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Immunomodulatory role of metalloproteases in chronic inflammatory diseases

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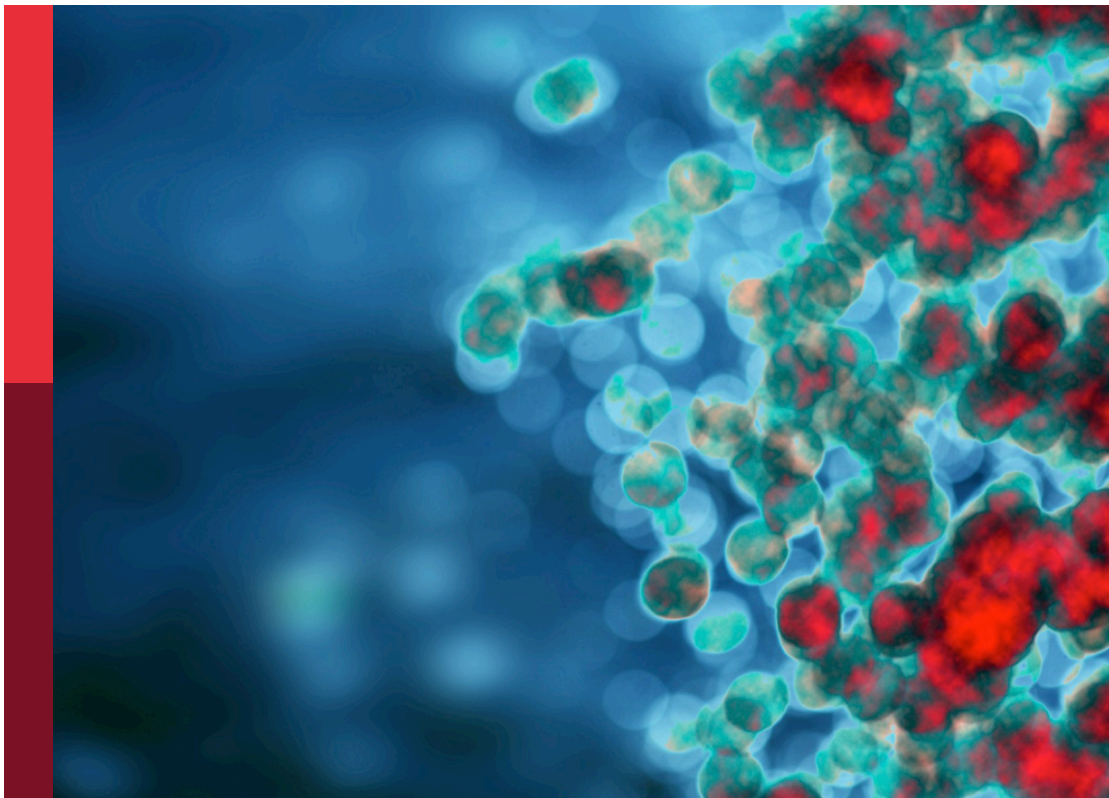

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Immunomodulatory role of metalloproteases in chronic inflammatory diseases

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Table of contents

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Editorial: Immunomodulatory role of metalloproteases in chronic inflammatory diseases

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Editorial on the Research Topic
Immunomodulatory role of metalloproteases in chronic inflammatory diseases

Metalloproteases are a diverse class of enzymes involved in the regulation of numerous pathological and physiological processes. Evidence has shown that metalloproteases could either directly or indirectly regulate the secretion of chemokines and the differentiation and/or activation of immune cells, thereby mediating many inflammatory and innate immune responses (1, 2). While different metalloproteases could have substantially different primary structure, their active center which contains metal ions (e.g. iron, zinc, cobalt, nickel ions) is relatively conservative. Metalloprotease relies on metal ions to maintain its catalytic function. Studies have shown that metal chelators such as EDTA could completely inactivate metalloproteases (3–6; Liu et al.). Under inflammatory conditions, metalloproteases are constitutively activated or deactivated in multiple immune- or non-immune cells and could contribute to a variety of inflammatory diseases, such as rheumatoid arthritis (RA) (Li et al.), chronic enteritis (Deng et al. and Mei et al.), allergic dieseases (Wang and Wang and Bendavid et al.), diabetes (Chen et al.), and cancers (He et al.), etc. Mutiple chronic inflammatory diseases could even hijack various metalloproteases to promote and exacerbate inflammation (1, 2). Numerous preclinical and clinical studies have shown that metalloprotease modulators, including lysine-specific demethyalses (KDMs) inhibitors (3–5, 7), histone deacetylases (HDACs) inhibitors (8), and matrix metalloproteinases (MMPs) inhibitors (6), and a disintegrin and metalloproteinases (ADAMs) inhibitors (9), possess *in vitro* and *in vivo* anti–inflammatory activities. Therefore, understanding the roles of metalloproteinases in the immune system may potenitally uncover new targets for the diagnosis and treatment of chronic inflammatory diseases.

This Research Topic contributes to a better understanding of immunomodulatory role of the metalloproteases in several chronic inflammatory diseases and highlights the clinical significance of the immunomodulatory role of metalloproteases in disease diagnosis and drug discovery. This Research Topic accepted a total of 12 articles from 75 authors. All contributions to this Research Topic focus on one or more of the following research areas:

MMPs

MMPs are a family of zinc-dependent proteases playing the role of targeting and cleaving extracellular proteins. They are involved in the occurence and progression of multiple chronic inflammatory diseases, including colitis [Chen et al. and Deng et al.], rheumatoid arthritis (RA) (Li et al.), diabetes (Chen et al.), and cancers (10), etc. Mei et al. identifed five MMPs-realted genes (TLR5, CD160, MMP-9, PTGDS, and SLC26A8) as the biomakers of inflammatory bowel disease (IBD) using machine learning by screening from public Gene Expression Omnibus datasets and functional enrichment analysis. *In vivo* study using sodium dextran sulfate (DSS)-induced colitis indicated that the level of TLR5 was significantly reduced in the model group and the levels of other four protiens were significantly increased. Futher studies have shown that MMPs modualate intestinally inflammatary and immune responses mainly through CD8+ cells in colitis. This study reveals the crucial roles of MMPs in the pathogenesis of IBD and provides insights into the molecualr mechanism and theranostical targets of IBD. Deng et al. suggests that MMPs-related modules are the main differential gene sets between Crohn's disease and ulcerative colitis based on integrated analysis of multiple microarray. RA is an autoimmune disease caused by a variety of factors (Yang et al.). MMPs were found to play a crucial role in the pathogenesis of RA. Mutiple herbal medicines can inhibit the inflammatory responses of RA and thus alleviate RA through modulating MMPs and the associated signaling pathways (Li et al.). Diabetic ulcer is a serious complication of diabetes characterized by recalcitrant wounds, which could tremendously affect the quality of life of patients and impose a substantail medical and economic burden on a country. Chen et al. found that many natural products including flavonoids, alkaloids, polysaccharides, and polypeptides, etc. are effective to treat diabetic ulcer through regulation of the MMPs-mediated pathways. Furthermore, MMPs also contribute to many cancers. He et al. found that MMPs mediate the progression of colitis-associated cancer by regulating the expression of each member of MMPs precisely and thus promoting cell proliferation and differentiation, angiogenesis, and extracellular matrix remodeling. Wang et al. also suggested that MMPs could be theranostic targets of cancers and could potentially be applied in cancer diagnosis and treatment.

ADAMs

ADAMs are a family of transmembrane and secreted metalloproteases. They are involved in many chronic inflammatory diseases through modulating proteolysis and the related signalling pathways (11). Wang et al. sumarized the structure and immunoregulatory roles of ADMAD17 in tumorigenesis and highlighted that aborgating ADMAD17 using small inhibitors or monoclonal antibodies is an effective strategy to combat cancers. In addition, Wang et al. also showed that ADAMs modulate the adhesion and migration of cancer cells *via* releasing the proteolytic cell surface molecules including adhesion molecules, growth factors, and precursor forms of cytokines. Devel et al. suggested that both MMPs and ADAMs are involved in regulating CD95/CD95L signaling in proteolytic enzyme-dependent manner and targeting this signalling is a potential strategy for fighting cancer. Bendavid et al. explored the role of ADMAD28 in asthma using an OVA-induced asthma model. This study found that ADMAD28 could increase collagen deposition, smooth muscle hyperplasia, mucous hyperplasia, suggesting that ADMAD28 could promote the progression of asthma through regulating airway remodeling.

Others

Some enzymes such as Jumonji C (JmjC) demethylases, ten-eleven-translocation (TET) enzymes, COX2, and HDACs are also metalloproteases and are invlvoled in modulating several chronic inflammatory diseases [(5, 7), Jin et al. and Wang et al.]. Our previous studies showed that KDM5A or LSD1 could inhibit the progression of triple-negative breast cancer or acute leukemia *via* inducing cell cycle arrest and senescence, leading to cell apostosis *in vitro* and *in vivo* (12–15). Jin et al. systematically sumarized the emerging role of TET enzymes in the immune microenvironment at the maternal-fetal interface during decidualization and early pregnancy, providing an insight into the future implications in disease diagnosis or treatment. Zhou et al. revealed that the aqueous extract of *Dendrobium officinale* flowers exhibit anti-glycation, anti-cyclooxygenase, and anti-skin aging activity. HDAC1, which is a type of class I HDACs, is a crucial enzyme modulating the progression of chronic inflammatory diseases, including allergic diseases (16–19). Wang and Wang sumarized the roles of HDAC1 in allergic diseases. After stimulated by allergen, HDAC1 upregulates the levels of T helper 2 cytokine, reduces the number of Th1/Th17 cells and Interleukin-10, and downregulate the expression of TWIK-related potassium channel-1. This review hightlights the functions and regulatory roles of HDAC1 in allergic diseases, aids the understanding of allergic multimorbidity relationships, as well as provides insight into the feasibility of using HDAC1 as a molecular target for the diagnosis and treatment of allergic diseases.

In summary, we anticipate that this Research Topic will inspire future research on the immunoregulatory roles of metalloproteinases in chronic inflammatory diseases. Understanding the function and regulatory mechanisms of metalloproteinases may provide insights into the future development of diagnostic and therapeutic approaches (e.g. nanomaterials or metal-based probe targeting these enzymes) for chronic inflammatory diseases (20–24).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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The emerging role of histone deacetylase 1 in allergic diseases

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Histone deacetylase 1 (HDAC1) is a unique member of the classes I HDACs and helps to regulate acute and chronic adaptation to environmental stimuli such as allergen, stress. Allergic diseases are complex diseases resulting from the effect of multiple genetic and interacting foreign substances. Epigenetics play an important role in both pathological and immunomodulatory conditions of allergic diseases. To be consistent with this role, recent evidence strongly suggests that histone deacetylase 1 (HDAC1) plays a critical role in allergic response. HDAC1 expression is stimulated by allergen and attributes to increase T helper 2 (Th2) cytokine levels, decrease Th1/Th17 cells and anti-inflammatory cytokine Interleukin-10 (IL-10), and TWIK-related potassium channel-1 (Trek-1) expression. This review focuses on the contribution of HDAC1 and the regulatory role in characterizing allergic endotypes with common molecular pathways and understanding allergic multimorbidity relationships, as well as addressing their potential as therapeutic targets for these conditions.

KEYWORDS

epigenetic modifications, allergic diseases, HDAC1, Th2 cytokines, IL-10, Trek-1

Introduction

Epigenetics includes the heritable alterations in gene expression without any changes in a deoxyribonucleic acid (DNA) sequence, which is crucial in the pathophysiology of many diseases (1, 2). Multiple enzymes have been extensively studied that induce epigenetic changes, such as DNA methylation and histone acetylation of DNA regions. Histone deacetylases (HDACs) are the enzymes that catalyze lysine deacetylation of both histone and non-histone proteins. HDACs increase the positive charge on histones after removing acetyl groups from lysine residues, thus increasing the affinity of positively charged histones for negatively charged DNA (3). HDACs lead to the condensation of the chromatin and then reduces the accessibility of transcriptase, and finally leads to an overall suppression of gene transcription (4). HDAC family has four subclasses including I, II, III and IV. Classes I, II, and IV HDACs utilize a zinc-dependent mechanism and belong to the Zn2+ superfamily, while class III HDACs require nicotinamide adenine dinucleotide(NAD)+ for catalytic activity.

Histone deacetylase 1 (HDAC1) is a unique member of the classes I HDACs that has been shown to be involved in gene transcription, transcriptional regulation, cell cycle progression and developmental events by controlling both enzyme activity and epigenetics of key proteins (5). HDAC1 is the most abundant member of the class I HDACs in pulmonary endothelial cells (6), regulating the enzymatic activity and epigenetics of key proteins to adapt to external stimuli. It can efficiently decrotonylate this relatively less abundant histone modification (7). Moreover, HDAC1 is the key regulators of T cell subset differentiation and T cell-mediated immune diseases (8) that helps to regulate acute and chronic adaptation to environmental stimuli such as allergen, stress (9). Allergic diseases represent a collection of disorders such as allergic rhinitis, asthma, that mostly characterized by a type 2 immune response involving Th2 cells, eosinophils and mast cells, and M2 macrophages. T cell specific loss of HDAC1 leads to an increase in Th2 type allergic airway inflammation, such as enhanced secretion of Th2 type cytokines, eosinophil recruitment to the lung (10).For example, HDAC1 is highly expressed and the most abundant member of the class I HDACs in allergic rhinitis and severe asthma (3, 11, 12). Studies show that HDAC1 is localized within most airway cells and infiltrating inflammatory cells of asthmatic lung tissues (13). HDAC1 is significantly upregulated in the murine AR model while H3 acetylation is decreased at lysine 9 (H3AcK9) (14). The HDAC1 inhibitor sodium butyrate exhibits a preventive effect by decreasing HDAC1 expression and increasing H3 acetylation at lysine 9. Herein, we made a thorough review of recent studies and summarized the emerging functions of HDAC1 by regulating histone modifications and gene transcription in allergic disease.

Allergic diseases

Generalized allergic diseases include allergic rhinitis, asthma, Immunoglobulin E(IgE)-mediated food allergy, eosinophilic esophagitis, drug allergy, atopic dermatitis, and urticaria/angioedema. These different allergic diseases share several overlapping inflammatory pathways concerning with the hypersensitivity of the individual to foreign substances (15–18). Allergic diseases are a type 2 immune disorder classically characterized by high levels of IgE-mediated inflammation and Th1/Th2 cells imbalance (19–21). The Th 2 immune response involves Th2 cells, type 2 innate lymphoid cells, mast cells, eosinophils, and M2 macrophages (22). Th2 cytokines, particularly IL-4, are essential in the pathophysiology of allergic rhinitis and asthma (23, 24). In type I immediate allergic responses, naïve T cells is activated by dendritic cells to differentiate, proliferate and clonally expand into Th2 cells (23, 25). Enhanced Th2 cytokines induce IgE synthesis in B cells in an indirect manner (26, 27). In turn, IgE can also enhance Th2-cell response after sensitization (28). However, the aberrant immune responses in atopic disorders are not fully understood yet.

Epigenetics plays a major pathogenetic role in the development and management of allergic diseases by superimposing its effects above the DNA molecule through interaction with susceptibility genes, environmental factors, and immunologic influences (29). Epigenetics holds the key to unravel the complex associations between phenotypes and endotypes of allergic disease by identifying effective therapies and diagnosis (30). Epigenetic modifications of genes are contributing to asthma induced by allergens, such as DNA methylation changes in DCs, can be passed to future generations (31, 32). Histone modifications and DNA methylation represent the classical epigenetic mechanisms. Histone modifications participate in airway remodeling by regulation of T cells and macrophages. Inhibitors of histone-modifying enzymes may potentially be used as anti-allergic drugs (33).

The role of HDAC1 in allergic diseases

HDAC1 displays compensatory or specific roles in different cell types or in response to different stimuli and signaling pathways of atopic disorders. The expression level of HDAC1 in the nasal epithelia is elevated in allergic rhinitis (34), and HDAC1 inhibitors reduce the symptoms of allergic rhinitis (3, 12, 35). Immunohistochemical results also demonstrate the high HDAC1 expression in nasal epithelium of patients with sinusitis and nasal polyps (36). The differentially expressed genes (DEGs) analysis of 1,662 nasal–epithelium tissue samples and 572 DEGs from peripheral blood samples shows that HDAC1 is hub genes and serves an important role in the process of asthma (37). HDAC1 expression is enhanced in patients with severe asthma compared with healthy volunteers (11). Moreover, expression of HDAC1 is upregulated by the stimulation of dermatophagoides pteronyssinus allergen (Der p 1) in peripheral blood mononuclear cells of patients with severe and non-severe asthma (38). Animal models of allergic asthma exhibits significantly higher expression of HDAC1 compared to control. Selective targeting of HDAC1 may improve therapeutic effects of asthma (39). One single nucleotide polymorphism (SNP) in HDAC1 (rs1741981) is closely associated to asthma severity in a recessive model and increases the sensitivity to systemic corticosteroids treatment in asthmatic patients (40, 41). Besides, in epidermal keratinocytes, HDAC1 expression and activity are upregulated by the aryl hydrocarbon receptor nuclear translocator (ARNT or HIF1β) (42).

Regulation of inflammatory cytokines and downstream protein by HDAC1

A number of studies have shown that exposure to allergens would increase HDAC1 expression, leading to significantly advanced Th2 cytokine levels, reduced Th1/Th17 cells and anti-inflammatory cytokine IL-10, and Trek-1 expression (Figure 1). In the mouse model of allergic rhinitis, epigenetic regulation of HDAC1 produce an imbalance in Th1/Th2 by decreasing the secretion of interferon(IFN)-γ, increasing the secretion of IL-4 and IL-6 (14). Moreover, the transcriptional activity of forkhead box P3(Foxp3) is restrained that decreases T regulatory cells (43). As the number of Th1 cells decreases, the number of Th2 cells correspondingly increases, and subsequently the secretion of IL-4 increases to promote the activation of IgE released by B cells (44). Additionally, murine models of asthma confirm the upregulation of HDAC1 could increase airway inflammation, Th2 cytokine level, IgE and goblet cell metaplasia dramatically (45). Indeed, treatment with HDAC1 inhibitor trichostatin A(TSA) significantly attenuate airway hyper-responsiveness, mucus occlusions in lung tissue and the numbers of eosinophils and lymphocytes in bronchoalveolar lavage fluid. The infiltration of CD4+ and the expression of IL-4, IL-5, and IgE in BALF are also restrained by TSA (13). Particularly, Th2 cytokine interleukin 4 (IL-4) plays a key role in the pathogenesis of allergic disorders (46). HDAC1 can be recruited to the IL-4 gene locus in CD4(+) T cells, thereby promoting the immunoactivity of CD4 positive T cells to increase Th2 cytokine levels (47–49). The IL-4-induced rat nasal epithelial barrier dysfunction is blocked by HDAC1 inhibitor (Trichostatin A), or sodium butyrate (NaB), or administration of Clostridium Butyricum (Table 1) (14, 62). A non-secreted IL-4 variant (IL-4δ13) expression in human γδ T-cells is also stimulated by another HDAC inhibitor valproic acid (VPA) (Table 1) (58). The Induction of IL-4δ13 increases cytoplasmic IL-4Rα and decreases mature IL-4 (59). Along with the role of HDAC1 in altering the Th2 cytokine profile, it is reported that HDAC1 is recruited to change the euchromatin into tightly-packed heterochromatin to repress its expression in Th17 cells through production of cytokine IL17 (63). HDAC1 inhibitor sodium butyrate increases IL−17、interleukin 2 (IL−2) and interferon γ and decreases the expression of IL−4 and IL−5 (50). HDAC1 regulates the retinoic acid-related orphan receptor-mediated transcriptional activation of IL-17 (64).

Apart from the studies showing the Th1/Th2 imbalance and inhibition of IL17, histone deacetylation is an important mechanism that regulates the expression of anti-inflammatory cytokine IL-10 (65). HDAC1 represses IL-10 transcription activity by reducing chromatin accessibility and recruiting histone H3 acetylation at IL-10 regulatory regions (66). Sodium butyrate restrains the activation of HDAC1 in the antigen specific B cells to induce the expression of IL-10 and decrease the production of IgE in allergic rhinitis model (51). Another HDAC inhibitor entinostat stimulates the formation of IL-10 positive Breg cells to suppress contact hypersensitivity *in vivo* (54). Indeed, the administration with Clostridium butyricum (C. butyricum) enforces the effect of specific immunotherapy on intestinal allergic inflammation by

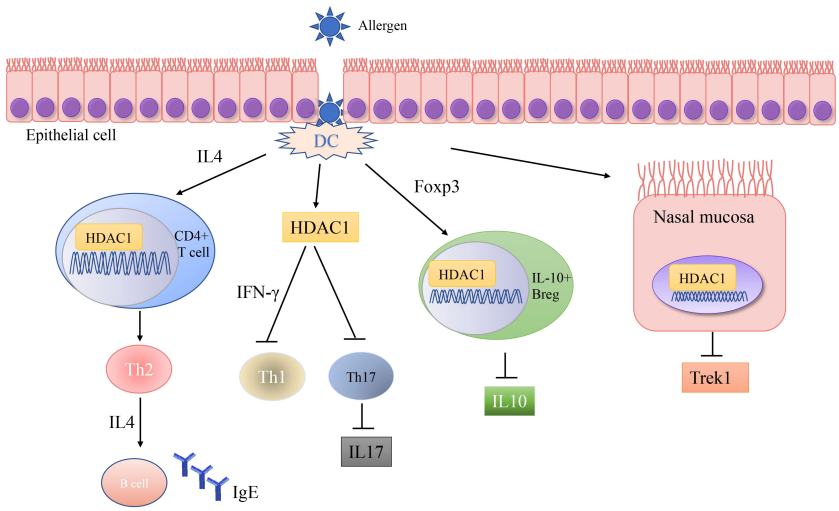
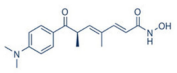
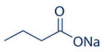
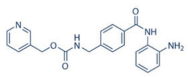


FIGURE 1
Schematic representations of HDAC1 related mechanism in allergic diseases. Allergic disease patients have an epithelial barrier suffering from allergen stimulation. Exposure to allergens activate dendritic cell and increase HDAC1 expression, leading to significantly increase Th2 cytokine levels, decrease Th1/Th17 cells and anti-inflammatory cytokine IL-10, and Trek-1 expression.

TABLE 1 The role of HDAC inhibitor in allergic diseases.

HDACI inhibitor		Structure	Model	Clinical application	Allergic Diseases	References
Trichostatin A (TSA)	pan-inhibitors		Ovalbumin-induced mouse asthma model;	Phase I clinical trials in hematologic malignancies	Asthma	(13)
Sodium butyrate (SoB, NaB)	selective inhibitors		Mouse model of allergic rhinitis	Phase 2 clinical trials in Shigellosis; Randomized controlled trial in inflammatory Bowel Diseases;	Allergic rhinitis	(50–53)
Entinostat	selective inhibitors		Mouse model of oxazolone-induced contact hypersensitivity	Phase 3 clinical trials in cancer;	Contact hyper sensitivity	(54–57)
Valproic acid (VPA)	selective inhibitors		Asthmatic mouse model; Peripheral blood mononuclear cell	Phase 2 clinical trials in cancer	Asthma healthy donors	(58–61)

increasing the phosphorylation of HDAC1, the expression of IL-10 and the IgE-producing plasma cells (67).

There are some studies documenting the role of Trek-1 in the maintenance of epithelial cell barrier function (62, 68). The allergic responses induce an insufficiency of Trek1 expression (69). Enhanced IL-4 markedly suppresses the expression of Trek1 *via* upregulating the expression of the HDAC1 in the nasal mucosa of allergic rhinitis (62). The treatment with antigen-specific immunotherapy and administration of probiotic C. butyricum reduce the serum levels of Th2 cytokines by increasing Trek-1 expression levels and decreasing HDAC1 in the nasal mucosa of allergic rhinitis patients (23). Allergic responses markedly suppress the expression of Trek1 in the intestinal epithelia *via* increasing the expression of HDAC1 (70).

HDAC1 is regulated by exposure to stimuli and is associated with gut microbiome

Different stimuli includes temperature, particles containing hazardous chemicals, and small chemical molecules that exhibits an impact on the expression of HDAC1. Particulate matter (PM) 2.5 exposure and cold stress (PMCS) exposures promote inflammation and redox levels in asthmatic mice through increasing the percentage of Th2 T cells and decreasing Th1 T cells, thereby decreasing HDAC1 expression and hyperacetylation of H3K9 and H3K14 in IL-4 gene promoter of CD4+T cells (71). Mechanically, HDAC1 helps maintain DNA-binding sites (response elements) for redox-sensitive transcription factors by co-repressor complexes (72). Besides, exposure to diesel exhaust particulate matter (DEP) causes degradation of histone deacetylase 1 (HDAC1), thus recruiting histone acetyltransferase (HAT) p300 to the promoter of the Cyclooxygenase-2 (COX-2) gene *in vitro* human bronchial epithelial cell line (BEAS-2B) (73). In addition, chronic exposure to alcohol decreases HDAC1 expression (74). Trichostatin A alleviates tissue damage that is caused by cigarette smoke exposure (75, 76).

On the other hand, HDAC1 is modulated by upstream transcription factors and signaling pathway in allergic diseases. Previous studies have shown that the transcription factor c-Myc-interacting zinc finger protein-1 (Miz1) was upregulated in allergic asthma, which in turn prevented the pro-Th1 skewing through the recruitment of histone deacetylase 1 (HDAC1) and transcriptional repression of IL-12 (77). HDAC1 expression is also increased by the advanced glycation end products *via* the phosphatidylinositol 3-kinase(PI3K)/AKT pathway through promoting the airway inflammation (45).

Moreover, gut microbiome is associated with allergic diseases (78–81). Sodium butyrate treatments lead to increase the richness in the stomach and colon and modify colonic microbial composition in pigs by decreasing HDAC1 (82, 83). The intestinal epithelial cells specific HDAC1 support intestinal homeostasis by controlling specific biological processes including oxidation-reduction, survival and translation processes, differentiation and lipid-related metabolic pathways *via* Janus kinase(JAK)/signal transducer and activator of transcription (STAT) pathway and steroid receptor pathway (84–86).

Potential of HDAC1 inhibitors as treatments

A large body of evidence shows that HDAC1 is a potential clinical target for treatment of allergic diseases. At present, numerous questions remain regarding to the precise functions of HDAC1 in allergic inflammation. The HDAC inhibitors such as trichostatin A (TSA) have a bidentate cheator, which binds to catalytic Zn2+ (87). The broad-spectrum HDAC1 inhibitor

trichostatin A has a hydroxamic acid based structure that affects the expression of thousand genes in the human genome. There is still no clinical application of these HDAC1 inhibitors. Thus, there is an ongoing discussion whether selective HDAC inhibitors have advantage for clinical use. These small-molecule compounds targeting HDAC1 have no serious toxicities.

There are many HDAC inhibitors in ongoing clinical trials (Table 1). The study on the tolerance of trichostatin A in patients with recurrent or refractory hematological malignancies is still in progress. Genetic and pharmacological studies have confirmed that HDAC1 is the key enzyme to reverse tumor immune escape. Entinostat selectively promotes the immune editing of new tumor antigens, leading effectively reshaping the tumor immune microenvironment (55). The randomized phase III trial of endocrine therapy confirms target inhibition in entinostat-treated breast cancer patients (56). Valproic acid and entinostat exhibit synergy in preclinical models when combined with rituximab in Non-Hodgkin's lymphoma (57). On the other hand, Valproic acid is the first-line drug for tonic clonic seizures (60). Besides, Valproic acid induces apoptosis of activated T cells to maintain immune homeostasis, which may be a safe and effective treatment for autoimmune diseases, such as multiple sclerosis (61). Entinostat and valproic acid can potentially be repurposed for treating asthma (88). However, there is no clinical trials to determine the role of entinostat and valproic acid in asthma. These findings highlight the need for further exploration of HDAC inhibitors in allergic diseases.

Sodium butyrate therapy during shigellosis leads to early reduction of inflammation and enhanced antimicrobial peptides (LL-37) expression in the rectal epithelia (52). The double-blind randomized controlled trial shows that sodium-butyrate supplementation in 49 inflammatory bowel diseases patients increases the growth of bacteria able to produce short-chain fatty acids (SCFA) with potentially anti-inflammatory action (53). These results support the potential effect of sodium butyrate in modulating gut microbiota, which anyway requires further confirmatory data including more patients. In considering future potential clinical application in allergic diseases, more studies are still needed to develop new HDAC1 specific selective inhibitors. HDAC1 specific selective inhibitors may provide a new starting point for the treatment of allergic diseases.

Prospective and conclusion

Allergic diseases comprise some of the most common chronic disorders in both childhood and adulthood. Allergic conditions are influenced by epigenetic elements which ultimately affect multiple molecular pathways (89, 90). Accumulating evidences have established in HDAC1 as a critical regulator of immune response in terms of imbalance in Th1/Th2, change in anti-inflammatory cytokine IL-10/IL-17 and Trek-1 expression. Over the past decades, histone deacetylase inhibitors are being evaluated in clinical trials for their safety and efficacy (91, 92). HDAC1 has become an attractive target to treat a wide range of diseases. However, these HDAC inhibitors do not display high selectivity and may restrain related HDACs. The potential side effects due to inhibition of systemic immune response are an urgent problem to be solved. Besides, additional work is required to examine the expression and activity of HDAC1 in allergic diseases. The development of selective HDAC1 inhibitors may lead to new therapeutic agents for allergic diseases, particularly in situations where current therapies are suboptimal.

Author contributions

All authors contributed to the search and collation of literature. YW contributed to the manuscript preparation and the revision of the manuscript. All authors read and approved the final manuscript.

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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.

Publisher's note

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Targeting matrix metalloproteases: A promising strategy for herbal medicines to treat rheumatoid arthritis

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As a type of metalloproteinase, matrix metalloproteinases (MMPs) can be divided into collagenase, gelatinase, stromelysins, membrane-type (MT)-MMPs and heterogeneous subgroups according to their structure and function. MMP contents in the human body are strictly regulated, and their synthesis, activation and inhibition processes should be kept in a certain balance; otherwise, this would result in the occurrence of various diseases. Rheumatoid arthritis (RA) is a known immune-mediated systemic inflammatory disease that is affected by a variety of endogenous and exogenous factors. In RA development, MMPs act as important mediators of inflammation and participate in the degradation of extracellular matrix substrates and digestion of fibrillar collagens, leading to the destruction of joint structures. Interestingly, increasing evidence has suggested that herbal medicines have many advantages in RA due to their multitarget properties. In this paper, literature was obtained through electronic databases, including the Web of Science, PubMed, Google Scholar, Springer, and CNKI (Chinese). After classification and analysis, herbal medicines were found to inhibit the inflammatory process of RA by regulating MMPs and protecting joint structures. However, further preclinical and clinical studies are needed to support this view before these herbal medicines can be developed into drugs with actual application to the disease.

KEYWORDS

matrix metalloproteinases, rheumatoid arthritis, herbal medicines, therapeutic, strategy

1 Introduction

Rheumatoid arthritis (RA) is an immune-mediated systemic inflammatory disease that is affected by a variety of endogenous and exogenous factors, and is characterized by synovial hyperplasia and progressive joint destruction (1). The prevalence of RA in the population is approximately 0.5-1%. The incidence of RA peaks between the ages of 40 and 60 years, and its prevalence is significantly higher in women than in men (2). In existing studies, it is generally believed that RA is a rare and nonfatal disease. However, during development of the disease, joint tissues, including cartilage and bone, experience nonnegligible damage, which can seriously affect the life quality and could even reduce the life expectancy of patients (3, 4). Unfortunately, the pathogenesis of RA has not been fully elucidated up to now, which brings great challenges to the cure of RA (5). Retrospective studies on the pathogenesis of RA have found that genetic and environmental factors may be important inducers of RA (6, 7). Among the current available treatment modalities, nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to suppress inflammation and relieve pain, while glucocorticoids are used to prevent long-term joint erosion (8). In addition, disease-modifying anti-rheumatic drugs (DMARDs), which exert anti-inflammatory and immunomodulatory effects through different pharmacological mechanisms, are often used as mainstay treatments in newly diagnosed RA cases. It is worth noting that since DMARDs have no direct anti-inflammatory or analgesic effects, there are no immediate effects (9). At the same time, biological agents that can selectively inhibit some specific molecules in the immune system have gradually been applied to RA (9). In RA treatments, high doses of drugs are often used to enable drugs to reach diseased joints and exert their curative effects, which are accompanied by toxicity and side effects. For example, NSAIDs and conventional DMARDs have apparent gastrointestinal and hepatorenal toxicity (10). Glucocorticoids can cause adverse reactions including osteoporosis, hypertension, and hyperglycaemia, while biologics may lead to autoimmune syndromes (11). Therefore, the development of new adequate pharmaceutical preparations is of great significance for conquering RA diseases.

To date, researchers have identified approximately 600 proteases in humans, including endopeptidases and exopeptidases (12). They are an indispensable part of life due to their strict regulation of various physiological processes in the human body, including autophagy, protein degradation, cell death, immune response and signal transduction (13, 14). In contrast, when protease activities are out of balance, this will result in many diseases (15). As a type of endopeptidase, metalloproteinases are related to extracellular pathways and can participate in the hydrolysis of the internal peptide bonds of polypeptide chains. Among them, matrix metalloproteinases (MMPs), as the most important metalloproteinases, are involved in the pathogenesis of various diseases, including RA (16). Synovial joint lesions are often accompanied by abnormally elevated MMP levels, suggesting that MMPs are closely related to the development of RA. This conclusion has been continuously confirmed in recent decades (16, 17). Meanwhile, with the deepening of research, we have been able to determine that MMPs are mainly responsible for the irreversible destruction of cartilage, bone and tendons in joints. Moreover, RA can be partially relieved after the use of tissue inhibitors of MMPs (TIMPs) (18). Therefore, MMPs can be considered important therapeutic targets for RA. Furthermore, increasing evidence has suggested that herbal medicines have many advantages in treating RA. They have promising roles in improving RA by participating in multiple pathways such as immune regulation, the inflammatory response, and angiogenesis (19). In this review, we focused on the regulation of MMPs by herbal medicines in RA to contribute to the development of new therapeutic drugs targeting MMPs in RA. Information on regulation of MMPs and treatment of RA by herbal medicines through MMPs was obtained through electronic database searches, including the Web of Science, PubMed, Google Scholar, Springer, and CNKI (Chinese). “Herbal medicine”, “matrix metalloproteinases” and “rheumatoid arthritis” were used for keyword screening, and the searched literature was classified and managed.

2 Classification and structure of MMPs

MMPs are zinc-dependent proteolytic enzymes, that can participate in various physiological and pathological processes, such as extracellular matrix remodeling, cell migration and angiogenesis, and are well-known extracellular modulators (20). MMPs belong to the metzincin clan of metalloendopeptidases along with ADAM (a disintegrin and a metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with a thrombospondin motif), which contain zinc at the catalytic site for the hydrolysis of peptide bonds (21). Figure 1 shows that 23 different MMPs have been found in humans, which can be roughly divided into 5 categories according to their different functions and structures: (1) collagenase (MMP-1, MMP-8 and MMP-13); (2) gelatinase (MMP-2 and MMP-9); (3) stromelysins (MMP-3, MMP-10 and MMP-11); (4) membrane-type (MT)-MMPs, which can be divided into two types: transmembrane-types (MMP-14, MMP-15, MMP-16 and MMP-24, also known as MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP) and glycosylphosphatidylinositol (GPI)-anchored types (MMP-17

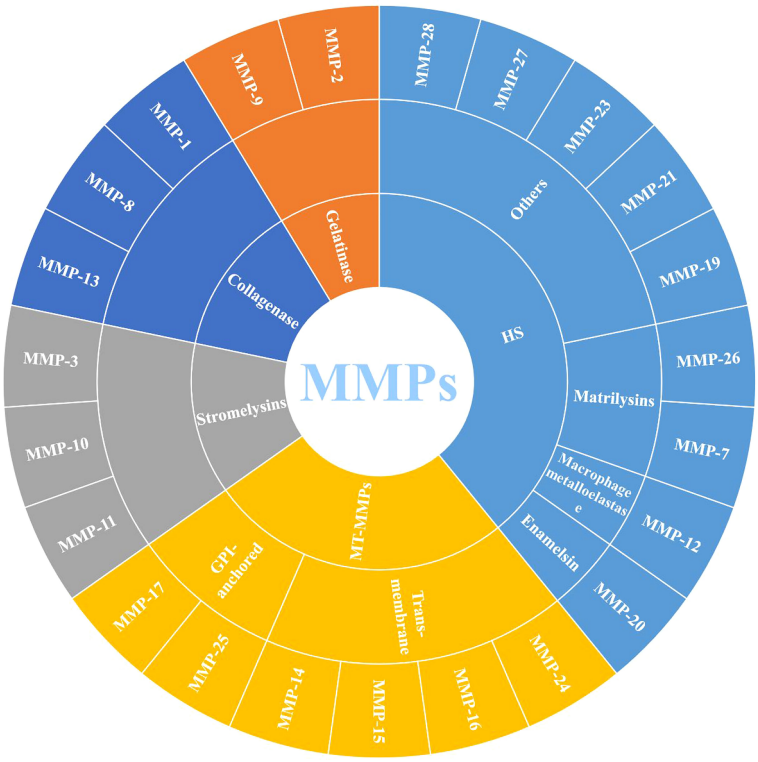


FIGURE 1
Classification of MMPs (MMPs: Matrix metalloproteinases; MT-MMPs: Membrane-type MMPs, and HS: Heterogeneous subgroups).

and MMP-25, also known as MT4-MMP and MT6-MMP) and (5) heterogeneous subgroups (HS), such as matrilysins (MMP-7 and MMP-26), enamelsin (MMP-20), macrophage metalloelastase (MMP-12) and others (MMP-19, MMP-21, MMP-23, MMP-27 and MMP-28) (6, 22).

Structurally, MMPs have similar components, including signal peptide (SP), amino (NH2)-terminal propeptide domains (Pro) and zinc-containing catalytic domains, which are shown in Figure 2. When MMPs migrate to the endoplasmic reticulum, signal peptidases cleaves SP. This basic structure is commonly found in MMP-7 and MMP-26, which also make them the smallest MMPs. In MMP-23, the type II transmembrane domain replaces the SP, making it a type II transmembrane protein. Meanwhile, the cysteine residues in Pro interact with zinc ions to inactivate MMP-23. However, there is also an Arg-X-Lys-Arg motif at the C-terminal of Pro, which can be recognized and cleaved by the pro-protein convertase furin to activate MMP-23. The catalytic domain of MMP-23 is followed by a cysteine array and an immunoglobulin-like domain (6). Except for the above MMPs, other MMPs, such as MMP-1, MMP-3, MMP-8, MMP-10, MMP-12, MMP-13, MMP-19 and MMP-20, also contain the hinge region and hemopexin (Hpx) C-terminal domain. The hinge region connects the Hpx domain to the catalytic domain. Among them, the catalytic substrate diversity is determined by the inclusion of four Hpx-like repeats in the Hpx domain. Meanwhile, the degradation of collagen and gelatin by MMP-2 and MMP-9 is due to the presence of three repeats of the fibronectin type II motif in the catalytic domain. The Pro of MMP-11, MMP-21 and MMP-28 also contain cysteine residues and Arg-X-Lys-Arg motifs. MT-MMPs, on the other hand, connect to the type I transmembrane domain or glycosylphosphatidylinositol (GPI) anchor after the Hgx domain on the basis of the MMP-11 structure (23).

3 Regulation of MMPs

To maintain a balance between anabolism and catabolism of joint tissues, the synthesis, activation and inhibition of MMPs are strictly regulated (24). In existing studies, it has been found that regulation of MMPs is mainly achieved by regulation of transcriptional and posttranscriptional activities.

Genes encoding MMPs are mainly expressed in connective tissue fibroblasts but also in monocytes, macrophages, endothelial cells and neutrophils. In normal tissues, MMP expression are maintained at constant low levels, while under pathological conditions such as RA, MMP expression increase sharply (25). The mechanisms that regulate the transcription of MMPs are

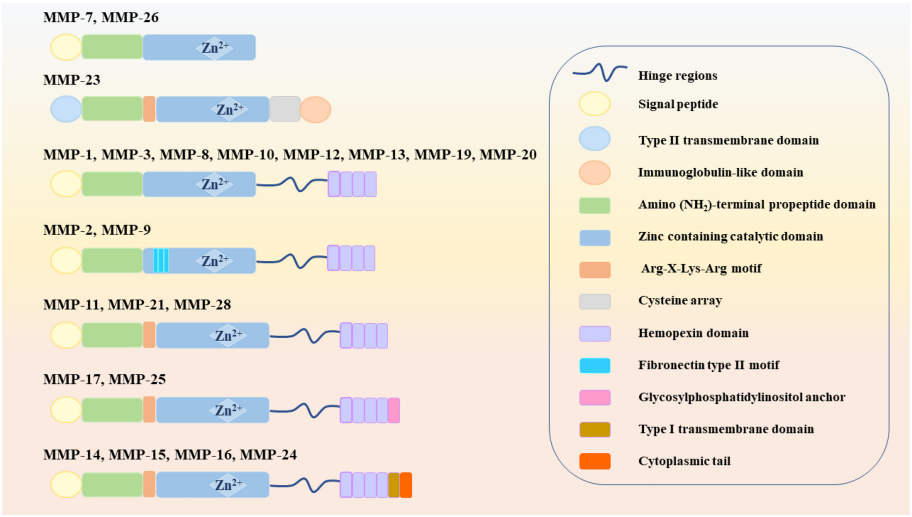


FIGURE 2
Structure of MMPs (MMPs: Matrix metalloproteinases).

extremely complex. Among them, the activator protein 1(AP-1) binding site at 73 bp or 1602 bp is a key regulator of MMP transcription, which can be activated by Jun and Fos family transcription factor proteins to form c-Jun/c-Jun homodimers or c-fos/c-Jun heterodimers, which in turn induce transcription of MMPs (26). Meanwhile, in MMP-1 and MMP-13, AP-1 cooperates with polyomavirus enhancer activator-3 (PEA3) to participate in the transcriptional activation of MMPs (26). Gene transcription of MMPs can also be induced by proinflammatory cytokines (e.g., IL-Iβ and TNF), growth factors (including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF-β)), among which, TGF-β can promote or inhibit transcription of MMPs in different cell types or cell states (27). In addition, many signaling pathways can also regulate the gene expression of specific MMPs through signal transduction pathways. For example, activation of NF-κB leads to transcriptional activation of MMP-1, MMP-3 and MMP-9, while MAPK, JNK and p38 can promote transcription of MMPs by increasing AP-1 levels (28). Notably, in some MMPs, such as MMP-1, MMP-3 and MMP-13, due to the presence of the AUUUA sequence in their 3' untranslated region genes, the posttranscriptional mRNA half-lives are extremely short, thus ensuring that MMPs can be maintained at low levels (29).

When MMPs are synthesized, the cysteine residues located in Pro need to be removed to become active. In the present study, activation of MMPs can be broadly divided into two pathways, namely intracellular activation and extracellular activation. In the intracellular pathway, Arg-X-Lys-Arg motifs can be recognized and activated by furin in the Golgi apparatus, and then transferred to the cell surface to activate other MMPs (30). In contrast, the extracellular pathway mainly occurs on the surfaces of cell membranes or in tissues. Among them, MMP-2 and MMP-13 are mainly activated by activated MT-MMPs on cell membrane surfaces (31). In tissues, a variety of enzymes are involved in activating MMPs, including cathepsin B and plasminogen activator urokinase type (uPA) (32, 33). In addition, some active MMPs in tissues are the activating enzymes of other MMPs. For example, MMP-10 can activate pro-MMP-8, while MMP-13 can activate pro-MMP-9, and other enzyme activation relationships are shown in Figure 3 (34). In addition to the above processes, there are endogenous inhibitors in the body that can block the activity of MMPs. Among them, TIMPs are closely related to joints. To date, researchers have identified four TIMPs, among which TIMP-2 can inhibit the activation process of MMPs and activity of MMPs after activation (35). In addition to its MMP inhibitory activity, TIMP-3 has a wide range of inhibitory effects. For example, TIMP-3 can inhibit the activity of ADAM-17 (also known as TNF-α converting enzyme, TACE) and ADAMTS-4 and -5 (aggrecan enzymes) (36–38). However, the activity of TIMPs remains controversial in some current studies, and these results showed that although TIMPs were able to reduce the effect of MMPs, their active effects were low. When TIMPs are overexpressed, they can also promote cell invasion and apoptosis, thereby promoting the development of RA (39–41). In addition, clinical attempts to use exogenous TIMPs to inhibit MMPs have generally failed (42).

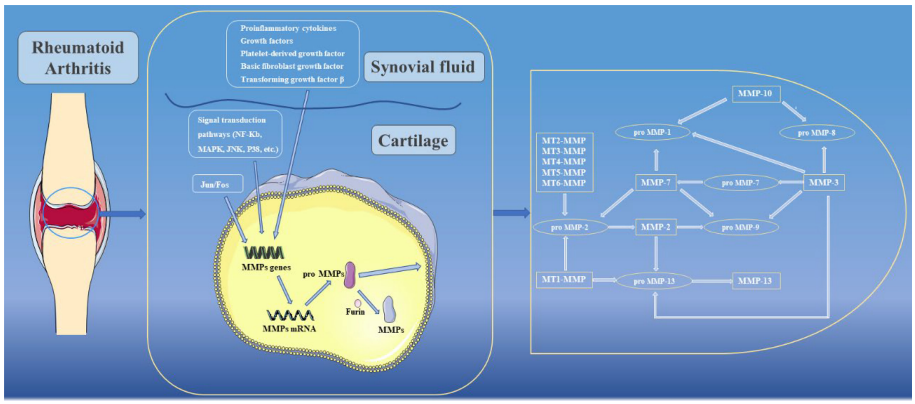


FIGURE 3
Regulation of MMPs and enzyme activation relationships between MMPs.

4 The role of MMPs in RA

4.1. MMPs act as mediators of inflammation

RA is an autoimmune disease that is associated with a chronic inflammatory process that affects multiple joints throughout the body. Although many molecular mechanisms have been used to explain the pathogenesis of RA, the exact etiology of RA is not well understood. It is generally accepted that genetic susceptibility and some stimulating events can induce an initial immune or inflammatory response in joints. Subsequently, inflammatory cells, which consist of neutrophils and macrophages, are recruited into the joints and release large amounts of inflammatory cytokines, such as IL-Iβ, TNF (also known as TNF-α), IL-6, and CXCL8. On the one hand, the released inflammatory cytokines can promote the proliferation of synovial fibroblasts, recruit macrophages and immune cells to form pannus and jointly invade and destroy cartilage, tendon and bone. On the other hand, they can further induce migration of inflammatory cells to the joints and aggravate immune and inflammatory responses in the body (43, 44). In addition, activated inflammatory cells and cytokines can also induce expression and secretion of MMPs, which in turn affect the actions of chemokines and cytokines. However, previous studies have shown that MMPs may promote or inhibit inflammation through different effects. For example, MMP-1, MMP-2, MMP-13 and MMP-14 can inactivate chemokines by cleaving monocyte chemoattractant protein-3 (MCP-3), thereby losing their ability to recruit monocytes and leukocytes (45, 46). At the same time, when MMP-8 expressions were restricted, neutrophil infiltration increased and RA manifestations were aggravated in mice (47). In contrast, MMP-7 can increase infiltration of inflammatory cells and promote the inflammatory response by shedding the ectodomain of syndecan-1 (48). Similarly, overexpression of MMP-12 in macrophages significantly enhanced the inflammatory response in RA, which was accompanied by increased synovial infiltration (49). Therefore, MMPs have dual roles in inflammation, and the balance between proinflammatory and anti-inflammatory signaling needs to be tightly regulated.

4.2. MMPs destroy joint structure

Complete joints consist of articular bones, articular cartilage, fibrous capsules, and synovial membrane. Articular cartilage is attached to the apex of the contact surface of two or more articular bones, and there is no vascular distribution. Synovial membranes can secrete lubricating synovial fluid and provide nutrients to cartilage through microcirculation. During the development of RA, in addition to the inflammatory response, RA is characterized by pathological changes in synovial tissue, cartilage and bone (50, 51). Previous studies have confirmed that activated osteoclasts can change the local environment to an acidic pH while secreting cathepsin K, which can degrade bone in an acidic environment. MMP activities are inhibited in acidic environments and therefore have no apparent effect on the calcified bone matrix (52). However, in RA, degradation of the cartilage matrix is largely attributed to MMPs. In existing studies, the expressions of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13 and MT1-MMP are closely related to RA.

