptosis in GPX4 knockout cells (27). Subsequent research showed that liproxstatin-1 can inhibit ferroptosis by inducing GPX4 expression, reducing cyclooxygenase-2 (COX-2) expression and inhibiting the kidney inflammation activation in the IRI model (129). Furthermore, another study demonstrated that liproxstatin-1 ameliorates ferroptosis-mediated renal IRI with decreased lipoperoxidation (LPO), MDA and lactate dehydrogenase (LDH) levels while simultaneously increasing the GSH level (229). Furthermore, liproxstatin-1 significantly prolonged the erythrocyte enucleation process in a Fe(II)-dependent renal tubular injury model. The study also highlights the involvement of ferroptosis in various biological processes, including embryonic erythropoiesis and the aging process. Therefore, liproxstatin-1 is suitable for further investigation and evaluation (230). Furthermore, liproxstatin-1 treatment reduced serum amylase levels, as well as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), Cr and BUN levels in rats with SAP. Additionally, it reduced lipid peroxidation in the kidneys and mitigated histopathological damage in pancreatic and renal tissues. These findings unequivocally demonstrate the therapeutic potential of liproxstatin-1 in the treatment of SAP-induced AKI (187).

Another common antioxidant in kidney disease is Tectorigenin, which exhibits antioxidant activity (193). The protective effect of tectorigenin against obstructive nephropathy is elucidated in a study that demonstrates its inhibitory effect on Smad3-mediated ferroptosis and fibrosis in a UUO mouse model (231).

Considering the potential contribution of ferroptosis and necroptosis to kidney disease mortality, employing a combination strategy could enhance the efficacy of kidney disease management (9). Nec-1f can target receptor-interacting protein kinase 1 (RIPK1) and ferroptosis in cell lines, improving survival in a mouse model of renal IRI (176).

5.2 Iron chelators and iron chelation therapy

Iron chelators bind to both circulating and intracellular iron, facilitating the elimination of iron through urinary and fecal routes. Most animal models have shown the efficacy of iron chelation therapy in mitigating kidney diseases.

Deferoxamine (DFO) was the initial iron chelator approved by the Food and Drug Administration (FDA). DFO binds to iron in the circulation, forming the DFO-iron complex (known as ferrioxamine), which is eliminated through urine and feces (183). The upregulation of COX-2, 4-HNE, TNF-α, MCP-1, and IL-6 levels induced by cisplatin was hindered by DFO. DFO also alleviated cisplatin-induced kidney injury and renal dysfunction. The protective effect of DFO against cisplatin-induced nephrotoxicity (CIN) may be partially attributed to the inhibition of ferroptosis (232). Moreover, in mice with UUO, DFO effectively averts renal tubulointerstitial fibrosis by modulating TGF-β/Smad signaling, mitigating oxidative stress, and suppressing inflammatory responses (233). Another study demonstrated that treatment with DFO effectively suppressed renal injury and fibrosis by influencing ferroptosis induction in the remnant kidney following partial nephrectomy, thereby elucidating the underlying pathogenesis (234).

In a CKD rat model, DFX administration was found to alleviate renal fibrosis by suppressing the TGF-1/Smad3 signaling pathway, reducing inflammation, and mitigating oxidative stress (201). A subsequent study found that DFX and vitamin D3 co-therapy mitigates iron-induced renal injury in rats by effectively modulating cellular anti-inflammatory, anti-oxidative stress, and iron regulatory pathways (236).

Deferiprone (DFP), a novel iron chelator administered orally, has been shown to attenuate AKI by inhibiting ferroptosis in two studies: CIN in rats (235) and an aluminum chloride-induced AKI model in mice (237). A recent study found that combination therapy with DFP and DFO significantly enhanced renal protection compared to DFP alone (237).

Cysteine is the rate-limiting precursor in GSH biosynthesis. Studies have demonstrated that adding cystine or cysteine to cell culture media *in vitro* effectively inhibits ferroptosis (115). N-acetylcysteine (NAC) can improve the bioavailability of cysteine and contribute to positive outcomes in kidney disease (238). NAC could ameliorate CIN in mice by inhibiting the activation of kidney inflammation and the complement system, thereby exerting protective effects (239). Moreover, NAC treatment has effectively decreased circulating markers of oxidative stress and lowered serum creatinine levels. In addition, patients treated with NAC had a significantly lower incidence of severe acute kidney injury than those receiving a placebo (238, 240, 244). These findings strongly suggest that NAC holds promising clinical potential for kidney disease.

5.3 Other compounds that can affect kidney ferroptosis

Moreover, there are additional compounds that can effectively suppress kidney ferroptosis by targeting alternative molecular pathways. For example, our study shows that the novel third-generation ferrostatin (SRS 16-86) is more stable and potent than the first-generation compound ferrostatin-1. Ferrostatin-1 protects against IRI by inhibiting mitochondrial permeability transition and preventing postischemic and toxic renal necrosis in ferroptosis (120). Moreover, ACSL4 inhibitor rosiglitazone significantly improved renal function

inhibited kidney damage, and decreased inflammatory factors and immune cell infiltration in the kidney of the I/R-induced AKI mice (128). In streptozotocin (STZ) induced DN mice, the inhibition of NF-kB activation and MCP-1 expression by rosiglitazone has been shown to alleviate DN by preventing ferroptosis (241). However, rosiglitazone has been restricted due to adverse reactions in clinical trials (245). Furthermore, treatment with ciclopirox olamine (CPX-O), an iron chelator, suppressed ferritin accumulation in ADPKD and induced ferritinophagy in an iron-independent manner. Consequently, this led to a decrease in cyst growth in ADPKD mice (246). In a mouse model of RCC, it has been observed that L-buthionine-S and R-sulfoximine (BSO) can inhibit the GSH/GPX pathway, leading to the prevention of lipid peroxidation and ferroptotic cell death (210). Furthermore, artesunate effectively inhibits the growth of sunitinib-resistant RCC by inducing cell cycle arrest and ferroptosis (247). Furthermore, administering additional iron-binding compounds such as apotransferrin and neutrophil gelatinase-associated lipocalin has been demonstrated to mitigate the severity of renal injury following IRI (248, 249).

Although numerous drugs targeting ferroptosis have demonstrated promising outcomes in preclinical studies involving cells and animals, clinical trials for kidney disease remain scarce. Notably, DFO (NCT00870883, NCT04633889), DFP (NCT01146925, NCT01770652), and NAC (NCT01218178, NCT00780962) have entered clinical trials (244, 250, 251). Future strategies aimed at inhibiting ferroptosis must prioritize high specificity and selectivity to mitigate off-target effects and potential toxicity. Additionally, antioxidant-based approaches to inhibit ferroptosis may inadvertently impact other cell death pathways, such as apoptosis and necroptosis (176, 252).

6 Conclusion and future directions

Ferroptosis is a regulated cell death mediated by iron-dependent accumulation of lipid peroxidation. An increasing body of evidence substantiates ferroptosis's involvement in diverse kidney diseases. However, there are still some questions that need to be addressed.

Firstly, the crucial mechanisms that regulate kidney ferroptosis still need to be determined. Furthermore, the absence of specific biomarkers for ferroptosis has persisted for an extended period. Therefore, it is imperative to identify distinct markers that can accurately predict the occurrence of ferroptosis in kidney diseases. Thirdly, it has been discovered that various genes are involved in regulating ferroptosis. Therefore, there is an urgent need to develop effective strategies specifically targeting ferroptosis to prevent and treat kidney diseases associated with this process. Furthermore, despite the demonstrated improvement in renal function in animal models through selective inhibition of ferroptosis, there have been no clinical trials to date using ferroptosis-specific inhibitors for the treatment of kidney disease. Therefore, it is necessary to further investigate the effectiveness of these inhibitors through clinical trials. Finally, it is imperative to investigate the molecular mechanisms underlying the interplay between ferroptosis and other types of cell death in the progression of kidney diseases. This exploration holds promise for developing novel treatment strategies targeting diverse forms of cell death in kidney diseases.

In summary, ferroptosis is essential in the pathogenesis of kidney diseases. Excess-free reactive iron can result in tissue damage, thus leading to the widespread recommendation of iron chelation therapy for the treatment of patients with kidney disease associated with iron overload. Further study will propose an effective targeted-ferroptosis strategy for the treatment of kidney diseases.

Author contributions

WJW: Writing – review & editing, Data curation, Investigation, Project administration, Resources, Software, Validation, Visualization. JC: Investigation, Resources, Visualization, Writing – review & editing, Conceptualization. LZ: Conceptualization, Resources, Visualization, Writing – review & editing, Supervision, Project administration, Software, Validation. HZ: Formal Analysis, Resources, Visualization, Supervision, Writing – review & editing. LW: Formal Analysis, Resources, Data curation, Software, Writing – original draft. MG: Software, Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – review & editing. HG: Conceptualization, Visualization, Resources, Writing – original draft. JX: Conceptualization, Resources, Writing – original draft, Methodology. WW: Conceptualization, Methodology, Writing – original draft, Formal Analysis, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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IFNβ drives ferroptosis through elevating TRIM22 and promotes the cytotoxicity of RSL3

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Background: Cyclic GMP-AMP synthase (cGAS)-stimulator-of-interferon genes (STING) pathway is a cytosolic DNA sensor system. The production of this pathway, interferon-β (IFNβ), could suppress the growth of tumor cells, yet it is unclear whether ferroptosis is involved in IFNβ-induced cell death.

Methods: The effects of IFNβ on ferroptosis were analyzed in HT1080, 4T1, HCT116 and 786-O cells. HT1080 and 4T1 cells treated with IFNβ were subjected to RNA-Seq analysis. STAT1, STAT3, TRIM21, and TRIM22 were silenced by siRNAs to examine their effects on IFNβ-induced ferroptosis. The cGAS-STING signaling pathway-activated mice were used to evaluate the effects of IFNβ on ferroptosis *in vivo*. HT1080 cells, three-dimensional (3D) spheroids, and the xenograft mouse models were treated with IFNβ, RSL3, or IFNβ combination with RSL3 to analyze whether IFNβ enhances RSL3-induced ferroptosis.

Results: Here, we found that IFNβ could promote intracellular Fe^{2+} and lipid peroxidation levels, and decrease GSH levels in tumor cells. RNA sequencing data revealed that IFNβ induced a transcriptomic disturbance in ferroptosis-related genes. Knockdown of tripartite motif-containing 22 (TRIM22) suppressed the levels of intracellular Fe^{2+} and lipid ROS. It also reduced heme oxygenase (HMOX1) protein levels and increased ferroptosis suppressor protein 1 (FSP1) levels in HT1080 cells treated with IFNβ. Furthermore, our results illustrated that IFNβ enhanced the RAS-selective lethal 3 (RSL3)-induced ferroptosis and the inhibitory effect of RSL3 on GPX4. Meanwhile, compared to the groups treated with either IFNβ or RSL3 alone, the combination treatment of IFNβ and RSL3 significantly inhibited the growth of HT1080 three-dimensional (3D) spheroids and tumor in a mouse xenograft model.

Conclusions: Our work reveals a role for IFNβ in promoting ferroptosis and provides evidence that IFNβ could be used with RSL3 to increase cytotoxic effects in tumor cells.

KEYWORDS

IFNβ, ferroptosis, cGAS-STING, TRIM22, RSL3

1 Introduction

Ferroptosis is a recently discovered form of programmed cell death characterized by iron-dependent lipid peroxidation (1). Several small-molecule ferroptosis inducers promote cell death by depleting intracellular glutathione (GSH), directly targeting GPX4 activity or increasing intracellular Fe^{2+}, such as RSL3, which inhibits GPX4 activity and leads to the accumulation of intracellular lipid peroxide (2). Tumor cells are susceptible to ferroptosis inducers, as they have higher levels of Fe^{2+}, increasing the promise of these inducers to be applied in cancer therapy (3).

The cGAS-STING signaling pathway detects intra-cytoplasmic DNA in mammalian cells to trigger an innate immune reaction (4). Within this pathway, double-stranded DNA (dsDNA) activates the catalytic activity of cGAS. It induces the production of cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), a second messenger molecule and agonist of STING (5). cGAMP activates the endoplasmic reticulum transmembrane protein STING, then recruits TANK-binding kinase 1 (TBK1) to activate interferon regulatory factor 3 (IRF3) via phosphorylation. Activated IRF3 enters the nucleus to promote IFNβ expression (6). STING agonists or cGAMP have potent anti-tumor effects *in vivo* (7). However, whether the cGAS-STING signaling pathway acts against tumors by mechanisms other than enhancing natural immunity remains to be established.

IFNβ is the production of the cGAS-STING pathway (8). Besides antiviral activity, IFNβ could also reduce tumor growth by inducing anti-proliferative and apoptotic effects and promoting systemic immunity against the tumor targets (9). However, the role of ferroptosis in IFNβ-mediated cell death and tumor suppression has not been identified. The aims of this study were: 1) to verify whether IFNβ promotes ferroptosis in tumor cell lines; 2) to investigate which proteins play essential roles in IFNβ-mediated ferroptosis; 3) to observe whether IFNβ can synergize with classical ferroptosis inducer RSL3 to exert anti-tumor effects.

2 Materials and methods

2.1 Cell culture and treatment

HT1080 (human fibrosarcoma cell line) (cat number: CL-0117), 4T1 (mouse mammary tumor cell line) (cat number: CL-0007), HCT116 (human colorectal carcinoma cell line) (cat number: CL-0096), and 786-O (human clear cell renal carcinoma cell line) (cat number: CL-0010) were obtained from Procell Co., Ltd. (Wuhan, China). HT1080 and HCT116 were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific). 4T1 and 786-O were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific). All the cells were cultured at 37°C in a humidified incubator with 5% CO_2. To induce ferroptosis, 4T1 cells were treated with mouse IFNβ, while other cells were treated with human IFNβ at a concentration of 20 ng/mL (Sinobiological, China) for 48 hours. In the combination treatment, RSL3 (Med Chem Express, cat number: HY-100218A) (0.5 μM) was added to the cultures with 20 ng/mL IFNβ.

2.2 Cell viability assay

Cell viability was detected using a cell counting kit (CCK8) (MedChemExpress, cat number: HY-K0301). In brief, cells were seeded into 96-well cell plates and treated with IFNβ or other compounds the next day. After 48 h, CCK8 (10 μL/well) was added, and the cells were incubated for one hour. The optical density of each well was measured by a spectrophotometer (Multiskan GO, Thermo Fisher Scientific) at a wavelength of 450 nm. Six repeat wells were set for each group. To evaluate the rescue effect of ferroptosis inhibitor, liproxstatin-1 (10 μM) (MedChemExpress, cat number: HY-12726) was added to the cultures with 20 ng/mL IFNβ.

2.3 Lipid peroxide level assay

Cells were seeded and treated with IFNβ (20 ng/mL) or combined with RSL3 (0.5 μM) the next day. Lipid peroxides were detected by C11-BODIPY 581/591 (cat number: D3861, Thermo Fisher Scientific) 24 h later. The cells were incubated with a C11-BODIPY medium (10 μM) at 37°C for 30 min. FACScan (Becton Dickinson; FACSAriaII) measured the C11-BODIPY fluorescence intensity. The results were analyzed with FlowJo V10 software.

2.4 Intracellular iron assay

Intracellular Fe^{2+} levels were assessed using a FerroOrange (Fe^{2+} indicator) kit (cat number: F374, Dojindo, Japan). Cells were seeded in 6-well plates and treated with IFNβ (20 ng/mL), RSL3 (0.5 μM), or their combination the next day. FerroOrange (1 μM), dispersed in serum-free medium, was added to the cells 24 h later, and the cells were incubated for 30 min at room temperature and protected from light. The absorbance was subsequently observed by a fluorescence microscope or measured by flow cytometry. Fluorescence microscopy images were quantitatively analyzed using ImageJ software, examining six regions per sample. The images were converted to RGB format, and thresholding was applied to distinguish positively stained cells from the total cell population. Using ImageJ's "Analyze" menu and "Measure" function, we obtained AOD (% Area) values. The proportion of positive cells was determined by comparing AOD values between the experimental and control groups.

2.5 Viability staining

Live cells were stained with calcein AM showing green fluorescence, while dead cells were stained with propidium iodide (PI) showing red fluorescence. Following the kit instructions (cat number: C2015M, Beyotime, China), 3D spheroids were stained and photographed under a fluorescence microscope after 30 minutes. Fluorescence quantitative analysis of microscope images was performed using ImageJ software to analyze positively stained cells in 6 spheroids. The images were converted to RGB format, and thresholding was applied to distinguish positively stained cells from the total cell population. Using ImageJ's "Analyze" menu and "Measure" function, we obtained AOD (% Area) values. The proportion of positive cells was determined by comparing AOD values between the experimental and control groups.

2.6 GSH assay

According to the manufacturer's instructions, individual levels of GSH were measured using a GSH assay kit (Colorimetric) (cat number: ab239727). The optical density of each well at a wavelength of 450 nm was measured by a spectrophotometer (Multiskan GO, Thermo Scientific).

2.7 Cell transfection

For knockdown of STAT1, STAT3, TRIM21 and TRIM22, siRNAs or a non-targeting control siRNA were purchased from HippoBio (Huzhou, China). The selected siRNA sequences are listed in Supplementary Table S1. The cells were transfected with Lipofectamine RNAiMAX (cat number: 13778075, Thermo Fisher Scientific, Inc.) using 3 μL (30 pmol) of siRNA per well in a 6-well plate. Transfection was performed when the cell confluence reached 60-80% following the manufacturer's instructions.

2.8 Reverse-transcription quantitative polymerase chain reaction

Total cellular RNA was extracted using Total RNA Kit II (cat number: R6934, Omega Bio-Tek, Inc., Norcross, GA, USA). The RNA samples were treated with DNase. After RNA quantification, 3 μg of total RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit, following MIQE guidelines (10). PowerUp SYBR Green mix (cat number: A25741, Applied Biosystems, Thermo Fisher Scientific, Inc.) was used for qPCR. PCR was carried out for 40 cycles under the following conditions: 30 seconds at 94°C and 60 seconds at 58°C. Data were normalized to ACTB. The relative expression levels of genes were calculated according to 2-ΔΔCT methods (11). Primers used in the qPCR assays are shown in Supplementary Table S2.

2.9 RNA sequencing analysis

For RNA sequencing, total RNA was extracted from HT1080 and 4T1 cells treated with or without 20 ng/mL mouse IFNβ for 24 h. The enriched mRNA is then converted into complementary DNA (cDNA) through reverse transcription. Then, the cDNA is fragmented and adapters are ligated to its ends for library preparation. Following library preparation, fragment distribution and quantification performed to ensure optimal library quality. The library is sequenced using the Illumina Novaseq 6000 sequencing platform. Raw sequencing reads were demultiplexed using bcl2fastq software. Reads were aligned to the human or mouse reference genome. Differential expression analysis is performed using the DESeq2 library in the R statistical environment to identify genes with significant expression changes between IFNβ treated and untreated HT1080 or 4T1 cells. The enrichment analysis of differentially expressed genes (DEGs) was analyzed by Metascape (https://metascape.org).

2.10 Western blotting

The concentrations of proteins were quantified by a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific). Cellular proteins (15 μg/sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). PVDF membranes were blocked in a blocking solution (in PBS with 0.1% Tween 20 and 2% BSA) for 1 h and then incubated with primary antibodies at 4°C overnight. The membranes were washed and incubated with horseradish peroxidase (HRP)-labeled antibody at 37°C for 1 h. Immunostaining was developed using the enhanced chemiluminescence detection system (WesternBright ECL, Advansta) and recorded by Alliance Q9 Advanced (UVItec, ltd. Cambridge). All primary antibodies used in this study are listed in Supplementary Table S3.

2.11 Animal experiments

All mouse procedures in this study were approved by the Animal Care and Use Committee of the 900th hospital (approval number: 2021-025) on December 31, 2021. These experiments have been performed in accordance with the Basel Declaration (http://www.basel-declaration.org/basel-declaration/). To stimulate the cGAS-STING pathway in mouse models, C57 mice were injected with cGAMP (MedChemExpress, Shanghai, China) from the tail vein. Twelve male C57 mice aged 8 weeks were randomly divided into two groups (n = 6 per group): 1) vehicle control; 2) cGAMP-infused group (6 mg/kg). The mice received cGAMP every other day for four times.

Six-week-old male BALB/c nude mice were provided by the Shanghai Laboratory Animal Center (Shanghai, China). HT1080 cells (5×10^5) suspended in 100 μL PBS/Matrigel (1:1) were injected into the hind flank of the mice. Then, the mice were randomly divided into four groups (n = 5 per group): 1) vehicle control; 2) IFNβ (mouse IFNβ, 100 ng per mouse, administered by intra-tumor injection); 3) RSL3 (2.5 mg/kg, administered by intraperitoneal injection); 4) or IFNβ co-treatment with RSL3. The mice received drugs every other day for seven consecutive times. At the endpoints, tumor tissues were removed to analyze intracellular Fe^{2+}, lipid peroxidation, and GSH.

2.12 Immunohistochemical staining

Tumor tissues were fixed, embedded into the paraffin, and sectioned in standard procedure. After antigen retrieval and blocking, the slides were incubated with Ki67 antibody (1:800; Cell Signaling Technology) at 4°C overnight. Slides were washed and incubated with a secondary antibody (horseradish-peroxidase–conjugated, 1:500; ZSGB-BIO, Beijing, China). After DAB staining, the slides were counterstained with hematoxylin. Images were captured under a microscope (Olympus, BX43). Ki67-positive nuclei were quantified in five regions, with approximately 2000 cells analyzed per image using ImageJ. The images were converted to RGB stack format, and a threshold was set to distinguish between total nuclei and the positively stained nuclei. The measurements were obtained by selecting the "Analyze" menu in ImageJ and choosing the "Measure" option, which provided the AOD (%Area) value. The AOD value of Ki67-positive nuclei was then compared to the total nuclei AOD value to calculate the percentage of Ki67-positive cells.

2.13 Statistical processing

The data obtained are presented as mean ± S.D. Statistical significance was measured by oneway ANOVA (for more than two comparisons) and Student's t-test (comparison of two groups) (*P* values). *, $P < 0.05$; **, $P < 0.01$; n.s., non-significant. All the statistical graphs were performed using GraphPad Prism software version 8.0. The results of *in vitro* experiments were collected from at least three independent biological replicates.

3 Results

3.1 IFNβ induces tumor cell death partially through ferroptosis

Human HT1080 fibrosarcoma cells and murine 4T1 breast tumor cells were treated with IFNβ for 48 h. We observed IFNβ increased cell death in both cell lines (Figures 1A, B). Figure 1C shows that the ferroptosis inhibitor liproxstatin-1 reduced the cytotoxic effect of IFNβ in HT1080 and 4T1 cells. Then, we quantified the iron level in HT1080 and 4T1 cells using a Fe^{2+} iron probe named FerroOrange. Consistent with our expectation, IFNβ increased the intracellular Fe^{2+} concentrations within a dose-dependent manner (Figure 1D). Flow cytometric results also demonstrated an increment of Fe^{2+} in IFNβ-treated HT1080 and 4T1 cells (Figure 1E). Moreover, IFNβ treatment enhanced lipid peroxidation as assessed by the lipid peroxydation sensor BODIPY-C11 (Figures 1F, Supplementary Figure S1), but decreased glutathione (GSH) levels to 40% in HT1080 and 4T1 cells (Figure 1G). Human renal cell carcinoma cell line 786-O and colon cancer cell line HCT116 cells were treated with IFNβ for 48 h. IFNβ treatment increased cell death, intracellular Fe^{2+} and lipid peroxidation levels (Supplementary Figure S2). Transmission electron microscopy analysis revealed that IFNβ significantly induced mitochondrial cristae disappearance and outer membrane rupture in 4T1 and HT1080 cells (Supplementary Figure S3). Collectively, these findings suggested that IFNβ induced ferroptosis in tumor cells.

3.2 IFNβ treatment stimulates ferroptosis regulators

To further decipher the molecular mechanism that may account for IFNβ stimulation of ferroptosis, we performed whole-transcriptome sequence analysis (RNA-seq) to detect the gene expression changes after IFNβ treatment. IFNβ exposure caused significant alterations in gene expression compared to untreated HT1080 cells (Figure 2A). A set of ferroptosis-related genes was obtained from the FerrDb database (http://www.zhounan.org/ferrdb/current/). We identified 25 ferroptosis-related differentially expressed genes (DEGs) (Figure 2B, Supplementary Table S4), among which 21 were upregulated, four were downregulated in HT1080 cells (Figure 2C). Similar results were identified in 4T1 cell (Supplementary Figure S4A), IFNβ changed 18 ferroptosis-related genes (Supplementary Figure S4B, Supplementary Table S5), among which 16 were upregulated, two were downregulated (Supplementary Figure S4C). RT-qPCR assays proved that PML, PTGS2, CHAC1, PARP9, PARP12, PARP14, ATF3 and TRIM21 were upregulated in both IFNβ-treated HT1080 and 4T1 cells (Figures 2D, E). Western blot analysis demonstrated that IFNβ treatment led to rapid activation of signal transducer and activator of transcription 1 (STAT1) and STAT3 in both cell lines, as evidenced by enhanced phosphorylation levels within 30 minutes of exposure. Furthermore, IFNβ exposure resulted in upregulated protein expression of several key factors, including COX2, ATF3,

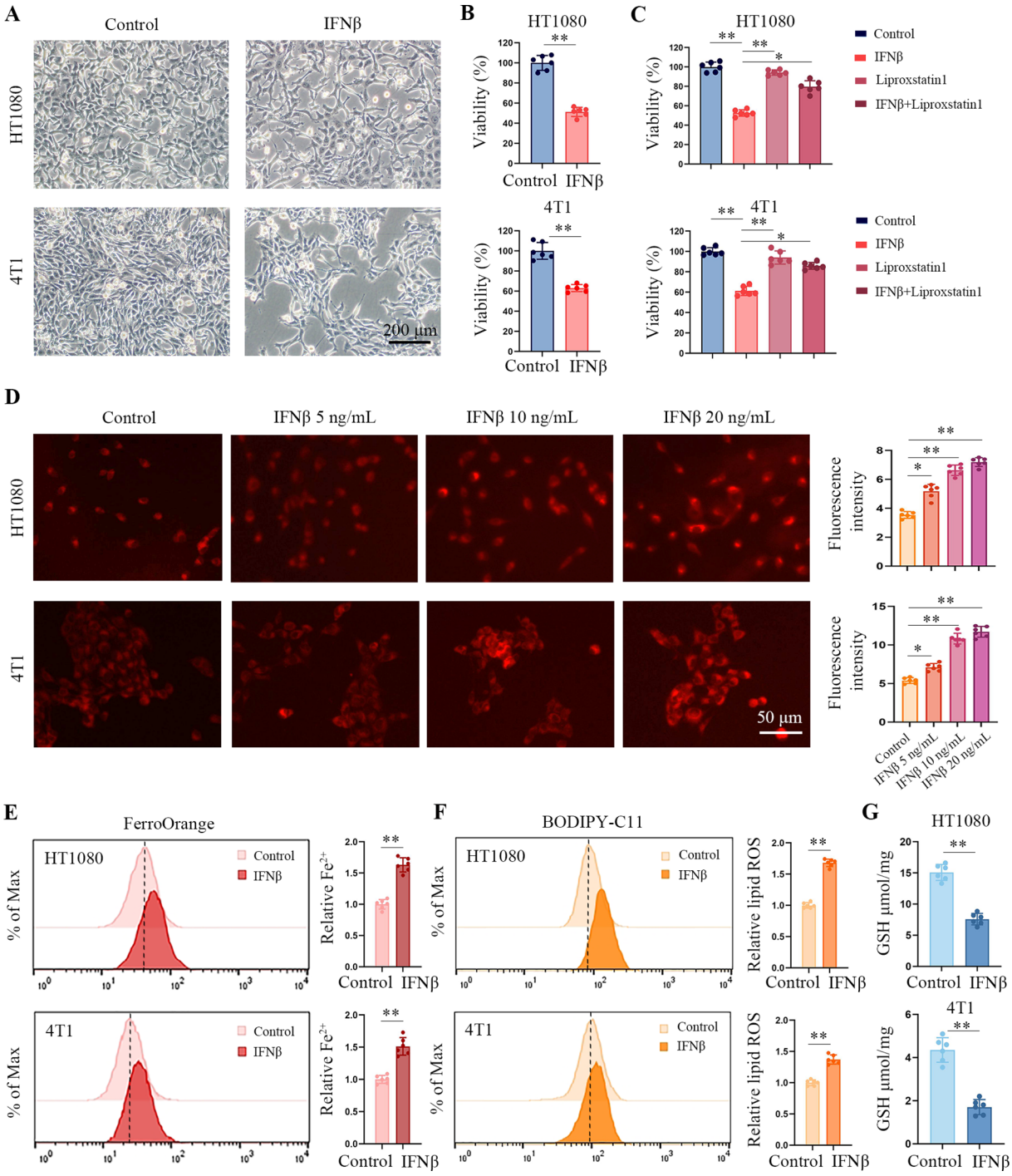

FIGURE 1
IFNβ treatment induces ferroptosis in tumor cells. **(A)** Representative images by inverted light microscopy show morphology changes in HT1080 and 4T1 cells treated with IFNβ (20 ng/mL) for 48 h. Scale bar: 200 μm. One representative experiment of three independent experiments is shown. **(B)** The CCK-8 assay determined the inhibitory effects of IFNβ (20 ng/mL). Data indicated as mean ± S.D. (n = 6 replicates). One representative experiment of three independent experiments is shown. **(C)** CCK-8 assay showing the response of HT1080 and 4T1 cell lines to IFNβ (20 ng/mL) with or without liproxstatin-1 (10 μM) for 48 h. Data indicated as mean ± S.D. (n = 6 replicates). One representative experiment of three independent experiments is shown. **(D)** Representative images show intracellular Fe^{2+} levels in HCT1080 and 4T1 cells treated with various concentrations of IFNβ (0 ng/mL, 5 ng/mL, 10 ng/mL, and 20 ng/mL) for 24 h. Scale bar: 50 μm. Data indicated as mean ± S.D. (n = 6 experiments). **(E, F)** Flow cytometric analysis of intracellular Fe^{2+} and lipid peroxydation levels in HCT1080 and 4T1 cells treated with or without 20 ng/mL IFNβ for 24 h. Data indicated as mean ± S.D. (n = 6 experiments). **(G)** GSH levels of HCT1080 and 4T1 cells primed with or without 20 ng/mL IFNβ for 24 h. Data indicated as mean ± S.D. (n = 6 experiments). Statistical significance relative to mock conditions are indicated as $^*P < 0.05$, $^{**}P < 0.01$.

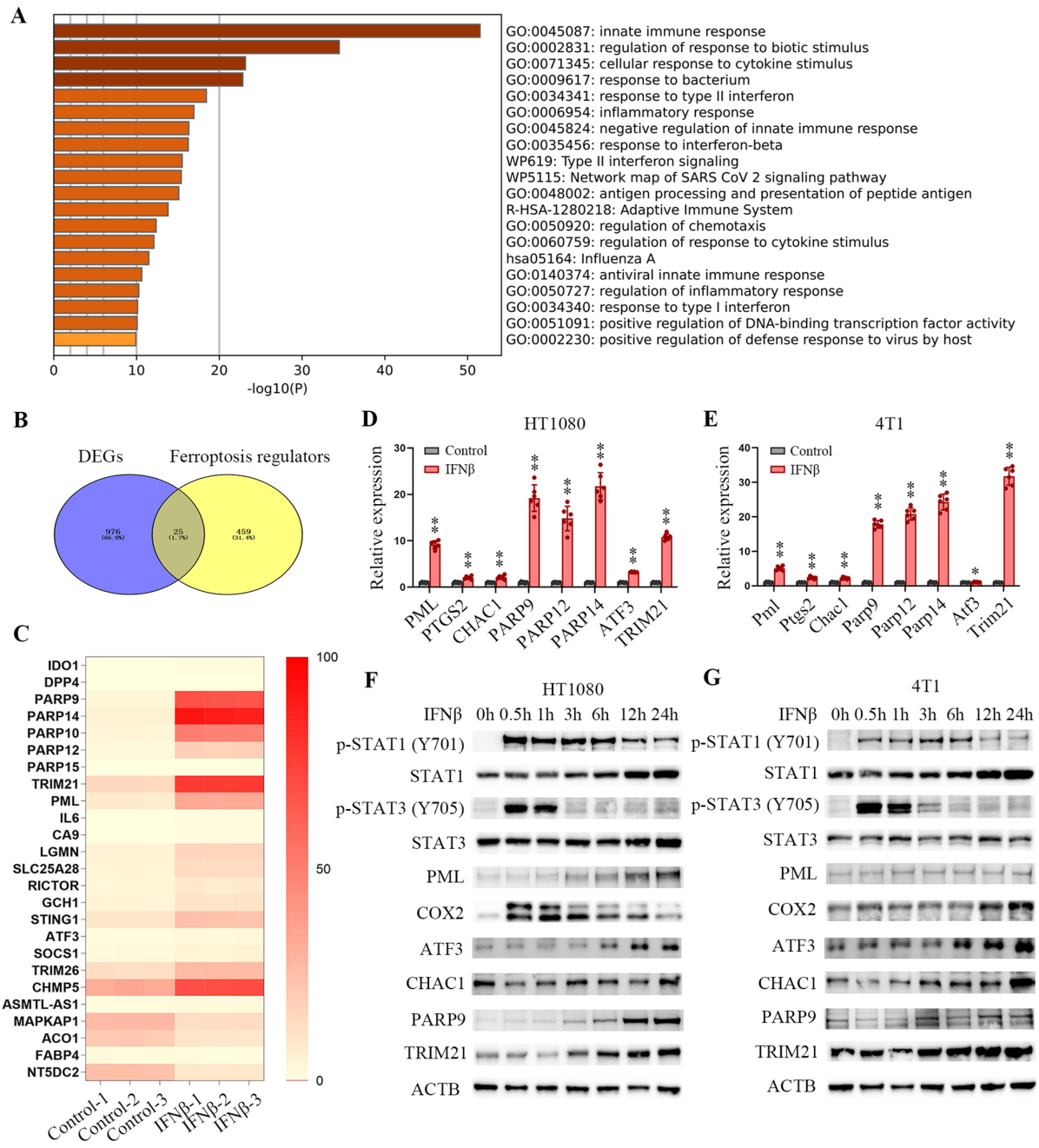

FIGURE 2
IFNβ treatment changes ferroptosis-related gene expression. **(A)** Bar graph of functional enrichment analysis by Metascape. **(B)** Venn diagrams showing the differentially expressed genes (DEGs) that overlapped among ferroptosis regulators. **(C)** Heatmap of ferroptosis regulators detected by the RNA-seq. The heatmap shows a fold-change of ferroptosis genes that range from zero to 100 between IFNβ untreated and treated HT1080 cells (n = 3 samples). **(D, E)** mRNA levels of ferroptosis regulators normalized to ACTB and determined by RT-qPCR. Bar plots represent the fold-change of each gene relative to mRNA levels from IFNβ-untreated HT1080 or 4T1 cells. Data indicated as mean ± S.D. (n = 6 experiments). **(F, G)** Representative images of western blotting analysis show the protein levels of STAT1, STAT3, PML, COX2, ATF3, CHAC1, PARP9, TRIM21 and phosphorylation status of STAT1 and STAT3 in HT1080 and 4T1 cells treated with 20 ng/mL IFNβ for the indicated time points. Statistical significance relative to mock conditions is indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

CHAC1, PARP9, and TRIM21 (Figures 2F, G). These findings suggest that besides the immune response genes, IFNβ disturbed several ferroptosis-related gene expressions in tumor cells.

To investigate the ferroptosis effect induced by IFNβ *in vivo*, we treated the mice with cGAMP to stimulate the cGAS-STING pathway. ELISA analysis shows cGAMP induced the amount of IFNβ in serum (Supplementary Figure S5A). As the heart is sensitive to ferroptosis (12), we focused on cardiac tissues to investigate the influence of IFNβ. We found that cGAMP-injection promoted the expression level of Ifnb1 in the heart (Supplementary Figure S5B). The activation of STAT1/STAT3 pathways suggested that cardiac cells were attacked by IFNβ (Supplementary Figure S5C). Compared

to non-injected mice control, cGAMP treatment enhanced intracellular Fe^{2+} and lipid peroxidation levels (Supplementary Figures S5D, E) and significantly decreased GSH levels in heart tissues (Supplementary Figure S5F). Moreover, cGAMP-infusion induced overexpression of ferroptosis-related genes in the heart (Supplementary Figure S5G). These results supported the notion that systemic activation of the cGAS-STING pathway could induce ferroptosis.

3.3 Inhibition of TRIM22 mitigates IFNβ-mediated ferroptosis

Signal transducers and activators of transcriptions (STATs) play a crucial role in the cellular response to IFNβ. Recent studies have described how STAT1 and STAT3 participate in ferroptosis, but their roles need to be more consistent (13–15). We observed that STAT1 knockdown did not affect Fe^{2+} levels and lipid peroxydation accumulation in IFNβ-treated HT1080 cells (Figures 3A–D). STAT3 inhibition upregulated the level of intracellular Fe^{2+} and the product of lipid peroxidation and reduced cell viability in HT1080 cells (Figures 3E–H). These results suggested that inhibition of STAT1 did not affect IFNβ-mediated ferroptosis, and inhibition of STAT3 even enhanced ferroptosis in HT1080 cells.

Tripartite motif-containing (TRIM) family proteins play a crucial role in regulating various substrates and signaling pathways. Previous studies show that TRIM21 is involved in ferroptosis (16, 17). Hence, we supposed TRIM21 activity was required for IFNβ-induced ferroptosis. However, TRIM21 deficiency did not affect Fe^{2+} levels or lipid peroxydation accumulation in IFNβ-treated HT1080 cells (Figures 4A–D). TRIM22 is another member of the IFNβ-induced protein and a transcriptional target of TP53 (18). We found that the expression of TRIM22 was enhanced in IFNβ-treated HT1080 cells (Figure 4E). TRIM22 was not detected in 4T1 cells because TRIM22 is not expressed in mice. TRIM22 depletion reduced intracellular Fe^{2+} levels and lipid peroxydation accumulation in HT1080 cells (Figures 4F–I). Western blotting demonstrated that TRIM22 inhibition significantly decreased heme oxygenase (HMOX1) protein levels, while increasing the protein levels of ferroptosis suppressor protein 1 (FSP1, also known as AIMF2), both of which are regulatory genes of ferroptosis (Figure 4J). Besides TRIM proteins,

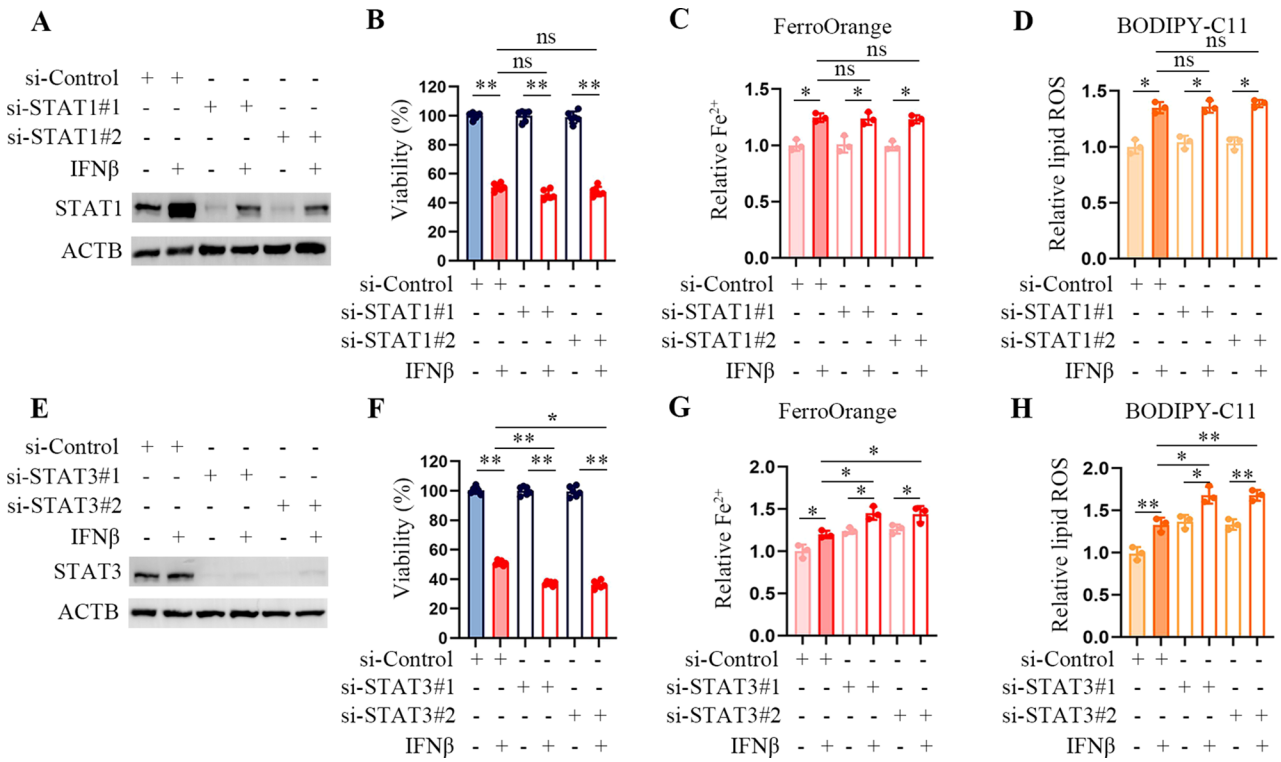

FIGURE 3
STAT3 downregulation enhances IFNβ-mediated ferroptosis. **(A)** Representative images of western blotting analysis of STAT1 in IFNβ untreated and treated HT1080 cells transfected with control or STAT1 siRNAs. **(B)** CCK-8 assay of HT1080 cells transfected with control or STAT1 siRNAs in the presence or absence of 20 ng/mL IFNβ for 48 h. Data indicated as mean ± S.D. (n = 6 replicates). One representative experiment of three independent experiments is shown. **(C, D)** Flow cytometric analysis of intracellular $Fe2^{+}$ and lipid ROS levels in HCT1080 cells transfected with control or STAT1 siRNAs followed by treatment with or without IFNβ for 24 h. Data indicated as mean ± S.D. (n = 3 experiments). **(E)** Representative images of western blotting analysis of STAT3 in IFNβ untreated and treated HT1080 cells which transfected with control or STAT1 siRNAs. **(F)** CCK-8 assay of HT1080 cells transfected with control or STAT3 siRNAs in the presence or absence of 20 ng/mL IFNβ for 48 h. Data indicated as mean ± S.D. (n = 6 replicates). One representative experiment of three independent experiments is shown. **(G, H)** Flow cytometric analysis of intracellular Fe^{2+} and lipid peroxydation levels in HCT1080 cells transfected with control or STAT3 siRNAs followed by treatment with or without IFNβ for 24 h. Data indicated as mean ± S.D. (n = 3 experiments). Statistical significance relative to mock conditions is indicated as ns, non-significant, $*P < 0.05$, $**P < 0.01$.

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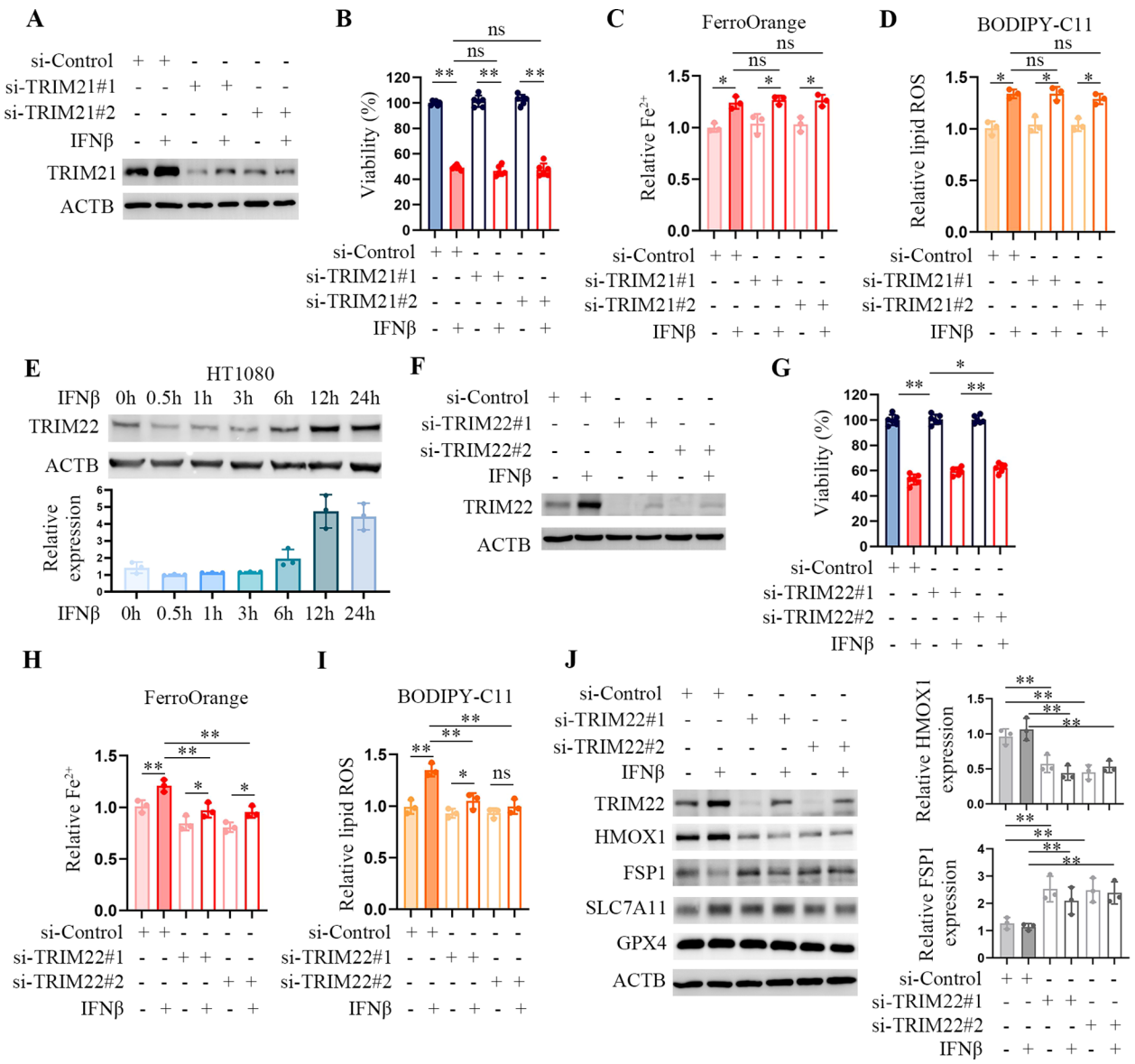

FIGURE 4
TRIM22 downregulation mitigates IFNβ-mediated ferroptosis. **(A)** Representative images of western blotting analysis of TRIM21 in IFNβ-treated or untreated HT1080 cells transfected with control or TRIM21 siRNAs. **(B)** CCK-8 assay of HT1080 cells transfected with control or TRIM21 siRNAs in the presence or absence of 20 ng/mL IFNβ for 48 h. Data indicated as mean ± S.D. (n = 6 replicates). One representative experiment of three independent experiments is shown. **(C, D)** Flow cytometric analysis of intracellular Fe^{2+} and lipid ROS levels in HCT1080 cells transfected with control or TRIM21 siRNAs followed by treatment with or without IFNβ for 24 h. Data indicated as mean ± S.D. (n = 3 experiments). **(E)** Representative images of western blotting analysis showing the levels of TRIM22 in HT1080 cells treated with 20 ng/mL IFNβ for the indicated time points. **(F)** Representative images of western blotting analysis of TRIM22 in HT1080 cells transfected with control or TRIM22 siRNAs in the presence or absence of IFNβ for 24 h. **(G)** CCK-8 assay of HT1080 cells transfected with control or TRIM22 siRNAs in the presence or absence of 20 ng/mL IFNβ for 48 h. Data indicated as mean ± S.D. (n = 6 replicates). One representative experiment of three independent experiments is shown. **(H, I)** Flow cytometric analysis of intracellular Fe^{2+} and lipid ROS levels in HCT1080 cells transfected with control or TRIM22 siRNAs followed by treatment with or without IFNβ for 24 h. Data indicated as mean ± S.D. (n = 3 experiments). **(J)** Representative images of western blotting analysis showing the protein levels of TRIM22, HMOX1, FSP1, SLC7A11 and GPX4 in HCT1080 cells transfected with control or TRIM22 siRNAs followed by treatment with or without IFNβ for 24 h. Statistical significance relative to mock conditions is indicated as ns, non-significant, *$P < 0.05$, **$P < 0.01$.

we individually knocked down PML, PARP9, PTGS2, CHAC1, and ATF3 in HT1080 through siRNA silencing. We observed that depletion of these proteins did not affect or enhance intracellular Fe^{2+} levels and lipid peroxydation accumulation in IFNβ-treated HT1080 cells. These results indicate that TRIM22 plays an essential role in IFNβ-mediated ferroptosis.

3.4 IFNβ enhances RSL3-induced ferroptosis in HT1080 cells

Our data shows that IFNβ only induces ferroptosis up to a certain amount. We wondered if IFNβ could augment the cytotoxicity of the ferroptosis inducer. HT1080 cells were treated with IFNβ, RSL3, or

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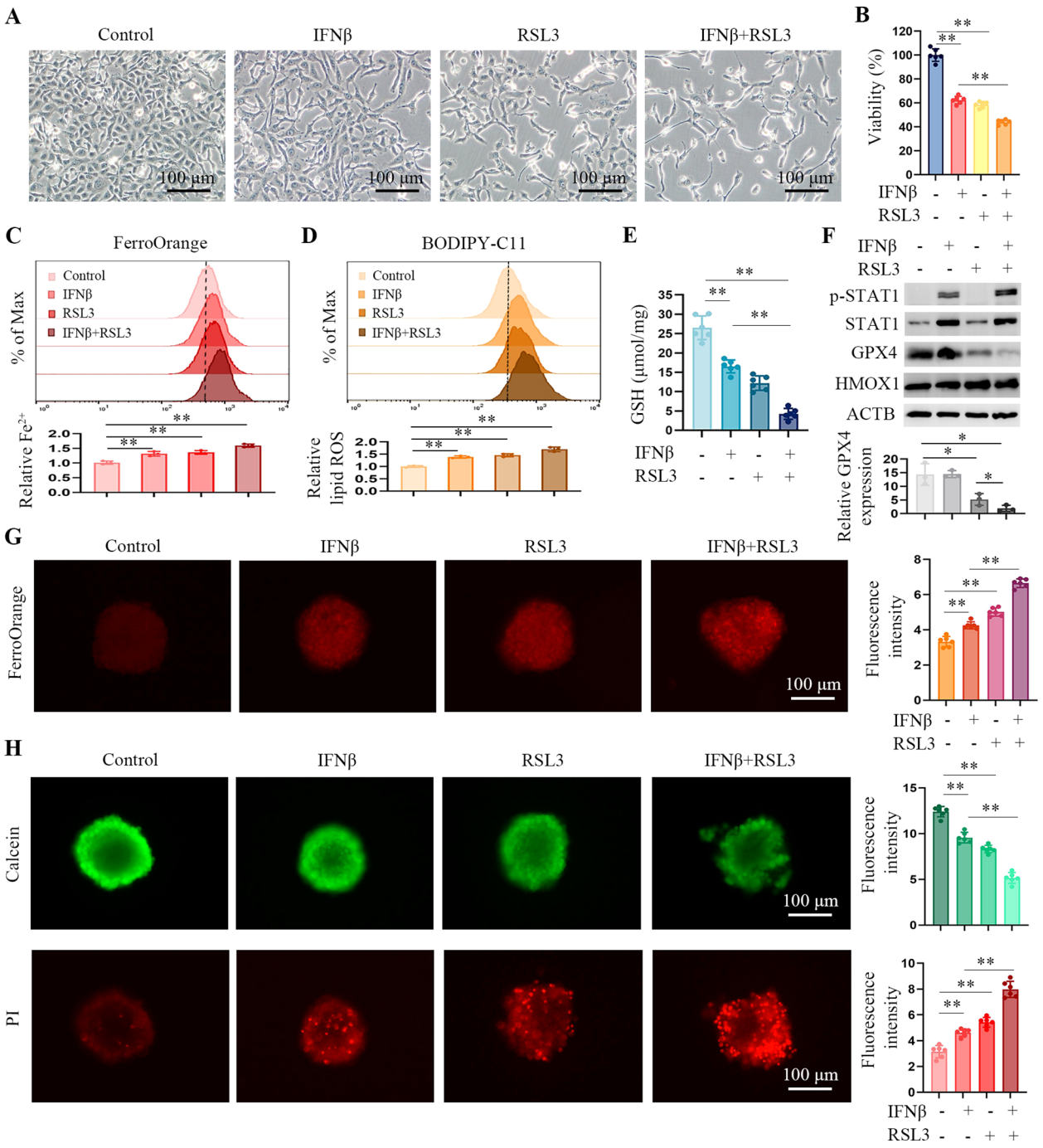

FIGURE 5
IFNβ treatment enhances RSL3-induced ferroptosis in HT1080 cells. **(A)** Representative images by inverted light microscopy showing morphology changes in HT1080 cells treated with IFNβ (20 ng/mL), RSL3 (0.5 μM), or their combination for 48 h. Scale bar, 100 μm. One representative experiment of three independent experiments is shown. **(B)** CCK-8 assay showing the response of HT1080 cells to IFNβ or RSL3 or their combination for 48 h. Data indicated as mean ± S.D. (n = 6 replicates). One representative experiment of three independent experiments is shown. **(C, D)** Flow cytometric analysis of intracellular Fe^{2+} and lipid ROS levels in HCT1080 cells treated with IFNβ, RSL3, or their combination for 24 h. Data indicated as mean ± S.D. (n = 3 experiments). **(E)** Levels of GSH in HCT1080 cells treated with IFNβ, RSL3, or their combination for 24 h. Data indicated as mean ± S.D. (n = 6 experiments). **(F)** Representative images of western blotting analysis showing the protein levels of STAT1, GPX4 and HMOX1 in HCT1080 cells treated with IFNβ, RSL3, or their combination for 24 h. **(G)** Representative images by immunofluorescence showing intracellular Fe^{2+} levels in HT1080 spheroids treated with IFNβ, RSL3, or their combination for 6 h. Bar plots represent the fluorescence intensity of five spheres in one experiment. Data indicated as mean ± S.D. (n = 6 experiments). **(H)** Representative images of HT1080 spheroids stained with Calcein-AM (green) and PI (red). Data indicated as mean ± S.D. (n = 6 experiments). Statistical significance relative to mock conditions is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$.

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IFNβ combination with RSL3. Compared with IFNβ alone, the combination treatment enhanced the death of HT1080 cells (Figures 5A, B). Furthermore, the combination treatment increased intracellular Fe^{2+} (Figure 5C) and lipid peroxidation (Figure 5D). The GSH levels were significantly lower in the two compounds' co-treated cells (Figure 5E). GPX4 is targeted by the ferroptosis inducer RSL3 (2). Figure 5F demonstrates that while IFNβ treatment alone did not significantly affect GPX4 levels, simultaneous exposure to IFNβ and RSL3 further inhibited GPX4 levels compared to RSL3 treatment alone. This suggests that IFNβ may enhance RSL3-induced ferroptosis, possibly due to the increased inhibition of GPX4. Moreover, we observed similar results in 786 cells, IFNβ enhanced the ferroptosis inducing effect of RSL3 (Supplementary Figure S6).

In vitro, 3D cancer cell culture exhibits mimic profiles to solid tumors. 3D spheroids are more appropriate for cancer drug screening (19). Therefore, we used ultra-low attachment round-bottom 96-well plates to develop HT1080 3D spheroids. Intracellular iron levels were determined in HT1080 spheroids by using a FerroOrange probe. The spheroids co-treated with IFNβ and RSL3 had stronger fluorescence than those treated with IFNβ or RSL3 alone (Figure 5G). To further confirm the proportion of live/dead cells in the cell spheres, we stained spheroids with calcein-AM (green) and PI (red). As shown in Figure 5H, compared to the IFNβ or RSL3-treated spheroids, co-treated spheroids caused higher levels of PI (dead cells) and lower calcein-AM (live cells) signal. These findings suggest that following treatment with IFNβ and RSL3 increased ferroptosis in HT1080 cell lines.

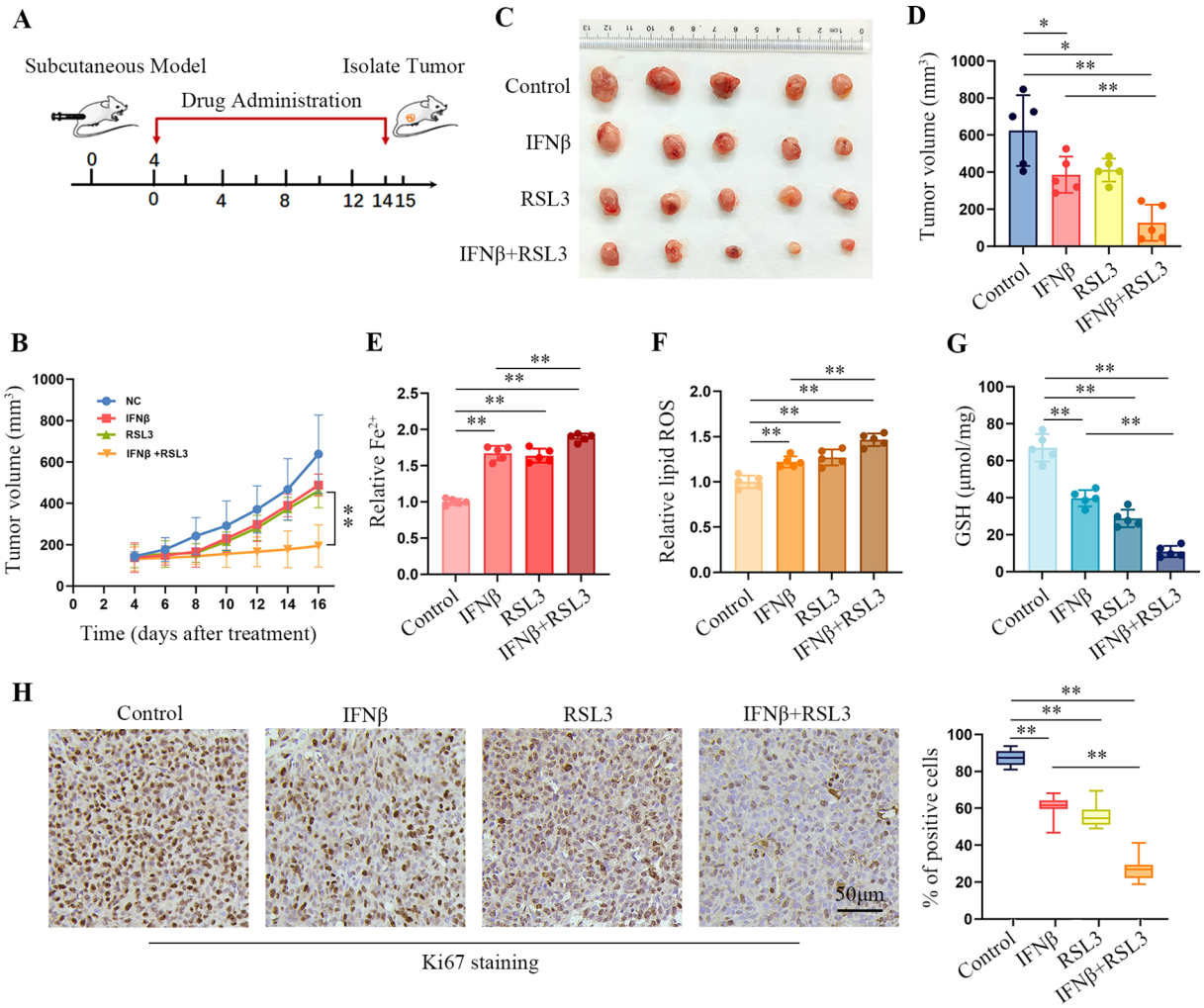

FIGURE 6
IFNβ enhances the toxicity of RSL3 in HT1080-derived xenograft tumors. **(A)** Schematic of the anti-tumor effect of the combination of IFNβ (100 ng per mouse) and RSL3 (2.5 mg/kg) in an HT1080 subcutaneous tumor model. **(B)** Growth curves of the xenograft tumors in BALB/c nude mice (n = 5 mice per group). **(C)** Representative images of HT1080 xenograft tumors in BALB/c nude mice injected with IFNβ, RSL3, or their combination. **(D)** Tumor volume of HT1080 xenograft tumors in BALB/c nude mice injected with IFNβ, RSL3, or their combination. n = 5 mice per group, mean ± SD. **(E–G)** Levels of intracellular Fe^{2+}, lipid ROS, and GSH in HT1080 xenograft tumors treated with IFNβ, RSL3, or their combination. n = 5 mice per group, mean ± SD. **(H)** Representative image of Ki67 immunohistochemical staining in the HT1080 xenograft tumor tissues treated with IFNβ, RSL3, or their combination. Scale bars: 50 μm. n = 5 mice per group. Statistical significance relative to mock conditions is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$.

3.5 Combination treatment with IFNβ and RSL3 promotes ferroptosis in the HT1080 xenograft nude mouse model

We established an HT1080 xenograft nude mouse model to explore whether IFNβ and RSL3 combination treatment *in vivo* enhanced ferroptosis. Figure 6A shows the schematic of tumor inoculation and systemic injection. All the mice survived after cell implantation and drug injection. After 16 days, the tumor size gradually increased in the control group. However, administration of IFNβ, RSL3, or IFNβ in combination with RSL3 led to a decrease in tumor size (Figure 6B). Moreover, combination treatment improved the efficacy of RSL3 in suppressing tumor growth (Figures 6C, D). In addition, we found that co-treatment significantly enhanced the intracellular Fe^{2+} and lipid peroxydation levels (Figures 6E, F) but decreased the amount of GSH compared to IFNβ or RSL3 administration (Figure 6G). Immunohistochemical staining assays also show lower levels of Ki67 in the co-treatment group (Figure 6H). These results further support that combination treatment with IFNβ and RSL3 increased HT1080 ferroptosis.

4 Discussion

The cGAS–STING pathway implicates host defense, inflammation, and tumor immunity (4, 7, 20). Previous studies have reported that this pathway also links to cell death processes, including apoptosis, pyroptosis, and necroptosis (21). Our study found that the cGAS–STING pathway produced IFNβ sensitized tumors to ferroptosis. IFNβ induced intracellular Fe^{2+} and lipid peroxydation levels while decreasing GSH levels in cancer cells.

Cytokines, such as interferons (IFNs), interleukin 6 (IL-6), and platelet-derived growth factor (PDGF), could activate the STAT1/STAT3 pathway (22). IFNβ binds to a heterodimeric receptor consisting of IFNα receptor 1 (IFNAR1) and IFNAR2, and then engages the JAK kinases to phosphorylate STAT1/STAT3 (23). Our study used the activation of the STAT1/STAT3 pathway to indicate IFNβ exposure. STAT1 has been reported to be a ferroptosis inducer protein, which induces the expression of cyclooxygenase-2 (COX-2) (14). In contrast, STAT1 inhibition observed here did not affect IFNβ-mediated intracellular Fe^{2+} and lipid peroxydation level increment. Pharmacological or genetic inhibition of STAT3 could block erastin-induced ferroptosis in pancreatic ductal adenocarcinoma (PDAC) cells (24). However, Ouyang et al. reported that knockdown of STAT3 promotes ferroptosis in gastric cancer cells (13). Our data show that STAT3 inhibition could enhance IFNβ-mediated ferroptosis in HT1080 cells. These findings are in line with Ouyang colleagues, but the modest difference may also suggest that the impact of STAT3 on ferroptosis isn't as clear, and more data is still needed to confirm.

Most TRIM family proteins have E3 ubiquitin ligase activities (25). IFNs have been reported to stimulate several TRIM genes, such as PML, TRIM8, TRIM21, and TRIM22 (26). Our data showed that IFNβ treatment significantly stimulated TRIM21 expression. TRIM21 depletion has been reported to suppress doxorubicin-induced ferroptosis through mediating activation of the p62-Keap1-NRF2 antioxidant pathway (27). We silenced the expression of TRIM21 and found that TRIM21 silencing did not affect IFNβ-mediated intracellular Fe^{2+} and lipid peroxydation level increment.

Like TRIM21, TRIM22 is induced by IFNβ. We demonstrated that IFNβ strongly induced the expression of TRIM22. Knockdown TRIM22 reduced IFNβ-induced intracellular Fe^{2+} levels, lipids oxidation rates, and HMOX1 protein levels. However, it led to an increase in FSP1 protein levels. HMOX1 plays a role in iron and ROS homeostasis. Excessive activation of HMOX1 led to heme degradation and the release of free iron (28, 29). FSP1 is an inhibitor of ferroptosis (30, 31). Ubiquitination modification is one of the regulatory mechanisms of FSP1 (32). The ubiquitin ligase TRIM22 could possibly be involved in maintaining the stability of the FSP1 protein. Although further research is needed to clarify how TRIM22 was upregulated by IFNβ and the role of TRIM22 in HMOX1 and FSP1 regulation, we hypothesized that TRIM22 served as a regulator of IFNβ-mediated ferroptosis.

RSL3 is a known ferroptosis inducer that reduces GPX4 activity and inhibits the clearance capacity of lipid peroxides (33). Our results demonstrate that the combination treatment of IFNβ and RSL3 leads to increased intracellular Fe^{2+} levels and lipid peroxidation in HT1080 cells, both *in vitro* and *in vivo*. In addition, IFNβ can enhance the effect of RSL3 in reducing GPX4 levels. These findings suggest that IFNβ enhances the cytotoxic effect of ferroptosis-inducing compounds, providing a strong rationale for considering IFNβ in cancer therapy. Future research should focus on monitoring and evaluating how IFNβ enhances the cytotoxic effect of other ferroptosis inducers. Furthermore, beyond ferroptosis, IFNβ triggers apoptosis as a cell death mechanism, as confirmed by previous studies (9). While our investigation specifically examined the ferroptotic pathway induced by IFNβ, we acknowledge the scope for exploring additional cell death mechanisms. Future therapeutic strategies may benefit from combining IFNβ with both ferroptosis inducers and apoptosis-promoting agents to enhance tumor treatment efficacy.

5 Conclusion

In summary, our study reveals that IFNβ promotes ferroptosis in cancer cells. IFNβ disturbs the expression of several ferroptosis-related genes. TRIM22 inhibition moderates IFNβ-mediated ferroptosis. In addition, we report that IFNβ enhances RSL3-induced ferroptosis. These findings expand the knowledge on IFNβ role in cancers.

Data availability statement

The data presented in this study are deposited in the GEO repository under accession number GSE288379.

Ethics statement

The animal study was approved by the Animal Care and Use Committee of the 900th hospital. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HD: Investigation, Methodology, Resources, Writing – original draft. LZ: Investigation, Methodology, Validation, Writing – original draft. JS: Data curation, Investigation, Writing – original draft. QC: Validation, Writing – review & editing. PL: Methodology, Resources, Writing – review & editing. WZ: Validation, Writing – review & editing. HZ: Validation, Writing – review & editing. RL: Validation, Writing – review & editing. ZY: Formal analysis, Visualization, Writing – review & editing. JL: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1535554/full#supplementary-material

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Exosomes secreted by ATF3/Nrf2-mediated ferroptotic renal tubular epithelial cells promote M1/M2 ratio imbalance inducing renal interstitial fibrosis following ischemia and reperfusion injury

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Background: Severe renal ischemia and reperfusion injury (IRI) progresses to renal interstitial fibrosis (RIF) with limited therapeutic strategies. Although ferrptosis and macrophage polarization both play important roles in this model, their specific pathogenesis and interactions have not been elucidated. Therefore, we aimed to explore the mechanisms by which ferrotosis occurs in renal tubular epithelial cells (RTECs) and ferroptotic cell-derived exosomes induce macrophage polarization in IRI-related RIF model.

Methods: *In vivo*, C57BL/6J mice were randomly divided into four groups: sham group, ischemia and reperfusion (IR) group, IR + Ferrostatin-1 (Fer-1) group, and IR +ATF3 knockdown (ATF^{KD}) group. *In vitro*, RTECs were divided into control (CON) group, hypoxia/reoxygenation (HR) group, HR +Fer-1 group, HR + siRNA-ATF3 (siATF3) group.

Result: Compared with the sham group, the IR group showed more severe kidney injury in HE staining, more collagen fibers in Masson staining, and higher α-SMA expression levels in immunohistochemistry. Total iron and MDA content increased while GSH content decreased. The IR group had more significant mitochondrial damage and higher PTGS2 and TFRC mRNA levels than those in the sham group. Compared with the IR group, the above indexes were all alleviated in the IR+Fer-1 or IR+$ATF3^{KD}$ groups. In addition, the protein expressions of ATF3, Nrf2 and HO-1 in the IR group were increased than those in sham group. Compared with the IR group, ATF3 expressions in the IR+Fer-1 or IR+$ATF3^{KD}$ groups were decreased, and the protein contents of Nrf2 and HO-1 were further increased. Moreover, there were higher levels of M2 markers (Arg1, TGF-β and IL-10 mRNA) in the IR group than those in the sham group, and lower levels in the IR+Fer-1 group or in the IR+$ATF3^{KD}$ group compared with the IR group. The results of *in vitro* experiment are consistent with those of *in vivo* experiment. Mechanistically, the release of exosomes carrying miR-1306-5p by the HR group promoted more M2 macrophage.

Conclusion: ATF3 might accelerate the ferroptosis by inhibiting Nrf2/ARE pathway, and exosomes from ferroptotic cells reduced the M1/M2 macrophage ratio, promoting fibrosis.

KEYWORDS

ischemia and reperfusion, renal interstitial fibrosis, ferroptosis, activation transcription factor 3, exosome, macrophages

1 Introduction

Severe renal ischemia and reperfusion injury (IRI) is a risk factor for the development and progression of chronic kidney disease (CKD) and end-stage renal disease, which will further increase the global morbidity and mortality (1). Long-term IRI causes persistent non-specific inflammation, drives immune cell infiltration and cytokine release, and causes excessive accumulation of extracellular matrix, which leads to irreversible renal interstitial fibrosis (RIF) and decreases the long-term survival rate of transplanted kidney (2, 3). Persistent inflammation and RIF are two pathophysiological processes involved in the maladaptive repair process of IRI to CKD transformation (4, 5). During IRI-related RIF, severe and persistent inflammatory injury leads to excessive repair of M2 macrophages, which not only releases more pro-fibrotic factors to further activate myofibroblasts, but also encourages macrophages to directly transform into myofibroblasts (6). Therefore, macrophage polarization and its functional diversity are important factors of IRI-related RIF in transplanted kidney.

Activation transcription factor 3 (ATF3) is a basic leucine zipper (bZIP) DNA binding protein induced by oxidative stress and a member of the transcription factor family of ATF/cAMP responsive element binding proteins. In recent years, studies on fibrosis of heart, liver, lung and other organs have shown that ATF3 could accelerate extracellular matrix accumulation and down-regulation of ATF3 expression could ameliorate fibrosis (7–9). Although Takumi Yoshida et al. demonstrated that renal IRI and oxidative stress during this period significantly enhanced ATF3 expression in the acute phase (10), there is a lack of research in the field of severe IRI on interstitial fibrosis of transplanted kidneys in the chronic phase. ATF3 was initially identified through our previous analysis of ferroptosis-related genes before and after renal ischemia and reperfusion (11), which confirmed that ATF3 is highly expressed following renal IRI. Building on this, we further discovered that ATF3 is significantly upregulated in renal IRI-related renal interstitial fibrosis. This finding led us to initiate a focused investigation into the role of ATF3 in this pathological process. Notably, ATF3 upregulation restricts the activation of Nrf2 signaling pathway, thus aggravating ferroptosis (12), which may be due to the fact that ATF3-Nrf2 heterodimer formed on the promoter of Nrf2 target gene ARE directly inhibits the antioxidant stress ability of Nrf2 (13). In addition, renal tubular epithelial cells (RTECs) are considered to be the most sensitive cells to ferroptosis in the kidney, and are usually the most serious parenchymal cells of IRI (14, 15). Inhibition of ferroptosis in RTECs is helpful to alleviate RIF (16), so it's suspected that ATF3 may promote ferroptosis in RTECs by inhibiting Nrf2 signaling pathway in IRI-related RIF of transplanted kidney.

Exosomes is an important regulator of transplanted kidney and immune system, and promote the repair and fibrosis of kidney tissue suffering from IRI (17). Intriguingly, Dai EY et al. proved for the first time in 2020 that there is a connection between ferroptotic cells and macrophages, that is, the exosomes released by ferroptotic cancer cells carry KRAS, which led to M2 macrophages polarization (18). Subsequently, in 2022, Qiying Chen et al. further proved that exosomes derived from ferroptotic cardiomyocytes affected M1 macrophages polarization, contributing to the pathological progression of myocardial infarction (19). Although the exosomes secreted by RTECs can promote RIF following IRI (20), it is still unknown whether exosomes derived from ferroptotic RTECs can induce macrophage polarization and the internal mechanism. Hence, this study aimed to investigate that ATF3 promoted ferroptosis in RTECs by inhibiting Nrf2 pathway, and the exosomes derived from ferroptotic RTECs induced M1/M2 ratio imbalance, offering a potential therapeutic target for decelerating the progression of renal IRI towards RIF.

2 Methods

2.1 RIF mouse model associated with IRI

Six to eight weeks C57BL/6 mice (Experimental Animal Center, Renmin Hospital of Wuhan University, No. 430727230101780713) were housed alone in an animal laboratory without specific pathogens, with a temperature of 22-25℃, a relative humidity of 60-65% and a light/dark cycle of 12 hours (free to eat and drink). The experimental protocol of the present study was approved by the Ethics Committee of Renmin Hospital of Wuhan University and in accordance with the principles of Laboratory Animal Care by the National Institutes of Health (permit no. 20210124). Mice were adapted for a week before the experiment, and then randomly divided into four groups (n=5): sham group, ischemia and reperfusion (IR) group, IR+Fer-1 group and IR +ATF3 knockdown ($ATF3^{KD}$) group. 1% pentobarbital sodium was injected intraperitoneally to anesthetize mice. After the midline of the

abdomen was cut to expose the abdomen, two renal pedicles were separated and clamped with capillary clamps. After 30 minutes, the capillary clamps were removed from the renal pedicles for reperfusion. During ischemia, the body temperature was maintained at 37-38°C by using a heating blanket with steady-state control through a rectal temperature probe. After sewing the incision, the mice were allowed to eat at will. The mice in the sham group received the same operation but did not use capillary forceps. 100 μL adenovirus carrying sh-ATF3 was injected through the tail vein with a titer of 1×10^{11} TU. Fer-1 was injected intraperitoneally at a dose of 5 mg/kg/day. On the 14th day of IR, mice were euthanized under the anesthesia of 1% pentobarbital sodium. Some renal tissues were fixed in 4% paraformaldehyde, and the rest were frozen at -80°C.

2.2 Measurement of blood urea nitrogen and serum creatinine levels

1 ml blood was centrifuged at 3,000 × g for 10 mins at 4°C, and then serum was separated and stored at -20°C. serum creatinine and BUN were measured using an Olympus automatic analyzer (Chemray 800; Rayto Life and Analytical Sciences).

2.3 Hematoxylin-eosin staining

Tissue was fixed in buffered 4% formalin solution for 24 hours and embedded in paraffin. Then, 4μm thick slices were dewaxed in xylene, rehydrated with gradient ethanol, and stained with hematoxylin for 5 minutes. Next, 1% hydrochloric acid-ethanol was used to distinguish the slices for 3 s, and 5% eosin was used to dye for about 3 minutes, then dehydrated, removed and embedded. Finally, the slices were observed under a microscope. HE staining was evaluated for renal tubular injury based on brush border loss, vacuolization, cellular desquamation, tubular dilatation, and tubular degeneration (all scored on a scale ranging from 0-3), with a maximum tubular injury score of 15 (9).

2.4 Immunohistochemical staining

Paraffin-embedded renal tissue slices with a thickness of 4μm were heated in EDTA buffer for 20 minutes for antigen recovery. Next, the slices were treated with 0.3% H_2O_2 for 10 minutes to eliminate endogenous peroxidase activity, and then washed with PBS. After blocking with 5% BSA for 20 minutes, the slices were incubated with the primary antibody against α-SMA (1: 1000, GB12044, Servicebio) at 4°C overnight. After that, the sections were incubated with HRP Goat Anti-Mouse lgG (1:3000, GB23301, Servicebio) for 1 hour. Finally, the slices were developed with 3,3'- diaminobenzidine tetrahydrochloride for 3 minutes and analyzed under a microscope. Quantitative analysis of immunohistochemistry was completed by two researchers who were unaware of the experimental conditions using ImageJ software to analyze five high magnification fields of view of each randomly selected mouse (21).

2.5 Masson staining

Paraffin-embedded renal tissue slices with a thickness of 4μm were dewaxed and hydrated, and then stained with iron hematoxylin after overnight with potassium dichromate. Next, ponceau acid fuchsin-phosphomolybdic acid dyeing and aniline blue dyeing were carried out, and finally the film was dehydrated and sealed. Microscopic examination showed that the collagen fibers were dyed blue and the nucleus was dyed blue-brown. The positive blue tissue in each image was quantitatively analyzed by ImageJ software (22).

2.6 Mouse renal tubular epithelial cell line cells culture and treatment

RTECs were carried out according to the following protocol: Control group (CON), hypoxia/reoxygenation (HR) group, HR +Fer-1 group, HR + siATF3 group. RTECs was cultured in 5% CO_2 incubator at 37°C, supplemented with 10% fetal bovine serum in Dulbecco modified Eagle medium. In HR experiment, RTECs were exposed to serum-free medium for 48 hours of hypoxia (5% CO_2, 1%O_2 and 94%N_2), and replaced with normal medium for 48 hours of reoxygenation (5%CO_2, 21%O_2 and 74%N_2). It was then incubated with Fer-1(10μM) for 24 hours. For ATF3 silencing, RTECs grown under normal conditions were transfected with siATF3 according to the manufacturer's guidelines.

2.7 Cell activity assay

RTECs with 5×10^3 cells/well were inoculated on a 96-well plate, CCK8 reagent was added according to the manufacturer's requirements, and incubated at 37°C for 4 hours.

2.8 Exosomes extraction

Extracellular vesicles were extracted by ultracentrifugation. Cultivate RTECs using serum without extracellular vesicles, collect the supernatant, centrifuge at 10, 000 g for 30 minutes to remove fragments, then centrifuge at 100, 000 g for 120 minutes in an ultracentrifuge. Discard the supernatant, collect extracellular vesicles from the sediment, and resuspend in PBS. Store in a refrigerator at -80°C. All steps were carried out in a 4°C; environment.

2.9 Nanoparticle tracking analysis

Resuspension the exosomes in PBS at a concentration of 5 mg/L, and further dilute the suspension by 100-500 times. Manually inject the sample into the sample chamber at ambient temperature. Each sample is equipped with a 488 nm laser and a high sensitivity sCMOS camera. By collecting scattered light signals from

nanoparticles, an image of the Brownian motion of the nanoparticles in the solution is captured to track the Brownian motion of each particle. Finally, calculate the radius and concentration of exosomes using NTA analysis software.

2.10 Exosome uptake assay

The green fluorescent dye PKH67 (UR52303, Umibio) was co-cultured with exosomes from RTECs cells to label exosomes. Macrophages were adhered to laser confocal dishes and co-cultured with PKH67-labeled exosomes for 2 hours. RAW 264.7 cells were incubated dropwise with DAPI for 5 min away from light for nuclear staining, and RAW 264.7 cellular uptake of exogenous exosomes was detected by laser confocal microscopy (Olympus, UltraVIEW VoX & IX81).

2.11 miRNA-seq analysis in exosomes derived from RTECs

Differentially expressed miRNAs in exosomes were screened via the edgeR package (version 4.0.16) in the R software (version 4.3.1) with a critical criterion of $|\log2FoldChange| > 1$ and $P < 0.05$, and were demonstrated by volcano plotting with the ggplot2 package (version 3.5.0). Their expression levels were demonstrated by heatmaps drawn with the pheatmap package (version 1.0.12). Intersections were taken for target genes predicted by miranda, RNAhybrid and miRWalk and visualized by the VennDiagram package (version 1.7.3). Finally, gene ontology (GO) annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed on the intersected target genes via the clusterProfiler package (version 4.10.1).

2.12 Flow cytometry

After exosome incubation, RAW 264.7 cells were centrifuged at 350g for 5 min to remove medium and resuspended in PBS. RAW 264.7 cells were then incubated with CD86 antibody (ab239075, abcam) or with CD206 antibody (18704-1-AP, proteintech) for 30 min at 4°C away from light. Then incubated with Alexa Fluor 488 - conjugated Affinipure Goat Anti-Rabbit IgG (H+L) for 30min at 4°C away from light. After centrifugation and resuspension, RAW 264.7 cells were analyzed by CytoFLEX LX flow cytometer (Beckman, USA).

2.13 Detection of total iron content, malondialdehyde and glutathione

Tissue iron assay kit (A039-2-1, Nanjing Jiancheng Bioengineering Institute, China) was applied for iron content assay. According to this scheme, kidney tissue was homogenized in phosphate buffered saline, and then iron chromogenic agent was added. After vortexing, the mixture was clarified by centrifugation at 1500 g for 10 minutes. Finally, the absorbance was measured at the wavelength of 520 nm. Similar to iron content assay, MDA (A003-1-1) and GSH (A006-2-1) assay kit were both built in Nanjing Jiancheng Bioengineering Institute and carried out under the manufacturer's instructions. The results were normalized by protein content.

2.14 Transmission electron microscope

Kidney tissues from mice were harvested and fixed with 5.4% glutaraldehyde at 2°C overnight, and then fixed with 1% osmium tetroxide at 2°C for 37 hours. Acetone gradient (50%, 70%, 90% and 100% respectively) was dehydrated and then embedded in epoxy resin. The ultrathin sections were dyed with uranyl acetate/lead citrate for visualization by TEM.

2.15 Western blot

Total protein was obtained from renal tissue by lysis buffer and its concentration was measured by Bicinchoninic Acid Protein Kit. Protein samples were separated on sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene fluoride membrane, then blocked with 5% BSA and incubated with β-actin antibody (1: 1000, Servicebio, GB11001-100, China), ATF3 antibody (1: 1000, CST, 33593S, USA), Nrf2 antibody (1: 1000, protein, 16396-1-AP, USA). After washing with TBST for 30 minutes, PVDF membrane was incubated with HRP conjugated Goat Anti-Rabbit IgG (1: 3000, Servicebio, GB23303, China) for 1 hour at room temperature. ECL reagents were applied to generate chemiluminescence signals, which were detected by ChemiDoc™ system (Bio-Rad, USA). The image is used to analyze the intensity of protein bands.

2.16 Quantitative real-time PCR

Total RNA was extracted from renal tissue and complementary DNA was synthesized by reverse transcription kit. Finally, RT-qPCR was carried out on Lightcycler 480II real-time fluorescence quantitative PCR system (Roche, Germany). The β-actin is used for expression standardization. The primers are listed in Table 1.

The relative expression of genes was detected by $2^{-\Delta\Delta CT}$ method.

2.17 Statistical analysis

Data are presented as the means ± standard deviation (SD). GraphPad Prism 9.0 were for statistical analysis and $P < 0.05$ was considered statistically significant. Student's t-test was used to compare with two groups and ANOVA followed by Tukey's test was for multiple-group comparisons. The Kruskal-Wallis test was applied to analyze data that does not follow a normal distribution. All experiments and assays were independently repeated at least three times.

TABLE 1 Gene-specific primers used for RT-qPCR.

Gene	Forward primer	Reverse primer
TFRC	CTTCCTGTCGCCCTATGTATCT	TCCCTGAATAGTCCAAGTAGCC
PTGS2	GTGATGAGCAACTATTCCA	GGTGAATGACTCAACAAAC
iNOS	TTGGCTCCAGCATGTACCCT	TCCTGCCCACTGAGTTCGTC
IL-1β	TCAGGCAGGCAGTATCACTC	AGCTCATATGGGTCCGACAG
TNF-α	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG
Arg1	CAGAGTATGACGTGAGAGACCA	GCATCCACCCAAATGACACATA
IL-10	GCTGGACAACATACTGCTAACCG	CACAGGGGAGAAATCGATGACAG
COL1α1	AAGAAGCACGTCTGGTTTGGAG	GGTCCATGTAGGCTACGCTGTT
FN1	AAGGCTGGATGATGGTGGACT	TCGGTTGTCCTTCTTGCTCC
β-Actin	GTGGGAATGGGTCAGAAGGA	TCATCTTTTCACGGTTGGCC

3 Results

3.1 RIF evolved from severe bilateral IRI

As shown in Figures 1A, B, compared with sham group, HE staining in IR group indicated renal tubular dilatation, renal tubular epithelial injury and lumen destruction, accompanied by immune cell infiltration, while renal injury in IR+Fer-1 and IR+AKF3^{KD} groups was alleviated. Meanwhile, compared with sham group, serum creatinine and BUN were significantly higher in the IR group, while both were significantly lower in the IR+Fer-1 and IR +AKF3^{KD} groups (Figures 1C, D). These results imply that renal tissue damage still exists in the 14days following IR and can be ameliorated by ferroptosis inhibitors.

As shown in Figures 1E, F, histological analysis of masson staining revealed that there were obvious extracellular matrix deposition and fibrosis formation in IR group. The mRNA levels of COL1a1 and FN1 in the IR group were increased than those in the sham group (Figures 1G, H). As shown by immunohistochemistry in Figures 1I, J, α-SMA, a myofibroblast marker, was expressed more in IR group than in sham group. Compared with IR group, the above fibrosis indexes were significantly reduced in IR+Fer-1 and IR+AKF3^{KD} groups. In conclusion, the successful establishment of a RIF model with 30 minutes of ischemia and 14 days of reperfusion is available for further study, which was ameliorated by ferroptosis inhibitors.

3.2 Ferroptosis occurred in IRI-related RIF model

Compared with sham group, mitochondrial crista disappeared, and outer membrane ruptured in IR group, but it was obviously ameliorated in IR+Fer-1 and IR+AKF3^{KD} groups (Figure 2A). Next, iron content was significantly increased in the IR group compared to the sham group, and Fer-1 treatment significantly reduced abnormal iron accumulation, as the same as IR+AKF3^{KD} groups (Figure 2B). A decrease of GSH levels and an increase of MDA content were observed in the IR group compared to the sham group (Figures 2C, D). A decrease in MDA content and an increase in GSH levels were observed in IR+Fer-1 and IR+AKF3^{KD} groups compared with the IR group. In addition, the mRNA expression levels of PTGS2 and TFRC, the molecular markers of ferroptosis, were significantly upregulated in IR group but were limited in IR+Fer-1 and IR+AKF3^{KD} groups, suggesting that ferroptosis occurred in IRI-related RIF model (Figures 2E, F). *In vitro* experiments showed the same trend for the above mortality parameters (Figures 2G–K). In summary, the above results demonstrate that ferroptosis takes place among renal RTECs in IRI-related RIF model.

3.3 ATF3/Nrf2 pathway regulates ferroptosis *in vivo and in vitro*

In vivo, the increase of ATF3 expression was identified in the RIF mouse model (Figures 3A–D). Similarly, the expression levels of Nrf2 and its downstream gene HO-1 in IR group were increased compared with those in sham group. In IR+Fer-1 and IR+AKF3^{KD} groups, the expression of ATF3 decreased, while the expression of Nrf2 and HO-1 further increased. *In vitro* experiments also revealed that the expression levels of ATF3, Nrf2 and HO-1 in HR group were higher than those in CON group, but the expression levels of ATF3 in HR+Fer-1 or HR+siATF3 group were lower than those in HR group, and the expression levels of Nrf2 and HO-1 were further increased (Figures 3E–H). These results indicate that ferroptosis in RTECs during RIF following renal IRI was mediated by ATF3 inhibiting the Nrf2/ARE pathway.

3.4 Ferroptosis promotes M2 macrophage polarization in IRI-related RIF model

Immunofluorescence of CD206 (surface marker of M2 macrophages) showed a significant accumulation of M2 macrophages in the IR group compared to the sham group and a

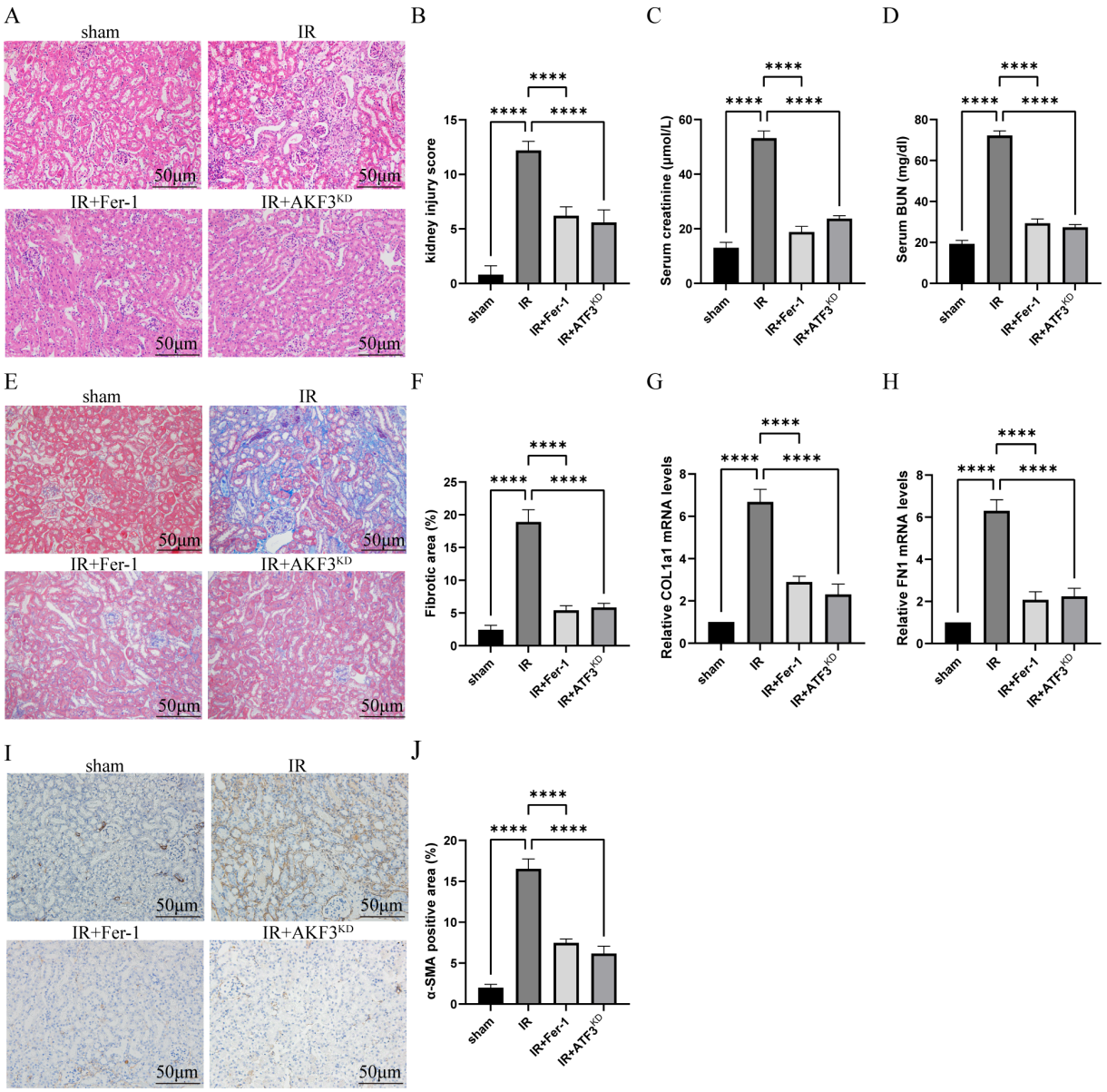

FIGURE 1
Establishment of RIF model following severe bilateral IRI. **(A)** Representative HE staining images of kidneys for the assessment of IRI. The scale bar represents 50 μm. **(B)** Pathologic scoring of renal HE staining. **(C, D)** Serum creatinine and BUN levels. **(E)** Representative Masson staining images of kidneys to evaluate the area size of collagen fibers. The scale bar represents 50 μm. **(F)** Quantification of Masson staining. **(G, H)** Relative level of mRNA expression of COL1a1 and FN1 in the kidneys detected by RT-qPCR. The mRNA level of sham group was normalized as 1. **(I)** Representative Immunohistochemistry staining images of α-SMA expression level in the kidneys. The scale bar represents 50 μm. **(J)** Quantification of α-SMA positive cells in IHC. Data are expressed as means ± SD, n=5, *****P* < 0.0001.

significant reduction of M2 macrophages in the IR+Fer-1 and IR +ATF3^{KD} groups compared to the IR group (Figure 4A). Although the trend of CD86 (surface marker of M1 macrophages) was similar to that of CD206, the magnitude of change was not as large as that of M2 macrophages (Figure 4E). As shown in Figures 4B–D, RT-qPCR analysis revealed that M2 markers (Arg1, TGF-β, and IL-10) showed the same trend as CD206. typical M1 markers (iNOS, TNF-α, and IL-1β) showed the same trend as CD86 (Figures 4F, H). Taken together, these data suggested that ferrotosis might accelerate the transformation of macrophages into M2 in renal IRI-related RIF.

3.5 Characterization of exosomes derived from ferroptotic RTECs

According to TEM (Figure 5A) and NTA (Figure 5B), the exosome particles of each group were about 50 nm to 200 nm in diameter, with elliptical spheres and central depression. Western blot analysis in Figure 5C revealed that these particles expressed CD63 expression, a widely recognized molecular marker of exosomes, was significantly increased in the HR group compared to the sham group, and Fer-1 treatment significantly reduced the

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exosomes content, as the same as HR+siATF3 groups. It should be mentioned that control refers to the exosomes isolated from the corresponding group, while control cell refers to the cell supernatant from the same group, which serves as a negative control to exclude potential contamination or infection from the supernatant. These results propose that HR can induce RTECs to generate more exosomes, and that these HR-RTECs are closely associated with ferroptosis, which can be hypothesized to be ferroptosis-related exosomes

3.6 Exosomes derived from ferroptotic RTECs promote M2 macrophage polarization

Subsequently, we co-cultured exosomes derived from CON or HR groups with RAW264.7 cells (CON-EXO or HR-EXO). Intracellular transport of the exosomes into RAW264.7 cells was shown in Figure 6A, as PKH67-labeled exosomes (green fluorescence) were taken up by RAW264.7 cells and colocalized with DAPI-labeled

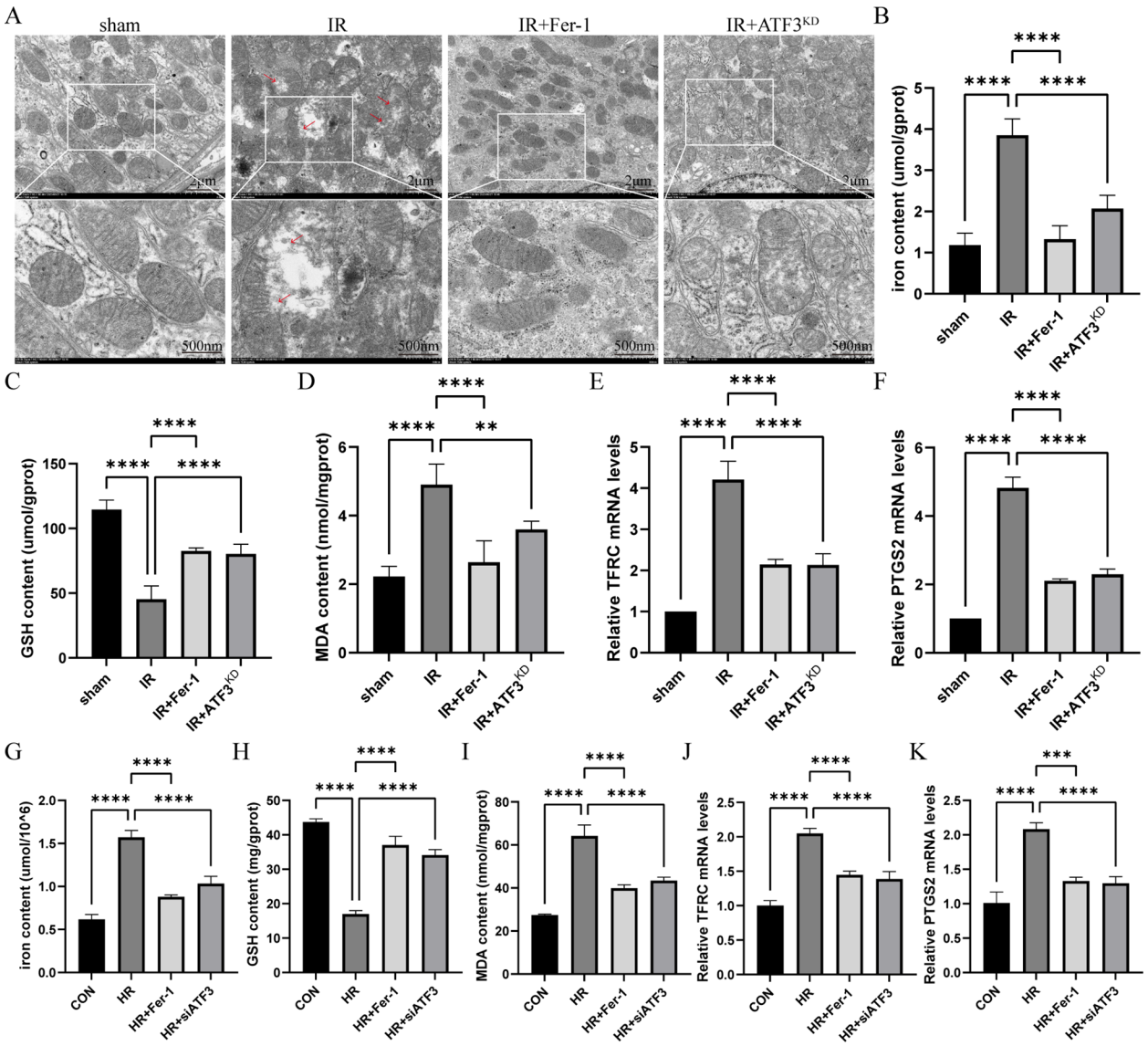

FIGURE 2
Ferroptosis in renal IRI-related RIF model. **(A)** Representative pictures acquired by transmission electron microscopy. The scale bar represents 2 μm and 500nm. **(B-D)** Total iron, GSH and MDA content in renal tissue. **(E, F)** Relative level of mRNA expression of TFRC and PTGS2 in the kidneys detected by RT-qPCR. The mRNA level of sham group was normalized as 1. **(G-I)** Total iron, GSH and MDA content in RTECs. **(J, K)** Relative level of mRNA expression of TFRC and PTGS2 in RTECs detected by RT-qPCR. Data are expressed as means ± SD, n=5, $^{**}P < 0.01$, $^{****}P < 0.0001$.

nuclei (blue fluorescence). We detected the concentration of M1 markers (iNOS, IL-1β, and TNF-α) and M2 markers (Arg1, IL-10, and TGF-β) in the culture supernatant, and found that exosomes inhibited the production of iNOS, IL-1β, and TNF-α (Figures 6B–D), and enhanced Arg1, IL-10, and TGF-β (Figures 6E–G). Flow cytometry results also support that exosomes cause an increased proportion of $CD206^+$ M2 phenotype macrophages (Figures 6H and I). In summary, these data indicated that exosomes secreted by ferroptotic RTECs promoted the polarization of macrophages towards M2.

3.7 miRNA-seq analysis of exosomes derived from ferroptotic RTECs

As shown in Figure 7A, the miRNA detection of exosomes secreted from HR-treated RTECs was analyzed. The heatmap showing the expression results of 33 miRNAs indicated that 18 miRNAs were significantly up-regulated in HR-EXO compared to CON-EXO, with the highest fold up-regulation of miR-1306-5p ($P < 0.05$; Figure 7B). As Figures 7C, D showed, potential targets of miR-1306-5p were predicted from three databases (miRWalk, miRanda, and RNAhybrid), and overlap analysis revealed 85 potential targets. As Figures 7E–H showed, they were significantly enriched in three major categories: BP (lipoprotein catabolic process, regulation of inclusion body assembly, cGMP-mediated signaling), CC (nuclear envelope, myosin filament, nuclear DNA-directed RNA polymerase complex), and MF (protein-containing complex destabilizing activity, nucleotidyltransferase activity, signal sequence binding). KEGG analysis revealed the major involvement in pathways such as Polycomb repressive complex, Glycerophospholipid metabolism, Other glycan degradation.