Healthy articular cartilage is mainly composed of collagen and proteoglycan aggregates. Among them, a single aggregate of proteoglycan aggregates consists of approximately 100 core protein units called aggrecans. It is well known that aggrecans possess three globular domains (e.g., G1, G2, and G3) and can

covalently bind to negatively charged glycosaminoglycans (GAGs), such as chondroitin sulphate, keratan sulphate, and dermatan sulphate. Subsequently, single aggregates bind noncovalently to hyaluronic acid consisting of repeated glucuronic acid and N-acetylglucosamine disaccharides, and eventually form proteoglycan aggregates. In proteoglycan aggregates, GAGs can attract water due to their negative charge, providing cartilage protection and reducing the friction coefficients of joint surfaces (53, 54). In RA, ADAMTs are the major enzymes involved in the degradation of proteoglycan aggregates in cartilage, while only some MMPs, such as stromelysin, MMP-1, MMP-2 and MMP-3, can participate in the cleavage of proteoglycan aggregates (55, 56). When MMPs act on proteoglycans, the G1 domain dissociates from the aggregates and diffuses into the synovial fluid, thereby losing its structural function.

As another important component of articular cartilage, collagen is also the most abundant protein in the animal kingdom. In articular cartilage, type II collagen is the most common collagen, while type IX and XI collagen are only a minority. Notably, type II collagen is a unique component of cartilage, consisting of three Alpha1 (type II) chains that support the rigid structure of cartilage (57). In organisms, collagen degradation is almost exclusively mediated by MMPs. First, collagenase (e.g., MMP-1, MMP-8 and MMP-13) can target the collagen triple helix between Gly775 and Leu776 to cleave collagen, producing a 1/4 C-terminus and 3/4 N-terminus (56, 58). Subsequently, these deformed molecules are further degraded by gelatinases (MMP-2 and MMP-9) (59). Although all three collagenases can cleave the triple helix structure of collagen and due to their different locations and substrate preferences, the degradation effects of these three collagenases are also different. Among the three collagenases, MMP-1 and MMP-8 are localized to the superficial surfaces of cartilage. MMP-1 is stably expressed in a variety of cells and has a certain ability to cleave type I, II, III, VII and X collagens, while MMP-8 can cleave type I, II and III collagens. Notably, MMP-8 is mainly stored in neutrophils, and its activity is highest when cleaving type I collagen (60, 61). In addition, MMP-13, as the main force in the cleavage of type II collagen, is mainly distributed in chondrocytes, and its activity in cleavage of type II collagen is 5-10 times that of MMP-1. In some studies, MT1-MMP was shown to directly degrade collagen and indirectly participate in collagen degradation through proMMP-13 activation (53).

MMPs can degrade all components of the extracellular matrix, and most MMPs can degrade a variety of extracellular matrices. Gelatinases degrade proteoglycans in addition to denatured collagen (62, 63). Stromelysins have a wide range of specificity for fibronectin, laminin, and elastin. Stromelysin-1 (MMP-3) not only can damage the specificity of proteoglycans, but also activate proMMP-1 to degrade collagen, so stromelysin-1 plays a dual role in RA (56).

In addition to the above methods, synovial tissue invasion, which is closely related to MMP expression, is another important pathway of cartilage destruction and is closely related to the inflammatory process. In addition to MMP-12, MMP-13 is also thought to be involved in the invasion of RA synovial fibroblasts (RASFs) into cartilage. Direct evidence showed that cartilage erosion was significantly alleviated after the use of a specific MMP-13 inhibitor in vivo (64). Interestingly, selective silencing of MMP-13 in an in vitro model had no apparent effect on RASF invasion (65). Moreover, MT1-MMP not only directly degrades collagen, but also promotes synovial invasion. After MT1-MMP was specifically inhibited or selectively knocked out, the invasion effect of RASF on cartilage was significantly alleviated (66, 67). In addition, MT1-MMP may contribute to cartilage destruction by promoting angiogenesis and endothelial cell migration (68).

5 Herbal medicines can treat RA by regulating MMP

In recent years, the existing drugs have shown certain deficiencies in treating RA, but the incidence of RA is still high. Therefore, it is urgent to find new therapeutic drugs. Herbal medicines are derived from plants that exist in nature. They have a wide range of sources and various types, and many have multitarget effects, making them excellent source of drugs.

5.1 Extracts from herbal medicines

5.1.1 Herbal medicine formulas

Qing-Luo-Yin (QLY) is an herbal medicine formula containing four Chinese herbs, namely *Sophora flavescens* Aiton, *Phellodendron amurense* Rupr., *Sinomenium acutum* (Thunb.) Rehder & E.H.Wils. and *Dioscorea collettii* var. *hypoglauca* (Palib.) S.J.Pei & C.T.Ting. QLY is commonly used to treat RA in China and has achieved remarkable results. As early as 2002, Li et al. first used a collagen induced arthritis (CIA) model to confirm that QLY had significant effects on the arthritis index, pain, and ankle swelling in CIA rats (69). Subsequently, Li et al. continued to use the CIA model for in-depth explorations. The results showed that QLY could reverse imbalances between MMP-3 and TIMP-1 expressions in RA by inhibiting the expression of MMP-3 and promoting TIMP-1 production, thereby inhibiting angiogenesis in the synovium, and finally playing a role in improving the joint morphology of rats (70). More details are shown in Table 1.

Tongbiling (TBL) is a Chinese herbal formula that has anti-arthritic effects and has been used clinically for many years. It consists of *Neolitsea cassia* (L.) Kosterm., *Paeonia lactiflora* Pall., *Aconitum carmichaeli* Debeaux, *Achyranthes bidentata* Blume,

TABLE 1 Herbal medicine formulas that can treat rheumatoid arthritis by regulating MMPs.

Components	Plant source	Experimental model		Effective dose	Effect	Mechanism	Ref
		In vivo	In vitro				
Qing-Luo-Yin	*Sophora flavescens* Aiton, *Phellodendron amurense* Rupr., *Sinomenium acutum* (Thunb.) Rehder & E.H.Wils. and *Dioscorea collettii* var. *hypoglauca* (Palib.) S.J.Pei & C.T.Ting.	CIA mice		0.3 g/kg/d	Suppressing angiogenesis	MMP-3↓, TIMP-1↑	(69, 70)
Tongbiling	*Neolitsea cassia* (L.) Kosterm., *Paeonia lactiflora* Pall., *Aconitum carmichaeli* Debeaux, *Achyranthes bidentata* Blume, *Celastrus orbiculatus* Thunb, *Wisteriopsis reticulata* (Benth.) J.Compton & Schrire	CIA mice		300 mg/kg/d	Decreasing inflammation	IL-1β↓, TNF↓, MMP-2↓, MMP-3↓, MMP-9↓, IgG2a type anti-CII antibody↓	(71)
Alkaloids of *jeevaneeya rasayana*	*Cyperus rotundus* L., *Boerhaavia diffusa* L., *Tribulus terrestris* L., *Curculigo Orchioides* Gaertn., *Mucuna Pruriens* (L.) DC., *Withania somnifera* (L.) Dunal, *Asparagus racemosus* Willd., *Hygrophila auriculata* (Schumach.)	FCA-induced rat		10 mg/kg/day	Decreasing inflammation	PGE_2↓, NO↓, COX-2↓, TNF, IL-6↓, MMP-9↓	(72)
Ruteng	*Boswellia carterii* Birdw, *Tinospora sinensis* (Lour.) Merr., *Cassia obtusifolia* L, *Abelmoschus manihot* (L.) Medic, *Terminalia chebula* Retz., *Lamiophlomis rotata* (Benth.) Kudo, *Pyrethrum tatsienense* (Bur. et Franch.) Ling	CIA mice		95.0, 190.0, 285.0 mg/kg/d	Decreasing inflammation; Decreasing oxidative	MMP-1↓, MMP-3↓, MMP-13↓, TNF↓, COX-2↓, iNOS↓, IL-1β↓, IL-6↓, SOD↑, MDA↓	(73)
Fufang Shatai Heji	*Glycyrrhiza Uralensis* Fisch., *Ophiopogon japonicus* (Thunb.) Ker Gawl., *Astragalus mongholicus* Bunge, *Pseudostellaria heterophylla* (Miq.) Pax, *Adenophora triphylla* (Thunb.) A.DC., *Rehmannia glutinosa* (Gaertn.) DC., *Triticum aestivum* L, *Prunella vulgaris* L. and *Dendrobium nobile* Lindl.	CIA mice		10 mL/kg/d	Alleviating synovial hyperplasia and cartilage destruction	ADAMTS-4↓, ADAMTS-5↓, MMP-9↓, MMP-13↓	(74)

ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; CIA, collagen-induced arthritis; COX, cyclo-oxygenase; FCA, Freund's Complete Adjuvant; IL, Interleukin-1; MMPs, matrix metalloproteinases; PGE, prostaglandin E; TIMP, tissue inhibitors of MMP; TNF, tumor necrosis factor.

Celastrus orbiculatus Thunb and *Wisteriopsis reticulata* (Benth.) J.Compton & Schrire. Shen et al. used CIA mice as a model to validate the therapeutic effects of TBL on RA. It was found that TBL suppressed inflammation and attenuated cartilage and bone destruction, and the underlying mechanisms were corrected by decreasing the amounts of IL-1β and TNF and reducing the expressions of MMP-2, -3, and -9 (71).

Jeevaneeya rasayana (JR) is an anti-arthritic ayurvedic polyherbal formulation that consists of *Cyperus rotundus* L., *Boerhaavia diffusa* L., *Tribulus terrestris* L., *Curculigo Orchioides* Gaertn., *Mucuna Pruriens* (L.) DC., *Withania somnifera* (L.) Dunal, *Asparagus racemosus* Willd. and *Hygrophila auriculata* (Schumach.) Heine. Shyni et al. studied the anti-arthritic effects of the alkaloid fraction of Jeevaneeya Rasayana (AJR) in an adjuvant-induced rat model and found that AJR attenuated paw oedema. Furthermore, the levels of PGE2 and serum NO, activity of COX-2 and mRNA expressions of TNF, IL-6 and MMP-9 were downregulated, suggesting that AJR has pharmacological anti-arthritis activity (72).

Compound Ruteng (CRT) is a Chinese herbal formula that has been used to treat rheumatism for centuries in the Tibetan area of China. It contains seven herbal medicines, including *Boswellia carterii* Birdw, *Tinospora sinensis* (Lour.) Merr., *Cassia obtusifolia* L, *Abelmoschus manihot* (L.) Medic, *Terminalia chebula* Retz., *Lamiophlomis rotata* (Benth.) Kudo, *Pyrethrum tatsienense* (Bur. et Franch.) Ling. To reveal the mechanisms of the anti-arthritis effect of CRT, Huang et al. used network pharmacology and experimental validation. Their results showed that CRT attenuated inflammation in paw swelling, synovial joints and cartilage in collagen-induced arthritic (CIA) rats and downregulated MMP-1, MMP-3, MMP-13, TNF, COX2 and iNOS, suggesting that CRT may exert anti-arthritis effects by inhibiting inflammatory cytokines, suppressing oxidative stress, and balancing MMPs (73).

Another herbal medicine formula, Fufang Shatai Heji (FST), also protects joint structures. It is mainly composed of *Glycyrrhiza Uralensis* Fisch., *Ophiopogon japonicus* (Thunb.) Ker Gawl., *Astragalus mongholicus* Bunge, *Pseudostellaria heterophylla* (Miq.) Pax, *Adenophora triphylla* (Thunb.) A.DC., *Rehmannia glutinosa* (Gaertn.) DC., *Triticum aestivum* L, *Prunella vulgaris* L. and *Dendrobium nobile* Lindl. First, Fan et al. found that FST could protect CIA mice from spleen injury, and they subsequently speculated that FST might also protect against cartilage injury in CIA mice. Their conjecture was verified after a tissue staining analysis of knee and ankle joints. Cartilage destruction was significantly suppressed in CIA mice after FST treatment. After further exploring the underlying mechanism, it was found that FST could inhibit collagen degradation by downregulating the expressions of MMP-9 and MMP-13 and downregulating the expressions of ADAMTS-4

and ADAMTS-5 to inhibit aggrecan degradation, and the two acted together to protect the structure of cartilage (74).

5.1.2 Plant extracts

In addition to herbal medicine formulas, researchers have also explored the effects of single herbal extracts on MMP expression in RA (shown in Table 2). In 2001, Sylvester studied the effect of *Tripterygium wilfordii* Hook. F. (TWHF) extract on TNF/IL-1β/IL-17-induced femoral head primary chondrocytes/confluent primary bovine chondrocytes/human synovial fibroblasts and TNF-induced human chondrocytes, and found that a TWHF extract showed significant anti-inflammation activity and could inhibit cartilage matrix resorption by MMP-3 and MMP-13 by interfering with the DNA binding ability of the AP-1 and NF-kB transcription factors (75).

In the experiment conducted by Shin et al., the researchers screened 12 herbs that may have potential therapeutic effects on RA, including *Chaenomelis speciosa* Nakai, *Achyranthes bibentata* Blume, *Angelica sinensis* Oliv., *Cnidium officinale* Makino, *Gastrodia elata* Blume, *Acanthopanax senticosus* Maxim., *Carthamus tinctorius* L., *Cinnamomum aromaticum* Nees, *Gentiana macrophylla* Pall., *Ledebouriella seseloides* Wolff, *Clematis chinensis* Retz., and *Phlomis umbrosa* Turczaninow. These herbs were extracted in 25% ethanol and then administered to CIA mice. After histopathological observation of the knee joint, it was found that articular cartilage loss was alleviated after drug treatment. At the same time, the content of TIMP-2 in the serum was significantly increased, and the ratios of TIMP-2 to MMP-2 also increased. After the detection of anti-inflammatory factors in serum, the results showed that the IL-4 levels increased after administration, while the IL-10 levels did not change significantly. In conclusion, the above extracts can affect MMP balance in vivo, inhibit inflammation and protect joint structure (76).

Chang et al. used n-butanol to extract *Panax notoginseng* (Burk.) F. H. Chen and named the extract BT-201. In vitro, BT-201 inhibited inflammation by inhibiting the NF-KB and MAPK signaling pathways, resulting in reduced secretion of TNF and IL-1β and a similar reduction in MMP-13 secretion. CIA mice were used to evaluate the efficacy of BT-201 in vivo. The results showed that the onset time of arthritis was delayed after BT-201 treatment, and cartilage destruction, bone erosion and synovial hyperplasia were alleviated, indicating that BT-201 had dual anti-inflammatory and joint structure protection effects in RA (77).

Choi et al. reported that an ethanol extract of *Ligularia fischeri* (Ledeb.) Turcz. (EELFL) decreased the amounts of TNF, IL-6 and MMP-3 in IL-1β–treated SW982 cells, which demonstrated anti-inflammatory effect of EELFL. In addition, the expressions of p-JNK, p-p38, NF-κB and AP-1 were downregulated, which could be the mechanism by which EELFL exerts anti-inflammatory effects (78).

Ra et al. used IL-1β stimulated fibroblast-like synoviocytes (FLSs) as a model to screen 35 medicinal plants in 2011. The results showed that six species, including *Artemisiae Capillaris* Thunb, *Phyllostachys nigra* var. *henonis* (Mitford) Rendle, *Senna tora* (L.) Roxb., *Cornus officinalis* Siebold & Zucc., *Leonurus cardiaca* L. and *Sesamum indicum* L., effectively inhibited the expression of MMP-3, but the pharmacodynamic effects of these six species on RA were not further investigated (79).

Aflapin contains *Boswellia serrata* Roxb. extract enriched in 3-O-acetyl-11-keto-β-boswellic acid and non-volatile oil portion. The experimental results of Sengupta et al. showed that aflapin significantly reduced the paw oedema volume of SD rats induced by Freund's complete adjuvant (FCA) and played an anti-inflammatory role. Subsequently, the model rats were treated with drug-containing serum, and the results showed that aflapin could exert an anti-inflammatory effect by reducing the content of TNF. In IL-1β-treated human primary chondrocytes, different aflapin concentrations increased the glycosaminoglycan contents in a dose-dependent manner. In addition, aflapin also decreased the level of MMP-3 in TNF-induced SW982 human synovial cells. Taken together, these data collectively suggest that aflapin may have multiple effects in RA, both anti-inflammatory and anti-protein degradation (80).

Sekiguchi et al. used hot-water extracts of *Salacia reticulata* Wight (HSR) to explore their potential effects on RA. They obtained a synoviocyte-like cell line from mice with type II collagen antibody-induced arthritis and named it MTS-C H7. In this study, they found that HSL inhibited both IL-1β-induced cell proliferation and MMP-13 expression. At the same time, HESR was isolated and the isolated components were applied to cells, resulting in inhibition of cell proliferation by only the low molecular weight protein. Therefore, researchers hypothesized that the low molecular weight proteins in HSL may have the potential to inhibit cell proliferation and MMP expression (81).

Celastrus orbiculatus Thunb. (CLO) is a Chinese herb that has been widely used in folk medicine to treat inflammation. In the study of Li et al., after applying its extract to FLSs stimulated by IL-1β and TNF, it was found that CLO could inhibit the transcriptional activity of MMP-9 by inhibiting the binding activity of NF-κB in the MMP-9 promoter and the phosphorylation and nuclear translocation of NF-κB, thereby downregulating the expression and activity of MMP-9. In turn, invasion of FLSs was inhibited (82).

The roots of *Angelica sinensis* (Oliv.) Diels (AS) are a widely used herbal medicine with a protective effect on AR. Lee et al. found that the ethyl acetate fraction from Angelica sinensis (EAAS) could suppress the expression of COX-2, MMP-1 and MMP-3, decrease PGE2 production, and inhibit the activation of NF-κB and phosphorylation of MAPK pathways in IL-1β-induced RASFs, suggesting that EAAS treated RA via anti-inflammation effects (83).

Salvia plebeia R. Br. has also been considered in recent years for treating RA due to its excellent anti-inflammatory effects. In a 2015 study, an extract of Salvia plebeia R. Br. (ESP) was administered to CIA mice and TNF-stimulated RA synovial

TABLE 2 Plant extracts that can treat rheumatoid arthritis by regulating MMP.

Components	Plant source	Experimental model		Effective dose	Effect	Mechanism	Ref
		In vivo	In vitro				
Extract of *Tripterygium wilfordii* Hook F	*Tripterygium wilfordii* Hook F		TNF/IL-1β/IL-17-induced femoral head primary chondrocytes/ confluent primary bovine chondrocytes/ human synovial fibroblasts	2.5 and 5 ng/mL	Decreasing inflammation; Blocking cartilage matrix resorption	MMP-3↓, MMP-13↓	(75)
			TNF-induced human chondrocytes	5 ng/mL		AP-1↓, NF-κB↓	
Ethanol extract from 12 herbs (PG201)	*Chaenomelis speciosa* Nakai, *Achyranthes bibentata* Blume, *Angelica sinensis* Oliv., *Cnidium officinale* Makino, *Gastrodia elata* Blume, *Acanthopanax senticosus* Maxim., *Carthamus tinctorius* L., *Cinnamomum aromaticum* Nees, *Gentiana macrophylla* Pall., *Ledebouriella seseloides* Wolff, *Clematis chinensis* Retz., and *Phlomis umbrosa* Turczaninow	Collagen-induced arthritis (CIA) mice		0.2 mg/d	Decreasing inflammation	TNF↓, IL-1β↓, TIMP-2↑, TIMP-2/MMP-2↑, IL-4↑	(76)
N-butanol extract of *Panax notoginseng* (Burk.) F. H. Chen (*P. notoginseng*) (BT-201)	*Panax notoginseng* (Burk.) F. H. Chen	CIA mice		15 mg/kg/d	Decreasing inflammation	TNF↓, IL-1β↓, iNO↓, MMP-13↓, p-IKKb↓, p-ERK↓, p-p38↓, p-JNK↓	(77)
			LPS-induced THP-1 cells	0.125, 0.25, 0.5 mg/mL			
Ethanol extract of *Ligularia fischeri* (Ledeb.) Turcz	*Ligularia fischeri* (Ledeb.) Turcz		IL-1β–induced SW982 cells	10 and 50 μg/ml	Decreasing inflammation	TNF↓, IL-6↓, MMP-3↓, p-JNK↓, p-p38↓, NF-κB↓, AP-1↓	(78)
Water extract of six traditional medicinal plants	*Artemisiae Capillaris* Thunb, *Phyllostachys nigra* var. *henonis* (Mitford) Rendle, *Senna tora* (L.) Roxb., *Cornus officinalis* Siebold & Zucc., *Leonurus cardiaca* L. and *Sesamum indicum* L.		IL-1β-induced FLSs	1, 10 and 100 μg/mL	Decreasing inflammation	MMP-3↓	(79)
Aflapin	*Boswellia serrata* Roxb.	Freund's Complete Adjuvant (FCA)-induced rats		100 mg/kg/d	Decreasing inflammation	TNF↓	(80)
			IL-1β-induced HCH cells	0.25, 0.5 and 1 μg/ml	Improving cell proliferation; Improving glycosaminoglycans production		(80)
			TNF-induced SW982 cells	1 μg/ml	Inhibiting secretion of collagen degrading enzyme	MMP -3↓	(80)
Water extract of *Salacia reticulata* Wight	*Salacia reticulata* Wight		IL-1β-induced MTS-C H7 cell	50 μg/mL	Inhibiting cell proliferation	MMP-3↓, MMP-13↓	(81)
Celastrus orbiculatus Thunb. extract	*Celastrus orbiculatus* Thunb.		IL-1β and TNF combination-	5, 10 and 20 μg/ml	Inhibiting cell invasion	MMP-9↓, p-IκBα↓, NF-κB↓	(82)

(Continued)

TABLE 2 Continued

Components	Plant source	Experimental model		Effective dose	Effect	Mechanism	Ref
		In vivo	In vitro				
			stimulated RA-FLSs				
Ethyl acetate fraction from Angelica sinensis	The root of *Angelica sinensis* (Oliv.) Diels		IL-1β-induced RASFs	100 μg/mL	Inhibiting cell proliferation	MMP-1↓, MMP-3↓, COX-2↓, PGE2↓, p-ERK-1/2↓, p-p38↓, p-JNK↓, NF-κB↓	(83)
Extract of *S. plebeia*	*Salvia plebeia* R. Br.	CIA mice		2, 10, 50 mg/kg	Suppressing the development of CIA; Decreasing inflammation	MMP-1↓, MMP-3↓, IL-1β↓, IL-6↓, NF-κB↓, Akt↓	(84)
Methanol extract of *C. ternatea*	*Clitoria ternatea* Linn. *(C. ternatea)*	CIA mice		50 mg/kg/d	Decreasing inflammation; Decreasing oxidative stress	MPO activity↓, TNF↓, IL-1β↓, IFNγ↓, IL-6↓, IL-12p4↓, CXCL8↓, MCP-1↓, ROS↓, TNFR1↓, TLR2↓, iNOS↓, COX-2↓, MMP-2↓	(85)
quercetin-3ß-D-glucoside				2.5 mg/kg/d			
Ethanol extract of *Gastrodia elata* Blume	*Gastrodia elata* Blume		TNF-induced RA-FLS	1, 5 and 10 μg/ml	Decreasing inflammation	IL-6↓, CXCL8↓, MMP-3↓, MMP-13↓, p-p65↓, IkBα↑	(86)
			TNF-induced RASFs				
Extracts from *Strychnos nux-vomica* L.	*Strychnos nux-vomica* L.		SW982 cells	10 μg/ml	Inhibiting cell proliferation; Inhibiting cell migration	Wnt5a↓, Runx2↓, MMP-3↓, Bmp2↑, p-JNK↓, p- p65↓	(87)
Polyphenolic extract from extra virgin olive oil	Extra virgin olive oil		IL-1β-induced SW982 cell	12.5, 25 and 50 μg/mL	Decreasing inflammation	TNF↓, IL-6↓, COX-2↓, PGE synthase-1↓, MMP-1↓, MMP-3↓, p-JNK↓, p-p38↓, p-ERK↓, IκB-α↑	(88)
Aqueous extract of *Cinnamomi ramulus*	*Cinnamomum cassia* Presl. (Lauraceae)		TNF-induced MH7A cells	0.2, 0.4 and 0.6 mg/mL	Inhibiting cell migration and invasion	MMP-1↓, MMP-2↓, MMP-3↓,	(89)
Extractive of *Stachys inflata* var. *caucasica* Stschegl	*Stachys inflata* var. *caucasica* Stschegl.	44 women (age: 30–65 years) diagnosed with moderately active RA	Triple-blind, randomized controlled	2.4 g/day SSC + 2.4 g/day black tea 8 weeks	Reducing the number of tender, swollen joints and DAS28	HS-CRP↓, IL-1β↓, MMP-3↓	(90)

LPS, lipopolysaccharide; RA-FLSs, rheumatoid arthritis fibroblast-like synoviocytes.

fibroblasts. The results showed that EPS could inhibit the expression of proinflammatory factors and MMP-3 by inhibiting the NF-κB, Akt and MAPKs signaling pathways, thereby delaying the occurrence and development of RA (84).

Adhikary et al. investigated the effects of *Niphogeton ternata* (Willd. ex Schult.) Mathias & Constance (C. ternateaand) and its main active ingredient (quercetin-3ß-D-glucoside, QG) on CIA mice, and the results revealed that C. ternateaand and QG suppressed the release of proinflammatory cytokines, chemokines and reactive oxygen species, while they could also inhibit MMP-2 expression (85).

Gastrodia elata Blume (GE) is a traditional Chinese herbal medicine with anti-inflammatory activity, and according to a study, GE significantly decreased the IL-6, CXCL8, MMP-3 and

MMP-13 levels in TNF-induced rheumatoid arthritis fibroblast-like synoviocytes (RA-FLSs). At the same time, this decrease in inflammatory factors was accompanied by a decrease in p-p65 expression and an increase in IκBα. Therefore, GE might be a potential herbal therapy to treat RA by suppressing the inflammatory response by inhibiting the NF-κB pathway (86).

Strychnos nux-vomica L. (SL) has been used as medicine for thousands of years in China, and it has been gradually extended to treat RA in modern applications. In experiments by Deng et al., it was found that an alkaloid extract from SL without lappaconitine (ASLL) could significantly inhibit the proliferation and migration of SW982 cells. In a qPCR assay, ASLL showed inhibitory effects on Wnt5a, Runx2 and MMP-3 mRNA and increased the expression of Bmp2 mRNA. In addition, ASLL inhibited phosphorylation of JNK and NF-κB p65 and MMP-3 expression. In conclusion, ASLL may inhibit the proliferation and migration of FLSs by inhibiting the Wnt5A-mediated JNK and NF-κB pathways and has a certain therapeutic potential for RA (87).

Another study by Rosillo et al. reported that a polyphenolic extract (PE) from extra virgin olive oil (EVOO) showed an anti-inflammatory effect by inhibiting production of IL-6, CXCL8, MMP-3 and MMP-13, and the mechanisms might be related to suppress the phosphorylation of MAPK and the activation of NF-κB (88).

In a later study by Liu and Zhang et al., it was found that an aqueous extract of *Cinnamomi ramulus* (ACR), which was derived from the dry twigs of *Cinnamomum cassia* Presl. (Lauraceae), may have potential therapeutic effects on RA. In this study, TNF-induced RA-derived FLS MH7A cells were used as the research object. The results showed that ACR could effectively promote apoptosis of MH7A cells by increasing the expressions of BAX and caspase-3 and inhibiting the expression of Bcl-2. On the other hand, it could induce G2/M phase arrest of MH7A cells by upregulating P53, P21, cyclin D and downregulating cyclins B1, CDC2, CDK4. More importantly, ACR can inhibit the expression of MMP-1, MMP-2 and MMP-3 in MH7A cells, and effectively prevent the invasion and migration of synovial fibroblasts, thereby protecting cartilage and bone from injury (89).

In addition, a clinical trial examining the anti-arthritic effects of *Stachys inflata* var. *caucasica* Stschegl. (SS) was conducted. A triple-blind, randomized controlled clinical trial involving 44 female patients diagnosed with RA showed that SSC might decrease the number of tender and swollen joints by decreasing serum levels of IL-1β and MMP-3 (90). Other details are shown in Table 3.

5.2 Monomers from herbal medicines

5.2.1 Terpenoids

Triptolide is a diterpenoid triepoxide that is extracted from the traditional Chinese herbal *Tripterygium wilfordii* Hook. f. (TWHF), which has been reported to have therapeutic effects on RA. Lin et al. initially explored the molecular mechanisms of triptolide in treating RA, and found that triptolide effectively inhibited messenger RNA levels and production of proMMP-1 and -3 while inhibiting PGE2 production by suppressing COX-2 expressions in IL-1α-treated human synovial fibroblasts. In addition, triptolide decreased the levels of IL-1β and IL-6 in LPS-treated mouse macrophages. These results suggested that the anti-RA activity shown by triptolide is due at least in part to anti-inflammatory activity (91). In the experiments of Liacini et al., triptolide was found to inhibit the expressions of MMP-3 and MMP-13 in a variety of cartilage destruction models, such as primary human OA chondrocytes, SW1353 cells, bovine chondrocytes and human synovial fibroblasts stimulated by cytokines and human and bovine cartilage explants stimulated by IL-1. In addition, triptolide also inhibited ADAMTS-4 expressions in bovine chondrocytes induced by IL-1-, IL-17- and TNF. All of these results suggest that triptolide has the potential to protect cartilage (92). Other details are shown in Table 3.

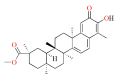
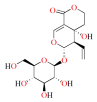
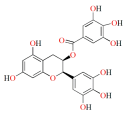
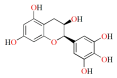
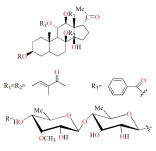
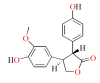
Pristimerin is a natural triterpenoid product that is derived from the family *Celastraceae*. In Tong et al. 's experiment, pristimerin effectively inhibited the inflammatory response in arthritic rats by inhibiting the proinflammatory cytokines IL-6, IL-17, IL-18 and IL-23 and promoting the anti-inflammatory factor IL-10. Subsequently, decreases in IL-6 and IL-17 directly led to a decrease in MMP-9 activity, thus causing pristimerin to have a protective effect on articular cartilage and bone (93). However, whether pristimerin directly affects MMP-9 could not be confirmed in this study.

Swertiamarin, another terpenoid with therapeutic potential for RA, was derived from *Enicostema axillare* (Lam.) A. Raynal (Gentianaceae). When swertiamarin was used to treat arthritic rats, it significantly reduced rat paw thickness, inhibited synovial monocyte infiltration, and protected joint tissues. After treatment, the plasma levels of IL-1, TNF and IL-6 were decreased, which were accompanied by decreased mRNA levels of MMP-9, iNOS, PGE2, PPARγ and COX-2, while IL-10 and IL-4 were increased. Further exploration of the mechanism behind swertiamarin showed that swertiamarin could inhibit RA development by inhibiting the NF-κB/IκB and JAK2/STAT3 signaling pathways (94).

5.2.2 Flavonoids

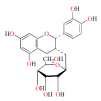
Green tea is a popular beverage worldwide, and it is clear that catechins are the most active ingredients in green tea. Fechtner et al. studied whether epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), and epicatechin (EC), three catechins in green tea, had anti-RA effects. Based on their results, EGCG and EGC suppress IL-6, CXCL8 and MMP-2 production, and selectively decrease the expression of COX-2 in IL-1β–treated RASFs. However, EC did not show the same effects as described above. Thus, GCG and EGC partly contributed to the anti-inflammatory effect of green tea (95).

TABLE 3 Monomers from herbal medicines that can treat rheumatoid arthritis by regulating MMP.

Family of compounds	Components	Plant source	Structure	Experimental model		Effective dose	Effect	Mechanism	Ref
				In vivo	In vitro				
Terpenoids	Triptolide	*Tripterygium wilfordii* hook f,			IL-1α-induced human synovial fibroblasts	28, 56 and 140 nM	Decreasing inflammation	proMMP-1↓, proMMP-3↓, TIMP-1↑, TIMP-2↑, PGE2↓, COX-2↓	(91)
					LPS-induced mouse macrophages	28 nM		IL-1α↓, IL-1β↓, TNF↓, IL-6↓	
					IL-1/IL-17/TNF-induced SW1353 cells	125 and 250 nM	Decreasing inflammation	MMP-3↓, MMP-13↓, ADAMTS-4↓	(92)
	Pristimerin	Celastraceae family		Mycobacterium tuberculosis H37Ra (Mtb)-induced rat		1 mg/kg/d	Decreasing inflammation	IL-6↓, IL-17↓, IL-18↓, IL-23↓, pSTAT3↓, ROR-γt↓, IL-10↑, IFN-γ↑,	(93)
	Swertiamarin	*Enicostema axillare* (Lam.) A. Raynal (Gentianaceae)		FCA-induced rats		2, 5 and 10 mg/kg/d	Inhibiting the levels of paw thickness, lysosomal enzymes; Increasing the body weight; Alleviating bone destruction	IL-1↓, TNF↓, IL-6↓, MMP-9↓, iNOS↓, PGE2↓, PPARγ↓, COX-2↓, IL-10↑, IL-4↑, p65↓, p-IκBα↓, p-JAK2↓, p-STAT3↓	(94)
				LPS-induced RAW264.7 cells	10, 25 and 50 μg/mL		p65↓, p-IκBα↓, p-JAK2↓, p-STAT3↓		
Flavonoids	Epigallocatechin-3-gallate	Tea			IL-1β–induced RASFs	5, 10 and 20 μM	Decreasing inflammation	IL-6↓, CXCL8↓, MMP-2↓, COX-2↓, TAK-1↓, p-p38↓, NF-κB↓	(95)
	Epigallocatechin	Tea			IL-1β–induced RASFs	5, 10 and 20 μM	Decreasing inflammation	IL-6↓, CXCL8↓, MMP-2↓, COX-2↓, TAK-1↓	(95)
Polysaccharides	*Lycium barbarum* L. polysaccharide	*Lycium barbarum* L.		CIA mice		25, 50, 100 mg/kg/d	Reducing paw thickness and CIA score; Attenuating joint damage; Decreasing inflammation	TNF↓, IL-6↓, IL-17↓, PGE2↓, MIP-1↓, anti-type II collagen IgG↓, MMP-1↓, MMP-3↓	(96)
Glycoside	Polyoxypregnane glycoside	*Dregea volubilis* (L.f) Benth. ex Hook. f			IL-1β–induced human articular chondrocyte (HAC)	6.25, 12.5 and 25 μM	Inhibiting cartilage degradation	MMP-1↓, MMP-3↓, MMP-13↓, IKKα/β↓, IκBα↑	(97)
	Cinnamomulactone	The dried twigs of *Cinnamomum cassia* (L.) J. Presl			TNF-stimulated RASFs	0.1 μM		MMP-1↓, MMP-3↓, IL-1β↓	(98)

(Continued)

TABLE 3 Continued

Family of compounds	Components	Plant source	Structure	Experimental model		Effective dose	Effect	Mechanism	Ref
				In vivo	In vitro				
	(-)-Epicatechin-3-O-β-d-allopyranoside	*Davallia formosana* Hayata		CIA mice		50 and 100 mg/kg/d	Suppressing the development of CIA; Decreasing inflammation	MMP-9↓, IL-1β↓, TNF↓, IL-17↓, IL-10↑, IL-4↑, IgG1↓, IgG2a↓, $CD4^+CD25^+$ regulatory T cells↓	(99)

5.2.3 Polysaccharides

After treating CIA mice with *Lycium barbarum* L. polysaccharide (LBP), Liu et al. found that LBP could exert anti-RA effects by downregulating inflammatory mediators and inhibiting joint bone damage through MMPs. Specifically, LBP reversed abnormal increases in inflammatory factors such as TNF-, IL-6 and IL-17 in CIA mice, decreased the protein expressions of MMP-1 and MMP-3, alleviated the ankle swelling in CIA mice, and increased the bone volume (96).

5.2.4 Glycosides

Polyoxypregnane glycoside (PPG), which was extracted from the roots of *Dregea volubilis* (L.f) Benth. ex Hook. f, may have therapeutic potential for RA. Itthiarbha et al. showed that PPG inhibited the expression of MMP-1, MMP-3 and MMP-13 by inhibiting NF-κB activation in IL-1β-treated human articular chondrocytes. In addition, they further examined the mRNA levels of MMP-1, -3 and -13, and concluded that PPG could directly downregulate MMP expression by reducing mRNA levels, thus inhibiting the degradation of type II collagen (97).

In addition, in Kim's experiments, a new butyrolactone compound, cinnamomulactone, was isolated from *Cinnamomum cassia* (Lauraceae) for the first time. In a quantitative real-time PCR (qPCR) assay, cinnamomulactone was found to effectively reduce the gene expressions of MMP-1 and MMP-3 in TNF-stimulated synovial fibroblasts, suggesting a potential therapeutic effect on RA (98).

(−)-Epicatechin-3-O-β-D-allopyranoside (ECAP) is a glycoside isolated from *Davallia formosana*. ECAP significantly inhibited knee cartilage erosion and reduced arthritis scores in CIA model mice. In addition, ECAP also reduced the levels of TNF and IL-17, increased the levels of IL-10 and IL-4, and inhibited IL-1 and MMP-9 expressions in CIA mice (99).

6 Conclusion and perspectives

As mentioned above, although RA is a nonfatal disease, RA seriously threatens the quality of life of patients and may even reduce their life expectancy due to its non-negligible damage to the joints. Previous studies have confirmed that MMPs, as important proteolytic enzymes, are involved in multiple RA processes, in which articular cartilage matrix destruction is particularly important. Therefore, targeted regulation of MMPs has become a research hotspot in the prevention and treatment of RA.

Disappointingly, there are limitations to the existing therapeutic approaches that target MMPs. For example, some endogenous proteins such as $alpha_2$-macroglobulin, which can block the activity of MMPs, are present in the plasma after being secreted by the liver. However, because this is a large tetrameric glycoprotein, it cannot cross blood vessels to enrich in the cartilage, limiting its action to the inflammatory fluid around the joint. At present, the development of MMP inhibitors is mainly focused on inhibiting the active effects of MMPs. Although much research has been devoted to small molecule inhibitors, single-target inhibitors have not yet been developed. Non-selective MMP inhibitors may decrease multiple MMPs, inhibit the low levels of MMPs required for the normal physiological turnover of connective tissue, and thus cause significant side effects in the organism. Therefore, the effects of targeted MMPs in treating RA have not reached expectations.

Due to the unique multitarget effects of herbal medicines, they have gradually attracted the attention of researchers. In recent years, an increasing number of studies have focused on RA treatment that regulate MMPs. In existing studies, researchers have attempted to explore the efficacy and underlying mechanisms of herbal medicine in RA treatment through in vivo and in vitro experiments. To a certain extent, they have found some drugs with therapeutic potential. After a relevant comprehensive analysis, it was found that there are also some problems that cannot be ignored. In this paper, we found that dozens of herbal medicines can exert therapeutic effects on RA. Among these, some studies have confirmed that herbal medicines can directly inhibit collagen or proteoglycan degradation by affecting MMPs, thereby protecting joint structure. Remaining studies have focused more on alleviating the development and progression of RA by suppressing inflammation. In addition,

some studies have shown that herbal medicines can protect joint structure, but this protection is only an indirect result after inhibiting inflammation, rather than directly protecting joint structure through MMPs. In addition, some MMPs, including MMP-3 and MMP-13, have been suggested for use as serum markers for RA diagnosis. Therefore, in these studies, MMPs were used only as biomarkers to compare their changes before and after treatment to determine the effects of herbal medicines. However, in this process, some researchers have ignored the specific roles of MMPs in RA, so they have not further explored the manner in through which MMPs are regulated by herbal medicines in experiments. In addition, although these herbal medicines have been experimentally validated in vitro and in vivo, most of them remain in preclinical research, and only a few herbs have been tested in clinical trials. More work needs to be done before herbal medicines can actually be applied in clinical trials. Therefore, this review only provides a reference for the majority of researchers, so that will have more possibilities for treating RA.

Author contributions

All authors contributed to the article and approved the submitted version. WP, S-QL and C-JW conceived this paper. R-LL, H-XD and QL took part in drafting, revising and critically reviewing the article. YH and QZ gave final approval of the version to be published. WP, S-QL and C-JW have agreed on the journal to which the article has been submitted and agree to be accountable for all aspects of the work.

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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.

Publisher's note

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Glossary

ACR	aqueous extract of Cinnamomi ramulus
ADAM	a disintegrin and a metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin type 1 motif
AP-1	activator protein 1
AS	Root of Angelica sinensis (Oliv.) Diels
ASLL	alkaloid extract from SL without lappaconitine
bFGF	basic fibroblast growth factor
CIA	collagen-induced arthritis
CLO	Celastrus orbiculatus Thunb.
CRT	compound Ruteng
DMARDs	disease-modifying anti-rheumatic drugs;
EAAS	ethyl acetate fraction from Angelica sinensis var. sinensis
EC	epicatechin
ECAP	(−)-epicatechin-3-O-b-D-allopyranoside
EGC	epigallocatechin
EGCG	epigallocatechin-3-gallate
EGF	epidermal growth factor
ESP	extract of Salvia plebeia R. Br.
EVOO	extra virgin olive oil
FCA	Freund's complete adjuvant
FST	Fufang Shatai Heji
GAGs	glycosaminoglycans
GE	Gastrodia elata Blume
GPI	glycosylphosphatidylinositol
Hpx	hemopexin
HS	heterogeneous subgroups
HSR	hot-water extracts of Salacia reticulata Wight
JR	Jeevaneeya rasayana
LBP	Lycium barbarum L. polysaccharide
MCP-3	monocyte chemoattractant protein-3
MMPs	matrix metalloproteinases;
MT	membrane-type
NSAIDs	nonsteroidal anti-inflammatory drugs;
PDGF	platelet-derived growth factor
PE	polyphenolic extract
PEA3	polyomavirus enhancer activator-3
PPG	polyoxypregnane glycoside
QG	quercetin-3ß-D
QLY	Qing-Luo-Yin
qPCR	quantitative real-time PCR
RA	rheumatoid arthritis
RASF	RA synovial fibroblasts
SL	Strychnos spuxvomica L.
SP	signal peptide
SS	Stachys inflata var. caucasica Stschegl
TACE	TNF-a converting enzyme
TBL	Tongbiling
TGF-b	transforming growth factor b
TIMPs	tissue inhibitors of MMPs
TWHF	Tripterygium wilfordii Hook. F.

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Immunomodulatory role of metalloproteinase ADAM17 in tumor development

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ADAM17 is a member of the a disintegrin and metalloproteinase (ADAM) family of transmembrane proteases involved in the shedding of some cell membrane proteins and regulating various signaling pathways. More than 90 substrates are regulated by ADAM17, some of which are closely relevant to tumor formation and development. Besides, ADAM17 is also responsible for immune regulation and its substrate-mediated signal transduction. Recently, ADAM17 has been considered as a major target for the treatment of tumors and yet its immunomodulatory roles and mechanisms remain unclear. In this paper, we summarized the recent understanding of structure and several regulatory roles of ADAM17. Importantly, we highlighted the immunomodulatory roles of ADAM17 in tumor development, as well as small molecule inhibitors and monoclonal antibodies targeting ADAM17.

KEYWORDS

ADAM17, tumor microenvironment, shedding activity, immune response, inflammation

Introduction

Transmembrane proteolysis is a post-translational modification that plays an important role in cellular biological processes, such as signal transduction and immune responses (1–3). Many transmembrane proteins need to be cleaved from the cell surface and released in a soluble form to initiate cellular or intercellular signal transduction (4–6). ADAM17, also known as tumor necrosis factor (TNF)-α converting enzyme (TACE), CD156B, NISBD1, and snake venom-like protease (cSVP), is a member of the disintegrin and metalloprotease family. ADAM17 exists in two forms: precursor and activated ADAM17. Activation of ADAM17 is required for the cleavage of its prodomain and exposure of the active site. In response to the inflammatory stimuli,

activated ADAM17 prompts multiple receptor-mediated signal transduction by cleaving ectodomains of membrane proteins, including inflammatory cytokines, growth factors, receptors, and adhesion factors (7). The expression of ADAM17 in mouse articular cartilage is positively correlated with the development of arthritis, and its deletion attenuates articular cartilage degeneration (8). Moreover, ADAM17 is associated with glomerular inflammation and fibrosis (9). In diabetic mice, ADAM17 deletion in the proximal tubules improves glucose tolerance, prevents podocyte loss, and inhibits the accumulation of glomerular macrophages and collagen (9). More importantly, ADAM17 is contributory to the occurrence and development of cancers, including lung carcinoma (10), ovarian carcinoma (11), breast carcinoma (12–14), gastric carcinoma (15), and cervical carcinoma (16). Interestingly, it regulates some immune signaling pathways through the shedding activity, which may facilitate the inflammatory response in tumor development (17–19). However, the study of the relationship between the abnormal expression of this metalloproteinase in tumors and its immune regulation is still not well studied. Herein, we summarized and updated multiple regulatory roles of ADAM17 as well as the development of ADAM17 inhibitors with a focus on the immunomodulatory role of ADAM17 in tumor development, which may provide reasonable insights for the prevention and treatment of cancer diseases.

Characterization of ADAM17

ADAM17 is a widely distributed transmembrane protein that is involved in different physiological processes such as inflammation, cell proliferation and apoptosis by its hydrolysis of various precursor membrane proteins, such as TNF-α, TNFRII, HB-EGF, IL-1R1, etc. It is localized in the membranes and cytoplasm of normal and tumor tissues and expressed in human lung, bronchus, nasopharynx, placenta, and lymphoid tissues (20, 21). In lung or respiratory tissues, activation of ADAM17 may contribute to the shedding of the collectrin-like part of ACE2, leading to the formation of soluble ACE2 (sACE2) (22, 23) and the development of inflammatory response (24). Furthermore, in distinct cells from the lung, ADAM17 expression is relatively high in pneumocytes and endothelial cells (20), suggesting that ADAM17 may be participating in the cleavage and shedding of key proteins in lung tissues. Activation of ADAM17 promotes the release of soluble fms-like tyrosine kinase 1 (sFlt1) in the placenta and induces preeclampsia (25). ADAM17 also induces T-cell activation in lymphoid tissues through the promotion of L-selectin hydrolysis and shedding (26). ADAM17 is lowly expressed in NK cells and its activation by IL-15 obstructs the proliferation of NK cells (19). Among multiple immune cells, ADAM17 is relatively high expressed in granulocytes and monocytes (20). ADAM17 mediates IL-6R shedding from neutrophils and induces apoptosis (27), which may be associated with a pro-inflammatory response mediated by the sIL-6R/IL-6 trans-signaling pathway (28).