4 Discussion

4.1 Interpretation of key findings

In this study, we report the following (1): ATF3/NRF2 pathway-mediated ferroptosis in RTECs acts as an important role in the IRI-associated RIF model, which is accompanied by M1/M2 ratio imbalance; (2) HR induces the secretion of ferroptosis-related exosomes by RTECs, and correlates with ATF3-mediated ferroptosis; (3) ferroptosis-related exosomes increase the relative proportion of M2 macrophages which secretes more fibrosis-relative factors. In conclusion, persistent activation of ATF3 by renal IR exacerbates ferroptosis in RTECs possibly by further inhibiting the Nrf2 pathway, and secretion of ferroptosis-related exosomes by RTECs contributes to hyperpolarization of M2 macrophages, which initiates IRI-related RIF occurrence.

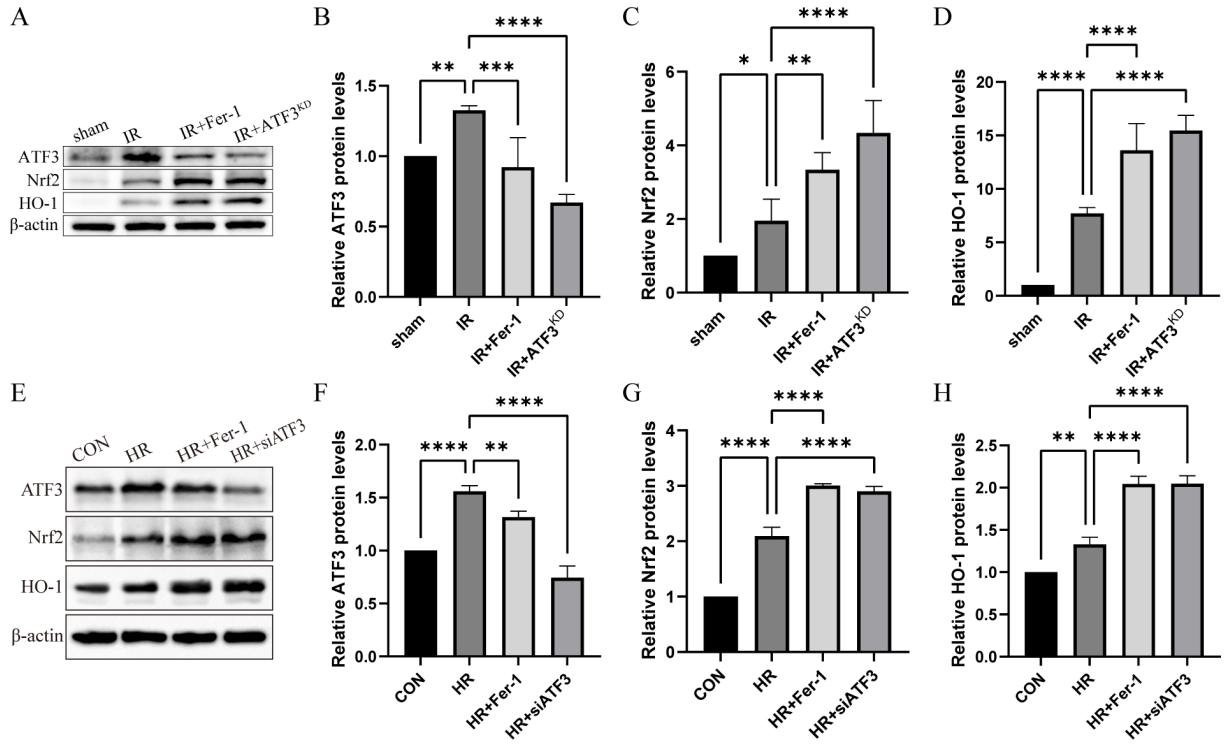

FIGURE 3
ATF3/Nrf2 pathway regulates ferroptosis in RTECs in the renal IRI-related RIF model. **(A)** Relative level of protein expression of ATF3, Nrf2 and HO-1 in the kidneys detected by Western blot. **(B-D)** Quantification of ATF3, Nrf2 and HO-1 protein expression in the kidneys detected by Western blot. **(E)** Relative level of protein expression of ATF3, Nrf2 and HO-1 in RTECs detected by Western blot. **(F-H)** Quantification of ATF3, Nrf2 and HO-1 protein expression in RTECs detected by Western blot. The protein level of CON group was normalized as 1. Data are expressed as means ± SD, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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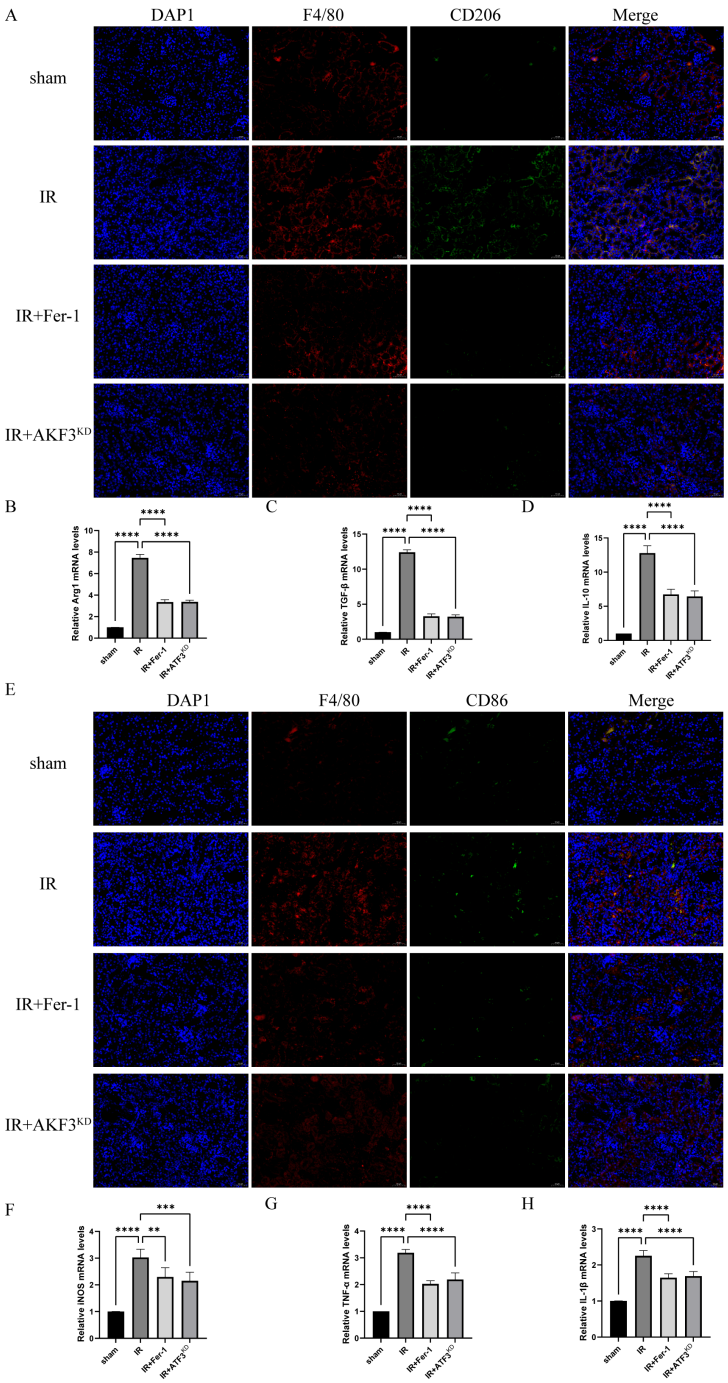

FIGURE 4
Ferroptosis promotes polarization of M2 macrophages in renal IRI-related RIF model. **(A)** Representative images of immunofluorescence co-staining of F4/80 (red signal) and CD206 (green signal) in the kidneys. Cell nuclei were stained with DAPI (blue signal). The scale bar represents 50 μm. **(B-D)** Relative level of mRNA expression of Arg1, TGF-β, and IL-10 in the kidneys detected by RT-qPCR. **(E)** Representative images of immunofluorescence co-staining of F4/80 (red signal) and CD86 (green signal) in the kidneys. Cell nuclei were stained with DAPI (blue signal). The scale bar represents 50 μm. **(F-H)** Relative level of mRNA expression of iNOS, TNF-α, and IL-1β in the kidneys detected by RT-qPCR. The mRNA level of sham group was normalized as 1. Data are expressed as means ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

4.2 Comparison with previous studies

Previous studies have shown that RTECs are the initiation site for the severe IRI-driven transition to RIF and that ferroptosis predominates in RTECs injury (23, 24). Renal tubules do not sensitize to necrotic apoptosis after selective removal of FAD or caspase-8, and the RIPK1 inhibitor necrostatin-1 (Nec-1) cannot protect freshly isolated tubules from hypoxic damage (25). An interesting feature of ferroptosis is that it can rapidly propagate between RTECs in a wave-like manner, which can be explained by the diffusion of the NADPH gradient formed by the reduced redox capacity within injured RTECs through intercellular junctions (26, 27).

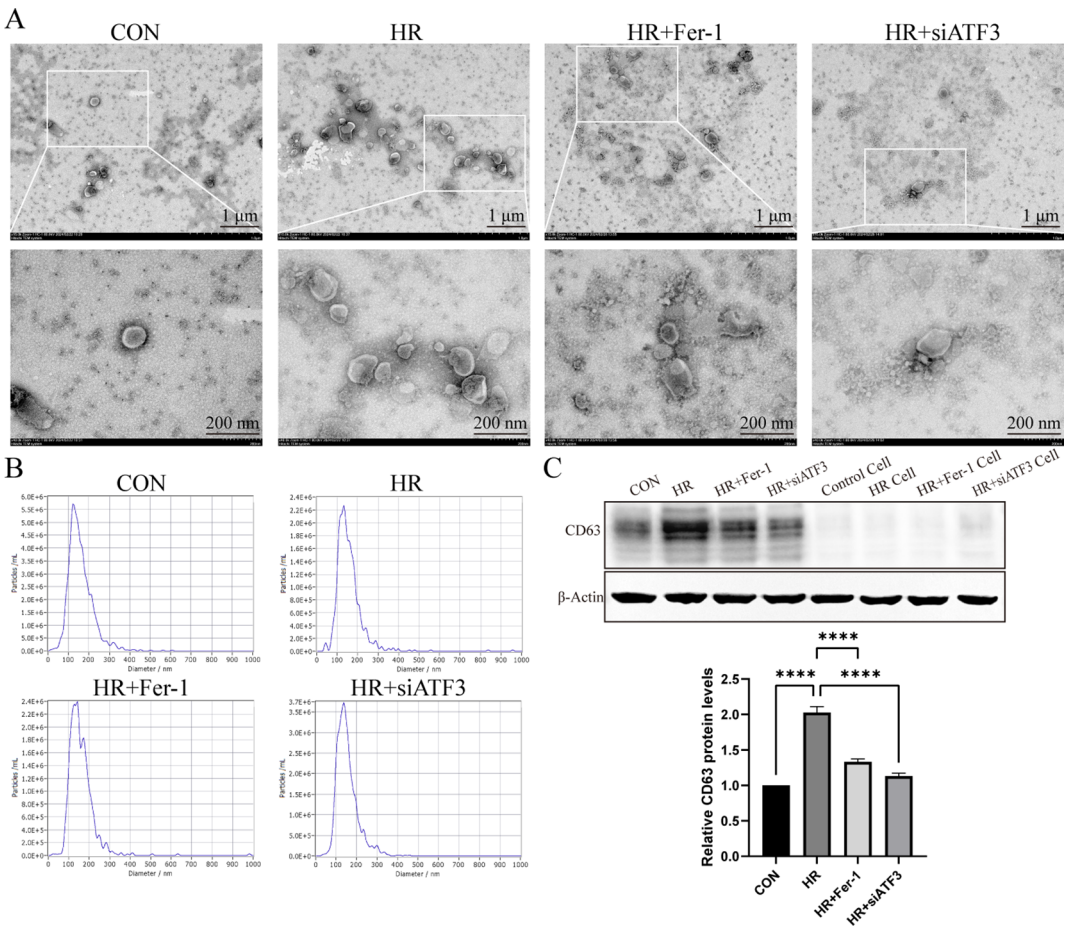

FIGURE 5
Characterization of exosomes derived from ferroptotic RTECs. **(A)** Representative pictures acquired by transmission electron microscopy (TEM). The scale bar represents 1 μm and 200 nm. **(B)** Average size distribution curve of exosomes as determined by nanoparticle tracking assay (NTA). **(C)** Relative level of protein expression of CD63 in the kidneys detected by Western blot. The protein level of sham group was normalized as 1. Data are expressed as means ± SD, *****P* < 0.0001.

Notably, ferroptosis drives the accumulation of injured RTECs, which underlie renal interstitial fibers (14, 28). Therefore, this study focused on RTECs and demonstrated that ferroptosis occurs in RTECs to exacerbate RIF and that ferroptosis inhibition ameliorates renal tissue injury and attenuates renal fibrosis. Based on our previous analysis of ferroptosis-related genes before and after IR confirming that ATF3 is one of the genes abundantly expressed after IR (11), the present study also demonstrated that ATF3 is abundantly expressed in IRI-related RIF. ATF3 has been reported to inhibit system Xc- and deplete intracellular GSH, which promotes lipid peroxidation, contributing to ferroptosis (29). Furthermore, we demonstrated that ATF3 knockdown attenuated ferroptosis in RTECs, improved IRI-related RIF, and was accompanied by an increase in Nrf2 and HO-1 expressions. To summarize, one of our most important findings is the bioinformatic analysis-based confirmation that the ATF3/NRF2 signaling pathway regulates ferroptosis in RTECs, which not only refines a novel molecular mechanism of ferroptosis, but also provides a therapeutic target for the future treatment of renal fibrosis.

As is well known, ferroptosis not only contributes to the damage of renal parenchymal cells, but can also be recognized by immune cells, leading to a series of inflammation or specific reactions (30). During RIF, the most prominent immune cells are macrophages, which exhibit different polarization states at different stages due to phenotypic heterogeneity and functional diversity, playing an important role in the progression of IRI to CKD (31). iNOS^{+}M1 macrophages are recruited into the kidney during early IRI, induced by exposure to IFNγ, LPS, TNF-α, or GM-CSF, and express pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (32). During the IRI recovery phase, IL-4 and/or IL-13 induce polarization of M2 macrophages, inhibite the expression of these pro-inflammatory markers, and activate the expression of arginase-1, mannose-receptor (MR), and IL-10. M2 macrophages produce pro-fibrotic factors (such as transforming growth factor (TGF) -β1, fibroblast growth factor 2 (FGF-2), platelet-derived growth factor (PDGF), and galactogenin 3) under the guidance of a variety of factors, which regulate epithelial and endothelial cell proliferation, myofibroblast activation, stem and tissue progenitor cell differentiation, and angiogenesis (33). Although M1 and M2 macrophages are the most commonly studied pro-inflammatory and anti-inflammatory phenotypes, macrophages are also shown to promote wound healing, pro-fibrosis, anti-fibrosis, pro-regression, and tissue regeneration (34, 35). Another important finding of the present study was that ferroptosis inhibition in RTECs involved the polarization

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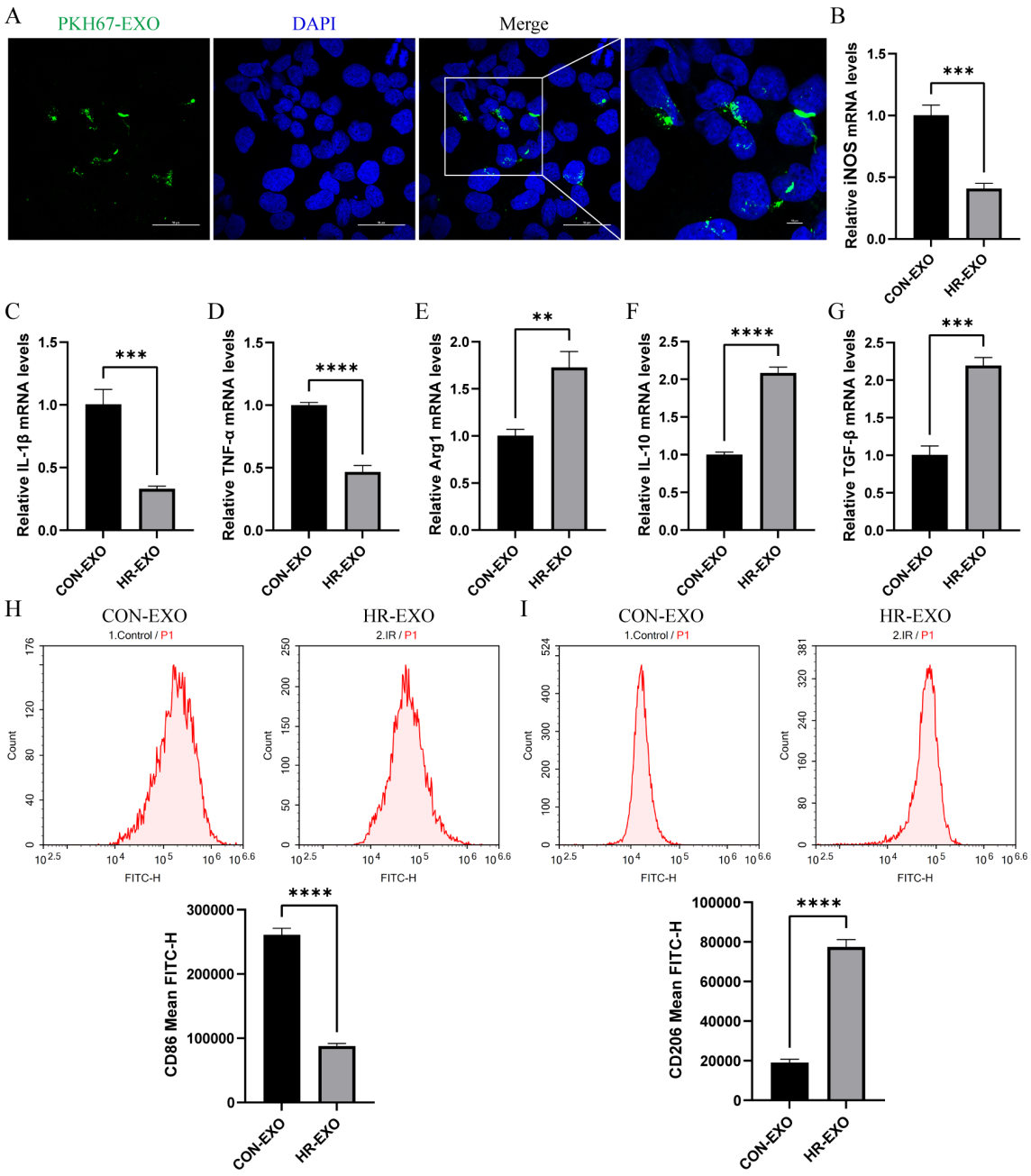

FIGURE 6
Exosomes derived from ferroptotic RTECs promote M2 polarization of macrophages. **(A)** Representative immunofluorescence staining images of RAW264.7 cells with exosomes derived from ferroptotic RTECs. The scale bar represents 50 μm and 10 μm. **(B-G)** Relative level of mRNA expression of TFRC and PTGS2 in RAW264.7 cells detected by RT-qPCR. **(H)** Mean FITC-H of CD86 antibody-labeled M1 phenotype macrophages in the CON-EXO and HR-EXO groups detected by flow cytometry. **(I)** Mean FITC-H of CD86 antibody-labeled M1 phenotype macrophages in the CON-EXO and HR-EXO groups detected by flow cytometry. Data are expressed as means ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

of M2 macrophages, suggesting a potential relationship between ferroptotic RTECs and macrophages that needs to be further explored.

Exosomes are nanoscale (50 to 200 nm) extracellular vesicles, which, in addition to serving as biomarkers of disease, transmit a variety of signaling substances, including proteins, lipids, and sugars, as well as genetic signals such as DNA, mRNA, and microRNA (36). In acute and chronic renal injury models, RTECs release exosomes containing CCL2 mRNA and are taken up by macrophages, thereby exacerbating tubule interstitial inflammation (37). Hypoxic RTECs secret exosomes rich in miR-23a, which activate macrophages and promote tubulointerstitial inflammation (38). Exosomes from RTECs can activate fibroblasts by blocking the miR-21/PTEN/Akt pathway, thereby accelerating the development of renal fibrosis (39). miR-150-5p delivered by tubular cell-derived exosomes can negatively regulate the expression of cytokine signaling 1 inhibitor, which activates fibroblasts and prevents IRI from developing into renal fibrosis (20). Although the current studies began to explore exosomes secreted by RTECs affecting the progression of IRI-related RIF, few of them

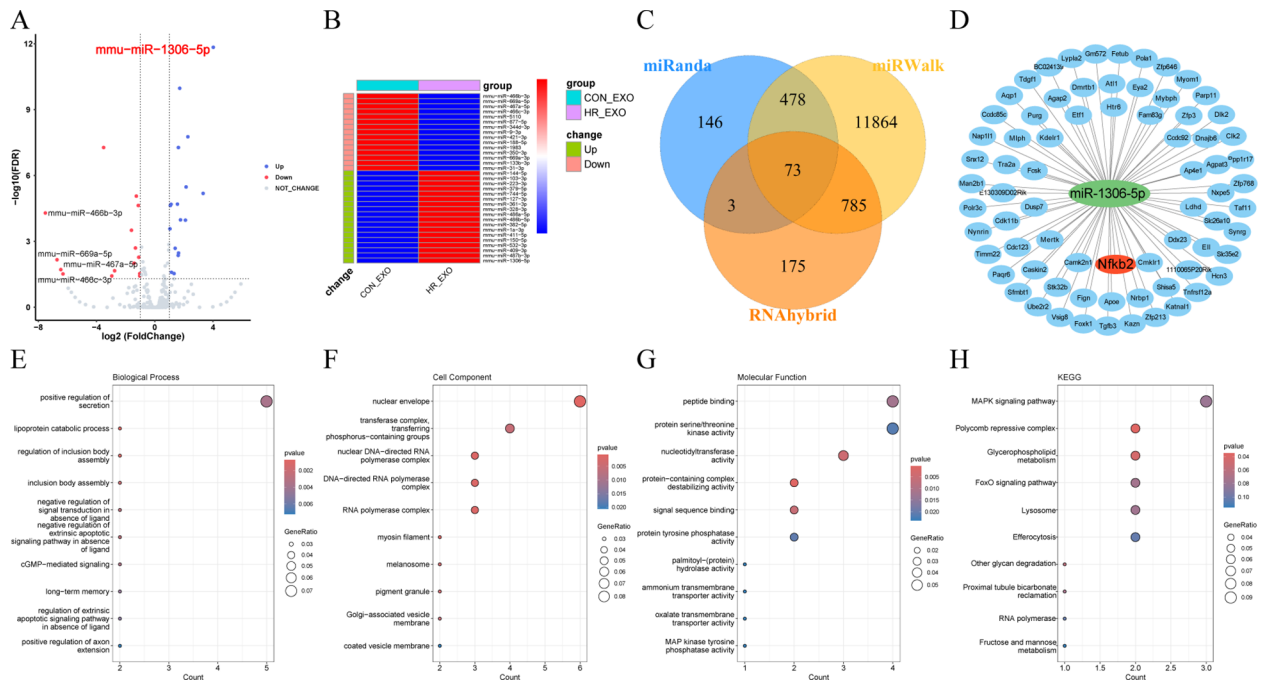

FIGURE 7
miRNA-seq analysis of exosomes derived from ferroptotic RTECs. **(A)** Volcano plots showing variable miRNAs in exosomes. **(B)** Heatmaps showing expression levels of differential miRNAs **(C)** Venn diagrams showing predicted target genes crossed over in miRWalk, miRanda and RNAhybrid. **(D)** Network diagram of miR-1306-5p and crossed predicted target genes shown by cytoscape software. **(E-H)** Dot plots showing the GO (biological process, cellular component and molecular function) and KEGG enrichment analysis results of miR-1306-5p target genes.

focused on the ferroptotic RTECs and exosomes. In the present study, we found that HR promoted cellular ferroptosis accompanied by increased exosome secretion, which could also be ameliorated by ferroptosis inhibitors, laterally revealing increased exosome release from ferroptotic RTECs. To further understand the functional specificity of ferroptosis-related exosomes in inducing M2 macrophage polarization, we identified exosome content components by miRNA-seq, with the highest expression being miR-1306-5p. Other previous studies have shown that miR-1306-5p was positively associated with adverse clinical outcomes in patients with acute heart failure (40, 41), and was also involved in other diseases such as glaucoma, human enamelogenesis imperfecta, and atherosclerosis (42–44). Of interest, miR-1306-5p has a target gene, Nfkb2, which is one of the important components of the transcription factor NF-kB that promotes the release of various cytokines and chemokines (e.g., TNFα, IL1β, IL6, IL12, CXCL9, etc.) from M1 macrophages via the non-classical NF-κB pathway (45). Therefore, we hypothesized that miR-1306-5p binding to Nfkb2 via base complementary pairing inhibits Nfkb2 expression at the post-transcriptional level, ultimately leading to an increase in the relative proportion of M2 macrophages. Anyway, our study demonstrated a novel mechanism involved in IRI-related RIF from the perspective of iron metabolism, i.e., exsomes derived from ferroptotic RTECs contained miR-1306-5p and were indeed picked up by macrophages, which were transformed into fewer M1 macrophages and more M2 macrophages that secreted more pro-fibrotic factors. However, this study has not explored the specific mechanism by which miR-1306-5p causes the decrease in the relative ratio of M1/M2 macrophages after miRNA-seq analysis, which will be further investigated by our next study.

4.3 Study limitations and future directions

Our study has several limitations. First, the lack of clinical data is a major limitation. Although our experiments were conducted using murine and cell-based models, these findings have yet to be validated in human clinical samples. The absence of clinical data limits the generalizability and translational potential of our results, and future research incorporating clinical samples is needed to confirm their relevance to human disease. Second, the exact mechanism through which miR-1306-5p regulates macrophage polarization remains unclear. While our study suggests that miR-1306-5p is involved in this process, further investigation is required to identify its direct targets and the specific pathways through which it exerts its effects on macrophage polarization. This will be an important direction for future research.

4.4 Conclusion

Briefly, this study has confirmed that ATF3 promotes ferroptosis possibly by regulating Nrf2, and that ferroptotic cells-derived exosomes carrying miR-1306-5p led to an increase in the relative proportion of M2 macrophages, which ultimately exacerbates RIF

following renal IRI. Therefore, ATF3 is a potential therapeutic target for the treatment of renal IRI-related RIF.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the Ethics Committee of Renmin Hospital of Wuhan University and in accordance with the principles of Laboratory Animal Care by the National Institutes of Health. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QT: Conceptualization, Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. YW: Investigation, Methodology, Writing – original draft, Writing – review & editing. CD: Funding acquisition, Resources, Writing – original draft, Writing – review & editing. QS: Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. JX: Data curation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dicoumarol sensitizes hepatocellular carcinoma cells to ferroptosis induced by imidazole ketone erastin

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Introduction: Ferroptosis, an iron-dependent form of regulated cell death, is characterized by the lethal accumulation of lipid peroxides on cellular membranes. It not only inhibits tumor growth but also enhances immunotherapy responses and overcomes drug resistance in cancer therapy. The inhibition of the cystine-glutamate antiporter, system Xc−, induces ferroptosis. Imidazole ketone erastin (IKE), an inhibitor of the system Xc− functional subunit solute carrier family 7 member 11 (SLC7A11), is an effective and metabolically stable inducer of ferroptosis with potential in vivo applications. However, tumor cells exhibited differential sensitivity to IKE-induced ferroptosis. The intrinsic factors determining sensitivity to IKE-induced ferroptosis remain to be explored to improve its efficacy.

Methods: Bulk RNA-sequencing data from hepatocellular carcinoma (HCC) and normal liver tissues were collected from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases. Differentially expressed genes were identified and intersected with the ferroptosis-related genes (FRGs) listed in the FerrDb database, yielding the identification of 13 distinct FRGs.

Results: A ferroptosis signature index model (Risk Score) was developed to predict HCC prognosis. And SLC7A11 and NAD(P)H quinone dehydrogenase 1 (NQO1) were identified as candidate FRGs indicating poor prognosis of HCC. Dicoumarol (DIC), an inhibitor of NQO1, was subsequently employed to assess its sensitizing effects on IKE in HCC treatment. In HCC cell lines and the subcutaneous xenograft model, the combined suppression of SLC7A11 and NQO1 significantly enhanced the inhibitory effect on tumor growth by inducing ferroptosis.

Discussion: In conclusion, our findings demonstrate that DIC sensitized HCC cells to IKE-induced ferroptosis in HCC. Moreover, the identification of potential drugs that enhance the susceptibility of HCC cells to ferroptosis could provide novel therapeutic strategies for the treatment of HCC.

KEYWORDS

hepatocellular carcinoma, ferroptosis, NRF2, NQO1, SLC7A11, prognostic model

1 Introduction

Hepatocellular carcinoma (HCC), the most prevalent form of liver cancer, ranks as the fourth leading cause of cancer-related mortality globally. It is characterized by a heterogeneous prognosis due to varying tumor burdens and the severity of chronic liver disease (1). Typically, HCC originates in cirrhotic livers, often associated with chronic liver diseases such as chronic hepatitis B or C infection, alcohol-related liver disease, and metabolic dysfunction-associated steatotic liver disease (2). For early-stage HCC, surgical therapies such as resection and transplantation offer substantial survival benefits. For intermediate-stage HCC, intra-arterial therapies, including transarterial embolization (TAE), transarterial chemoembolization (TACE), and transarterial radioembolization (TARE), serve as first-line treatments or bridging therapies before transplantation. However, HCC is often detected at advanced stages. Although tyrosine kinase inhibitors (TKIs)-based systemic therapies are widely used in advanced HCC, their clinical benefit is limited due to drug resistance (3). Immune checkpoint inhibitors have been approved for advanced HCC treatment, but the strong immunosuppressive tumor microenvironment inhibits cytotoxic T lymphocyte infiltration, restricting the responsiveness to immune checkpoint blockade in a minority of patients. Investigating resistance mechanisms and identifying novel therapeutic targets is critical for enhancing HCC therapeutic efficacy (2).

Tumor cells can develop resistance to antitumor drugs by promoting cell survival pathways, preventing apoptosis, and facilitating epithelial-mesenchymal transition. Defects in apoptosis contribute to resistance against cancer treatments and play a role in tumorigenesis. In human hepatocarcinogenesis, the dysregulation of the balance between cellular proliferation and death is a pro-tumorigenic principle (4). Some HCC patients exhibit poor responses to systemic therapies due to acquired or intrinsic resistance to apoptosis (5, 6). As resistance to apoptosis is a hallmark of cancer cells, the induction of non-apoptotic regulated cell death, such as ferroptosis, pyroptosis, and necroptosis, is emerging as a novel approach for cancer treatment (7). These targeted therapies have shown significant potential in enhancing therapeutic efficacy by bypassing apoptosis resistance and exhibiting synergistic antitumor immune responses (8).

In recent years, ferroptosis has emerged as a significant area of research due to its role as a natural tumor-suppressive mechanism and its potential to enhance antitumor immunity (9). Cancer cells, which require elevated levels of iron for survival, are particularly susceptible to ferroptosis, and this susceptibility is closely linked to the progression, treatment response, and metastasis of various cancer types (10). Notably, mesenchymal and dedifferentiated cancer cells, which are often resistant to apoptosis and conventional therapies, demonstrate a remarkable vulnerability to ferroptosis. The induction of ferroptosis can also restore the sensitivity of drug-resistant cancer cells to standard treatments (11). However, some cancer cells mitigate their susceptibility to ferroptosis by downregulating the ferroptosis pathway, leading to resistance to anticancer therapies. Furthermore, ferroptosis has been implicated in numerous oncogenic pathways, suggesting its potential as a target for novel cancer therapeutics (12). Emerging evidence indicates that ferroptosis exerts its anti-tumor effects by interacting with various tumor suppressor genes, highlighting its role as a tumor suppressor mechanism (13–16). Ferroptosis interacts with the tumor microenvironment (TME) in complex ways, influencing the immune response within TME. It has been reported that ferroptotic cells release pro-inflammatory damage-associated molecular patterns (DAMPs), which can trigger the innate immune system (17). High mobility group box 1 (HMGB1), one of the best-characterized DAMPs involved in immunogenic cell death, triggers inflammation and immune responses during ferroptosis induced by RAS-selective lethal 3 (RSL3) and erastin *in vitro* (18) (19). The glutathione (GSH)/glutathione peroxidase 4 (GPX4) axis is known to control the activities of lysyl oxidase (LOX) and prostaglandin-endoperoxide synthase (PTGS) *via* the peroxide (17). The enzyme PTGS2 serves as an effective marker of ferroptosis (20). The sensitivity of immune cells to ferroptosis in TME varies significantly; thus, regulating ferroptosis sensitivity may aid in the discovery of novel therapeutic strategies to improve cancer treatment (12). Pro-ferroptosis systems, which produce lipid peroxides, and ferroptosis defense systems, which detoxify these peroxides, exist in a delicate balance (21). When pro-ferroptosis activities exceed ferroptosis defense systems, excessive accumulation of lipid peroxides on cellular membranes can damage membrane integrity, ultimately leading to ferroptosis (21, 22). Oncogenes could cause ferroptosis resistance in cancer cells by

stimulating antioxidant defense systems that prevent lipid peroxidation or by suppressing cellular metabolic processes that promote lipid peroxidation, including the formation of labile iron pools, the composition of polyunsaturated fatty acids (PUFA)-containing phospholipids, and the synthesis of mitochondrial reactive oxygen species. Identifying reliable biomarkers that can amplify pro-ferroptotic effects or increase the susceptibility of HCC tumors to ferroptosis may significantly contribute to a more accurate prognosis of HCC. This advancement could lead to the formulation of more efficacious therapeutic strategies, thereby enhancing survival rates among HCC patients (23). For example, activation of Yes-associated protein (YAP) signaling can sensitize HCC to ferroptosis *via* arachidonate lipoxygenase 3 (ALOXE3)-mediated lipid peroxidation accumulation (24).

Enhancing sensitivity to ferroptosis is critical for the application of ferroptosis-based therapeutic strategies. Bioinformatic analysis plays a significant role in studying cancer and ferroptosis. This study aims to identify ferroptosis-related genes (FRGs) and associated pathways in tumors, elucidate the regulatory mechanisms of ferroptosis within these malignancies, and assess the therapeutic efficacy and safety profiles of drugs targeting ferroptosis. Multiomics has identified the correlation between intratumor steatosis and the exhausted tumor immune microenvironment in HCC (25). Previous research has shown that a novel, integrated cell death index model predicts the prognosis and responsiveness to immune checkpoint inhibitors in patients with oesophageal squamous cell carcinoma (26). In a study of non-small cell lung cancer, thorough multi-omics analysis clarified the biology of cancer resulting from genetic aberration (27). Proteogenomic profiling of small cell lung cancer has been instrumental in uncovering distinct biological mechanisms and identifying subtype-specific therapeutic strategies (28).

Further investigation on the effectiveness of imidazole ketone erastin (IKE) in other animal cancer models would be beneficial. In this study, we identified two ferroptosis suppressor genes, solute carrier family 7 member 11 (SLC7A11) and NAD(P)H quinone dehydrogenase 1 (NQO1), associated with the prognosis of HCC by bioinformatics analysis. The combined inhibition of SLC7A11 and NQO1 had a more significant suppressive effect on tumor growth in the subcutaneous HCC model. These findings may inform whether ferroptosis stimulation can yield favorable therapeutic outcomes in specific cancer cases.

2 Materials and methods

2.1 Cell culture

Hep 3B cell line was obtained from Suzhou Starfish Biotechnology Co., Ltd. (TCH-C195). HCCLM3 cells were obtained from Be Na Culture Library (BNCC102270). Huh-7 and Hep G2 liver cancer cell lines were sourced from the American Type Culture Collection (ATCC, United States). Mycoplasma contamination testing confirmed all cell lines to be negative. The cells were maintained in a humidified incubator at 37°C with 5% CO2 and cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS).

2.2 Data acquisition and preprocessing

RNA-sequencing (RNA-seq) data of 50 normal tissues and 371 HCC patients were downloaded from The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga/) database. RNA-seq data of 110 normal tissues from the Genotype-Tissue Expression (GTEx) (https://www.genome.gov/) database and 369 overall survival (OS) data of HCC patients were obtained from the University of California Santa Cruz (UCSC) (https://xenabrowser.net/) database.

2.3 Identification of differently expressed FRGs in HCC

Three bioinformatics methods—DESeq2, edgeR, and limma—were employed to identify differentially expressed genes in 371 HCC cases and 160 normal samples. A significance threshold of $p < 0.05$ and $\log_2$ fold change (FC) > 1 were utilized as cut-off criteria. Principal component analysis (PCA) and heatmap visualizations of differential gene expression between tumor and normal tissues were generated using the R package "tinyarray." Using differential expression analysis methods—DESeq2, edgeR, and limma—we identified genes that were significantly up-regulated and down-regulated. Subsequently, we utilized the R package "tinyarray" to determine the intersection of these gene sets. A Venn diagram, representing 356 up-regulated genes and 154 down-regulated genes, was constructed using the R package "tinyarray." The intersection of these 510 genes with FRGs (functionally relevant genes) from FerrDb (http://www.zhounan.org/ferrdb/current/operations/download.html) was considered, leading to the identification of 13 differentially expressed FRGs in HCC. Volcano plots and heatmaps of the 13 FRGs were created using "tinyarray."

2.4 Enrichment analysis of differently expressed FRGs

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG) enrichment analysis of 13 differently expressed FRGs was performed using "clusterProfiler," "org.Hs.eg.db," "enrichplot," "ggplot2," and "GOplot" R packages. We performed Gene Set Enrichment Analysis (GSEA) to further understand 13 differently expressed FRGs. Furthermore, GSEA analysis was also performed on the signaling pathways that were enriched in the high-risk and low-risk groups in HCC. Significance was set at a p-value threshold of less than 0.05. A thousand permutation analyses were run in order to assess the significance levels. We visualized co-expression potential with the R package "corrplot" to assess co-expression and anti-correlation between 13 differently expressed FRGs.

2.5 Protein-protein interaction (PPI) network and identification of hub genes and key molecules

The PPI network was utilized to identify functional modules based on 13 differently expressed FRGs. This was accomplished

using Cytoscape (version 3.8.1) and the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org). The Molecular Complex Detection (MCODE) algorithm within Cytoscape and the CytoHubba plugin were employed to identify hub genes or significant PPI network modules.

2.6 Identification of prognostic genes and establishment of a prognostic model

A risk model incorporating univariate, multivariate, and lasso regression analysis was developed using the "ggExtra" and "survival" R packages. The model was then applied to stratify the patients into high-risk and low-risk groups.

2.7 Verification of the prognostic model

The correlation between gene expression levels and overall survival (OS) in HCC patients was investigated using data from the TCGA, International Cancer Genome Consortium (ICGC), and Gene Expression Omnibus (GEO) databases (GSE14520). Furthermore, receiver operating characteristic (ROC) curves for the 1-, 2-, and 3-year survival periods were employed to establish the gene expression cutoff that distinguishes high-risk or low-risk patients using the "forestplot" package's functionality for generating forest plots.

2.8 Assessment of risk genes

A nomogram, developed from a multivariate regression analysis, served as a prognostic tool using the "survival," "rms," and "regplot" R packages. This nomogram enabled the calculation of individual probabilities for clinical events by integrating various prognostic and determinant variables, such as age, gender, stage, and Risk Score, thereby facilitating the prediction of diverse prognoses influenced by gene expression. The calibration curve illuminated the accuracy of the model's probability estimation. A scatter plot, a common graphical representation of the calibration curve, depicted the model's predicted probabilities or scores on the x-axis and the empirically observed event rates on the y-axis. The "Corrplot" R package was utilized to evaluate the correlation between the Risk Score and expression of specific genes, including E-twenty-six-specific sequence variant 4 (ETV4), kinesin family member 20A (KIF20A), cyclin-dependent kinase inhibitor A (CDKN2A), SLC7A11, and NQO1.

2.9 Differential expression of the hub gene in different stages and screening of target genes

After identifying the Hub gene, we utilized the Gene Set Cancer Analysis (GSCA) database to compile and illustrate the progression of mRNA expression levels between early- and late-stage cancers (http://bioinfo.life.hust.edu.cn/GSCA/#/). Employing Origin's box plot functionality, we delineated the correlation between clinical stages and gene expression levels. Through an integrative approach involving PPI network analysis, risk assessment modeling, and a thorough review of pertinent literature, we identified three pivotal target genes. The "survival" R package was utilized to find the association between prognosis and three target gene expressions. In order to ascertain how genes interact, the Gene Transcription Regulation Database (http://gtrd20-06.biouml.org/) was utilized to investigate the transcription factors' downstream regulation of genes.

2.10 Drug resistance analyses

Utilizing the CellMiner tools, we rapidly retrieved transcript data for a comprehensive set of 22,379 genes and 360 microRNAs. Additionally, the platform offered activity reports for 20,503 chemical compounds, including 102 medications approved by the U.S. Food and Drug Administration. By translating the differential expression levels into quantifiable patterns across the National Cancer Institute (NCI)-60, we improved data organization and enabled sophisticated cross-comparisons through the application of an innovative pioneering pattern match tool. Utilizing samples from the CellMiner database, we quantified the relationship between drug sensitivity profiles and gene expression patterns. The drug binding affinity, depicted by the Z score, was plotted on the y-axis, with the x-axis indicating the gene's relative expression levels. The violin plot of the Z score under high and low gene expression was shown.

2.11 Multiplex immunohistochemistry (mIHC)

The high-throughput tissue microarray was procured from Shaanxi Avila Biotechnology Co., Ltd. (Cat. No. DC-liv11047). Within the 96 cases of the tissue microarray, 10 cases were normal liver tissue, and 86 cases were cancer patients (71 patients with HCC, 15 with intrahepatic cholangiocarcinoma, and 1 with mixed carcinoma). There were two panels of five biomarkers examined in this study, including panel 1: SLC7A11 (ab307601, Abcam, 1:100), NQO1 (393700, Thermofisher, 1:100), and NF-E2 p45-related factor 2 (NRF2) (R1312-8, Huabio, 1:100). Another tissue came from a subcutaneous tumor. And panel 2: HMGB1 (ab79823, Abcam, 1:300), PTGS2 (12282s, CST, 1:500). Formalin-fixed and paraffin-embedded (FFPE) samples were cut from subcutaneous tumors, sections of 5 μm thickness. The slides were stained manually according to the instructions using the Opal seven-color IHC Kit (NEL861001KT, PerkinElmer). Stained slides were scanned by the Vectra (Vectra 3.0.5, PerkinElmer). Representative images were used for analysis by the inform software after scanning (inform 2.3.0, PerkinElmer).

2.12 Western blotting

Briefly, cells were scraped into ice-cold radioimmunoprecipitation assay (RIPA) buffer, supplemented with a 1% final concentration of the Halt protease and phosphatase inhibitor cocktail. Cell lysates were

centrifuged at 14,000 rpm for 15 min at 4°C, and supernatants were collected. Protein concentrations were determined using the Bradford method. Cellular proteins were separated by 10% SDS-gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 μM, IPVH00010, Millipore), and blocked with 5% skim milk at room temperature for 1 h. Membranes were then incubated with the following primary antibodies overnight at 4°C: anti-cystine glutamate reverse transporter (xCT) (ab307601, Abcam, 1:1000), anti-NQO1 (393700, Thermofisher, 1:500), anti-NRF2 (R1312-8, Huabio, 1:1000), and anti-β-actin (ab8226, Abcam, 1:3000). They were washed and incubated with horseradish peroxidase conjugated goat anti-mouse IgG (H+L) (31430, Thermofisher, 1:5000) and goat anti-rabbit IgG (H+L) (31460, Thermofisher, 1:5000).

2.13 Immunofluorescence staining

HCCLM3 cells were seeded in 6-well plates at a density of 4×10^5 cells per well. Following treatment with IKE, DIC, or IKE + DIC, cells were subjected to immunofluorescence analysis. They were fixed with 4% paraformaldehyde for 15 minutes and then washed with PBS. Subsequently, cells were permeabilized with 0.2% Triton X-100 to facilitate antibody penetration. Non-specific binding was blocked using a blocking buffer consisting of 5% normal goat serum (NGS) in PBS with 0.1% Tween-20 for 1 hour at room temperature. Primary antibodies, diluted in PBS with 1% BSA and 0.1% Tween-20, were applied to cells at 4°C overnight. The antibodies used were anti-cystine glutamate reverse transporter (xCT) (ab307601, Abcam, 1:100) and anti-NQO1 (393700, Thermo Fisher Scientific, 1:100). After incubation, unbound primary antibodies were washed away with PBS. Fluorochrome-conjugated secondary antibodies, compatible with the primary host species, were then applied for 1 hour at room temperature in the dark. Following further washing, cell nuclei were counterstained with DAPI. Immunofluorescence was visualized using a confocal microscope with appropriate filters for the fluorochromes.

2.14 Measurement of cell death

Four HCC cell lines (Hep G2, Hep 3B, HCCLM3, and Huh-7) were seeded in 96-well plates (3×10^3 cells/well). IKE (TS#2301, TargetingScience) was diluted to 8 μM, 4 μM, 2 μM, and 1 μM, respectively. Dicoumarol (DIC) (HY-N0645, MCE) was diluted to 4 μM. Ferrostatin-1 (HY-100579, MCE) was used as a specific ferroptosis inhibitor. Cells were collected for SYTOX Green (S7020, Invitrogen) and Hoechst33342 (C1029, Beyotime) staining for analyzing cell death by microscopy, and the number of dead cells and total cells was counted using ImageJ software.

2.15 *In vivo* xenograft mouse model

Male nude mice (7 weeks old) were injected with 3.0×10^6 HCCLM3 suspension cells subcutaneously. Tumor size was measured by an electronic caliper every 2 days and calculated using the formula: tumor size (mm^3) = 0.5 × length × width^2. Mice were randomly separated into 4 groups, 8 mice per group. When the average tumor volume of each group of mice reached 100 mm^3, drug intervention was started. One group of mice was injected with the solvent (5% DMSO + 5% Tween-80 + 40% PEG-300 + 50% ddH_2O) as a control. Two groups of mice were injected with IKE or DIC, respectively. One group of mice was injected with IKE and DIC at the same time. All three drugs were injected intraperitoneally. IKE was injected continuously for two weeks, 100 μL (30 kg/ml) each time. DIC was injected every other day for two weeks, 100 μL (30 kg/ml) each time.

2.16 Statistical analysis

Statistical analyses were performed using R software (version 4.2.3) and GraphPad Prism 8 (GraphPad Software Inc., USA). Comparisons between two groups were made for normally distributed variables using an independent Student's t-test, while non-normally distributed variables were assessed using the Mann-Whitney U test (Wilcoxon rank-sum test). The experimental data are presented as the mean ± SEM. Descriptive statistics were calculated using GraphPad Prism. Statistical significance was defined as $p < 0.05$.

3 Results

3.1 Identification of 13 differently expressed FRGs in HCC

Figure 1 provides a comprehensive overview of the bioinformatics analysis workflow employed in this study. FRGs were extracted from the FerrDb V2 database. Liver cancer-related genes were identified in 110 normal tissues from GTEx and in 50 normal tissues and 371 HCC cases from the TCGA database. Three distinct bioinformatics methods were utilized to analyze differentially expressed genes in HCC, with results depicted in a heatmap and a volcano plot. Using the R package "DESeq2," we identified 316 down-regulated and 818 up-regulated differently expressed FRGs (Figure 2A). With the R package "edgeR," 196 down-regulated and 952 up-regulated genes with differential expression related to ferroptosis were found (Figure 2B). The "limma" package revealed 688 down-regulated and 616 up-regulated FRGs (Figure 2C). Principal component analysis (PCA), a dimensionality reduction technique, distinctly showed the distribution patterns between HCC patients and healthy individuals (Figure 3A). Heatmaps were used to illustrate the gene expression profiles of HCC patients and healthy individuals (Figure 3B). A Venn diagram highlighted the overlap and uniqueness of up-regulated and down-regulated, revealing differential expression patterns. By integrating results from DESeq2, edgeR, and limma, we identified a common set of 356 up-regulated and 154 down-regulated genes in HCC, indicating significant alterations in gene expression (Figures 3C, D). The intersection of these genes with FRGs yielded 13 differentially expressed FRGs in HCC. These genes were visualized using a

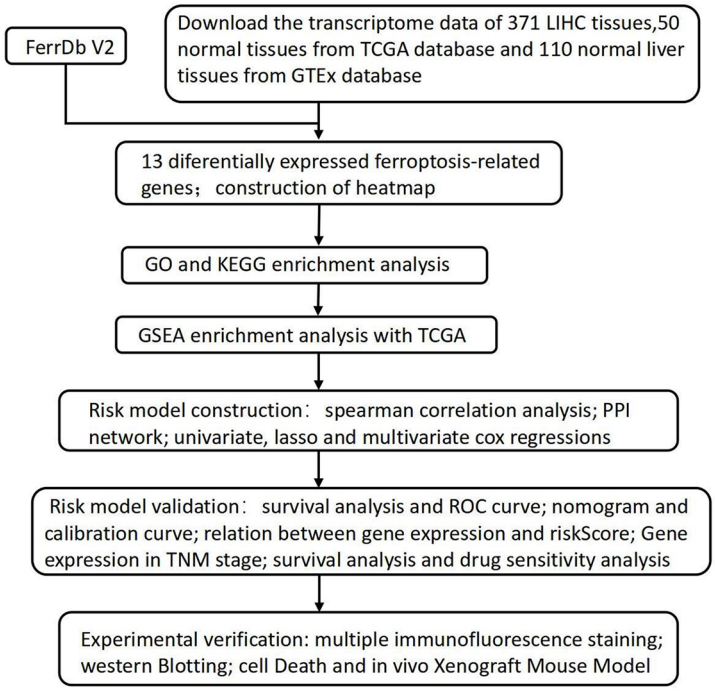

FIGURE 1
The flowchart of the research process.