Structure of ADAM17

ADAM17 is a member of the adamalysins subfamily of metzincin metalloproteinases consisting of 824 amino acids with zinc-dependent catalytic activities (29). The human ADAM17 protein sequence contains an N-terminal signal sequence (SS), a prodomain (PD), a catalytic metalloprotease domain (MD), a disintegrin domain (DD), a membrane-proximal protein domain (MPD), a conserved ADAM17 interaction sequence (CANDIS), a transmembrane domain (TM), and a C-terminal cytoplasmic domain (CD), which are located at amino acid residues 1-17, 18-216, 217-474, 480-559, 581-642, 643-666, 672-694, and 695-824, respectively (7, 30) (Figure 1A). Among them, the first five protein sequences that make up its extracellular domain may be involved in regulating multiple biological functions, including angiogenesis, cell migration, cell proliferation, inflammation, and immune responses. SS transfers the newly synthesized ADAM17 protein (110 kDa) to endoplasmic reticulum and Golgi apparatus (32). The PD obstructs the catalytic activity of metalloproteinases based on the cysteine-switch mechanism (33) (Figure 1B). During activation, furin, PC7 and PC5B pro-protein convertases are able to remove the prodomain of ADAM17 and induce production of the matured protein (80 kDa) (34). The cysteine-switch mechanism is not essential for the maintenance of inactivated ADAM17, which may be due to the presence of subdomains in the amino-terminal region of the prodomain (35). The MD serves as the main catalytic region of ADAM17 that contains a zinc-dependent HexGH-XXGXXHD motif (36). Amino acid residues His^{405}, His^{409} and His^{415} located in this motif bind to zinc ions and determine the activity of the ADAM17 enzyme (31). The curved "Met turn" structure consisting of amino acid residues Tyr^{433}, Val^{434}, Met^{435}, Tyr^{436}, also known as 1,4-β-turn, is prone to ADAM17 cleavage and its mutations (37, 38). The DD can impair multiple functions of integrins, thereby affecting cell-cell/extracellular matrix interactions (20). In contrast to other members of ADAMs family, ADAM17 shows disulfide bonds in the MD, but its DD lacks typical calcium binding sites (39, 40). ADAM17 MPD plays crucial roles in substrate recognition and protein shedding. Due to the dimerization of ADAM17 and its substrate specificity, cysteine-rich and epidermal growth factor (EGF)-like domains are considered important components of MPD (41, 42). The hypervariable region of the former contributes to substrate recognition and shedding of extra-substrate domains and the latter affects the protein regulation of ADAM17 activity (43, 44). However, the existence of EGF-like domain remains controversial (45). In

addition, the positively charged motif (Arg^{625}-Lys^{628}) in MPD binds to phosphatidylserine in the outer membrane, affects the conformation of ADAM17, and induces its activation (46). TM and CD mainly regulate the response of exocytodomain signaling molecule-related events (7, 38, 47), which may be attributed to the functional assembly of the Src SH3-binding motif (20). The CANDIS domain lies between MPD and TM, consisting of amino acid residues 643-666 (48), which binds to the type I transmembrane protein IL-6R but not the type II transmembrane protein TNF-α (49). As shown in Figure 1C, the visualized crystal structure of the catalytic domain of ADAM17 has five α-helices and five highly distorted β-sheet structures. The N-terminus binds to β1 and β3 sites, and the C-terminus binds to the α5 sites (31, 50). ADAM17 has shallower hydrophobic S1' and very deep hydrophobic S3' pockets linked by water channels, which facilitate the binding of the hydroxamic acid-based inhibitor TAPI-1 (also called an ADAM17 inhibitor) to the isobutyl side chain S1' pocket and its other long chain to the S3' pocket (31, 45). The structure and function of MD and MPD catalyzed by ADAM17 have been studied extensively, but the crystal structure and exact function of the remaining domains are still unclear.

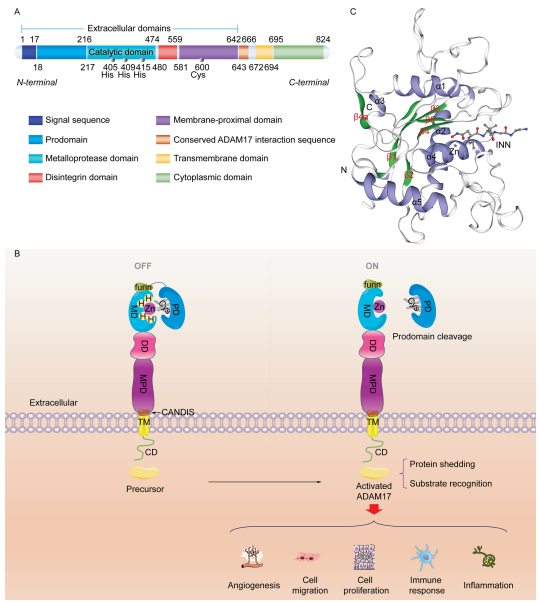

FIGURE 1
Molecular structure of the ADAM17 protein. **(A)** Sequence and structure of ADAM17. ADAM17 protein mainly comprises five extracellular domains, a transmembrane domain, and a cytoplasmic domain. **(B)** The classic cysteine-switch mechanism. The conserved cysteine switch is located in the prodomain. It coordinates with Zn^{2+} at the catalytic site of the metalloproteinase domain to produce an inactivated enzyme (ADAM17 precursor). Once its prodomain is cleaved, the adjacent furin site (RVKR sequence) is responsible for catalyzing the separation of zinc from cysteine, ultimately leading to ADAM17 activation. **(C)** The 3D catalytic structure of ADAM17 (PDB CODE: 1BKC) (31) with a hydroxamic acid-based inhibitor INN and Zn^{2+} shows N-terminal domains, α-helix (blue), β-sheet (green), and C-terminal domains. INN stands for N-{(2R)-2-[2-(hydroxyamino)-2-oxoethyl]-4-methylpentanoyl}-3-methyl-L-valyl-N-(2-aminoethyl)-L-alaninamide with the chemical structure of C19H37N5O5. INN, also known as TAPI-2, is an analogue of TAPI-1. This 3D image was made with the SWISS-MODEL Expasy.

Regulatory roles of ADAM17

ADAM17 regulates post-translational modification

Post-translational modification of precursor proteins includes proteolysis, phosphorylation, glycosylation, methylation and acetylation (51). It can regulate the hydrolysis and cleavage of proteins, affect their activities, localization and interaction with other cellular molecules. As an irreversible post-translational modification, proteolysis/cleavage of transmembrane proteins is responsible for activating multiple cytokine-mediated signal transduction pathways. ADAM17 was first identified as the TNF-α converting enzyme, and its transmembrane proteolysis is related to inflammation (52) and immune regulation (26). TNF consists of TNF-α and TNF-β, to be secreted by macrophages and/or T lymphocytes (53, 54). TNF-α interacts with its receptors TNFR1 and TNFR2. TNFR1 is widely expressed in various human cells and is involved in cell survival and cellular damage (55, 56). The death domain of TNFR1 is occupied by the silencer of death domains (SODD) which blocks the binding of TRADD to TNFR1 and suppresses the TNFR1 signaling pathway (57). The binding of TNF-α and TNFR1 enables the shedding of SODD from the death domain of TNFR1 and leads to the formation of the TNFR1-TRADD-RIP1-TRAF2 complex, thus promoting cell survival (57). In addition, TNFR1 is also internalized by the clathrin protein, which subsequently triggers the assembly of intracellular death-inducing signaling complex and activation of caspase8, leading to apoptosis or necrosis (57, 58). TNFR2 is mainly distributed in immune cells and plays a role in regulating the function of the immune system (59). Numerous studies have shown that furin endopeptidase close to the prodomain can remove the NH2-terminus of ADAM17 by proteolysis/protein cleavage (60), thereby activating it and inducing shedding of pro-TNF-α, TNFR1, and TNFR2 and subsequent pro-inflammatory response. Besides, ADAM17 triggers the hydrolysis and release of more than 90 substrate proteins. These have been further discussed in "ADAM17 Mediates Substrate Shedding Activity" section. Phosphorylation of ADAM17's cytoplasmic tail is another post-translational modification. ADAM17 is often hyperphosphorylated in patients with emphysema (61). As shown in Figure 2, ADAM17 can be phosphorylated by various protein kinases, such as PKC (3), PKL2 (3), PTK2 (18), MAPKs (3, 62), Akt/GSK (63), and Smad2/3 (64). Recent studies have

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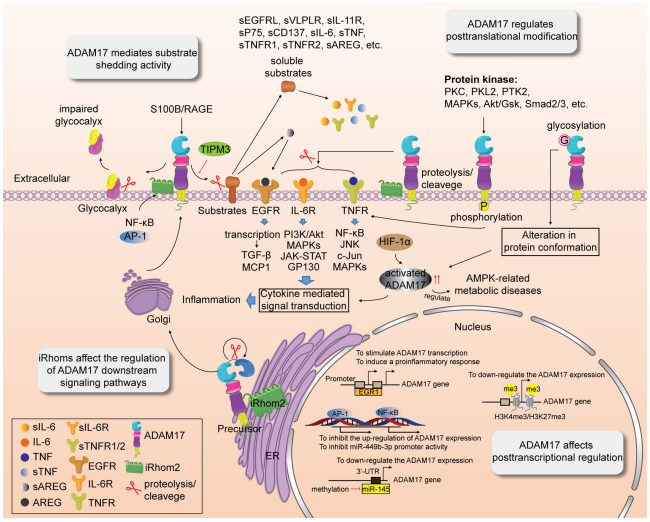

FIGURE 2
Multiple regulatory roles of ADAM17. ADAM17 activity is affected by transcriptional regulation, and post-transcriptional and post-translational modification. ADAM17 activity is also associated with substrate shedding. iRhoms affect the shedding of ADAM17 and regulation of its downstream signaling pathways.

shown that the extracellular domain of ADAM17 with a homodimer structure can bind tightly to selective ADAM17 inhibitors, while serine 819 (Ser^{819}) and threonine 735 (Thr^{735}) in the cytoplasmic tail release selective ADAM17 inhibitors, which activate ADAM17 by inhibiting its phosphorylation-induced dimerization (65, 66). In addition, ADAM17 phosphorylation further promotes the shedding of TNF-α and two TNF receptors (32). Short term pro-TNF-α shedding by ADAM17 substrate does not depend on rapid phosphorylation of pro-TNF-α or the cytoplasmic tail of ADAM17 and it is mainly regulated by serine/threonine kinases (67). It has been reported that serine phosphorylation of ADAM17 substrate NRG1-ICD can restrain its cleavage of these post-translational modified substrates to some extent (68). Glycosylation of ADAM17 plays an important role in regulating enzyme activity or binding to substrates (69). ADAM17 glycosylation is significantly different between mammalian and insect cells (69). The glycosylation of ADAM17 cannot be detected in CRIB-1 cells (70). ADAM17-mediated TNF-α shedding is associated with O-glycosylation in the extracellular proximal membrane region (71). O-glycosylation at Ser41, however, prevented ADAM17-dependent cleavage of β1-AR (72). Glycosylation not only alters protein folding and conformation and affects ADAM17 activity, but also regulates receptor-mediated signal transduction (73, 74) and facilitates drug interventions targeting non-zinc-binding exosome sites of ADAM17 (69). Chen et al. found that zidovudine-based treatment inhibited the glycosylation of ADAM17 and the lysis of monocyte CD163 (75), indicating the important role of glycosylation in ADAM17 activity and disease progression.

ADAM17 affects post-transcriptional regulation

In addition to post-translational modifications, ADAM17 also affects post-transcriptional regulation. ADAM17 is highly expressed or upregulated in cancer (76, 77) and other inflammation-related diseases, including kidney disease (78), sepsis (79), cicatrization (80), diabetic retinopathy (81), myocardial fibrosis (82), aortic dissection (83), arthritis (84) and atherosclerosis (7). The guanine-cytosine (G-C) sequences in the promoter region of ADAM17 are capable of binding specifically to many transcription factors (85–87). The gain- or loss-of-function of ADAM17 is attributed to the regulation of the following transcription factors, such as NF-κB (77, 88, 89), AP-1 (77, 88), SP1 (85), HIF-1α (82, 83), C/EBP-β (76), EGR1 (79), Sim1 (90), RUNX2 (91). For instance, inflammatory induction of inactive rhomboid protein 2 (iRhom2) stimulated by TNF and IFN-γ drives the activation and upregulation of ADAM17 expression and subsequent shedding of cell-surface molecules (77, 88, 89), which is blocked by NF-κB and AP-1 (77, 88, 89). However, ADAM17 can negatively regulate miR-449b-3p expression and its promoter activity *via* activating NF-κB transcription. MiR-449b-3p is a downstream target of ADAM17 and has a binding site of NF-κB in its promoter (77). He et al. found that EGR1 is bound to the ADAM17-172A>G (rs12692386) promoter region with affinity, leading to upregulation of ADAM17 promoter activity and transcription (79). However, the loss of EGR1 function prevents ADAM17 expression and induces a pro-inflammatory response. HIF-1α is

an upstream target of ADAM17, and the transcriptional activation of HIF-1α promotes the upregulation of ADAM17 expression (82, 83). The latter regulates AMPK metabolism-related diseases through the adrenergic receptor (ADRA1A) (82). In addition, miR-145 downregulates ADAM17 expression by binding to the 3'-UTR of ADAM17, which leads to activation of the ADAM17-EGFR-Akt-C/EBP-β feedback loop and induction of tumor invasion (76). Epigenetic regulation of histone post-transcriptional modifications also plays a pivotal role in the post-transcriptional regulation of ADAM17. Recruitment/deletion of histone H3K4me3/H3K27me3 at the ADAM17 gene promoter downregulates ADAM17 expression (92), suggesting that dynamic chromatin modifications at this site lead to inflammatory responses.

ADAM17 mediates substrate shedding activity

Due to the shedding activity, ectodomains of many transmembrane proteins are hydrolyzed and released by ADAMs metalloproteinases. Studies over the past five years revealed that ADAM17 has more than 90 substrates (7, 32) with distinct functions (Table 1), which are involved in various cellular processes, including cell adhesion, migration, development, inflammation, immune response, tumorigenesis, signal transduction. The cleavage and release of substrates (inflammatory cytokines, growth factors, receptors, adhesion molecules, and others) for ADAM17 may result in different functions of substrate proteins. Some substrate proteins, such as glycocalyx (104), TNFR (173, 178), and JAM-A/FIIR (156), are shed by ADAM17 in the form of active molecules. Glycocalyx is a polysaccharide protein complex that covers the aperture membrane surface of vascular endothelial cells and regulates the homeostasis of the cytoplasmic membrane through proteoglycan-glycoprotein attachment to endothelial cells. Recent studies have shown that activation of S100B/RAGE signaling by traumatic brain injury contributes significantly to ADAM17-mediated endothelial calyx shedding, which aggravates blood-brain barrier dysfunction and increased vascular permeability (104). The sheddase activity of ADAM17 drives scramblase-dependent phosphatidylserine (PS) exposure to the membrane surface, allowing the substrate to be cleaved and shed at the membrane surface (178, 179). The inability of ADAM17 to interact directly with PS may be due to the ability of the ortho-phosphorylserine form of PS to competitively inhibit the shedding of ADAM17 substrates (179). ANO6 facilitates the regulation of phosphatidylserine on the plasma membrane due to its scramblase activity. Veit M et al. found that downregulation of ANO6 expression by RNA interference significantly reduced the cleavage and release of TNFR1 by ADAM17 in HUVECs (178) and that free TNFR1 promotes TNF-induced cell necrosis (173). ADAM17-mediated JAM-A/FIIR shedding is responsible for aging-related abnormal endothelial remodeling (156). However, other substrates, like EGFR ligands (17, 97, 180), E-cadherin (124), VLDLR (4), IL-11R (5), CD137 (94), P75 (11), GPIBα (6), HPP1 (119), and NRG1 (10) are precursor proteins or fusion proteins that can yield active components or soluble active receptors only after cleavage and release by ADAM17 (Figure 2). Evidence suggests that ADAM17 promotes tumor-associated macrophage polarization and angiotensin II-mediated pro-growth and pro-migration signals by shedding EGFR ligands, including heparin-binding EGF-like growth factor (HB-EGF) and AREG (members of the EGF family), from the cell membrane (17, 32). E-cadherin is a key substrate for ADAM17, which is conducive to epigenetic regulation, endocytosis and efflux of cells by cleaving and shedding E-cadherin. Once ADAM17 binds to CD82, ADAM17 metalloproteinase activity is inhibited, leading to a reduction in E-cadherin cleavage products (124). IL-11 is a member of the IL-6 family that binds to IL-11R and forms a complex with CP130 to mediate anti-inflammatory signal transduction. On the other hand, IL-11R is hydrolyzed to soluble IL-11R (sIL-11R) *via* ADAM17 overexpression, mediating IL-11 trans-signaling pathway (5), which confers pro-inflammatory cytokine activity. Similarly, the bidirectional regulation of CD137/CD137L-mediated cellular responses has been implicated in the development of tumors and autoimmunity. The shedding protease ADAM17 triggers the production of soluble CD137 (sCD137), a spliceosome of CD137, which subsequently enhances T cell proliferation, whereas inhibition of ADAM17 activity intercepts the sCD137 production (94). VLDLR, an apolipoprotein receptor, plays an important role in foam cell formation, plasma triglyceride metabolism and inflammation. Its soluble ectodomain-mediated anti-inflammatory effect is related to the activation of the Wnt signaling pathway. ADAM17 induces the release of soluble VLDLR (sVLDLR), which inhibits the Wnt pathway and leads to macular degeneration in eye tissue, whereas the shedding of sVLDLR is blocked by selective ADAM17 inhibitors (4). Carrido et al. revealed that tumor formation mechanisms were probably caused by ADAM17-mediated cleavage of the P75 ectodomain (11). In addition, the increased ectodomain cleavage of other ADAM17 substrates (GPIBα and HPP1) may be required for immune platelet clearance and tumor suppression (119, 181). However, in another study related to oncogenic KRAS, KRAS mutations triggered enhanced ADAM17-mediated NPG1 shedding of the SLC3A2-NPG1 fusion protein, which in turn promoted tumor cell growth (10). Collectively, the pro-inflammatory and anti-inflammatory effects induced by ADAM17 substrate shedding may be related to distinct regulatory effects and functions of the substrates.

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TABLE 1 Updated ADAM17 substrates (7, 32).

Cytokines	Growth factors	Receptors	Adhesion molecules	Others
CSF1 (93)	AREG (17)	4-1BB/CD137 (94)	ALCAM/CD166 (95, 96)	EGFRL (97)
CX3CL1 (98)	Epigen (99)	ACE2 (100, 101)	CD44 (102, 103)	Glycocalyx (104)
FLT-3L (105)	Epiregulin (106)	APP (107)	CD62L/L-selectin (108)	Klotho (109)
INFγ (110)	HB-EGF (111)	CA IX (112)	Collagen XVII (113–115)	Pref1 (116, 117)
Jagged1 (118)	HPP1/TMEFF2/Tomoegulin2 (119)	CD163 (75)	Desmoglein2 (95, 120)	SEMA4D (121)
Kit-ligand l and 2 (122)	NRG1 (10)	CD30 (123)	E-cadherin (124)	VASN/Vasorin (125)
LAG-3 (126)	TGFα (127)	CD40 (128, 129)	EpCAM (130)	
MICA (131)		CD89/FcαR (132)	ICAM1 (133)	
MICB (134)		c-MET (38, 135)	L1-CAM (136)	
RANKL (7, 137)		EMMPRIN/CD147 (138)	NCAM (139)	
TNFα (140)		EPCR (141)	Nectin4 (142)	
TNFβ (143)		ErbB4 (96)	PTP-LAR (144)	
		GHR (145)	VCAM1 (146)	
		GPIbα (6)		
		GPV (147)		
		GPVI (148)		
		IGFR1 (138)		
		IGF2R (149)		
		IL-11R (5)		
		IL-1RII (150, 151)		
		IL-6R (152, 153)		
		Integrin β1 (154, 155)		
		JAM-A/FIIR (156)		
		KIM1 (157)		
		LeptinR (158)		
		LOX1 (159, 160)		
		LRP1 (46, 161)		
		MEGF10 (162)		
		MerTK (163)		
		Notch1 (164)		
		NPR (32, 165)		
		p55TNFαR1 (140)		
		P75 (11)		
		p75 TNFR (127)		
		Ptprz (166)		
		PTPRA/PTPα (167)		
		sVLDLR (4)		
		Syndecan-1 and -4 (168)		
		TGFβR1 (169)		
		TIL4 (170)		
		TIM-3 (171, 172)		
		TNFR1 (173)		
		Trop2 (174, 175)		
		VEGFR2 (176)		
		VPS10P (177)		

ADAM17 participates in the regulation of its downstream signaling pathways

ADAM17 regulates signal transduction in many pathophysiological processes, including inflammation, immunity and tumor. The upregulation of ADAM17 expression leads to increased EGFR ligand release and polarization of the EGFR signaling, which is responsible for cell proliferation, invasion, and migration (182, 183). However, downregulation of the ADAM17 expression urges the opposite effect by suppressing the EGFR/ERK, EGFR/Akt/C/EBP-β or EGFR/ErbB signaling pathways (76, 184). ADAM17-mediated EGFR signaling increases the levels of TGF-β and accumulates extracellular matrix (185), implying the role of TGF-β in the regulation of multiple immune cells under pro-inflammatory conditions. Emerging evidence suggest that blocking ADAM17 expression effectively alleviates inflammatory responses, which may be relevant to the regulation of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (186, 187). However, the loss of ADAM17 function with gene mutations triggers the development of inflammatory diseases (48, 188). Based on the aforementioned discussion, we suggest that ADAM17's critical role in various signaling pathways ensures its activity is strictly regulated.

iRhoms, lacking the catalytic motif GxS, are members of the rhomboid protein family with important biological functions (189). Recently, iRhoms have been identified as key regulators of ADAM17 activation. In different tissues, iRhoms appear to form proteolytic complexes with ADAM17 sheddase, but not other ADAMs (190), thus mediating ADAM17 cell membrane surface transport. iRhoms contribute to the activation of ADAM17-dependent shedding events and substrate recognition, while deletion of iRhoms hinders ADAM17 activation, suggesting that iRhoms are required for ADAM17 maturation (190). iRhoms contain two inactive homologs, iRhom1 and iRhom2, also known as RHBDF1 and RHBDF2, respectively. iRhom1 is barely expressed in inflammatory/immune cells and yet iRhom2 is highly expressed in these cells and is responsible for ADAM17 activation (89). iRhom2 deficiency inhibits ADAM17-dependent substrate release, including bidirectional regulators and TNFs (191, 192). In iRhom2-mutated macrophages, ADAM17 remains in endoplasmic reticulum (ER), and cannot be activated by lysis of its prodomain (193). The cytoplasmic domain of iRhom2 participates in the regulation of ADAM17-dependent shedding events (189). Shed ADAM17 triggers phosphorylation of the N-terminus of iRhom2 and promotes the separation of ADAM17 from the iRhom2/ADAM17 complex by recruiting 14-3-3 protein (194). Despite the loss of protease activity, iRhom1 and iRhom2 maintain critical non-protease activities in regulating EGF and TNF-α signaling pathways (41, 195). Upregulated expression of iRhom1 in ER may enhance proteasome activity *via* the PAC1/2 pathway rather than *via* EGF signaling. Mice with iRhom2 deficiency had severe immunodeficiency and could neither produce the main inflammatory cytokine, TNF, nor could they respond to lipopolysaccharide-induced inflammation and immune responses. Therefore, iRhoms play an integral role in ADAM17-mediated downstream signal regulation. Hence, targeting iRhoms and ADAM17 may provide new strategies for anti-inflammatory treatment.

Immune regulation of ADAM17 in cancers

Abnormal expression of ADAM17 in cancers

Due to the shedding activity, ADAM17 is closely related to the formation and development of distinct cancer types, including lung cancer, ovarian cancer, breast cancer, stomach cancer, colorectal cancer, bladder cancer, melanoma, cervical cancer, pancreatic cancer, etc.

ADAM17 in lung cancer

Lung cancer has the highest incidence and mortality rate in the world. ADAM17 is usually an oncogene and its upregulation is associated with the progression of lung cancer. In LUAD, KRAS mutation contributes to the phosphorylation of ADAM17 threonine *via* p38 MAPK, thereby driving ADAM17 to selectively promote its substrate IL-6R shedding and subsequent ERK1/2 MAPK-IL-6-mediated trans-signal transduction, leading to malignant progression of the cancer (152). Enhanced ADAM17 activity mediated by KRAS mutation also facilitates the shedding of S-N (SLC3A2-NRG1) fusion protein NRG1 and the release of soluble NRG1 (sNRG1), which contributes to the increase in ERBB2-ERBB3 heterocomplex receptors and the activation of the downstream PI3K-AKT-mTOR pathway, leading to the growth of lung cancer cells (10). In addition, iRhom2, as a key binding protein for ADAM17, further promotes KRAS-induced tumor cell growth by modulating the release of ERBB ligands (196). However, the efficacy of radiotherapy for non-small cell lung cancer was enhanced when blockade of ADAM17 function with the neutralizing antibody (197). These findings suggest that ADAM17 is a cancer-promoting gene and a potential target for anti-lung cancer therapies.

ADAM17 in ovarian cancer

Fabbi et al. found that ADAM17 is significantly upregulated in ovarian cancer, and its high-expression is associated with poor clinical prognosis in ovarian cancer patients (198). High levels of ADAM17 in serum and ascites fluid of patients with ovarian cancer may be used as a hematologic tumor marker for the detection of ovarian cancer (199). ADAM17 promotes the

malignant progression of ovarian cancer and causes chemo-resistance by mediating ADAM17-dependent shedding of AREG, HB-EGF, IL-6Rα, TNF, TNFR1-α, TGFα and activating the EGFR signaling pathway (198, 200). Deletion of ADAM17 or treatment with selective ADAM17 inhibitor GW280264X is capable of declining substrate cleavage/release and promoting chemo-sensitization (198, 201). ADAM17-induced P75 cleavage may also be responsible for ovarian cancer-promoting activities (11).

ADAM17 in breast cancer

ADAM17 functions as one of the highly expressed genes in breast cancer that plays an important role in the development of breast cancer. ADAM17 promotes cleavage of PD-L1 on the surface of breast cancer cells (12), regulates the interaction between PD-L1 and PD-1 (12), and may contribute to immune escape of triple-negative breast cancer cells (12, 202). ADAM17 can mediate the release of sTNFR1 and sTNFR2, which inhibit the secretion of metastasis-promoting chemokines (CXCL8, CCL5, CXCL) and induce anti-metastasis effects in triple-negative breast cancer cells (203). An earlier study indicated that breast cancer-associated fibroblasts stimulated breast cancer cell proliferation through ADAM17-mediated cleavage of TGF-α (204). Interestingly, ADAM17 is also present in platelets and is involved in tumor immune escape. It was found that downregulation of ADAM17 in activated platelets from breast cancer patients was associated with tumor metastasis and clinical stage of breast cancer (14). D8P1C1, an anti-ADAM17 monoclonal antibody, remarkably inhibited tumor growth in triple-negative breast cancer mouse models (205). Similar results were reported in another published paper (13). In summary, the critical role of ADAM17 in breast cancer makes it a potential target for breast cancer therapy.

ADAM17 in gastric cancer

ADAM17 is probably associated with aggressive metastasis and poor prognosis of gastric cancer. A meta-analysis associated with gastric cancer indicated that ADAM17 might be a tumor marker for poor prognosis in gastric cancer, and high expression of ADAM17 is associated with lymph node metastasis and clinical staging of lymph node metastasis in gastric cancer (15). ADAM17 promotes gastric cancer cell metastasis by activating the Notch-Wnt signaling pathway (206). Epithelial-mesenchymal transition (EMT) is a transformation of cell morphology that occurs in the development of tumors, including gastric cancer. It was reported that ADAM17 promotes EMT in gastric cancer cells (33, 64). The mechanism of ADAM17 in gastric cancer may be through TGF-β/p-Smad2/3-mediated EMT activation (207, 208).

ADAM17 in other cancers

ADAM17 is also highly expressed in cervical cancer, liver cancer, colorectal cancer and bladder cancer. ADAM17-modified bone marrow mesenchymal stem cells may stimulate the malignant growth of drug-resistant cervical cancer cells by activating the EGFR/PI3K/Akt pathway (16). ADAM17 is thought to cleave the Notch receptor and inactivate Notch signaling, thereby impeding the GPR50/ADAM17/Notch axis-mediated development of liver cancer (85). ADAM17 can interact with cellular integrin α5β1 to promote the binding and uptake of exosomes derived from colorectal cancer (209). Newly formed exosomes are associated with the malignant phenotype of tumors. In addition, ADAM17 also promotes STAT3 activation by induction of EGFR/IL-6 transduction signaling pathways, which ultimately lead to tumor progression; inhibition of the ADAM17/IL-6/STAT3 signaling axis significantly attenuated the growth of colon cancer cells (210). The ADAM17/EGFR/AKT/GSK3β axis plays a key role in regulating melanoma cell proliferation, migration, and cell sensitivity to chemotherapeutic drugs (211). ADAM17 is also involved in immune-related autocrine and paracrine regulation (40). However, knockdown of ADAM17 or treatment with anti-ADAM17 antibody MEDI3622 resulted in regression of pancreatic tumors, accompanied by down-regulated EGFR/STAT3 signaling, increased cytotoxic T cells, and decreased granulocyte-like medullary inhibitory cells in mouse models of pancreatic cancer (212).

Regulation of macrophages by ADAM17

Tumor microenvironment (TME) refers to a complex environment closely related to tumorigenesis, tumor growth and its metastasis, which is composed of a variety of cells (including macrophages, fibroblasts, lymphocytes, endothelial cells, etc.), extracellular matrix, and multiple signaling molecules (cytokines, growth factors, chemokines, hormones, etc.) (213). Autocrine and paracrine are conducive to the activation of multiple signaling pathways in tumor cells and non-tumor cells (e.g., macrophages, lymphocytes, endothelial cells) (214–216). In this way, the dynamic interaction between tumor cells and their surrounding matrix triggers tumor cell proliferation, immune evasion, distal metastasis, and drug resistance, and angiogenesis as well (217, 218).

Tumor-associated macrophage (TAM) is derived from mature monocyte in peripheral blood. Monocytes are recruited to TME through chemokines and cytokines secreted by tumor cells and become TAMs. TAMs are the most abundant immune cells in the TME and are closely relevant to tumor growth, invasion and metastasis (219). For one thing, macrophages serves as an important component of tumor stromal cells that are able to gather around blood vessels and promote angiogenesis, tumor invasion and metastasis (220, 221). For another thing, it also has the ability to remove tumor cells and reshape the TME (222). Due to the influence of cytokines in the TME, TAMs can be divided into two distinct polarized forms,

M1 and M2 macrophages. The former is responsible for killing tumor cells; the latter is able to promote tumor growth (223). Macrophage M1/M2 polarization is adjustable and reversible. Increased M2-polarized TAMs are often associated with cytokines and growth factors, e.g., IL-4 (224), IL-10 (225), CSF-1 (226), TGF-β (227) secreted by tumor cells or Th2 cells in the TME, indicating a poor prognosis for tumor patients.

Metalloproteinase ADAM17 can shed distinct signaling proteins on the cell surface, making it a mediator for intercellular signal transduction (7, 20). Our previous study showed that the expression of ADAM17 was associated with infiltration of multiple immune cells, including macrophages (20), in TCGA pan-cancer samples and yet the specific regulatory mechanism of ADAM17 is unknown. Recently, Gnosa et al. have revealed the positive roles of ADAM17 in regulating the polarization of TAMs (17). By using bioinformatics analysis based on the TCGA dataset and immunohistochemical analysis from triple-negative breast cancer cohort, the authors first confirmed that highly expressed ADAM17 in tumors is positively correlated with tumorigenic macrophage markers CD163 or CD206. Deletion of ADAM17 gene inhibited tumor growth, increased the survival in tumor-bearing mouse models, and resulted in a significant decrease in CD163+ cell population. In a co-cultured mouse bone marrow-derived macrophages with ADAM17-WT or ADAM17-KO tumor cells, knockdown of ADAM17 significantly diminished the expression of CD163 or CD206, IL-6, IL-10, and CCR7 in bone marrow-derived polarized macrophages, suggesting an important role of ADAM17 in tumorigenic macrophages. Furthermore, the authors used cellular co-culture and zebrafish embryo propagation models to demonstrate that tumor cells, in an ADAM17-dependent manner, drive macrophage polarization into a tumor-promoting phenotype and accelerate tumor cell invasion. Based on the sheddase activity of ADAM17 (38), this macrophage polarization is regulated by ADAM17-mediated shedding of EGFR ligands (HB-EGF, AREG). Actually, the mechanism of macrophage polarization driven by tumor cells has been reported in many previous works. For instance, the EGFR/PI3K/AKT/mTOR axis plays an important role in promoting TAM M2 polarization by secreting EGF from colon cancer cells (228). Pancreatic cancer triggers the polarization of TAM M2 by secreting REG4 through the EGFR/AKT/CREB pathway (229). These findings further indicate that EGFR ligand shedding mediated by ADAM17 may be beneficial to activating the EGFR signaling pathway and inducing the polarization of tumor-promoting TAMs. Finally, they further demonstrated the promoting effect of macrophage-derived CXCL1 secretion on tumor cell invasion by RNA-seq analysis of transcriptome data from co-cultured macrophages. Taken together, these findings suggest a critical role of the ADAM17-EGFR (HB-EGF/AREG) axis in the polarization of TAMs (Figure 3), which also provides a new strategy for the anti-tumor immunotherapy.

Regulation of NK cells by ADAM17

Natural killer (NK) cells are important lymphocytes to fight against tumor escape or immune evasion. A large number of studies have shown that the activity and function of NK cells in peripheral blood of some cancer patients are significantly reduced (230), which may be conducive to the development of

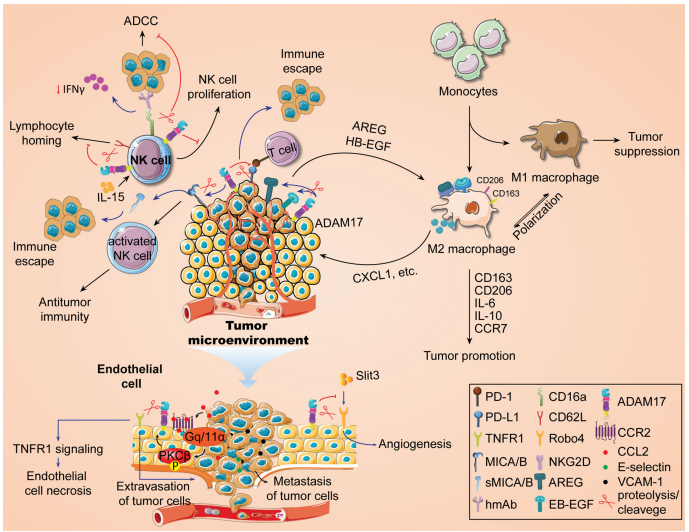

FIGURE 3
Immunomodulatory role of ADAM17 in tumor development.

malignant tumors. In non-small cell lung cancer, the function of NK cells has been shown to be significantly impaired. Therefore, immunotherapy targeting NK cells has become a therapeutic concept for this type of cancer (231). One of the reasons for the anti-tumor immune activity of NK cells is attributed to the binding of its surface-activated receptor natural killer cell group 2D (NKG2D) to MHC class I chain-related protein A/B (MICA/B), an NKG2D ligand on the surface of tumor cells, thus activating NK cell function and enables NK cells to kill tumor cells (232). Studies have shown that inhibition of the ADAM9 activity significantly blocked MICA shedding and affected the immune killing effect of NK cells on tumor cells (233). ADAM17 is also a member of the metalloproteinase family that may have a similar function. Recently it was found that ADAM17 has the ability to hydrolyze MICA/B on the surface of tumor cells to generate soluble MICA/B (sMICA/B) (52), the latter of which alters the conformation of NKG2D on the surface of NK cells (234) and affects the recognition and binding of membranous MICA with NKG2D, thereby inhibiting NK activation signals and reducing the killing sensitivity of NK cells to tumor cells (235). Knockdown of ADAM17 prohibits MICA shedding and boosts MICA expression on the surface of hepatocellular carcinoma cells (131). In addition, hypoxia-induced shedding of MICA on the surface of pancreatic cancer cells enables tumor cells to evade NK cell immune killing (235). The function of MICA/B monoclonal antibodies is to inhibit MICA/B shedding by binding antibodies at key epitopes in the MICA/M proximal membrane domain, and its antitumor immunity activity is associated with NKG2D and CD16 Fc receptor activation (236). Inhibition of MICA/B shedding with monoclonal antibodies drives NK cell-mediated antitumor immunity (237), suggesting that the sMICA levels may be correlated with decreased NK cell function. Therefore, blocking ADAM17-mediated hydrolytic activity to inhibit MICA shedding may be one of the ways to improve NK cell killing of tumor cells (Figure 3).

The antitumor immune activity of NK cells is also related to the antibody-dependent cell-mediated cytotoxicity (ADCC) induced by CD16 Fc receptor (Figure 3). ADCC is a key cytolytic mechanism of NK cells. NK cells, on the one hand, interact with the Fc region of antibodies that recognize proteins on the surface of tumor cells through their IgG Fc receptors to target tumor antigens and produce cytotoxic effects. On the other hand, it also mediates adaptive immune responses. In human beings, IgG's FcR family consists of six receptors, including FcγRI (CD64), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc (CD32c), FcγRIIIa (CD16a), and FcγRIIIb (CD16b), of which CD16a is primarily responsible for triggering NK cell-mediated ADCC. Therefore, exploring the mechanism of CD16a contributes to the development of anti-tumor immunotherapy drugs that enhance ADCC activity. The metalloprotease ADAM17 has been reported to shed CD16a (238), leading to decreased ADCC activity and reduced IFN-γ production (239). However, Blocking CD16 shedding or avoiding cleavage prompted a stronger tumor cell killing by NK cells (240, 241) and increased IFN-γ production (242). Paradoxically, treatment with an ADAM17 inhibitor did not increase IFN-γ levels induced by stimulated NK cells (242). CD16a is a hot topic discussed in recent NK cell anti-tumor immunity, and more information about the role of ADAM17 in the regulation of CD16a in NK cells can be seen in some recent studies (238, 243).

In addition, IL-15, an immunomodulatory factor, also plays a key role in the development, homeostasis, activation and proliferation of NK cells (244). IL-15 can differentiate hematopoietic progenitor cells into CD56+ NK cells to induce pro-proliferative responses. In NOG-IL-15 Tg mice expressing transgenic human IL-15, there is a significant increase in transplanted NK cells from healthy subjects' peripheral blood (19, 245). In different tumor-bearing animal models, IL-15 treatment contributes to tumor regression, reduction of tumor metastasis, and improvement of animal survival. Currently, the developed IL-15 mutant (IL-15N72D) or its stable soluble complex, ALT-803, has been shown to have similar functions as IL-15 and significantly improved the antitumor activity of anti-CD20 monoclonal antibody in NK cells and the immunotherapeutic efficacy of PD-1/PD/L1 monoclonal antibody (232). ADAM17 is present in various immune cells, including NK cells (20), which mediates lysis and shedding of cell surface receptors. CD62L/L-selectin is an immune cell homing receptor that regulates the migration of white blood cells to sites of inflammation. It was found that CD62L expression is increased in IL-15-stimulated NK cells (19). Expression of ADAM17 on NK cells promotes the downregulation of CD62L expression (242). Mishra et al. first indicated that ADAM17 reduced IL-15-stimulated NK cell proliferation with the participation of CD62L (19). The blockade of ADAM17 reversed this event. Overall, IL-15-mediated NK cell proliferation promotes an increase in CD62L levels, while prolonged activation of ADAM17 leads to CD62L shedding and impaired NK cell proliferation stimulated by IL-15 (Figure 3).

Regulation of endothelial cells by ADAM17

Metastasis is a form of tumor progression. 90% of tumor-related deaths are caused by metastasis of tumor cells. The process includes: 1) the shedding of tumor cells from the primary tumor; 2) intravasation; 3) survival in the blood circulation; 4) extravasation of blood vessels and metastases. The interaction between endothelial cells and tumor cells is an important step in tumor metastasis. Tumor cell-endothelial cell tight contacts promote tumor cell adhesion to the vascular wall through justacrine or paracrine signaling (246). As shown in

Figure 3, endothelial cells secrete a series of adherent molecules, such as E-selectin, VCAM-1, etc., to increase the adhesion of tumor cells with endothelial cells, and further promote tumor metastasis. The mechanism of tumor metastasis may be related to EMT, angiogenesis, tumor stem cell characteristics, and the increase of circulating tumor cells. ADAM17 is widely present in endothelial cells and is positively correlated with immune infiltration levels of endothelial cells in multiple cancer species (20, 88, 173). It is speculated that endothelial ADAM17 may help tumor metastasis. Recent emerging evidence supports this speculation (173). Julia et al. also confirmed that endothelial ADAM17 is required for endothelial necrosis, tumor cell extravasation and metastasis (247). ADAM17-dependent death receptor TNFR1 ectodomain shedding promotes endothelial cell necrosis and tumor cell extravasation (173, 247). In addition, CCL2 secreted by tumor cells and macrophages promotes PKCβ activation by binding to endothelial CCR2, which further leads to ADAM17 activation (247). ADAM17 appears to be closely associated with pathological angiogenesis (138). In ADAM17flox/flox/Tie2-Cre mice, loss of endothelial ADAM17 inhibits chord formation and impedes ectopic injected tumor growth (138). In endothelial cells, soluble Robo4 (sRobo4) is shed and released by ADAM1, which subsequently inhibits SLIT3-induced angiogenesis (248). Meanwhile, SLIT3 obstructs Robo4 shedding and enhances its signal transduction (248). ADAM17 may disrupt the barrier effect of vascular endothelial cells by affecting their attachment and tight junctions (249). Beyond vascular endothelial cells, ADAM17 is also important in lymphatic endothelial cell-induced tumor migration and metastasis. Sun et al. indicated that ADAM17 activation by MAPK14/T180 promoted the secretion of soluble CX3CL1, which further led to malignant metastasis of liver cancer cells (18). In addition, Macrophage M2 polarization is also associated with ADAM17-dependent CX3CL1 secretion (18). As a critical binding protein for ADAM17, iRhom1 has been found to promote independent regulation of ADAM17 under physiological shear stress (88). However, there is no report yet on the regulation of ADAM17 by iRhom1 in endothelial cells and its effect on tumor malignant progression, which may be an interesting topic.

ADAM17 inhibitors

ADAM17 has over 90 substrates, some of which are mediators of cancer diseases, which implies that substrate based ADAM17 inhibitors have the potential to be used for the treatment of malignant tumors. In this section, we outline recent advances in potent and selective ADAM17 inhibitors containing hydroxamate and non-hydroxamate moieties, as well as anti-ADAM17 monoclonal antibodies (Figure 4, Table 2).

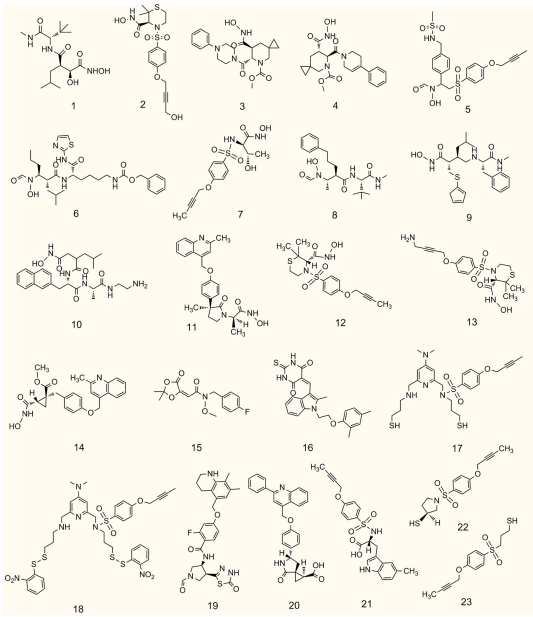

FIGURE 4
Chemical structures of representative small molecule ADAM17 inhibitors.

Hydroxamate-based small-molecule inhibitors

The metalloproteinase domain of ADAM17 has a catalytic site containing a sequence of zinc-dependent amino acid residues that can bind to zinc ions to interfere with ADAM17 enzyme activity. The hydroxamate moiety is a common zinc-binding motif, and hydroxamate-based small-molecule inhibitors targeting the catalytic site may be an effective strategy against tumors. Marimastat and apratastst are the earliest synthesized hydroxamate-based inhibitors with limited selectivity. Marimastat inhibits the cleavage of TNF-α and CD44 and reduces the invasion of tumor cells with an IC_{50} of 4.75 μM (102). Shu et al. found that apratastst significantly inhibited TNF-α cleavage with IC_{50} of 81.7 ng/mL *ex vivo* and 144 ng/mL *in vitro*, respectively (250). INCB7839 is not ideal as a single agent, but it enhances the efficacy of trastuzumab in metastatic HER2-positive breast cancer. INCB7839 suppresses ADAM10/17-dependent EGFR ligand shedding and potentiates the antitumor effects of the recombinant peptidase PEPDG278D (251). Since January 2009, INCB7839 has been used in Phase I/II clinical trials alone or in combination with rituximab/trastuzumab + vinorelbine/trastuzumab + docetaxel for the treatment of diffuse large B cells non-hodgkin lymphoma gliomas, breast cancer or solid tumors (Table 3). In a subset of subjects, INCB7839 at a dose of 300 mg b.i.d. (Phase II) in combination with rituximab resulted in a range of serious side

TABLE 2 Summary of the inhibitory activities of ADAM17 inhibitors.

Compound Number[a]	Chemical Name or Product Name	IC_{50} Value[b]	Reference
Hydroxamate-based small-molecule compounds:			
1	Marimastat	4.75 μM	(102)
2	Apratastat	144 ng/mL (*in vitro*); 81.7ng/mL (*ex vivo*)	(250)
3	Aderbasib/INCB7839	*N.D.*	(251)
4	INCB3619	14 nM	(252)
5	KP-457	10.6 nM	(253)
6	GW280264X	*N.D.*	(4, 201, 254)
7	PF-5480090/TMI-002	~1696.5 RFu/mg	(255)
8	GI254023X	541 μM	(256)
9	Batimastat	*N.D.*	(257)
10	TAPT-1	8.09 μM	(107)
11	(2R)-N-hydroxy-2-[(3S)-3-methyl-3-{4-[(2-methylquinolin-4-yl)methoxy]phenyl}-2-oxopyrrolidin-1-yl]propanamide	*N.D.*	(20)
12	(3S)-4-{[4-(but-2-ynyloxy)phenyl]sulfonyl}-N-hydroxy-2,2-dimethylthiomorpholine-3-carboxamide	*N.D.*	(20)
13	(3S)-4-{[4-(but-2-ynyloxy)phenyl]sulfonyl}-N-hydroxy-2,2-dimethylthiomorpholine-3-carboxamide	*N.D.*	(20)
14	Methyl (1R,2S)-2-(hydroxycarbamoyl)-1-{4-[(2-methylquinolin-4-yl)methoxy]benzyl} cyclopropanecarboxylate	*N.D.*	(20)
15	BMS-561392	0.20 nM	(258)
Non-hydroxamate-based small-molecule compounds:			
16	ZLDI-8	6.85 μM	(259)
17	SN-4	3.22 μM	(102)
18	SN-4$(Nps)_2$	*N.D.*	(102)
19	JTP-96193	5.4 nM	(258)
20	(1S,3R,6S)-4-oxo-6-{4-[(2-phenylquinolin-4-yl)methoxy]phenyl}-5-azaspiro[2.4]heptane-1-carboxylic acid	*N.D.*	(20)
21	N-{[4-(but-2-yn-1-yloxy)phenyl]sulfonyl}-5-methyl-D-tryptophan	*N.D.*	(20)
22	(3S)-1-{[4-(but-2-yn-1-yloxy)phenyl]sulfonyl}pyrrolidine-3-thiol	*N.D.*	(20)
23	3-{[4-(but-2-yn-1-yloxy)phenyl]sulfonyl}propane-1-thiol	*N.D.*	(20)
Anti-ADAM17 monoclonal antibodies:			
	A300E	~0.7 μg/mL	(260)
	A9(B8)	0.22 nM (human); 0.25 nM (mouse)	(261)
	D1(A12)	4.7 nM	(262)
	MEDI3622	39 pmol/L (human); 132 pmol/L (mouse)	(263)

[a]See Figure 4; [b]N.D. refers to not detected.

effects, including thromboembolism, pain, and infections (NCT02141451). However, other anticancer clinical trials associated with INCB7839 were terminated for some reason or were not conducted or not yet reported (NCT01254136; NCT00864175; NCT0429575; NCT00820560). INCB3619, an early hydroxamate-based inhibitor with the IC_{50} value of 14 nmol/L, significantly inhibits tumor cell survival by blocking the shedding of ErbB ligands (252). INCB3619 also enhances the sensitivity of gefitinib (264), cisplatin (252), and lapatinib (265), and acts synergistically with CD16 × 33 bispecific killer cell conjugates against acute myelogenous leukemia (266). KP457 increases the production of platelets derived from functional human induced pluripotent stem cells by inhibiting the exodomain shedding of platelet glycoprotein Iba (GPIba), with an IC_{50} value of 10.6 nmol/L for KP457 (253). GW280264X facilitates the anti-ovarian cancer effect of cisplatin (201) and restrains the development of lung adenocarcinoma cells (254). The IC_{50} value of PF-5480090/TMI-002 in MDA-MB-468 cells is approximately 1696.6 RFU/mg, which reduces the release of TGF-α and increases the cytotoxic effects of anti-EGFR/HER drugs (255). GI254023X is a selective inhibitor of ADAM10 and ADAM17, but its selectivity for ADAM10 is 100 times higher

TABLE 3 Currently approved clinical trials using ADAM17 inhibitors for tumor treatment.