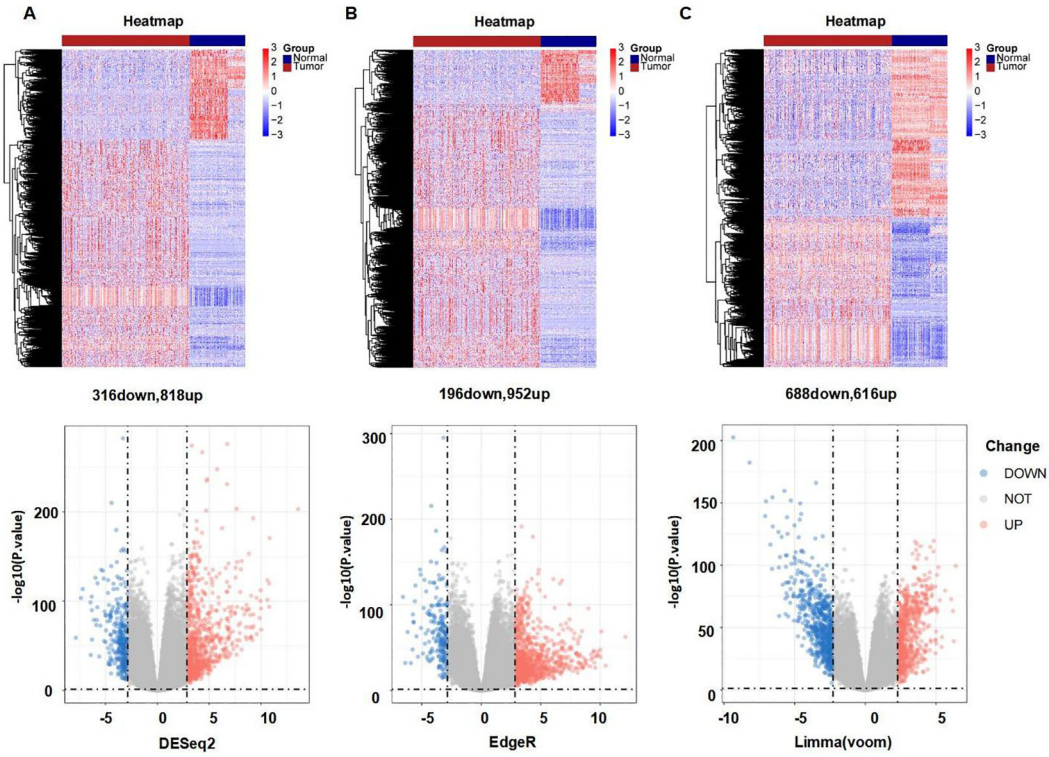

FIGURE 2
Differently expressed FRGs between HCC tissues and normal tissues analyzed with three data analysis methods. **(A)** Heatmap and volcano plot indicating FRGs using "DESeq2." **(B)** Heatmap and volcano plot indicating FRGs using "edgeR." **(C)** Heatmap and volcano plot indicating FRGs using the "limma" R package. Red indicating high expression, blue indicating low expression, "Normal" representing normal tissues, and "Tumor" representing tumor tissues. Volcano plots of differently expressed FRGs, with red indicating up-regulated, blue indicating down-regulated, "Up" representing up-regulated genes, "Down" representing down-regulated genes, and "NOT" representing neither up-regulated genes nor down-regulated genes.

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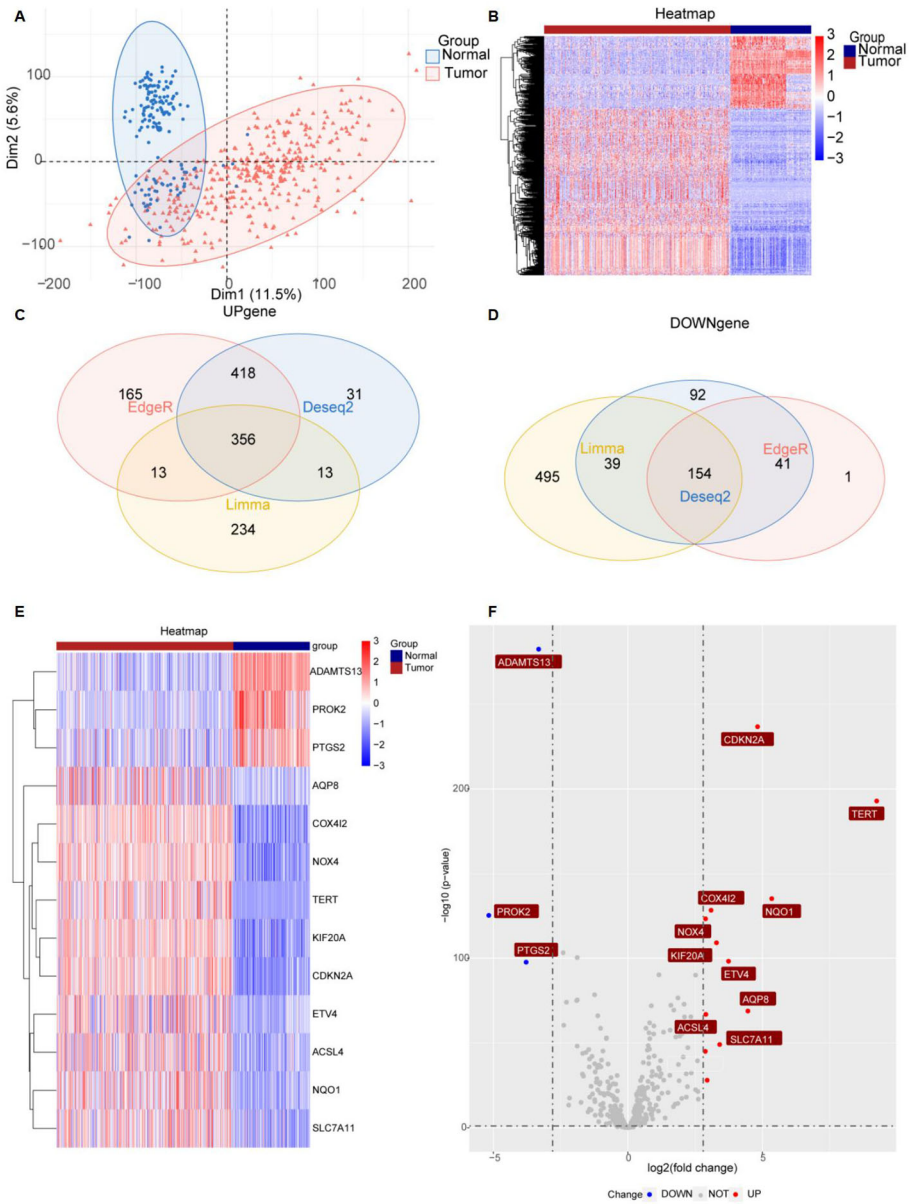


FIGURE 3
Significantly differently expressed FRGs are screened between HCC tissues and normal tissues. **(A)** PCA diagram of dimensionality reduction analysis, with red indicating tumor, blue indicating normal, "Normal" representing normal tissues, and "Tumor" representing tumor tissues. **(B)** Heatmap of differently expressed FRGs, with red indicating high expression, blue indicating low expression, "Normal" representing normal tissues, and "Tumor" representing tumor tissues. **(C, D)** Venn diagram showing the overlap of differentially expressed genes in the TCGA and GTEx databases. **(E, F)** Heatmap and volcano plots of 13 differently expressed FRGs, with red indicating high expression, blue indicating low expression, "Normal" representing normal tissues, and "Tumor" representing tumor tissues. "Up" representing up-regulated genes, "Down" representing down-regulated genes, and "NOT" representing neither up-regulated genes nor down-regulated genes.

heatmap and a volcano plot to show their expression patterns and the significance of their expression changes (Figures 3E, F). The heatmap indicated that aquaporin-8 (AQP8), cytochrome c oxidase subunit 4 isoform 2 (COX4I2), NADPH oxidase 4 (NOX4), telomerase reverse transcriptase (TERT), KIF20A, CDKN2A, ETV4, acyl-CoA synthetase long-chain family member 4 (ACSL4), NQO1, and SLC7A11 were down-regulated in tumors relative to normal tissues, whereas a disintegrin and metalloproteinase with thrombospondin type 1 motif, 13 (ADAMTS13), prokineticin 2 (PROK2), and PTGS2 were up-regulated.

3.2 Identification of gene correlation through functional enrichment analysis, gene set enrichment analysis, and gene correlation analysis

GO and KEGG pathway enrichment analyses were performed to investigate the functions of the differentially expressed FRGs. Treemap visualizations demonstrated that these FRGs were predominantly linked to replicative senescence, inflammatory cell apoptosis, sulfur amino acid metabolism, and regulation of myeloid

cell apoptosis (Figure 4A). The data were graphically represented using a treeplot for hierarchical detail and a barplot for an overview of enrichment scores (Figures 4A, B). GSEA was applied to identify the most relevant phenotypic pathways among the differentially expressed FRGs. The ridge diagram indicated significant enrichment of these genes in various biological pathways, including the cell cycle, complement and coagulation cascades, drug metabolism (cytochrome P450), retinol metabolism, coronavirus disease (COVID-19), carbon metabolism, and retinol metabolism (Figure 4C). A cnetplot visually depicted significant gene-gene interactions, particularly the strong association between SLC7A11 and CDKN2A (Figure 4D). In the TCGA HCC patient cohort RNA-seq data, which comprised 371 subjects, pairwise Pearson correlation coefficients were calculated to evaluate gene-gene relationships and were visualized in a matrix format for gene-by-gene comparison. A subsequent correlation analysis

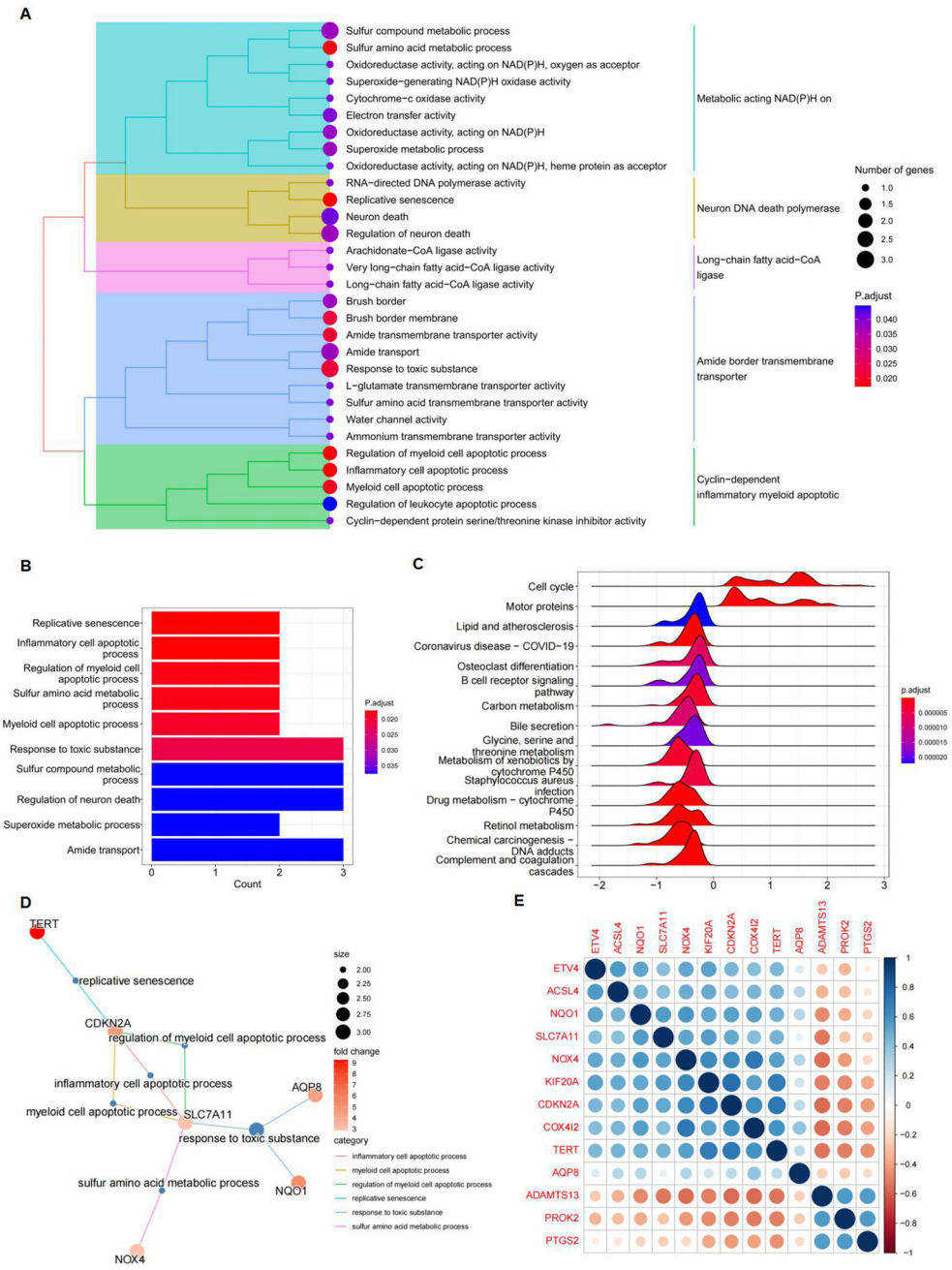
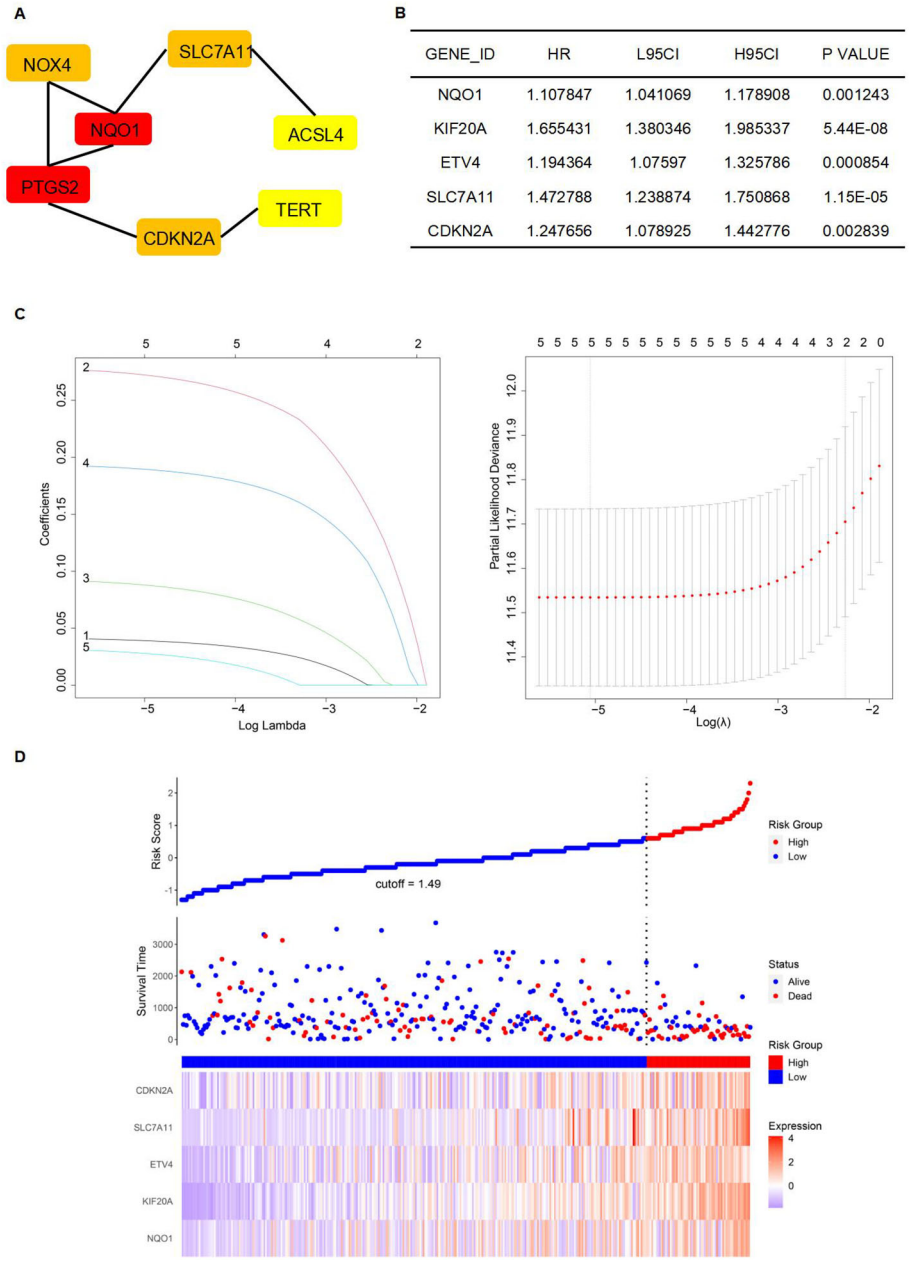


FIGURE 4
Gene function analysis of significantly differently expressed FRGs. **(A)** A treeplot of GO and KEGG enrichment analysis. **(B)** Barplot of GO and KEGG enrichment analysis. **(C)** Ridge diagram of 13 differently expressed FRGs. **(D)** Cnetplot of GO and KEGG enrichment analysis. **(E)** A positive correlation between NQO1 and SLC7A11. Gene-by-gene correlation matrix visualizing the pairwise Pearson correlation coefficients in bulk RNA-seq TCGA data from patients with HCC (n = 371). Genes are favorably connected if their respective circles are near blue; on the other hand, genes are negatively correlated if their respective circles are near red.

focused on the 13 differentially expressed FRGs and confirmed the correlation between ferroptosis and these genes (Figure 4E).

3.3 Establishment of a prognostic model and identification of prognostic FRGs

A protein-protein interaction (PPI) network was constructed to identify key genes and visualize their interactions. The string interaction network of genes with varying expression levels was investigated using Cytoscape. Utilizing the MCODE plugin within Cytoscape, we identified highly interconnected subnetworks, suggesting the presence of functionally related genes. Specifically, we selected seven genes to construct a PPI network relevant to ferroptosis: SLC7A11, NQO1, TERT, NOX4, ACSL4, PTGS2, and CDKN2A (Figure 5A). Among these, SLC7A11, NQO1, and TERT could inhibit ferroptosis (29–31). To identify FRGs whose expression levels correlate with the overall survival (OS) of HCC patients, we conducted a univariate Cox proportional hazards regression analysis. This approach allowed us to assess the association between gene expression and survival outcomes, applying a threshold p-value to refine potential prognostic FRGs. Notably, NQO1, KIF20A, ETV4, SLC7A11, and CDKN2A exhibited significant differential expression (Figure 5B). Lasso regression, incorporating a regularization



GENE_ID	HR	L95CI	H95CI	P VALUE
NQO1	1.107847	1.041069	1.178908	0.001243
KIF20A	1.655431	1.380346	1.985337	5.44E-08
ETV4	1.194364	1.07597	1.325786	0.000854
SLC7A11	1.472788	1.238874	1.750868	1.15E-05
CDKN2A	1.247656	1.078925	1.442776	0.002839

FIGURE 5
Establishment of a prognostic model. **(A)** The establishment of the PPI network. **(B)** Genes were significantly expressed in the univariate Cox regression model. **(C)** Lasso regression of 5 genes in **(B)**. **(D)** High-risk and low-risk groups according to Risk Score.

parameter λ, was then applied to identify the optimal predictive model, reducing regression coefficients β and eliminating variables with minimal impact. Lasso regression mitigates multicollinearity and overfitting by penalizing regression coefficients. Cross-validation indicated improved predictive accuracy as the deviation from the partial likelihood ordinate decreased. NQO1, KIF20A, ETV4, SLC7A11, and CDKN2A were identified as having the best fit within the model (Figures 5B, C). Furthermore, the multivariate Cox regression analysis identified NQO1, KIF20A, ETV4, SLC7A11, and CDKN2A as statistically significant predictors of increased mortality risk in HCC patients, suggesting their potential as independent risk factors and therapeutic targets. Subsequently, a stepwise multivariate Cox regression analysis was used to develop prognostic indicators, identifying genes with significant prognostic value. The prognostic Risk Score for each patient was calculated as the weighted sum of gene expression levels, each multiplied by its corresponding regression coefficient from the multivariate Cox regression analysis: Risk Score = 0.042*NQO1+0.281*KIF20A +0.095*ETV4+0.195*SLC7A11+0.034*CDKN2A (Figure 5D).

3.4 Assessment and verification of the prognostic model

After establishing the prognostic risk model, we validated its performance using three independent datasets: TCGA, ICGC, and GEO. The Risk Score for each patient was calculated using the multivariate Cox regression method, which was followed by survival analyses. Patients were subsequently stratified into high- and low-risk groups based on their Risk Score. To evaluate the predictive accuracy of the model, we constructed a ROC curve. The area under the curve (AUC) value represents the model's ability to distinguish between high-risk and low-risk groups within each corresponding database. It is observed that the AUC varies under different survival times for patients. The survival time with the highest AUC value can be selected to assess patient risk, indicating the optimal discrimination between the high- and low-risk groups. The AUC values for the 1-year, 2-year, and 3-year survival predictions for HCC patients were 0.801, 0.705, and 0.690, respectively, demonstrating significant model performance with a p-value of less than 0.0001 for the Kaplan-Meier (Km) survival analysis within the TCGA database (Figure 6A). Similarly, the AUC values for the HCC patients' 1-year, 2-year, and 3-year survival predictions were 0.722, 0.724, and 0.720, respectively, confirming the model's robustness with a p-value of less than 0.0001 for the Km survival analysis under the ICGC database (Figure 6B). Lastly, the AUC values for the 1-year, 2-year, and 3-year survival predictions were 0.613, 0.648, and 0.644, respectively, with a p-value of less than 0.067 for the Km survival analysis in the GEO database (Figure 6C).

3.5 Assessment of the risk genes by Risk Score

The nomogram was designed into three main components (1): Predictive model variables, including age, gender, and stage, were clearly labeled and accompanied by a visual depiction of the Risk Score. Each variable was allocated a line segment on the nomogram, calibrated with a scale that matched its value range. The length of these line segments represented the relative impact of each variable on the final outcome (2). The total score, labeled as "Total Points" on the nomogram, was determined by aggregating the individual scores from the variable values. Each variable's contribution was quantified by a score, graphically displayed as a point along the corresponding line segment (3). The nomogram detailed survival probabilities, noting that for the "High Risk Stratification" group, the 1-year survival rate was the primary endpoint, with the 5-year survival rate being less probable. Conversely, the "Low Risk Stratification" group was characterized by significant 3- and 5-year survival rates as the main outcomes. (Figure 7A). Ideally, the model's scatter points should align with a 45-degree line, indicating a close match between predicted and observed outcomes. Our analysis showed increasing predictive accuracy for 5-year, 1-year, and 3-year survival probabilities (Figure 7B). Using the TCGA database, we developed a nomogram model to calculate the Risk Score for HCC patients and correlated it with the expression levels of five pre-screened genes. Each gene exhibited a significant correlation: ETV4 (correlation coefficient R = 0.60, p-value = 7.1e-38), KIF20A (R = 0.81, p-value = 2e-87), CDKN2A (R = 0.45, p-value = 1.3e-19), SLC7A11 (R = 0.65, p-value = 6.6e-46), and NQO1 (R = 0.42, p-value = 4e-17), highlighting their potential as prognostic biomarkers (Figures 7C–G).

3.6 Higher expression of NQO1 and SLC7A11 is correlated with a poorer prognosis in HCC

After identifying the 13 differentially expressed FRGs, we employed the GSCA database to analyze mRNA expression trends from the early to late stages of HCC. The trend maps exposed distinct patterns of change at different stages, with a focus on the five risk genes: ETV4, KIF20A, CDKN2A, SLC7A11, and NQO1. It was observed that the expression levels of these genes collectively increased from stage I to stage II. Between stage II and stage III, the expression of KIF20A and SLC7A11, among the five risk genes, exhibited a significant increase. Finally, from stage III to stage IV, an increase in expression was observed solely for ETV4. (Figure 8A). Among the five risk genes identified, SLC7A11 and NQO1 have been recognized as critical players in conferring resistance to ferroptosis (32, 33). SLC7A11 is essential for regulating GSH synthesis, which confers resistance to ferroptosis. NQO1, an enzyme, protects cells from oxidative stress, thereby reducing their susceptibility to ferroptosis. Both genes facilitate tumor growth by inhibiting ferroptosis. We categorized samples into high- and low-expression groups based on the levels of NFE2L2, SLC7A11, and NQO1, with higher expression levels being associated with poorer survival outcomes in the TCGA database, showing significant p-values for SLC7A11 ($p < 0.0001$) and NQO1 (p = 0.00089) (Figures 8B–D). The NFE2L2 gene encodes NRF2, a regulator of cellular antioxidant responses. Analysis of NRF2 chromatin immunoprecipitation followed by sequencing (ChIP-seq) data revealed that NRF2 binds in the vicinity of key antioxidant target gene loci, specifically NQO1 and

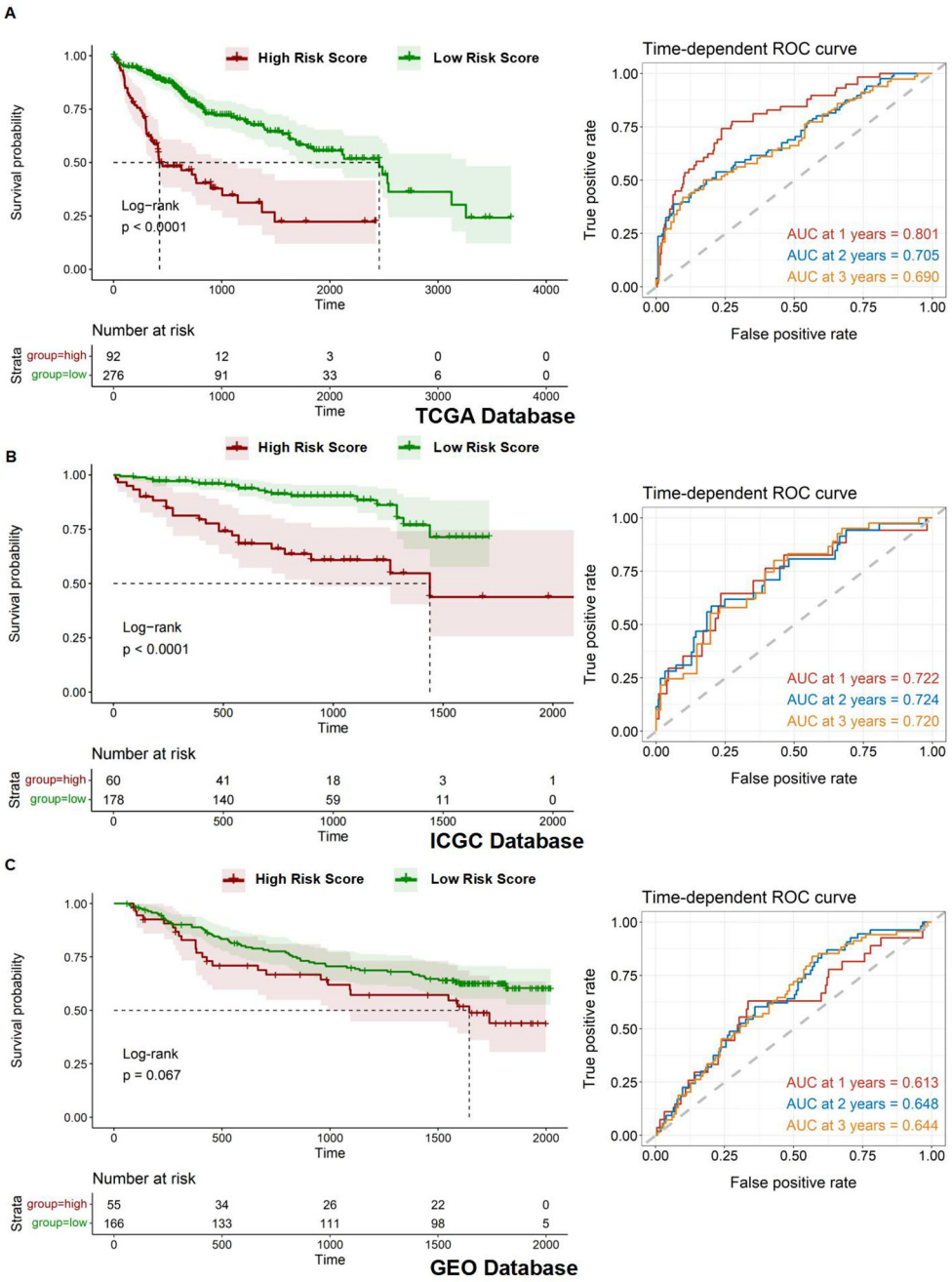


FIGURE 6
Assessment of prognostic model. **(A-C)** Kaplan-Meier overall survival (OS) curves for patients with high and low Risk Scores and time-dependent ROC curves in the TCGA, ICGC, and GEO databases.

SLC7A11, as documented in the Gene Transcription Regulation Database, indicating NRF2's role in their transcriptional regulation, which is essential for combating oxidative stress (Figure 8E).

3.7 Drug resistance analyses in high- and low-expression groups of genes that are sensitive to ferroptosis

We retrieved data from the CellMiner database, which included drug activity measurements (drug-tolerant persister NCI-60-Average z score) and gene expression profiles (RNA-seq composite expression) for NFE2L2, NQO1, KIF20A, ETV4, SLC7A11, and CDKN2A in HCC cell lines. Employing the "pattern comparison" tool, we conducted comparative analyses to explore the relationship between gene expression and drug activity. We found that an improved prognosis is correlated with increased drug binding sensitivity, potentially mitigating drug resistance. These six genes, known to inhibit ferroptosis, display significant expression variation within our HCC gene dataset. As gene expression in liver cancer cells increases, so does the binding affinity between the drugs and the cancer cells.

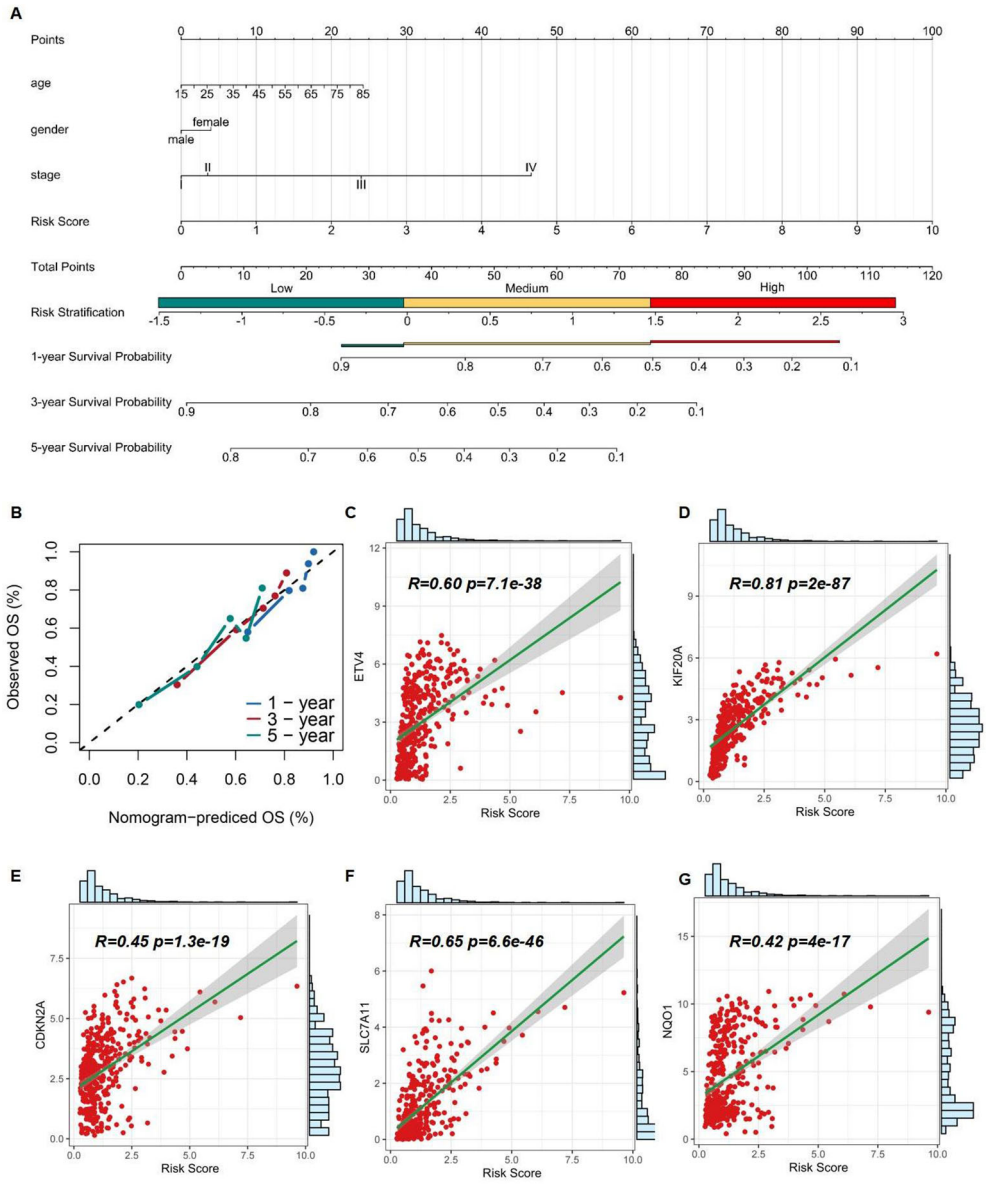


FIGURE 7
Assessment of 5 risk genes. **(A)** A nomogram based on multivariate regression. **(B)** Calibration curve of the risk model. **(C-G)** Correlation of Risk Score and expression of ETV4, KIF20A, CDKN2A, SLC7A11, and NQO1.

Consequently, a positive correlation between the binding potency of certain drugs and gene expression levels was established. We prioritized drugs with the strongest binding affinity, suggesting their potential for the most significant therapeutic impact (Figure 9).

3.8 Validation of the expression levels of NRF2, SLC7A11, and NQO1 in human liver and HCC tissues

The general and clinical characteristics of the study participants, as determined by the high-throughput tissue microarray, were presented in Table 1. mIHC analysis confirmed the expression patterns of NRF2, SLC7A11, and NQO1 proteins in HCC tissues compared to normal liver tissues. Notably, NRF2, SLC7A11, and NQO1 co-localized, with an increased positive rate for SLC7A11 and NQO1 associated with a high NRF2 positive rate in patients (Figure 10A). The positive rates for NRF2 and NQO1 were significantly higher in liver cancer tissues than in normal liver tissues. However, in the results of the local scan analysis, SLC7A11 did not exhibit this differential expression between all liver cancer and healthy individuals. Consequently, in subsequent analyses, it is necessary to either examine the differential positive rate for each cell or increase the sample size (Figure 10B). The positive rates for NRF2 and NQO1 in HCC tissue microarrays were also significantly greater than those in normal liver tissue microarrays. SLC7A11 did

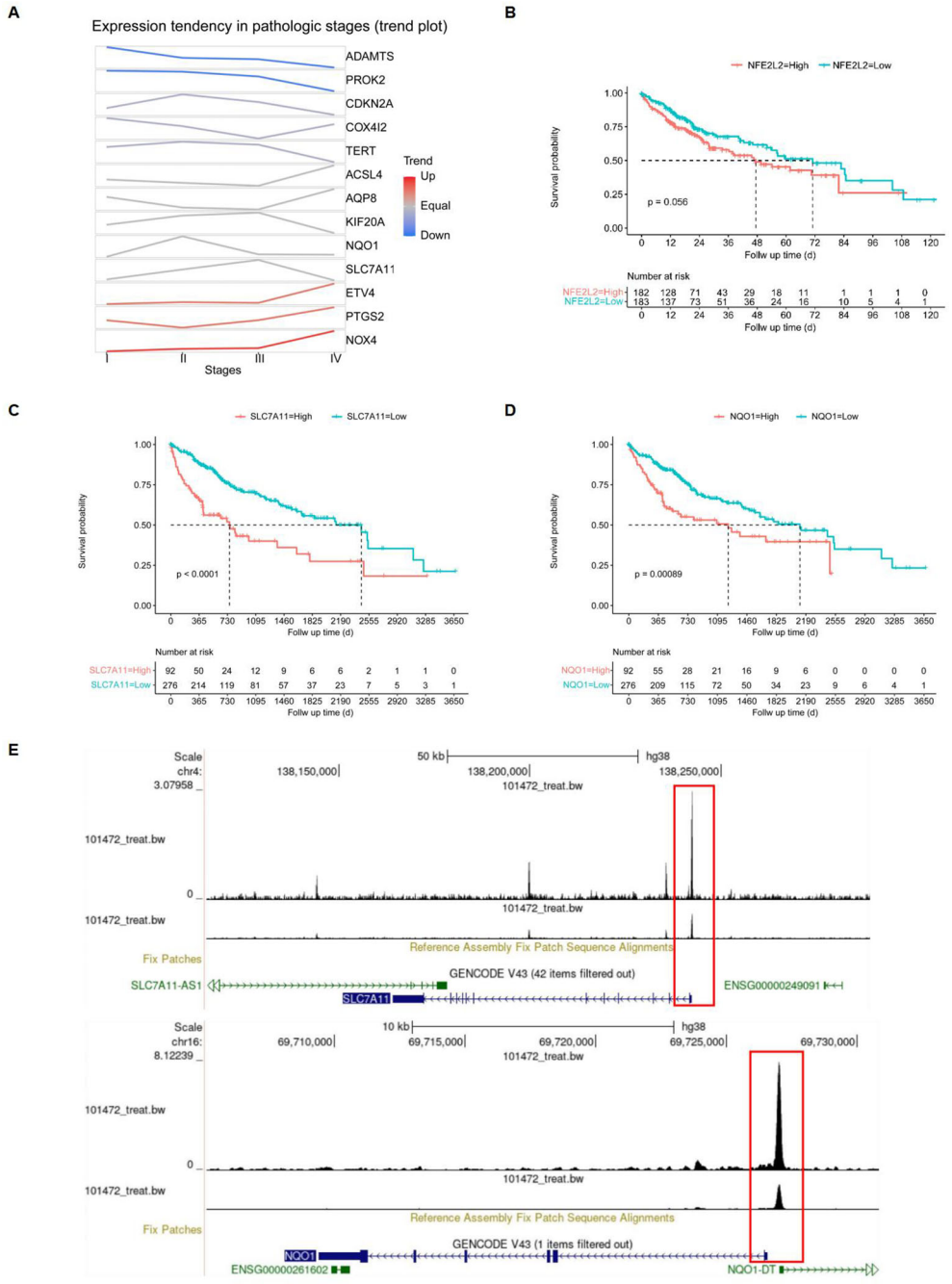


FIGURE 8
Screening of target genes. **(A)** FRGs expression tendencies in pathologic stages. **(B-D)** Kaplan-Meier overall survival (OS) curves for patients with high- and low-expressing NFE2L2, SLC7A11, and NQO1, with red indicating high expression and blue indicating low expression. **(E)** Gene transcription regulation of NFE2L2.

not demonstrate a significant difference between HCC patients and healthy individuals in the local scan analysis (Figure 10C). We combined the mean fluorescence intensity (MFI) values from the local scan areas with clinical data to evaluate whether the expression levels of NRF2, NQO1, and SLC7A11 varied significantly across different grades and tumor-node-metastasis (TNM) stages. However, no statistical differences were observed (Figure 10D).

3.9 Correlation of NRF2 with NQO1 and SLC7A11 in human liver and HCC tissues

We analyzed the correlation between NRF2 and NQO1, SLC7A11 in LIHC using data from the analysis data by the inform software after scanning. The expression levels of NRF2 showed a positive correlation with both NQO1 (r = 0.6436, $p < 0.0001$) and

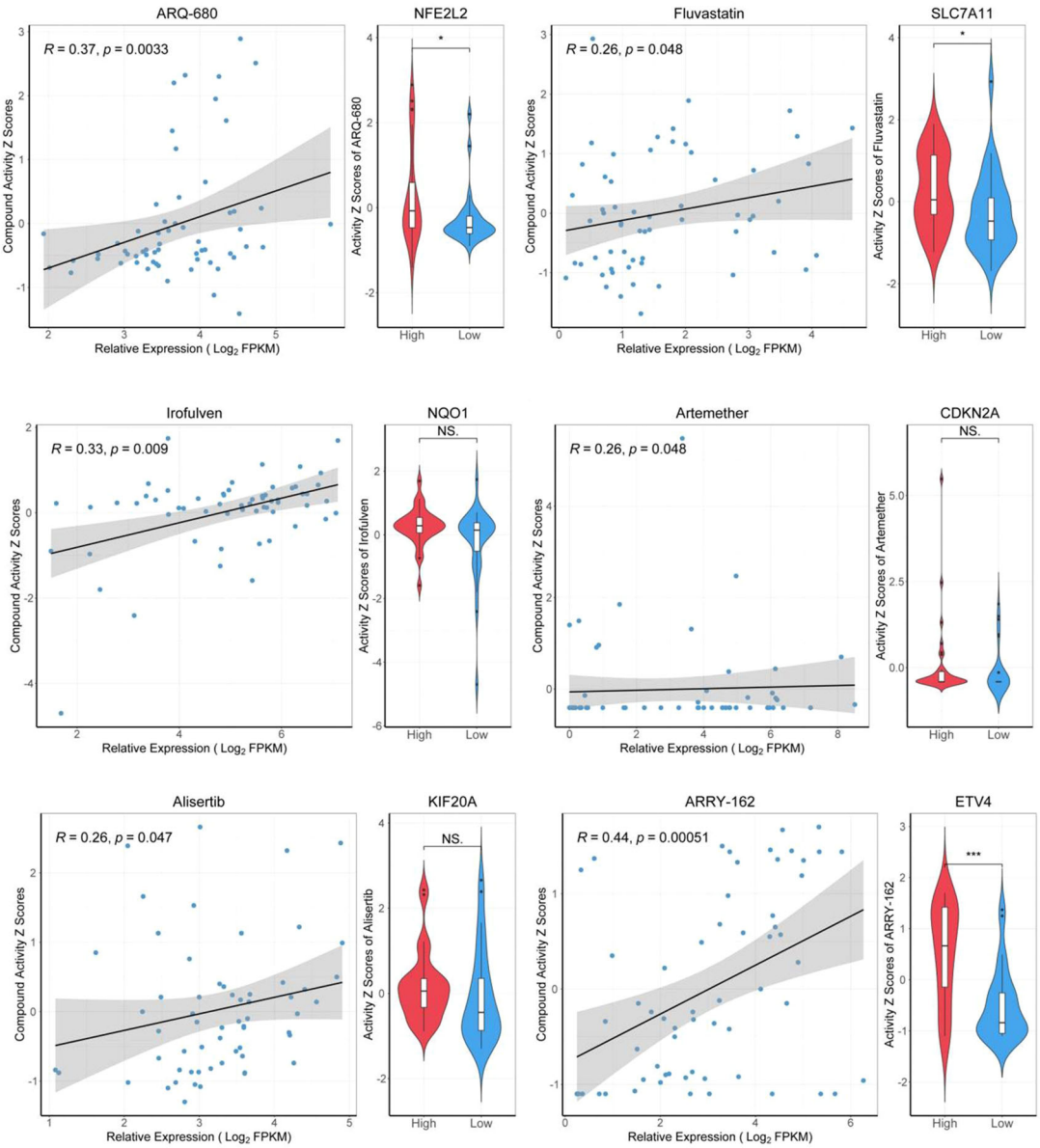


FIGURE 9
Drug sensitivity analysis of target genes. "High" representing high-expressed genes, and "Low" representing low-expressed genes. *p<0.05, ***p<0.001.

SLC7A11 (r = 0.6125, $p < 0.0001$) in LIHC patients (Figure 11A). This correlation was also observed in HCC patients, where NRF2 expression levels were positively correlated with NQO1 (r = 0.6585, $p < 0.0001$) and SLC7A11 (r = 0.7039, $p < 0.0001$) (Figure 11B). We categorized the MFI data of NRF2 in the cytoplasm of LIHC patients and healthy individuals into "NRF2 high" and "NRF2 low" based on the median MFI values of local scan slices. Elevated MFI of NRF2 in the cytoplasm was found to correlate with increased MFI of SLC7A11 in the membrane and NQO1 in the cytoplasm (Figure 11C). This pattern was consistent when analyzing HCC data alone, indicating a uniform association across patient samples (Figure 11D). At the single-cell level, significant differences ($p < 0.0001$) in the NRF2 expression patterns were identified between all liver cancer patients and healthy individuals (Figure 11E). We then classified the single-cell mean MFI data of NRF2 in the cytoplasm for both HCC patients and healthy individuals, selecting the highest MFI values from five HCC patients and the lowest from five healthy individuals. This approach confirmed that elevated MFI of NRF2 in the cytoplasm correlates with increased MFI of SLC7A11 in the membrane and NQO1 in the cytoplasm (Figure 11F).

3.10 DIC sensitizes IKE-induced ferroptosis in HCC cell lines and leads to tumor regression

Cell death induced by IKE was suppressed by the ferroptosis inhibitor Ferrostatin-1 (Fer-1). All cell lines showed sensitivity to

TABLE 1 General and clinical characteristics of liver cancer patients (N=96).

	Fall Group (n=86)		Control Group (n=10)	
Characteristics	Mean±SD or n (%)			
Age	50.95±9.95		35.60±10.16	
Sex				
F	15	(0.17)	7	(0.70)
M	71	(0.83)	3	(0.30)
Grage				
2	42	(0.51)	–	–
2-3	2	(0.02)	–	–
3	38	(0.46)	–	–
Stage				
I	10	(0.12)	–	–
II	62	(0.72)	–	–
III	9	(0.10)	–	–
IV	5	(0.06)	–	–

IKE-induced ferroptosis in a dose-dependent manner, with Hep G2 cells being the most sensitive and HCCLM3 cells the least sensitive (Figure 12A). To investigate whether DIC can enhance IKE-induced ferroptosis, we selected HCCLM3 cells for our further research (Figure 12B). NRF2, NQO1, and SLC7A11 were confirmed to be expressed in HCC cell lines, including Hep G2, Hep 3B, HCCLM3, and Huh-7. (Figure 12C). We verified the effects of IKE and DIC on the expression of SLC7A11 and NQO1 *in vitro* using western blot and immunofluorescence experiments (Figures 12D, E). A subcutaneous tumor model was established using HCCLM3 cells in nude mice to investigate the impact of NQO1 and SLC7A11 on tumor growth (Figure 12F). The combination of DIC with IKE significantly decreased tumor growth (Figure 12G). The expression levels of SLC7A11 and NQO1 were significantly reduced in groups treated with IKE, DIC, or both, with the combined treatment showing a more pronounced decrease compared to single treatments (Figures 12H–J). These findings indicated that the combination of SLC7A11 and NQO1 inhibitors is a promising therapeutic approach for HCC.

The combination of IKE and DIC significantly reduced tumor volume and weight. Our research confirmed that DIC inhibited the expression of NQO1 and IKE inhibited the expression of SLC7A11, both of which are known to suppress ferroptosis. Importantly, DIC sensitized HCCLM3 cells to IKE-induced ferroptosis. To investigate how DIC sensitizes tumor cells to IKE-induced ferroptosis *in vivo*, we used DAB-enhanced Prussian blue staining to identify iron deposits within cells and tissues. Our analysis revealed that the treatment groups—IKE, DIC, and the combination of IKE and DIC—increased the level of iron. The hepatic non-heme iron in sections from mice that IKE and DIC were higher than in those treated with IKE or DIC alone and control mice, as determined by DAB-enhanced Prussian blue staining (Figure 13A). mIHC showed that the expression levels of HMGB1 and PTGS2 were significantly elevated in tumors treated with IKE and DIC together (Figures 13B, C).

4 Discussion

Ferroptosis, an iron-dependent type of programmed cell death (PCD) marked by increased lipid peroxidation, is implicated with tumor growth and therapeutic responses across various cancers. While the precise effect of ferroptosis in tumor biology is not fully understood, evidence suggests a link between therapeutic induction of ferroptosis and mutations in cancer-related genes, particularly those involved in stress response pathways (34). It is hypothesized that IKE and DIC might induce ferroptosis and modulate other biological processes by regulating SLC7A11 and NQO1 expression, potentially explaining the observed reduction in tumor volume in our experiment.

The translation of ferroptosis-targeted therapies from the bench to bedside is crucial yet challenging. Several treatments, including radiotherapy, immunotherapy, chemotherapy agents (like altretamine), and targeted therapy agents (like sorafenib), are known for their potential to induce or sensitize cancer cells to ferroptosis (21). However, extensive clinical trials are necessary to confirm the safety and efficacy of these methods in combination with other ferroptosis-inducing treatments and to determine their ability to replicate preclinical successes in overcoming therapeutic resistance (34).

We employed three distinct analytical methods to assess differential gene expression in HCC using transcriptomic data from both the TCGA and GTEx databases. The intersection of differentially expressed genes from HCC with the FRGs in the FerrDb database identified a set of 13 differentially expressed FRGs. Following a comprehensive bioinformatics analysis, including functional enrichment, gene set enrichment, gene correlation assessments, the construction of a PPI network, and various regression analyses such as univariate Cox, Lasso, and multivariate regression, we developed a prognostic risk model. Within this model, SLC7A11 and NQO1 were identified as the key target genes for this investigation.