Diseases	ADAM17 inhibitors	Phase for trial	Trial ID	First Posted date	Recruitment Status	Last Update Posted
Diffuse Large B Cell Non-Hodgkin Lymphoma	INCB7839 + Rituximab	Phase I/II	NCT02141451	May 19, 2014	Completed	Feb 19, 2020
Gliomas	INCB7839	Phase I	NCT04295759	Mar 4, 2020	Active, not recruiting	Aug 16, 2022
Breast Cancer	INCB007839 + Trastuzumab and Vinorelbine	Phase I/II	NCT01254136	Dec 6, 2010	Terminated	Jan 25, 2012
Breast Cancer	INCB007839 + trastuzumab and docetaxel	Phase I/II	NCT00864175	Mar 18, 2009	Terminated	Jan 18, 2018
Solid Tumors and Hematologic Malignancy	INCB007839	Phase I	NCT00820560	Jan 12, 2009	Completed	Jan17, 2018

(Source: the U.S. National Library of Medicine, https://clinicaltrials.gov/).

than that of ADAM17, with IC_{50} values of 5.3 μM and 541 μM for ADAM10 and ADAM17, respectively (256). The hydroxamate derivative batimastat inhibits ADAM17 shedding (267) and has prevented the progression of multiple tumors in clinical trials, particularly the formation of peritoneal carcinomas (268). TAPI-1 with IC_{50} value of 8.09 μM is capable of inhibiting matrix metalloproteinase and blocking the shedding of cytokine receptors (107). Recent studies have shown that TAPI-1 appreciably restrains ADAM17 activation during pseudomonas aeruginosa infection (269). Additionally, we previously retrieved four novel hydroxamate-based small molecule compounds 11-14 targeting ADAM17 from the DrugBank database, but no *in vitro* and *in vivo* experimental data were reported (20). BMS-561392 reduced ADAM17 activity with an IC_{50} of 0.2 nM. Overall, most hydroxamate-based inhibitors exhibit potent ADAM17 shedding activity and resist tumor progression. Compounds with the hydroxamate group, however, are usually poorly bioavailable and produce toxic hydroxylamine through metabolism, which somewhat limits the clinical use of these compounds (270).

Non-hydroxamate-based small-molecule inhibitors

To avoid side effects and toxicity caused by the hydroxamate group and to improve bioavailability, research on new ADAM17 inhibitors has been directed toward non-hydroxamate-based small-molecule compounds (46). By searching the literature published in the last five years, we have selected the following four new compounds for description. With computerized virtual screening, Lu et al. identified a non-hydroxamate-based inhibitor, called thioxodihydro pyrimidindione ZLDI-8, which reversed taxol resistance, displayed an IC_{50} value equal to 6.85 μM against ADAM17 (259), and inhibited metastasis of hepatocellular carcinoma (271). It also enhanced the antitumor effects of sorafenib and 5-fluorouracil (272, 273). Another non-hydroxamate-based inhibitor, SN-4 specifically impedes ADAM17-mediated cleavage of TNF-α and CD44 with a higher activity than malistamate and an IC_{50} value of 3.22 μM (102). SN-4$(Nps)_2$, a prodrug of SN-4, can markedly enhance its bioavailability. A thiadiazolone derivative JTP-96193 showed 1800 times more selectivity toward ADAM17 over other matrix metalloproteinases with an IC_{50} value of 5.4 μM (258). Compounds 20-23 are novel non-hydroxamate-based small molecules targeting ADAM17 from the DrugBank database, whereas their ADAM17 inhibitory activity and potential mechanism remain to be further explored (20).

Anti-ADAM17 monoclonal antibodies

The development of anti-ADAM17 monoclonal antibodies has accelerated the progress of innovative ADAM17 inhibitors. Anti-ADAM17 monoclonal antibodies include A300E, A9 (B8), D1 (A12), MEDI3622, etc. A300E is rapidly internalized by ADAM17-expressing cells (274), and its IC_{50} against ADAM17 is approximately 0.7 μg/mL (260). Trad et al. suggested that A300E plays a role in cancer cells by transporting a conjugated toxin to target cells (260). A9 (B8) cross-reacts with both human and mouse ADAM17, whereas D1 (A12) binds only to human ADAM17. D1 (A12) is bound to both the catalytic and non-catalytic domains of ADAM17. Yang et al. found that A9 (B8) conferred EGFR-TKI-mediated antitumor effects in NSCLC cells with IC_{50} values of 0.22 nM and 0.25 nM against human and murine ADAM17, respectively (261). Ye et al. revealed that A9 (B8) inhibited the shedding of ADAM17 substrate and contributed to the growth inhibition of pancreatic ductal adenocarcinoma *in vivo* and *in vitro* (275). D1 (A12) at 4.7 nM inhibits 50% TNF-α shedding and induces anti-ovarian cancer effects (262). Besides, D1 (A12) restrains the progression of head and neck squamous cell carcinoma by reducing HERS-transactivation induced by retarded hormone and even has therapeutic prospects for EGFR TKI-resistant head and neck squamous cell carcinoma (276). Another anti-ADAM17 monoclonal antibody, MEDI3622, inhibits tumor-dependent

EGFR activity with IC_{50} values of 39 pmol/L and 132 pmol/L against human and murine ADAM17, respectively (263). In esophageal and colorectal tumors, the antitumor effect of MEDI3622 was superior to that of the EGFR/HER pathway inhibitor, suggesting that MEDI3622 inhibits tumor growth by partially modulating non-EGFR-mediated pathways (263). In addition, MEDI3622 enhances the release of antibody-bound tumor cells binding IFN-γ in NK cells by blocking CD16A shedding (239).

To date, there are no clinically available ADAM17 inhibitors. The high toxicity and low selectivity of existing ADAM17 inhibitors and the high structural homology between the catalytic domain of ADAM17 and other metalloproteases (e.g., ADAM10) have limited the development of selective ADAM17 inhibitors. However, the advent of small molecule compounds and anti-ADAM17 monoclonal antibodies targeting the non-catalytic domain of ADAM17 or the catalytic and non-catalytic domains of ADAM17 (44, 46, 277–279) further overcome these problems and improve bioavailability, which may provide a new strategy for the development of the highly effective low-toxicity ADAM17 inhibitors. In addition, as iRhom2 is a specific binding protein of ADAM17, targeting iRhom2 to inhibit ADAM17 activity is also a trend in the development of ADAM17 inhibitors.

Discussion

Metalloproteinase ADAM17 holds a vital role in post-translational protein modification, gene transcription and post-transcriptional regulation, and is closely associated with tumors and inflammation. ADAM17 regulates cell membrane protein shedding and subsequent signal transduction. It can also be impacted by the interacting proteins and thus participate in the regulation of its downstream signaling pathways. ADAM17 has been implicated in immune regulation of tumor development. However, its immunomodulatory functions and mechanisms in cancer diseases are not well studied, and therefore more studies are needed to further determine the role of ADAM17 in tumor development. In this article, we summarized the structure and multiple regulatory roles of ADAM17, the latest immune regulation of ADAM17 in tumor formation and development, as well as the progress in the development of ADAM17 inhibitors. On the one hand, although the regulatory effect of ADAM17 on macrophages, NK cells, and endothelial cells has been confirmed in tumor, more key proteins or genes related to ADAM17 need to be identified, and the immune response involved in TME needs to be further explored. In addition, the role of ADAM17 in post-translational modifications, such as proteolysis, phosphorylation, glycosylation, and post-transcriptional regulation in cancer progression remains unclear. On the other hand, due to the structural homology of ADAM17's catalytic domain with other metalloproteinases, more three-dimensional crystal structures associated with ADAM17 need to be uncovered to make them more conducive to highly selective and toxic drug design for ADAM17 inhibitors. How to reduce or avoid the toxic side effects of ADAM17 is also a potential research direction. Therefore, studying the key role and immunomodulatory mechanisms of ADAM17 in tumor development will provide new strategies for the prevention, diagnosis and treatment of cancer diseases.

Author contributions

KW and HW conceived and designed this work. KW drafted the manuscript. KW and ZX edited and revised the manuscript. CY, XL, and MZ provided constructive suggestions for the manuscript and literature. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Integrated analysis of multiple microarray studies to establish differential diagnostic models of Crohn's disease and ulcerative colitis based on a metalloproteinase-associated module

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Background: The ulcerative colitis (UC) and Crohn's disease (CD) subtypes of inflammatory bowel disease (IBD) are autoimmune diseases influenced by multiple complex factors. The clinical treatment strategies for UC and CD often differ, indicating the importance of improving their discrimination.

Methods: Two methods, robust rank aggregation (RRA) analysis and merging and intersection, were applied to integrate data from multiple IBD cohorts, and the identified differentially expressed genes (DEGs) were used to establish a protein–protein interaction (PPI) network. Molecular complex detection (MCODE) was used to identify important gene sets. Two differential diagnostic models to distinguish CD and UC were established *via* a least absolute shrinkage and selection operator (LASSO) logistic regression, and model evaluation was performed in both the training and testing groups, including receiver operating characteristic (ROC) curves, calibration plots and decision curve analysis (DCA). The potential value of MMP-associated genes was further verified using different IBD cohorts and clinical samples.

Results: Four datasets (GSE75214, GSE10616, GSE36807, and GSE9686) were included in the analysis. Both data integration methods indicated that the activation of the MMP-associated module was significantly elevated in UC. Two LASSO models based on continuous variable (Model_1) and binary variable (Model_2) MMP-associated genes were established to discriminate CD and UC. The results showed that Model_1 exhibited good discrimination in the training

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and testing groups. The calibration analysis and DCA showed that Model_1 exhibited good performance in the training group but failed in the testing group. Model_2 exhibited good discrimination, calibration and DCA results in the training and testing groups and exhibited greater diagnostic value. The effects of Model_1 and Model_2 were further verified in a new IBD cohort of GSE179285. The MMP genes exhibited high value as biomarkers for the discrimination of IBD patients using published cohort and immunohistochemistry (IHC) staining data. The MMP-associated gene levels were statistically significantly positively correlated with the levels of the differentially expressed cell types, indicating their potential value in differential diagnosis. The single-cell analysis confirmed that the expression of ANXA1 in UC was higher than that in CD.

Conclusion: MMP-associated modules are the main differential gene sets between CD and UC. The established Model_2 overcomes batch differences and has good clinical applicability. Subsequent in-depth research investigating how MMPs are involved in the development of different IBD subtypes is necessary.

KEYWORDS

crohn's disease, ulcerative colitis, robust rank aggregation, microarray, metalloproteinases, differential diagnostic models

Introduction

Inflammatory bowel disease (IBD) leads to chronic intestinal inflammation, is associated with significant morbidity, and results from the intersection of genetic and environmental factors that influence immune responses (1). Crohn's disease (CD) and ulcerative colitis (UC) are the two main types of inflammatory bowel disease. Despite certain common pathological and clinical characteristics, CD and UC have several differences that indicate they are two distinct disease types. CD is characterized by fissuring ulceration, granulomatous inflammation and submucosal fibrosis. However, the characteristic histological findings in UC include crypt distortion, lymphocyte infiltration and chronic inflammation of the rectum, which is usually limited to the lamina propria (2, 3). Clinically, the diagnosis of IBD is usually established by a collective assessment of the clinical presentation and endoscopic, histopathological, radiographic and laboratory findings (4, 5).

An objective and clear discrimination between CD and UC diagnoses in patients with IBD colitis is currently vital for a tailored treatment plan since each disease involves different therapeutic and coping mechanisms (6–9). However, the differential diagnosis of these subtypes remains a remarkable clinical challenge since there is no single diagnostic gold standard for either UC or Crohn's colitis (6–11). According to the public literature, approximately 5% to 15% of patients do not meet the strict criteria for either UC or CD (12–14), and the diagnoses of up to 14% of patients change over time (15–19). Therefore, the diagnostic assessment of IBD is often challenging; discriminating between CD and UC can be particularly challenging in patients in whom the inflammatory lesions are limited to the colon (20).

In recent years, with the development of high-throughput microarray technology, several studies have reported miRNAs as candidate biomarkers in IBD diagnostic assessment (21); however, few studies reported that mRNAs can be directly used for the differential diagnosis of CD and UC. Metalloproteinases (MMPs) belong to a large group of zinc-dependent proteolytic enzymes involved in degrading and remodeling the extracellular matrix (ECM) by cleaving specific components (22). In this study, we integrated data obtained from multiple IBD cohorts using two methods to identify important functional gene sets that differed between CD and UC and identified that the activation of the MMP-associated module was significantly elevated in UC. Furthermore, using different analytical methods, we established two differential diagnostic models to distinguish CD and UC *via* a least absolute shrinkage and selection operator (LASSO) logistic regression and further verified the models' efficiency in several

different cohorts. Finally, we explored the roles of these genes in immunity during the progression of UC, providing evidence that the expression of MMP-associated genes is correlated with the presence of multiple immune cell types. Thus, our diagnostic models provide promising diagnostic tools that might soon improve clinical practice.

Materials and methods

Search strategy for microarray datasets

In total, 139 datasets were collected from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) by systematic retrieval using the following keywords: ("Inflammatory Bowel Diseases"[MeSH Terms] OR Inflammatory Bowel Diseases [All Fields]) AND "Homo sapiens"[porgn] AND ("Expression profiling by array"[Filter] AND ("2008/01/01"[PDAT]: "2021/01/01"[PDAT]). The inclusion criteria were (1) a sample size > 15 (2); the inclusion of both CD and UC samples (3); sample sources of "ileum/colon"; and (4) available gene annotation information. To further verify the effectiveness of the models, a newly published IBD cohort, GSE179285, was used to evaluate the two models simultaneously. Another dataset, GSE125527, generated from single-cell sequencing was analyzed to investigate the role of MMP-associated genes in different cell clusters. The flow of the experimental design and data analysis is shown in Figure 1. The detailed information of the characteristics of the included datasets is shown in Table 1.

Robust rank aggregation analysis and identification of differentially expressed genes in the integrated cohort

Using RRA, all genes in each dataset were sorted and ranked based on their log fold-change (logFC) values using the limma package. The DEGs were then ranked using the ranked list and aggregated using the RRA package in R software. Using this method, an adjusted P value determines the likelihood that DEGs will be identified in the datasets with highly ranked genes. LogFC values > 0.7 and adjusted P values < 0.05 were set as the criteria for identifying DEGs.

Identification of DEGs by merging and intersection

To increase the sample size of the IBD cohort, three datasets (GSE10616, GSE36807, and GSE9686) from the same platform were merged and named Combined Datasets. The ComBat function was used to remove batch effects using the SVA package. Then, the DEGs were identified in the Combined Datasets and GSE75214 with the criteria of LogFC values > 0.6 and adjusted P values < 0.1. The final DEGs were identified by considering the intersection of the DEGs between the two IBD cohorts.

Functional and pathway enrichment analyses

We performed a Gene Ontology biological process (GO-BP) analysis and Kyoto Encyclopedia of Genes and Genomes

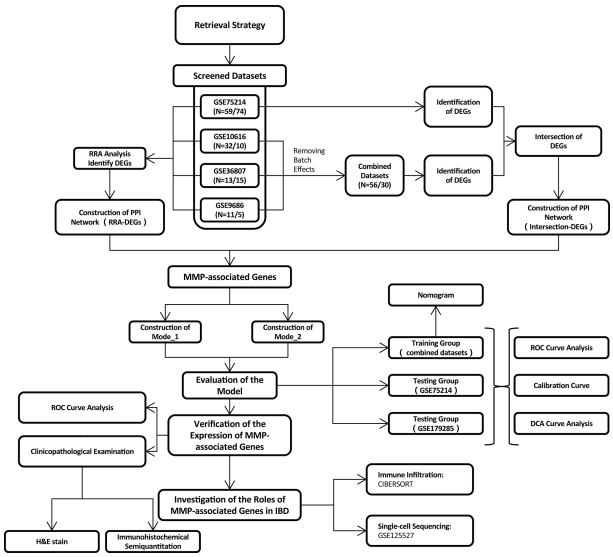

FIGURE 1
Flowchart of the overall study design.

TABLE 1 Characteristic of the included microarray datasets.

GSE ID	Participants (CD/UC)	Analysis type	Platform	Year	Tissues	Sex (Male: Female)	Included in the RRA analysis	Network address
GSE75214	59/74	Array	GPL6244	2017	8 Colon and 51 Ileum in CD/74 Colon in UC	Not determined	Yes	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75214
GSE10616	32/10	Array	GPL5760 (identical to GPL570)	2009	14 Colon and 18 Ileo-colonic in CD/10 Colon in UC	Not determined	Yes	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10616
GSE36807	13/15	Array	GPL570	2013	Not determined	9:4 in CD/ 8:7 in UC	Yes	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36807
GSE9686	11/5	Array	GPL5760 (identical to GPL570)	2008	11 Colon in CD/5 Colon in UC	Not determined	Yes	https://www.ncbi.nlm.hih.gov/geo/query/acc.cgi?acc=GSE9686
GSE179285	37/23	Array	GPL6480	2021	14 Colon and 33 Ileo in CD/23 Colon in UC	Not determined	No	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179285
GSE125527	7/7	Single-cell Sequencing	GPL20301	2020	7 Rectum in CD/7 Rectum in UC	Not determined	No	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125527

(KEGG) analysis of the DEGs identified by the RRA analysis using the limma and clusterProfiler packages. The enrichment analysis of the DEGs was performed with the criteria of adjusted P values of < 0.05 (23).

Establishment of a PPI network and MCODE analysis

Using the DEGs obtained by the RRA analysis/intersection, a PPI network was established using the STRING website (https://cn.string-db.org/), with a parameter of confidence of > 0.4. Visualization of the PPI network was performed by Cytoscape (v3.7.2), and molecular complex detection (MCODE) (a plug-in in Cytoscape) was used to identify the functional modules (24).

Establishment of model_1 *via* LASSO logistic regression

For the logistic regression, to achieve a high performance, LASSO was applied to reduce the dimensions of the analysis. The candidate genes were collected from the combination of two MMP-associated modules identified in the PPI network, including MMP3, MMP1, MMP12, PLAU, MMP9, CXCL1, MMP10, PTGS2, TIMP1, MMP7, CXCL13, S100A12, S100A8, S100A9, and ANXA1. The combined datasets were set as the training group, while GSE75214 was used as the testing group to verify the effect of the model.

The LASSO procedure involved performing a logistic regression with an L1 regularization penalty, which has the effect of shrinking the regression weights of the least predictive features to 0. To determine the coefficients of optimal penalty, we performed tenfold cross-validation, and binomial deviation was used as a performance measure. Therefore, the function cv.glmnet was used with the following parameters: alpha = 1, nfolds = 10, and type.measure = "deviance". To obtain parsimonious models, the largest lambda, which was within one standard error of the minimum training deviance as recommended, was used to establish the final model.

Establishment of model_2 *via* LASSO logistic regression

To correct the model application problems caused by the batch differences across different platforms, we used another method to generate the model. A binary variable translation was performed with 15 candidate MMP-associated genes to obtain a new index for each MMP-associated gene in each sample. For genes with increased expression in UC, the binary variable of MMP-associated genes was assigned a value of 1 if the expression value of a gene was greater than the median of the expression value of that gene in all samples; otherwise, the index was defined as 0. For genes with increased expression in CD, the binary variable of MMP-associated genes was assigned a value of 1 if the expression value of a gene was less than the median of the expression value of that gene in all samples; otherwise, the index was defined as 0. Therefore, the expression values of 15 genes were converted from continuous variables into binary variables. Similar to the method used to establish Model_1, the combined datasets were set as the training group, while GSE75214 was used as the testing group to verify the effect of the model.

Model_2 was generated using a different method than model_1. To determine the coefficients of optimal penalty, we performed 8-fold cross-validation, and the area under the receiver operator characteristics (ROC) curve was used as a performance measure. Thus, the function cv.glmnet in the package glmnet v2.0-16 was used with the following parameters: alpha = 1, nfolds = 8, and type.measure = "auc". To obtain parsimonious models, the largest lambda, which was within one standard error of the maximal training AUC as recommended, was used to generate the final model.

Evaluation of the differential diagnostic models

The models were developed and strictly validated according to the Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis (TRIPOD) guidelines (25). Specifically, we used ROC analyses and the AUC to assess the discriminatory ability of the model in discriminating CD from UC cases. Calibration plots were drawn to assess the goodness of fit of each model. A decision curve analysis (DCA) was performed to assess the clinical net benefit of each model and compare the model with the use of all strategies and random chance. The equations used in the final models are presented as nomograms. Importantly, the ROC analysis, calibration plot assessments and DCA were performed in both the training and testing groups, while the nomogram was illustrated only in the training group according to the TRIPOD guidelines. All analyses were conducted using R version 4.1.3.

Evaluation of immunohistochemical staining

To validate the results of the genetic analysis at the transcriptional level, human intestinal mucosal tissues were collected from the Department of Gastroenterology at the Second Hospital of Hebei Medical University between 2020-2021. The histopathologic diagnosis was made by two pathologists, and the sample set included 15 CD samples and 23 UC samples.

The collected intestinal mucosa of the patients were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. IHC staining was performed as previously described (26). The following antibodies were used: MMP7 (A20701), ANXA1 (A1118), MMP10 (A3033), HRP-labeled goat anti-rabbit antibody (AS014; 1:200 dilution, all from ABclonal, Wuhan, China), CXCL13 (bs-2553R), and CXCL1 (bs-10234R, all from Bioss, Beijing, China; 1:200 dilution).

The samples were scored by two trained pathologists according to the percentage contribution of the high positive, positive, low positive, and negative samples. The immunoreactive score (IRS) was evaluated as follows: 4, high positive; 3, positive; 2, low positive; and 1 negative (27).

Landscape of immune cell infiltration

To evaluate immune cell infiltration, CIBERSORT was used to quantify 22 tumor-infiltrating immune cell subgroups in the CD and UC groups in both the Combined Datasets and the GSE75214 dataset. Because the MMP-associated genes exhibited a higher level in UC in this study, the relationships between the expression of immune cell subgroups and MMP-associated genes in UC were further examined by a Spearman correlation analysis.

Single-cell sequencing analysis

The single-cell sequencing analysis was based on public data (GSE125527, including 7 CD and 7 UC patients) downloaded from the GEO website and analyzed *via* R software according to the following steps: 1) the "Seurat" package was adopted to convert 10× scRNA-seq data as a Seurat object; 2) the "FindVariableFeatures" function was adopted to filter the top 2000 highly variable genes; 3) a principal component analysis (PCA) was performed based on the 2000 genes, and the data from different samples were further merged *via* harmony integration; 4) a clustering analysis was performed to find subtypes, and uniform manifold approximation and projection (UMAP) was used for dimensionality reduction and cluster identification.

Statistical analysis

All statistical tests were implemented using R software 4.1.3. A Wilcoxon test was used to analyze the significance of the differences between the groups. Spearman's correlation test was used to determine the correlation between the variables. Statistically significant results were defined as those with P values < 0.05.

Results

Characteristics of the included microarray datasets

After conducting a systematic search based on the inclusion criteria described in the Materials and Methods, in total, four microarray datasets of IBD patients were retrieved from the GEO database, including GSE75214 (N=59/74, x/y=the number of CD/UC included in the research) (28), GSE10616 (N=32/10)

(29), GSE36807 (N=13/15) (30), and GSE9686 (N=11/5) (31). Therefore, in total, 115 cases of CD and 104 cases of UC were finally included in the analysis.

Identification of DEGs by an RRA analysis and functional enrichment analysis

Considering that the collected data were generated from different microarray platforms, combining the datasets by using direct merging inevitably led to erroneous conclusions caused by bias. Therefore, the RRA method was first applied to identify DEGs in the four GEO datasets.

First, the datasets were standardized to correct batch differences within the datasets, and the results showed that the homogeneity of the data met the requirements (Supplementary Figure 1). A volcano map of each dataset was produced using the Limma package in R software, and the DEGs are indicated as green and red points in Figures 2A-D. The results showed that the DEGs greatly differed across the different datasets. In the GSE75214 dataset, many DEGs were identified, while few DEGs were identified in the GSE10616, GSE36807, and GSE9686 datasets as indicated in the figures, suggesting that the data collected from the cohorts across different sources were heterogeneous.

After the RRA analysis, in total, 141 DEGs (86 overexpressed in UC and 55 overexpressed in CD) were finally identified, and a heatmap of the expression data of the top 20 DEGs is shown in Figure 2E. The top 10 significant genes that were aberrantly expressed in UC included five genes [SLC6A14 (P = 2.78E-08), TNIP3 (P = 1.03E-07), MMP7 (P = 4.45E-07), MMP10 (P = 1.68E-06), and CXCL13 (P = 2.09E-06)], and five genes were overexpressed in CD [GBA3 (P = 7.48E-07), PCK1 (P = 3.27E-06), AQP8 (P = 8.98E-06), MEP1B (P = 1.82E-05), and GUCA2A (P = 3.48E-05)]. The overall results of the RRA analysis are listed in Supplementary Table 1.

The DEGs were subjected to a GO-BP analysis and KEGG analysis, and the top five results are listed in Figures 2F, G. The results showed that the short-chain fatty acid metabolic process, cellular lipid catabolic process, drug catabolic process, fatty acid metabolic process and cellular response to xenobiotic stimulus were the top five enriched BPs, while bile secretion, drug metabolism - cytochrome P450, tyrosine metabolism, steroid hormone biosynthesis, and phenylalanine metabolism were the top five enriched KEGG pathways in CD. In the UC samples, negative regulation of proteolysis, extracellular matrix organization, collagen catabolic process, extracellular matrix disassembly, and extracellular structure organization were the top five enriched BPs, while the IL-17 signaling pathway, rheumatoid arthritis, cytokine–cytokine receptor interaction, NF-kappa B signaling pathway and TNF signaling pathway were the top five enriched KEGG pathways. The detailed results are listed in Supplementary Table 2.

Identification of DEGs by merging and intersection and a functional enrichment analysis

To identify the DEGs more comprehensively, we adopted another method. Because the data in the GSE10616, GSE36807 and GSE9686 datasets were collected using the same platform, combined datasets (named Combined Datasets, N=56/30) were generated by merging the GSE10616, GSE36807 and GSE9686 datasets by removing batch effects *via* the SVA package in R software. The distribution of the data before and after correction was tested by a principal component analysis (PCA) dimensionality reduction (Figures 3A, B). The results showed that the distribution of each dataset was quite different before the batch correction, while the distribution of the data after correction overlapped well.

The differentially expressed genes in the GSE75214 dataset and Combined Datasets were analyzed (Figures 3C, D). The final DEGs were determined by considering the intersection of the differentially expressed genes, and in total, 65 DEGs were identified as listed in Figure 3E and Supplementary Table 3.

The DEGs, including 45 overexpressed in UC and 20 overexpressed in CD, were subjected to a GO-BP analysis and KEGG analysis, and the top five results are listed in Figures 4F, G. The results showed that bicarbonate transport, positive regulation of guanylate cyclase activity, regulation of guanylate cyclase activity, chloride transport, and positive regulation of cyclase activity were the top five enriched BPs, while nitrogen metabolism, proximal tubule bicarbonate reclamation, glycolysis/gluconeogenesis, the PPAR signaling pathway, and bile secretion were the top five enriched KEGG pathways in the CD samples. In the UC samples, humoral immune response, neutrophil degranulation, neutrophil activation involved in immune response, antimicrobial humoral response, and granulocyte chemotaxis were the top five enriched BPs, while the IL-17 signaling pathway, amoebiasis, TNF signaling pathway, cytokine–cytokine receptor interaction, and complement and coagulation cascades were the top five enriched KEGG pathways. The detailed results are listed in Supplementary Table 4. Notably, although we used two different methods to identify the DEGs, the results showed that the identified enriched signaling pathways were highly similar, including the IL-17 signaling pathway, TNF signaling pathway, and cytokine–cytokine receptor interaction in UC.

MMP-associated module is the most important network module in both PPI networks

For clarity, we named the DEGs identified in the RRA analysis RRA-DEGs, and the DEGs identified from the

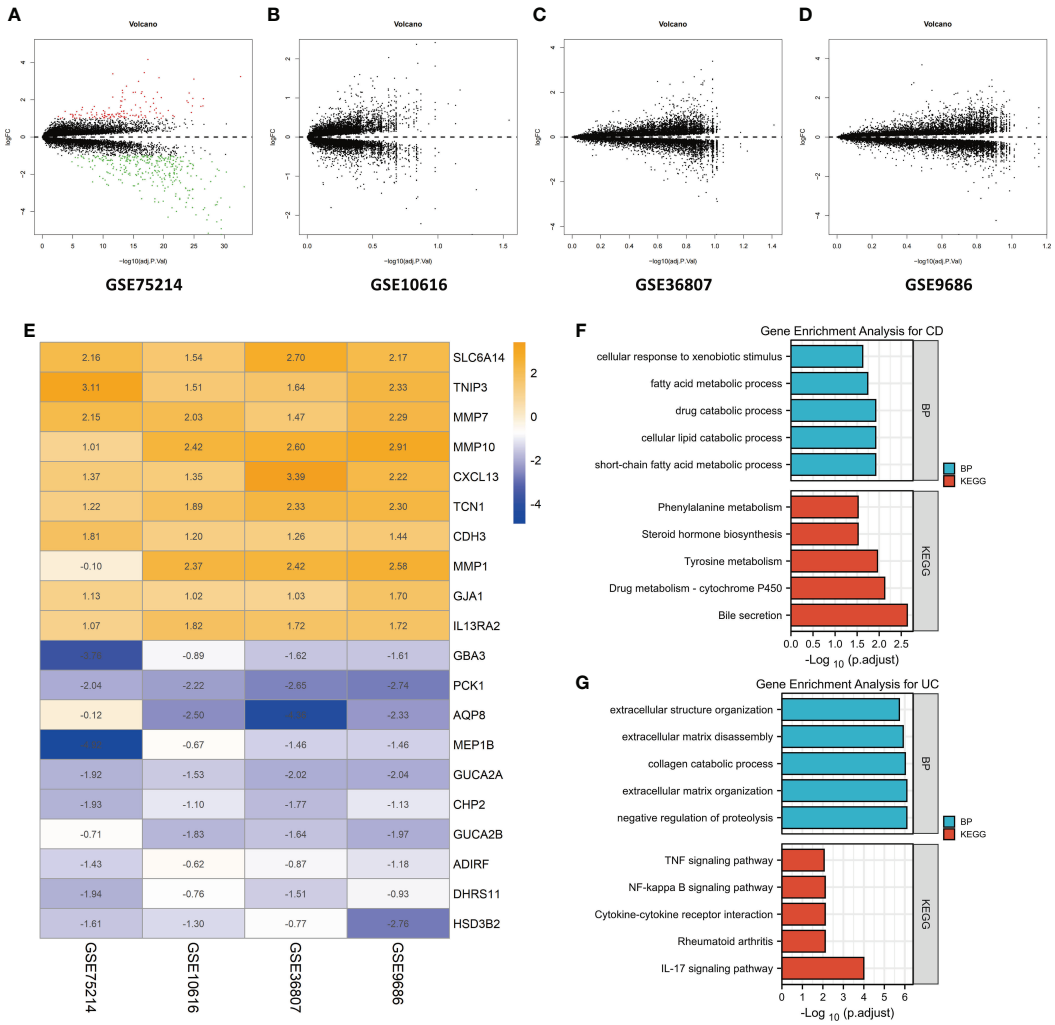

FIGURE 2
Identification of DEGs by an RRA analysis and functional enrichment analysis. Volcano plots of the DEG distributions in the GSE75214 **(A)**, GSE10616 **(B)**, GSE36807 **(C)**, and GSE9686 **(D)** datasets. Upregulated genes are indicated by red points, while downregulated genes are indicated by green points. Genes with no significant differences in levels are indicated by black points. **(E)** Heatmap of the expression data of the top 10 DEGs identified in the RRA. The genes upregulated in CD are indicated in blue, and those upregulated in UC are indicated in orange. Functional enrichment analysis of DEGs, including **(F)** a GO-BP analysis and **(G)** a KEGG pathway analysis.

intersection of the GSE75214 dataset and Combined DataSets were named Intersection-DEGs. Two PPI networks based on the RRA-DEGs and intersection-DEGs were generated to better explore the differences in complex regulatory mechanisms between CD and UC using the STRING website, and the results were visualized by Cytoscape software.

The network generated by using the RRA-DEGs is shown in Figure 4A and includes 101 nodes and 464 edges. The Molecular Complex Detection (MCODE) function module is a commonly used module in the establishment of PPI networks. This module can be used to identify important subnetworks and genes in a large PPI network according to the relationship between the edges and nodes to facilitate the following analysis. Through a MCODE analysis, we observed that the most important subnetworks mainly involved MMP family genes, including MMP1, MMP12, PLAU, MMP9, CXCL1, MMP10, PTGS2, TIMP1, and MMP7, with MMP3 as the seed gene (Figure 4B). Through a gene enrichment analysis, we observed that extracellular matrix disassembly, collagen catabolic process, collagen metabolic process, extracellular matrix organization, and extracellular structure organization were the top five enriched BPs, while the IL-17 signaling pathway, TNF signaling pathway, rheumatoid arthritis, prostate cancer, and NF-kappa B signaling pathway were the top five enriched KEGG pathways (Figure 4C and details in Supplementary Table 5).

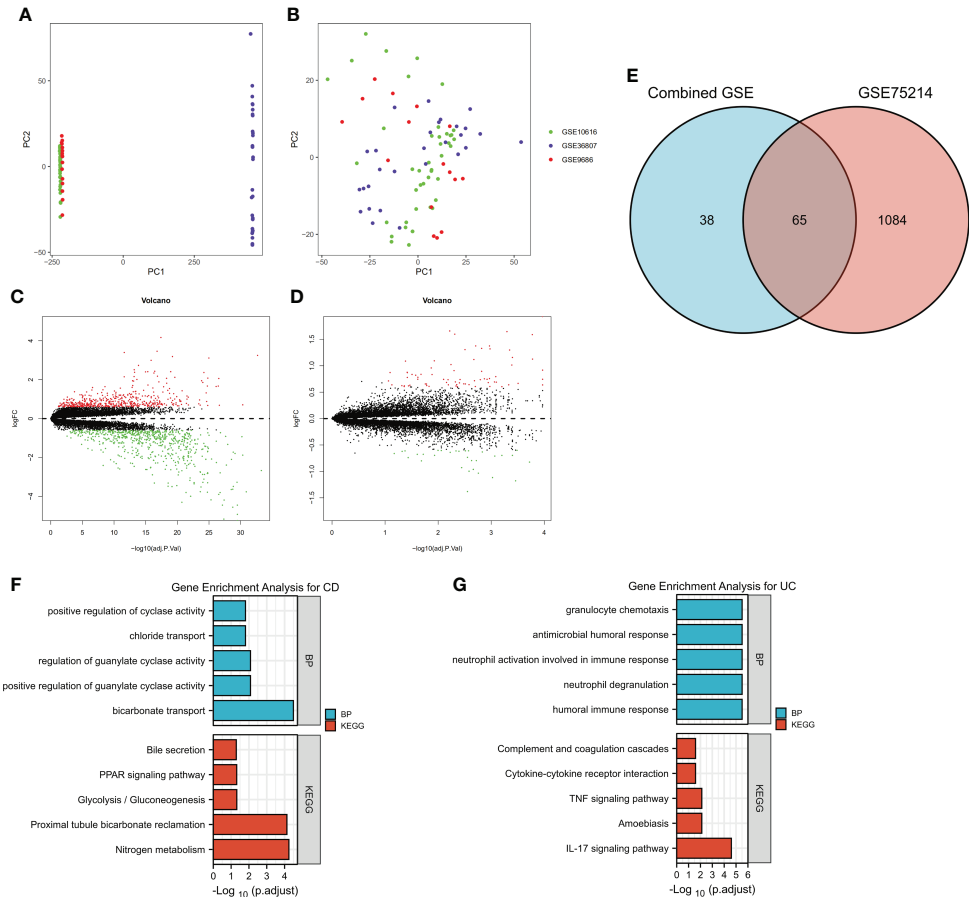

FIGURE 3
Identification of DEGs by merging and intersection and a functional enrichment analysis. The GSE10616, GSE36807 and GSE9686 datasets were merged, and batch effects were further removed. PCA plots of different datasets are illustrated before **(A)** and after **(B)** batch effects were removed. Volcano plots of the DEG distributions in the GSE75214 dataset **(C)** and Combined Datasets **(D)**. **(E)** The intersection of DEGs in the GSE75214 dataset and Combined Datasets is displayed as a Venn diagram. Functional enrichment analysis of DEGs, including **(F)** a GO-BP analysis and **(G)** a KEGG pathway analysis.

Then, we used the intersection-DEGs to generate another PPI network. The results are shown in Figure 4D, which shows 49 nodes and 190 edges. As before, the MCOD plug-in was used to identify important subnetworks and key genes. Although fewer DEGs were used to generate the PPI network in this analysis, the key subnetworks finally identified still mainly involved the MMP family, including MMP12, MMP10, MMP3, MMP9, TIMP1, CXCL1, PLAU, S100A9, CXCL13, S100A8, ANXA1 and S100A12, with MMP7 as the seed gene (Figure 4E). Through a gene enrichment analysis, we found that extracellular matrix disassembly, collagen catabolic process, granulocyte chemotaxis, granulocyte migration, and myeloid leukocyte migration were the top five enriched BPs, while the IL-17 signaling pathway, prostate cancer, TNF signaling pathway, transcriptional misregulation in cancer, and rheumatoid arthritis were the top five enriched KEGG pathways (Figure 4F and details in Supplementary Table 5).

Notably, the results of both PPI networks independently identified the MMP family as a pivotal module, and the results of the gene functional enrichment analysis of both MMP modules were quite similar to those obtained by analyzing all DEGs in the RRA/intersection analysis as shown in Figures 2F, G and Figures 3F, G; this finding indicates that the module of the MMP family is the main differential gene set between CD and UC.

Establishment of model_1 based on the MMP-associated module *via* a LASSO logistic regression

Based on the above results, we speculated that the MMP family module was the main differential gene set between UC and CD. Therefore, the genes in two MMP subnetworks were

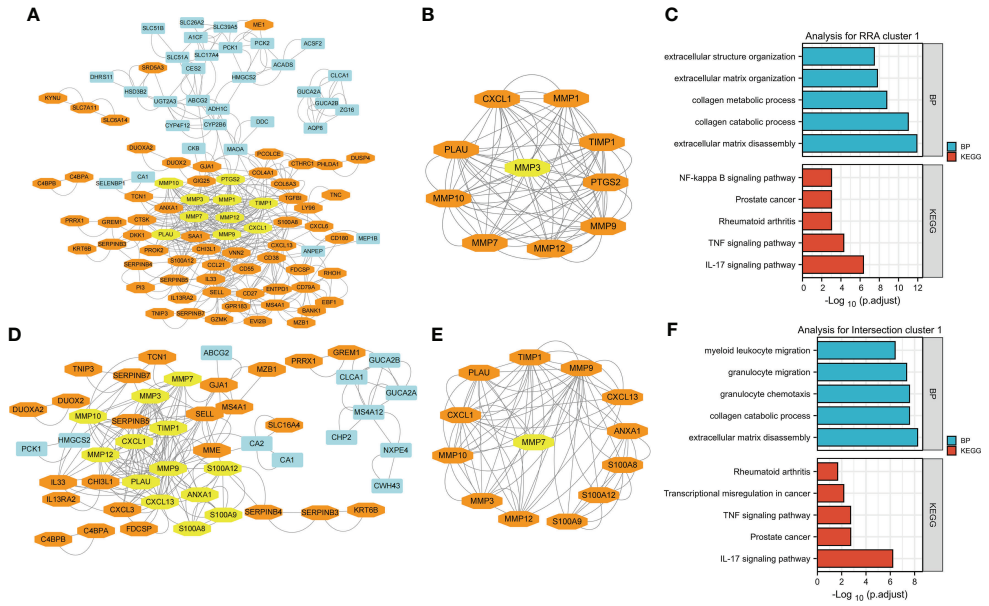

FIGURE 4
Visualization and module identification using two different PPI networks. **(A)** RRA-DEGs were mapped using Cytoscape software (genes mainly expressed in CD are indicated in blue, and genes mainly expressed in UC are indicated in orange). Using the MCODE plug-in, the module with the highest scores was identified (indicated in yellow). **(B)** The identified module comprised MMP1, MMP12, PLAU, MMP9, CXCL1, MMP10, PTGS2, TIMP1, and MMP7 (indicated in orange), with MMP3 as the seed gene (indicated in yellow). Functional enrichment analyses of the genes in the MMP-associated module were performed **(C)**. **(D)** The intersection-DEGs were mapped using Cytoscape software (genes mainly expressed in CD are indicated in blue, and genes mainly expressed in UC are indicated in orange). Using the MCODE plug-in, the module with the highest scores was identified (indicated in yellow). **(E)** The identified module comprised MMP12, MMP10, MMP3, MMP9, TIMP1, CXCL1, PLAU, S100A9, CXCL13, S100A8, ANXA1 and S100A12 (indicated in orange), with MMP7 as the seed gene (indicated in yellow). Functional enrichment analyses of the genes in the MMP-associated module were performed **(F)**.

merged and served as candidates for establishing the LASSO logistic regression model. The combined datasets were set as the training group, while the GSE75214 dataset was set as the testing group. After the model reached the minimum+1 standard error lambda, an MMP-related signature with 4 components was built discriminate between CD and UC (Figure 5A). The diagnostic score was defined as -3.7469+[expression level of ANXA1 × (0.4213)]+[expression level of MMP10 × (0.2944)]+[expression level of MMP1 × (0.0826)]+[expression level of CXCL13 × (0.0351)], and the optimal features and their coefficient values are shown in Figure 5B. Notably, the diagnostic score was a scoring system used only for differential diagnosis, and the score was positively correlated with UC and negatively correlated with CD.

In the next investigation, we evaluated the diagnostic efficiency of Model_1 in discriminating between CD and UC. The methods of the evaluation strictly complied with the guidelines of the TRIPOD (Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis) Statement (25), including discrimination (ROC curve and AUC), calibration (calibration plot), decision curve analyses and a nomogram. The results of the ROC curve showed that the AUC achieved in the training group was 0.839, and that achieved in the testing group was 0.815 (Figures 5C, D). In addition, the calibration curve showed that the diagnostic score had a better prediction accuracy in the training group and poor calibration in the testing group (Figures 5E, F). Similarly, the DCA results showed that the diagnostic score in the training group served as a better indicator during clinical decision-making than that in the testing group (Figures 5G, H). The nomogram for the differential diagnosis of CD and UC is illustrated in Figure 5I. For example, for a patient with a CXCL13 value of 1.0416, MMP1 value of 2.6360, ANXA1 value of 1.9737, and MMP10 value of 1.5244, the predicted probability of the patient having UC was 0.0987, while the predicted probability of the patient having CD was 0.9013. According to a cutoff value of 0.5, the patient was identified as having CD based on Model_1.

Establishment of model_2 based on the MMP family module *via* a LASSO logistic regression

Based on the poor calibration and clinical applicability of Model_1, a better method for establishing a diagnostic model

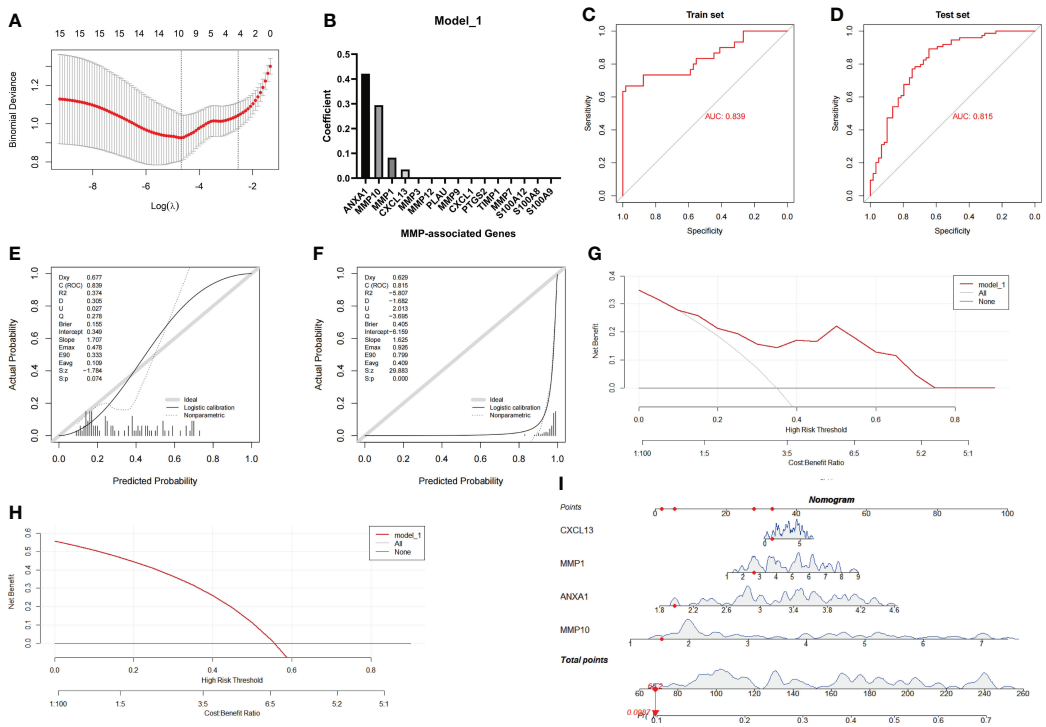

FIGURE 5
Establishment and evaluation of Model_1 with the MMP-associated module. **(A)** Selection of the optimal parameter (lambda) in the LASSO model. **(B)** Plot of coefficients obtained by LASSO. ROC curve **(C, D)**, calibration curves **(E, F)**, and decision curve analysis (DCA) **(G, H)** of the training and testing cohort models. **(I)** Nomogram of Model_1 for the predictive value of CD/UC is illustrated.

was applied as described in the Methods and Material. The main difference was that the continuous variables representing gene expression were transformed into binary variables, leading to a reduction in distribution differences due to specific expression values.

Similar to the method used to assess Model_1, the Combined Datasets were set as the training group, while the GSE75214 dataset was set as the testing group. After the model reached the minimum+1 standard error lambda, an MMP-related signature with 4 components was generated to discriminate between CD and UC (Figure 6A). The diagnostic score was defined as -1.3813+ [value of ANXA1×(0.6358)]+ [value of CXCL13×(0.1000)]+ [value of MMP1×(0.2507)]+ [value of CXCL1×(0.4478)], and the optimal features and their coefficient values are shown in Figure 6B.

Similar to the method used during the investigation of Model_1, we further evaluated the diagnostic efficiency of Model_2 in identifying CD and UC. The results of the ROC curve showed that the AUC achieved in the training group was 0.801, and that achieved in the testing group was 0.811 (Figures 6C, D). In addition, the calibration curve showed that the diagnostic score had a better prediction accuracy in both the training group and the testing group (Figures 6E, F). Similarly, the DCA results showed that the diagnostic score in both the training group and the testing group served as a better indicator in clinical decision-making (Figures 6G, H). The nomogram for the differential diagnosis of CD and UC is illustrated in Figure 6I. For example, for a patient with a CXCL13 value of 0, an MMP1 value of 1, an ANXA1 value of 0, and a CXCL1 value of 1, the predicted probability of a UC diagnosis was 0.336, while the predicted probability of a CD diagnosis was 0.664. According to a cutoff value of 0.5, the patient was identified as having CD based on Model_2. Overall, these results suggest that Model_2 based on the MMP-associated module has good prediction ability.

Verification of the effectiveness of model_1 and model_2 in a new IBD cohort

Although the effects of Model_1 and Model_2 were tested in the above studies, considering that the GSE75214 cohort was included in the RRA analysis, strictly speaking, the GSE75214 cohort is not a complete test queue. Therefore, a recently published IBD cohort, GSE179285, which was not included in the RRA analysis, was used for a more rigorous evaluation of the effects of the two models.

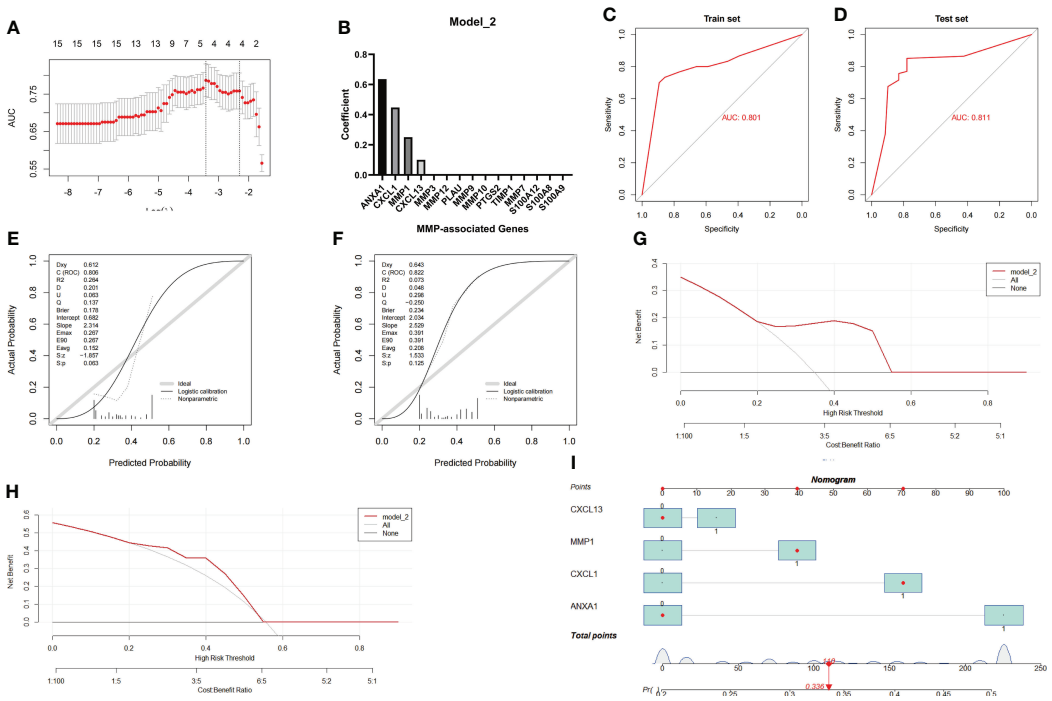

FIGURE 6
Establishment and evaluation of Model_2 with the MMP-associated module. **(A)** Selection of the optimal parameter (lambda) in the LASSO model. **(B)** Plot of coefficients obtained by LASSO. ROC curve **(C, D)**, calibration curves **(E, F)**, and decision curve analysis (DCA) **(G, H)** of the training and testing cohort models. **(I)** Nomogram of Model_2 for the predictive value of CD/UC is illustrated.

The evaluation results of Model_1 are shown in Figures 7A-C. The results show that the ROC curve, calibration curves and decision curve analysis of Model_1 are very poor likely because GSE179285 was generated from GPL6480, a platform different from the other queues. In addition, since the GSE179285 cohort contains a large number of colon tissue from CD cases, we used Model_1 to evaluate CD and UC in colon tissue only and showed that the differentiation of Model_1 was still poor (Figure 7D, AUC=0.509). In contrast, Model_2 still shows good ROC curves, calibration curves and decision curve analysis in the new cohort (Figures 7E-G), although the data were derived from a platform that the model never faced before. Furthermore, Model_2 still shows a good ROC curve between the UC and CD samples from only colon tissue, highlighting its great application value as a clinical diagnostic model (Figure 7H, AUC=0.730). As we know, the real challenge of differential diagnostic of two subtypes of IBD is between colonic dominant CD versus UC, which reflects the potential clinical value of the Model_2. We performed statistical analysis on CD and UC samples from colonic tissue in the former IBD cohort. The results showed that the model had an AUC of 0.674 in GSE75214 (CD/UC=8/74), 0.754 in GSE10616 (CD/UC=14/10), and 0.900 in GSE9686 (CD/UC=11/5, Supplementary Figure 3).

Verification of MMP-associated genes in different IBD cohorts

After establishing and evaluating the model, we explored the function and diagnostic value of MMP-associated genes, including the seed genes identified in the MCODE analysis (MMP3 and MMP7) and 5 genes assessed in Model_1 and Model_2 (ANXA1, MMP10, MMP1, CXCL13 and CXCL1). We first examined the correlation between the internal MMP-associated genes in both the Combined Datasets and the GSE75214 dataset using a Spearman correlation analysis, and the results are illustrated in Figures 8A, B. The results showed that multiple strong positive correlations were detected between the levels of the MMP-associated genes. In addition, the differences in the MMP-associated gene levels between CD and UC were examined, and the results showed statistically significant differences between the two groups in the expression of most MMP-associated genes, including MMP3, MMP7, ANXA1, MMP10, CXCL13 and CXCL1, except for MMP1 in the GSE75214 dataset (Figures 8C, D).

To further verify the value of the MMP-associated genes as diagnostic markers, we explored their ROC curves in different cohorts (Figures 8E, F). The results showed that the AUCs of ANXA1 and MMP10 were greater than 0.75, and those of

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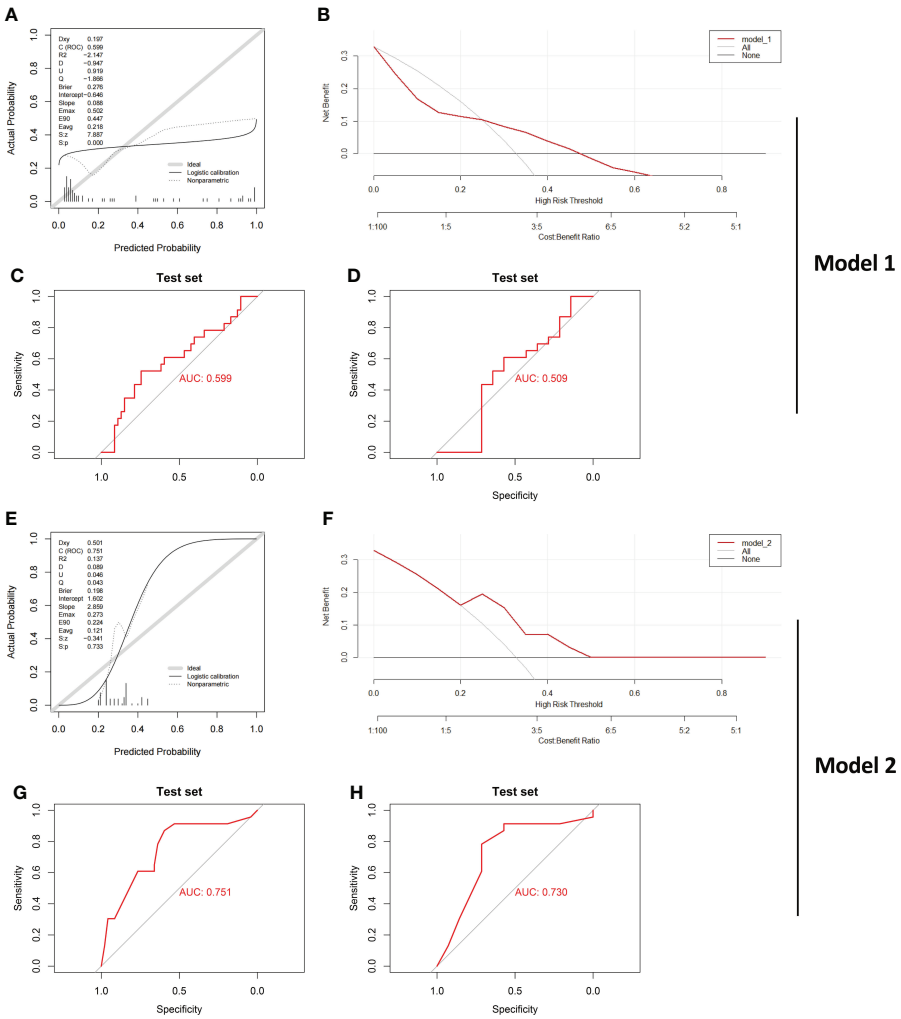

FIGURE 7
Verification of the effect of both Model_1 and Model_2 in a new IBD Cohort Calibration curves **(A)**, DCA curve **(B)** and ROC curve **(C)** testing the effect of Model_1 in GSE179285 are illustrated. CD and UC samples from colon tissue only were further examined by Model_1, and **(D)** ROC curve is shown. Calibration curves **(E)**, DCA curve **(F)** and ROC curve **(G)** testing the effect of Model_2 in GSE179285 are illustrated. CD and UC samples from colon tissue only were further examined by Model_2, and **(H)** ROC curve is shown. CD/UC=37/23 is the sample size in GSE179285, and CD/UC=14/23 is the sample size in GSE179285 including only colon tissue.

MMP7, CXCL13 and CXCL1 were greater than 0.70 in the two cohorts, CD and UC. Therefore, these genes showed high value as biomarkers for the discrimination of IBD patients.

Validation of the expression levels of MMP-associated genes in clinical samples

To verify the reliability of the above results obtained from a public database, we collected intestinal mucosa samples from 38 patients (CD/UC =15/23) to test the expression levels of the MMP-associated genes. As shown in Figure 9, the expression levels of ANXA1, MMP10 and CXCL13 did not differ between CD and UC samples. However, MMP7 and CXCL1 showed higher levels in the UC patients than in the CD patients. After the scoring data were summarized and analyzed, the results showed that the differences in the MMP7 and CXCL1 levels between the CD and UC groups were statistically significant. However, due to the complexity of the etiology of IBD, we believe that IHC of a single index cannot accurately provide a reference for disease diagnosis, especially based on the judgment of a small clinical sample size.

Analysis of the correlation between MMP-associated gene levels and the levels of infiltrating immune cells

By performing CIBERSORT, we compared the infiltration levels of most immune cell populations between CD and UC

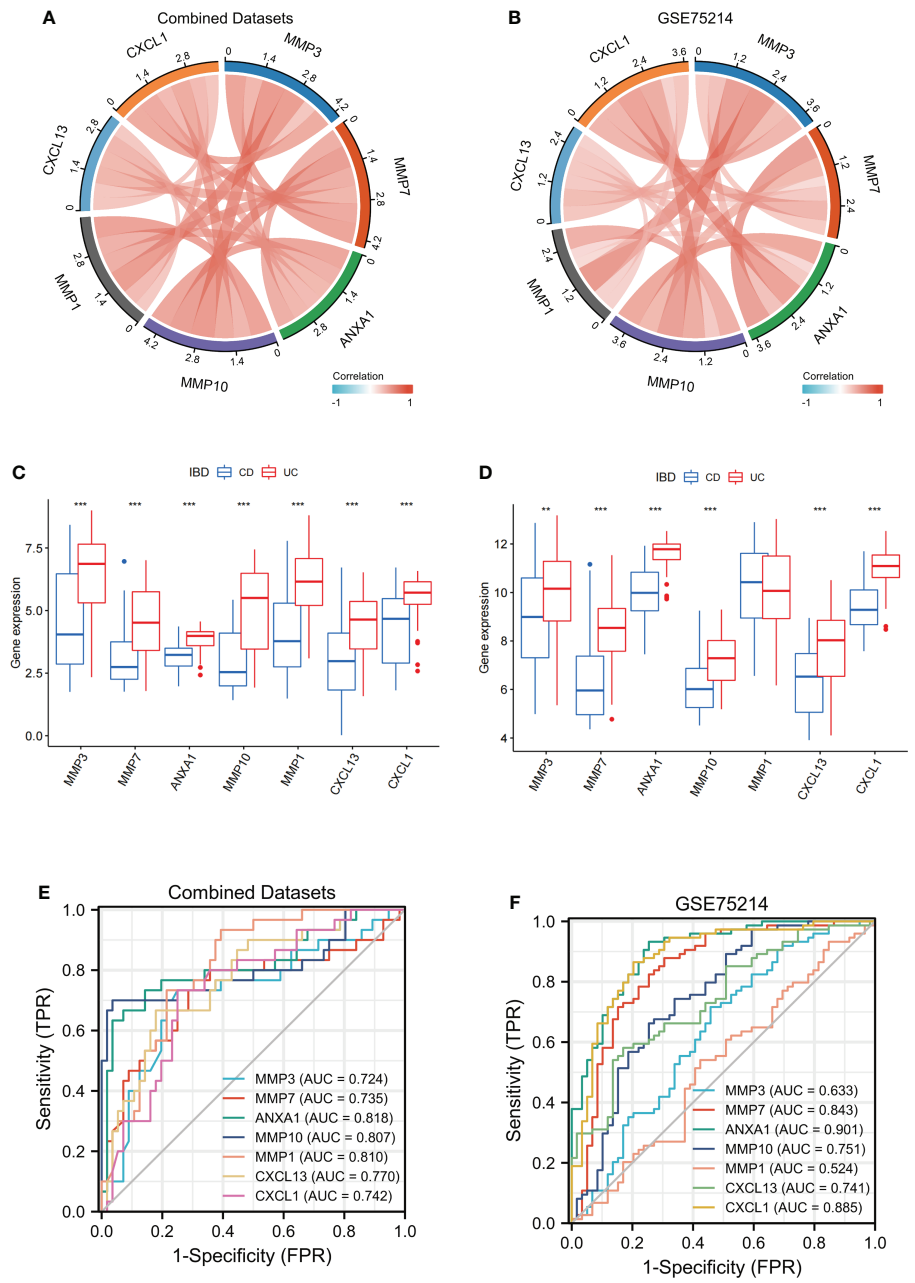

FIGURE 8
Verification of MMP-associated genes in different IBD cohorts The correlation between the levels of internal MMP-associated genes in both Combined Datasets **(A)** and the GSE75214 dataset **(B)** using a Spearman correlation analysis. The differences in MMP-associated genes between CD and UC were examined in both the Combined Datasets **(C)** and the GSE75214 dataset **(D)** using the limma package. ROC curves estimating the diagnostic performance of the MMP-associated genes ANXA1, MMP10, MMP1, CXCL13 and CXCL1 in the discrimination of IBD patients in the Combined Datasets **(E)** and GSE75214 **(F)**. ** indicates P<0.01, *** indicates P<0.001.

patients in both the Combined Datasets and the GSE75214 dataset (Figures 10A, B). The results showed that several immune cell types, including neutrophils and humoral immune cells (naive B cells and follicular helper T cells), were more abundant in UC patients, while a higher level of Treg cells was observed in the CD group. In addition, the correlations between the MMP-associated gene levels and the levels of various immune cell populations were further examined by a

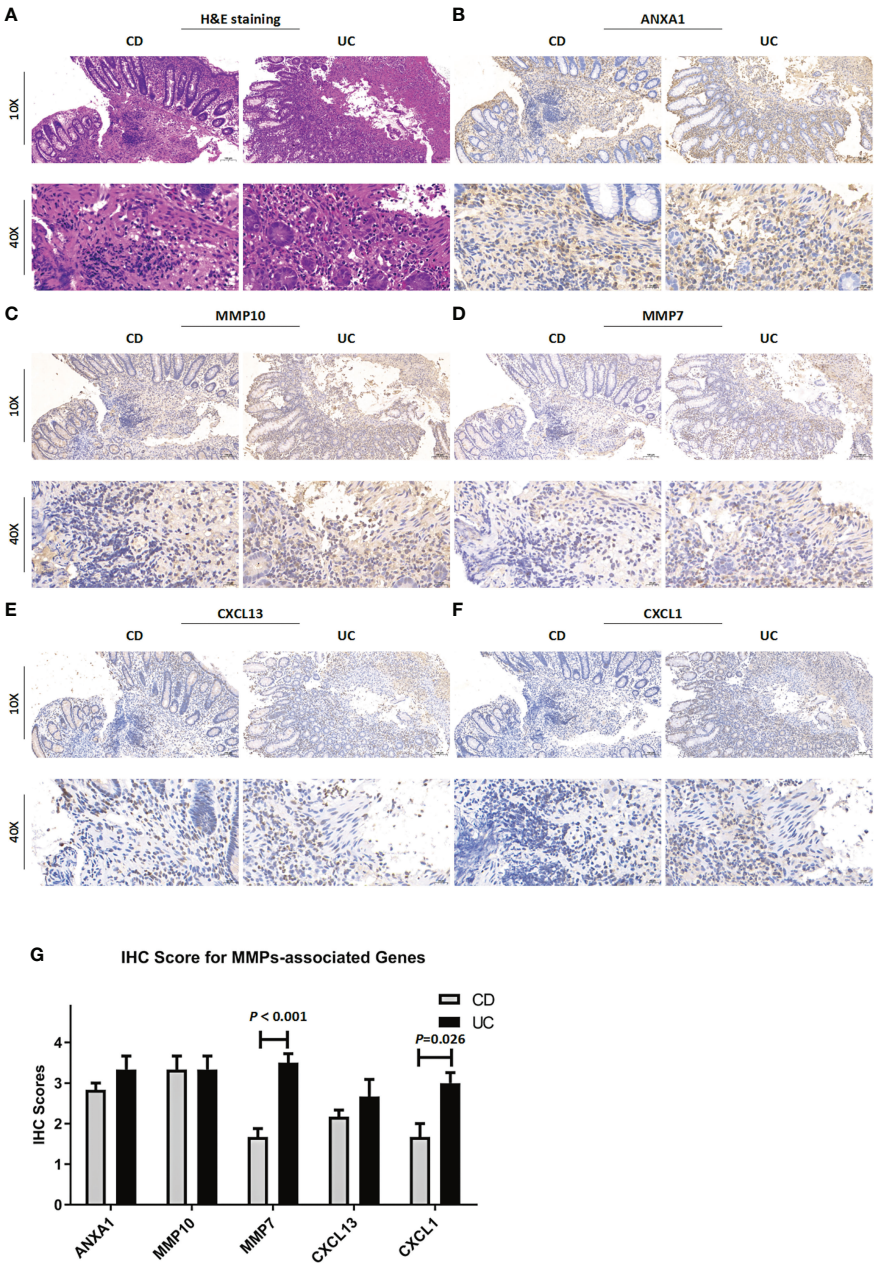

FIGURE 9
Clinicopathological examination of MMP-associated genes in tissue samples. **(A)** Representative images of H&E staining of samples from CD and UC patients. The IHC staining images are representative images of the expression levels of ANXA1 **(B)**, MMP10 **(C)**, MMP7 **(D)**, CXCL13 **(E)** and CXCL1 **(F)** in CD and UC samples. **(G)** IHC score difference analysis of MMP-associated genes in IBD patients. Statistical differences were analyzed by a Mann–Whitney U test.

Spearman correlation analysis (Figures 10C, D, and Supplementary Figure 2), and the results showed that the levels of all MMP-associated genes were statistically significantly positively correlated with the levels of the differentially expressed cell types, indicating their potential value as biomarkers during differential diagnosis.

Investigation of MMP-associated genes in CD and UC *via* single-cell sequencing

Finally, we used single-cell sequencing technology to explore the expression of MMP-associated genes in IBD. Using GSE125527, we analyzed a total of 14 samples, including 7 UC

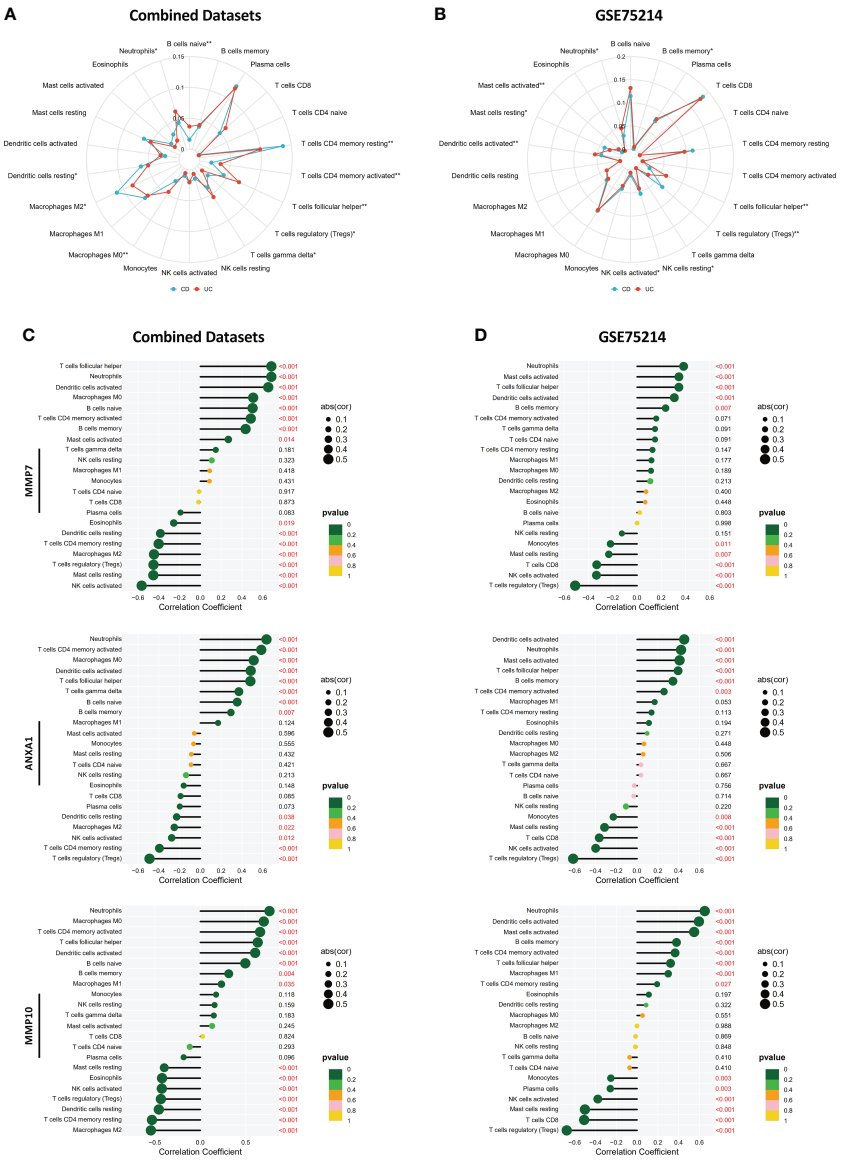

FIGURE 10
Correlations between MMP-associated gene levels and the levels of infiltrating immune cells determined by CIBERSORT. Differences in the levels of infiltrating immune cells between the CD and UC groups in the Combined Datasets **(A)** and the GSE75214 dataset **(B)**. Correlations between the MMP-associated gene levels and immune cell levels were further examined by a Spearman correlation analysis in the Combined Datasets **(C)** and the GSE75214 dataset **(D)**, and results with significant differences are shown (*p < 0.05, **p < 0.01). The results, including data regarding other MMP-associated genes, including CXCL13, MMP3, MMP1 and CXCL1, are illustrated in Supplementary Figure 2.

patients and 7 CD patients. Through harmony integration technology, we reduced the batch differences between the different samples to better analyze gene expression (Figure 11A). Due to the limitation of single-cell sequencing technology in sequencing depth, most MMP-associated genes were not detected, except for ANXA1. The results showed that the expression of ANXA1 in UC was higher than that in CD, and the cells with a high expression of ANXA1 were mainly monocytes, NK cells and γδ T cells (Figure 11B).

Discussion

UC and CD are subtypes of inflammatory bowel disease and are autoimmune diseases influenced by multiple complex factors, including the environment, genetic factors, and the gut microbiota. The specific pathogenesis underlying IBD remains unclear despite extensive research investigating the disease over many years. The clinical treatment strategies used for UC and CD patients are often different. For example, it is recommended that

aminosalicylates should be used as a first-line approach for treating and maintaining remission in UC, but they play a much smaller role in the management of CD (6, 7, 32, 33). In contrast, methotrexate has shown a higher rate of response in CD patients than in UC patients (34–36). However, there remains a large number of IBD patients who are difficult to identify in clinical practice, indicating that it is important to improve the discrimination of the two different subtypes, especially since the results of differential diagnosis affect clinical management (21).

In this study, we systematically collected and integrated published microarray data obtained from CD and UC patients. The first challenge we faced was determining the best strategy for integrating these data to best identify the DEGs. Importantly, the choice of integration method has an impact on the analysis results. If bias is introduced during the integration of the data, the results and their interpretation are inevitably affected. An RRA analysis was adopted to integrate the data to identify the DEGs. RRA is based on a comparison of actual data with a null model that assumes a random order of input lists; then, a P value is assigned to the difference in the levels of each gene in the aggregated list that describes how much better a gene ranked than expected. As an algorithm that is both computationally efficient and statistically stable, RRA has obvious advantages. First, due to scoring based on the order of gene expression, this method is very robust and can accommodate the variable gene content that results from the use of different microarray platforms. Second, even if a gene is not indicated in one platform, it is not eliminated due to the combination of multiple datasets to prevent losing information regarding important genes. This conjecture can also be confirmed from the analysis results. For example, in the GSE75214 dataset, we identified many differentially expressed genes, while relatively few DEGs were identified in the other three datasets, proving the heterogeneity of the data obtained from different central sources (Figures 2A-D).

However, RRA also has shortcomings. For example, when the final results from different datasets are summarized by an RRA, the results obtained from large sample sizes and small sample sizes are given the same weight, which may lead to issues in the representativeness of the final results. Therefore, after conducting the RRA, we used another method of data consolidation. Datasets from the same gene platform were merged with the removal of batch variance. Then, the DEGs were identified in the Combined Datasets and the GSE75214 dataset, and the final DEGs were identified by considering the intersection of the DEGs between the two IBD cohorts. The gene enrichment analysis showed that the pathways enriched by the DEGs identified by the two integration methods were similar, indicating the robustness of the DEG screening results. Another noteworthy finding was that the IL-17 signaling pathway was the most significant pathway enriched in UC. This result was similar to the results of a recently published single-cell sequencing study that reported that IL17A+ T cells are mainly enriched in UC (37).

The DEGs identified by the two different filtering strategies were further used to generate PPI networks, and pivotal gene modules were identified through MCODE. Interestingly, the results from both sets of DEGs indicated that the MMP-associated module was the most important gene set differing between CD and UC patients, providing credible evidence that the MMP-associated module serves as the main functional group associated with the difference between CD and UC.

In future studies, we plan to establish a differential diagnosis model based on the MMP-associated module to assist in the diagnosis of the CD and UC subtypes in IBD patients. To the best of our knowledge, this study offers the first publicly reported mRNA model for the differential diagnosis of IBD, although some studies have reported the role of miRNAs in the differential diagnosis of IBD (20). In this study, combined datasets were used as the training group, and the GSE75214 dataset was used as

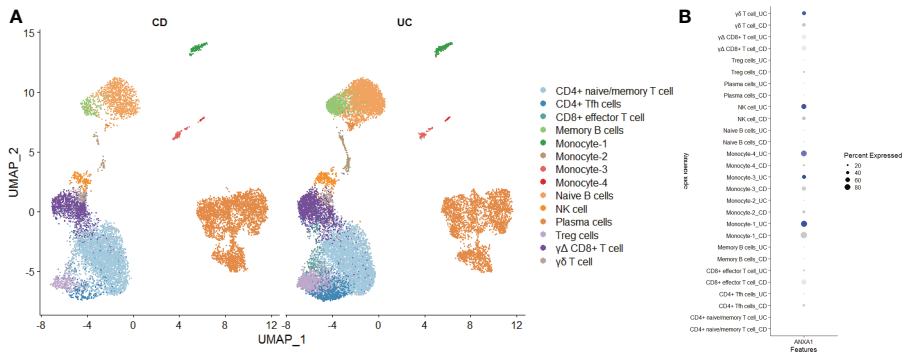

FIGURE 11
Exploring the Expression of MMP-Associated Genes by Single-cell Sequencing **(A)** Intestinal mucosa samples from 14 IBD patients (7 UC and 7 CD) were integrated through the Harmony method and are displayed in a UMAP reduction diagram. **(B)** ANXA1 expression in different cell clusters.

the testing group to generate Model_1 *via* a LASSO logistic regression. From the results of the model evaluation, Model_1 showed good discrimination in both the training group and the testing group. Further studies using a calibration analysis and DCA showed that Model_ 1 exhibited good performance in the training group but failed in the testing group. We believe the reason is that the data obtained from the training set and the testing set were collected from different microarray platforms, resulting in large batch differences between the two IBD cohorts. Although Model_ 1 based on the training set could better distinguish the data obtained from other platforms, it was difficult to meet higher accuracy requirements. This challenge is an inherent defect of diagnostic models obtained based on microarray technology, which also limits the application of such diagnostic models; thus, a diagnostic model can only be applied to clinical data collected from the same microarray platform in real clinical diagnosis.

Based on the above, another model named Model_ 2 was generated. In the new model, we omitted the specific expression values of the genes and converted them into binary variables; thus, we did not need to consider the problem of batch differences. Subsequently, we used the Combined Datasets as the training group and the GSE75214 dataset as the testing group to generate a LASSO logistic regression model. Interestingly, although the continuous variables in the matrix were transformed into binary variables, the genes identified using Model_2 were generally the same as those identified using Model_1 (ANXA1, MMP10, MMP1 and CXCL13 in Model_1 *vs.* ANXA1, MMP1 CXCL13 and CXCL1 in Model_2). In the subsequent model evaluation, we found that Model_2 showed good discrimination in both the training set and the testing set. More importantly, Model_2 also performed well in both the training group and testing group based on the results of the calibration analysis and DCA. Notably, because Model_2 only included 4 genes and the levels of each gene were represented by a binary variable, the actual values obtained using Model_2 included only 16 different values at most, further indicating the reliability of the analysis based on the included genes. Most importantly, the newly included cohort GSE179285 contains CD samples from colon tissue. In the newly added validation results, we performed a model validation using IBD cases containing colonic/ileal CD and IBD cohorts containing colonic CD only, and the validation results were consistent with our expected results. In the subsequent experiment, we explored the diagnostic value of the MMP-associated genes. The results showed that the use of a single-gene diagnostic strategy performed better, and these results were further confirmed in clinical samples.

Matrix metalloproteinases (MMPs) constitute a group of zinc-dependent neutral peptidases that can degrade all components of the extracellular matrix (ECM) and are associated with extensive mucosal degradation and tissue remodeling, which ultimately favor the development of ulcers, fistulae and strictures (38). According to their primary substrate, MMPs can be divided into various subclasses. The genes identified in this research included stromelysins (MMP-3, MMP-7, and MMP-10) and collagenases (MMP-1). To date, sufficient evidence suggests that IBD-related mucosal inflammation is associated with an enhanced induction of several MMPs. For example, a series of pioneering studies documented the abundant expression of MMP-1 (39–42) and MMP-3 (39, 43–46) RNA in gastrointestinal tissue surrounding ulcers, including those present in the gut of IBD patients. Subsequent research has further proven that MMP-7 (47–51) and MMP-10 (47, 48, 52–54) RNA expression levels are increased in the inflamed tissue of UC patients. Considering that adequate functional studies support the involvement of MMPs in IBD-related mucosal degradation, several inhibitors of MMPs have been developed and used to attenuate gut inflammation in animal models of IBD (55–57), and 3 clinical trials investigating MMP inhibitors have been performed in the context of IBD treatment (58–61). However, our research indicates that the MMP-associated module was also the main differential gene set between CD and UC. CIBERSORT showed that MMP-associated genes were closely associated with unique immune characteristics in UC, including higher levels of neutrophils and humoral immune cells (naive B cells and follicular helper T cells) and lower levels of Treg cells than in CD patients.

Regarding the other genes included in the models, chemokine C-X-C motif ligand-1 (CXCL1) is widely known as a strong neutrophil chemoattractant that participates in inflammation in multiple tissues. MMP3-CXCL1 (62) and MMP7-CXCL1 (63) often function as partners in neutrophil activation or as biomarkers of the dysplasia-carcinoma transition in sporadic colorectal cancer. The chemokine CXC ligand 13 (CXCL13), also named B-cell-attracting chemokine-1 (BAC-1) or B-lymphocyte-chemoattractant (BLC), is a CXC subtype member of the chemokine superfamily that serves as an inflammatory mediator linked to B lymphocyte activity and lymphoid-neogenesis (64). Recently, UC was characterized as exhibiting a plasmablast-skewed humoral response associated with disease activity, and a subset of intestinal CXCL13-expressing TFH-like T peripheral helper cells was identified to be associated with the pathogenic B-cell response (65). Another molecule that deserves attention is annexin A1 (ANXA1) because it played an important role in both models. According to early studies of UC, one of the characteristics of an active episode of US is the intense mucosal infiltration of leukocytes, and the proresolution mediator ANXA1 exerts counterregulatory effects on leukocyte recruitment and exhibits elevated levels in sera isolated from active IBD patients (66). Further studies reported that ANXA1 was packaged in extracellular vesicles (EVs) derived from IECs (67), indicating that an analysis of the increasing levels of ANXA1 in IEC-derived EVs may become a specific diagnostic approach for IBD clinical diagnosis (68–71). By conducting a single-cell sequencing analysis,

we searched for ANXA1 expression traces more precisely. The results showed that ANXA1 is mainly expressed in monocytes, including macrophages and dendritic cells. More studies are needed to explore the biological functions of ANXA1 in IBD.

The advantages of this study include the following: 1) to the best of our knowledge, this study is the most recently published study using CD and UC microarray data with the largest sample size and the first to offer an mRNA-based model for differential diagnosis; 2) this study strictly followed the guidelines of the TRIPOD and performed a comprehensive evaluation of the generated model; and 3) the generated Model_2 overcame the problem of batch differences and had good clinical applicability. However, this study also had some shortcomings. The data used during the model establishment were obtained from a public database, and the results need to be verified using a larger amount of clinical data. Although we collected a certain number of clinical samples for IHC testing in our article, the number was small. In particular, due to the lack of examination of difficult cases, it is not possible to assess whether the model can be used as an aid in the diagnosis of difficult IBD cases.

Conclusion

Although both CD and UC are types of IBD and exhibit similar clinical symptoms, there are many differences in the immune landscape. This finding can explain to some extent why CD and UC patients exhibit different responses after receiving the same treatment. In recent years, single-cell sequencing has been used to describe the immune landscape of CD and UC patients, which has enabled a more accurate identification of immune cell differences (37). However, due to the heterogeneity and complexity of IBD, data analyses based on larger sample sizes and multicenter data through high-throughput microarray remains important.

Our study revealed that the MMP-associated module is an important differential functional set in CD and UC, and based on this, we established two models to assist in the differential diagnosis of CD and UC in the clinic. The comprehensive model evaluation demonstrated that the model based on the MMP-associated module had good application value. Subsequent in-depth research investigating how MMPs are involved in the development of different subtypes of IBD is necessary.

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 authors.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of the second hospital of Hebei Medical University. The ethics committee waived the requirement of written informed consent for participation.

Author contributions

JD collected the papers, analyzed the data, and drafted the manuscript. NZ and L-pL analyzed the data. PM and Y-yaZ reviewed the data and conclusions. J-bX and X-pZ contributed to manuscript writing. Z-aC and Y-yuZ presented the idea for this manuscript, supported the funding, analyzed the conclusions, and drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1022850/full#supplementary-material

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Role of metalloproteases in the CD95 signaling pathways

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CD95L (also known as FasL or CD178) is a member of the tumor necrosis family (TNF) superfamily. Although this transmembrane ligand has been mainly considered as a potent apoptotic inducer in CD95 (Fas)-expressing cells, more recent studies pointed out its role in the implementation of non-apoptotic signals. Accordingly, this ligand has been associated with the aggravation of inflammation in different auto-immune disorders and in the metastatic occurrence in different cancers. Although it remains to decipher all key factors involved in the ambivalent role of this ligand, accumulating clues suggest that while the membrane bound CD95L triggers apoptosis, its soluble counterpart generated by metalloprotease-driven cleavage is responsible for its non-apoptotic functions. Nonetheless, the metalloproteases (MMPs and ADAMs) involved in the CD95L shedding, the cleavage sites and the different stoichiometries and functions of the soluble CD95L remain to be elucidated. To better understand how soluble CD95L triggers signaling pathways from apoptosis to inflammation or cell migration, we propose herein to summarize the different metalloproteases that have been described to be able to shed CD95L, their cleavage sites and the biological functions associated with the released ligands. Based on these new findings, the development of CD95/CD95L-targeting therapeutics is also discussed.

KEYWORDS

ADAM, CD95L, cancer, cleavage, inflammation, MMP

Introduction

Different environmental factors (infection, pollution, UV …) involved in chronic inflammatory disorders and cancers affect the expression level and/or the interaction of different receptors and ligands, which in turn alter intracellular signaling pathways, subsequently leading to pathophysiological phenotypic changes. Death receptors (DR) are transmembrane receptors that can implement cell death signals *via* apoptosis,

necroptosis, pyroptosis or ferroptosis. Ligands of the tumor necrosis factor (TNF) family and their receptors (TNF-R) are cytokines contributing to the induction of a caspase-dependent apoptotic death. Interestingly, these so-called "death receptors" can also trigger non-apoptotic signaling pathways involved in cell migration, differentiation, survival, and proliferation (1–5).

Six human death receptors (DRs) have been identified, TNF-R1 (6, 7), CD95 (Fas/APO-1/TNFRSF6) (8, 9), TRAIL-R1 (DR4) (10), TRAIL-R2 (DR5) (11, 12), DR3 (TRAMP) (13–16), and DR6 (also known as TNFRSF21 (17)). These death receptors are activated by TNF (18), CD95L (also known as FasL or CD178) (19), TRAIL (20), and TL1A, respectively (21), with the ligand for DR6 remaining to be confirmed even if amyloid precursor protein represents a solid option (22, 23). Apoptosis is finely regulated by these DRs, and mutations or expression deregulation of these receptors lead to various diseases (auto-immune, neurodegenerative, heart diseases or cancer) and development of chemoresistance (24).

CD95 and CD95L

CD95 is a ubiquitously expressed transmembrane receptor, which belongs to the TNF-R family (8). Its natural ligand, CD95L is a transmembrane protein involved in the induction of a caspase-dependent apoptotic signal (8, 25, 26). The CD95/CD95L pair contributes to immune homeostasis and surveillance, and different mutations mainly localized within the CD95 death domain (DD), an intracellular region involved in the recruitment of the adaptor protein Fas-Associated protein with Death Domain (FADD), have been associated with breakdown of self-tolerance in autoimmune lymphoproliferative syndrome (ALPS) patients (27, 28) and Lpr^{Cg} mice (29, 30). CD95 mutations have also been detected in lymphoma pushing the authors to classify CD95 as a tumor suppressor gene (31, 32). Although DD-localized CD95 mutations foster tumor progression by rendering tumor cells resistant to the apoptotic response (33), new and accumulating evidence support that this receptor exerts more complex biological functions, and might promote oncogenesis and inflammation/auto-immunity independently of its ability to trigger cell death (3, 34–36).

For CD95L, rare mutations have been reported in human and are associated with lupus (37) or ALPS type Ib (38, 39) pathologies. The *gld* (for *generalized lymphoproliferative disease*) mice also display a lupus-like phenotype and harbor a mutation in CD95L with the replacement of its phenylalanine 273 by a leucine (F273L). This mutation reduces the efficiency of CD95/CD95L interaction (40).

Interestingly, CD95L might also interact with another TNFR member, DR5 (41). The authors show that, although the CD95L affinity for DR5 was weaker than that for CD95 ((K_D was $1.23x10^{-12}$ M for DR5–CD95L *versus* $6.01x10^{-13}$ M for DR5–TRAIL), CD95L can compete TRAIL for DR5 binding, suggesting that both ligands share a similar interaction region in DR5 (41). More importantly, the CD95L/DR5 interaction has been suggested to promote arthritis severity in a mouse model (*i.e.*, autoantibody-induced arthritis). Surprisingly, the K_D of CD95L for DR5 assessed in this study is far higher than that currently measured for CD95 (K_D comprised between and $7x10^{-8}$ and $2x10^{-9}$M (42–45), suggesting that CD95L would possess a stronger affinity for DR5 than for its own receptor. This conclusion remains to be strengthened with structural methods to definitively validate the CD95L/DR5 interaction.

At least, two main forms of CD95L exist. The transmembrane CD95L (m-CD95L) triggers cell death when it interacts with CD95-expressing cells, while metalloproteases can release soluble CD95L (s-CD95L) (46–48). Expressed by activated B and T-cells, m-CD95L contributes to the immune contraction (49) and its expression by myeloid cells participates in tissue inflammation by recruiting macrophage in damaged spinal cord (50). In this latter study, the role of m-CD95L and/or s-CD95L in the inflammatory process remains to be addressed. Contradicting studies exist on s-CD95L; while soluble CD95L can trigger apoptosis and promote lung damage in acute lung injury (ALI) (51, 52), it fails to induce cell death but rather stimulates inflammation in chronic autoimmune disorders such as systemic lupus erythematosus (SLE) (34, 48) and metastasis occurrence in cancers (53–57). Such a discrepancy might be ascribed to the stoichiometry of s-CD95L (43, 58), which seems to rely on the presence or absence of juxtamembrane N-terminal end (51, 59). In this respect, metalloproteases involved in the m-CD95L shedding as well as their preferential cleavage sites within the stalk region will directly impact the N-terminal length of s-CD95L (Figures 1A–C) end and thereby, its biological function as discussed below. It has been reported that m-CD95L can be shed close to its transmembrane domain releasing a s-CD95L encompassing a stalk region both in mouse (43, 60) and human (43, 61). This stalk region promotes the aggregation and the cytotoxic activity of s-CD95L. These observations point out that the presence or absence of certain metalloproteases involved in the CD95L shedding, might be responsible for the release of different ligands that either trigger cell death or aggravate inflammation or oncogenesis.

Cloning

CD95L/FasL, cloned in 1993 (19), is a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Northern hybridization revealed that the ligand is mainly expressed in activated splenocytes and thymocytes, consistent with its involvement in T cell-mediated cytotoxicity and immune homeostasis. This ligand is also detected in several nonlymphoid tissues, such as testis (19). In 1989, a monoclonal antibody (mAb) APO-1 isolated by Peter Krammer's group killed many tumor cells (25). This antibody recognizes CD95, a transmembrane receptor cloned in 1991 by the Nagata's team (8, 9).

CD95L and CD95 structures

As aforementioned, CD95L is a type II transmembrane protein that encompasses a long cytoplasmic intracellular domain, a transmembrane (TM) domain, a stalk region and a TNF homology domain (THD) (Figure 1A). The THD adopts a 'jelly-roll' topology that participates in the ligand homotrimerization and its interaction with CD95 (62). CD95L can be cleaved within its stalk region (amino acid residues 103 to 143) by different proteases (Figure 1B). Of note, only 3 cleavage sites over 5 are conserved between human and mouse (Figure 1C) suggesting that either different proteases or different sites are involved between these two species or that the main cleavage sites correspond to the three conserved sequences. The intracellular N-terminal region of CD95L is long and contains different domains including a casein kinase I (CKI) substrate motif (SSASS in human) and a proline-rich domain (PRD) (63)(Figure 1A). CD95L PRD interacts with proteins containing SH3 and/or WW domains (*i.e.*, SH3 domain of Src kinase $p59^{Fyn}$) and these interactions seem to regulate the expression level and stability of CD95L (64, 65). In addition, PRD contributes to the CD95L-mediated reverse signaling (66, 67). Like TNF (68), the CKI domain of CD95L might also participate in the reverse signaling. In addition, the intracellular region of CD95L can be cleaved by signal peptide peptidase-like 2a (SPPL2a) releasing an intracellular peptide, trafficking to the nucleus to inhibit transcription (69). The biological role of SPPL2a cleavage and its cleavage site remain to be elucidated.

CD95 contains three extracellular cysteine-rich domains (CRDs) (70). While CRD1 is responsible for pre-association of the receptor at the plasma membrane and has been named the pre-ligand binding assembly domain (PLAD) (71–73), both CRD2 and CRD3 regions contribute to ligand binding (74). Although CD95 does not possess any enzymatic activity, its cytosolic region encompasses a death domain (DD) (75) involved in the apoptotic signal, and a juxtamembrane domain interacting with ezrin (76) and phospholipase Cγ1 (48, 77, 78) to promote neurite growth or cell migration, respectively. Through protein-protein interactions (PPIs), the 80-amino acid containing DD recruits the Fas-Associated protein with Death Domain (FADD), which in turn binds and aggregates the pro-caspase-8 (79). This complex, designated death inducing signaling complex (DISC), initiates apoptosis (79). The juxtamembrane region interacts with different partners to trigger the motility-inducing signaling complex (MISC) formation implementing a Ca^{2+} response, and the subsequent induction of non-apoptotic signaling pathways (2, 76, 80, 81).

Extracellular matrix and metalloproteases

Extracellular matrix (ECM) is composed of a large number of structural and functional components that includes enzymes, collagens and proteoglycans, which are secreted and self-assembled into the immediate cellular environment (82). Other non-proteoglycan matrix components include hyaluronic acid, fibronectin, elastin, and laminin. This ECM provides structural support to cells and an integral signaling network through the action of different cytokines and growth factors interacting with the matrix components (83–86).

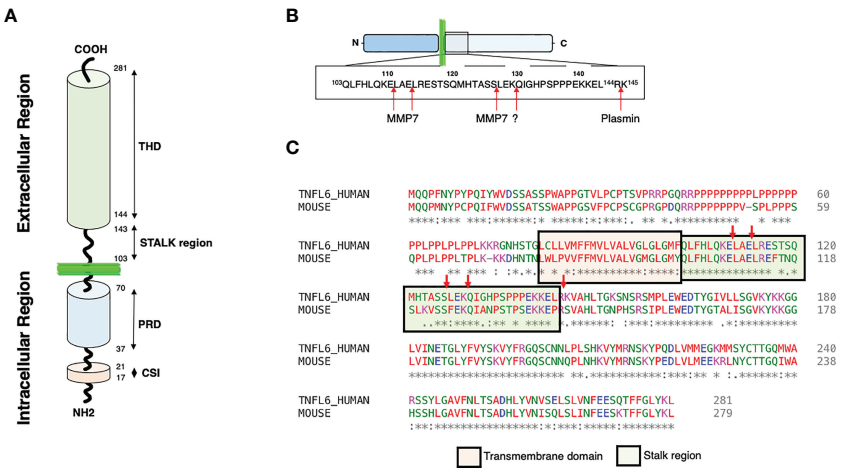

FIGURE 1
CD95L structures and cleavages sites. **(A)** Representation of CD95L domains. Proline rich domain: PRD; Casein kinase I substrate motif: CSI; TNF homology domain: THD. **(B)** Representation of the different cleavage sites described within the CD95L stalk region. **(C)** Alignment of human and mouse CD95L protein sequence using Clustal omega (1.2.4). The transmembrane and the stalk domains are represented.

Forinstance, binding of the s-CD95L to ECM, and more specifically to fibronectin, transforms the non-apoptotic ligand into a potent death inducer (87) suggesting that immobilization and/or aggregation of the s-CD95L homotrimer can foster the induction of the apoptotic response. In agreement with this observation, although a soluble and homotrimeric CD95L fails to trigger apoptosis, its hexameric counterpart (58) can do it. We also observed that the more CD95L is aggregated, the more its ability to induce apoptosis is increased (88).

Most of the ECM protein components are processed by matrix metalloproteinases (MMPs). In human, this family of zinc-dependent endopeptidases englobes 23 members sharing structural domains (89, 90). These proteases are mainly secreted within the pericellular and extracellular space (61) but can also be anchored to the cell surface (91) or adopt an intracellular localization, that has been correlated in certain cases to non-proteolytic functions (90, 92). Except during specific stages of development involving tissue remodeling (*e.g.*, embryogenesis) and wound healing processes, there is no constitutive expression of MMPs at homeostasis. Once secreted, these enzymes coexist within the extracellular space under latent forms (zymogens) and active forms, whose proteolytic activity is finely tuned by endogenous inhibitors such as tissue inhibitors of metalloproteases (TIMPs) or alpha-macroglobulin.

Recent N-terminomics and proteomics techniques have been used to profile hundreds of cleavage sites in proteomes associated with MMP activity, which reveal that more than two-third of MMP substrates are non-ECM proteins. Accordingly, far beyond their capacity to collectively cleave the ECM substrates, MMPs can process chemokines, cytokines, cell-surface receptors, growth factors, and nuclear proteins. Thus, MMPs are involved in inflammatory response, angiogenesis, cell-to-cell communication and cell proliferation, and the deregulation of their activity contributes to the progression of many diseases including cancer, chronic inflammatory disorders, vascular and central nervous system diseases (90).