NRF2, a master regulator of cellular redox homeostasis and xenobiotic detoxification (35), is often associated with elevated NFE2L2/NRF2 expression, oncogenic activity of NRF2, tumor growth, metastasis, and resistance to anticancer treatments, primarily due to Kelch-like ECH-associated protein 1 (KEAP1) mutations (36–38). Clinical studies have correlated high NRF2 expression with poor prognosis in various malignancies (39). As cancer cells can develop resistance to apoptosis-inducing therapies, alternative PCD mechanisms, such as ferroptosis, warrant exploration (40). The expression of SLC7A11 and NQO1 is positively regulated by the transcriptional factor NRF2, whereas they are negatively regulated by the NRF2 suppressor gene KEAP1 (41). NRF2, a transcription factor, plays a crucial role as a major regulator of the antioxidant response. It promotes the transcription of SLC7A11 and NQO1 under conditions such as oxidative stress,

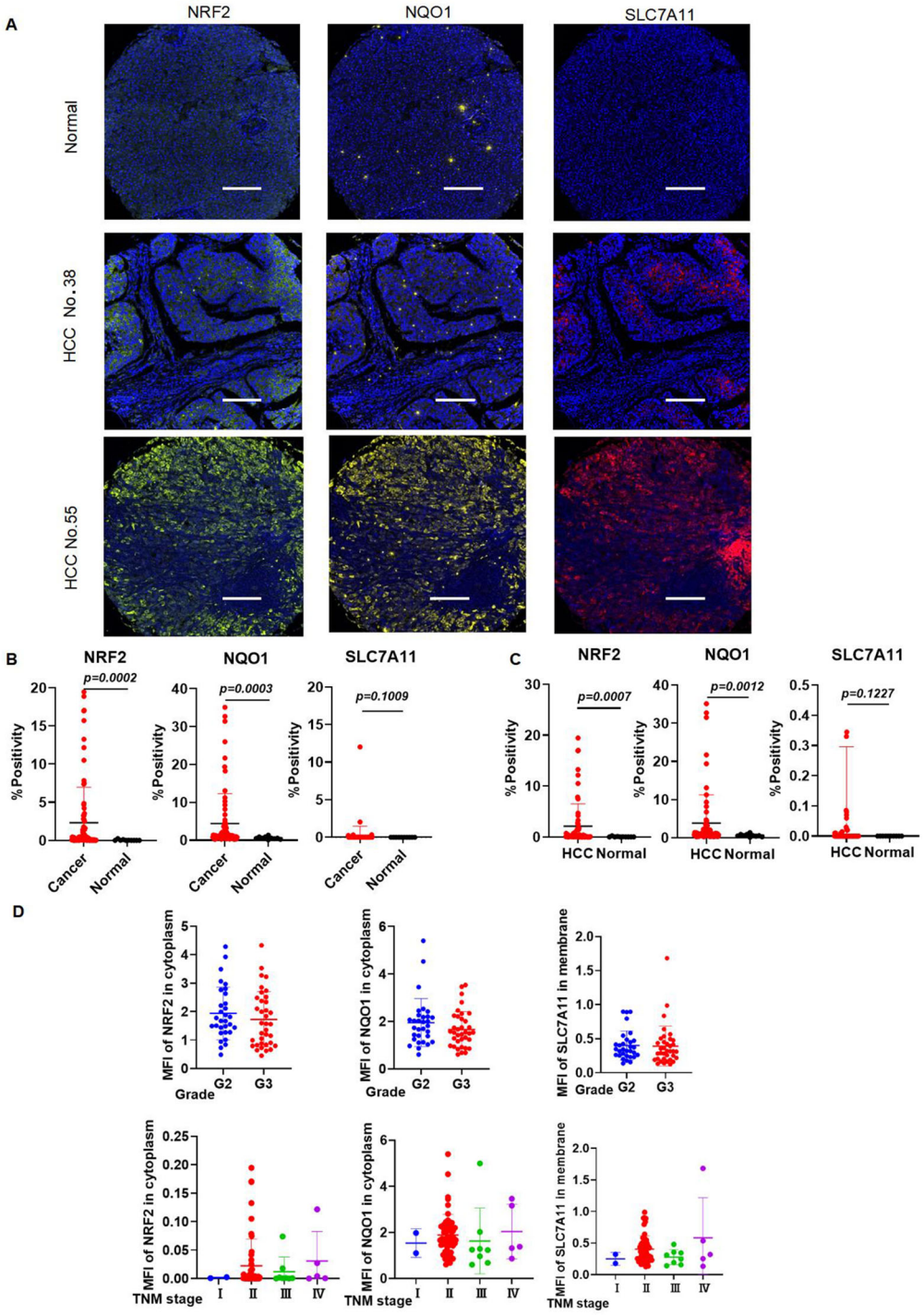


FIGURE 10
The expression levels of NRF2, SLC7A11, and NQO1 in HCC are higher in HCC than those in healthy individuals. **(A)** Representative images of mIHC staining of NRF2, SLC7A11, and NQO1. **(B)** mIHC statistical analysis of NRF2, SLC7A11, and NQO1 in all liver cancer and healthy individuals. **(C)** mIHC statistical analysis of NRF2, SLC7A11, and NQO1 in HCC and healthy individuals. **(D)** The expression of NRF2, NQO1, and SLC7A11 in different grades and TNM stages.

as evidenced by references (42, 43). Notably, NRF2 enhances the mRNA level of xCT by binding to the antioxidant response element (ARE), which is also recognized as the electrophilic response element (EpRE), located in the proximal promoter region of the xCT gene (43). Upon receiving oxidative signals, NRF2 translocates to the nucleus, where it binds to ARE to enhance the mRNA level of NQO1 within the promoter regions of numerous phase II detoxification and antioxidant genes (44). Furthermore, NRF2 and the inflammasomes it activates are responsible for inflammasome-dependent HMGB1 release (45). HMGB1, in turn,

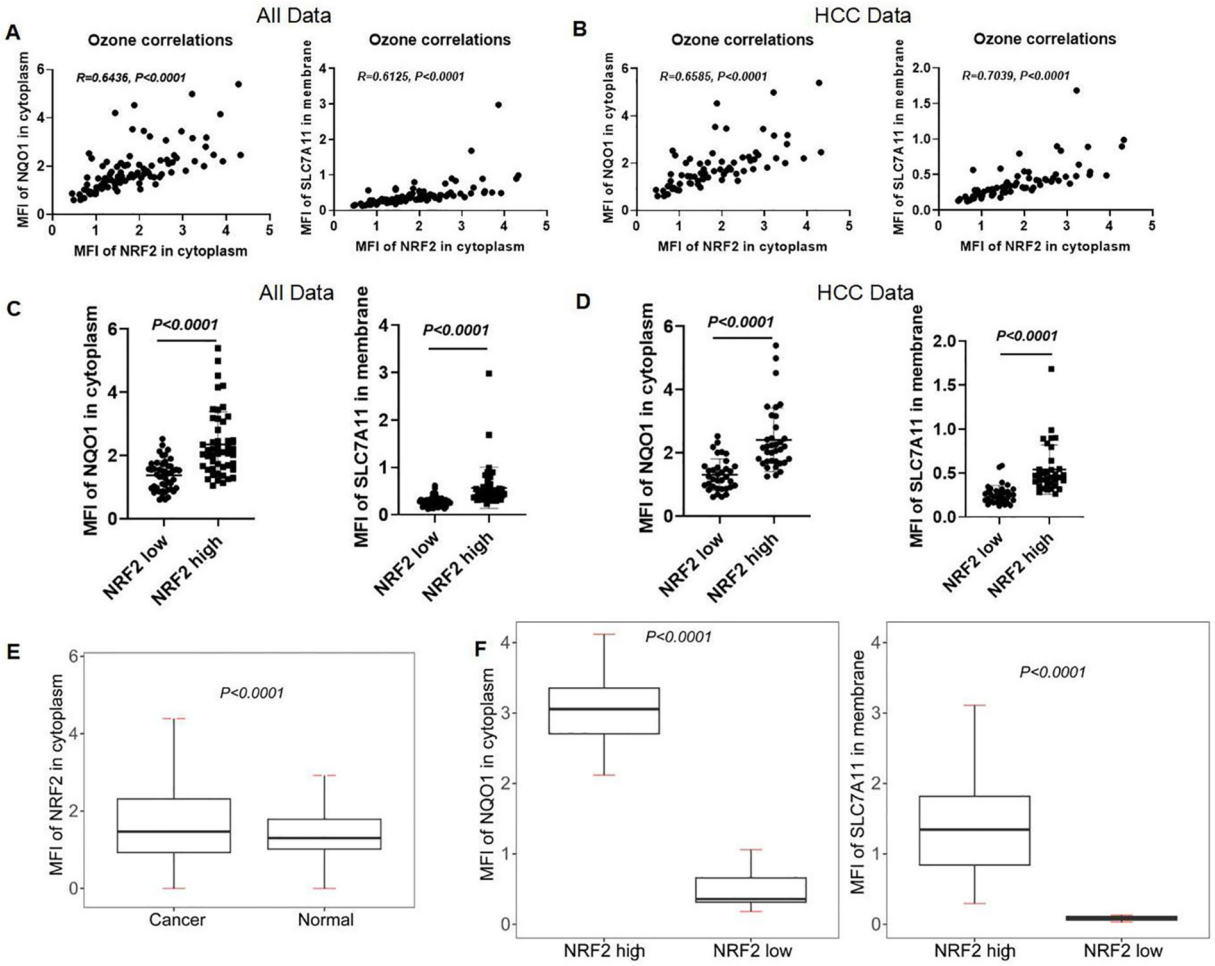


FIGURE 11
Correlation of NRF2 with NQO1 and SLC7A11 in human liver and HCC tissues. **(A, B)** The correlation analysis of NRF2, NQO1, and SLC7A11 in all subjects and HCC patients. **(C, D)** mIHC MFI statistical analysis of NQO1 and SLC7A11 in the local scan of all subjects and HCC patients with high and low expression of NRF2. **(E)** mIHC MFI statistical analysis of NRF2 in full scan between all liver cancer patients and normal people. **(F)** The different MFIs of NQO1 and SLC7A11 in HCC patients with high and low expression of NRF2.

promotes ferroptosis by modulating the Nrf2/HO-1 pathway (46). It is also worth mentioning that the inhibition of the p53/SLC7A11/GPX4 pathway, which is mediated by HMGB1, can effectively inhibit ferroptosis (47). Additionally, the inhibition of PTGS2 expression, coupled with the activation of the NRF2 signaling pathway and its downstream ferroptosis-related proteins, such as SLC7A11, can lead to a reduction in lipid peroxidation. This, in turn, alleviates ferroptosis induced by iron overload (45).

Our study seeks to confirm the regulatory role of NRF2 on SLC7A11 and NQO1 during HCC development and to evaluate the potential of DIC to enhance HCC susceptibility to IKE-induced ferroptosis. SLC7A11 and NQO1 have been identified as inhibitory genes of ferroptosis in previous studies. System Xc-, composed of SLC3A2 and SLC7A11 (xCT), facilitates cystine import and glutamate export. Elevated SLC7A11 expression in myeloma cells increases susceptibility to erastin-induced ferroptosis (48). Overexpression of SLC7A11 can promote tumor growth by inhibiting ferroptosis (49) and evade ferroptosis through post-transcriptional mechanisms. The inhibition of SLC7A11 has been shown to induce ferroptosis in tumor cells (30, 50). Activation of mTORC1 enhances ferroptosis resistance and tumor progression by up-regulating SLC7A11 (51). Decreased sensitivity to ferroptosis activators such as erastin has been observed in myocardial infarction models (52). IKE, an erastin analog, is recognized as an effective and metabolically stable inhibitor of the system Xc-, which can serve as a novel anti-tumor drug to inhibit tumor growth by inducing ferroptosis. The use of polyethylene glycol-poly (lactic-co-glycolic acid) nanoparticles (PEG-PLGA NPs) to facilitate IKE delivery has been highlighted in previous studies, demonstrating low toxicity in diffuse large B cell lymphoma (DLBCL) xenograft models (53). These findings suggest that the efficacy of IKE can be enhanced and its toxicity can be reduced through a specific drug delivery system, which is crucial for clinical application. NQO1, a cytoplasmic flavoprotein, is overexpressed in various cancers, including breast, pancreatic, hepatocellular, bladder, ovarian, thyroid, colorectal, cholangiocarcinoma, cervical, melanoma, and lung (54–58). Its overexpression is associated with larger tumor sizes, advanced stages, and poor survival rates (56, 58, 59).

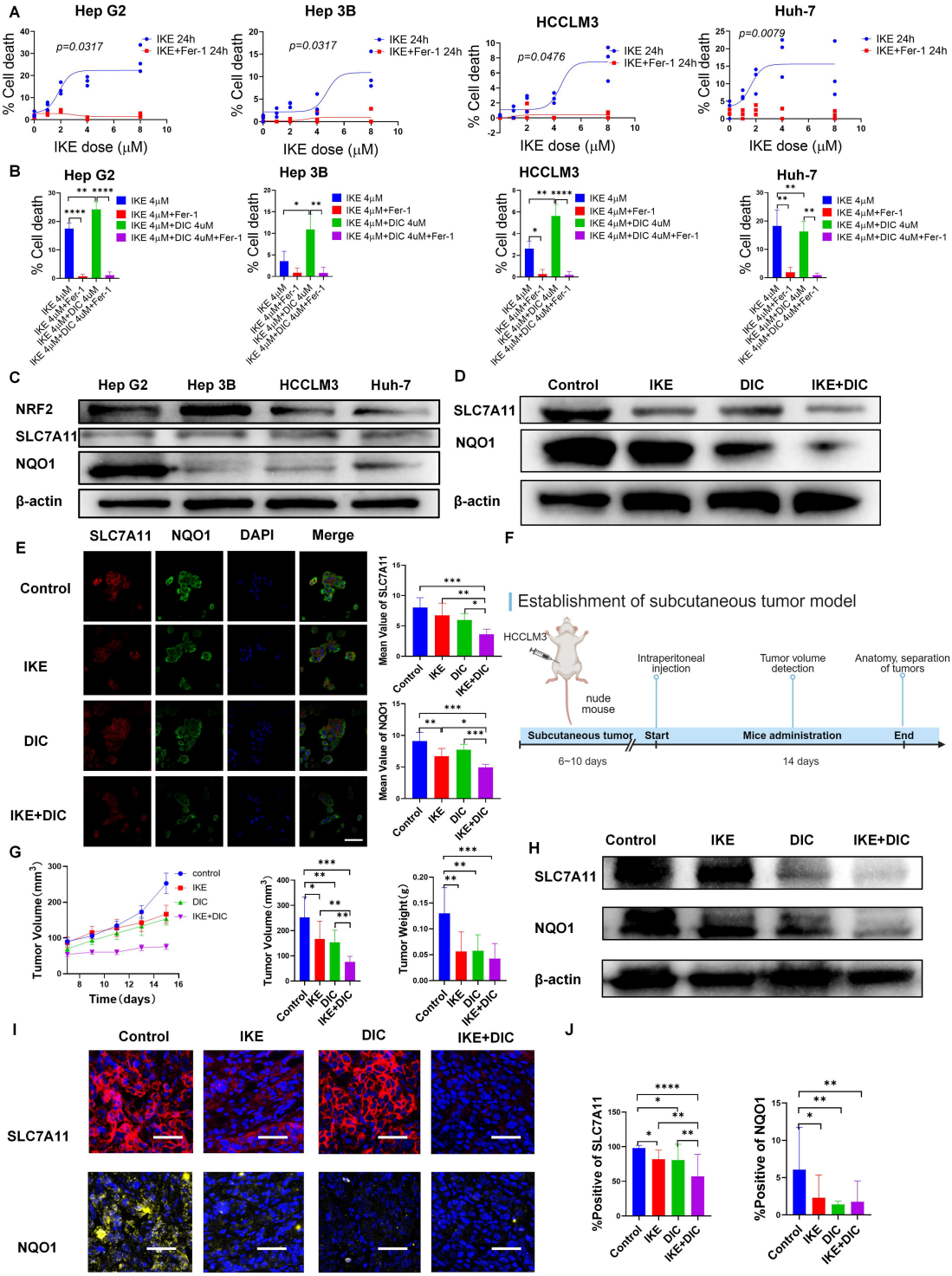


FIGURE 12
Establishment of subcutaneous tumor model. **(A)** The image shows the Hep G2, Hep 3B, HCCLM3, and Huh-7 were treated with IKE (0, 1, 2, 4, 8 μM) and IKE (0, 1, 2, 4, 8 μM) + Fer-1 (2 μM) respectively. Cell death was measured following 24-h treatment of IKE and Fer-1. **(B)** The image shows the Hep G2, Hep 3B, HCCLM3, and Huh-7 were treated with IKE (4 μM), IKE (4 μM)+Fer-1 (2 μM), IKE (4 μM)+DIC (4 μM), and IKE (4 μM)+DIC (4 μM)+ Fer-1 (2 μM) respectively. Cell death was measured following 24-h treatment of IKE, DIC, and Fer-1. n = 3 biologically independent samples per condition. **(C)** The expression of target protein in Hep G2, Hep 3B, HCCLM3, and Huh-7. **(D)** Protein expression of target molecules of HCCLM3 cells. **(E)** Immunofluorescence staining of SLC7A11 and NQO1 in HCCLM3 cells. Scale bars for others, 100 μm. **(F)** The establishment of a subcutaneous tumor model schematic diagram. **(G)** Subcutaneous tumor volume monitoring. **(H)** Protein expression of target molecules in subcutaneous tumor. **(I)** Multiple immunohistological staining of SLC7A11 and NQO1 in subcutaneous tumor. Scale bars for others, 100 μm. **(J)** Multiple immunohistological staining positive rate statistics of SLC7A11 and NQO1. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

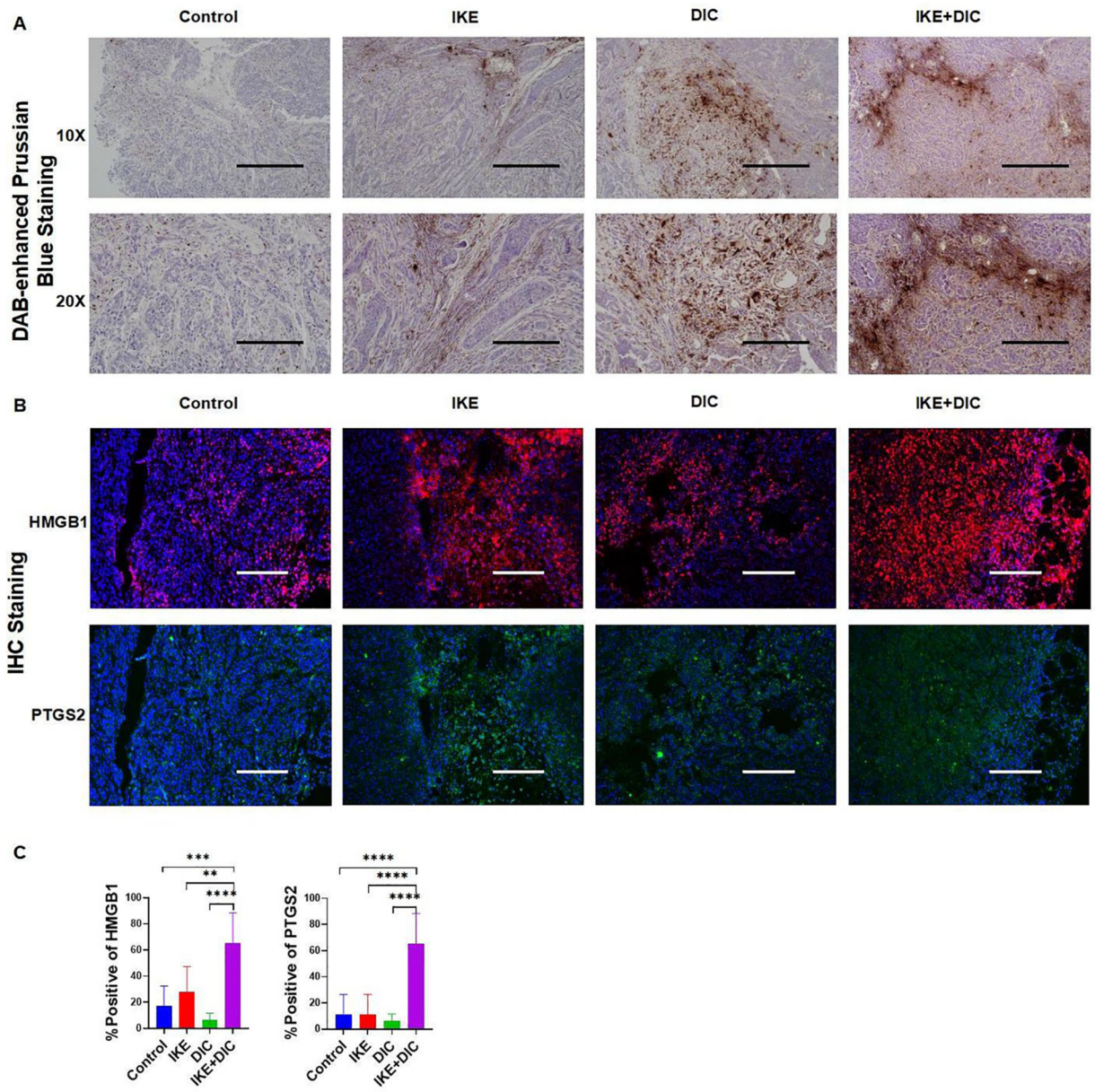


FIGURE 13
DIC enhances susceptibility to IKE-induced ferroptosis by increasing non-heme iron. **(A)** DAB-enhanced Prussian blue staining for iron in sections obtained from subcutaneous tumors. **(B)** Representative images of mIHC of HMGB1 and PTGS2 in subcutaneous tumors. **(C)** mIHC positivity statistical analysis of HMGB1 and PTGS2 in a partial scan of mice. **p<0.01, ***p<0.001, ****p<0.0001.

Triggering ferroptosis *via* NQO1 can also combat tumor drug resistance (30). DIC, as an NQO1 inhibitor and an FDA-approved drug, was identified as a potential therapeutic agent targeting core ferroptosis-related genes in polycystic ovary syndrome (60). Our study explores the potential of DIC to reduce IKE resistance and enhance HCC susceptibility to ferroptosis.

However, no literature has reported the synergistic effect of combining these two inhibitors to enhance the susceptibility of HCC cells to ferroptosis, thereby exerting an inhibitory effect on tumor growth. We hypothesize that the mechanism by which DIC enhances ferroptosis sensitivity to IKE may be related to the increase in non-heme iron levels. It is crucial to acknowledge that the subcutaneous xenograft model may not perfectly mimic the complex tumor microenvironment. Furthermore, since these models are typically established in immunodeficient mice, they may give rise to tumors that exhibit characteristics distinct from those of human cancers (61). Our subsequent studies will further verify the roles of IKE and DIC through patient-derived xenograft (PDX) models and patient-derived organoid (PDO) models. These models are expected to provide a more accurate representation of human cancers, allowing for a deeper understanding of the mechanisms and therapeutic potential of ferroptosis in HCC.

Although we have shown that DIC can reduce IKE resistance and increase HCC susceptibility to ferroptosis, it is essential to further verify the safety and efficacy of the combination of DIC and IKE in a larger cohort of clinical trials. This verification process

should include evaluating drug interactions, determining maximum tolerated doses, assessing pharmacokinetic and pharmacodynamic properties, and considering possible long-term side effects.

In this study, we have successfully developed a nomogram model that is designed to calculate the Risk Score for HCC patients based on the expression levels of five genes that have been rigorously pre-screened for their relevance in HCC. Application of this model provides a comprehensive elucidation of the molecular signaling events that are pivotal in the pathogenesis of HCC, and it offers valuable insights into the realm of targeted therapy. Furthermore, it identifies potential biomarkers that could be utilized for the prediction of therapeutic efficacy. While significant strides have been made in the development of targeted therapies for HCC, there are still formidable challenges to be addressed in the implementation of therapeutic strategies that are both effective and precise. We put forward the hypothesis that the application of DIC could enhance the sensitivity of HCC cells to ferroptosis, a form of regulated cell death that has been implicated in cancer treatment. This enhancement could potentially serve as a critical reference point in the development of novel treatment approaches for HCC, offering a new avenue for improving patient outcomes.

Data availability statement

The data presented in the study are deposited in Figshare, accession link: https://figshare.com/s/93882e56b8a8f153ba87.

Ethics statement

The animal study was approved by the ethics and welfare of Institutional Animal Care and Use Committee (IACUC) of National Translational Science Center (Approval No.: 2023-NTSCMM-ID008), National Translational Science Center for Molecular Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZY: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. TH: Conceptualization, Data curation, Investigation, Software, Visualization, Writing – original draft. RY: Writing – original draft. YZ: Writing – original draft. YQ: Writing – original draft. JH: Writing – original draft. FH: Writing – original draft. ZF: Writing – original draft. YD: Writing – original draft. JY: Writing – original draft. GZ: Writing – original draft. SW: Writing – original draft. XX: Writing – original draft. PL: Writing – original draft. Z-NC: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing. JW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Integrated UPLC-ESI-MS/MS, network pharmacology, and transcriptomics to reveal the material basis and mechanism of Schisandra chinensis Fruit Mixture against diabetic nephropathy

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Backgrounds: It has been regarded as an essential treatment option for diabetic nephropathy (DN) in Traditional Chinese medicine. Previous studies have demonstrated the anti-DN efficacy of Schisandra chinensis Fruit Mixture (SM); however, a comprehensive chemical fingerprint is still uncertain, and its mechanism of action, especially the potential therapeutic targets of anti-DN, needs to be further elucidated.

Objective: Potential mechanisms of SM action on DN were explored through network pharmacology and experimental validation.

Methods: The chemical composition of SM was analyzed using UPLC-ESI-MS/MS technology. Active bioactive components and potential targets of SM were identified using TCMSP, SwissDrugDesign, and SymMap platforms. Differentially expressed genes were determined using microarray gene data from the GSE30528 dataset. Related genes for DN were obtained from online databases, which include GeneCards, OMIM and DisGeNET. PPI networks and compound-target-pathway networks were constructed using Cytoscape. Functional annotation was performed using R software for GO enrichment and KEGG pathway analysis. The DN model was built for experimental validation using a high-sugar and high-fat diet combined with STZ induction. Hub targets and critical signaling pathways were detected using qPCR, Western blotting and immunofluorescence.

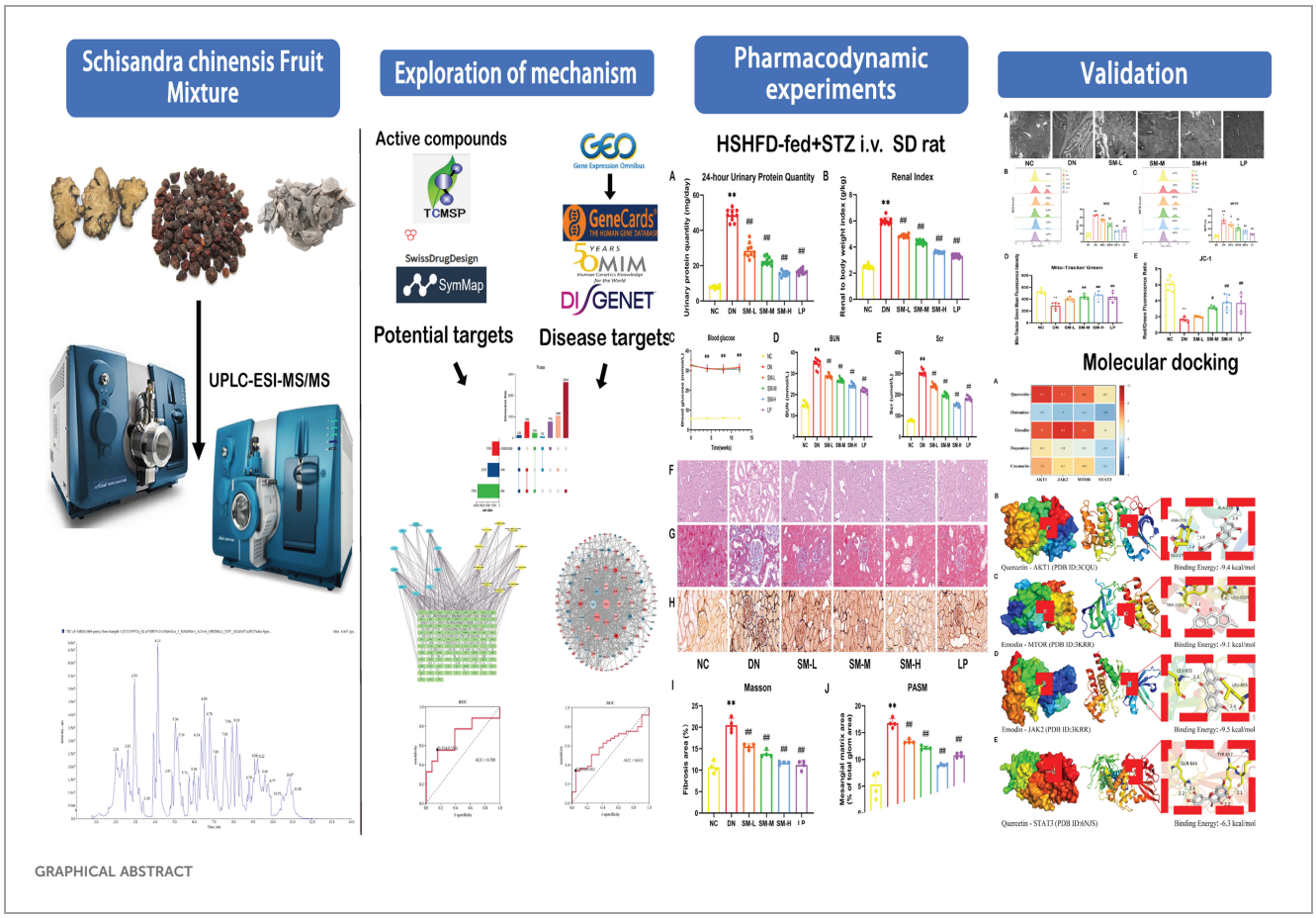
Results: Utilizing the UPLC-ESI-MS/MS coupling technique, a comprehensive analysis identified 1281 chemical components of SM's ethanol extract, with 349 of these components recognized as potential bioactive compounds through network pharmacology. Through this analysis, 126 shared targets and 15 HUB targets were

pinpointed. Of these, JAK2 is regarded as the most critical gene. Enrichment analysis revealed that SM primarily operates within the PI3K/AKT signaling pathway. *In vivo* experiments confirmed that SM improved pathological injury and renal function in rats with DN while improving mitochondrial morphology and function and modulating the expression of proteins linked to apoptosis (cleaved-caspase-3, Bax, and Bcl-2) and pro-inflammatory factors (IL-6 and TNF-α). Mechanistically, SM alleviates DN primarily by suppressing the PI3K/AKT/mTOR and JAK2/STAT3 signaling pathways to fulfill the energy needs of renal tissues. Furthermore, molecular docking analysis provided direct validation of these findings.

Conclusion: The findings of this study offer initial indications of the active component and robust anti-inflammatory and anti-apoptotic characteristics of SM in the mitigation of DN, along with its capacity to safeguard the integrity and functionality of mitochondria. This research unequivocally validates the favorable anti-DN effects of SM, indicating its potential as a viable pharmaceutical agent for the management of DN.

KEYWORDS

network pharmacology, diabetic nephropathy, Schisandra chinensis Fruit Mixture, JAK2/ATAT3 signalling pathway, inflammatory response, apoptosis



GRAPHICAL ABSTRACT

1 Introduction

Diabetic nephropathy (DN) is a significant microvascular complication arising from diabetes mellitus, which has emerged as a prevalent chronic metabolic disease globally, exhibiting a protracted clinical course and a substantial complication rate (1). Apoptosis, oxidative stress, and inflammatory responses have been implicated in the etiology of DN, ultimately leading to the deterioration of renal function (2). In clinical practice, DN is typically managed with a variety of drugs, including angiotensin-converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARBs), non-steroidal mineralocorticoid receptor antagonists (NS-MRAs), fexofenadine, and sodium-glucose cotransporter-2 (SGLT2) inhibitors. These drugs collectively aim to control blood pressure, reduce proteinuria, and preserve renal function (3). Prolonged use of these medications may lead to adverse effects such as persistent dry cough, urinary tract infections, and hypersensitivity reactions, which can limit their effectiveness (4, 5). Consequently, there is a pressing necessity to identify novel drugs.

Traditional Chinese medicine (TCM), specifically the Schisandra chinensis Fruit Mixture (SM) formulated by Professor Zhang Daning, is increasingly recognized as a beneficial strategy for the treatment of DN due to its empirical formula based on the principles of tonifying the kidney and activating the blood of the kidney (6). SM is a novel herbal formula comprising a combination of 3 herbal medicines: *Schisandra chinensis* fructus (Wuweizi; containing the dried fruiting body of *Schisandra chinensis* (Turcz.) Baill.), *Ligusticum chuanxiong Hort.* (Chuanxiong; containing dried roots and rhizomes of Ligusticum wallichii Franch), Ostreae concha (Muli; containing shells of three species of oyster: Crassostrea gigas Thunberg (Ostrea gigas Thunberg), Crassostrea talien-whanensis Crosse and Crassostrea rivularis Gould). Recent research has shown that herbal formulas with properties of kidney tonification and blood activation can effectively mitigate fibrotic injury in mice with streptozotocin (STZ)-induced DN, thereby preserving renal function (7). Previous investigations conducted by our research group have also demonstrated the ability of SM and its bioactive compounds to attenuate renal fibrosis in DN (8, 9). While these findings suggest the therapeutic potential of SM in treating DN, further research is needed to elucidate the specific components and mechanisms underlying its efficacy.

Phytochemical analysis utilizing ultrahigh-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) has proven to be a valuable analytical technique for identifying the bioactive compounds present in herbal remedies (10, 11). In addition, with the development of high-throughput sequencing technology, transcriptome and microarray profiling have been widely used in a variety of diseases, including DNA (12). In addition, with the development of high-throughput sequencing technology, transcriptome and microarray profiling have been widely used in a variety of diseases, including to find biomarkers of disease progression, gain a deeper understanding of the pathogenesis of DN, and develop new strategies for individualized treatments. In recent years, the integration of network pharmacology and transcriptomics has emerged as a significant approach for elucidating the mechanisms underlying the therapeutic effects of natural products. This method facilitates the identification of molecular and pharmacological pathways, thereby enhancing our understanding of the complex interactions involved in multi-targeted herbal formulations (13, 14).

This study utilized a combination of UPLC-ESI-MS/MS-based widely targeted metabolomic methods, network pharmacology, and transcriptomics to identify and predict the potential active components and targets of SM for the treatment of DN. Additionally, a rat model of DN induced by a high-sugar, high-fat diet (HSHFD) combined with STZ was established to confirm the predictive analyses and elucidate the underlying mechanisms of SM in treating DN. The Graphical Abstract is shown here.

2 Materials and methods

2.1 Preparation of SM ethanol extract and UPLC-ESI-MS/MS analysis

The herbs used in this study were sourced from the Tianjin Chinese Medicine Decoction Piece Co., Ltd., which included *Schisandra chinensis* fructus (batch number G2011040-03), *Ligusticum chuanxiong Hort.* (batch number 9210800101), and Ostreae concha (batch number G2104019-01), and verified by Professor Mianzhi Zhang. The authenticated samples were stored at the Experimental Centre of Dongfang Hospital, Beijing University of Chinese Medicine. The composition of SM was determined based on the specifications outlined in Supplementary Table 1. The extraction process for the specific drugs is detailed as follows. Initially, the mixed herbs were subjected to extraction by soaking them 8 times the volume of 95% ethanol (v/w) for a duration of half an hour, followed by heating and refluxing for 3 hours. The resulting solution was then filtered, and the residue from the filtration process was subjected to re-extraction by heating and refluxing with six times the weight of the original herb in 95% ethanol for 2 cycles, each lasting 3 hours. Subsequently, the solutions obtained from the three extraction processes were combined and concentrated to a relative density of 1 g/ml before being stored at a temperature of 4°C.

The 2 ml sample was weighed and placed in a 50 ml centrifuge tube, which was extracted with 1200μl of 70% methanol. After 1 minute, the sample underwent centrifugation at 12000 r/min, maintained at 4°C, for 3 minutes. Subsequently, the supernatant was filtered through a microporous filter membrane, possessing a pore size of 0.22 μm, and was subsequently analyzed using UPLC-ESI-MS/MS.

2.2 Strategy of identification and characterization of compounds

The analytical conditions were as follows: UPLC column, Agilent SB-C18 (1.8 μm, 2.1 mm * 100 mm); the mobile phase consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. Sample measurements were performed with a gradient program that employed the starting conditions of 95% A, 5% B. Within 9 min, a linear gradient to 5% A,

95% B was programmed, and a composition of 5% A, 95% B was kept for 1 min. Subsequently, a composition of 95% A and 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was 0.35 mL per minute, and the column oven was set to 40°C. The injection volume was set to 2 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear iontrap (QTRAP)-MS. The ESI source operation parameters were as follows: source temperature 550°C; ion spray voltage (IS) of 5500 V (positive ion mode) and -4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 50, 60, and 25 psi, respectively; the collision-activated dissociation (CAD) was high.

2.3 Target prediction of SM bioactive ingredients

Composition was performed on the screened substances using TCMSP (https://www.tcmsp-e.com//), SwissDrugDesign and SymMap (http://www.symmap.org/) databases, respectively, with the following screening criteria: the GI absorption has been set to "HIGH," and two or more compounds can be considered active ingredients if they pass all five predictive properties (Lipinski, Ghose, Veber, Egan and Muegge) by SwissADME program (http://swissadme.ch/index.php); exhibiting oral bioavailability (OB) of at ≥ 30% and drug-likeness (DL) of at ≥ 0.18 in the TSMSP database were deemed as the bioactive ingredients of SM. Then, we searched the TCMSP database, SymMap (http://www.symmap.org/), and SwissTargetPrediction database for predicted mapped targets. Next, the UniProt database (https://www.uniprot.org/) was applied by entering the target name and limiting the species to human. All retrieved targets were corrected to the gene symbol and the target corresponding to the compound was obtained.

2.4 Analysis of transcriptomics data and gene set enrichment analysis

The GSE30528 dataset was obtained from the GEO public database. It includes genes from the glomerular tissue of nine DN patients and thirteen normal controls on the GPL571 platform. To identify the corresponding genes, we utilized probe IDs within the Bioconductor package of the R software. In cases where a gene had multiple probes on the same chip, the average expression value of all those probes was considered representative of the gene's expression level. To identify differentially expressed genes (DEGs), we utilized the Benjamini-Hochberg corrected two-tailed t-test to control for false discovery rates and detect genes with significant expression changes. DEGs were further filtered ($p < 0.05$ and $|\log2$ fold change (FC)$| > 1$) using the Limma package (15) in R. Visualization of DEGs expression patterns was achieved through the generation of heatmaps and volcano plots using the 'Pheatmap' and 'ggplot2' packages in R (16), respectively. Analyzing and interpreting pathway-level changes between normals and DNs are done using gene set enrichment analysis (GSEA) (17). It was performed with the R package clusterProfiler (18), using the MSigDB GeneSet Database [H: hallmark gene sets (50)] as the reference genome and 10,000 alignments, with a significance threshold of 10. We visualized the results with the R package "ggplot2".

2.5 Acquisition of disease-related targets

DN-associated genes were obtained from GeneCards (https://www.genecards.org) (screening threshold: relevance score greater than 0), OMIM (http://www.omim.org), and DisGeNET (https://www.disgenet.org) online databases using the keyword "diabetic nephropathy".

2.6 Functional enrichment analysis of potential targets

GO and KEGG analyses were conducted using the R software package (org.Hs.eg.db, colorspace, stringi, DOSE, clusterProfile, ggplot2), which can be downloaded from the Bioconductor online site (https://www.bioconductor.org/) (19), to performed ID conversion, GO functional analysis, and KEGG pathway analysis. The screening criteria were set at a significance level of $p < 0.05$ to examine the biological processes (BP), molecular function (MF), and cellular components (CC) associated with the DEGs.

2.7 Construction of a PPI network and hub gene screening

The genes with overlapping regions were inputted into the STRING database (https://cn.string-db.org/) with a confidence score of 0.4, leading to the construction of a PPI network by removing unconnected nodes. The interactions among the various genes were retrieved, and the PPI network was refined utilizing Cytoscape software (v.3.8.2). The structure of the PPI network was assessed using the Network Analyzer plug-in, and an examination of node degree values was conducted to identify the central target gene network. JAK2 was identified as a HUB gene and subsequently verified in the GSE96804 dataset. To estimate the discriminatory capability of JAK2 in distinguishing DN patients from healthy controls, the ROC (20) was plotted utilizing the "pROC" package in R software.

2.8 Active ingredient-target-disease network diagram construction

The Cytoscape software was utilized to construct a 'component-target-pathway' network to investigate the anti-DN mechanism of active compounds in SM.

2.9 Molecular docking studies

It was determined whether there are any potential interactions between the target and key bioactive components through molecular docking (21). The crystal structures of the key target

genes were retrieved from the PDB online database (22). Details of the critical proteins are shown in Supplementary Table 1. Subsequently, the 3D structural representations of the ligands were obtained from the PubChem database (23) (https://pubchem.ncbi.nlm.nih.gov/), which were comprised of Coumarin (PubChem CID: 323), the primary active component of SM, Dopamine (PubChem CID: 681), Emodin (PubChem CID: 3220), Histamine (PubChem CID: 774), and quercetin (PubChem CID: 5280343). Next, the small molecule structure was optimized using the MMFF94 force field of the OpenBabel toolbox (24) to finally obtain the optimal molecular structure for the lowest energy state. Proteins were hydrogenated using AutoDock Tools 1.5.6, and small molecules were hydrogenated and determined to be torsionally bonded and saved as pdbqt files. Use the Grid plate to set the molecular docking range parameters, and the docking range parameters for each protein are shown in Supplementary Table 2. Subsequently, Auto Dock Vina 1.2.0 software (25) was run to docking model to calculate the affinity between receptor and ligand. The results were visualized using PyMOL (v.2.3.0) software (26).

2.10 Experimental validation *in vivo*

2.10.1 Experimental animals

Sixty-five male Sprague-Dawley (SD) rats of clean-grade quality, aged 6 weeks with a body mass of 200 ± 20 g, were procured from Beijing Viton Lihua Laboratory Animal Technology Co. Ltd (Laboratory Animal License No. SYXK (Beijing) 2019-0013). The SD rats were housed in a specific pathogen-free (SPF)-grade animal facility at the Experimental Animal Centre of Dongfang Hospital, University of Chinese Medicine, Beijing, China. The housing conditions included a temperature of 23 ± 2°C, humidity maintained within the 40% - 60% range, light and dark cycle of 12 hours, and ad libitum access to water and food. All procedures in this study followed internationally recognized principles for using and caring laboratory animals and were approved by the Animal Protection and Use Committee of the Oriental Hospital of Beijing University of Chinese Medicine ((approval number: DFYY202102R).

2.10.2 Experimental method

Following a one-week acclimatization period, the rats were divided into the normal group (NC, n=10) and the model group (M, n=55). The M group was subjected to an HSHFD consisting of a 67% maintenance diet, 10% lard, 20% sucrose, 2.5% cholesterol, and 0.5% sodium cholate, while the NC group continued with a standard diet. After six weeks of unrestricted feeding, diabetes was induced in the M group through intraperitoneal injection of 35 mg/kg STZ (Sigma-Aldrich, S0130-1G) freshly dissolved in sodium citrate buffer (0.1 mmol/L, pH 4.5, Solarbio, C1013). In contrast, rats in group NC were injected with an equal volume of sodium citrate buffer intraperitoneally.

Random blood glucose levels were assessed using a Roche blood glucose meter after 72 hours. Rats were deemed to have effectively simulated DN if they exhibited a random blood glucose concentration of ≥ 16.7 mmoL/L (300 mg/dL) for three consecutive days and a 24-hour urine protein quantification of ≥ 30 mg/24h (27). Five rats died in the process and were excluded, and rats with successful modelling (n = 50) were used for further studies. Next, M group was randomly divided into 5 groups: (1) DN (n=10), (2) SM-L (1.5 g/kg/d, n = 10), (3) SM-M (3 g/kg/d, n = 10, (4) SM-H (6 g/kg/d, n = 10), (5) Losartan Potassium (LP) (20 mg/kg/d, n = 10), which was administered continuously for 12 weeks. Gastric gavage gave equal volumes of double-distilled water in the DN and NC groups. As illustrated in Figure 1A, the experimental methodology involved gavage administration at specified time intervals, with regular monitoring of body weight and random blood glucose levels every 4 weeks. Upon reaching week 20, all rats were humanely euthanized, and serum and tissue samples were obtained. A portion of the kidney was preserved in 4% paraformaldehyde for histological examination, while the remaining portion was promptly frozen for subsequent molecular analysis. The experimental design is shown in Figure 1A.

2.10.3 Measurement of 24-hour quantitative urine protein

The 24-hour urine of rats in each group was collected in metabolic cages and mixed well. The supernatant was aspirated after centrifugation (3500 rpm × 20 min at four °C), and the quantification of 24-h urinary protein was determined by a urinary protein kit (Nanjing JianCheng, C035-2-1). The 24-hour urinary protein was calculated and detected every 4 W.

2.10.4 Measurement of biochemical indicators

Blood samples were obtained from the abdominal aorta of all rats and subsequently centrifuged (3500 rpm × 15 min at 4°C) after a 3-hour incubation at room temperature to isolate serum samples to assess baseline renal function. Serum urea nitrogen (BUN) levels were determined using a blood urea nitrogen kit (NanJing JianCheng, C013-2-1) and serum creatinine (Scr) was measured using a blood creatinine kit (NanJing JianCheng, C011-2-1) as per the specifications provided by the reagent vendors.

2.10.5 Measurement of the kidney to body weight ratio

After execution, both kidneys were removed and weighed, as described previously, and the ratio of total kidney weight (mg)/rat body weight (g) was calculated. A kidney-to-body weight ratio can also indirectly reflect the degree of renal fibrosis as a quantitative indicator of kidney hypertrophy.

2.10.6 Histological analysis

The kidneys were harvested and immersed in a 4% paraformaldehyde solution for 48 hours, followed by dehydration, paraffin embedding, and sectioning at a thickness of approximately 3 μm. Subsequently, staining techniques, including hematoxylin and eosin (H&E), periodic acid silver methylamine (PASM), and Masson's trichrome (Masson), were conducted.

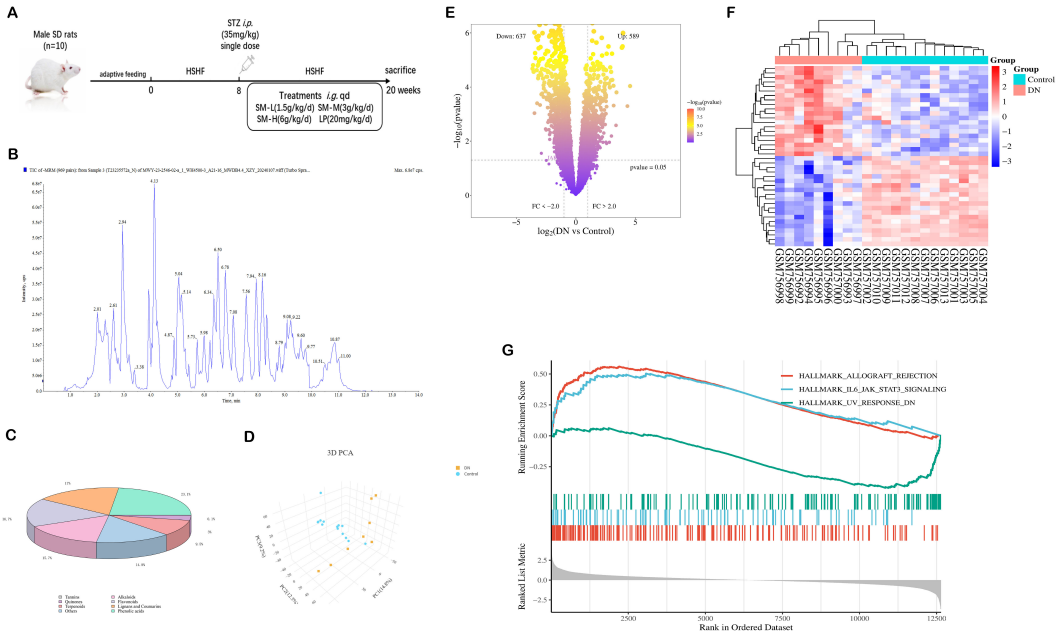


FIGURE 1
Identification of SM chemistry and acquisition of DN targets. **(A)** Experimental design. **(B)** The total ion current is based on a UPLC-ESI-MS/MS chromatogram of SM's ethanol extract. **(C)** Categorized pie chart of SM components. **(D)** PCA diagram of GSE30528. **(E)** Volcano map of DEGs in GSE30528. **(F)** Heat map of DEGs in GSE30528. **(G)** GSEA Analysis in GSE30528.

2.10.7 Transmission electron microscopy observation of mitochondria

Fresh kidney tissue samples measuring 1 mm^3 were fixed in 2.5% glutaraldehyde solution prepared in 0.1 mol/L phosphate buffer (pH = 7.4) for 2 hours at 4°C using a pre-chilled blade. Subsequently, the samples underwent three washes with 50 ml of phosphate buffer (pH = 7.4, 0.1 mol/L). The kidney tissues were extracted and reintroduced into 1% osmium tetroxide, fixed at 4°C for 2 hours, followed by two washes with phosphate buffer. Dehydration was carried out in a series of graded ethanol concentrations. Next, immersion, embedding, and polymerization were performed. Finally, the ultrathin sections (50 nm thick) were observed and photographed using a TEM after double staining with dioxiranyl acetate and lead citrate for 20 min.

2.10.8 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining

Apoptosis was detected in kidney tissues with the One-step TUNEL *In Situ* Apoptosis Kit (Elabscience, E-CK-A320) following the manufacturer's instructions. Then, the nuclei were stained with DAPI. Finally, images were acquired with a fluorescence microscope (Nikon, Japan).

2.10.9 Measurement of mitochondrial content (MitoTracker Green), the mitochondrial membrane potential, mitochondrial permeability transition pore and reactive oxygen species

Fresh kidney tissues were obtained, and single-cell suspensions of *in vivo* kidney tissues were prepared. The cells were then incubated in Calcein AM staining solution and DCFH-DA for 30 minutes at 37°C respectively, shielded from light, following the established protocols of the MPTP assay kit (Beyotime, C2009S) and ROS assay kit (Beyotime, S0033S). Subsequently, after washing, centrifugation, and resuspension, the average fluorescence intensity was quantified using flow cytometry to evaluate the extent of MPTP and ROS opening.

Mitochondrial activity was measured utilizing the MitoTracker Green kit (Yeasen, 40742ES50).