MMPs are classified according to their linear sequence similarity, domain organization and substrate specificity (90). All the MMPs share a minimal N-terminal region, consisting in a signal peptide, a pro-domain and a metalloprotease/catalytic domain (90) (Figure 2A). Except for MMP-7, -26 and -23, all MMPs encompass a hemopexin-like C-terminal region, which is important in determining substrate specificity and interaction with tissue inhibitors of metalloproteinases (TIMPs). This C-terminal domain plays also an important role in cell migratory function of certain MMPs. Gelatinase-A (MMP-2) and gelatinase-B (MMP-9) contain fibronectin type-II inserts within their catalytic domain. These inserts confer the ability to bind and cleave gelatin and collagen.

Membrane-type MMPs (MT-MMPs) are embedded in the plasma membrane of the cells *via* a transmembrane domain or a glycosylphosphatidylinositol (GPI)-anchor (Figure 2A). This family includes the transmembrane proteins MMP-14, MMP-15, MMP-16, and MMP-24, and the GPI-anchored proteins MMP-17 and -25

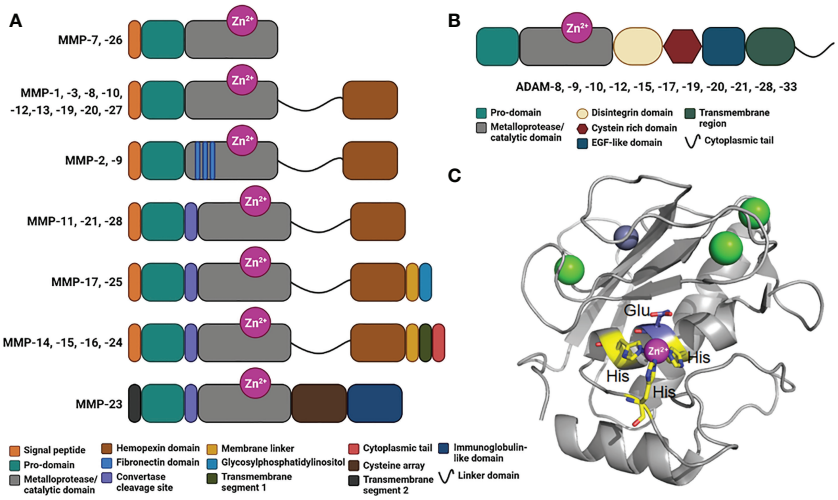

FIGURE 2
Domains in human MMPs and ADAMs. **(A)** Schematic representation of the domains in human MMPs consisting in a signal peptide, a prodomain, a metalloprotease/catalytic domain, a linker domain, a hemopexin domain, fibronectin inserts, a convertase cleavage site, a membrane linker, a glycosylphosphatidylinositol, a transmembrane segment 1, a cytoplasmic tail, a transmembrane segment 2, a cysteine array and immunoglobulin-like domain. **(B)** Schematic representation of ADAMs organized in modules consisting in a prodomain, a metalloprotease/catalytic domain, a disintegrin domain, a cysteine rich domain, an EGF-like domain, a transmembrane region and a cytoplasmic tail. **(C)** Crystal structure of a typical Metalloprotease/catalytic domain in cartoon representation (hMMP-12, PDB code: 4GQL), with catalytic zinc ion as magenta ball, His residues chelating the catalytic zinc ion in yellow stick, catalytic glutamic acid residue in blue stick, and structural zinc and calcium ion as grey and green balls, respectively.

(93). Some MMPs harbor a furin-like convertase cleavage site (Figure 2A), which is intracellularly cleaved to activate the protease and promote its distribution at the cell surface. MMP-23 is a unique MMP that contains a cysteine array and immunoglobulin-like domain, whose exact role remains elusive.

Within the extracellular space, a disintegrins and metalloproteinases (ADAMs) family can also exert a proteolytic activity (94–96). The main substrates for ADAMs are type I and II transmembrane proteins, which make them as shedding proteases. However, these proteases are also capable of processing cytokines and growth factors (95). Interestingly, in the case of transmembrane proteins, the cleavage consistently occurs between 10 and 15 amino acids from the plasma membrane. Like MMPs, ADAMs possess several domains, including a pro-domain, a metalloprotease/catalytic domain, a disintegrin domain, a cysteine rich domain, an EGF-like domain, a transmembrane domain and a C-terminal cytoplasmic tail (Figure 2B). All ADAMs contain a disintegrin domain, which can bind to integrins from adjacent cells, with potential consequences in cell adhesion and migration. These metalloproteases are implicated in different diseases including cancer (95), systemic inflammation (96), cardiovascular diseases and atherosclerosis (97, 98). A critical role in kidney pathologies (99) and in immunity (100) has also been documented.

Both MMPs and ADAMs belong to the superfamily of metzincin proteases. These metzincins share a conserved HEXXHXXGXXH motif within their metalloprotease/catalytic domain, where the three histidine residues bind to the catalytic zinc ion and the glutamate, as a general acid base, and activates a water molecule required for the peptide bond hydrolysis (Figure 2C).

MMPs, ADAMs and CD95L regulation

CD95L can be cleaved by several metalloproteases, including MMPs and ADAMs, to release different soluble CD95Ls (s-CD95Ls), which have been reported to induce cell proliferation, migration, survival (36) but also cell death (51, 59). Rendering more complex to predict the biological outcome of s-CD95L, this ligand can also interact with other TNFR members, including as aforementioned, DR5 (41) or the soluble receptor DcR3 (44). Despite the complexity of the signaling pathways induced by the different forms of s-CD95L and their implication in the progression of different pathologies including chronic inflammatory disorders and cancers only a limited structural knowledge exists on these s-CD95Ls.

Metalloproteases and CD95L

Thirty years after CD95L cloning, it remains difficult to address what are the MMP/ADAMs responsible for the cytokine shedding, where the protease cleaves m-CD95L and whether the released soluble factor triggers non-apoptotic (34, 41, 55, 57, 78, 80, 101) or apoptotic outcome (51, 59, 102).

Some ADAM members have been described to contribute to the generation of s-CD95L. Indeed, both ADAM10 (69, 103) and ADAM17 (104) can cleave m-CD95L to release s-CD95L. ADAM10 can also shed the transmembrane TNFα (105). As aforementioned, a second step occurs following ADAM10-mediated cleavage, with the SPPL2a-mediated cleavage of the CD95L intracellular region to release a cytosolic domain modulating gene expression (69). MMP7 also cleaves the transmembrane CD95L but the biological role of the released ligand remains difficult to apprehend. While from prostate epithelial cells, MMP7 can release a soluble and cytotoxic CD95L, which is involved in the involution of the organ in rat (106), the same metalloprotease in human sheds membrane-bound CD95L from tumor cells to protect them from doxorubicin or oxaliplatin-induced cell death in human (107, 108). S-CD95L is increased in sera of human idiopathic pulmonary fibrosis (IPF) and bleomycin-induced lung fibrosis in mice and this ligand prevents the elimination of fibrotic-lung myofibroblasts by CD95L-expressing T cells (109). Accordingly, MMP-7 knock-out mice exhibit resistance to the bleomycin-induced lung fibrosis, probably because these animals fail to cleave CD95L and generate the anti-apoptotic soluble ligand (109). Of note, MMP7 also cleaves the receptor of CD95L, CD95 and by doing so, promotes its ability to implement non-apoptotic signaling pathways in cancer cells (45, 110).

Regarding the cleavage positions within the CD95L stalk region, *in vitro* analyses revealed that MMP-7 is likely to cleave before the two leucine residues in the amino acid residues ^{110}ELAELR^{115} conserved between human and mouse sequences (Figures 2B, C) (111). This sequence is at proximity of the plasma membrane bilayer suggesting that the released ligand might exert an apoptotic function because it conserves a full-length stalk region. As above mentioned, the stalk region of CD95L seems to exert a pivotal role in the apoptotic property of the soluble ligand (59). For instance, conservation of the stalk region (Figures 1A, C) in the soluble CD95L dosed in acute respiratory distress syndrome (ARDS) engenders a cytotoxic ligand killing the alveolar epithelial cells by apoptosis (51). Mutations of the ^{110}ELAELR^{115} sequence do not completely abrogate the release of s-CD95L, because MMP7 might process m-CD95L at an additional position between ^{126}SL^{127} (111), which, in this case, generate a non-apoptotic cytokine regarding the loss of the stalk region. Tschopp's team also highlighted a cleavage of the transmembrane CD95L between amino acid residues ^{126}SL^{127} (47), while Nagata's team observed a processing between ^{129}KQ^{130} (46, 112). The protease(s) involved in these shedding was/were not identified and although the cleavage sites diverge, both groups came with the conclusion that the metalloprotease-cleaved CD95Ls do not trigger apoptosis.

In rheumatoid arthritis (RA), MMP3 has also been suggested to cleave m-CD95L and accumulate s-CD95L in the synovial fluid of these patients (113). The role of s-CD95L in RA remains to be elucidated.

In neuronal and glial cells, preclinical studies showed that MMP9 contributes to the motor neuron cell death in amyotrophic lateral sclerosis (ALS) patients by regulating TNF-α and CD95L expression (114). Selective inhibition of MMP-9 activity has also been shown to increase in the m-CD95L/s-CD95L ratio on neonatal monocytes (115). Macrophages exposed to bacteria (*i.e.*, Escherichia coli infection) undergo an increase in CD95L expression (115) and the up-regulation of MMP-9 in these cells protects them from an autocrine and/or paracrine precocious phagocytosis-induced cell death by shedding the transmembrane CD95L.

Plasmin, a serine protease, can also cleave CD95L between amino acid residues Arg^{144} and Lys^{145} (Figure 2B) and although the released CD95L is devoid of its stalk region, it can still trigger cell death in endothelial cells (102). In conclusion, not only the identification of the amino acid sequence, but also the structure and stoichiometry of the soluble CD95Ls present in the different chronic inflammatory disorders and cancers must be realized to apprehend the biological role of each CD95 ligand.

MMPs and cancer

Many studies have reported the expression of MMPs in human cancers. However, what was originally thought about their detrimental roles has been challenged these two last decades. Indeed, an overexpression of certain MMPs does not necessarily imply the promotion of tumor or metastasis. In this respect, at least 10 MMPs have been reported to have protective roles in cancer (116). Among the "oncogenic" MMPs, MMP-2 and MMP-9 have been implicated as the most important prognostic factor in cancer microenvironment (117, 118). MMP-2 is correlated with the development of different types of cancers and associated with poor prognosis (119, 120). MMP9 contributes to the ECM remodeling and the release of membrane-bound proteins and thereby, might favor cell invasion and poor prognosis (121, 122). Other MMPs such as MMP3, MMP-7, MMP-11, and MMP-13 also participate in cancer development (123–128). With MMPs, ADAM10 is up-regulated in gastric cancer lesions compared with adjacent non-cancerous tissues (129). It remains to evaluate whether these metalloproteases could affect oncogenesis by reducing the quantity of membrane-bound CD95L or increasing the concentration of soluble CD95L. Numerous small-molecule MMP inhibitors (MMPi) have been developed but systematically failed in late-stage clinical studies (91, 130). Beside their poor pharmacokinetics and low oral availability/inability, this major failure has been mainly attributed to their lack of specificity within the MMP family and towards other metalloenzymes. Benefiting from a better understanding of MMP biology that emphasizes the necessity to selectively target one single MMP in a given pathological context, a new generation of selective MMPi has emerged recently (131). To achieve a better selectivity, several strategies have been deployed. Regarding the small-molecule inhibitors they mainly consist in either replacing the hydroxamic acid group found in most of broad spectrum MMPis by a weaker Zn^{2+} chelating moiety (132, 133) or targeting exclusively the S1' pocket which significantly differ between the MMPs (131, 132). Alternatively, the development of surrogates of MMPs endogenous inhibitors such as TIMP analogs or targeting MMP gene expression using mRNAs have also been explored. Despite these improvements, finding the right balance between activity, selectivity and ADMET parameters still remain challenging and the timing of MMPi application is critical to achieve the desired therapeutic effect, as the "window of opportunity" is often in premetastatic disease (91, 130, 134).

CD95L, metalloproteases and cancer

Accumulating evidence highlight the pro-oncogenic role of CD95 and CD95L pair. Although the elimination of CD95 expression in some colorectal tumors was reported to predict metastatic tumor recurrence (135), most of the analyses indicate that CD95 expression is maintained in these tumors and contributes to activate pro-oncogenic signaling pathways (136). On the other side, the expression of membrane CD95L and CD95 expression is gradually increased during progression from (early) adenoma to colorectal carcinoma (56, 137). Overexpression of CD95 in apoptosis-resistant 3LL cells makes them apoptosis-sensitive *in vitro* (138) but, transplantation of these cells into mice, reveals a tumor growth advantage as compared to control cells. This underscores the importance of investigating a mechanism within an environment that resembles the clinical situation as much as possible. The seminal experiments establishing the oncogenic role of CD95 came from the elimination of the receptor in two mouse models of cancers (*i.e.*, ovarian and liver cancers), which was associated with the significant reduction of cancer occurrence and growth (35). More recently, we observed that the expression of CD95 is maintained in triple negative breast cancer (TNBC) cells to regulate the NF-κB signaling pathway (139). Accordingly, CD95 loss in TNBC cells stimulates an inflammatory signal, which contributes *in vivo* to the anti-tumor activity of natural killer (NK) cells (140). Therefore, although soluble CD95L is an attractive target to develop drugs and prevent metastasis dissemination of TNBC cells (57), it might be more appropriate to develop therapeutics targeting CD95.

Accumulating evidence support that s-CD95L promotes tumor development and metastasis but the MMPs or ADAMs involved in this process remain to be elucidated. The identification

of i) the MMPs/ADAMs and ii) their cleavage sites in CD95L will help us to identify how many s-CD95Ls exist *in vivo*, and anticipate their stoichiometry to better predict their biological effects on the immune response and the tumor progression.

Targeting CD95/CD95L in clinic, what next?

As aforementioned, CD95 can induce a broad range of signaling pathways, with different biological outcomes. This is related to a fine-tuned control of CD95 aggregation, conformation, distribution within plasma membrane sub-domains and post-translational modifications. These parameters rely on the quality of the CD95/CD95L interaction (141). MMPs and ADAMs are responsible for the generation of soluble CD95L, that might promote metastatic occurrence in cancers or stimulate trafficking/activation of immune cells in chronic inflammatory disorders and thus, inhibiting MMP or ADAM activity could represent an attractive therapeutic strategy in these pathologies (Figure 3). In addition, inhibition of the non-apoptotic signaling pathways downstream s-CD95L/CD95 interaction might also represent an attractive option to treat certain cancers and chronic inflammatory disorders. Asunercept (initially called APG101) is a decoy receptor encompassing the extracellular region of CD95 fused to the Fc domain of human IgG1. APG101 interacts with CD95L, both transmembrane and soluble forms (Figure 3), and abrogates all signals induced by these ligands. Asunercept in phase I/II clinical trials exhibits encouraging therapeutic effect on myelodysplastic syndromes (142) and glioblastoma (143, 144). In addition, the therapeutic value of this drug is also under evaluation (NCT04535674) in COVID-19 patients, in whom CD95L inhibition might protect against the macrophage/neutrophil-driven damage of epithelial cells (145). Although the clinical outcomes of these trials are motivating, it remains that APG101 blocks both apoptotic and non-apoptotic signals, rendering difficult to discriminate the role of each cellular response in the pathogenesis. We recently developed a drug (i.e., peptidomimetic) neutralizing in a selective fashion, the CD95 non-apoptotic pathway (78). This drug, designated DB550, disrupts the CD95/PLCγ1 interaction and the subsequent calcium signaling pathway, which is mandatory for cell migration (77). DB550 injection in SLE-prone mice prevents Th17 cell transmigration in inflamed kidneys and alleviates clinical symptoms (78). These findings support that the selective inhibition of CD95-mediated non apoptotic pathways might turn out sufficient to treat cancers and chronic autoimmune disorders in which s-CD95L is up-regulated (36).

Regarding m-CD95L shedding, another alternative to selectively block the CD95-mediated non-apoptotic signal is to prevent the generation of s-CD95L by inhibiting metalloproteases. Beyond the fact that metalloproteases are pleotropic enzymes, whose inhibition will engender clinical outcomes difficult to predict, an additional concern is the accumulation of membrane-bound CD95L that, might favor the elimination of certain cancer or immune cells, but might also engender undesired tissue damage (Figure 3). Finally, another therapeutic approach

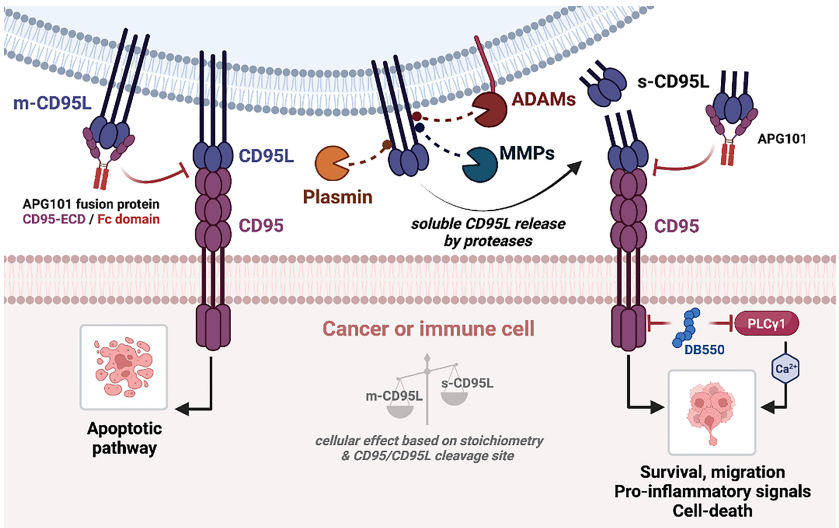

FIGURE 3
CD95/CD95L-mediated signaling pathways. (Left) Binding of m-CD95L to CD95 induces an apoptotic signaling pathway. (Right) m-CD95L processing by proteases (ADAMs, MMPs, plasmin) leads to the release of different s-CD95L in the extracellular environment. Depending on the ratio m-CD95L/s-CD95L, and the shedding sequence, several signaling pathways can be triggered: cell survival, migration (promotes the development of metastases), chemoattraction and pro-inflammatory signal, or cell death. Blocking of CD95L binding to CD95 by APG101 (Asunercept) blocks both apoptotic and non-apoptotic signaling pathways.

for cancer patients could be to develop methods to extinguish the CD95 expression itself. Indeed, we recently observed that the elimination of CD95 in triple negative breast cancers induces a pro-inflammatory signal and promote the anti-tumor activity of NK cells (139, 140).

Author contributions

LD, NG, SB, AC, MJ and PL wrote the original draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

PL and MJ are involved in patents protecting the use of CD95 or CD95L in chronic inflammatory disorders and cancers WO2014118317; WO2015189236; WO2015158810; WO2015104284; WO2017149012; WO2018130679.

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

Publisher's note

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Immunomodulatory role of metalloproteases in cancers: Current progress and future trends

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Metalloproteinases (MPs) is a large family of proteinases with metal ions in their active centers. According to the different domains metalloproteinases can be divided into a variety of subtypes mainly including Matrix Metalloproteinases (MMPs), A Disintegrin and Metalloproteases (ADAMs) and ADAMs with Thrombospondin Motifs (ADAMTS). They have various functions such as protein hydrolysis, cell adhesion and remodeling of extracellular matrix. Metalloproteinases expressed in multiple types of cancers and participate in many pathological processes involving tumor genesis and development, invasion and metastasis by regulating signal transduction and tumor microenvironment. In this review, based on the current research progress, we summarized the structure of MPs, their expression and especially immunomodulatory role and mechanisms in cancers. Additionally, a relevant and timely update of recent advances and future directions were provided for the diagnosis and immunotherapy targeting MPs in cancers.

KEYWORDS

metalloproteases, immunomodulatory, cancers, diagnosis, therapy

1 Introduction

Metalloproteinases (MPs) efficiently hydrolyze proteins and peptides. MPs comprise the largest of the five groups of proteases in the human genome and can be divided into two subgroups: endopeptidase and exopeptidase. Endopeptidases are split into three main families; matrix metalloproteinases (MMPs), A disintegrin and metalloproteases (ADAMs), and ADAMs with thrombospondin motifs (ADAMTS) (1, 2). It is well known

that metalloproteinases initially produced were in inactive zymogens form, which were activated by intracellular and extracellular proteins such as plasmin and even active members of their family (3). And recently, several new mechanisms have been found increasing a few complications of the regulation of metalloproteinases activity, including binding to the molecules of ECM or some specific cells, such as cancers cells and immune cells (4). For instance, the proteolytic activity of MMP7 can be increased after it's catalytic site interacted with cholesterol sulfate on the surface of colon cancer cells (5). Active metalloproteinases are dependent on metal ions that are inhibited by tissue inhibitors of metalloproteases (TIMPs) as well as other metal chelating agents such as ethylene diamine tetra-acetic acid and phenanthroline (2, 6, 7).

Studies have shown that MPs are involved in multiple biological and pathological processes including inflammatory and immune interactions, protein homeostasis, processing of peptide hormones, release of cytokines and growth factors, metabolism of antibiotics, cell migration and invasion and tissue morphogenesis (8, 9). Recent studies have demonstrated that MPs and their inhibitors are closely related to the diagnosis, treatment and prognosis of cancers, especially in immunomodulation during tumorigenesis and cancer development (10–12). In fact, MPs not only regulate tumor immunomodulation through signal transduction pathways, but also influence the tumor microenvironment (TME) (13, 14). Most cancers are associated with a TME rich in various immune-infiltrating cells and factors produced by these cells that induce host cells to differentiate and produce growth factors, cytokines, and chemokines that are conducive to tumor cell survival and metastasis (15). MPs can modulate immunoregulatory factors and other immune-related proteins containing cytokine receptors, notch receptors, phagocytic receptors and cell adhesion molecules, all of which play significant roles in immune system function (11, 16).

MPs are also responsible for matrix protein degradation and remodeling of the extracellular matrix (ECM), either directly or through the liberation of growth factors and cell surface receptors (14). The ECM, a non-cellular three-dimensional macromolecular network structure secreted by cells, is composed of collagen, proteoglycan/glycosaminoglycan, elastin, fibronectin, laminin and other glycoproteins (17). Proteoglycan and hyaluronic acid, which are rich in the ECM, have been found to be elevated during inflammatory progression in a variety of diseases, including cancers (18, 19). The ECM becomes highly dysregulated in tumors and the loss of matrix homeostasis is considered a hallmark of solid tumors (20). Moreover, the ECM contains bioactive motifs that can directly modulate immune responses in cancer (21). For a long time, studies of tumor invasion and metastasis have focused on the inherent adhesion and migration ability of tumor cells themselves. MPs are known to assist tumor cells to break through the tissue barrier *via* adhesion and protease hydrolysis, and facilitate ECM remodeling during the migration process, leading to invasion and metastasis (22, 23).

In this review, we provide an overview of MPs structure, expression and regulation in various tumors, principally MMPs, ADAMs and ADAMTS, with a focus on their emerging immunomodulatory role in cancers. We also review investigational MP-targeting therapies.

2 The structure, expression and role of MPs in cancer

There are numerous members of the MP family that have similar but not identical structure. Importantly, each MP has a varying but equally important roles in the occurrence and development of various cancers (Figure 1 and Table 1).

2.1 Matrix metalloproteinases

The MMP family consists of 28 members, at least 23 of which are expressed in human tissues (83). Typically MMPs comprise a pro-peptide of about 80 amino acids maintaining the stability of zymogen, a catalytic metalloproteinase domain of about 170 amino acids with zinc ion binding sites, a linker peptide (hinge region) of variable length rich in proline, and a hemopexin domain of about 200 amino acids in length (84, 85). Although MMPs have a common core structure, they are classified either as collagenases (MMP1, MMP8, MMP13), gelatinases (MMP2, MMP9), stromelysins (MMP3, MMP10, MMP11), matrilysins (MMP7, MMP26), membrane-type MMPs (MT-MMPs) or other MMPs according to the structure of their substrates and structural domains (86). PRCGXPD is a cysteine-switch motif in the MMP pro-peptide that is responsible for the characteristic proteolytic activity (84). Different types of MMPs have specific structural characteristics that are different from typical MMPs (86). For instance, MT-MMPs lack the pro-domain, while MMP7 (matrilysin 1), MMP26 (matrilysin 2) and MMP23 lack the Hpx domain and the linker peptide. In addition, MMP2 and MMP9 contain three repeats of a fibronectin. These various domains, modules, and motifs in MMPs are involved in interactions with other molecules, thereby influencing or determining MMP activity, substrate specificity, cellular and tissue localization (87). MMPs participate in both broad-spectrum turnover and some proteolysis of extracellular proteins, which includes ectodomain shedding at the plasma membrane in a complementary fashion to ADAMs (24). In addition, MMPs can degrade the ECM and participate in almost the whole process of tumor growth and development, including tumor invasion, metastasis and angiogenesis (38).

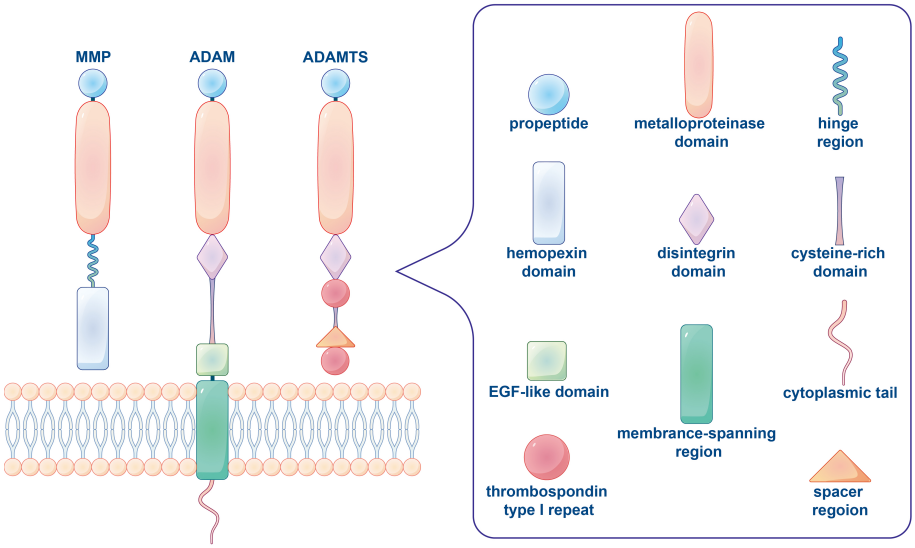

FIGURE 1
The typical domain structure of human metalloproteinases (MMP, ADAM, ADAMTS). An archetypal MMP orderly contains a propeptide, a catalytic metalloproteinase domain, a linker peptide (hinge region) and a hemopexin domain. ADAM is a class of transmembrane protein with characterized structure including a propeptide, a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a membrane-spanning region and a cytoplasmic tail. The basic domain organization of ADAMTS comprises a propeptide, a metalloproteinase and disintegrin-like domain, a thrombospondin type 1 repeat (TSR), a cysteine-rich domain and a spacer region, while lacked a transmembrane region, cytoplasmic domain and EGF-like domain.

Reportedly, MMPs have been detected in a variety of human cancers, and high expression of MMPs is usually associated with poor survival in most cancers (25) including colorectal cancer (39), lung cancer (40), breast cancer (26), ovarian cancer (41), and gastric cancer (70). However, upregulation of a specific MMP does not always lead to promotion of tumor growth or metastasis. The gelatinase protein family including MMP2 (gelatinase A) and MMP9 (gelatinase B) are able to degrade type IV collagen in basement membranes and are the most extensively studied metalloproteinases that are associated with disease progression and poorer survival in patients with various cancers (88). A meta-analysis comprising 41 studies and 6517 patients with primary breast cancer reported that MMP2 and MMP9 overexpression conferred a higher risk of distant and lymph node metastasis, respectively, and were both associated with higher clinical stage and histological grade (26). Similarly, high levels of MMP12 are strongly associated with poor survival in patients with gastric cancer. In contrast, MMP12 overexpression is related to increased survival in patients with colorectal cancer (70, 89).

2.2 A disintegrin and metalloproteases

Also known as Metalloprotease Disintegrin Cysteine-rich (MDC), ADAMs are type I transmembrane proteins anchored to cell surface membranes. Over 30 types of ADAM have been discovered so far (90). Similar to MMPs, ADAMs include a pro-domain and a zinc-binding metalloprotease domain. ADAMs also include a disintegrin domain, which is unique among cell surface proteins (91). The metalloproteinase domain of ADAM is highly conserved, and most ADAMs have an EGF-like domain adjacent to a cysteine-rich domain and a membrane-spanning region, followed by a cytoplasmic tail that varies widely in length and sequence between different ADAM family members (90–92). Due to the presence of these domains, ADAMs can bind substrates and affect changes in cell adhesion and migration, and the proteolytic release of cell surface molecules. Their major substrates are intact transmembrane proteins such as growth factors, adhesion molecules, and precursor forms of cytokines (71).

Cancer cells often express high levels of ADAMs, suggesting a selective advantage for tumors. Interestingly, overexpression of ADAMs is not carcinogenic (52). There are two functional attributes of ADAM proteins, namely proteolytic activity and cell adhesion, which supports the hypothesis that ADAMs may have a crucial role in cell migration as well as extracellular remodeling (93) ADAM17 is the most widely studied of all the ADAM proteins. One study evaluating ADAM17 as a potential blood biomarker for ovarian cancer showed that ADAM17 levels are significantly higher in culture medium supernatants of cultured ovarian cancer cell lines and also in the serum and ascites of patients with ovarian cancer, compared with controls (94). Toshie et al. reported that ADAM10 could be a potential

TABLE 1 The prognostic role and functions of several representative metalloproteinases involved in pan-cancers.

Type of MP	Representatives	Cancers	Prognostic role	Functions	References
Matrix Metalloproteinase (MMPs)	MMP2	Most types of cancer	Decreasing OS	Degrading collagen and extracellular matrix; Involving TGF-β signaling pathway; Increasing cytokine production; Enhancing cancer cell invasiveness; Promoting angiogenesis;	(24–37)
	MMP9	Most types of cancer	Decreasing OS	Degrading collagen and extracellular matrix; Involving TGF-β signaling pathway; Facilitating the migration of immune cell; Hydrolyzing cytokines; Promoting cancer migration, invasiveness and metastasis; Promoting angiogenesis;	(24–29, 31–35, 38–50)
	MMP14	Ovarian cancer, Breast cancer, etc.	Decreasing OS	Degrading extracellular matrix; Cleaving and activating cytokines; Activating metalloproteinase; Promoting angiogenesis;	(27–29, 31–35, 39, 51)
A Disintegrin and Metalloprotease (ADAMs)	ADAM10	Lung cancer, glioma, head and neck squamous cell carcinoma(HNSCC), etc.	Most in decreasing OS (controversy in HNSCC)	Involving Notch signaling pathway; Involving formation of cancer-associated fibroblasts; Regulating immune cells; Inhibiting antitumor immunity;	(13, 52–69)
	ADAM17	Most types of cancer	Decreasing OS	Involving TNF and Notch signaling pathway; Regulating immune cells; Regulating cytokines; Inhibiting antitumor immunity;	(12, 13, 53, 54, 58–63, 67, 68, 70–78)
ADAMs with Thrombospondin Motifs (ADAMTS)	None	Many cancers	Uncertainty	Dual function (Promoting and inhibiting cancer progression)	(79–82)

lung cancer biomarker through investigating enzyme-specific proteolytic activities, rather than ADAM17 (95). Proteomics technologies can be used to identify ADAM proteins that are shed by tumor cells (52).

2.3 ADAMs with thrombospondin motifs

As close relatives of ADAMs, ADAMTS also belong to the Adamalysins family of proteins (79, 80). Unlike ADAMs, ADAMTS are secreted metalloproteinases characterized by an ancillary domain containing a thrombospondin type 1 repeat (TSR) and a spacer region, and the absence of a transmembrane region, cytoplasmic domain and (EGF)-like (81). The human ADAMTS family includes 19 proteins that can be sub-grouped on the basis of their known substrates, namely aggrecanases or proteoglycanases (ADAMTS1, 4, 5, 8, 9, 15 and 20), procollagen N-propeptidases (ADAMTS2, 3 and 14), cartilage oligomeric matrix protein (also known as thrombospondin-5), cleaving proteinases (ADAMTS7 and 12), von Willebrand factor (VWF), cleaving proteinase (ADAMTS13) and a group of orphan enzymes (ADAMTS6, 10, 16, 17, 18 and 19) (81, 82). ADAMTS proteases are involved in the maturation of procollagen and von Willebrand factor, as well as in ECM proteolysis relating to morphogenesis, angiogenesis and cancer (82, 96, 97).

Although major ADAMTS members were identified as suppressors or oncogenes in cancers, studies have shown that different ADAMTS exhibit diverse biological functions and that individual ADAMTS can play distinct roles in different cancers or depending on the clinical setting (98). For example, high expression of ADAMTS8 was associated with better survival in patients with lung cancer by inhibiting cell proliferation and promoting apoptosis.ADAMTS8 overexpression was also associated with decreased levels of vascular endothelial growth factor A (VEGFA), which is a major regulator of angiogenesis and contributes to tumor growth and metastasis (99). A study using topological data analysis identified 38 elusive cancer-related genes, including an inactivating mutation in ADAMTS12 in lung adenocarcinoma. Mice with ADAMTS12 deletion mutations have a five-fold increased susceptibility to lung cancer, confirming the role of ADAMTS12 as a tumor suppressor gene (100). In general, the involvement of ADAMTS in the TME is less well studied compared with MMPs and ADAMs, and studies systematically characterizing their functions in cancers are urgently needed.

3 The relationship between metalloproteases and immunomodulation in cancers

3.1 Signal pathway involving MPs related to immunity in cancer cells

Signal transduction pathways are comprised of multiple molecules recognizing and interacting with each other and transmitting signals to regulate many important biological processes such as tumor cell proliferation, metastasis and immune regulation. Three signaling pathways in particular are closely related to MPs in immunomodulation and are described below (Figure 2).

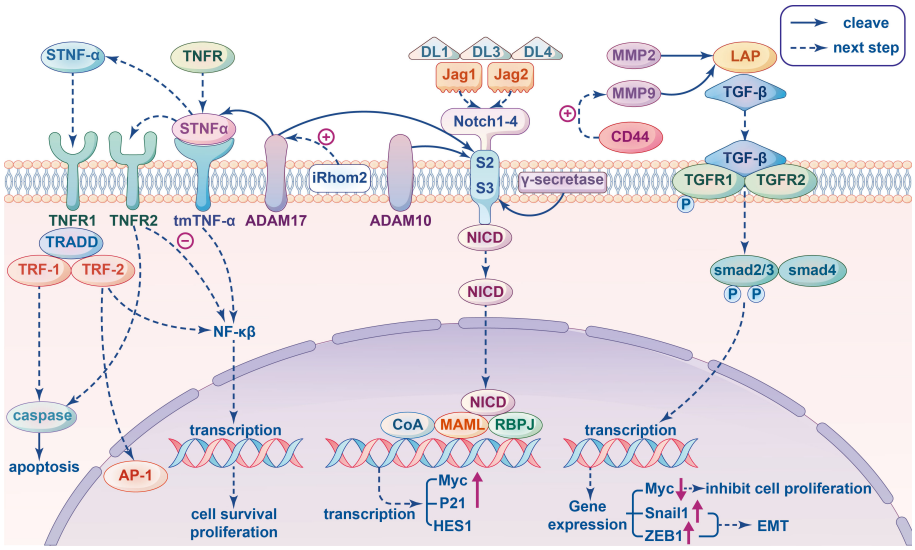

FIGURE 2
Signal pathway involving metalloproteinases related to immunity in cancer cells. Tumor Necrosis Factor (TNF) signal pathway: The transmembrane TNF-α (tmTNF-α) cleaved by proteolytic enzyme ADAM17 produced soluble TNF-α (sTNF-α), which binds to TNFR1 and then recruits TNFR-associated death domain (TRADD), TNFR-associated factor (TRF)-1 and TRF-2 generating two different results: mediating caspase activation to apoptosis or leading to activation of the NF-κB and AP-1 for tumor cell proliferation. When tmTNF-α binds to TNFR2 as ligands to inhibite NF-κB mediated activation of anti-apoptotic genes, then apoptosis and tumor suppression will be triggered; as a receptor tmTNF-α transect the reverse signal to promote tumor proliferation by constitutively activating NF-κB. The activity of ADAM17 can be stimulate by pseudoprotease enzyme iRhom2. Transforming growth factor β (TGF-β) signal pathway: MMP9 and MMP2 cleave the inactive latent TGF-β propeptide (LAP) and produce different activated TGF-β proteolytic cleavage products. In the canonical pathway, active TGF-β triggers TGFβRII to phosphorylate TGFβRI, which in turn recognizes and phosphorylate SMAD2 and SMAD3 proteins to interact with SMAD4 to form a complex that can enter the nucleus and regulate the transcription of target genes. For example, down-regulating the expression of proto-oncogene Myc to inhibit cell proliferation, inducing the Snail1 and ZEB1 to promote epithelial mesenchymal transition (EMT) in tumors. The co-aggregation of CD44 and MMP9 can promoted the protein activity of MMP9. Notch signaling pathway: ADAM10 and ADAM17 have been indicated in many studies that they can act on cleaving S2, and subsequent cleavage S3 mediated by γ-secretase occurs in the transmembrane region, leading to the release of Notch intracellular domain (NICD), which translocate into the nucleus and combines Mastermind-like (MAML) and DNA-binding protein Recombination Signal-binding Protein for Immunoglobulin kappa J region (RBPJ) to recruits additional coactivators (CoA) triggering the transcription of target genes, such as Myc, P21, HES1 and so on.

3.1.1 Tumor necrosis factor signaling

Tumor necrosis factor-α (TNF-α) is an important proinflammatory cytokine that is involved in the maintenance and homeostasis of the immune system, as well as inflammation and host defense (72). Substantial experimental and clinical data have been shown that TNF-α is involved in the promotion and progression of cancer (72, 101–104). TNF-α is found in both a soluble and transmembrane form. Soluble TNF-α is cleaved from transmembrane TNF-α (tmTNF-α) by proteolytic enzyme ADAM17, also known as TNF-a-converting enzyme (TACE), which can orchestrate immune and inflammatory responses *via* activation of TNF-α (73, 90). Both tmTNF-α and soluble TNF-α initiate signaling cascades by binding to TNF-α receptors. There are two types of TNF-receptors: TNF receptor 1 (TNFR1), which is activated by soluble ligands, and TNF receptor 2 (TNFR2) that binds primarily to tmTNF-α. The shedding of tmTNF-α and TNFR both require pseudoprotease enzyme iRhom2, which is an important cofactor of ADAM17 and can stimulate the activity of ADAM17 on the cell surface to control the specificity of ADAM17 protein breakdown (105).

Soluble TNF-α and tmTNF-α all possess dual abilities to promote tumor growth and survival, while tmTNF-α has much broader anti-proliferative capabilities (106). The binding of soluble TNF-α to TNFR1 generates two different out comes. First, TNFR1 recruits TNFR-associated death domain (TRADD), TNFR-associated factor (TRF)-1 and TRF-2. If the complex activates nuclear factor κB (NF-κB), tumor cell proliferation is observed. Otherwise, the complex enters the cytoplasm and the recruitment of FAS-associated *via* death domain (FADD) mediates caspase8 activation leading to apoptosis, which was found to contribute to ADAM17-mediated shedding of TNFR1 (74, 107). It should also be noted that the activity of ADAM17 plays a crucial role in TNFR1-dependent tumor cell–induced endothelial cell death. This is because ADAM17-mediated ectodomain shedding and subsequent γ-secretase-mediated regulated intramembrane proteolysis (RIP) of TNFR1 is a prerequisite for TNF-induced cell death (108). TmTNF-α can be transduced bidirectionally as a ligand or membrane receptor. Apoptosis and tumor suppression are triggered when TmTNF-α binds to TNFR2, leading to the inhibition of NF-κB-mediated activation of anti-apoptotic genes, which is regulated in part by the actin cytoskeleton. However, if tmTNF-α is expressed on the cell surface, it will act as a receptor and transect the reverse signal to promote tumor proliferation by constitutively activating NF-κB (106). Although the exact mechanism has not been elucidated, TNF-signaling in cancer cells has an overall pro-tumor effect, promoting survival, proliferation, and evasion of immune surveillance (109, 110). Therefore, in view of the action of ADAM17 on the receptors and ligands of TNF-signaling pathway, ADAM17 is deemed to affect TNF-α signaling in a variety of ways. For example, a decrease in the production of soluble TNF-α would lead to accumulation of tmTNF-α, which would bind to TNFR2 and lead to a different biological outcome (111).

3.1.2 Transforming growth factor–β signaling

As a key regulator of tumor behavior, transforming growth factor- β (TGF-β) plays an important role in tumor invasion and metastasis, immune regulation and therapeutic resistance (42). TGF-β is also the core of immune suppression in the TME, which has bidirectional effects on the immune system depending on the specific situation (43). In general, TGF-β inhibits cell proliferation during the early stages of cancer development. Inactivation of the TGF-β pathway or decoupling from tumor suppressor effects can promote tumor progression, which affects ECM and cell adhesion molecules, promotes metastasis and angiogenesis, and induces immune suppression. Through a classic membrane-to-nucleus signaling process, the TGF-β pathway involves direct receptor-mediated activation of small mother against decapentaplegic (SMAD) transcription factors (43). TGF-β1, TGF-β2, and TGF-β3 are three closely related isoforms secreted by an inactive complex that covalently combine mature TGF-β with the latent TGF-β pro-peptide (LAP) *via* disulfide bonds in the endoplasmic reticulum (112). There are also three known TGF-β receptors (TGFβRI, TGFβRII and TGFβRIII). In the canonical pathway, active TGF-β triggers TGFβRII to phosphorylate TGFβRI, which in turn recognizes and phosphorylates SMAD2 and SMAD3 proteins to interact with SMAD4 to form a complex that can enter the nucleus and regulate the transcription of target genes (113).

MMP9 and MMP2 are two metalloproteinases known to cleave the inactive latent TGF-β and produce different TGF-β proteolytic cleavage products, which leads to transforming growth factor-B activation (114). Hyaluronic acid-mediated CD44 cross-linking induced co-aggregation of CD44 and MMP9 promotes the protein degradation activity of MMP9. Moreover, degradation of fibronectin by MMP9 bound to CD44 results in release of the active TGF-β (115). The levels of MMP9 in cancer cells may not only influence the proteolysis of TGF-β, but also the expression of TGF-β and substances downstream of the TGF signaling pathway. A study on the relationship between MMP9 and the TGF signaling pathway in breast cancer showed that overexpression of MMP9 in breast cancer cells not only significantly up-regulated the expression of SMAD2, SMAD3 and SMAD4, but also enhanced the phosphorylation of SMAD2 (116). Subsequently, the target gene KLF10 binds to the promoters of SMAD2 and TGF-β1 and to form a positive feedback loop regulating the TGF-β signaling pathway by inducing SMAD2 expression (117). In addition, decorin, which is expressed in the stroma of various cancers and can be cleaved by MMP2, 3, 7 and MT1-MMP, recognizes and binds to all isoforms of TGF-β to form an inactive complex, which inhibits TGF-β signaling *in vitro* and indirectly attenuates downstream signaling pathways (118).

3.1.3 Notch signaling pathway

Notch signaling is involved in multiple aspects of tumor biology, and its role in the development and regulation of immune responses is complex, including shaping the immune system and components of the TME, such as intricate crosstalk between antigen presenting cells, T-cell subsets and cancer cells (119). In particular, Notch plays a crucial role in the development and maintenance of different immune cells such as adaptive T-cells, Natural killer cells and innate immune myeloid cells e.g., granulocytes, macrophages, and dendritic cells (53). Several studies have found that Notch is a target of tumor-mediated immune suppression, and reactivation of Notch in T cells may protect T cells from tumor-mediated immune suppression and enhance their anti-tumor activity (54, 119). The Notch signaling pathway mediates the activation effect after two cells come into contact with each other. Notch receptors comprise four isoforms (Notch1–4), which are single-pass transmembrane proteins that receive signals from transmembrane ligands comprised of three delta-like ligands (DLL1, DLL3, and DLL4) and two jagged ligands (Jag1 and Jag2) expressed on neighboring cells (75). Following binding of transmembrane ligands to Notch receptors, downstream signaling is mediated by some proteases including members of the ADAM family (55).

Firstly, the receptor/ligand interaction exposes the proteolytic cleavage site, S2, which is cleaved by ADAM metalloproteases. Subsequent cleavage at S3, mediated by γ-secretase occurs in the transmembrane region, leading to the release of Notch intracellular domain (NICD), which translocated into the nucleus and combines Mastermind-like (MAML) with DNA-binding protein Recombination Signal-binding Protein for Immunoglobulin kappa J region (RBPJ) to recruit additional coactivators (CoA), triggering the transcription of target genes such as Myc, P21, and HES1 (120). ADAM10 and ADAM17 are known to be involved in cleaving S2, while ADAM17 leads to ligand-independent Notch activation, and ADAM10 causes ligand-dependent activation (27, 121, 122). One study tested whether restoring Notch signaling in ADAM10-deficient mice would block tumor development and showed that the loss of ADAM10 promotes head and neck squamous cell carcinoma (HNSCC) tumorigenesis by impairing Notch signaling (28).

3.2 Tumor microenvironment regulation by MPs

The TME refers to the surrounding microenvironment of tumor cells including blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, various signaling molecules and the ECM. Previous studies have shown that tumors can modulate their microenvironment, and in turn, the TME can influence tumor growth and spread. The TME plays a key role in regulating the immune response in cancers. Tumor cells and their microenvironment typically produce multiple immunomodulatory molecules that have either negative or positive effects on immune cell function. Thus, the TME is able to switch the immune response from tumor-destructive mode to tumor-promoting mode depending on the composition of the TME.

3.2.1 The influence of MPs on the ECM

The ECM is a non-cellular component of the TME stroma, and remodeling of the ECM plays a significant role in the development and homeostasis of cancers, as well as immune cell recruitment and tissue transfer. Extensive remodeling of the ECM during cancer progression leads to changes in its density and composition, and ECM degradation is an important consequence (14). Specifically, protease-induced breakdown of ECM components is essential for tumor cells to cross tissue barriers. MMPs and ADAMs are the main enzymes involved in ECM degradation, either directly or through the release of growth factors and cell-surface receptors (29, 123). The MMPs involved in ECM degradation can be broadly divided into membrane-anchored MMPs and soluble MMPs (Figure 3). They are first synthesized as inactive precursors (zymogens) in the endoplasmic reticulum and then transported to the Golgi apparatus, where they are sorted and transported to specific membrane domains on the cell surface (56). Although membrane-anchored MMP14, which also called membrane-type 1 matrix metalloproteinase (MT1-MMP), localizes preferentially at membrane protrusions called invadopodia where it plays a central role in degradation of the surrounding ECM. ECM degradation is mainly achieved by MT1-MMP-activated soluble MMPs such as soluble gelatinases MMP2, MMP9 and soluble collagenase MMP13, and there is a significant decrease in total ECM degradation when soluble MMP dynamics are switched off (124–126).

The ECM has three main components: fiber, proteoglycans and polysaccharides. MMPs play an important role in tissue remodeling by binding to these substrates to promote turnover of various ECM proteins. The catalytic activity of metalloproteinases usually requires zinc ions and water molecules, and water bound to zinc ions performs a nucleophilic attack on the substrate, causing it to rupture and release the water molecules (86). Matrix degradation can also remove physical barriers (such as basement membranes), and destruction of the normal matrix facilitates malignancy and metastatic dissemination. The specific mechanism by which MMPs degrade the ECM remains unclear, although multiple studies have identified a role for MMPs in ECM degradation (125).