The staining working solution was prepared according to the Mito Tracker® Green FM kit, and after washing with PBS, the treated cells were stained with Mitotracker Green FM. Fluorescence intensity was scanned and analyzed at excitation/emission wavelengths (490/523 nm) using a fluorescent enzyme marker (Molecular Devices, USA).

According to the literature, Mitochondria were extracted and purified from kidney tissue using the Mitochondrial Extract Kit (Servicebio, SM0020). Then, changes in MMP were detected using the MMP assay kit (Beyotime, C2003S) containing JC-1. Purified mitochondria were stained with JC-1 staining solution at 37°C for 20 min. Fluorescence intensity was scanned and analyzed using a fluorescence enzyme marker (Molecular Devices, USA). JC-1 monomer expression was detected at an excitation wavelength of 490 nm and an emission wavelength of 530 nm. Red fluorescent JC-1 aggregates are observed in hyperpolarized membranes, while the green fluorescent monomeric form indicates membrane depolarization. Thus, MMP was determined by analyzing the reduced red/green fluorescence intensity ratio, with higher values indicating a more structurally intact mitochondrial membrane.

2.10.10 Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted using Trizol reagent following the manufacturer's instructions, and RNA integrity was evaluated by spectrophotometry at 260 nm. Subsequently, RNA was reverse transcribed to cDNA utilizing a reverse transcription kit (ExonScript RT SuperMix with dsDNase) with reaction conditions set at 25°C for 10 min, 55°C for 15 min, and 85°C for 5 min. The cDNA product was used as a PCR template, and the reaction conditions for real-time PCR were as in Supplementary Table 3. The relative expression levels of target genes (Caspase-3, Bcl-2, and Bax m RNA) were determined using the 2-ΔΔCt method with β-actin as the internal reference. The amplification was carried out using a fluorescence quantitative PCR instrument. Primer sequences can be found in the Supplementary Table 4.

2.10.11 Enzyme-linked immunosorbent assay

The levels of IL-6 (Lianke, EK306) and TNF-α (Lianke, EK382) in renal tissues were determined by ELISA kits according to the instructions of the reagent vendor.

2.10.12 Western blot analysis

Remove the unnecessary punctuation and clarify the steps more formally: 100 mg of rat kidney tissue was removed from a -80°C freezer and added to RIPA tissue lysate. The mixture was homogenized on ice and allowed to stand for 10 minutes before being centrifuged at 12,000 rpm for 15 minutes at 4°C to collect the supernatant. The total protein content was determined using the BCA Protein Quantification Kit, and separation gels and stacked gels were prepared. The protein was transferred to a PVDF membrane via SDS-PAGE electrophoresis. The gel blocking was performed with 5% nonfat dry milk in Tris-buffered saline containing 0.1% TBST at room temperature for 1 h, followed by incubation with primary antibodies overnight at 4°C. Primary antibodies include AKT (1:1000, Proteintech, 60203-2-Ig), p-AKT (1:1000, Proteintech, 80455-1-RR), mTOR (1:1000, Proteintech, 28273-1-AP), p-mTOR (1:1000, Biodragon, BD-PP0176), JAK2 (1:1000,Biodragon, BD-PT2426), p-JAK2 (1:1000, Biodragon, BD-PP1374), STAT3 (1:1000, Biodragon, BD-PT4443), p-STAT3 (1:1000, Biodragon, BD-PP1513), cleaved-Caspase-3 (1:1000, proteintech, 25128-1-AP), Bc1-2 (1:1000, Biodragon, BD-PT0470), Bax (1:1000, Biodragon, BD-PT0455) and β-actin (1:3000, Affinity, AF7018). Following three washes with TBST, the membrane was incubated with the appropriate secondary antibody for 1 hour at room temperature. Protein bands were visualized and quantified using ECL and Image J software.

2.10.13 Immunofluorescence

Frozen renal tissues were sectioned (5 μm), fixed, and washed with PBS buffer before incubating with a 5% sealing serum at room temperature for 1 hour. The sealing solution was then removed, and the tissues were incubated overnight at 4°C with primary antibodies (anti-p-AKT, 1:100; anti-p-mTOR, 1:200; anti-p-JAK2, 1:50; p-STAT3, 1: 50). The following day, the sections were washed with PBS and incubated with corresponding secondary antibodies at room temperature for 1 hour in the dark. After the second antibody incubation, the tissue sections were washed with PBS and incubated with DAPI in the dark for 10 minutes. Next, the sections were sealed by adding an anti-fluorescent bursting agent and visualized by fluorescence microscopy.

2.11 Statistical analysis

Experimental data were collected with the mean expressed as ± standard deviation (SD). Each independent experiment was replicated a minimum of three times to determine the mean. Student's t-test was utilized for comparisons between two groups, while one-way ANOVA and *post hoc* Tukey's test were employed for comparisons among multiple groups. Wilcoxon paired-signs rank test was used for comparisons between groups with non-parametric data distributions. Statistical analyses and graphical representations were performed using GraphPad Prism 8.0. A significance level of $P < 0.05$ was considered statistically significant.

3 Results

3.1 Results of the identification of the components of the alcoholic extract of SM

3.1.1 Identification and characterization of constituents of SM

The chemical composition of SM was thoroughly analyzed using UPLC-ESI-MS/MS, with the total ion chromatogram in negative ion mode depicted in Figure 1B. A total of 1281 chemical components in SM were identified based on the MS data and standard fragmentation pattern. These constituents were further categorized into 8 classes, as illustrated in Figure 1C.

3.2 Results of network pharmacological analyses

3.2.1 Results of prediction and enrichment analysis of the active components of SM

The 1281 compounds were subsequently inputted into TCMSP, SwissDrugDesign, and SymMap databases for active ingredient screening and target prediction of SM. A total of 349 SM bioactive components (Supplementary Table 5) and 2025 corresponding targets were annotated.

3.2.2 The results of prediction for biomarkers of DN

The results of principal component analysis (PCA) depicted in Figure 1D revealed significant distinctions between the populations of the DN and Control groups within the GSE30528 dataset, suggesting the credibility of the sample sources. Subsequently, a total of 1 226 DEGs were obtained (Figure 1E). As shown in

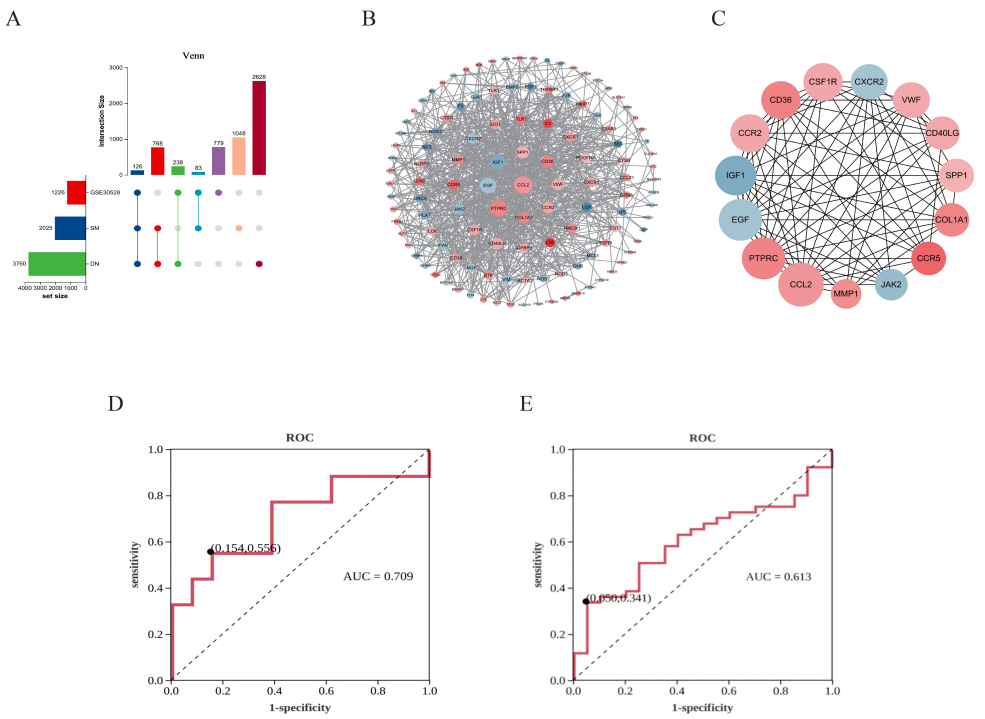


FIGURE 2
Identification results of critical targets for SM therapy DN. **(A)** UpSet diagram. **(B)** PPI network. **(C)** Network of HUB genes. **(D)** ROC analysis of JAK2 in GSE30528. **(E)** ROC analysis of JAK2 in GSE96804.

Figure 1F, the results of the clustered heatmap indicated that the DEGs were able to clearly differentiate between DN tissues and normal tissues, and thus, the DEGs were representative of the whole sample. GSEA (Figure 1G) showed that the DEGs from the GSE30528 dataset were mainly involved in pathways related to inflammation, energy metabolism and immunity, including IL6/JAK-STAT3 signaling, inflammation response, oxidative phosphorylation, epithelial-mesenchymal transition, UA response DN, TGF - β signaling, etc.

3.2.3 Prediction for DN-related target genes of GeneCards, OMIM, and DisGeNET

Three databases were consulted to collect 3760 DN-related target genes: GeneCards, OMIM, and DisGeNET.

3.2.4 Construction of PPI network, identification and validation of HUB genes

The UpSet plot revealed that a collective sum of 126 shared genes was identified from SM, DN, and GSE30528 datasets (Figure 2A). Subsequently, the significance of these potential targets was assessed through network topology analysis in PPI analysis. After removing isolated nodes, the network comprised 120 nodes and 769 edges. The significance of nodes within the network was assessed based on their degree. Furthermore, in the PPI network graph (Figure 2B), red nodes denote up-regulated genes, while blue nodes represent down-regulated genes. The intensity of the color is directly proportional to the absolute value of the log2 FC, and the size of the nodes is determined by their degree (higher-degree nodes are positioned closer to the network center and have larger areas). The top 15 protein targets with mean degree values greater than 24 included CCL2, PTPRC, EGF, IGF1, SPP1, CD36, VWF, COL1A1, CCR2, CD40LG, CSF1R, JAK2, CCR5, CXCR2, and MMP1, which were identified as HUB genes (Figure 2C). The ROC curve of JAK2 in GSE30528 exhibited an AUC of 70.9%, specificity of 84.6%, and sensitivity of 55.6% (Figure 2D). Additionally, a validation set (GSE96804) was utilized to enhance the accuracy and reliability of the results, confirming the diagnostic ability and expression level of JAK2. The AUC of JAK2 was determined to be 61.3% (Figure 2E), indicating its importance as a biomarker for DN with significant diagnostic potential. These results demonstrated that JAK2 is a crucial biomarker for DN, with significant diagnostic value for DN. Detailed information on the core targets is provided in Supplementary Table 6.

3.2.5 Functional analysis of DN-related genes of SM

To elucidate the potential functions of the overlapping genes, gene annotation and functional enrichment analyses were performed using R software (Figures 3A–D). GO enrichment analysis were mainly involved in treating 2 major aspects of DN injury, including inhibition of apoptosis and inhibition of inflammation. The KEGG pathways related to these targets ($p < 0.05$) included pathways in (Figures 3B–D): PI3K/AKT signaling pathway, Rap1 signaling pathway, MAPK signaling pathway, Calcium signaling pathway, HIF-1 signaling pathway, etc. These

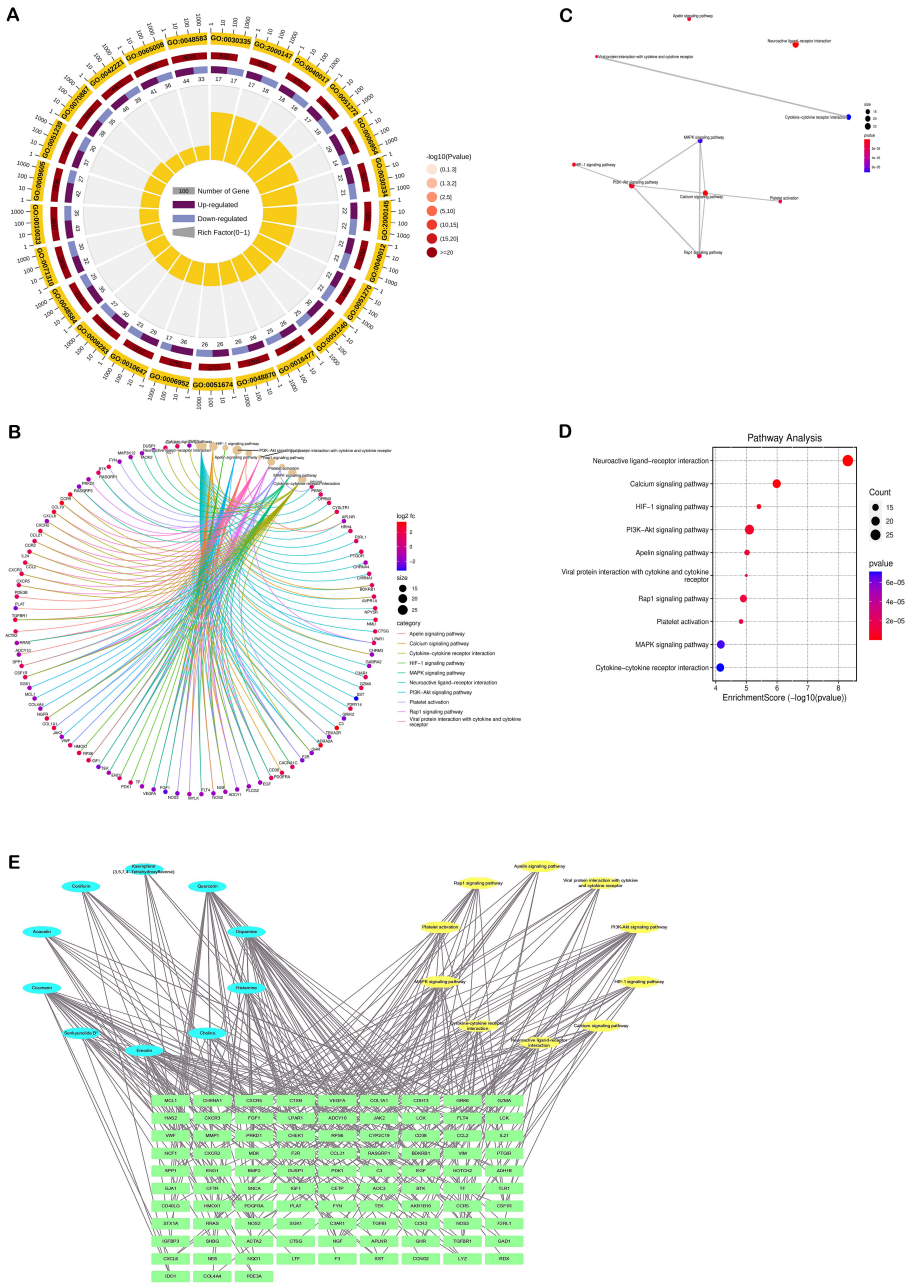


FIGURE 3
Results of enrichment analysis of the DN-related targets of SM. **(A)** Chord diagram of GO pathway. **(B-D)** KEGG analysis. **(E)** Network of component-target-pathway. The blue ovals represent the active ingredients of the SM, the yellow oval represents the pathway, and the green diamond represents the target.

pathways are mostly associated with apoptosis, inflammatory response, and mitochondrial function.

3.2.6 Component-target-pathway maps and key components

The 'component-target-pathway' network (Figure 3E) showed that Quercetin was the most active component of SM, followed by Dopamine, Coumarin, Emodin, and Histamine, with degree values of 511, 320, 320, 237, and 182, respectively.

3.3 Experimental verification

3.3.1 Therapeutic effect of SM in STZ-induced DN rats

The findings indicated a significant increase in 24-hour urinary protein and kidney weight/body weight ratio in DN rats compared to NC rats, while DN rats treated with SM/LP exhibited a reduction in these parameters (Figures 4A, B). Additionally, the M group, after STZ interventions, displayed blood glucose levels exceeding

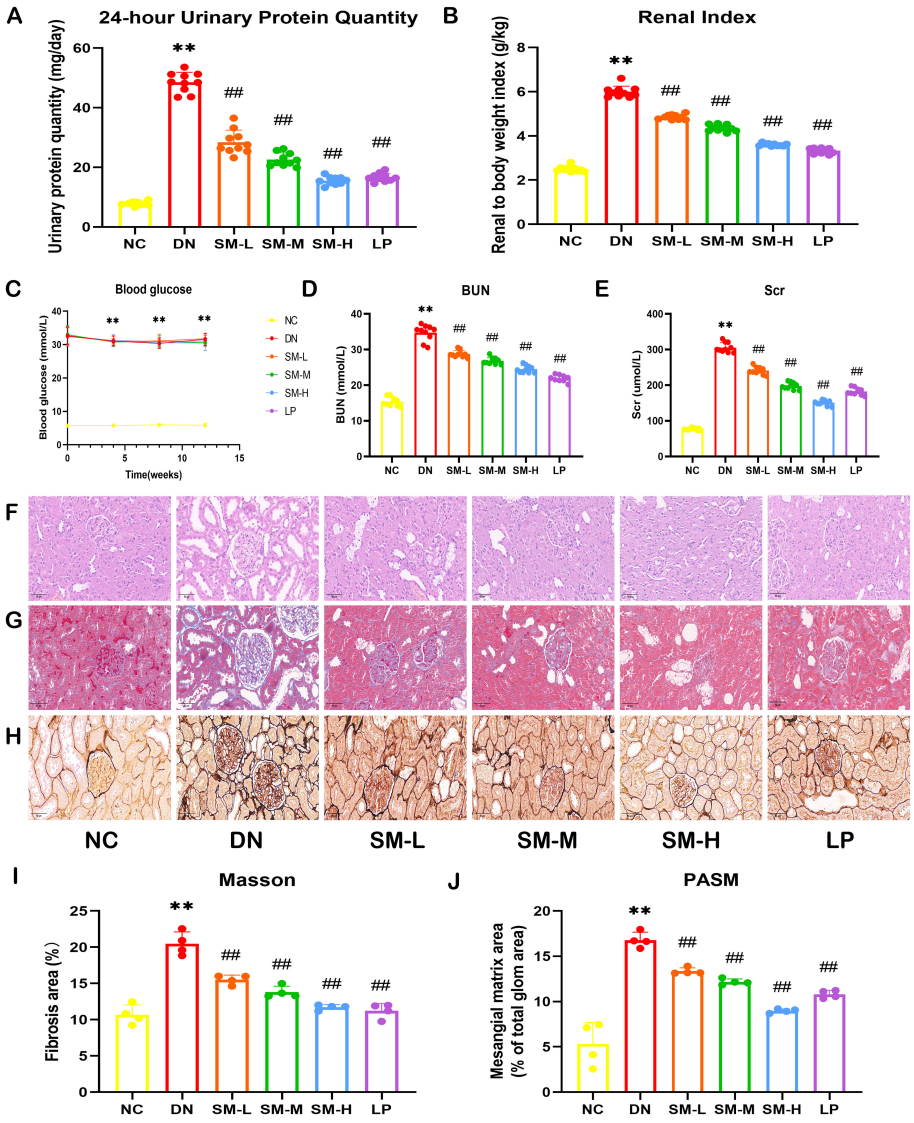


FIGURE 4
Effects of SM on DN rat. **(A)** 24-h-urine protein levels. **(B)** Renal Index. **(C)** Blood glucose levels. **(D)** BUN levels. **(E)** Scr levels. **(F)** Representative images of H&E staining (200 ×; scale bar = 50 μm). **(G)** Representative images of PASM staining (400 ×; scale bar = 20 μm). **(H)** Representative images of Masson staining (400 ×; scale bar = 20 μm). **(I)** Mesangial matrix area. **(J)** Fibrosis area. The data are X ± SEM. ** $p < 0.01$ *vs.* normal control (NC) group; $^{\#\#}p < 0.01$ *vs.* diabetic nephropathy (DN) group.

16.7 mmoL/L (Figure 4C). Furthermore, following a 12-week period of SM treatment, our study revealed that there was no statistically significant variance in blood glucose levels between the DN group and the SM group, suggesting that SM did not confer a notable advantage in terms of blood glucose regulation in DN rats. Analysis of Figures 4D, E indicated that levels of BUN and Scr were markedly elevated in the DN group. Still, following treatment with the drug, there was a significant decrease in Scr and BUN levels in the SM/LP-treated groups. Subsequently, the impact of SM on morphological alterations in DN rats was assessed through HE, MASSON and PAS staining (Figures 4F–J). Our findings indicate that SM mitigated renal injury in DN rats dose-dependently. Furthermore, the therapeutic efficacy of SM was comparable to that of LP, a commonly prescribed medication for the clinical management of DN. HE, PAS and MASSON staining confirmed these findings, demonstrating a reduction in glycogen deposition in the glomeruli of DN rats following SM/LP treatment. Notably, the improvements were more pronounced in the SM-H and LP groups. These results provide compelling evidence that SM possesses significant potential for treating DN.

3.3.2 Effects of SM on mitochondrial morphology and function in DN rats

The alterations in mitochondrial ultrastructure were elucidated through TEM. The TEM images revealed that the mitochondrial morphology and structure in the kidney tissues of DN rats and NC rats were predominantly normal. However, compared to the NC group, the DN group exhibited decreased mitochondrial volume, the darker coloration of mitochondria, ruptured mitochondrial membranes, and reduced cristae. SM/LP-treated groups were

improved when compared with those in the DN group, and the most noticeable improvement was found in the SM-H and LP groups (Figure 5A).

Mitochondrial dysfunction was identified by decreased mitochondrial content, elevated ROS production, heightened MPTP opening, and diminished MMP.

Mitotracker Green indicated changes in mitochondrial content, DCFH-DA staining assessed changes in ROS, JC-1 probes determined changes in MMP, and Calcein AM staining suggested the degree of MPTP opening. In the DN group, it was observed that excessive ROS production correlated with reduced MMP, increased MPTP opening, and significantly decreased mitochondrial content (Figures 5B–E). However, treatment with SM-H/LP partially reversed the decline in MMP and mitochondrial content, inhibited MPTP opening, and reduced ROS production (Figures 5B–E). In summary, SM-H treatment mitigated mitochondrial damage.

3.3.3 SM ameliorated STZ-induced inflammatory response in the rats with DN

To assess the impact of SM on the inflammatory response in DN *in vivo*, the expression levels of inflammatory factors in renal tissues post-modeling were analyzed using ELISA. The findings indicated a notable upregulation and enhanced expression of IL-6 and TNF-α in the DN group ($p < 0.05$). Subsequent intervention with SM and LP significantly mitigated this effect ($p < 0.05$). Consequently, SM demonstrates efficacy in suppressing the inflammatory response in DN, as evidenced by the decreased expression of IL-6 and TNF-α, suggesting its potential for inhibiting DN via the inflammatory signaling pathway (Figures 6A, B).

3.3.4 SM ameliorated STZ-induced apoptosis in the rats with DN

TUNEL staining showed that apoptotic cells increased in the DN group, while apoptotic cells decreased after SM-M and SM-H treatment (Figures 6C, D). Moreover, western blotting showed that cleaved-caspase-3 and Bax were up-regulated in the DN group compared with the NC group, in contrast to the down-regulation of Bcl-2. The administration of SM-H/LP treatment reduced the downregulation of anti-apoptotic proteins and the inhibition of the upregulation of pro-apoptotic factors. Consistent with the findings of protein blotting, PCR analysis demonstrated higher levels of cleaved-caspase3 and Bax expression in the DN group compared to the NC group ($p < 0.01$). In contrast, Bcl-2 expression was lower in the DN group ($p < 0.01$). Treatment with SM-H effectively modulated this specific expression pattern. These results indicate that SM-H may mitigate DN by suppressing apoptosis (Figures 6C–G).

3.3.5 Regulation of PI3K/AKT/mTOR signaling pathway and JAK2/STAT3 signaling pathway by SM

Western blot analysis was used to semi-quantitatively assess the expression levels of four proteins: AKT, p-AKT, mTOR, and p-mTOR. The findings indicated a notable increase in the phosphorylated protein

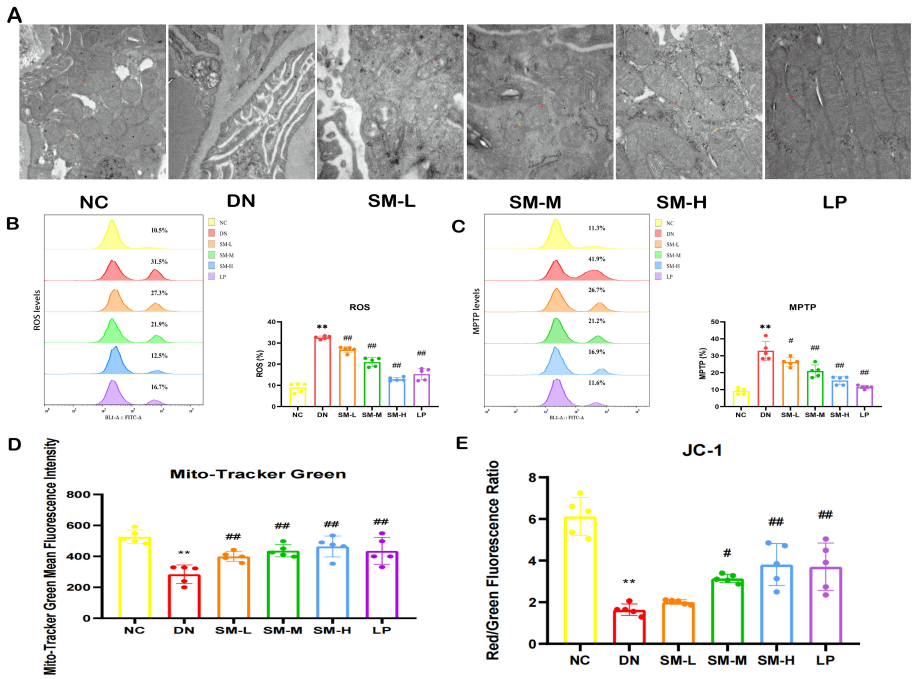


FIGURE 5
Effects of SM on mitochondrial morphology and function in DN rats. **(A)** Representative images of the ultrastructure of mitochondria on renal tissue (× 15000, Scale bar = 500 nm). Yellow arrows represent the outer mitochondrial membrane, red arrows represent the inner mitochondrial membrane, and black arrows represent cristae. **(B)** Flow diagrams of ROS (n = 5). **(C)** Flow diagrams of MPTP (n = 5). **(D)** Quantitative analysis of mitochondrial content by Mito Tracker Green (n = 5). **(E)** Quantitative analysis of MMP (n = 5). The data are X ± SEM. ** $p < 0.01$ *vs*. normal control (NC) group; $^{\#}p < 0.05$ *vs*. diabetic nephropathy (DN) group; $^{\#\#}p < 0.01$ *vs*. diabetic nephropathy (DN) group.

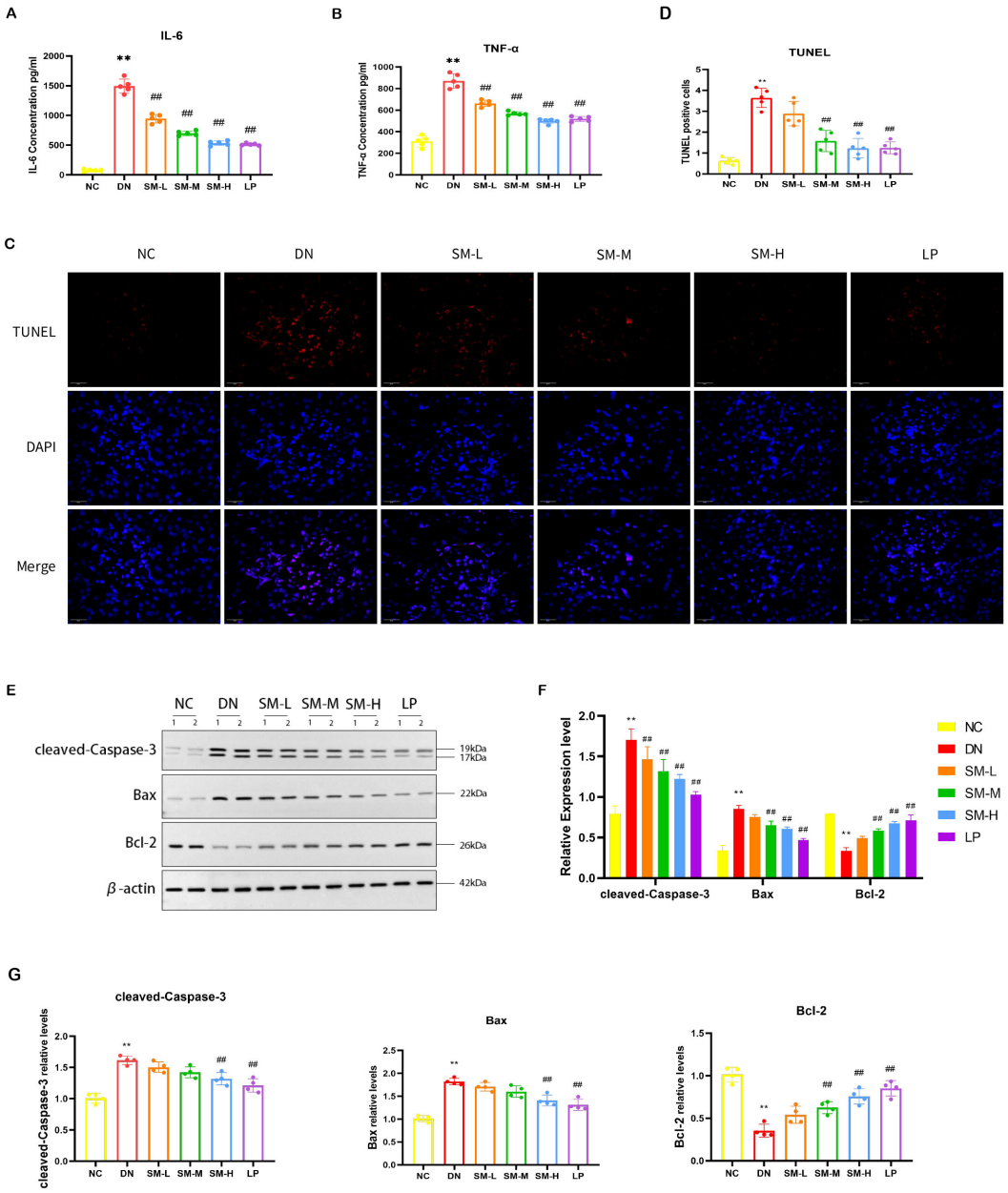


FIGURE 6
SM administration improves renal inflammation response and apoptosis in DN rats. **(A, B)** IL-6 and TNF-α levels in kidney tissue were detected by ELISA (n = 5). **(C)** Representative micrographs of TUNEL staining were given (200 ×; Scale bar = 50 μm). **(D)** Results of quantitative determination of TUNEL staining (n = 5). **(E)** The expression of cleaved-Caspase-3, Bax, and Bcl-2. β-actin was used as a loading control. **(F)** Results of quantitative determination of western blotting of cleaved-Caspase-3, Bax, and Bcl-2 (n = 4). **(G)** PCR analysis of Caspase-3, Bax, Bcl-2, and β-actin (n = 4). The data are X ± SEM. ** $p < 0.01$ *vs*. normal control (NC) group; $^{\#\#}p < 0.01$ *vs*. diabetic nephropathy (DN) group.

expression of AKT and mTOR in DN rats. At the same time, no significant differences were observed in the total levels of AKT and mTOR compared to the NC group (Figures 7A, B). The ratios of p-AKT/total AKT protein and p-mTOR/total mTOR protein were calculated, revealing a significant increase in the ratios of p-AKT/AKT and p-mTOR/mTOR in the DN group. As anticipated, phosphorylated AKT and mTOR protein levels were notably reduced in the treatment groups following 12 weeks of SM-H/LP administration ($p < 0.01$). IF analysis revealed significant expression of p-AKT and p-mTOR in the glomeruli and tubulointerstitium of the DN group, while a decreasing trend in p-AKT and p-mTOR expression was observed in the rats in the SM-M, and SM-H groups (Figures 7C–E).

In order to further explore the potential relationship between the anti-inflammatory and anti-apoptotic effects of SM on DN and the JAK2/STAT3 signaling pathway, Western blot analysis was conducted to assess the levels of JAK2, STAT3, and phosphorylation in rat kidney tissues. The experiment results indicated that, compared to the NC group, there was no significant difference in the total levels of JAK2 and STAT3 proteins in rat kidney tissues. Of the DN group. However, there was a substantial increase in the relative expression of p-JAK2/JAK2

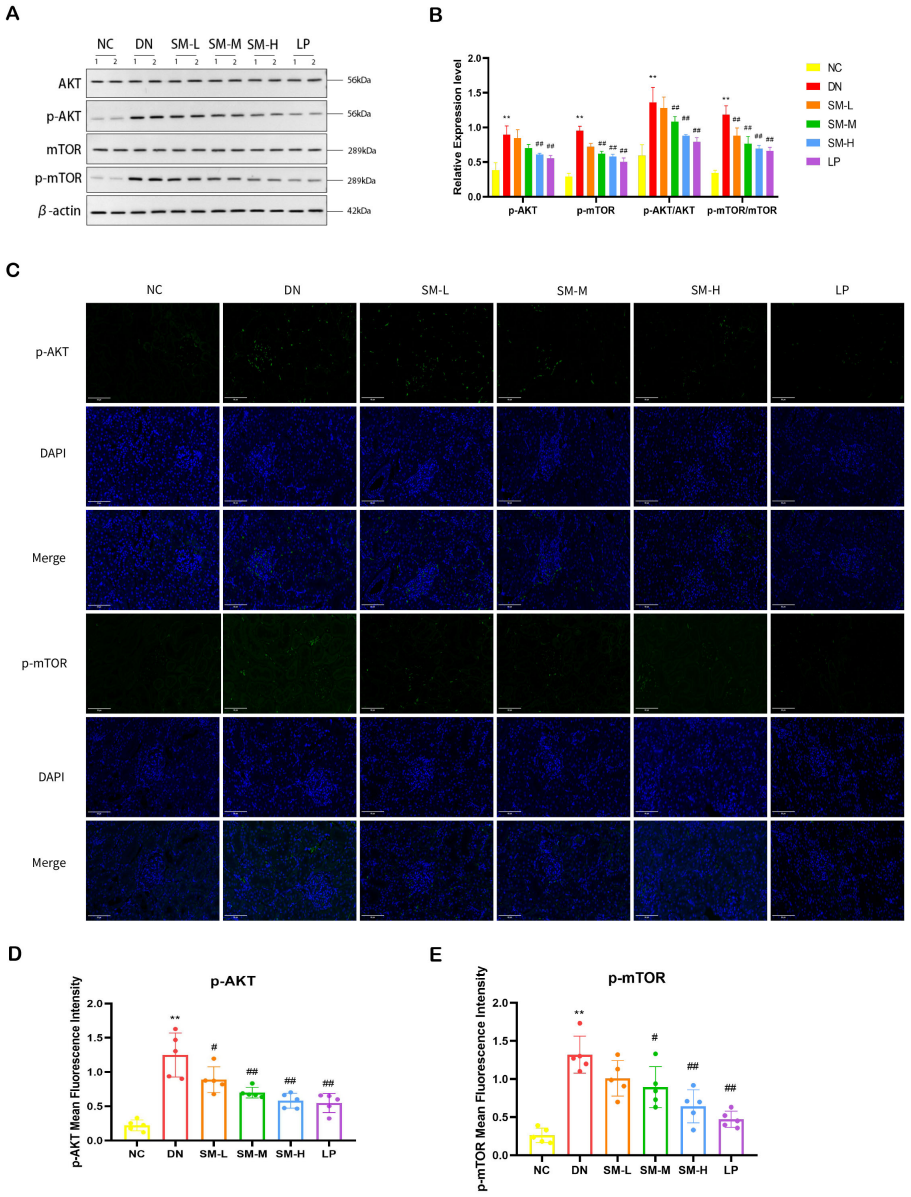


FIGURE 7
Effect of SM on PI3K/AKT/mTOR signaling pathway expression in rats with DN kidney tissues. **(A)** The expression of AKT, p-AKT, mTOR, and p-mTOR. β-actin was used as a loading control. **(B)** Quantitative determination of p-AKT, p-AKT, and p-mTOR's relative abundances (n = 4). **(C)** Immunofluorescence analysis that shows the abundance and distribution of p-AKT and mTOR. Representative micrographs were given (200 x; Scale bar = 50 μm). **(D, E)** The graph below shows quantitation data of an average of 5 independent p-AKT and p-mTOR immunofluorescence staining. The data are X ± SEM. $^{**}p < 0.01$ *vs.* normal control (NC) group; $^{\#}p < 0.01$ vs. diabetic nephropathy (DN) group; $^{\#\#}p < 0.01$ *vs.* diabetic nephropathy (DN) group.

and p-STAT3/STAT3, suggesting a potential activation of the JAK2/STAT3 pathway in DN. Compared to the DN group, the treatment group exhibited minimal changes in the total protein expression levels of JAK2 and STAT3 in renal tissues ($p < 0.01$). However, there was a noticeable reduction in the protein expression of p-JAK2 and p-STAT3 in the SM-H and LP groups, which demonstrated significant therapeutic effects as depicted in Figures 8A, B. Immunofluorescence analysis revealed a substantial expression of p-JAK2 and p-STAT3 in glomeruli and tubulointerstitium of the DN group, whereas the SM-H showed a marked decrease in the expression of p-JAK2 and p-STAT3 (Figures 8C–E).

In conclusion, SM-H can regulate the PI3K/AKT/mTOR signaling pathway and the JAK2/STAT3 signaling pathway to inhibit the inflammatory response and apoptosis.

3.4 Verification of the interaction between identified bioactive compounds and HUB targets via molecular docking

The mean affinity value of 20 groups was -6.55 kcal/mol and 14 groups had affinity ≤ -7.00 kcal/mol. Thus, the therapeutic effect of

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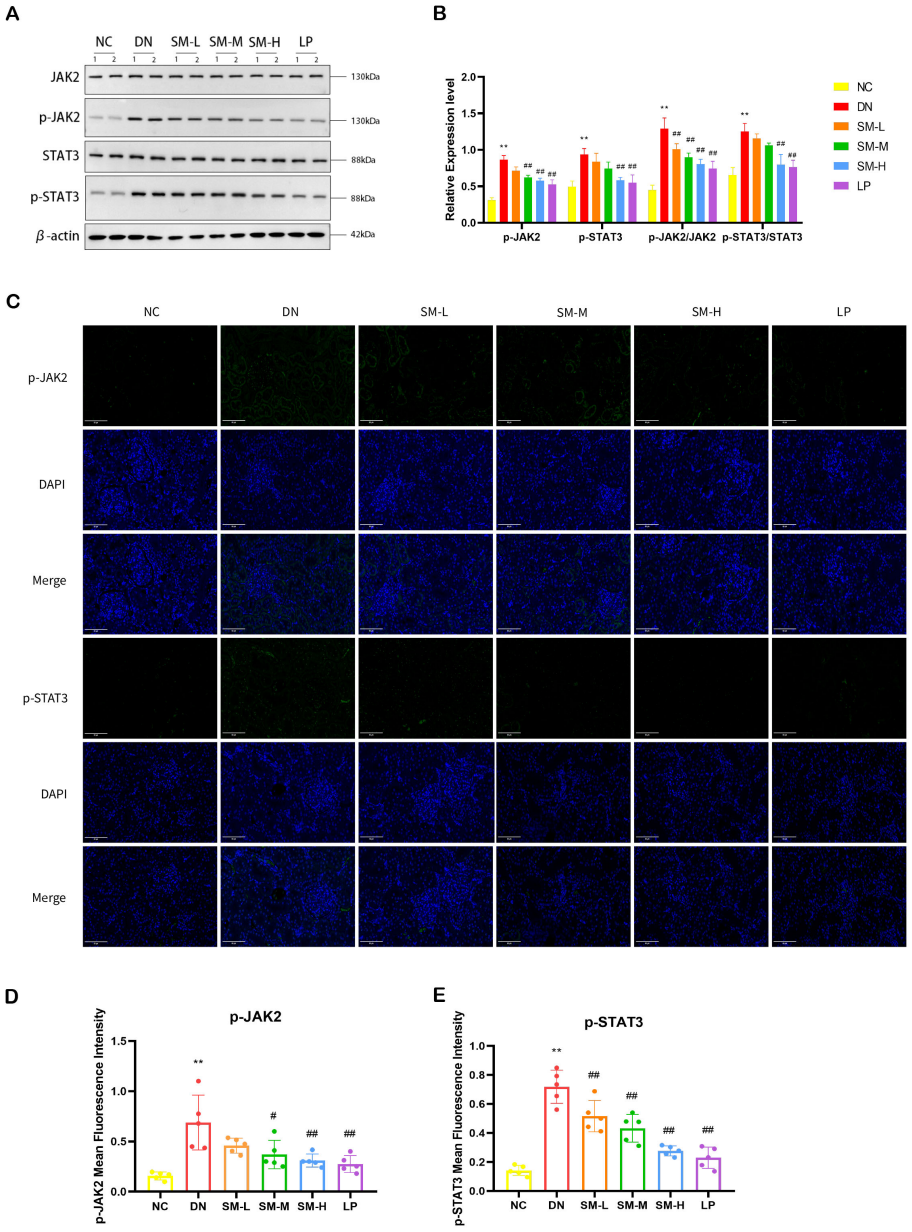

FIGURE 8
Effect of SM on JAK2/STAT3 signaling pathway expression in rats with DN kidney tissues. **(A)** The expression of JAK2, p-JAK2, STAT3, and p-STAT3. β-actin was used as a loading control. **(B)** Quantitative determination of the relative abundances of JAK2, p-JAK2, STAT3, and p-STAT3 (n = 4). **(C)** Immunofluorescence analysis that shows the abundance and distribution of p-JAK2 and p-STAT3. Representative micrographs were given (200 ×; Scale bar = 50 μm). **(D, E)** The graph below shows quantitation data of an average of 5 independent p-AKT and p-mTOR immunofluorescence staining. ** $p < 0.01$ *vs.* normal control (NC) group; $^{\#}p < 0.01$ vs. diabetic nephropathy (DN) group; $^{\#\#}p < 0.01$ *vs.* diabetic nephropathy (DN) group.

SM on DN was verified at the molecular docking level (Figure 9A). Additionally, the average binding energy with AKT1 was found to be the lowest among the five principal bioactive constituents (Figure 9A). The docking patterns of the four combinations of 'target protein-active molecule' with small binding energies were graphed, as depicted in Figures 9B–E. This finding provides additional evidence that the primary active constituents of SM interact with essential targets such as AKT1, mTOR, and JAK2, among others, and exhibit strong affinity towards these critical targets. This observation suggests that the primary active constituents of SM may have therapeutic potential for the treatment or amelioration of DN through interactions with core targets.

4 Discussion

Initially, a total of 1281 constituents of SM ethanol extract were identified using the UPLC-ESI-MS/MS coupling technique. Subsequently, three databases were consulted to collect 3760 DN-

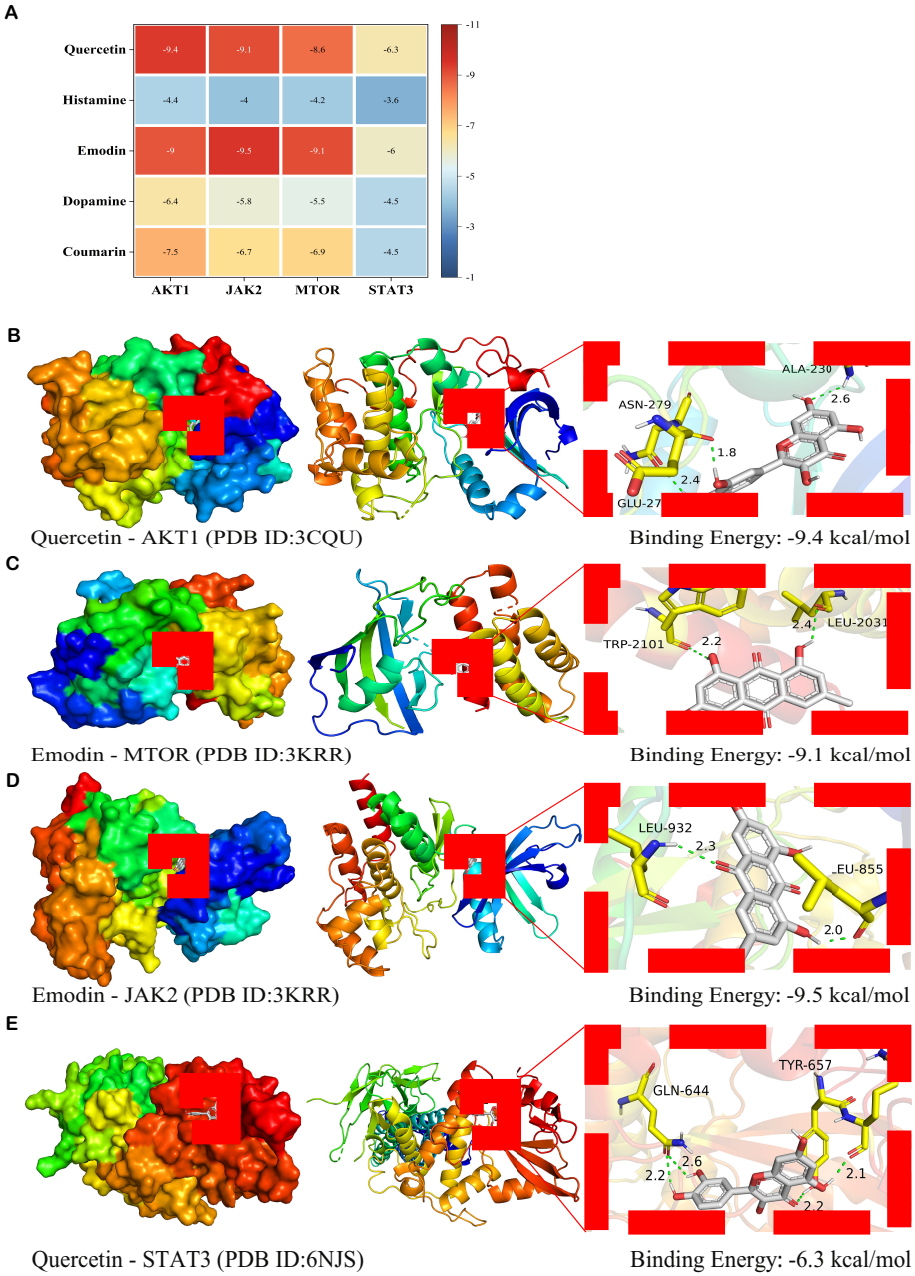

FIGURE 9
Molecular docking analysis of the main active ingredients with essential proteins **(A)** Heat map of binding energy (kcal/mol). The vertical axis represents the bioactive ingredient of SM, and the horizontal axis represents the core target protein receptor. Each value represents the binding energy of molecular docking; the darker the color, the greater the absolute value of the binding energy. **(B)** Docking analysis of Quercetin with AKT1 receptor. **(C)** Docking analysis of Emodin with mTOR receptor. **(D)** Docking analysis of Emodin with JAK2 receptor. **(E)** Docking analysis of Quercetin with STAT3 receptor. Green dashed lines, interacting hydrogen bonds.

related target genes: GeneCards, OMIM, and DisGeNET. Also, 1226 DEGs were identified from the GEO database through RNA sequencing analyses in this investigation. GSEA prioritizes gene sets with shared biological functions, chromosomal locations, and expression regulation within the gene matrix. To investigate the precise pathogenic mechanism of SM in DN, the GSE30528 dataset was chosen for additional examination. The GSEA findings of this study revealed enrichment of 50 SM-related gene sets in DN, notably including IL6/JAK-STAT3 signaling, inflammation response, oxidative phosphorylation, epithelial-mesenchymal transition, UA response DN, TGF-β signaling, etc. In addition, 2025 SM putative target genes were cross-combined with 1226 DEGs and 3760 DN-associated target genes, resulting in a total of 126 overlapping genes. These 126 target genes were considered potential therapeutic targets for the improvement of DN by SM. Then, we constructed a PPI network of overlapping genes and screened 15 key targets, including CCL2, PTPRC, EGF, IGF1, SPP1, CD36, VWF, COL1A1, CCR2, CD40LG, CSF1R, JAK2, CCR5,

CXCR2, and MMP1. One-third of the nodes are primarily associated with the PI3K-AKT signaling pathway in diabetic complications. The majority of the HUB genes target cellular signal transduction factors. Through the integration of GSEA and KEGG analyses, JAK2 is identified as a pivotal gene for the treatment of DN. The significant diagnostic utility of JAK2 for DN was confirmed through ROC curve analysis on the validation set (GSE96804). JAK2 is a non-receptor tyrosine kinase that is a crucial component in cytoplasmic signaling cascades initiated by cytokine receptors. Upon activation by cytokines, JAK2 initiates receptor phosphorylation and subsequently recruits and phosphorylates signal transducer and activator of transcription (STAT) proteins to facilitate intracellular signaling (28, 29). Research has indicated that various immune cytokines activate JAK, essential in cellular immunity, cell proliferation, differentiation, and apoptosis (30, 31). Investigations have revealed elevated levels of JAK2 expression in the glomerular and tubulointerstitial regions of patients with DN compared to controls, indicating a potential association with inflammation, immune injury, apoptosis, and other mechanisms of DN (32, 33). Subsequent studies have demonstrated that inhibiting the activity of JAK2 can lead to improved renal function and disease alleviation in DN patients (33, 34). These studies suggest that JAK2 is involved in specific pathogenic mechanisms of DN and could be a potential therapeutic target for DN.

The network analysis findings presented a broader and more holistic understanding of the therapeutic mechanism of tonifying the kidney and activating blood in the treatment of DN. An enrichment analysis was conducted on overlapping genes to further elucidate the potential molecular mechanisms of SM in treating DN. The KEGG results indicated that the PI3K-AKT signaling pathway, with a lower P value and strong connections to other pathways, was significantly enriched with the highest number of genes in the HUB genes, suggesting it is the most crucial pathway for SM in treating DN. The findings of these enrichment analyses indicate that the potential mechanisms underlying the therapeutic effects of SM on DN may involve the modulation of inflammatory responses, regulation of mitochondrial dysfunction, and inhibition of apoptosis.

To corroborate these bioinformatics results, a series of experimental studies were undertaken. Initially, the impact of SM on renal function and histopathology in rats with DN induced by a combination of HSHFD and STZ was examined. Key diagnostic biomarkers of DN, including blood glucose levels, serum creatinine, blood urea nitrogen, and 24-hour urinary protein excretion, were assessed to evaluate renal function (35).

Furthermore, pre-network pharmacology and bioinformatics analyses suggested that SM-H's anti-DN mechanism of action may involve the regulation of the PI3K/AKT/mTOR axis and JAK2/STAT3 axis. Subsequent *in vivo* experiments demonstrated that the therapeutic effect of SM-H (6 g/kg/d) on DN was stable and significant. In addition, renal tissue analysis through PCR, Western blotting, and immunofluorescence techniques indicated that the compound SM-H suppressed the expression of IL-6 and TNF-α, as well as Bax and BCL-1 while also reducing the activities of p-AKT, p-mTOR, p-JAK2, and p-STAT3, and increasing levels of cleaved-caspase 3.

Mitochondria are widely recognized as pivotal regulators of energy metabolism and apoptosis in organisms. The functionality of mitochondria is governed by Bcl-2 family proteins, which consist of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, as well as pro-apoptotic proteins like Bad, Bid, and Bax (36, 37). Furthermore, upon cellular stimulation, the excessive generation of ROS hinders the release of cytochrome C and other pro-apoptotic factors into the cytoplasm by impeding the extensive opening of the mitochondrial MPTP. In contrast, pro-apoptotic proteins reduce MMP, leading to heightened outer mitochondrial membrane permeability and subsequent liberation of apoptotic factors, such as cytochrome C, into the cytoplasm. Upon release, cytochrome C triggers the activation of apoptotic protease activating factor-1 (Apaf-1), forming apoptotic bodies composed of cytochrome C, Apaf-1, and pro-caspase-9. These bodies subsequently cleave pro-caspase-9 into cleaved-caspase-9, which then cleaves pro-caspase-3 into cleaved-caspase-3. The cleaved-caspase-3 enzyme hydrolytically cleaves various regulatory and structural proteins, thereby initiating the process of apoptosis (38–40). Various research studies have demonstrated the potential of Bcl-2 as a biomarker for renal function and its role in apoptosis during the progression of DN (41). In a survey by Xue-Qi Liu et al., it was demonstrated that wogonin treatment improved renal histopathological changes in high glucose-induced podocyte injury and STZ-induced diabetic mice by targeting the modulation of Bcl-2-mediated anti-autophagy and anti-apoptotic effects (42).

PI3K is commonly activated through ligand binding, leading to the activation of AKT, which subsequently phosphorylates mTOR. This activation of mTOR plays a crucial role in various cellular processes such as mitochondrial energy metabolism, inflammation, oxidative stress, apoptosis, epithelial-mesenchymal transition, and autophagy-related signaling pathways in DN, contributing significantly to the progression of the disease (43–45). Saikosaponin-A has been proposed as a potential treatment for mitigating mitochondrial dysfunction and apoptosis by inhibiting the PI3K/AKT signaling pathway (46). Furthermore, Yih-Gang Goan et al. demonstrated that targeted suppression of the PI3K/AKT/mTOR pathway can impede apoptosis and mitochondrial dysfunction (47). Emerging evidence suggests that an imbalance between pro- and anti-inflammatory factors in the kidney plays a significant role in the development of DN (48, 49). Pro-inflammatory factors, such as TNF-α and IL-6, normally exist in equilibrium. However, prolonged hyperglycemia and hyperlipidemia can lead to increased expression of ROS and pro-inflammatory factors in the body, damaging renal tubular epithelial cells and disrupting kidney glomerular function (50).

This can lead to the proliferation of thylakoid cells, renal fibrosis, or glomerulosclerosis. The activation of the JAK/STAT pathway has been implicated in the signaling of cytokines such as IL-6, TNF-α, Bax, and BCL-1, which in turn activate the expression of genes related to inflammatory infiltration, apoptosis of immune response, and cell proliferation, thus promoting the development of DN (51, 52). JAK2 and STAT3 are the most frequently studied

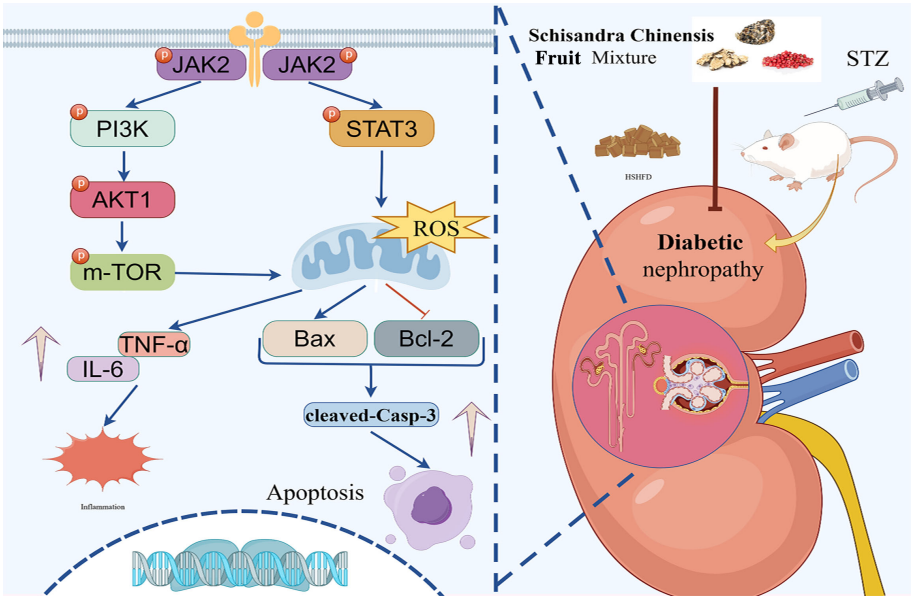

FIGURE 10
The illustration of the JAK2/STAT3 and PI3K/AKT signaling pathways implicated in the protective effect of SM on kidneys injured by DN. SM stimulated the JAK2/STAT3 pathway, subsequently stimulating the PI3K/AKT/mTOR signaling cascade, which resulted in elevated Bcl-2 levels and reduced Bax, IL-6, TNF-α, and cleaved caspase-3, so inhibiting apoptosis and inflammation.

isoforms associated with DN. Maolin Zhu et al. discovered that targeting the JAK2/STAT3 signaling pathway resulted in decreased levels of IL-6, ICAM-1, Bcl2, and Bax in renal tissues of rats with STZ-induced DN, leading to a reduction in inflammatory response, apoptosis, mitigation of pathological damage to the kidneys, and enhancement of renal function (53). Yu-Li Shen et al. demonstrated that herbal extracts could mitigate the inflammatory response and protect renal function in DN rats by targeting and inhibiting the CXCL6/JAK/STAT3 pathway (54).

Furthermore, research has shown that Chinese medicine can reduce proteinuria in mice with chronic renal failure by inhibiting the JAK2/STAT3 and PI3K/AKT signaling pathways (35). These findings collectively suggest that the protective effects of SM are attributed to the suppression of the PI3K/AKT/mTOR axis and the JAK2/STAT3 axis, which inhibit apoptosis and inflammatory responses, regulate mitochondrial dysfunction, and improve renal pathological damage.

Finally, the study confirmed the interactions of the identified compounds with the PI3K/AKT/mTOR and JAK2/STAT3 signaling pathways through molecular docking analysis. The results of the docking analysis suggested that multiple compounds exhibit stable binding to the validated targets. Specifically, the primary active components of SM, including Coumarin, Emodin, and Quercetin, demonstrated high affinity for these key targets, indicating their potential significance in the treatment of DN with SM. A literature search was conducted to investigate the potential efficacy of key compounds in protecting renal function and treating DN. Subsequent *in vivo* and *in vitro* studies demonstrated that quercetin could mitigate renal injury in DN by modulating the Nrf2/HO-1 signaling pathway, leading to improvements in blood creatinine levels and inhibition of ferroptosis in renal tubular epithelial cells (55). Additionally, the coumarin derivative has been shown to function as an Nrf2 activator, effectively inhibiting high glucose-induced oxidative stress and fibrosis in renal mesangial cells and exerting a protective effect on glomeruli (56). Current evidence indicates that treating a KK-Ay mouse model with DN using Emodin for 8 weeks significantly reduces urinary albumin, Scr, and BUN levels (57). Additionally, this treatment improves thylakoid matrix expansion, glycogen storage, and pedunculated synaptic fusion by inhibiting the activation of the PERK/eIF2α signaling pathway from mitigating apoptosis (57). These findings have been corroborated by *in vitro* studies. Further validation experiments are necessary to elucidate the potential roles and mechanisms of the monomers above. In general, the bioinformatics results were accurate and practical.

5 Conclusion

To sum up, our research verified that the JAK2/STAT3 and PI3K/AKT/mTOR pathways contribute to the anti-DN effects of SM (Figure 10).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. The GEO data provided in this study are deposited in the GEO repository under accession number GSE30528.The data presented in the study are publicly available. This data can be found here: https://github.com/1977426071/20250212. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Animal Protection and Use Committee of the Oriental Hospital of Beijing University of Chinese Medicine ((approval number: DFYY202102R). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

Y-YD: Writing – original draft, Writing – review & editing, Formal analysis, Methodology, Validation, Visualization. X-YM: Writing – original draft, Writing – review & editing, Methodology, Validation, Visualization. P-FH: Writing – original draft, Writing – review & editing, Validation, Visualization. ZL: Formal analysis, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. NT: Writing – review & editing. S-ND: Formal analysis, Data curation, Resources, Writing – review & editing. SZ: Data curation, Formal analysis, Validation, Writing – original draft. JP: Data curation, Formal analysis, Validation, Writing – original draft. P-WM: Data curation, Formal analysis, Validation, Writing – original draft. X-JL: Data curation, Formal analysis, Validation, Writing – original draft. CC: Data curation, Formal analysis, Validation, Writing – original draft. P-YZ: Data curation, Formal analysis, Validation, Writing – original draft. BP: Data curation, Formal analysis, Validation, Writing – original draft. JW: Data curation, Formal analysis, Validation, Writing – original draft. L-YZ Data curation, Formal analysis, Validation, Writing – original draft. X-KZ: Data curation, Formal analysis, Validation, Writing – original draft. M-YZ: Data curation, Formal analysis, Validation, Writing – original draft. M-ZZ: Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1526465/full#supplementary-material

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Glossary

AKT	protein kinase B
AUC	area under the curve
ACEi	angiotensin-converting enzyme inhibitors
ARBs	angiotensin receptor blockers
Bcl-2	B-cell lymphoma-2
Bax	bcl-2-associated X protein
BUN	blood urea nitrogen
DEGs	differentially expressed genes
DN	diabetic nephropathy
ELISA	enzyme-linked immunosorbent assay
GO	gene ontology
H&E	hematoxylin-eosin
IL-6	interleukin 6
IF	immunofluorescence
JAK2	Janus kinase 2
KEGG	kyoto encyclopedia of genes and genomes
mTOR	mammalian target of rapamycin
MPTP	mitochondrial membrane permeability transition pore
MMP	mitochondrial membrane potential
NS-MRAs	non-steroidal mineralocorticoid receptor antagonists
PASM	periodic acid-silver
PI3K	phosphatidylinositol 3 kinase metheramine
PPI	protein-protein interactions
ROS	reactive oxygen species
SM	Schisandra chinensis Fruit Mixture
STAT3	signal transducer and activator of transcription 3
Scr	serum creatinine
STZ	streptozotocin
SGLT2	sodium-glucose cotransporter-2
TCM	traditional Chinese medicine
TCMSP	TCM systems pharmacology
TNF-α	tumor necrosis factor alpha
UPLC-ESI-MS/MS	ultrahigh-performance liquid chromatography-electrospray ionization-tandem mass spectrometry
PPI	protein-protein interaction.

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Microglia-derived CXCL2 induced neuronal ferroptosis via CXCR2/Jun axis in sepsis-associated encephalopathy

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Background: Neuronal ferroptosis is a characteristic pathological change of sepsis-associated encephalopathy (SAE), which can be induced by activated microglia. CXCL2 is mainly secreted by inflammatory cells (neutrophil and microglia) and involved in neuronal damage. However, the specific mechanism behind microglia-neuron crosstalk in SAE remains unclear.

Method: This study is to explore in which way microglia-secreted CXCL2 induced neuronal ferroptosis. For this purpose, the present study used CXCL2 knockdown (KD) mice to generate SAE model and determined effects of CXCL2 on neuronal ferroptosis. Afterward, BV2 and HT22 were used to instead of microglia and neuron respectively and the co-cultured system was used to simulate their interaction *in vivo* environment. RNA-sequencing technology was applied to investigate the key mechanism and targets of CXCL2-induced neuronal ferroptosis. siRNA was used to evaluate the function of key molecules.

Results: Cecum ligation perforation (CLP) induced an obvious cognitive dysfunction, shorten the survival time and promoted the activation of microglia and neuronal loss. The level of inflammatory cytokines, ferroptosis-related markers and malonaldehyde was obviously lower and the level of glutathione was significantly higher in CXCL2 KD mice when compared with wide-type SAE mice. RNA-seq revealed that Jun is a potential target of CXCL2. The following experiments further demonstrated that microglia-secreted CXCL2 induced the neuronal ferroptosis, but siRNA-Jun in neuron can abolish this effect. In addition, siRNA-CXCL2 of microglia mitigated the neuronal ferroptosis induced by sepsis, while Jun agonist reversed this protective effect.

Conclusion: In conclusion, microglia-derived CXCL2 could induce the occurrence of neuronal ferroptosis by targeting Jun. Thus, regulating the expression and secretion of CXCL2 will probably be a crucially novel strategy for the treatment of SAE.

KEYWORDS

sepsis-associated encephalopathy, neuronal ferroptosis, microglia activation, CXCL2/CXCR2 axis, crosstalk

Background

Sepsis, a fatal organ dysfunction, is caused by a maladjusted host response to infection (1). According to statistics in 2017, there were approximate 48.9 million sepsis cases and 11.0 million deaths, accounting for 19.7% of all deaths worldwide (1). Sepsis-associated encephalopathy (SAE) is a common complication of sepsis, as characterized by confusion or even coma (2). Over 70% of sepsis patients are diagnosed with SAE, which is regarded as the most common reason of encephalopathy in intensive care unit (3). Unfortunately, sepsis-induced neurological dysfunction usually last for several years in many survivors and badly influence their quality of life, leading to a great social and economic burden. Although data on pathological mechanism contributing to SAE are scarce, several potentially factors have been determined, including blood–brain barrier (BBB) destruction, microglial activation and neuronal ferroptosis (4–6).

As the predominant immune cells in central nervous system (CNS), microglia have been demonstrated to exert a crucial role in the pathophysiology of SAE (7, 8). During sepsis, after periphery cytokines (IL-1β, IL-6 and TNF-α) enter into CNS via BBB, microglia are rapidly activated, displaying obviously functional changes and secreting a large number of aforementioned cytokines, which in turn further expands the neuroinflammatory response (9, 10). On the other hand, ferroptosis is a novel type of programmed cellular death, involving the accumulation of reactive oxygen species (ROS) caused by an increase in oxidative damage and intracellular iron ions induced by lipid peroxidation. Previous literatures have demonstrated the correlation between neuronal ferroptosis and SAE. For example, Chu et al. (11) demonstrated that sepsis induced the occurrence of neuronal ferroptosis and acetaminophen can reverse this process via the GPX4 signaling pathway. Xie et al (12) identified the association of neuronal ferroptosis with glutamate-mediated excitotoxic neuronal damage. Although these reports revealed the crucial role of neuron ferroptosis in SAE, they did not further explore the underlying mechanisms and primary "culprits". Recently, several literatures have indicated that microglial activation can facilitate neuronal ferroptosis in certain neurological disease, such as Alzheimer's disease (13). Thus, it is necessary to investigate whether microglial activated by sepsis promotes the progression of SAE via inducing neuronal ferroptosis and reveal the potential mechanism.

C-X-C motif chemokine ligand 2 (CXCL2), a member of CXC subfamily, is involved in inflammatory and immunoregulatory processes via targeting C-X-C motif chemokine receptor 2 (CXCR2). Recently, it has been demonstrated that overexpression of PDCD10 in glioblastoma recruits and activates microglia/macrophages via CXCL2 (14); in turn, activated BV-2 cells by ATP can also secrete and release CXCL2 (15). Importantly, previous studies have confirmed that CXCL2 can regulate the activity and axis growth of neuron. For instance, Teona Deftu et al. (16) indicated that CXCL2 stimulus significantly blocked the axon outgrowth, while blocking CXCR2 can reverse these effects. In addition, the short-term incubation of CXCL2 can effectively reduce TRPV1 desensitization in $TRPV1^{+}/IB4^{+}$ dorsal root ganglia neurons (17). Interestingly, CXCL2 overexpression was also found to induce the occurrence of ferroptosis in cancer cells. However, whether sepsis-activated microglia can secrete CXCL2 and thus promote the neuronal ferroptosis remains unclear.

This study is to explore the role and underlying mechanism of microglia-neuron crosstalk in SAE and determine whether activated microglia promote neuronal ferroptosis via secreting CXCL2. Finally, the potential mechanism by which microglia-secreted CXCL2 induce neuronal ferroptosis will be further investigated.

Abbreviations: ANOVA, One-way analysis of variance; AP-1, activator protein-1; BBB, blood–brain barrier; CLP, cecum ligation perforation; CNS, central nervous system; CXCL2, C-X-C motif chemokine ligand 2; C-X-C motif chemokine receptor 2 (CXCR2); DEGs, differentially expressed genes; FBS, fetal bovine serum; FRGs, ferroptosis-related genes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; hFRGs, hub ferroptosis-related genes; MDA, malondialdehyde; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SAE, Sepsis-associated encephalopathy.

Material and methods

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and approved by the Second Affiliated Hospital of Fujian Medical University (approval number: 2023156).

Establishment of SAE model

CXCL2 knockdown mice was purchased from Cyagen biosciences (Suzhou) lnc. A SAE model was prepared using the cecum ligation perforation (CLP) method. In summary, the mice were anaesthetized with 1% sodium pentobarbital. Following disinfection of the abdominal skin, a 1.5 cm incision was made in the midline of the abdomen to expose the cecum. The exposed cecum was then ligated with a 4-0 suture below the ileocecal valve, at a distance of 5 mm from the tip of the cecum. Subsequently, the cecum was repositioned within the abdominal cavity, with a minimal quantity of feces extruded through a permeable opening created with a 21-gauge needle. The incision was then sutured. Subcutaneous injection of 1 ml of saline was administered to all mice for resuscitation purposes. Following resuscitation, the mice were returned to their original cages. The mice in the sham-operated group underwent the same incision in the abdominal cavity as the other groups, but without ligation or perforation. All mice were permitted to eat and drink ad libitum, with automatic temperature control at 22 ± 2°C, humidity of 55-65%, and a light-dark cycle of 12 h - 12 h. Four to five mice were housed in each cage.

Barnes maze

The Barnes maze experiments were conducted on a circular platform with a diameter of 90 cm and a height of 90 cm above the ground. The mice were positioned at the center of a circular platform (SD Instruments, San Diego, CA) comprising 20 equally

spaced holes. One of the holes was connected to a dark chamber, designated as the "target box." The mice were stimulated to search for the target box using an obnoxious noise of 85 dB and bright light of 200 W. At the outset of each trial, the animals were subjected to a habituation phase in a start box situated at the center of the maze. After a 5s interval, the start box was removed, and the mouse was permitted to explore the area for 300 s. The entire procedure encompassed four training days, with each day comprising four trials of 5 min each, and a single test day. The behavioral responses were recorded and evaluated using a video tracking system (SD Instruments, San Diego, CA).

RNA extraction and RT-qPCR

Total RNA was extracted from cultured HT22 neurons and BV2 microglia using a Trizol kit (Thermo Fisher Scientific). cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions, followed by RT-qPCR reactions conducted on a StepOnePlus Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). The RT-qPCR reaction conditions were as follows: an initial denaturation at 94°C for 3 min, followed by 45 s of denaturation at 94°C, 30 s of annealing at 56°C, and 45 s of extension at 72°C, for a total of 45 cycles. The relative quantitative $2^{-\Delta\Delta Ct}$ method was employed to ascertain the expression level of the genes, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the housekeeping gene. The PCR primer sequences utilized are provided in Supplementary Table 1.

Western blot

The total protein was lysed with RIPA protein extract, which contains a mixture of protease and phosphatase inhibitors, and the protein concentration was measured using a BCA kit (Beyotime Institute of Biotechnology, Inc., Shanghai, China) in accordance with the manufacturer's instructions. The samples were separated by SDS-PAGE to ensure equal amounts of protein (20 μg per lane) and transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific). The membranes were sealed with 5% skimmed milk at room temperature for 1h. Subsequently, the membranes were incubated with the primary antibody TFRC (1:1000, A21622, ABclonal), PTGS2 (1:1000, CY5580, Abways), Jun (1:1000, 380397, Zenbio), and GAPDH (1:10000, ab181602, abcam) at 4°C overnight, followed by incubation with the anti-rabbit IgG secondary antibody at room temperature for 1h. Thereafter, the blots were developed using ECL reagent and the blots were imaged. Each experiment was repeated three times, and the mean values were calculated.

Immunofluorescence

Brain cryosections or treated cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for a period of 10 min at RT. Subsequently, the fixed tissues and cells were incubated with 3% bovine serum albumin (Sigma-Aldrich) for 30 min at 37°C. Next, the brain sections or cells were incubated with the primary antibodies overnight at 4°C. Following a rinse with PBS, tissues and cells were incubated with the corresponding secondary antibody at RT in the dark for one hour. Nuclear counterstaining with DAPI was thereafter performed. The samples were imaged under a fluorescence microscope (Nikon, Tokyo, Japan), and 15 fields of view (5 fields of view/slice × 3 slices/mouse) were randomly selected for the quantification of positive cells using ImageJ software.

Nissl staining

A Nissl staining assay (methylene blue method) was used to evaluate the hippocampal neuronal damage. All of procedures were performed according to the instructions of commercial kit (Cat.No, S1096; Bioss, Beijing, China). Briefly, the slices were dewaxed and hydrated, followed by staining with Methylene Blue Stain for 10 min. Subsequently, the sections were differentiated with Nissl Differentiation fluid for 5-10s and then treated by Ammonium Molybdate Solution for 5 min. Finally, the sections were dehydrated with 95% and 100% ethanol, and fixed with Neutral balsam. Images were captured using a light microscope (Nikon, Japan).

Cell culture (HT22 and BV2 cells)

The BV2 cell line was purchased from the Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China), while the HT22 mouse hippocampal neurons were sourced from Thermo Fisher Scientific Inc. (Waltham, MA, USA). BV2 microglia and HT22 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C and 5% CO_2 atmosphere for incubation.

siRNA transfection

BV2 microglia or HT22 neurons were plated in 6-well plates and grown to 50–60% confluency in medium containing 10% fetal bovine serum without 1% penicillin/streptomycin. Subsequently, the cells were transfected with siRNA targeting CXCL2 or Jun using the siRNA reagent system (HyCyte Biotechnology, Suzhou, China) according to the manufacturer' s protocol, with scrambled siRNAs (HyCyte Biotechnology, Suzhou, China) serving as a negative control.

CXCL2, malondialdehyde and glutathione measurement

A suspension of cells (4×10^5 cells per well) was introduced into 6-well plates and incubated at 37°C overnight. The cell supernatants were aspirated into 1.5ml EP tubes. The levels of MDA were evaluated using commercial kits following the manufacturer's instructions (Cat.No, BC0025; Beijing Solarbio Science & Technology Co., Ltd, Beijing, China). Then, absorbance was tested

at 532 nm and 600 nm. Additionally, the levels of GSH were evaluated with Glutathione Assay Kit (Cat.No, A006-2-1; Nanjing Jiancheng Bioengineering Research Institute, Naijing, China) according to the manufacturer's instructions, and the content of GSH was determined via calculating the absorbance values at 405 nm. CXCL2 in mice serum and medium supernatant of BV2 microglia were detected using the ELISA system (R&D systems, MN).

FerroOrange staining

Fe^{2+} levels were assessed using FerroOrange staining kit (Cat.No, F374; dojingo, Shanghai, China). HT22 cells were seeded in a confocal dish at 1×10^5 cells/ml density and cultured for 24 h. Based on the specific requirement, the cells were next treated for 24 h. Subsequently, a working solution of 1 μmol/l FerroOrange fluorescent probe was added to the cells. The cells were maintained at 37°C and 5% CO_2 atmosphere for incubation for 30 min. Finally, images were obtained using confocal microscopy (Nikon, Japan).

RNA-sequence analysis

For the purpose of RNA-seq analysis, the HT22 cells were treated with rmCXCL2 for 24 h. Whole genome RNA was extracted using the TRIZOL method, and the RNA concentration was quantified using a NanoDrop 2000 (Thermo Fisher Scientific). The RNA was then purified using the QIAgen RNEasy kit. Sequencing libraries were constructed using the NEBNext Ultra RNA Library Preparation Kit from NEB (USA), and the library quality was assessed using the Agilent Bioanalyzer 2100 system. The resulting samples were then analyzed on two channels using an Illumina 2000 Hi-seq machine, resulting in the generation of paired-end reads.

Statistics

All data were subjected to statistical analysis using SPSS Statistics 26 software, and graphs were generated using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) and Tukey's *post hoc* test were employed to perform statistical analyses. Additionally, an unpaired t-test was used to compare the difference between two groups. A p-value of less than 0.05 was considered statistically significant.

Results

SAE model stability

In order to closely mimic the progression and characteristics of human sepsis and SAE, we used the most quintessential CLP sepsis model and evaluated the survival status, body temperature, symptom score, and cognitive change of SAE mice after surgery. The mortality rate of mice in the CLP group is 67%, which was obviously lower survival rate than the sham mice (Figure 1A, $p < 0.05$). In addition, SAE mice displayed noticeably lower body temperature and severer symptom score when compared with sham mice (Figures 1B, C, $p < 0.05$). Subsequently, Barrns maze was performed to evaluate the cognitive change of SAE mice. After being trained for 4 days, the cognitive function of mice in both groups to enter in the target hole was obviously improved in the training period (Figures 1D, E, $p < 0.05$). Meanwhile, 7 days after surgery, the spatial learning ability of mice in SAE group was markedly impaired when compared with the mice in control group (Figure 1F, $p < 0.05$). To further depict the changes of hippocampus tissue at the cellular level (mainly microglia and neuron) after CLP surgery, CD68/Iba-1 staining and Nissl staining were performed to respectively evaluate the microglia activity and neuron death. Preliminary experiments displayed that the number of CD68/Iba-1 positive microglia obviously increased in SAE group (Figures 1G, H, $p < 0.05$); while neurons in hippocampal CA1 layers showed a significant decrease after treatment with CLP (Figures 1I, J, $p < 0.05$). All these changes in mice treated by CLP are consistent with the characteristic features of SAE, demonstrating the successful establishment of SAE mice.

CXCL2 expression was upregulated in the hippocampus tissue and microglia after sepsis/LPS stimulus

We detected the CXCL2 expression in the hippocampus 24 h after CLP at first. RT-qPCR and Elisa results demonstrated that the expression of CXCL2 mRNA and protein was obviously upregulated in SAE group, when compared with Con group (Figures 2A, B, $p < 0.05$). It has been reported that CXCL2 can be secreted by several inflammatory cells, including neutrophils, macrophages, astrocytes, or microglia (18). In CNS, microglia are the predominant immune cells. Thus, we next evaluated the CXCL2 expression in the BV2 microglia after LPS stimulus. The GSE171696 dataset, aiming at developing gene expression signatures for BV2 microglia activation, was firstly used to evaluate the expression difference of CXCL2 in BV2 microglia before and after activation. We found that the mRNA level of CXCL2 was significantly elevated in LPS-activated microglia compared with PBS-treated one (Figure 2C, $p < 0.05$). Subsequently, we performed a series of *in vitro* experiments to fully verify the aforementioned change of CXCL2 expression in microglia. The results from RT-qPCR, Elisa, and immunofluorescence further demonstrated the points that activated microglia by LPS displayed markedly higher expression level of CXCL2 in comparison with resting microglia (Figures 2D-G, $p < 0.05$).

CXCL2 knockdown alleviates sepsis-induced neuronal ferroptosis

CXCL2 was found to induce the occurrence of ferroptosis in cancer cell. To investigate the role of CXCL2 in neuronal ferroptosis induced by sepsis, CXCL2 knockdown mice were subjected to CLP. As expected, the expression of proinflammatory cytokines in hippocampus tissue, including IL-1β, IL-6, and TNF-α, in CXCL2 knockdown septic mice was strikingly lower than that in wide type

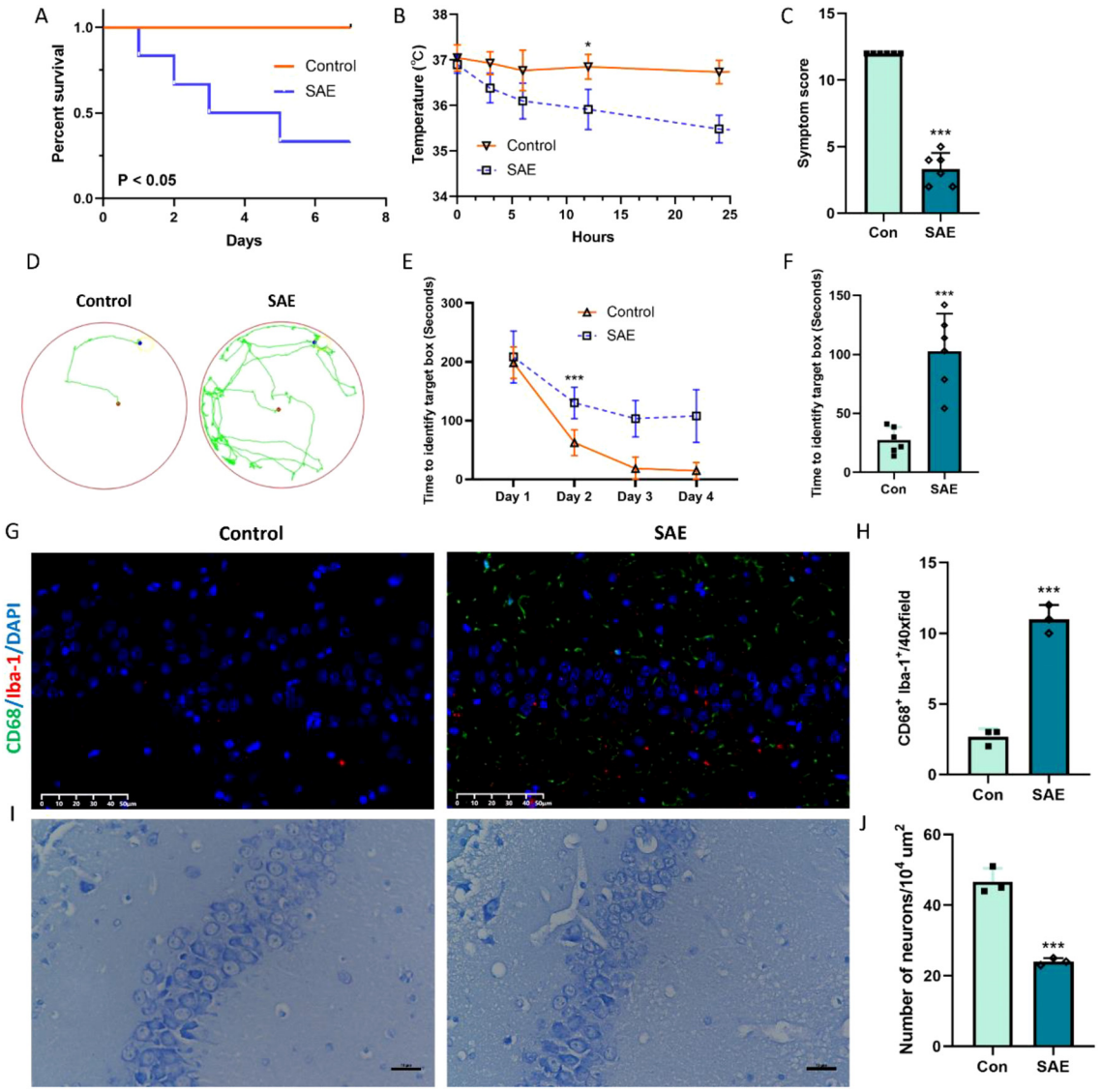

FIGURE 1
Representative change after CLP model. **(A)** Percentage of surviving animals each group 7 days after CLP (n=6); **(B)** Body temperature in each group 24 h after CLP (n=6); **(C)** Symptom score (n=6); **(D-F)** Barns maze (n=6); **(G, H)** Representative immunofluorescence images of microglial activation and the quantification of CD68/Iba-1-positive cells in the hippocampus (n=3). Scale bar, 50 μm; **(I, J)** Nissl-stained hippocampal sections indicating neuronal loss with quantification representing average number of Nissl positive cells (n=3). Scale bar, 10 μm. *p<0.05, ***p<0.001, vs. Con.

septic mice (Figures 3A-C, $p < 0.05$). Subsequently, CXCL2 knockdown was found to alleviate CLP induced neurons ferroptosis, as evidenced by higher GSH level, and lower ferroptosis-related markers expression (PTGS2 and TFRC) and MDA level (Figures 3D-J, $p < 0.05$).

Jun is a potential downstream target of CXCL2/CXCR2 in microglia-neuron crosstalk

To investigate the potential mechanism through which CXCL2 induces neuronal ferroptosis, RNA-seq analysis was conducted to determine the expression profile of HT22 hippocampal neurons following rmCXCL2 treatment. Compared with the neurons treated by PBS, a total of 239 differentially expressed genes (DEGs) were demonstrated in the samples treated by rmCXLC2, which contained 84 downregulated and 155 upregulated genes (Figure 4A). We then intersected these DEGs and ferroptosis-related genes (FRGs) to obtain seven hub FRGs (hFRGs) (Figure 4B). Figure 4C displayed the underlying connection among the hFRGs via PPI network. The CytoHubba plug was then utilized to calculated the betweenness value of hFRGs and Jun was found to be the top 1 key target with the highest score (Figure 4D). Subsequently, we performed several *in vitro* experiments to validate the expression change of Jun in HT22 hippocampal neuron before and after rmCXCL2 treatment. The results indicated that rmCXCL2 obviously upregulated the expression of Jun mRNA and protein (Figures 4E, F, $p < 0.05$). We

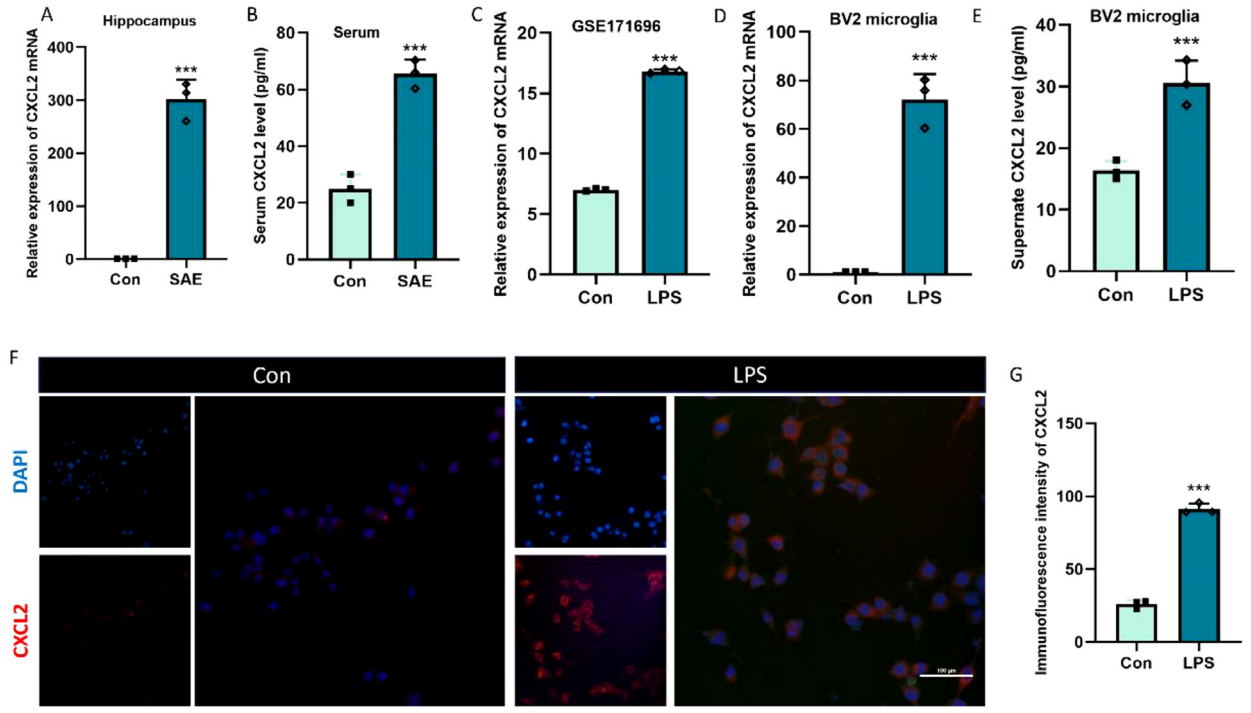

FIGURE 2
The expression of CXCL2 in hippocampus tissue and microglia after sepsis/LPS stimulus. **(A)** RT-qPCR results showing the levels of CXCL2 in hippocampus tissue; **(B)** ELISA results showing the levels of CXCL2 in mice serum; **(C)** The results showing the levels of CXCL2 in BV2 microglia from GSE171696 dataset; **(D)** RT-qPCR results showing the levels of CXCL2 in BV2 microglia; **(E)** ELISA results showing the levels of CXCL2 in BV2 microglia supernate; **(F, G)** Immunofluorescence results showing the levels of CXCL2 in BV2 microglia. Scale bar, 10 μm. n=3, ***p<0.001, vs. Con.

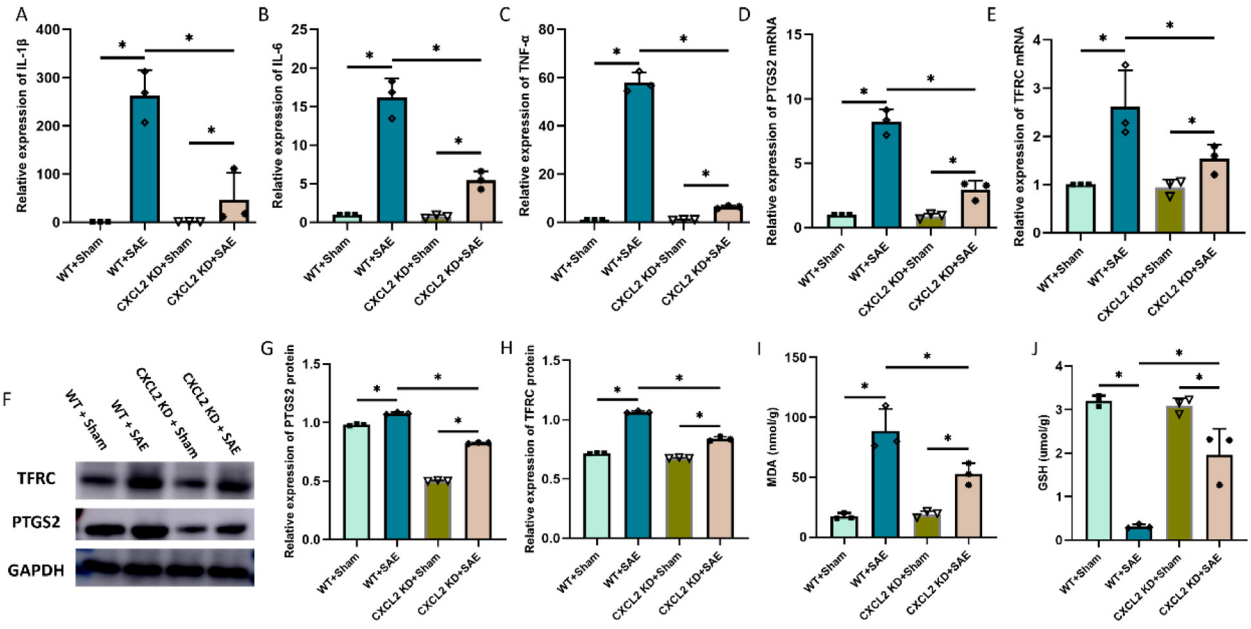

FIGURE 3
Effects of CXCL2 on neuronal ferroptosis and neuroinflammatory responses in sepsis associated encephalopathy (SAE) model. **(A-C)** RT-qPCR results showing the levels of inflammatory cytokines in hippocampus tissue; **(D, E)** RT-qPCR results showing the levels of ferroptosis-related markers (PTGS2 and TFRC) in hippocampus tissue; **(F-H)** Western blot results showing the levels of ferroptosis-related markers (PTGS2 and TFRC) in hippocampus tissue; **(I, J)** Quantitative analysis of GSH and MDA. n=3, *p<0.05.

further evaluated the effect of CXCL2 on Jun expression via *in vivo* experiments and found that CXCL2 knockdown could effectively downregulated the Jun expression at mRNA and protein level (Supplementary Figure 1, $p < 0.05$). These clues hinted that microglia-activated CXCL2/CXCR2 axis probably causes the neuronal ferroptosis via targeting Jun.

CXCL2/CXCR2 axis causes the neuronal ferroptosis via targeting Jun

In order to explore the function and effect of Jun in neuronal ferroptosis induced by microglia-secreted CXCL2, we transfected Jun siRNA to downregulate the expression of Jun in HT22 hippocampal neurons before rmCXCL2 treatment. The outcomes demonstrated that rmCXCL2-alone treatment promoted neuronal ferroptosis, as evidenced by the decreased GSH levels, elevated MDA levels, excessive iron uptake, and altered ferroptosis-related protein expression [specifically PTGS2 and TFRC]. However, when repressed Jun, the neuronal ferroptosis induced by rmCXCL2 was reversed compared to rmCXCL2-alone group ($p < 0.05$; Figures 5A-H). Additionally, we established a microglia-neuron coculture system to explore whether microglia activation induced neuronal ferroptosis via Jun. HT-22 cells treated with Jun siRNA or medium were placed in the lower chamber, and BV-2 cells treated with LPS or medium were added to the upper chamber for 24 hours. The results suggested that LPS-activated BV2 microglia can induce the occurrence of neuronal ferroptosis, but Jun siRNA transfection into HT22 neuron cells could curtail the ferroptosis of neuron ($p < 0.05$; Figures 6A-I).

Microglia-derived CXCL2 induces the neuronal ferroptosis via targeting Jun

Finally, we questioned whether microglia-derived CXCL2 induces the neuronal ferroptosis via targeting Jun. In the microglia-neuron coculture system, thus, we added BV-2 cells treated with LPS+CXCL2 siRNA in the upper chamber, while we added HT-22 cells treated with Jun agonist or medium in the lower chamber for 24 h. As shown by Figure 7, LPS-alone treatment promoted neuronal ferroptosis, but CXCL2 knockdown can revere the effects. However, when Jun agonist was added, the inhibitory effect of CXCL2 knockdown on neuronal ferroptosis was obviously hindered compared to LPS+CXCL2 knockdown group ($p < 0.05$; Figures 7A-I).

Discussion

It is a great truth that microglia are required for neural immunoregulation and the maintenance of neuronal homeostasis. To perform their functions, microglia, on the one hand, can sense neuronal activity via expressing various neurotransmitter receptors, whose activation triggers the key functions of microglia, including cellular motility, cytokines secretion and phagocytosis (19–21). On the other hand, they can also control the neuronal activity through activating a large variety of receptors expressed by neurons via releasing soluble molecules. Thus, the continuous microglia-neurons crosstalk contributes to the maintenance of neuronal activity and function. Undesirable activation of microglia probably impairs the normal communication with neurons, and

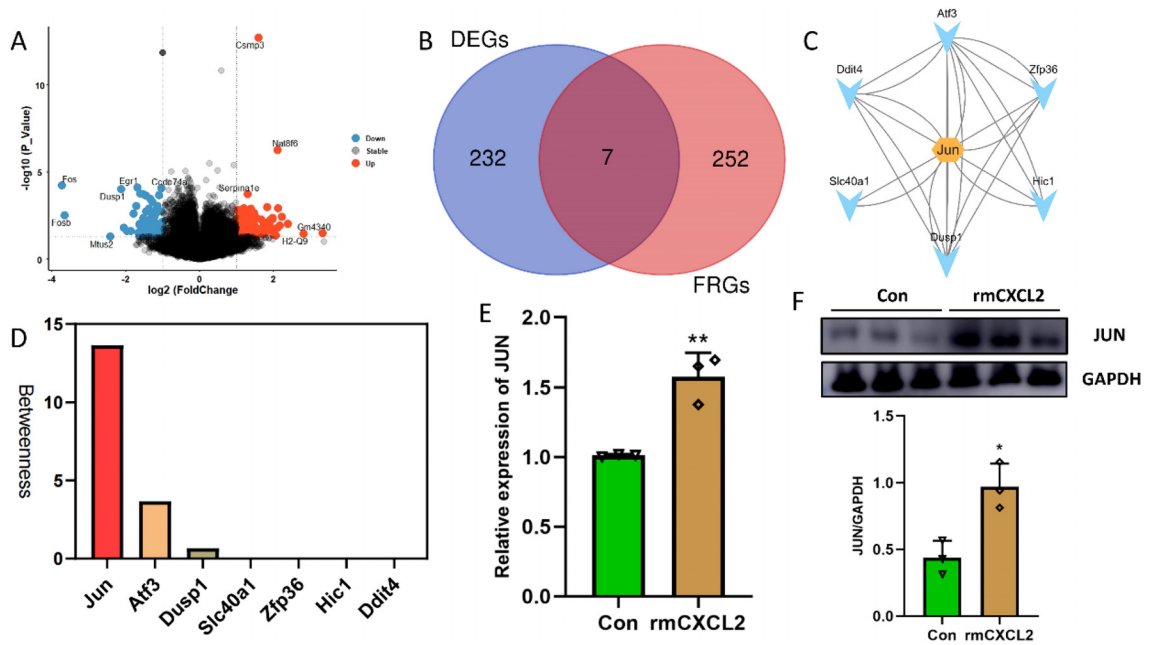

FIGURE 4
RNA-sequence analysis of HT22 neurons after rmCXCL2 stimulus. **(A)** Volcano plot displays differentially expressed genes (DEGs) of rmCXCL2-treated HT22 neurons compared with Con group. **(B)** Venn plot interesting DEGs with ferroptosis-related genes (FRGs). **(C)** Protein-protein interaction of hub FRGs; **(D)** The results from CytoHubba plug calculating the Betweenness value of hub FRGs; **(E)** RT-qPCR results showing the levels of Jun in HT22 neurons; **(F)** Western blot results showing the levels of Jun in HT22 neurons. n=3, *p<0.05, **p<0.01, vs. Con.

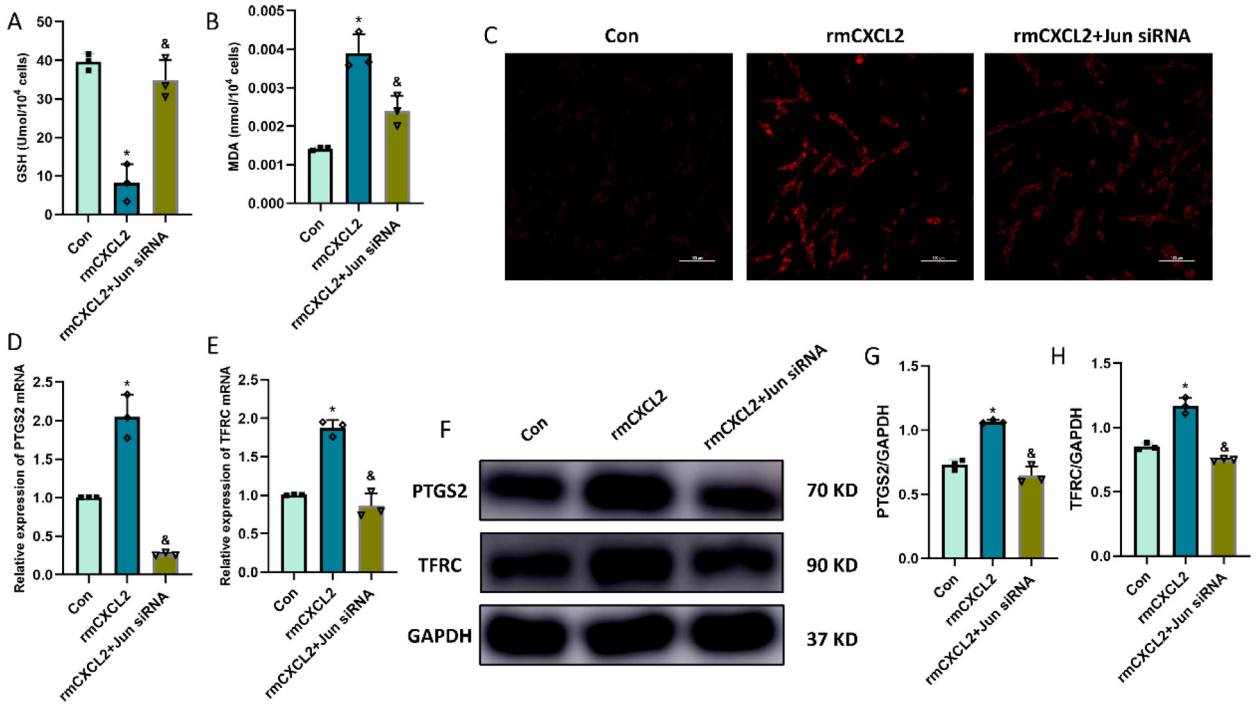

FIGURE 5
rmCXCL2 induced neuronal ferroptosis and activated Jun pathway in HT22 cells. **(A, B)** Quantitative analysis of GSH and MDA; **(C)** FerroOrange results showing the levels of Fe^{2+} in HT22 neurons. Scale bar, 100 μm; **(D, E)** RT-qPCR results showing the levels of ferroptosis-related markers (PTGS2 and TFRC) in HT22 neurons; **(F-H)** Western blot results showing the levels of ferroptosis-related markers in HT22 neurons. n=3, *p<0.05 vs. Con; $^{\&}$p<0.05 vs. rmCXCL2 group.