Cancer-associated fibroblasts (CAFs) are the main contributors to ECM stiffness and degradation, and alterations in CAFs contribute to tumor growth and dissemination as well as regulation of T-cell infiltration in cancers (14). High expression of TGF-β induces the transition of endothelial cells into mesenchymal cells, leading to the formation of CAFs and

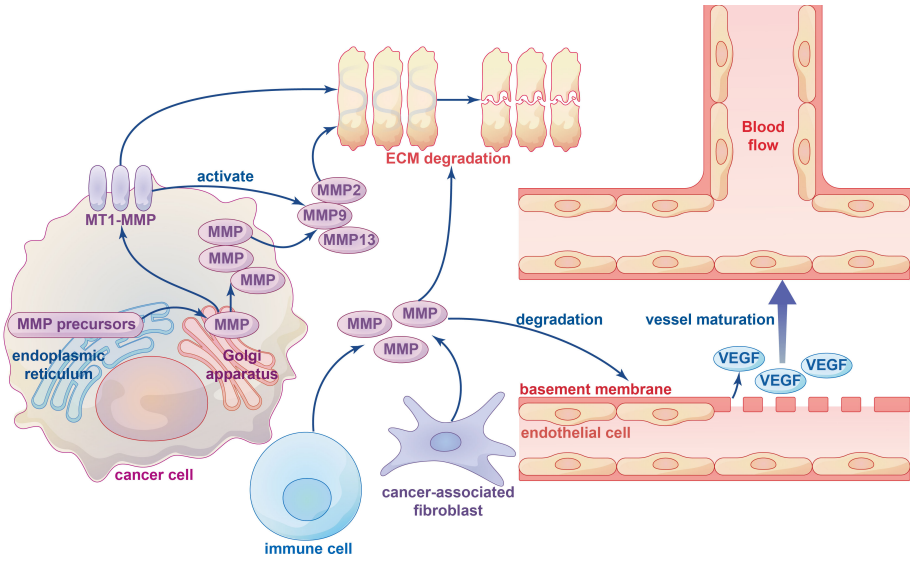

FIGURE 3
The influence of metalloproteinases on ECM degradation and angiogenesis. Various types of metalloproteinases in ECM-degrading are firstly synthesized as inactive precursors (zymogens) in the endoplasmic reticulum and then transported to the Golgi apparatus, which can be divided into membrane-type 1 matrix metalloproteinase (MT1-MMP) and other soluble MMPs. Several immune cells and cancer-associated fibroblasts (CAFs) can also produce metalloproteinases. ECM degradation is mainly performed by MT1-MMP-activated soluble MMPs, such as soluble gelatinases MMP2, MMP9 and soluble collagenase MMP13. The MMPs degrade the basement membrane structure of vascular endothelial cells and release VEGF bound to the extracellular matrix to initiate vessel maturation.

promoting tumor formation (127). In addition, a wide range of MPs controlled by the TIMP gene family influence the TME in cancer. Loss of TIMP1-4 in fibroblasts results in the acquisition of CAF-like features, manifested by increased collagen contractility and expression of activation markers such as A-SMA, stromal derived factor 1 (SDF-1), and TGF-β. ADAM10 inhibits RhoA and Notch activation induced by exonucleosome treatment, and down-regulation of ADAM10 expression in TIMP-free fibroblasts reduces their tumor-promoting and metastatic potential *in vivo* (128). Immune cells in the TME also release MMPs to assist with ECM degradation. For instance, mast cell precursors may spontaneously produce MMP9 during local tissue migration, which is directly or indirectly activated by MMP3 released from fibroblasts, chymase released from mast cells, and plasminogen activator released from microvascular endothelial cells, thereby causing degradation of the ECM (129).

3.2.2 The relationship between MPs and immune cells

Immune cells in the TME play an important role in tumorigenesis and possess tumor-antagonistic or tumor-promoting functions. Although anti-tumor immune cells in the TME tend to target and kill cancer cells in the early stages of tumorigenesis, cancer cells appear to inhibit the cytotoxic function of anti-tumor immune cells in a variety of ways, resulting in immune escape (130). Tumor-associated immune cells can be divided into two types according to their function: innate immune cells and adaptive immune cells. Innate immune cells, comprised of natural killer cells, eosinophils, basophils, and phagocytes, participate in tumor suppression by directly killing tumor cells or triggering adaptive immune responses. The adaptive immune system is comprised of lymphocytes (B cells and T cells), with B cells playing a major role in the humoral immune response and T cells participating in the cellular immune response (57). MPs play an important role in promoting immune cell activity and regulating immune cell migration (58, 59). The relationship between MPs and immune cells is depicted in Figure 4.

3.2.2.1 T cells

T cells are involved in the immune response through direct secretion of soluble cytokines or cell contact-dependent mechanisms. They also play an increasingly important role in tumor immunotherapy. T cells are complex, heterogeneous and are constantly regenerating. They can also be divided into several subpopulations according to their function. Helper T (Th) cells are central regulators of the adaptive immune response, (also known as CD4+ cells because they express CD4 on their surface). They are activated by engagement of the T-cell receptor (TCR) with the major histocompatibility complex (MHC II), which is expressed on the surface of antigen-presenting cells (APCs) (60, 61). ADAM10 and ADAM17 are expressed on the surface of

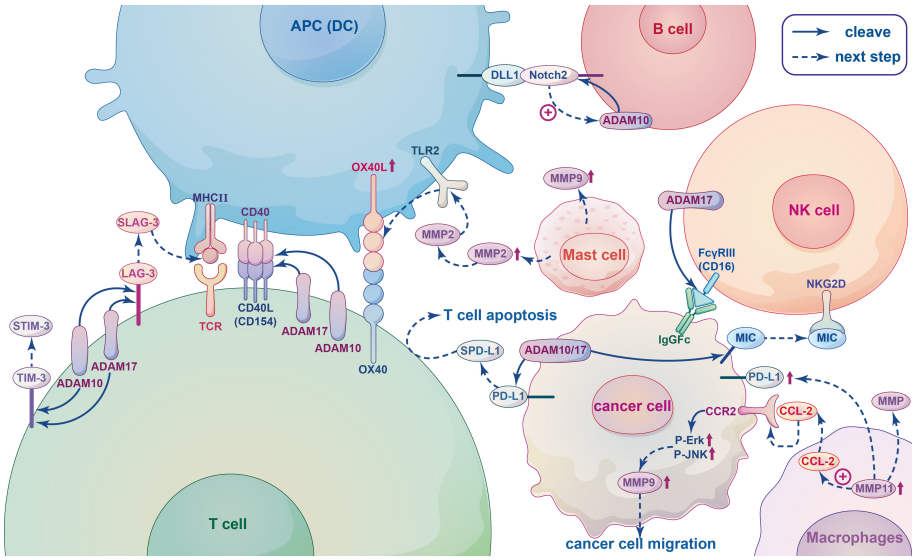

FIGURE 4
The relationship between metalloproteinases and immune cells. When T-cell receptor (TCR) of T cells interact with the major histocompatibility complex (MHC II) of antigen presenting cells (APCs), co-stimulatory receptor CD40L(CD154) expression is rapidly upregulated and linked to CD40, and subsequently released from the T cell surface by cleavage by ADAM10 and ADAM17. Lymphocyte Activation Gene-3 (LAG-3), T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3) are T-cell coinhibitory receptors can be cleaved by ADAM10 and ADAM17 yielding soluble form sLAG-3 and sTIM-3. SLAG-3 binds to MHCII inhibiting the activation of T cells and inducing dendritic cell maturation, sTIM-3 can impair the antitumor immune response of T cells. ADAM10 and ADAM17 can both produce different cleavage products of PD-L1, which called soluble PD-L1 (sPD-L1) and shed from the surface of tumor cells, inducing apoptosis of CD8+ T cells and inhibiting antitumor immunity. In B cells, Notch2 heterodimers bind to ligands DLL1 presented on antigen presenting cells (APCs), which initiates ADAM10 resulting in the release of the Notch intracellular domain that translocating to the nucleus to trigger the expression of downstream target genes. The IgG Fc receptor FcγRIII (CD16), recognizing blinding the Fc part of the IgG antibody of tumor cells and dissolving the cells by Ab-dependent cell-mediated cytotoxicity (ADCC), on NK cells can be cleaved by the metalloprotease ADAM17, leading to NK cell dysfunction and reduced ADCC capacity. MMPs and ADAMs can cleave ligands of the activated receptor NKG2D, such as MIC, on the surface of tumor cells, they bind to NKG2D inducing endocytosis this receptor and causing tumors to evade immune surveillance. Tumour-associated macrophages (TAM) can secret MMPs to promote tumor angiogenesis, invasion and regulate immune response. The chemokine CCL2 secreted by MMP11-overexpressing macrophages activates MAPK pathway, including Phosphorylation of ERK1/2 and JNK, through combined with its receptor CCR2, thereby promoting the migration of cancer cells by up-regulating MMP9. MMP11-expressing macrophages can also upregulate PD-L1 expression and induce immunosuppression of cancer cells. MMP2 and MMP9 released by mast cells (MCs) can promote tumor angiogenesis and tumor invasiveness, respectively. MMP2 as a physiological TLR2 ligand can specifically trigger TLR2 and then increase OX40 ligand (OX40L), which interacted with OX40, on dendritic cells (DCs) to drive T cell responses leading to modulation of immune responses.

resting CD4+ Th cells and are important for regulating the development and function of CD4+ Th cells (16). ADAM10/17 play crucial roles in shedding of the T-cell costimulatory receptor as well as co-inhibitory receptors (62). For instance, CD154 (CD40L) is a type II membrane co-stimulatory receptor that is expressed by all antigen-activated CD4+ Th cells with the exception of "regulatory" T cells (Tregs). Following the interaction between a T cell and an APC, CD154 expression is rapidly upregulated within a few hours and is subsequently released from the T cell surface following cleavage by ADAM10 and ADAM17 (131, 132). In addition, ADAM10 and/or ADAM17 also act on the costimulatory receptor CD137, which is expressed on CD4+ and CD8+ T cells following TCR activation (63) Lymphocyte Activation Gene-3 (LAG-3; CD223) and T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3) are both T-cell coinhibitory receptors acting as substrates for ADAM10/17 protease. The soluble form of each protein (sLAG-3 and sTIM-3, respectively) in humans are formed after proteolytic cleavage of ADAM10 and ADAM17 (15, 16). Hydrolytic cleavage of LAG-3 proximal linker peptides by ADAM10 and ADAM17 yields sLAG-3, which has been shown to bind to MHCII thereby inhibiting binding of LAG3,reducing the activation of T cells, and inducing dendritic cell maturation (133, 134). Anti-cleavage of LAG-3 can inhibit antitumor T-cell responses in mice, and sTIM-3 can impair the anti-tumor immune response in T cells by reducing the expression of Th1 cytokines in CD4 effector T cells (135). Similarly, CD8+ T cells are a subset of cells that express CD8 on the cell surface. Successful binding between naive CD8+ T cells and APCs stimulates immature T cells to become activated CD8+ T cells with cytotoxic capabilities (136). It is well known that the transmembrane glycoprotein PD-L1 is expressed on the surface of tumor cells and binds to the PD-1 receptor on the surface of immune cells to inhibit T cell proliferation, block cytokine production and inhibit T-cell survival. ADAM10 and

ADAM17 were recently shown to produce different cleavage products of PD-L1 that are shed from the surface of tumor cells, leading to apoptosis of CD8+ T cells and inhibition of anti-tumor immunity (137).

3.2.2.2 B cells

B cells are key cellular components of humoral immunity and play a role in immune regulation and tolerance induction through various mechanisms. Unlike T cells, B cells can respond directly to antigens. Activated B cells express both MHC class I and class II molecules on the cell surface; therefore, they can present intracellular and extracellular antigens to CD4+ Th and CD8+ T cytotoxic lymphocytes (64, 65). In particular, marginal zone B (MZB) cells located in the spleen express high levels of CD80/86 costimulatory molecules leading to activation of T cells (66). Notch2 signaling is required for the development of MZB cells, which play an important role in antigen trafficking and presentation. During the development of MZB, Notch2 heterodimers bind to ligands such as DLL1 on stromal cells and APCs, which initiates an unknown metalloproteinase hydrolytic receptor resulting in the release of the Notch intracellular domain that translocate to the nucleus and triggers the expression of downstream target genes (16, 138, 139). The unknown metalloproteinase may be ADAM10. Taok3 transports ADAM10 to the surface of immature B cells, which then promote the development of immature B cells into MZB cells (16, 76, 140). In addition, studies have shown that glioma cells produce ADAM10 upon activation, and ADAM10 can induce the development of regulatory B cells (Bregs) by converting latency associated peptide (LAP) into TGF-β in B cells. Bregs not only exert immunosuppressive effects by inhibiting the activity of CD8+ T cells, but also have the ability to induce the production of Tregs, which play an important role in the evasion of tumor cells from immune surveillance (77).

3.2.2.3 NK cells

Natural killer (NK) cells are specialized immune effector cells that are able to kill tumor cells and are the main source of cytokines and chemokines such as interferon (IFN)-γ and TNF-α, which regulate the function of lymphocytes and enhance the antigen-specific T-cell response (78). NK cells express an IgG Fc receptor FcγRIII (CD16). Activated NK cells can effectively recognize the Fc part of the IgG antibody that binds to tumor cells and dissolve cells by antibody-dependent cell-mediated cytotoxicity (ADCC) (141, 142). Notably, the CD16 molecule can be cleaved from the surface of activated NK cells by ADAM17, and inhibition of ADAM17 impairs the exocytotic abscission of CD16 and CD62L, which significantly increases intracellular levels of TNF-α and IFN-γ (143–145). In addition, the critical interaction between activating receptors on NK cells and MHC-I molecules (MIC) is important to prevent autoimmune destruction and facilitate evasion of immune surveillance by NK cells (146). For example, MMPs and ADAMS can cleave ligands of the activated receptor NKG2D from the surface of tumor cells. The soluble forms of these lysed proteins bind to NKG2D and induce endocytosis and degradation of this receptor, causing tumors to evade surveillance (147). Overall, there are multiple substrates lysed by ADAM17 that are associated with diverse effects on NK cells.

3.2.2.4 Macrophages

Among the immune inflammatory cells in the TME, macrophages are one of the most common (148). Although macrophages have anti-tumor effects as immune cells, experimental and clinical evidence suggests that tumor-associated macrophages (TAM) contribute to cancer initiation and malignant progression, and high levels of TAMs are associated with poor prognosis and reduced overall survival (44, 149–151). Activated macrophages include M1 and M2 subtypes. M1 macrophages can kill tumor cells, while M2 macrophages mainly play a role in promoting tumor growth. However, most of the macrophages in tumor tissues have the phenotype and function of M2 macrophages. In a variety of cancers, TAMs have been found to promote tumor angiogenesis and invasion as well as regulation of the immune response by secreting MMPs (45). MMP regulation is closely related to the chemokines secreted by TAMs. MMP11 expression on macrophages is an independent negative prognostic factor in breast cancer. The chemokine CCL2 secreted by MMP11-overexpressing macrophages activates the MAPK pathway, inducing phosphorylation of ERK1/2 and JNK and promotion of HER2+ breast cancer cell migration facilitated by the upregulation of MMP9. Additionally, MMP11-expressing macrophages play a role in promoting tumor through *via* the upregulation of PD-L1 expression and inducing immunosuppression in breast cancer cells (46). In GMB, CCL5 (derived from glioma-associated microglia/brain macrophages [GAMs]) enhances glioma cell invasiveness through a novel calcium-dependent MMP2 signaling pathway (47). SLIT2 has been found to be functionally deficient in breast cancer. Mechanistic studies have shown that SLIT2-activated macrophages have high phagocytic capacity, are polarized into an anti-tumor M1 phenotype, and inhibit tumor fibrosis by activating MMP13 secreted by macrophages (48). Although evidence has suggested that MT-MMPs are expressed in primary brain tumors as effective mediators of tumor cell infiltration into central nervous system tissues, recent studies have revealed that glioma cells, rather than macrophages/microglia, are the main source of MT-MMPs (152).

3.2.2.5 Mast cells

In the TME, MCs have both pro-tumor and anti-tumor properties. Once activated and degranulated, they recruit immune system cells to coordinate the anti-tumor immune

response. However, their presence may contribute to tumor progression by releasing vascular endothelial growth factor to support MMP9 degradation of the ECM (153, 154). In prostate cancer, infiltrating MCs can reduce androgen receptor (AR) transcription and increase the aggressiveness of prostate cancer cells by increasing MMP9 expression (155). The invasive ability of bladder cancer cells is enhanced by upregulation of estrogen receptor β(ERβ) expression in both MCs and bladder cancer cells, resulting in increased signaling related to CCL2, CCR2, EMT, and MMP9 (156). MC progenitors may spontaneously produce MMP9 during local tissue migration, and stem cell factors can down-regulate mast cell motility by reducing MMP9 production (129). In addition, MMP2 and MMP9 released by MCs can promote angiogenesis and tumor invasiveness, respectively (157).

3.2.2.6 Dendritic cells

Dendritic cells (DC)are APCs that can extract, process and present endogenous antigens to T and B lymphocytes (158, 159) Although DCs do not have the ability to kill tumor cells directly, they play a crucial role in the immune system. Recent studies have shown that in solid tumors, of the number of infiltrating DCs is directly proportional to prognosis, and DC-based vaccines have been applied in the study of tumor immunotherapy (49, 160, 161). Most DCs are in an immature state with strong antigen-phagocytosis ability. They eventually and evolve into mature DCs when they ingest antigens or are stimulated. During maturation, DCs migrate from antigen-exposed peripheral tissues into secondary lymphoid organs, where they present antigen peptides on the surface of MHC molecules to antigen-specific cognate responder T cells through the TCR, which stimulates immune responses (162, 163). The ability of mature DCs to migrate to secondary lymphoid tissues requires expression of a collagenase type IV protein, such as MMP9 (164). In a study of cervical cancer, monocyte-derived cells maintained MMP9 expression during differentiation into immature and phenotypically mature DCs (165). OX40 ligand (OX40L), which is expressed on DCs and modulated by molecules such as toll-like receptor 2 (TLR2), is a key costimulatory molecule that primes Th cells (166). MMP2 is a physiological TLR2 ligand/agonist that specifically triggers TLR2, leading to increased cytokine production and OX40L on DCs through activating components of the canonical NF-kB pathway, which results in modulation of immune responses (167). Additionally, ADAM23 expression on DCs partially governs antigen-presentation capacities to responder CD4+ T cells. Knockdown of ADAM23 in murine BMDCs did not alter the maturation profile of DCs but markedly depressed the activation, proliferation and total levels of cytokine production in CD4+ T cells, such as IL-2, IFN-γ, IL-4, and IL-17 (168). Notably, DCs have podosomes that can degrade the ECM and are proposed to be involved in cell migration (169). The podosome-related domains contain MMP14, which generates guidance tunnels within collagen gels in endothelial cells, and cancer cells use a similar mechanism as they move through the matrix (51).

3.2.3 Immunomodulatory substances associated with MPs

Cytokines are proteins secreted by immune and related cells that mediate and regulate immune processes (170). According to their structure and function, cytokines can be generally divided into interleukins, interferons, TNFs, colony-stimulating factors, chemokines, and growth factors. Cytokines coordinate the interaction between the TME and tumor immune cells, and their release can inhibit or promote tumor development (57). The interaction between cytokines and MPs plays an important role in regulating the TME. Inflammatory cytokines generally upregulate the expression, secretion and activation of MPs in immune cells (11, 171, 172). In addition, they are shed as substrates or become active after cleavage by MPs leading to various immune-inflammatory responses in multiple cancers (12, 173, 174).

A variety of cytokines derived from tumor cells, including TGF-β, EGF, HGF and TNF-α, mediate the expression of many MPs. The most important of these is MMP9, which is elevated in serum and tissues associated with tumors, and is involved in the degradation of the ECM to facilitate the migration of immune cells in cancer (11). A study on breast cancer showed that MMP9 was secreted predominantly by fibroblasts, and its expression in tumor fibroblasts is regulated by multiple cytokines and complex cellular signaling pathways (175). Interleukin, as one of the most widely studied cytokines, is associated with the occurrence and development of cancer. Various cell sources, receptors and signaling pathways determine that interleukins have pleiotropic effects in cancers, including participating in immune responses through interaction with MPs (173, 176). The members can be divided into several protein families according to their structural homology. Biochemical and cell-based assays suggest that IL-2 is subject to proteolytic processing by neutrophil MMP9. The proliferation of IL-2-dependent cells, including primary human regulatory T cells, significantly decreased after IL-2 was cleaved (173).

In addition, these cytokines must be cleaved by MPs to participate in tumor immune process. TmTNF-α, cleaved by ADAM17, generates active sTNF-α (106). IL-12 also plays a critical role in T-cell development and expansion and stimulates activated T cells and NK cells to release toxic enzymes or secrete effector cytokines in the TME, which are essential for tumor clearance (50). It was hypothesized that pro-IL-12 is mostly inactive before cleavage and switches to an active state in the TME after cleavage by MMP14. The amino acid sequence recognized by MMP14 (SGRSENIRTA) was chosen as the cleavable substrate linker. The hydrolysis efficiency of this peptide sequence is 79% for MMP14, 4% for MMP2 and 9% for MMP9. Therefore, pro-IL-12 was almost completely cleaved

and activity was significantly recovered after incubation with MMP14 (174).

3.2.4 Relationship between metalloproteinases and angiogenesis

Tumor blood vessels can provide oxygen and nutrients and remove waste products as well as serve as a conduit for tumor cell metastasis and immune cell infiltration. In terms of structure and function, these vessels are abnormal compared with those in non-malignant tissues, which promotes progression of cancers through impaired perfusion leading to hypoxia and low pH in the TME (30). The hypoxic microenvironment caused by impaired tumor blood perfusion can promote the invasion of tumor cells and hinder the anti-cancer effect of immune cells, which produce chemokines, cytokines, proteases and microvesicles. VEGF and inflammatory chemokines are not only major proangiogenic factors, but also immune modulators, increasing angiogenesis and immune suppression (177).

To date, several types of tumor angiogenesis have been reported, including sprouting angiogenesis and vasculogenic mimicry (VM) (178). Sprouting angiogenesis is achieved by the upregulation of various hydrolases such as MPs and tissue plasmin activators in the vascular basement membrane, which leads to degradation and remodeling of the basement membrane and the ECM (Figure 4) (179). MMPs secreted by CAFs degrade the basement membrane of vascular endothelial cells and release VEGF bound to the extracellular matrix to initiate angiogenesis. For example, in pancreatic neuroendocrine tumors, increased secretion of MMP9 releases sequestered VEGF from the matrix, which switches vascular quiescence to active angiogenesis (31). In lung cancer cells, inhibition of MMP2 activity reduces its interaction with integrin-AVB3, and inhibits the expression of VEGF mediated by downstream PI3K/AKT signaling, leading to decreased angiogenesis (32).

VM is a newer model for invasive tumors to form new blood vessels, which provides blood supply for tumor growth (33). Studies have shown that the initial hypoxic environment of solid tumors is inseparable from VM, and that hypoxia is closely related to the expression and activity of MMPs (34, 35, 180). Hypoxia-inducible factor-1α (HIF-1α) has been shown to directly regulate the expression of MMP14, MMP9 and MMP2 (36, 180, 181). In the early stage of VM formation, MMPs dissolve ECM adhesion proteins and connexins, leading to the release of individual epithelial cells from the epithelium. Some cell fragments then initiate signal transduction pathways, leading to extensive changes in gene transcription. MMP2, activated by MMP14, causes the lysis of Ln5γ2 (laminin) and promotes the formation of VM ducts. In addition, cell protrusions termed invasive pseudopodia, aggregate the proteolytic enzymes MMP2, MMP9, and MMP14 at their leading edge where they degrade collagen and the ECM basement membrane (182). However, other studies have shown that MMP2 and MMP9 are upregulated in cancer cells through a HIF1 -dependent mechanism, whereas MMP14 is upregulated in a HIF2-dependent manner, and their enhanced activity is due to increased expression of HIF-dependent urokinase-type plasminogen activator surface receptors (183).

4 Recent advances and future trends in application targeting MPs in tumors diagnosis and immunotherapy

4.1 The crucial role of metalloproteinases in cancers diagnosis

As MPs have been found to play an important role in the occurrence and development of tumors, several diagnostic methods involving MPs have emerged in recent years. Various molecular imaging techniques have been used in cancer diagnosis to show the activity of MPs *in vivo*. For the past decade or so, MP imaging has been limited to optical imaging (OIM), positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI), all of which have been inadequate in quantifying MP expression levels (184). Recently, a method has been developed for that precise quantification of MT1-MMP in cancer tissue sections using metal clusters composed of intrinsic red fluorescence and a specific mass signal. MMP14 can be directly observed *via* optical fluorescence microscopy and quantified by mass spectrometry 2D imaging (MSI) (185).

Highly selective fluorescent nanoprobes have also been developed to improve the diagnostic accuracy in early and metastatic cancers. MMP-2-responsive nanoprobes were prepared by immobilizing fluorescent fusion proteins, which consists of a fluorescent mCherry protein with a cell penetrating peptide (CPP) moiety with MMP-2 cleavage site, on nickel ferrite nanoparticles *via* the His-tag nickel chelation mechanism. The high selectivity of nanoprobes is due to the steric hindrance effect between nanoprobes and MMPs formed by hiding the cleavage site of MMP-2 substrates inside the system, which allows detection of soluble MMP-2 in the TME (186). Fluorescence nanoprobe technology can not only be used to accurately diagnose distant lymph node metastasis, but also as a prognostic tool for cancer treatment after treatment with photodynamic therapy (PDT) treatment (187). Rapid diagnosis during surgery has become an indispensable tool in cancer diagnostics. MP-mediated fluorescence nanoprobe technology has been proposed as a rapid and accurate method to assist decision-making during surgery (188).

4.2 Metalloproteinases inhibitors can be a potential partner for combination therapy in cancer immunotherapy

In view of the role of MPs in cancer immune regulation, it is conceivable that MPs can play a pivotal role in immunotherapy. The main immunotherapy modalities currently available are immune checkpoint blockade (ICB) therapy, chimeric antigen receptor (CAR)-T cell therapy, and cancer vaccines. Notably, the role of MP inhibitors or activators in different immunotherapy modalities is diverse. A variety of broad-spectrum MP inhibitors have emerged in clinical trials. However, due to the non-specificity of drugs and the complex role of MPs in immune regulation, MP inhibitors have so far failed to improve survival and prognosis of patients with cancer (10, 189). Recently, it has been reported that MP inhibitors can be used in combination therapy to improve the efficacy of immunotherapy (67–69).

4.2.1 The mechanism of MPs as an immunomodulator

ICB has been revolutionary for cancer treatment by suppressing immunosuppressive components in the TME including programmed cell death protein 1 and its ligand (PD-1/PD-L1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (190). Importantly, clinical response rates of ICB have been relatively low in some cancers, despite improved treatment outcomes; therefore, combination therapy has the potential to improve ICB therapy (191). SB-3CT, as an MMP2/9 inhibitor, has been suggested to improve the efficacy of anti-PD-1 and anti-CTLA4 treatment in mouse models of melanoma and lung cancer, as well as metastatic melanoma in the lung. SB-3CT treatment not only causes a reduction of PD-L1 expression through reducing multiple oncogenic pathways, but substantially improved immune cell infiltration and cytotoxicity of T cells in combination with anti-PD-1 treatment. In addition, the combination of SB-3CT with anti-CTLA-4 enhanced the downregulation of PD-L1 expression and increased the concentration of activated tumor-infiltrating CD8 + T cells in the tumor (67). Conversely, abundant expression of MMP2 in TME could trigger a gradual enzymatic-degradation of DOX-aTIGIT-GAB hydrogel that is composed of drugs including doxorubicin (DOX) and anti-TIGIT monoclonal antibody (aTIGIT) co-packaged in an injectable enzyme-responsive hydrogel. After being stimulated by released DOX, the immunogenic tumor recruits the infiltration of NK cells and effector T cells that could be further stimulated by the subsequently released aTIGIT to boost multilayered innate and adaptive immune responses (192).

As an emerging antitumor immunotherapy, tumor vaccines, including nucleic acid, DC-based, tumor cell, and synthetic long peptide (SLP) vaccines, have achieved notable therapeutic effects in several trials. The combination of tumor vaccines with immune checkpoint inhibition or other therapies may achieve superior therapeutic effects compared with single-agent treatment (37). Many studies in recent years have focused on the use of DC vaccines to initiate and shape an anti-tumor-specific immune response and/or boost existing spontaneous anti-tumor T-cell responses (193). However, the critical pathways by which DC-based vaccines activate effective immunity remain unknown (194). DCs from patients with melanoma have reduced expression of the cell surface inducible T-cell costimulator ligand (ICOSL), which plays an importance role in activating protective T-cell responses (195). Therefore, there is potential to improve therapeutic T-cell responses and treatment outcomes in patients with cancer through improving ICOSL expression on DCs. DCs express ADAM10 and significantly increase levels of ADAM17 after maturation, which can modulate availability of ICOSL co-stimulation during humoral immune activation by cleaving surface ICOSL (196, 197). In addition, inhibition of ADAM10/17 cleavage enzyme activity in DCs can increase surface expression of ICOSL, which yielded a vaccine with more effective anti-tumor capability (198).

CARs are synthetic receptors that enable T cells to recognize tumor-associated antigens (TAAs) independent of MHC (199). Although CAR-T cell therapy has been associated with clinical responses in subsets of B-cell leukemia or lymphoma, there are several challenges for CAR-T therapy in solid tumors and malignant hematological tumors, including tumor heterogeneity (200, 201). The development of CAR-T therapy for glioblastoma (GBM) has been limited by the scarcity and heterogeneity of GBM biomarkers. Chlorotoxin (CLTX), an acid peptide, has been studied in GBM and other neuroectodermal tumors as a method to weaken tumor cell migration and invasiveness, while exhibiting minimal cross-reaction with normal cells in the brain and elsewhere (202). CAR-T cells utilizing CLTX as the targeting domain (CLTX-CAR T cells) address two major hurdles in the way of effective immunotherapy for GBM: reduction of antigen escape and maintenance of tumor cell restriction (202). MMP2, a secreted MMP, specifically and selectively interacts with CLTX and high MMP2 expression facilitates the binding of CLTX (202–204). Accordingly, MMP2 knockdown in GBM cells substantially reduced CLTX-CAR T-cell activation and cytotoxicity (202). Additionally, MMP8 have been indicated that it was positive associated with good prognosis and survival of various cancers patients. The homing of CAR-T cells can be enhanced when CAR-T cells carrying overexpressing MMP8 because MMP8 can damage the collagen fibers surrounding the tumor (205).

4.2.2 Traditional and vanguard immunomodulatory drugs

4.2.2.1 Monoclonal antibodies

With a high target selectivity and favorable pharmacokinetic profiles, mAbs have shown promise for immunotherapy in cancer. These mAbs modulate the activity of these by barring

access to the active site, disrupting of exosite binding and preventing protease activation (206). Selective inhibition of single MMP isoforms has been previously demonstrated, e.g., the humanized monoclonal antibody Andecaliximab (GS-5745) that selectively inhibits MMP9 and Fab 3369 acting on MMP14 (207). Structural investigation revealed that GS-5745 inhibits MMP9 by binding to pro-MMP9 and preventing MMP9 activation, whereas binding to active MMP9 allosterically inhibits its activity (208). Fab 3369, derived from a synthetic humanized Fab library, intercepts endogenous MMP14 expressed on the cell surface and inhibits ECM degradation in triple-negative breast cancer (TNBC) (35). There are a variety of mAbs that effectively inhibit ADAM17, including first-generation Administration of D1(A12), second generation mAb A9 and MED13622 (206). mAbs targeting ADAMTS family members have also been studied in inflammatory and cardiovascular diseases, but not in cancer. There are also several small molecule inhibitors in clinical development that have shown positive effects in clinical trials (207).

4.2.2.2 Others

Engineered nanoparticles have also shown promise for the treatment of cancer (209, 210). O-NP, an intelligent nanocarrier, contains a cationic core and a molecule consisting of hydrophobic oleic acid, as well as a MMP9-cleavable peptide and a glutamate-rich segment (OMPE). Once exposed to MMP9 in the TME, OMPE is proteolytically processed, which leads to elimination of glutamic acid residues causing a charge reversal from anionic to cationic, which enhances endocytosis of the nanocarrier in cancer cells. When administrated systemically, this phenomenon results in efficient delivery to MMP9-overexpressing tumors (211). In addition, there is a growing body of research aimed at integrating multiple therapeutic tools into one for precise molecular sensing and site-specific cancer treatment. For instance, gold nanostars (GNS), which can be attached to MMP2 polypeptides (Ac-GPLGIAGQ) and IR-780 iodide, have been utilized for enhanced photothermal therapy (PTT)/PDT in lung cancer (212).

4.3 Current challenge of targeting MPs in the clinical applications

Despite a number of preclinical trials have suggested that targeting MPs can bring benefits to the diagnosis and treatment of cancer, they failed at different phases in researches, mostly because to the non-specificity of the drug and the complicated background for specific effects of MPs. While some studies have begun to test highly selective MP-targeting drugs, such as the monoclonal inhibitors against MPs mentioned above, the field is still at exploring and the efficacy and safety of this approach is not yet known. In addition, whether the addition of targeting MPs will bring some potential toxicity or immune-related adverse reactions while enhancing the efficacy of tumor immunotherapy still need to be explored with more studies in future. For example, inhibition of protease activity has been reported to produce significant joint pain and swelling, as well as myelosuppression and venous thromboembolism [7]. Remarkably, with the continuous breakthroughs in biotechnology, nanoparticles are particularly attractive as a new medium for targeting MPs for cancer diagnosis and treatment. While improving the specificity, the excellent targeting efficiency of nanoparticles is confronted with the selection of the nanocarriers, its stability and sustainability.

5 Conclusion and perspective

In this review, we highlighted the immunomodulatory roles of MPs in the TME including ECM remodeling, signal pathway transduction, cytokine shedding and release, and promotion of angiogenesis. MPs and some relating cleavage substrates may be prospectively used as predictive biomarker candidates of prognosis for certain cancer types; however, large, confirmatory studies are required. Emerging technologies and compounds related to MPs have been increasingly explored in cancer diagnosis and treatment. As such, it is difficult to develop highly selective drugs and nanoprobes targeted towards specific MPs. Better understanding of MP expression patterns and functions in the immunoregulation of cancer will contribute to the development of more effective therapeutic approaches for cancer diagnosis and immunotherapy. Evidence shows that combinations of biomedical technologies may be more efficient for cancer therapy compared with single agents. The new technologies based on MPs are of area constant exploration and great potential. If these technologies can be put into practice, they may provide effective strategies for the diagnosis and treatment of cancer in the future.

Author contributions

QW and KW jointly contributed to the first draft of the article. XT provided assistance in preparing figures and table. ZL and HW revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Role for the metalloproteinase ADAM28 in the control of airway inflammation, remodelling and responsiveness in asthma

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Background: Asthma is characterized by morphological modifications of the airways (inflammation and remodelling) and bronchial hyperresponsiveness. Mechanisms linking these two key features of asthma are still poorly understood. ADAM28 (a disintegrin and metalloproteinase 28) might play a role in asthma pathophysiology. ADAM28 exists as membrane-bound and soluble forms and is mainly expressed by lymphocytes and epithelial cells.

Methods: ADAM28$^{-/-}$ mice and ADAM28$^{+/+}$ counterparts were sensitized and exposed to ovalbumin (OVA). Airway responsiveness was measured using the flexiVent® system. After sacrifice, bronchoalveolar lavage (BAL) was performed and lungs were collected for analysis of airway inflammation and remodelling.

Results: The expression of the soluble form of ADAM28 was lower in the lungs of OVA-exposed mice (as compared to PBS-exposed mice) and progressively increased in correlation with the duration of allergen exposure. In lungs of ADAM28$^{-/-}$ mice exposed to allergens, the proportion of Th2 cells among CD_4^+ cells and the number of B cells were decreased. Bronchial responsiveness was lower in ADAM28$^{-/-}$ mice exposed to allergens and similar to the responsiveness of sham-challenged mice. Similarly, features of airway remodelling (collagen deposition, smooth muscle hyperplasia, mucous hyperplasia) were significantly less developed in OVA-exposed ADAM28$^{-/-}$ animals in sharp contrasts to ADAM28$^{+/+}$. In addition, we report the first evidence of ADAM28 RNA expression by lung fibroblasts and we unveil a decreased capacity of lung fibroblasts extracted from OVA-exposed ADAM28$^{-/-}$ mice to proliferate as compared to those extracted from OVA-exposed ADAM28$^{+/+}$ suggesting a direct contribution of this enzyme to the modulation of airway remodelling.

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Conclusion: These results suggest that ADAM28 might be a key contributor to the pathophysiology of asthma.

KEYWORDS

asthma, proteases, adamalysins, ADAM28, mouse model, airway remodelling

Background

Asthma is an inflammatory disease of the airways of increasing prevalence worldwide (1). The vast majority of asthmatics see their disease adequately controlled with currently available standard therapies and a minority of severe asthmatics remains uncontrolled and requires targeted treatments with biologicals aiming at interfering with the disease process (1). With an impaired quality of life and a number of disease-related complications, this subgroup of patients is responsible for the majority of asthma-related costs (healthcare resources, drugs, hospitalizations, working day loss, etc). Moreover, asthmatics displaying an accelerated decline of lung function during their lifetime or a fixed airway obstruction display significantly more profound airway remodelling (2, 3). Airway remodelling in asthma is a characteristic of the disease and includes Goblet cell hyperplasia, basement membrane thickening, collagen deposition around the airways as well as smooth muscle hyperplasia (4). The biological mechanisms leading to an established airway remodelling are still not fully unveiled but there is a strong influence of airway epithelial cells. Indeed, the airway epithelial cells collected from asthmatic donors display an enhanced expression of remodelling- related genes (5, 6). These characteristics of the asthmatic airway epithelial cells affect the profibrogenic potential of the airway fibroblasts that contribute to profoundly modify the extracellular matrix of the bronchi (7, 8). Although many different pathways might contribute to the activation of fibroblasts, transforming growth factor (TGF)-β induced fibroblast activation that triggers extracellular matrix production is key (9). The biology of TGF- β is complex since this mediator requires a post-translational activation that can be achieved by different mechanisms including a cleavage by matrix metalloproteinases (MMPs) (10).

A disintegrin and metalloproteinases (ADAMs) are membrane-bound or secreted enzymes that display the characteristics to play an important role in the regulation of inflammation and remodelling since they are able to process many soluble or membrane-bound mediators (including a number of chemokines/cytokines), hence modifying their biological activity (11). The prototypical example is the membrane-bound pro-TNF-alpha that requires a cleavage by ADAM proteases before being activated and released in a soluble form (11, 12). Some of these enzymes also cleave different components of the extracellular matrix. The ADAM proteins are matrix metalloproteinase (MMPs)-related enzymes, bearing a multi-domain structure. They have been associated to numerous physiological and pathological processes to date (11). Modulation of the expression of different ADAM(TS) in the bronchial tree of a cohort of human asthmatics has been reported (13, 14).

ADAM28 is a multipotent membrane-bound proteinase expressed by tissues derived from the foregut in embryo suggesting its involvement in respiratory tract organogenesis. Membrane-bound ADAM28 can be released after proteolytic cleavage by different proteinases including MMP7 resulting in the release of a soluble form of ADAM28 as it has been also shown for ADAM33 (15, 16). Interestingly, the soluble form of ADAM28 has been reported to enhance the α4β1-dependent cell adhesion to vascular cell adhesion molecule-1 (VCAM-1) and therefore it influences lymphocyte adhesion and trans-endothelial migration (17). Various ADAM28 splicing variants have been described. Notably, microarray studies show an upregulation of ADAM28 expression after induction of lung inflammation in a mouse model of chronic asthma (18) and ADAM28 is expressed by airway epithelial cells (19). Moreover, ADAM28 deficiency has been associated with impaired CD_8^+ T recruitment in lung and spleen contributing to a protective role for host ADAM28 against metastasis dissemination of cancer cells (20). This protease can therefore play different roles in the microenvironment either in its soluble or membrane-bound form.

In this article, we establish a strong relationship between the expression of ADAM28 and asthma-associated bronchial hyperresponsiveness and remodelling. ADAM28 deficient mice display lower infiltration of bronchial walls and bronchoalveolar lavage (BAL) by inflammatory cells, reduced features of airway remodelling, and lower hyperresponsiveness as compared to wild-type mice.

Materials and methods

Sensitization followed by allergen sensibilisation

Full Knock out mice (ADAM28$^{-/-}$) for ADAM28 BALB/c mice and Wild type (ADAM28$^{+/+}$) counterparts were previously described (20) and were enrolled in our studies according to "Principles of Laboratory Animal Care" (National Society for Medical Research). Experimental protocols described were approved by the animal ethical committee of the University of Liège (under the references #1597 and #2146). All experiments were performed on male and female mice. Animals were included in the asthma protocol aged 8 weeks and their weight was between 20 and 25 grams. Three protocols of asthma induction were used and referred to as a "short-term" (ST), "intermediate-term" (IT) and a "long-term" (LT) exposure protocol (Figure 1). Eight weeks-old BALB/c mice were sensitized on days 1 and 8 (ST model) or on days 1 and 12 (IT and LT models) by intraperitoneal injection of 10 µg of ovalbumin (OVA Grade V; ref#A5503 Sigma-Aldrich, Schnelldorf, Germany) emulsified in 2 mg aluminium hydroxide (AlumInject; ref#77161 Perbio, Erembodegem, Belgium). Mice were subsequently divided into 4 groups for daily nebulization in standard Plexiglas boxes (30 × 20 × 15 cm): 2 groups of mice (ADAM28$^{-/-}$ and ADAM28$^{+/+}$ mice) were only exposed to PBS (ref#17-516Q, Lonza, Verviers, Belgium) aerosol (control cohorts), and the 2 other groups (ADAM28$^{-/-}$ mice and ADAM28$^{+/+}$) were subjected to ovalbumin 1% aerosol for 30 min (OVA Grade III; ref#A5378 Sigma-Aldrich, Schnelldorf, Germany). Aerosols were generated daily by ultrasonic nebulizer from days 22 to 26 for the ST exposure model and from days 22 to 56 for the IT model (aerosols were continued until day 90 for the LT model) 5 days/week (every odd week).

Assessment of airway responsiveness

Mice were exposed to the 90 days model of exposition to OVA. At the day of sacrifice, they were anesthetized by intraperitoneal injection of pentobarbital (70 mg/kg, Nembutal®, Sanofi Animal Health, Belgium) and ketamin (75 mg/kg, Ketalar®, Pfizer, Brussels, Belgium). A surgical tracheotomy was performed and was followed by insertion of an 18-gauge polyethylene catheter into the trachea. Mice were ventilated by using a FlexiVent small animal ventilator

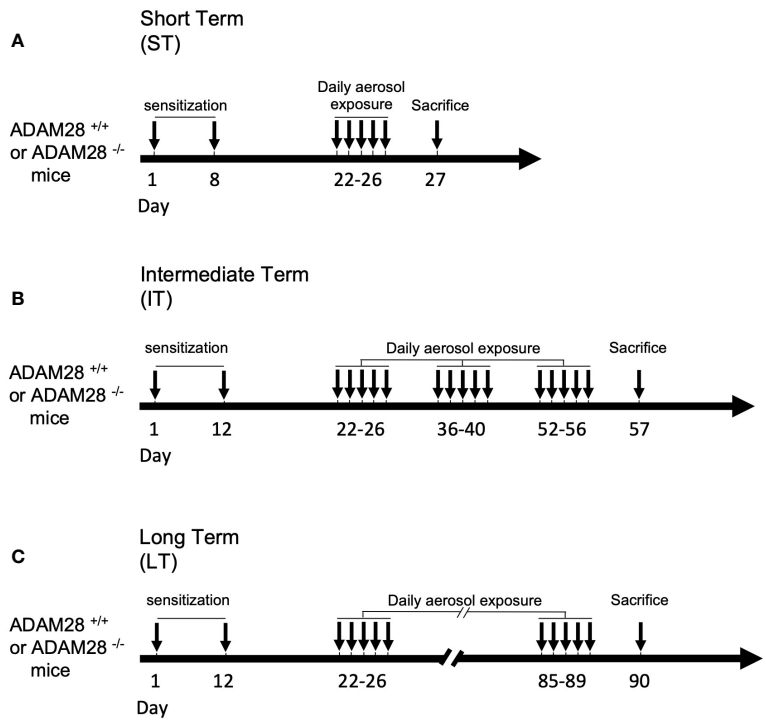

FIGURE 1
Experimental procedure. Short-term (ST) protocol **(A)**, intermediate-term (IT) protocol **(B)**, and long-term (LT) protocol **(C)**. PBS/ovalbumin (OVA) sensitization and exposure protocols. Mice were sensitized on days 1 and 8 by intraperitoneal injection of 10 µg of OVA. Mice were subsequently exposed to daily PBS aerosol or OVA 1% aerosol for 30 min for ST, IT, or LT duration (see materials and methods section for the detailed protocol).

(SCIREQ, Montréal, Québec, Canada). Baseline lung-function was evaluated during FlexiVent manoeuvres measuring lung compliance, tissue elastance, and tissue hysteresivity in basal conditions. Lung hysteresis, the area between the ascending and descending portions of the pressure-volume curve is a reflect of the property of the lung to dissipate the received energy (the energy applied to the lung in inspiration is not completely recovered in expiration). Hysteresivity (η) is the ratio of tissue damping (G) over tissue elastance (H). After basal evaluation, mice were exposed by inhalation to increasing doses of nebulized methacholine (1.25, 2.5, 5, 10, 20 mg/ml; ref#190231 ICN, Asse-Relegem, Belgium, PBS was used as a diluting solvent), and a dose-response (airway resistance) curve was obtained for each animal.

Measurement of airway remodelling and airway inflammation

Right main bronchus was clamped and right lung was excised and immediately frozen in liquid nitrogen. The remaining left lung was inflated with 4% paraformaldehyde (PFA, Ref#8.18715.1000, Sigma-Aldrich, Schnelldorf, Germany) and resected. After a night in PFA bath, lungs were embedded in paraffin. Five-µm sections were stained with haematoxylin–eosin. A peribronchial inflammation score was determined, related to cell infiltration around the bronchi, by quantification of peribronchial inflammatory cells (eosinophils, lymphocytes, macrophages, etc.), as previously described (21). When no inflammatory cells were detectable around the bronchi a value of 0 was given. A value of 1 was given when there were occasionally inflammatory cells, a value of 2 when most bronchi were surrounded by a thin layer (one to five cells) of inflammatory cells, and a value of 3 when most bronchi were surrounded by a thick layer (more than five cells) of inflammatory cells. The score was measured on seven randomly selected bronchial sections per mouse and peribronchial inflammation scores are expressed as a mean value per animal.

Masson's Trichrome staining was used to measure collagen deposition around the bronchi. As previously described, a collagen deposition score was applied (allowing to give a score from 0 to 3 to each observed bronchi) (18). A score of 0 was recorded when no collagen was stained by Trichrome Masson around the bronchi, a score of 1 for a thin layer of collagen, 2, for a cluster of collagens and 3, for a thick layer of collagen. Immunohistochemistry for alpha-smooth muscle actin (α-SMA) was performed using mouse primary antibody anti-α-SMA-FITC (ref#F3777 Sigma–Aldrich, Schnelldorf, Germany). Digitalized slides corresponding to 7 bronchi per mice were analysed. Smooth-muscle-cell layer was measured and reported to epithelial basement membrane perimeter. Glandular hyperplasia was evaluated by measuring the percentage of periodic acid-Schiff (PAS)-stained goblet cells per total epithelial cells (percentage of 300 randomly counted cells) (PAS ref#1.09034.1000, Merck, Hoeilaart, Belgium).

Lung fibroblasts: Culture, proliferation test and RNA analyse

Fibroblasts were isolated from the lung of ADAM28$^{+/+}$ mice exposed or non-exposed to allergens in LT model of asthma. They were cultured according to already validated protocols (22). Immediately after sacrifice, chests of mice were disinfected with 70% ethanol and lungs were excised using sterile tools. They were cut into small pieces by cutting with razor blades. Tissue digestion was performed by collagenase (Collagenase from Clostridium histolyticum, ref#C9891 Sigma-Aldrich). Fibroblasts were cultured in hypoxia (incubator at 37°C, 5% of CO2 and 3% of O2). Confluence of the fibroblasts was followed using microscope. When they covered 60% of the plate medium was changed to remove debris and unattached cells. Between days 7 and 14, according to fibroblasts confluence, cells were harvested and divided in several plates for analyse. One of them was reserved for further RNA extraction. A CyQUANT cell proliferation assay was performed following manufacturer's instructions. To evaluate fibroblasts growth rate, fluorescence was analysed 48 hours after first passage.