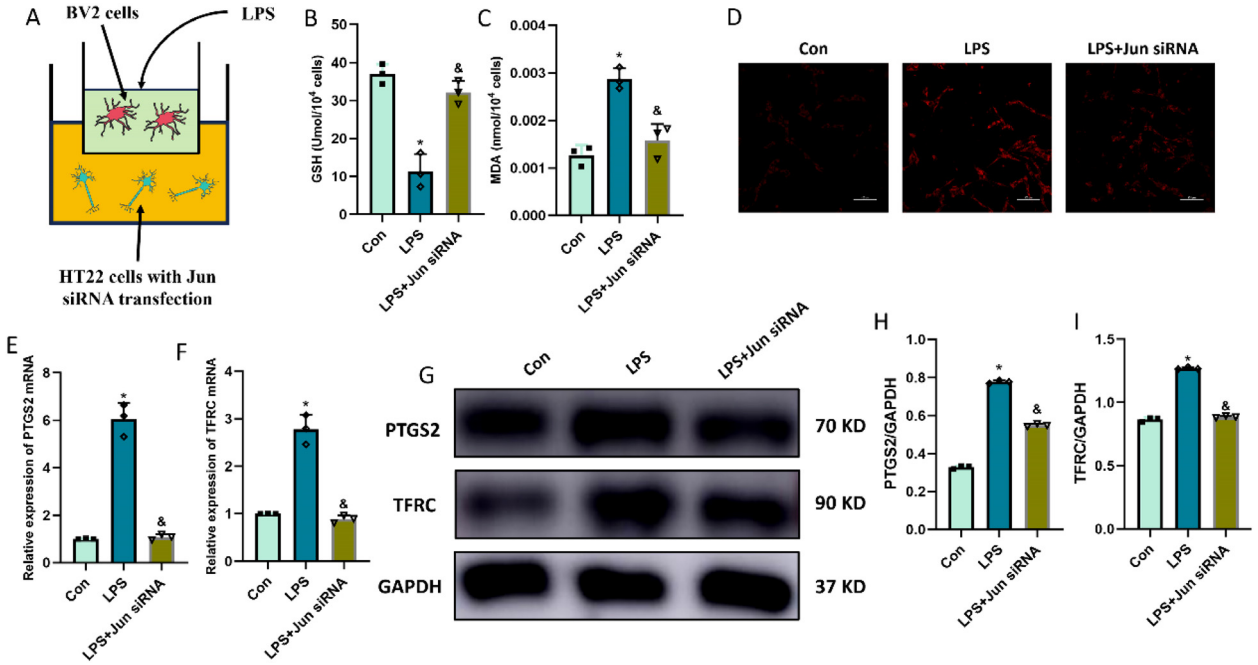

FIGURE 6
LPS-activated microglia induced neuronal ferroptosis in HT22 cells. **(A)** co-culture system of BV2 and HT22 cells; **(B, C)** Quantitative analysis of GSH and MDA; **(D)** FerroOrange results showing the levels of Fe^{2+} in HT22 neurons. Scale bar, 100 μm; **(E, F)** RT-qPCR results showing the levels of ferroptosis-related markers (PTGS2 and TFRC) in HT22 neurons; **(G-I)** Western blot results showing the levels of ferroptosis-related markers in HT22 neurons. n=3, *p<0.05 vs. Con; $^{\&}$p<0.05 vs. LPS group.

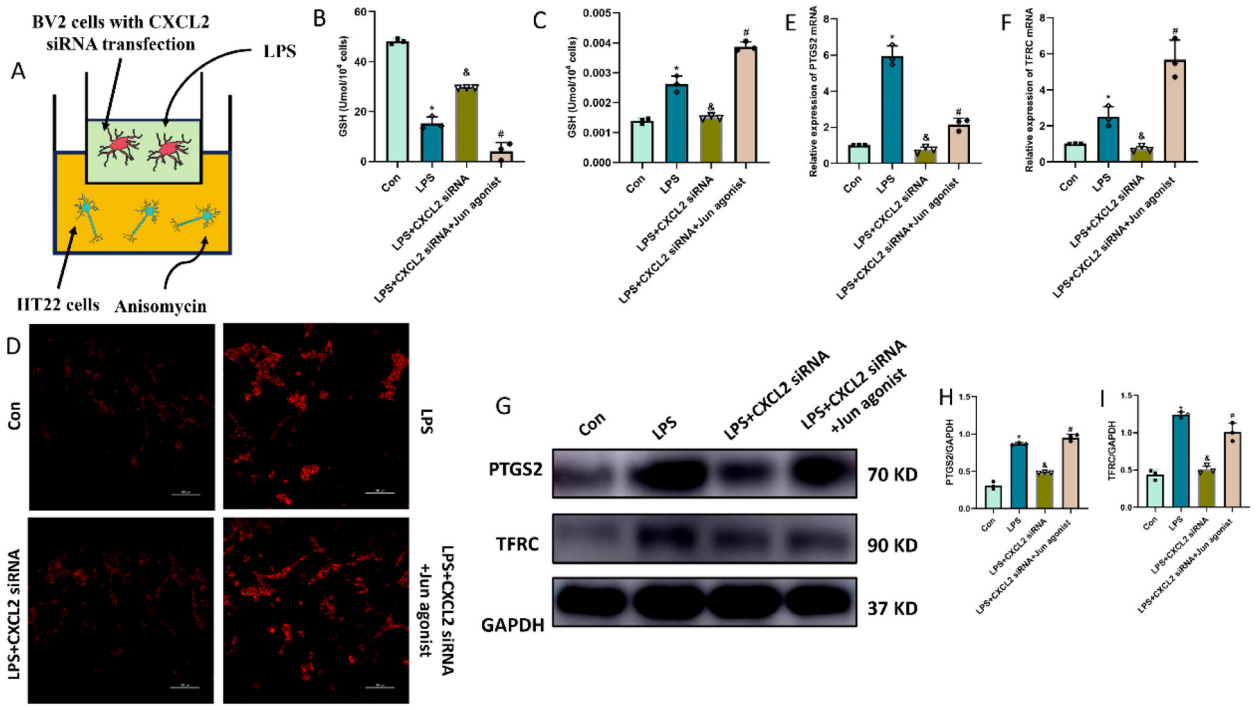

FIGURE 7
Microglia-secreted CXCL2 induced neuronal ferroptosis and activated Jun pathway in HT22 cells. **(A)** co-culture system of BV2 and HT22 cells; **(B, C)** Quantitative analysis of GSH and MDA; **(D)** FerroOrange results showing the levels of Fe^{2+} in HT22 neurons. Scale bar, 100 μm; **(E, F)** RT-qPCR results showing the levels of ferroptosis-related markers (PTGS2 and TFRC) in HT22 neurons; **(G-I)** Western blot results showing the levels of ferroptosis-related markers in HT22 neurons. n=3, *p<0.05 vs. Con; $^{\&}$p<0.05 vs. LPS group; $^{\#}$p<0.05 vs. LPS+CXCL2 siRNA group.

in consequence memory, learning, and other cognitive functions (22).

Recently, increasing evidence have demonstrated the abnormal microglia-neuron communication in many brain diseases. For example, cytokines released by microglia, including IL-1β (23), IL6 (24), TNFα (25), are associated with the regulation of neuronal functions, contributing to detrimental effects. Liu et al. revealed that the activated microglia facilitate the activation of $GRPR^+$ neurons via the NLRP3/caspase-1/IL-1β/IL1R1 axis, thus contributing to several different chronic itches (26). In addition, the reduction of microglia-neuron crosstalk caused by the decrease in CX3CR1/CX3CL1 axis leads to the homeostatic dysfunction, thus affecting physiological processes (27). Microglia activation could induce CCL2 expression and deletion of CCL2 from microglia facilitates VGluT1 synapse preservation and recovery (28). However, whether the altered crosstalk of microglia with neuron can be observed in SAE remains unclear. In this study, we reveal a previously unproven phenomenon and mechanism in which sepsis-activated microglia induces the occurrence of neuronal ferroptosis via the CXCL2/CXCR2/Jun axis.

Chemokines, an abbreviation of *chemotactic cytokines*, consist of a superfamily of small peptides (8-14 KD) that act via binding to G protein-coupled receptors. Its' function involves the activation of leukocyte chemotaxis and immune responses under pathological infection, as well as the regulation of the occurrence and metastasis of tumors (29–31). The chemokine superfamily is classified into four subgroups according to the configuration of the N-terminal cysteine residues, including CXC, CC, C, and CX3C subfamilies (32). Different chemokines have different physio-pathologic functions and many of them are correlated with a variety of neurological diseases. For example, CXCL12 overexpression was found to promote neuroinflammation and cognitive dysfunction in neuropathic pain via mediating monocyte transmigration into brain perivascular space (33). Additionally, Zhu et al. indicated that CCL2 probably mediates the local sympathetic effects on neuropathic pain and thus regulates the regeneration process (34). The link of CXCL12 and CX3CL1 expression to human nerve sheath tumors has also been reported in recent literature (35). These findings highlight the important tole of chemokines in neurological disease, thus promoting them to become a hot area of research for neurologist and psychiatrist. However, among them, the literatures revealing the association of CXCL2 with various neurological diseases remain tiny.

On the other hand, as one of the chemokine family members, CXCL2 has crucial physiological functions and exerts a crucial role in the inflammation response. Many literatures have indicated that CXCL2 is involved in the progression and prognosis of sepsis. For example, Villar et al. found that CXCL2 gene with a tandem repeat polymorphism (AC)n at position -665 is an independent factor of mortality and related to better prognosis in patients with severe sepsis (35). Flores et al. demonstrated that CXCL2 gene variants may enhance the susceptibility to sepsis and thus facilitate the development of severe sepsis (36). Furthermore, a recent original study revealed that macrophagic extracellular vesicle CXCL2 can aggravate the progression of sepsis via recruiting and activating the neutrophil CXCR2/PKC/NOX4 axis (37). Nevertheless, the role of CXCL2 in SAE is still unclear. In the present study, we found

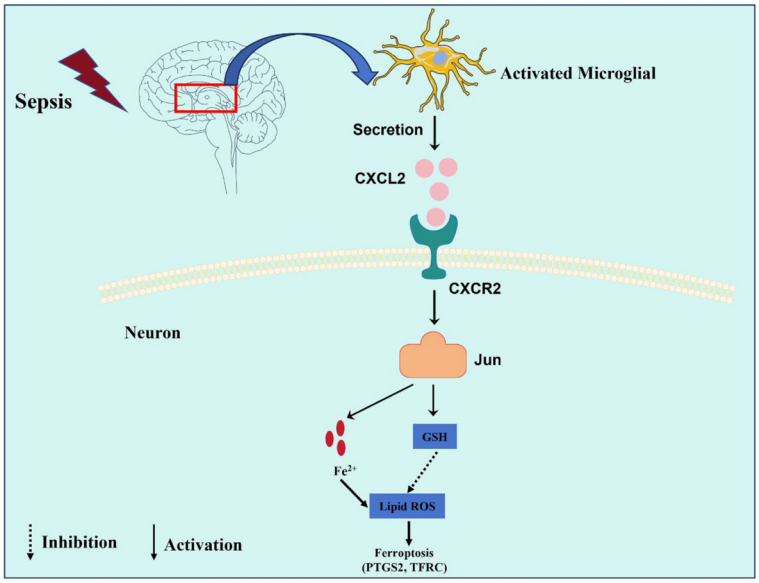

FIGURE 8
This graphic illustrating the role of the CXCL2/CXCR2/Jun signaling pathway in microglia-neuron crosstalk in SAE. Sepsis promoted the activation of microglia, which expresses and secreted a large amount of CXCL2. CXCL2 induced neuronal ferroptosis by activating the CXCR2/Jun signaling axis of hippocampus neurons, thus aggravates cognitive impairment in SAE.

CXCL2 expression was upregulated in the hippocampus tissue, blood serum, microglia and medium supernatant after sepsis/LPS stimulus. Importantly, we further revealed that microglia-secreted CXCL2 can induce the occurrence of neuronal ferroptosis in SAE.

In order to explore the mechanism by which CXCL2 leads to neuronal ferroptosis, RNA-seq analysis was constructed to depict the expression profile of HT22 hippocampal neuron after rmCXCL2 treatment. The results indicated that Jun is a potential downstream target of CXCL2/CXCR2 in microglia-neuron crosstalk. Jun, which is also known as activator protein-1 (AP-1) subunit, is the putative transforming gene of avian sarcoma virus 17 and encodes c-Jun protein [15]. The alteration of JNK/c-Jun signaling pathway in sepsis has been increasingly reported. Liu et al. found that the inhibition of the JNK/c-Jun signaling pathway by HIPK3 could potentially block the progression of sepsis (38). Furthermore, Kenzel et al. confirmed the importance of JNK/c-Jun signaling pathway in the transcriptional activation of proinflammatory cytokine genes responding to Group B streptococcus and further illustrated that the inhibition of JNK/c-Jun axis can retard GBS-induced cytokine formation and sepsis progression (39). On the other hand, Jun showed a close association with ferroptosis and iron metabolism. For example, Jun overexpression could alleviate erastin-induced ferroptosis in Schwann cells by the upregulation of GPX4 and the downregulation of PTGS2 (40). However, in pancreatic cancer, Jun overexpression was found to upregulate the mRNA transcription and protein expression of TFRC by binding to its' promoters, thus contributing iron metabolism (41). In this study, we also found that CLP-induced sepsis obviously upregulated the expression of Jun in hippocampus tissue, whereas the increasing tendency suffered a reversal after CXCL2 knockdown. Additionally, *in vitro* rmCXCL2 stimulation effectively induced the expression of Jun in HT22 neuron. Importantly, when Jun siRNA was transfected into HT22 hippocampal neurons to downregulate the expression of Jun, we observed that CXCL2-induced neuronal ferroptosis was effectively reversed. These findings indicated that microglia-derived CXCL2 induces the neuronal ferroptosis via targeting Jun.

Conclusions

Our study revealed that microglia-derived CXCL2 aggravated SAE progression by promoting neuronal ferroptosis via Jun, indicating a novel microglia-neuron circuit mediated by CXCL2/CXCR2/Jun signaling in SAE (Figure 8).

Data availability statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA023452) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Ethics statement

All animal care and experiments were performed according to the Institutional Animal Ethics Committee of the Second Affiliated Hospital of Fujian Medical University, and all efforts were made to minimize animal suffering. The study protocol was approved by the Animal Experimentation Committee of the Second Affiliated Hospital of Fujian Medical University (approval number: 2023156).

Author contributions

Y-SY: Conceptualization, Writing – original draft. J-WL: Conceptualization, Writing – original draft. M-QP: Formal analysis, Methodology, Writing – original draft. Y-MF: Formal analysis, Methodology, Writing – review & editing. Z-DS: Software, Writing – review & editing. H-FH: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1512300/full#supplementary-material

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Lipid metabolism in ferroptosis: mechanistic insights and therapeutic potential

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Ferroptosis, an iron-dependent form of regulated cell death driven by lipid peroxidation, plays a pivotal role in various physiological and pathological processes. In this review, we summarize the core mechanisms of ferroptosis, emphasizing its intricate connections to lipid metabolism, including fatty acid synthesis, phospholipid remodeling, and oxidation dynamics. We further highlight advancements in detection technologies, such as fluorescence imaging, lipidomics, and *in vivo* PET imaging, which have deepened our understanding of ferroptotic regulation. Additionally, we discuss the role of ferroptosis in human diseases, where it acts as a double-edged sword, contributing to cancer cell death while also driving ischemia-reperfusion injury and neurodegeneration. Finally, we explore therapeutic strategies aimed at either inducing or inhibiting ferroptosis, including iron chelation, antioxidant modulation, and lipid-targeted interventions. By integrating mechanistic insights, disease relevance, and therapeutic potential, this review provides a comprehensive perspective on ferroptosis as a crucial interface between lipid metabolism and oxidative stress.

KEYWORDS

ferroptosis, lipid peroxidation, iron metabolism, antioxidant defense, therapeutic targeting

1 Introduction

Ferroptosis is a non-apoptotic form of regulated cell death driven by iron-dependent membrane lipid peroxidation, which compromises plasma membrane permeability and integrity, ultimately leading to membrane rupture and cell death (1–3). A defining hallmark of ferroptosis is lipid peroxidative damage, which can occur either nonenzymatically or through enzyme-catalyzed processes (4). In both cases, iron availability and the

accumulation of lipid peroxides are crucial preconditions for the initiation of ferroptosis. As a self-protection mechanism, antioxidant enzymes such as glutathione peroxidase 4 (GPX4) and ferroptosis suppressor protein 1 (FSP1, also known as AIFM2) function to monitor and regulate cellular lipid peroxide levels, preventing them from reaching toxic thresholds. Inhibition of GPX4 results in a significant increase in lipid peroxidation (5–7). Conceivably, the fate of ferroptosis ultimately depends on the balance between lipid peroxidation and cellular surveillance mechanisms. Additionally, as ferroptosis is intrinsically intertwined with lipid metabolism, it is tightly regulated by cellular lipid composition, synthesis, storage, availability, and degradation. Among all lipid species, phospholipids (PLs) acylated with polyunsaturated fatty acids (PUFAs) are the most prone to lipid peroxidation, making them the primary driving force of ferroptosis (8).

Though ferroptosis is a relatively newly discovered form of regulated cell death, ferroptosis has gained considerable attention, and increasing evidence suggested that it plays a critical role in various diseases, including cancer, neurodegenerative diseases, and tissue ischemia injury (9). It has been shown that ferroptosis causes neuronal cell death and synaptic damage in Alzheimer's disease, and its inhibition mitigates disease progression and improves cognitive function in patients (10–12). In cancer treatment, drug-resistant cancer cells can be effectively eliminated by ferroptosis-inducing compounds (13, 14). Therefore, understanding the molecular mechanism of ferroptosis and elucidating its regulatory pathways in physiological and pathological conditions hold great potentials for developing new therapeutic strategies for human diseases. In this review, we summarize the underlying mechanisms of ferroptosis, with a particular focus on its intricate connections to lipid metabolic pathways and their regulation. We highlight advancements in detection techniques and methodologies for studying ferroptosis. Furthermore, we discuss the pathogenic roles of ferroptosis in different kinds of human diseases, as well as its therapeutic potentials.

2 Mechanisms of ferroptosis

Mechanistically, cellular lipid peroxidation occurs in three key steps: (1) Initiation, where reactive oxygen species (ROS) abstract a hydrogen atom from PUFAs-containing membrane phospholipids, generating lipid radicals ($L^{\bullet}$); (2) Propagation, where the lipid radicals react with oxygen, leading to production of lipid peroxyl radicals ($LOO^{\bullet}$), which further react with additional PUFA-containing phospholipids, producing lipid peroxides ((LOOH)) and propagating the lipid peroxidation chain reaction; (3) Termination, where antioxidants such as GPX4 neutralize oxidizing molecules, particularly lipid peroxyl radicals and lipid peroxides, preventing further oxidative damage (15, 16). Disruption in redox or lipid homeostasis, or deficiencies in antioxidant defense, result in an excessive accumulation of lipid peroxides, ultimately triggering ferroptotic cell death. Free intracellular iron or iron-containing enzymes serve as catalytic drivers of ferroptosis, actively participating in all three steps of lipid peroxidation (17, 18). In following subsections, we discuss iron dysregulation, lipid peroxidation, and the antioxidant defense system in detail. Figure 1 provides an overview of the three key steps of ferroptosis, highlighting crucial enzymes and compounds involved in the process.

2.1 Catalytic role of iron

As indicated by its name, ferroptosis is iron-dependent, and iron plays a pivotal role in both the initiation and regulation of ferroptosis. Within cells, most iron is bound in iron-sulfur clusters or stored in ferritin, an iron storage protein, since free intracellular iron is highly reactive and can catalyze the Fenton reaction, producing highly reactive hydroxyl radicals ($^{\bullet}OH$). These hydroxyl radicals then react with membrane PUFAs, generating lipid radicals that initiate lipid oxidation (19, 20). Ferrous ions (Fe2+) can also interact with lipid peroxides to produce peroxyl radicals, accelerating the propagation of lipid peroxidation and promoting ferroptosis (21). Additionally, iron overload inhibits GPX4, causing the accumulation of lipid peroxides to toxic levels (22). Dysregulated iron metabolism and overload are linked to aberrant ferroptosis, contributing to the pathogenesis of various diseases (23). Q-z Tuo and colleagues have demonstrated that ischemia-reperfusion injury induces ferroptotic iron accumulation in brain by impairing the iron efflux function (24). Studies also have revealed that deletion of the gene encoding iron storage protein ferritin enhances ferroptosis in cardiovascular cells and hepatocytes (25, 26).

2.2 Lipid peroxidation in ferroptosis

Due to the weak double bonds present in polyunsaturated fatty acids (PUFAs), hydrogen atoms are more easily abstracted from PUFAs than from saturated or monounsaturated fatty acids. Cellular or organelle membranes are rich in phospholipids that are incorporated with PUFAs, making them highly susceptible to peroxidation. The peroxidation of PUFAs-containing phospholipids in membranes compromises membrane integrity, increases permeability, and ultimately leads to membrane rupture and cell death. Lipid peroxidation also generates toxic byproducts, including 4-hydroxynonenal (HNE) and malondialdehyde (MDA), which further damage proteins, DNA, and lipids by forming adducts with these biommolecules (16).

Lipid peroxidation occurs through either nonenzymatic or enzymatic pathways. Nonenzymatically lipid peroxidation can be initiated by hydroxyl and/or peroxyl radicals, which are products of Fenton reaction that is catalyzed by labile iron. Once lipid peroxides are formed, they propagate the peroxidation to the neighboring PUFAs-containing phospholipids with the presence of ferrous iron, and produce more lipid hydroxyl and peroxyl radicals, unless they are rapidly neutralized (Figure 1) (27, 28).

Alternatively, enzymatic lipid peroxidation is mediated by various iron-dependent enzymes (18). Among them, lipoxygenases (LOXs) are the most well-characterized oxygenase

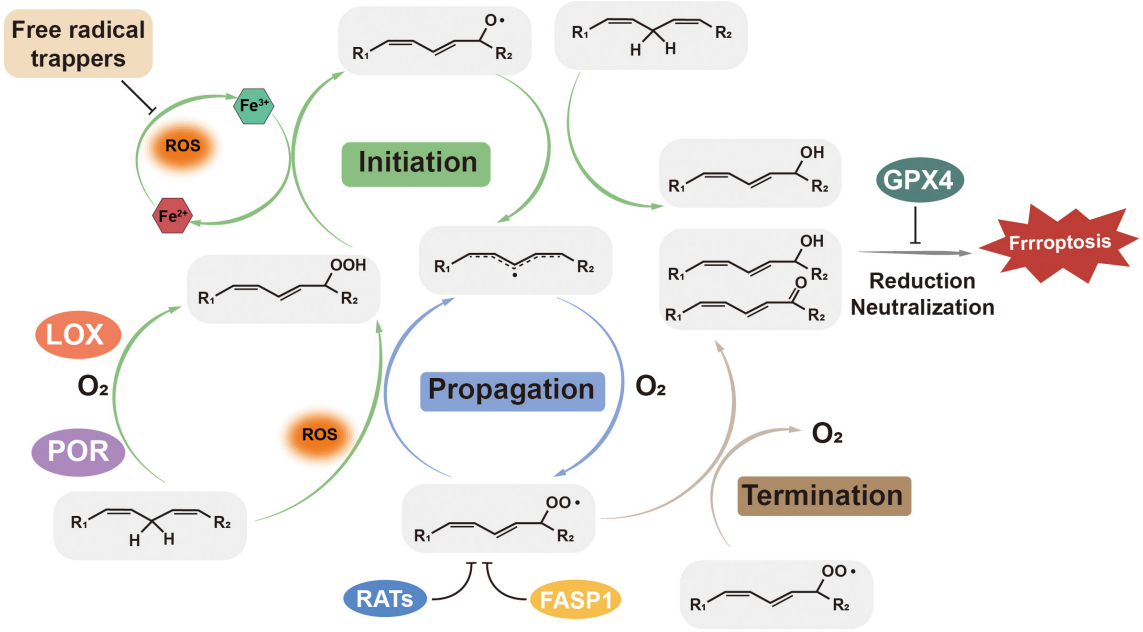

FIGURE 1
Lipid peroxidation: initiation, propagation, and termination. ROS, such as hydroxyl radicals (•OH) and peroxyl radicals (HOO•), initiate lipid peroxidation by attacking PUFA-PLs, forming lipid radicals (PLO•). These radicals propagate the reaction by reacting with oxygen to form lipid peroxyl radicals (PLOO•), which further oxidize adjacent PUFA-PLs. GPX4, Glutathione Peroxidase 4; LOX, Lipoxygenase; POR, P450 Oxidoreductase; RATs, Redox-Active Transporters; FASP1, Ferroportin 1.

involved in lipid peroxidation. LOXs are a family of non-heme iron-containing dioxygenases that insert oxygen into PUFAs, producing lipid radicals. There are six different LOXs in human, each with distinct substrate selectivity and oxidation site specificity (29). For example, arachidonate 5-lipoxygenase (ALOX5) preferentially oxidizes the PUFA arachidonic acid at carbon-5, resulting in the formation of 5- hydroperoxyeicosatetraenoic acid (5-HPETE) (30). Another key enzyme family involved in lipid peroxidation is cytochrome p450 (CYPs), a group of heme-containing monooxygenases capable of directly catalyzing lipid peroxidation. CYPs accept electrons transferred by NADPH-cytochrome P450 reductase (POR) and generate ROS to initiate the peroxidation (31). However, there enzymes are not indispensable in ferroptosis, as nonenzymatic lipid peroxidation can drive the ferroptosis independently (18).

2.3 Antioxidant defense systems

Antioxidant defense systems play a critical role in counteracting ferroptotic stress in cell. The well-known system Xc^-/GSH/GPX4 axis is an canonical mechanism that safeguards cells from lipid oxidation. Figure 2 displayed the antioxidant defense systems under the ferroptotic stress. In this pawthway, cystine/glutamate antiporter (system Xc^-) imports cystine, which is subsequently reduced to cysteine for glutathione (GSH) synthesis (29). GSH acts as a key cofactor for GPX4, the only known mammalian enzyme capable of reducing phospholipid hydroperoxides (PLOOH) into non-toxic phospholipid alcohol (PLOH), thereby preventing peroxidative damage to membranes (30–33). Disrupting this pathway—either by inhibiting system Xc^- with small molecule erastin or directly inactivating GPX4 with RSL3, depletes GSH, inactivates GPX4, and allows lethal LOOH accumulation (34, 35).

Beyond GPX4, the FSP1/CoQ10 system provides an independent line of defense. Ferroptosis suppressor protein 1 (FSP1) regenerates reduced ubiquinol (CoQ10H2) from oxidized ubiquinone (CoQ10) using NAD(P)H, enabling ubiquinol to act as a radical-trapping antioxidant that halts lipid peroxidation chain reactions (36). The mevalonate pathway further supports ferroptosis resistance by supplying CoQ10 precursors, linking cellular metabolism to antioxidant capacity (37).

Additionally, the GCH1/BH4 axis also contributes to lipid protection. GTP cyclohydrolase 1 (GCH1), the rate-limiting enzyme in tetrahydrobiopterin (BH4) biosynthesis, safeguards cellular membranes against ferroptosis by orchestrating antioxidant defense and lipid remodeling (38). By elevating intracellular levels of BH4 and its oxidized form BH2, GCH1 enables these metabolites to directly neutralize lipid radicals and selectively inhibit peroxidation of phospholipids. Moreover, the GCH1/BH4 axis promotes ferroptosis resistance by regenerating reduced CoQ10H2 (39, 40). The dual functionality of this pathway is evidenced by the ability of BH4/BH2 supplementation to restore cell viability under ferroptosis-inducing conditions.

Regulatory networks, such as the p62-Keap1-NRF2 pathway, integrate cellular stress signals to modulate ferroptosis sensitivity (41). Under oxidative stress, p62 sequesters Keap1, allowing nuclear

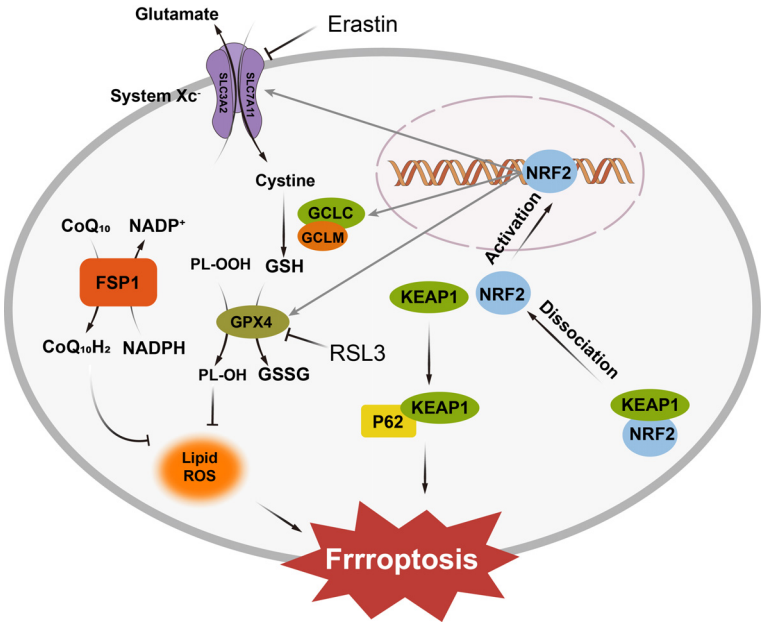

FIGURE 2
The System Xc^-/GSH/GPX4 axis imports cystine for glutathione synthesis, with GPX4 neutralizing lipid hydroperoxides. The FSP1/CoQ10 system regenerates reduced ubiquinol to halt lipid peroxidation, while the GCH1/BH4 axis protects membranes by neutralizing lipid radicals. Under oxidative stress, Nrf2 translocates to the nucleus and activates the expression of antioxidant enzymes, including GPX4 and glutathione synthase, enhancing the cell's ability to neutralize ROS and prevent ferroptosis. SLC3A2, Solute Carrier Family 3 Member 2; SLC7A11, Solute Carrier Family 7 Member 11; NRF2, Nuclear Factor Erythroid 2-Related Factor 2; KEAP1, Kelch-like ECH-associated Protein 1; GCLC, Glutamate-Cysteine Ligase Catalytic Subunit; GCLM, Glutamate-Cysteine Ligase Modulatory Subunit; GSH, Reduced Glutathione; GSSG, Oxidized Glutathione; GPX4, Glutathione Peroxidase 4; FSP1, Ferrostatin-1; CoQ10, Coenzyme Q10; CoQ10H2, Reduced Coenzyme Q10; $NADP^+$, Nicotinamide Adenine Dinucleotide Phosphate; NADPH, Reduced Nicotinamide Adenine Dinucleotide Phosphate; RSL3, Ras-selective lethal 3; P62, Sequestosome 1.

factor erythroid 2-related factor 2 (NRF2) to translocate to the nucleus and activate antioxidant and iron-regulatory genes. NRF2 upregulates enzymes involved in GSH synthesis (e.g., GCLC, GCLM), GPX4, and heme oxygenase-1 (HO-1) while simultaneously enhancing iron storage (via ferritin) and export (via ferroportin) (42). This dual regulation mitigates both oxidative damage and labile iron accumulation, highlighting NRF2 as a master regulator of ferroptosis resistance.

3 Interconnection between ferroptosis and lipid metabolism

How cells store, remodel, and metabolize lipids can either exacerbate or mitigate lipid peroxidation, directly affecting their sensitivity to ferroptosis (16). Lipid metabolic pathways, including fatty acid synthesis, uptake, β-oxidation, phospholipid synthesis and remodeling, lipid storage, and release, interact with the cell's antioxidant defenses to regulate ferroptosis sensitivity (43). These metabolic processes influence the availability of substrates for peroxidation and the capacity of cells to cope with oxidative stress. Understanding how lipid metabolism intersects with oxidative stress responses provides valuable insight into how cells balance lipid homeostasis, either promoting survival or triggering ferroptotic death in response to environmental stresses.

3.1 Fatty acid synthesis

The synthesis of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) is primarily mediated by enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). These enzymes convert acetyl-CoA into malonyl-CoA and further into palmitic acid, the SFA, which can be desaturated into MUFAs by stearoyl-CoA desaturase 1 (SCD1) (44, 45). In cancer cells, the overexpression of these enzymes supports fatty acid synthesis for energy and membrane biogenesis. However, the synthesis of SFAs and MUFAs generally confers resistance to ferroptosis, as these fatty acids are less susceptible to peroxidation than polyunsaturated fatty acids (PUFAs) (46). Conversely, the synthesis of PUFAs, which cannot be produced *de novo* in mammals, relies on dietary intake and subsequent desaturation and elongation reactions catalyzed by enzymes such as FADS1, FADS2, and ELOVL5. These PUFAs are incorporated into phospholipids, making cellular membranes more prone to ferroptosis due to their high susceptibility to lipid peroxidation (47).

3.2 Lipid uptake

Cells can absorb free fatty acids or lipoproteins from the extracellular environment through transporters such as CD36,

fatty acid transport proteins (FATPs), and fatty acid-binding proteins (FABPs) (48, 49). CD36-mediated FA uptake can induce LPO and ferroptosis in tumor-infiltrating $CD8^+$ T cells, blocking CD36 and restoring their antitumor activity (50). The cholesterol metabolite 27-hydroxycholesterol (27HC) enhances lipid uptake and metastatic capacity in aggressive breast cancer cells resistant to ferroptosis (51).

3.3 β-Oxidation

β-Oxidation is a process that breaks down fatty acids into acetyl-CoA units, primarily occurring in the mitochondria. This process is initiated by converting fatty acyl-CoA to carnitine esters by carnitine palmitoyltransferase 1 (CPT1), allowing the fatty acids to enter the mitochondria. Inside the mitochondria, fatty acyl-CoA is released from carnitine by CPT2 and undergoes a series of reactions that cleave two carbon units from the acyl chain (52, 53). The rate-limiting enzyme for *β*-oxidation of unsaturated fatty acids is 2,4-dienoyl-CoA reductase 1 (DECR1), which catalyzes the reduction of double bonds in fatty acids (54). *β*-Oxidation generally suppresses ferroptosis by reducing the availability of unesterified PUFAs, which are substrates for lipid peroxidation. In cancer cells, inhibiting *β*-oxidation can enhance ferroptosis by increasing the levels of free PUFAs and promoting lipid peroxidation (55, 56). Additionally, *β*-oxidation can influence the balance between fatty acid synthesis and degradation, affecting the overall lipid composition of cellular membranes and the susceptibility to ferroptosis (43).

3.4 Phospholipid synthesis and remodeling in ferroptosis

Phospholipid synthesis and remodeling dedicates structural and oxidative properties of cellular membranes, directly influencing their ferroptosis sensitivity. Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) are the two main players involved in such pathways (57). ACSL4 activates long-chain PUFAs, such as arachidonic acid (AA) and adrenic acid (AdA), by conjugating them to coenzyme A (CoA) to form PUFA-CoA derivatives (58). This activation step is necessary for their subsequent incorporation into phospholipids, leading to an increase in PUFAs-containing phospholipids in membranes (50). LPCAT3 functions in parallel to ACSL4 by catalyzing the reacylation of lysophospholipids to generating PUFA-containing phospholipids, including phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (59, 60). PUFA-PEs are highly reactive for LOX-driven lipid peroxidation (Figure 3), which triggers ferroptotic membrane destabilization greatly. Cells with a high expression profile of ACSL4 and LPCAT3 have a higher level of PUFA-containing phospholipids, predisposing them to ferroptotic damage due to the reactivity of these lipids toward oxidative stress (61). In contrast, inhibition of these enzymes diverts lipid metabolism toward monounsaturated fatty acids (MUFAs), which resist peroxidation and provide a survival advantage against ferroptotic stress (28).

3.5 Lipid storage

Cells store excessive fatty acids in lipid droplets in the form of triglycerides (TAGs), which can serve as a protective reservoir against lipid oxidation stress (62). Under metabolic stress, fatty acids are mobilized from lipid droplets through lipolysis, mediated by lipases, or lipophagy, a selective form of autophagy that degrades lipid droplets (63). The released fatty acids are readily available for phospholipid synthesis and remodeling, thereby alternating membrane lipid composition and ferroptosis susceptibility (64). Lipid storage in lipid droplets regulate ferroptosis in a dynamic way. In one hand, PUFAs can be stored in lipid droplets, rigorously controlling the incorporation of PUFAs to phospholipids in membranes to increase their resistance to lipid peroxidation; In the other hand, lipid droplets may facilitate the synthesis of PUFA-containing phospholipids as a PUFAs sources (37, 64).

4 Techniques for ferroptosis detection and analysis

Due to its complexity, a comprehensive characterization and understanding of ferroptosis require the implementation of diverse techniques across multiple scales, from subcellular structural changes to molecular alterations, lipidomic profiling, and *in vivo* validation. Microscopy and fluorescence imaging capture ferroptosis-associated morphological changes, while biochemical assays and immunodetection techniques provide insights into molecular pathways. Lipidomics and mass spectrometry-based approaches enable precise identification of oxidized phospholipids and lipid peroxidation byproducts, key markers of ferroptosis. Finally, *in vivo* models establish the physiological relevance of ferroptosis in disease contexts. Table 1 summarizes the commonly used approaches for ferroptosis research.

4.1 Subcellular structure changes

Ferroptosis is characterized by distinct subcellular structural changes at the organelle level, particularly in mitochondria and plasma membranes, which serve as key indicators of ferroptosis. Transmission electron microscopy (TEM) has also been widely used to visualize ferroptotic morphological features, including increased mitochondrial membrane density, cristae reductions, and outer membrane shrinkage—all of which differentiate ferroptosis from other types of cell death (68). Nuclear imaging can help distinguish ferroptosis from apoptosis and necrosis, as ferroptotic cells retain nuclear integrity, whereas nuclear fragmentation or condensation is a hallmark of apoptosis and necrosis (67). Immunohistochemistry (IHC) and immunofluorescence are widely employed to detect key proteins and markers involved in ferroptosis, including GPX4,

FIGURE 3
ACSL4 and LPCAT3 promote ferroptosis by facilitating the formation of polyunsaturated fatty acid phospholipids, thereby increasing susceptibility to lipid peroxidation. PUFA, Polyunsaturated Fatty Acid; ACSL4, Acyl-CoA Synthetase Long-chain Family Member 4; PUFA-CoA, Polyunsaturated Fatty Acyl-CoA; LPCAT3, Lysophosphatidylcholine Acyltransferase 3; PUFA-PL, Polyunsaturated Fatty Acid Phospholipid; LOX, Lipoxygenase; POR, P450 Oxidoreductase; PLLOH, Phospholipid Hydroperoxide.

SLC7A11, ACSL4, KEAP1, and p62 (41, 70–72). These techniques provide spatial and quantitative insights into the expression and localization of ferroptosis-related proteins at both the cellular and tissue levels, offering valuable tools for studying ferroptosis's molecular mechanisms and pathological roles.

Real-time imaging techniques further enhance ferroptosis detection by capturing membrane and organelle-specific biophysical alterations. Fluorescence imaging utilizing polarity-sensitive Mem-C1C18 and viscosity-sensitive MN-V probes allows researchers to monitor membrane integrity loss and mitochondrial viscosity shifts, providing dynamic insights into ferroptotic progression (74, 75).

4.2 Analyzing molecules and pathways in ferroptosis

At the molecular scale, ferroptosis is driven by reactive oxygen species (ROS), iron dysregulation, and lipid peroxidation, necessitating targeted analytical techniques for detection. Selective fluorescent probes have been developed to detect key ROS species, including hydrogen peroxide (HO) and hypochlorous acid (HClO), enabling real-time monitoring of oxidative stress within ferroptotic cells (73, 74).

Since iron catalyzes lipid peroxidation through the Fenton reaction, ferroptosis detection also involves tracking labile ferrous iron (Fe^{2+}) dynamics. Reactivity-based fluorescent probes have been designed to visualize intracellular iron fluctuations, providing high-resolution insights into iron homeostasis and ferroptotic vulnerability (75).

To assess the antioxidant response and redox balance, the System Xc^-/GSH/GPX4 axis can be monitored using fluorescence-based glutathione probes like RealThiol, which enable live-cell quantification of glutathione depletion (79).

Additionally, immunohistochemistry (IHC) and immunofluorescence are widely used to detect ferroptosis-related proteins, such as glutathione peroxidase 4 (GPX4), solute carrier family 7 member 11 (SLC7A11), acyl-CoA synthetase long-chain

TABLE 1 Tools and techniques for probing ferroptosis across subcellular, molecular, and *in vivo* levels.

Type	Approach	Target	References
Subcellular structure	Fluorescence Imaging	Membrane Integrity, Mitochondrial Viscosity	(65, 66)
	Nuclear Imaging	Nuclear Structure Integrity	(67)
	Transmission Electron Microscopy (TEM)	Mitochondrial Membrane Density, Cristae Reductions	(68)
	Immunohistochemistry (IHC)	GPX4, SLC7A11, ACSL4, KEAP1, p62	(41, 69–72)
Molecules and pathways	Endogenous Markers	ROS, Lipid Peroxidation, Iron Metabolism	(73, 74)
	Reactive Oxygen Species (ROS) Probes	Hydrogen Peroxide (H_2O_2), Hypochlorous Acid (HClO)	(73, 74)
	Reactivity-Based Fluorescent Probes	Labile Fe^{2+}	(75)
	Lipid Peroxidation Probes	C11-BODIPY, Oxidized Phospholipids	(76, 77)
	Chemoproteomic Tools	Protein Carbonylation	(78)
	System Xc^-/GSH/GPX4 Axis Probes	Glutathione Dynamics	(79)
	Flow Cytometry, Cell Activity Assays, Western Blotting	ROS, Lipid Metabolites (MDA), Mitochondrial Damage	(80–82)
Omics	lipidomics	Phospholipids	(83–86)
In Vivo Studies	Bioluminescence	Labile Fe^{2+} Levels	(87)
	Positron Emission Tomography (PET)	Ferroptosis Markers	(88)

family member 4 (ACSL4), Kelch-like ECH-associated protein 1 (KEAP1), and sequestosome 1 (p62) (41, 69–72). These techniques provide spatial and quantitative data, offering insights into both cellular and tissue-level ferroptosis regulation.

Complementary techniques such as flow cytometry, cell viability assays, and western blotting are frequently employed to quantify ROS levels, lipid peroxidation byproducts (e.g., malondialdehyde, MDA), and mitochondrial damage, further broadening the arsenal of ferroptosis detection tools (80–82).

4.3 *In vivo* studies

Advanced imaging technologies, including bioluminescence and positron emission tomography (PET), have become powerful tools for detecting ferroptosis in living organisms. Bioluminescent probes like ICL-1 enable real-time, longitudinal tracking of labile ferrous iron (Fe^{2+}) levels, offering dynamic insights into ferroptotic activity. Meanwhile, PET tracers such as ^{18}F-TRX allow high-resolution, three-dimensional imaging of ferroptosis markers, facilitating the study of ferroptotic processes in disease models and the assessment of therapeutic interventions (87, 88).

4.4 lipidomics and mass-spectrometry-based approach

Lipidomics and mass spectrometry-based techniques have become essential tools for investigating ferroptosis, enabling precise characterization of lipid composition, metabolic flux, and spatial lipid remodeling. Morgan's study demonstrated that PUFA-containing phospholipids (PUFA-PLs) determine ferroptosis susceptibility, with T cells being more vulnerable due to higher PUFA-PL levels, while myeloid cells resist ferroptosis due to lower PUFA-PL content. Wang et al. (2024) applied single-cell lipidomics to analyze lipid remodeling in foam cells, linking neutral lipid accumulation, sphingolipid depletion, and glutathione oxidation to ferroptosis progression (83).

Advanced isotope-resolved lipidomics by Reimers et al. (2023) revealed that phosphatidylethanolamines (PEs) are the primary peroxidation targets in ferroptosis (84). In an *in vivo* study, Gorman et al. (2024) employed spatial lipidomics with mass spectrometry imaging (MSI) to map iron and lipid distribution in ferroptotic ovarian tumors, showing localized accumulation of sphingolipids and triglycerides in iron-rich regions, while PUFA-containing phospholipids were enriched in peroxidized areas (85). Earlier, Doll and Kagan (2017) introduced redox phospholipidomics, identifying PEs as key substrates for lipid peroxidation upon GPX4 inhibition. Collectively, these studies highlight the power of lipidomics and mass spectrometry in deciphering ferroptotic lipid metabolism, advancing both mechanistic insights and biomarker discovery in ferroptosis research.

5 Ferroptosis in disease and therapeutic targeting

Ferroptosis plays a dual role in disease, acting as both a therapeutic target in cancer and a pathogenic driver in degenerative and ischemic conditions. Certain cancer cells, particularly mesenchymal-like subtypes, are highly susceptible to ferroptosis due to their elevated metabolic activity and reactive oxygen species (ROS) burden (89). Ferroptosis-inducing compounds, such as erastin, disrupt glutathione homeostasis by inhibiting system Xc^-, leading to lethal lipid peroxidation (90). Additionally, tumor suppressor p53 sensitizes cancer cells to ferroptosis by downregulating SLC7A11, further impairing antioxidant defenses (91).

Conversely, ferroptosis contributes to tissue damage in ischemia-reperfusion (I/R) injuries, such as stroke and myocardial infarction, where excessive ROS production triggers lipid peroxidation and cell death (92). Glutamate-induced excitotoxicity inhibits system Xc^-, driving ferroptosis in neurons during stroke, while in the heart, ferroptotic cardiomyocyte death worsens ischemic injury (93, 94). Ferroptosis is also implicated in neurodegenerative diseases, fibrosis, and autoimmune disorders, where iron dysregulation and lipid peroxidation drive cell death and disease progression (95–101).

Given its contrasting roles, therapeutic strategies targeting ferroptosis depend on disease context, aiming either to induce ferroptosis (e.g., in cancer) or inhibit it (e.g., in degenerative and ischemic diseases). Over the past decade, advances in iron metabolism regulation, antioxidant therapies, pathway modulation, and nanoparticle-based drug delivery have paved the way for clinical applications of ferroptosis-targeting strategies.

5.1 Regulating iron metabolism

Since iron accumulation catalyzes lipid peroxidation and ferroptosis, iron chelation therapy is widely used to prevent ferroptotic damage in conditions like thalassemia and hemochromatosis (102). Chelators such as deferoxamine (DFO), deferasirox (DFX), and deferiprone (DFP) bind excess iron, reducing oxidative stress and lipid peroxidation (103). In clinical settings, DFO has been shown to inhibit ferroptotic cardiomyocyte death, improving cardiac function in patients with iron overload, while DFX protects hepatocytes by preventing iron-driven ferroptosis (104–106).

5.2 Antioxidant-based ferroptosis inhibition

Since oxidative stress is central to ferroptosis, antioxidant-based therapies aim to restore redox balance. N-acetylcysteine (NAC), a glutathione (GSH) precursor, has demonstrated ferroptosis inhibition in diseases where GSH depletion exacerbates oxidative damage (107–109). Coenzyme Q10 (CoQ10), a radical-trapping antioxidant (RTA), has been studied for its protective effects in neurodegeneration, cardiovascular disease, and cancer (110). Clinical trials (NCT01964001) suggest that CoQ10 supplementation in hepatocellular carcinoma (HCC) patients improves antioxidant capacity and reduces inflammation, potentially by suppressing ferroptosis (111).

5.3 Targeting the SLC7A11/GPX4 axis

The SLC7A11/GPX4 pathway is a critical ferroptosis regulator, making it a key therapeutic target. GPX4 stabilizers and activators offer protection against ferroptotic damage in degenerative diseases (13, 112). Sulforaphane, a natural Nrf2 activator, enhances GPX4 expression, mitigating ferroptotic stress in various disease models (113). Clinical trials (NCT02255682) are currently evaluating CoQ10's role in ferroptosis prevention in diabetic cardiomyopathy (114).

5.4 Nanoparticle-based therapies

Nanotechnology provides targeted delivery of ferroptosis modulators, enhancing therapeutic efficacy and bioavailability. Triphenylphosphine-modified quercetin nanoparticles (TQCN) selectively inhibit ferroptosis in neurons by chelating iron and activating Nrf2-mediated antioxidant responses (115). Similarly, pH/GSH-responsive polyamino acid nanogels (NG/EDA) deliver edaravone to ischemic brain tissue, preventing ferroptotic damage and improving neurological recovery (116). These nanoparticle-based strategies hold significant potential for precisely modulating ferroptosis in clinical applications.

6 Conclusion

Ferroptosis represents a unique intersection of lipid metabolism, oxidative stress, and iron regulation, offering fresh insights into cell death mechanisms and their implications in disease. Characterized by the iron-dependent peroxidation of polyunsaturated fatty acids (PUFAs), ferroptosis is implicated in a wide range of pathological conditions, including cancer, neurodegenerative diseases, cardiovascular disorders, and metabolic syndromes. By unraveling its molecular underpinnings—such as the roles of ACSL4, GPX4, and lipid remodeling enzymes— researchers have identified promising opportunities for therapeutic intervention.

Modulating ferroptosis presents a dual therapeutic strategy: inducing ferroptotic cell death to eliminate cancer cells or inhibiting it to protect tissues from oxidative damage in diseases driven by lipid peroxidation. Advances in dietary, pharmacological, and genetic approaches targeting lipid metabolism and iron homeostasis further underscore its therapeutic potential. However, significant challenges remain, including understanding tissue-specific ferroptotic responses and overcoming resistance mechanisms in cancer therapy (117).

Future research should aim to refine *in vivo* models, identify reliable biomarkers, and develop precise, context-dependent therapeutic strategies. Integrating lipidomics and systems biology will be critical for deciphering ferroptosis regulatory networks, paving the way for innovative treatments. Ultimately, as a regulated cell death pathway driven by iron-dependent lipid peroxidation, ferroptosis bridges lipid metabolism, redox biology, and metal homeostasis. This intersection not only redefines our understanding of cellular demise but also unveils novel therapeutic avenues for diseases ranging from cancer to neurodegeneration (35, 118).

Author contributions

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Conflict of interest

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