Native lung fibroblasts cultured in bronchoalveolar lavage conditioned medium

Mice were sacrificed, and a bronchoalveolar lavage was immediately performed using 4 x 1 ml PBS–EDTA 0.05 mM (ref#324503 Calbiochem, Darmstadt, Germany) as previously described (13, 14). Cells were recovered by gentle manual aspiration. After centrifugation (1,200 rpm for 10 min, at 4°C), supernatant was collected and frozen at −80°C. BAL was collected from ADAM28$^{+/+}$ and ADAM28$^{-/-}$ mice exposed and non-exposed to OVA in a LT model of asthma. Cultured lung fibroblasts were isolated from the lungs of naïve ADAM28$^{+/+}$ mice. Enrichment of fibroblasts culture medium was performed by adding 10% of previously collected BAL (ADAM28$^{+/+}$ and ADAM28$^{-/-}$ exposed to OVA or to PBS) to culture medium and cell proliferation was measured by a CyQUANT assay (Ref#C7026, Invitrogen, Merelbeke, Belgium).

Tissue processing: RNA extraction and analyses

The right lobe of the lung previously frozen was disrupted with a Mikro-Dismembrator (Sartorius Stedim Biotech,

Vilvoorde, Belgium). Total lung RNA was extracted and purified using High Pure RNA Tissue Kit (Roche ref#12033674001, Mannheim, Germany) according to the manufacturer's instructions. Total ADAM28, membrane-bound (Variant 1, Var1) and soluble form (Variant 4, Var4) expressions were assessed by semiquantitative RT-PCR and normalized to the 28s rRNA. As 28S rRNA is the product of the precursor 45S rRNA, some portions of 45S rRNA are also amplified by the primers chosen to target 28S.

The following primers targeting respectively 28S, total ADAM28, ADAM28 Var1 and ADAM28 Var4 were used: (F) *5'- GTTCACCCACTAATAGGGAACGTGA -3'*, (R) *5'-GGATTC TGACTTAGAGGCGTTCAGT-3'*, (F) *5'-CTACTTGAGCT GCAAGTGTCCATC-3'* and (R) *5'-CAGGTCTTGCT CACAGCATTTG-3'*, (F) *5'-AGCCTCCACCTGATGTCCT AATCA-3'*, (R) *5'-TAACCCACTTTCCAGGGGTCAGTT-3'*, (F) *5'-AGCCTCCACCTGATGTCCTAATCA-3'*, and (R) *5'-cctgagggttaagagcgctagtaa-3'*. These latter primers amplifying ADAM28 variant 4 also amplify variant 5 that is a non-coding sequence.

For real-time RT-PCR, 100ng of cDNA were used, produced using RNA extracted, from whole lungs of OVA-exposed mice and control counterparts (PBS).

Flow cytometry

To assess and quantify inflammatory cells infiltration, lungs of ADAM28$^{+/+}$ and ADAM28$^{-/-}$ were harvested and digested in collagenase C (1mg/ml; Gibco) prior to red blood cell lysis (Red Blood Cell Lysis Buffer, Sigma Aldrich, Saint-Louis, Missouri). Cells were stained with fluorochrome-conjugated surface antibodies during 30 minutes, fixed and permeabilized using Cytofix/Cytoperm (ref#554714 BD Biosciences, Erembodegem, Belgium) before intracellular antigen staining. Antibodies used for flow cytometry analysis were: CD5-BV421 (ref#53-7.3, BioLegend), CD4-PERCP CY 5.5 (ref#RM4-5, BD Biosciences), CD8a-BB515 (ref#53-6.7, BD Biosciences), IFN-γ-PE CY7 (ref#XMG 1.2, BD Biosciences), IL-4-APC (ref#11B11, eBioscience, Thermo Fisher Scientfic), CD45R-APC-eFluor780 (B220) (ref#RA3-6B2, eBioscience). Data were acquired on FACS CANTO II flow cytometer (BD Biosciences) and analyzed using BD FACSDiva software (BD Biosciences).

Statistical analyses

Results are expressed as columns and scatter plots with means ± SE. Statistic comparison between groups was performed, using GraphPad InStat (GraphPad; http://www.graphpad.com). The D'Agostino Pearson and Bartlett's tests were performed to assess normality of the values and difference of variances. Non-normal law variables were compared by nonparametric Mann Whitney test, while normal-law variables were compared using the parametric t-test. When multiple comparisons were performed a one-way ANOVA was used. Values of $P<0.05$ were considered as significant.

Results

ADAM28 expression in lungs of OVA-exposed animals

In order to assess ADAM28 expression in lung in mouse models mimicking acute to chronic features of asthma, mRNAs corresponding to total ADAM28, soluble form (referred to as Variant 4: Var4) and membrane-bound form (or Variant 1: Var1) were measured by RT-PCR after ST, IT and LT allergen exposure (Figures 2A–C).

In the ST exposure model, total ADAM28 expression was lower in lungs of allergen-exposed mice as compared to PBS-exposed animals and ADAM28 expression correlated with the duration of exposure, i.e., animals challenged with OVA in the LT protocol had higher ADAM28 expression as compared to animals exposed to OVA in the ST protocol or in the IT protocol (Figure 2A). These results were confirmed by q-PCR (data not shown). Expression of the membrane-bound variant (var1) of ADAM28 was not significantly modulated at any stage of OVA exposure as compared to PBS (Figure 2B). On the other hand, expression of the soluble ADAM28 form (var4) was about twice higher when animals were exposed to OVA in the IT or LT protocol while not modulated in the ST protocol after OVA exposure (Figure 2C) and ADAM28 expression increased progressively according to the duration of allergen exposure, as it was observed for the total ADAM28.

Effects of ADAM28 depletion on allergen-induced inflammation in lungs in long-term asthma model

In order to assess a potential role for ADAM28 in airway remodelling, ADAM28$^{+/+}$ or ADAM28$^{-/-}$ mice were exposed to OVA in the LT protocol. OVA-exposed animals displayed increased levels of peribronchial inflammation as compared to PBS-exposed animals regardless of the ADAM28 expression (Figure 3A). Nevertheless, bronchial inflammation score was significantly lower in OVA-exposed ADAM28$^{-/-}$ as compared to OVA-exposed ADAM28$^{+/+}$ mice. In PBS-exposed animals, levels of bronchial inflammation were low and similar in ADAM28$^{-/-}$ and ADAM28$^{+/+}$ (Figures 3A–E). $CD4^{+}$T lymphocytes were measured in lungs of PBS- or OVA-exposed animals by flow cytometry. ADAM28$^{-/-}$ displayed a

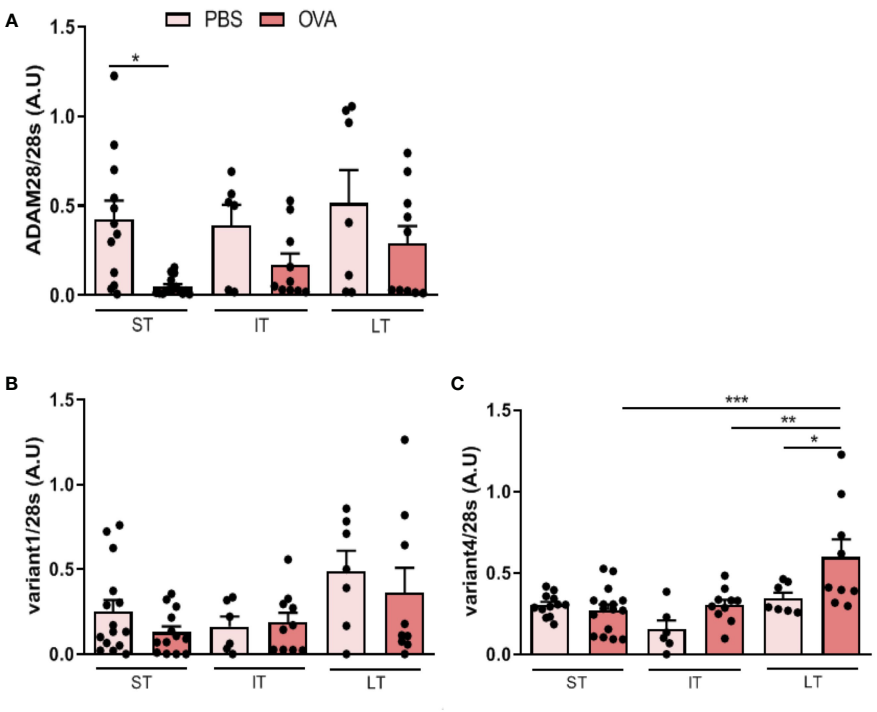

FIGURE 2
Effects of OVA exposure on ADAM28 RNA expression in the lung of mice assessed by RT-PCR on RNA extracted from lungs of mice exposed and non-exposed to OVA. Results are normalized to the 28s rRNA RT-PCR. **(A)** Quantification of relative total ADAM28 mRNA expression in lung of mice (N=2; PBS-ST n=12, OVA-ST n=15, PBS-IT n=6, OVA-IT n=10, PBS-LT n=7, OVA-LT n=10), **(B)** membrane-bound ADAM28 (Var1) mRNA expression (N=2; PBS-ST n=15, OVA-ST n=13, PBS-IT n=6, OVA-IT n=10, PBS-LT n=7, OVA-LT n=9), **(C)** secreted (Var4) ADAM28 mRNA expression (N=2; PBS-ST n=12, OVA-ST n=16, PBS-IT n=6, OVA-IT n=10, PBS-LT n=7, OVA-LT n=9). Results are represented as the mean ± SE, . *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA).

slightly higher recruitment of CD_4^+ T cells in the lungs upon allergen exposure as compared to ADAM28$^{+/+}$ (Figures 3F, G). In the meantime, levels of CD_8^+ T lymphocytes were lower in non-exposed ADAM28$^{-/-}$ mice as compared to the corresponding non-exposed ADAM28$^{+/+}$(Figure 3G). Nevertheless, Th_2 lymphocytes were significantly less recruited after OVA exposure in ADAM28$^{-/-}$ animals as compared to wild-type counterparts (Figures 3H, I). B lymphocytes failed to increase in the lungs of ADAM28$^{-/-}$ after OVA exposure in contrast with what was measured in ADAM28$^{+/+}$ mice (Figures 3J, K).

Measurement of lung function and airway responsiveness to methacholine after a LT allergen exposure

At baseline, i.e., in PBS-exposed mice, there were no significant differences between ADAM28$^{-/-}$ and ADAM28$^{+/+}$ animals regarding lung function parameters measured by Flexivent® (compliance (C), hysteresis and airway resistances) (Figures 4A–C). In the LT protocol, OVA-exposed ADAM28$^{+/+}$ mice displayed changes in lung function (significant decrease of compliance, and increase of hysteresis and airway resistances) when compared to PBS-exposed mice. In contrast, lung function measurements in the LT protocol (evaluated by compliance, hysteresis and airway resistances measurements) in ADAM28$^{-/-}$ were not modified after OVA exposure and remained similar to the values obtained from sham-exposed mice.

Bronchial responsiveness following exposure to increasing doses of methacholine was measured using the FlexiVent® system in ADAM28$^{+/+}$ or ADAM28$^{-/-}$ mice exposed for 90 days to OVA or PBS. During methacholine challenge, OVA-exposed ADAM28$^{+/+}$ animals displayed a classical increase in airway resistances when compared with PBS exposed counterparts while, in sharp contrast, OVA-exposed ADAM28$^{-/-}$ animals did not reach similar levels of airway resistance after being exposed to the highest doses of methacholine and displayed values similar to PBS-exposed animals (Figure 4D).

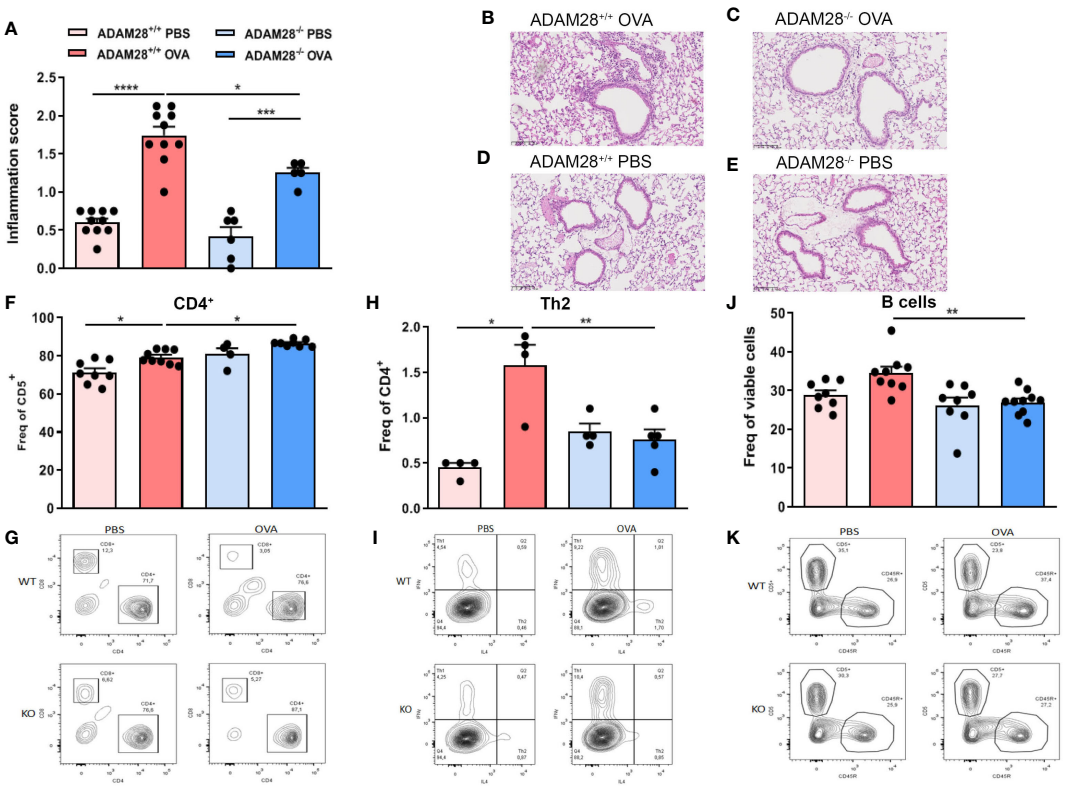

FIGURE 3
Effects of ADAM28 depletion on allergen-induced inflammation in lung tissue . Histological score estimating the peribronchial inflammation quantified from haematoxylin-eosin (H&E) stained lung section of mice lungs (N=3; ADAM28$^{+/+}$ - PBS n=10, ADAM28$^{+/+}$-OVA n=10, ADAM28$^{-/-}$ PBS n=6, ADAM28$^{-/-}$OVA n=5) **(A)**. Representative H&E staining of lung sections. Scale bar: 100µm. **(B-E)**. Measurement of cell numbers in digested lung: CD4^{+}/CD5^{+} ratio (N=2; ADAM28$^{+/+}$- PBS n=8, ADAM28$^{+/+}$-OVA n=9, ADAM28$^{-/-}$ PBS n=4, ADAM28$^{-/-}$OVA n=8) **(F)** and corresponding contour plot of one representative animal for each condition **(G)**; IL4^{+} and CD4^{+} cells that corresponds to the proportion of Th$_2$ among Th cells (N=1; ADAM28$^{+/+}$- PBS n=4, ADAM28$^{+/+}$- OVA n=4, ADAM28$^{-/-}$ PBS n=4, ADAM28$^{-/-}$ OVA n=5) **(H)** and corresponding contour plot of one representative animal for each condition **(I)**; CD45R^{+} positive and CD5^{+} negative cells that represents proportion of B cells among lymphocytes (N=2; ADAM28$^{+/+}$- PBS n=8, ADAM28$^{+/+}$- OVA n=9, ADAM28$^{-/-}$ PBS n=8, ADAM28$^{-/-}$ OVA n=10) **(J)** and corresponding contour plot of one representative animal for each condition **(K)**. Results are represented as mean ± SE, . *p < 0.05.

Allergen-exposed ADAM28$^{-/-}$ mice display significantly less features of airway remodelling in LT asthma model

Airway remodelling was evaluated in the LT protocol after allergen (OVA) or PBS exposure by histology and immunohistological analysis. Mucous hyperplasia was measured by Periodic Acid-Schiff (PAS) staining. Percentages of mucous cells in the airway epithelium were higher in OVA-exposed mice as compared to PBS-exposed counterparts (Figures 5A–C). Percentages of mucous cells after OVA exposure were significantly lower in ADAM28$^{-/-}$ as compared to ADAM28$^{+/+}$ animals (Figures 5A–C). Airway smooth muscle hyperplasia was quantified by the measurement of alpha-smooth muscle actin (α-sma) in immunohistochemistry (Figures 5D–F). In OVA-exposed ADAM28$^{+/+}$ mice, the area occupied by α-sma was significantly increased in airway walls as compared to PBS counterparts while this area did not increase in ADAM28$^{-/-}$ exposed to allergens (Figures 5D–F). ADAM28$^{+/+}$ mice exposed to allergens displayed a significantly increased collagen deposition in the airway walls as measured by Masson's trichrome staining. In contrast, the extent of collagen deposition in the airway walls was significantly lower in ADAM28$^{-/-}$ exposed to allergens (Figures 5G–I).

Lung fibroblasts express ADAM28 mRNA and their proliferation rate is lower after OVA exposure when they originate from ADAM28$^{-/-}$

In order to study the potential role of ADAM28 in the modulation of airway remodelling, fibroblasts were isolated from lungs of OVA- or PBS-exposed animals (LT protocol) and

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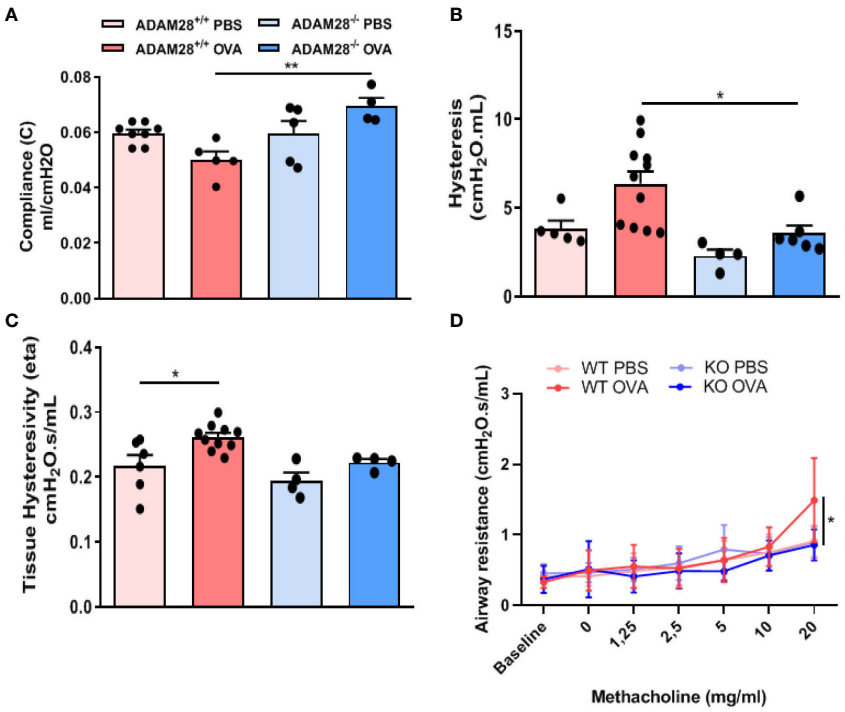

FIGURE 4
Measurement of airway function in ADAM28$^{-/-}$ and ADAM28$^{+/+}$ animals after allergen (OVA) or sham exposure (PBS). **(A)** Baseline lung compliance (N=2; ADAM28$^{+/+}$- PBS n=8, ADAM28$^{+/+}$- OVA n=5, ADAM28$^{-/-}$ PBS n=5, ADAM28$^{-/-}$ OVA n=4), **(B)** lung hysteresis (N=2; ADAM28$^{+/+}$- PBS n=5, ADAM28$^{+/+}$- OVA n=11, ADAM28$^{-/-}$ PBS n=4, ADAM28$^{-/-}$ OVA n=6) **(C)** lung hysteresivity (N=2; ADAM28$^{+/+}$- PBS n=6, ADAM28$^{+/+}$- OVA n=10, ADAM28$^{-/-}$ PBS n=4, ADAM28$^{-/-}$ OVA n=4). **(D)** Measurement of the airway responsiveness. The airway resistance was measured *via* the forced oscillation technique (Flexivent®) 24h after the last OVA or PBS exposure after exposure to increasing concentrations of methacholine. Results are expressed as mean ± SE, (N=2; ADAM28$^{+/+}$- PBS n=7, ADAM28$^{+/+}$- OVA n=10, ADAM28$^{-/-}$ PBS n=3, ADAM28$^{-/-}$ OVA n=6). *p < 0.05, **p < 0.01 (One way ANOVA).

cultured in order to evaluate their ability to produce ADAM28 mRNA and to proliferate. Proliferation rate of freshly isolated fibroblasts was evaluated after the first passage by measuring DNA levels in cell culture during 48h by Cyquant analysis. Fibroblast proliferation was significantly increased after OVA exposure, regardless of their extraction from ADAM28$^{+/+}$ or ADAM28$^{-/-}$ mouse lungs (Figure 6A). However, the proliferation of fibroblasts extracted from lungs of OVA-exposed ADAM28$^{-/-}$ animals was significantly lower as compared with OVA-exposed ADAM28$^{+/+}$ (Figure 6A). No significant difference was found regarding ADAM28 mRNA expression measured by RT-PCR in fibroblasts extracted from OVA- or PBS-exposed wild-type mice (Figure 6B).

Bronchoalveolar lavage from OVA-exposed ADAM28$^{-/-}$ fail to stimulate fibroblast proliferation

Lung fibroblasts were isolated from naïve ADAM28$^{+/+}$ mice and cultured with a medium containing 10% BAL. Cell proliferation was evaluated by measuring DNA levels using CyQUANT during 48 hours. Fibroblast proliferation was significantly increased when cells were cultured with BAL from OVA-exposed ADAM28$^{+/+}$ (LT model) in contrast to what was observed when fibroblasts were incubated with BAL collected from OVA-exposed ADAM28$^{-/-}$ (Figure 6C).

Discussion

We report here that total ADAM28 expression in lungs is lower after allergen exposure in a mouse model of asthma. However, the expression of the secreted variant of ADAM28 (var4) gradually increase with the duration of allergen exposure with levels that stay lower in allergen-exposed animals as compared to sham-exposed mice. As our working hypothesis was that ADAM28 could play a role in asthma-related airway remodelling, we focused on a long term allergen-exposure model (LT) obtained after 90 days of exposure to allergens where the highest levels of ADAM28 expression in the lung tissue were measured.

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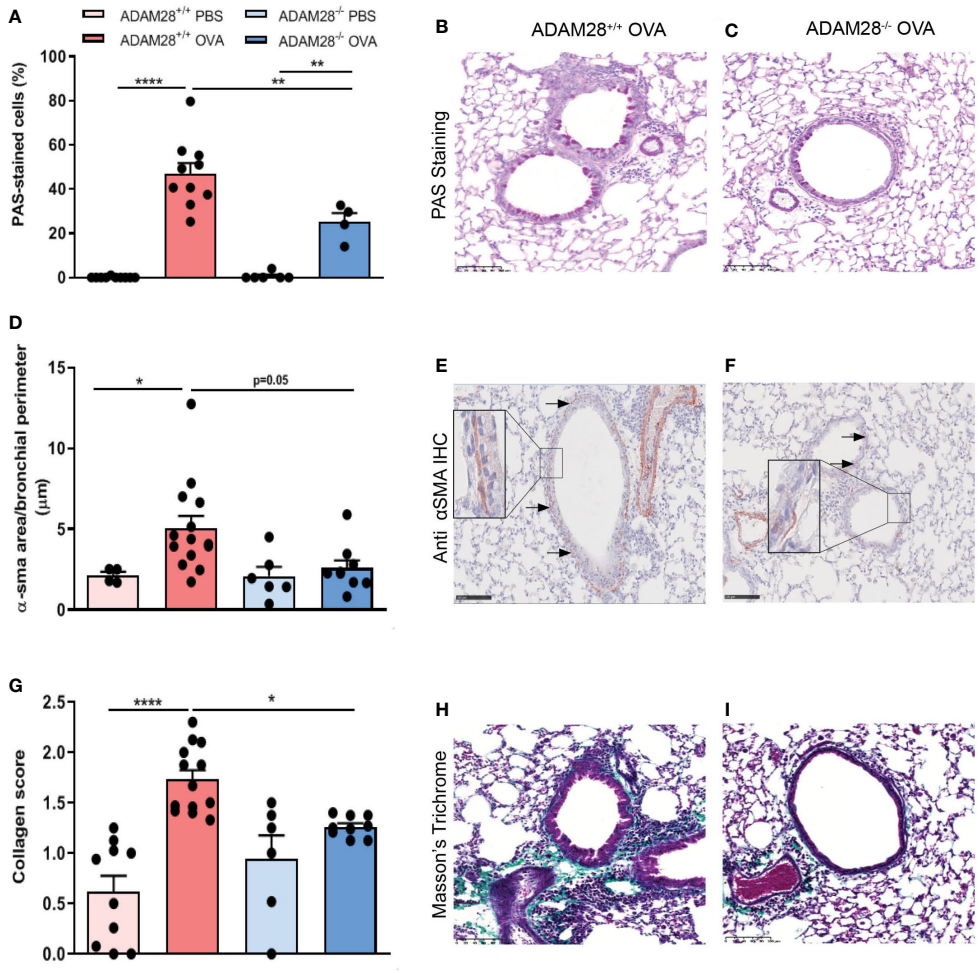

FIGURE 5
Assessment of airway remodelling in the lung of ADAM28$^{-/-}$ and ADAM28$^{+/+}$ mice exposed to allergens for 90 days (LT protocol). **(A)** Quantification of the percentage of PAS-positive epithelial cells per bronchi (N=2; ADAM28$^{+/+}$- PBS n=10, ADAM28$^{+/+}$- OVA n=10, ADAM28$^{-/-}$ PBS n=6, ADAM28$^{-/-}$ OVA n=5). **(B, C)** Representative PAS staining of lung sections of ADAM28$^{+/+}$ and ADAM28$^{-/-}$ mice exposed to OVA. Scale bar: 100 µm. **(D)** Assessment of smooth muscle area around the bronchi stained by IHC against α-SMA reported to perimeter of the epithelial basement membrane (mean of 7 bronchi per mouse) (N=2; ADAM28$^{+/+}$- PBS n=4, ADAM28$^{+/+}$- OVA n=14, ADAM28$^{-/-}$ PBS n=6, ADAM28$^{-/-}$ OVA n=9). **(E, F)** Representative IHC with an anti-α-SMA in lung sections of ADAM28$^{+/+}$ and ADAM28$^{-/-}$ mice exposed to OVA. Scale bar: 100 µm. **(G)**. Collagen deposition score related to the thickness collagen (mean of 7 bronchi randomly selected per mouse, each one scored from 0 to 3) (N=2; ADAM28$^{+/+}$- PBS n=10, ADAM28$^{+/+}$- OVA n=13, ADAM28$^{-/-}$ PBS n=6, ADAM28$^{-/-}$ OVA n=9). **(H, I)** Collagen stained by Masson's Trichrome staining of lung sections of ADAM28$^{+/+}$ and ADAM28$^{-/-}$ mice exposed to OVA. Scale bar: 100 µm. Results are represented as the mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001 (One way ANOVA).

Although its precise biological functions are still uncertain, ADAM28 might play a role in key mechanisms leading to asthma-related inflammation and airway remodelling. We show indeed that after OVA exposure, ADAM28$^{-/-}$ mice does not display the same level of inflammatory cells recruitment in the peribronchial area as observed in ADAM28$^{+/+}$ mice. Interestingly, Th_2 cell numbers fail to increase after allergen exposure in the lungs of ADAM28$^{-/-}$ suggesting a profound dysregulation of immunological pathways when these ADAM28 deficient mice are exposed to allergens. This is in line with the lower levels of CD8 cells reported earlier in cancer models in these mice (20). Together with lower levels of inflammation in ADAM28$^{-/-}$ animals, key features of airway remodelling (glandular hyperplasia, smooth muscle cell hyperplasia and collagen deposition) are significantly smaller after allergen exposure as compared to wild-type animals.

As we hypothesized that ADAM28 could cleave membrane-bound or soluble mediators and activate these molecules, we planned to assess the possible biological effects on fibroblasts (that could contribute to explain our findings regarding airway remodeling). One of the mechanisms that might account for these differences is an inhibition of fibroblast proliferation as

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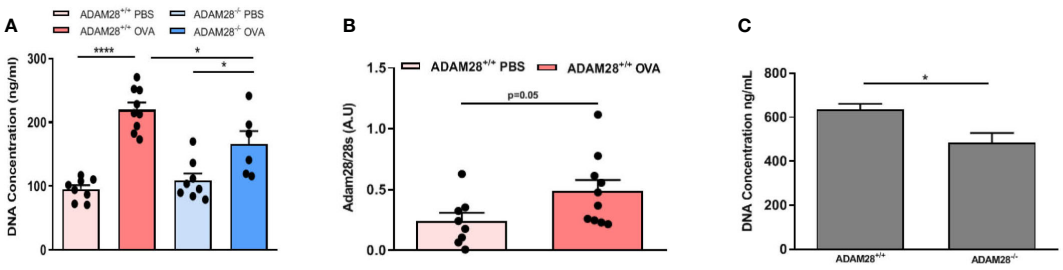

FIGURE 6
Study of lung fibroblasts. **(A)** CyQuant measurement of cell proliferation of fibroblasts extracted from lungs of ADAM28$^{-/-}$ and ADAM28$^{+/+}$ mice exposed to OVA or PBS. DNA levels were measured in cell culture during 48h (N=2; ADAM28$^{+/+}$- PBS n=8, ADAM28$^{+/+}$- OVA n=9, ADAM28$^{-/-}$ PBS n=8, ADAM28$^{-/-}$ OVA n=6). **(B)** RT-PCR measurement of ADAM28 mRNA expression in fibroblasts extracted from wild-type mice exposed to OVA or PBS for 90 days (N=2; ADAM28$^{+/+}$- PBS n=8, ADAM28$^{+/+}$- OVA n=10). **(C)** Measurement by CyQuant of cell proliferation of fibroblasts extracted from lungs of naive mice exposed during 48 hours to media enriched with 10% of BALF from ADAM28$^{+/+}$ or ADAM28$^{-/-}$ mice exposed to OVA in the LT model. DNA levels were measured in cell culture during 48h (N=1; ADAM28$^{+/+}$- OVA n=7, ADAM28$^{-/-}$ OVA n=7). Results are represented as the mean ± SE. *p < 0.05, ***p < 0.001 (Figure 6A: One way ANOVA, Figures 6B, C: Mann-Withney).

shown in this article on fibroblasts extracted from lungs of ADAM28$^{-/-}$ and wild-type counterparts. As fibroblasts incubated with the BAL from ADAM28$^{-/-}$ mice also displayed a lower proliferation rate, this suggests that a soluble factor in the BAL can stimulate fibroblast proliferation and is not produced nor activated in the absence of ADAM28. We chose to study effects of the BAL fluid since it contains a large number of mediators that have potentially been processed by ADAM28. However, the identification of such factor that might be a substrate for ADAM28 is not achieved yet. Specific techniques as iTRAQ-TAILS followed by mass spectrometry might be helpful to answer that question by unveiling cleavages performed by membrane-bound proteases (23).

In line with a significantly lower remodelling of the airways of ADAM28$^{-/-}$ animals after OVA challenge, we measured significantly lower airway responsiveness in these animals as compared to wild-type mice after allergen exposure. A possible protection of ADAM28$^{-/-}$ mice against structural changes of the airways is plausible since we measured significantly lower compliance, an increase of hysteresis and hysteresivity (η) in ADAM28$^{+/+}$ exposed to allergens. These parameters are closely related to the remodelling classically observed in models of asthma after 90 days allergen exposure therefore confirming our histological observations (lower airway remodelling in ADAM28$^{-/-}$ mice as compared to ADAM28$^{+/+}$ after OVA exposure).

The exact mechanisms linking ADAM28 and a modulation of airway remodelling and responsiveness in asthma are still to be unveiled but there are arguments to hypothesize that ADAM28 contributes to the events leading to the asthma phenotype. The ability of this protease to cleave the low affinity IgE receptor CD_{23} present at the surface of B-cells, monocytes, macrophages and eosinophils (24, 25) could interfere with different key processes. Indeed, this cleavage of CD_{23} generates a soluble form of CD_{23} as already demonstrated for other ADAM proteases including ADAM8 (26, 27). However, this effect on CD_{23} could be negligible since ADAM10 was recognized as the main sheddase for CD 23 (28). Nevertheless, control of CD23 is of key importance in mechanisms of asthma and allergy as this mediator modulates T cell activation (29, 30). As ADAM28 has the capacity to bind various integrins, it can also be hypothesized that this protease might interfere with inflammatory and stromal cells trafficking (17). For example, ADAM28 recognizes $\alpha_9\beta_1$ as well as $\alpha_4\beta_1$ which contribute to adhesion and transendothelial migration of neutrophils and lymphocytes, respectively (31–34). Also, it was shown that soluble ADAM28 is able to enhance α4β1-dependent cell adhesion to VCAM-1 (vascular cell adhesion molecule-1) therefore influencing lymphocyte adhesion to endothelium and trafficking across the capillary walls (17). This is possibly the cause of the lack of migration of Th_2 lymphocytes to the lung after allergen exposure in ADAM28$^{-/-}$ animals reported in this work.

We recently reported that ADAM28 depletion in mice causes increased tumour cell dissemination in lungs by decreasing the cancer cytotoxicity mediated by CD_8 lymphocytes (20). However, ADAM28 expression has been reported in thymic epithelial cells suggesting a role in T lymphocyte differentiation (32–35) but mice depleted for ADAM28 did not display any abnormalities in thymocytes and T lymphocytes differentiation (20). A drastic reduction of CD8$^+$ T cells was reported in spleen of ADAM28$^{-/-}$ (20). In this study, we report that CD8 lymphocytes are lower in ADAM28$^{-/-}$ as compared to ADAM28$^{+/+}$ animals. The role of CD8$^+$ lymphocytes in asthma is complex and has been nicely reviewed by Lourenço et al. (36). Inhibition studies using depleting antibodies suggest that CD_4^+ lymphocytes are not *per se* sufficient to induce airway remodelling (37) suggesting a role for other actors as CD_8^+ cells. These cells are heterogeneous and a subset of CD_8^+ cells was reported to produce IL13 and was

associated with airway obstruction suggesting a plausible role of these cells in airway remodelling (38). Moreover, IL13 plays a significant role in airway remodelling and was shown to increase the pro-fibrotic gene expression in fibroblasts (39).

Interestingly, ADAM28 might activate pathways leading to or supporting asthma-related inflammation by modulating the biological activity of mediators as TNF-α. Indeed, ADAM28 might activate TNF-α acting as a sheddase able to release mature TNF-α in the supernatant of ADAM28 transfected HEK-293 cells (40). Also, inhibition of endogenous ADAM28 in macrophages resulted in a reduced mature TNF-α release (40). Recently, it was suggested that ADAM28 might play a role in key immunomodulatory mechanisms since $CD20^+/CD22^+/ADAM28^+$ B cells were shown to promote response to immune checkpoint inhibitor therapy in non-small-cell lung cancer (41).

Regarding the differences of expression of ADAM28 Var4 in the mouse model, the primers used to amplify Var4 also recognize and amplify Var5 that is a non-coding sequence. However, we cannot rule out a possible interference with measurements of RNA corresponding to Var4.

Full ADAM28 depletion in mouse does not lead to any spontaneous phenotype and specifically no developmental abnormalities in bronchial tree, alveolae architecture, or bronchial epithelium (20). As previously reported for many other proteases, the stimulation of inflammation is able to unveil specific functions of ADAM28. Furthermore, our observations suggest that ADAM28 contributes to mechanisms leading to asthma-related airway inflammation and remodelling but it is probably not the final effector and further experiments are needed to fully unveil the precise molecular mechanism.

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 reviewed and approved by Comité d'éthique animal hospitalo-universitaire - Université de Liège et CHU Liège.

Author contributions

GB, CH, FP, AG, MJN, OC, CG performed the bench work and experiments described in the manuscript. GB drafted the figures. FP, DC, AG, and MJN edited the final version of figures. AN, PL, NR supervised the experiments and contributed to the interpretation of results and to manuscript redaction. NR created the ADAM28-/- mice. DC started the research program, applied for grants to fund the research program, supervised the bench work and the interpretation of results, finalized the manuscript and submitted the manuscript and the revised version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1067779/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Gating strategy for flow cytometry: Identification of IL4 and IFNγ production by CD4+ T cells. Debris and doublets were excluded based on FSC and SSC. B lymphocytes were defined as live cells positive for CD45R and negative for CD5. CD4+ T cells were identified as live CD5+ CD4+ cells. IL4 and IFNγ was then assessed in this population. CD8+ T cells were identified as alive CD5+ CD8+ cells.

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The emerging role of TET enzymes in the immune microenvironment at the maternal-fetal interface during decidualization and early pregnancy

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A dysregulated immune microenvironment at the maternal-fetal interface in early pregnancy may lead to early pregnancy loss, fetal growth restriction, and preeclampsia. However, major questions about how epigenetic modifications regulate the immune microenvironment during the decidualization process and embryo implantation remain unanswered. DNA methylation, the main epigenetic mechanism involved in the endometrial cycle, is crucial for specific transcriptional networks associated with endometrial stromal cell (ESC) proliferation, hormone response, decidualization, and embryo implantation. Ten-eleven translocation (TET) enzymes, responsible for catalyzing the conversion of 5-methylcytosine to 5-hydroxymethylcyosine, 5-formylytosine, and 5-carboxylcyosine to achieve the DNA demethylation process, appear to play a critical role in decidualization and embryo implantation. Here, we provide a comprehensive view of their structural similarities and the common mechanism of regulation in the microenvironment at the maternal-fetal interface during decidualization and early pregnancy. We also discuss their physiological role in the decidual immune microenvironment. Finally, we propose a key hypothesis regarding TET enzymes at the maternal-fetal interface between decidual immune cells and ESCs. Future work is needed to elucidate their functional role and examine therapeutic strategies targeting these enzymes in pregnancy-related disease preclinical models, which would be of great value for future implications in disease diagnosis or treatment.

KEYWORDS

DNA methylation, TET enzymes, decidualization, early pregnancy, microenvironment, immune cells

Introduction

The endometrium, regarded as one of the most dynamic tissues in the human body, undergoes periodic changes, including cell proliferation, differentiation, and apoptosis (1). This tissue is composed of luminal and glandular epithelial cells, stromal cells, immune cells, endothelial cells, and so on, participating in the formation of the microenvironment at the maternal-fetal interface and controlling the subsequent invasion of trophoblast cells and the establishment of the maternal-fetal interface immune tolerance (2, 3). Abnormal decidualization of the endometrium can lead to infertility and a variety of pregnancy-related diseases, including early pregnancy loss (EPL), fetal growth restriction (FGR), and preeclampsia (PE) (4, 5). At present, studies have shown that a variety of steroid hormones, transcription factors, lipids and cell cycle–related proteins regulate the process of decidualization and participate in early embryo implantation and pregnancy maintenance. Epigenetic modification is an important regulation mode that affects gene expression and cell function. It can occur at transcriptional, posttranscriptional, and posttranslational levels, involving DNA methylation, histone methylation, and histone acetylation and deacetylation. At present, there is a large amount of evidence that epigenetic modification is involved in the regulation of the decidualization process (6).

DNA methylation is one kind of chemical modification of DNA, by which the cytosine nucleotide is converted into 5mC by a family of DNA methyltransferases (7). Whereas DNA demethylation is generated by active enzymatic demethylation during which 5-methylcytosine (5mC) undergoes a series of oxidation reactions catalyzed by the methylcytosine dioxygenases ten-eleven translocation (TET) enzymes (8). High levels of CpG islands and methylation of these islands may result in transcriptional silencing or repressing (7, 9, 10). Thus, DNA methylation is considered to be a main mechanism behind many fundamental cellular processes, including the endometrium's cyclical changes (11). The tissue-specific variation in DNA methylation content across the menstrual cycle further suggests that DNA methylation regulates gene expression during the endometrial cycle (7, 12, 13).

Of note, the creation of an appropriate immune microenvironment is another key element for blastocyst implantation (14, 15). The decidual immune cells are mainly composed of lymphocytes (e.g., NK cells, T cells, dendritic cell and NK-T cells, etc.) and macrophages, and their proportion changes in the endometrial cycle (16). Dysregulation of the immune response and immune cell distribution may lead to placentation failure and reproductive decline (17, 18). Studies have shown that the genes affected by decreased methylation during decidualization were mainly associated with immune response regulation (19, 20). Furthermore, DNA methylation also plays a vital role in immune cell development and maturation, which contributes to decidual immune homeostasis (21–23). Altogether, we performed a comprehensive literature review concerning the roles of TET enzymes in the microenvironment at the maternal-fetal interface during decidualization and early pregnancy.

Common features of TET proteins

TET1, TET2, and TET3, which constitute a family of iron (II)/2-oxoglutarate-dependent dioxygenase, are responsible for catalyzing the conversion of 5mC to 5-hydroxymethylcyosine (5hmC), 5-formylytosine (5fC), and 5-carboxylcyosine (5caC) to achieve the DNA demethylation process (24–26). These enzymes are associated with several conserved signaling pathways in several kinds of organs or tissues during development, especially in embryo and cancer development (27, 28).

Structural similarities

TETs are all composed of an acatalytic region in their C-terminal that is responsible for 5mC dioxygenase activity termed the double-stranded β-helix domain and a conserved cysteine-rich domain, which is thought to be essential for proper folding. However, TET1 and TET3 carry a CXXC domain at the N-terminal region, which is not present in TET2. As a result, CXXC was separated and originates the IDAX gene, which acts as a negative regulator for TET2 (29).

Functions in DNA demethylation and decidualization and early pregnancy

During the DNA demethylation process, TET enzymes oxidize the methyl group to 5hmC, 5-formylcytosineand 5-carboxylcytosine (30–33). After being recognized and excised by the enzyme thymine DNA glycosylase, these bases are substituted by an unmodified cytosine by base excision repair and lose their indicated function (33). In other words, TET enzymes work as erasers in the DNA methylation machinery during the whole endometrial cycle (34). All three TET enzymes are detectable in both epithelium and stroma tissues during the cycle. Besides this, recent studies show that TET1 and TET3 are preferentially expressed in the midsecretory phase over the other phases (27). Moreover, progesterone induces expression levels of all TET enzymes in endometrial epithelial cells, whereas estradiol plus progesterone treatment increases the expression of TET3 in the same cell type, but estradiol only induces the expression of TET1 in stromal cells, indicating that sex hormones regulate the expression of TET genes in a dynamic and cell-specific manner in the human endometrium (27). Our previous study found that the expression of TET3 gradually decreases in the endometrial tissues of women in the

proliferative and secretory phases of the menstrual cycle as well as in the decidual tissues of early pregnancy, whereas it increases in the decidual tissues of women with EPL. Further mechanism studies indicate that TET3 negatively mediates miR-29a's role in promoting the decidualization of endometrial stromal cells (ESCs) *in vitro* and maintaining pregnancy *in vivo*, suggesting that TET3 inhibits decidualization of ESCs, which may be involved in the pathogenesis of EPL caused by abnormal decidualization (35).

It is worth noting that miR-29a can upregulate the levels of decidualization markers IGFBP1 and PRL, whereas TET3 inhibits this effect (35). The specific mechanism remains to be further explored. At present, there are two main mechanisms of action of TET3: first, TET3 α-ketoglutaric acid (α-ketoglutarate, α-KG) and Fe2+catalyze the conversion of 5mC to 5hmC, mediate the demethylation process, and finally promote gene expression (36). In addition to the above mechanisms, TET3 also combines O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) transferase (OGT), catalyses the *O*-GlcNAc glycosylation of histone serine and threonine residues (*O*-GlcNAcylation), and the final effect is to promote the downstream target genes transcription (37). However, these mechanisms are not enough to explain the phenomenon that TET3 downregulates the levels of IGFBP1 and PRL. There should be other mechanisms by which TET3 regulates the decidualization process of ESCs.

Based on the epigenetic modification mechanism, it can reduce or enhance the degree of DNA aggregation, thus regulating the expression of target genes at the transcriptional level (38–40). At the same time, other members of the TET family, TET1 and TET2, bearing certain structural homology with TET3, are also proved to be able to combine with multiple epigenetic regulatory molecules, for example, TET1 combines SIN3A, MeCP2, HDAC1/6/7, EZH2, LSD1, etc. (41, 42); TET2 combines Smarcb1/c2/e1, HDAC1/2, Ncor1/2, Baz1a/1b, Top2a/2b, Mbd2, Phf2, Ino80, Sap30bp, Trrap, Wdhd1, Chd8, Chaf1a, and Dnmt3a, etc. (43). At present, studies have shown that multiple molecules interacting with TETs play a role in the decidualization of ESCs, including SIN3A, EZH2, Dnmt3a, etc. (11, 44, 45), suggesting that members of the TET family can not only act as catalytic enzymes to affect epigenetic modification, but also act as anchor proteins for a variety of epigenetic modification enzymes.

Besides this, some studies also show TET expression in endometrial pathology. For example, a higher level of TET3 and lower levels of TET1 and TET2 were found in endometrial cancer compared with the normal endometrium, whereas endometrial cancer tissues showed lower levels of global hydroxymethylation at the same time (46). TET gene expression was also found dysregulated in the ectopic endometrial tissue of women with endometriosis, including decreased *TET1* levels (47). However, the expression and regulation of TETs in the endometrium is still not clear. Therefore, further studies are required to explore the mechanism by which and how TETs regulate the key processes during decidualization, embryo implantation, and placental growth.

Additional biological roles of TET enzymes in decidual immune tolerance

Decidual immune microenvironment and DNA methylation levels

Decidual immune cells are mainly composed of natural killer (NK) cells, macrophages, T cells, dendritic cells, and so on. Decidual NK (dNK) cells represent the largest population (50%–70%), whereas macrophages comprise approximately 10%–20% of whole decidual leukocyte populations, and the others are a very small minority (48–50). These immune cells, together with decidual stromal cells, cooperate to modulate trophoblast invasion, promote fetal growth, and regulate immune tolerance. Epigenetic modifications, including DNA methylation, are a key avenue for controlling immune responses, which can change the gene expression level without altering the underlying DNA sequence, thus allowing for a rapid adaptation of cells to the surrounding environment (51, 52). DNA methylation also provides an unexplored mechanism for immune regulation of decidual immune cells during the endometrial cycle, which could help explain how decidual immune cells are able to adapt and respond to the dynamic changes throughout the decidualization process. Interestingly, one recent study has identified low expression levels of genes that are related to NK cell function, such as KIR2DL3 and KLRC3, at the late proliferative phase, suggesting a decreased immune response mediated by NK cells at this phase of the endometrial cycle, which is consistent with the modulation of the immune response to favor embryo implantation (53). Besides this, another study also found the genes affected by decreased methylation were mainly associated with immune response regulation (FYN, BCL3, PVR, JAK3, IL1RL1, RFTN1, MYO1G, CXCL13, and C1S) (19).

Roles of TET enzymes in immune cell development and function

Whereas the implication of TET proteins in DNA demethylation is well-established, the mechanisms underlying TET proteins in immune cells is yet to be explored. Strikingly, TET loss of function is strongly associated with hematological malignancies. For example, TET2 loss-of-function mutations are frequently observed in myelodysplastic syndromes and myeloid malignancies as well as in certain peripheral T-cell lymphomas (54–57). The biological roles of TET proteins in immune cell

development, function, and malignant transformation have been unraveled in these studies.

T cells

In T cells, the loss of TET proteins may result in compromised immune function or malignant transformation. Of note, TET2/3 are preferentially expressed in T cells compared with TET1 and play central roles in 5hmC modification in these cells (58). Deletion of TET2 alone in the hematopoietic system or in T cells did not result in any defect in T-cell lineage fate, indicating a compensative relationship between TET2 and TET3 (59, 60). However, it is reported that lack of TET2 enhanced $CD8^+$ T-cell memory formation and differentiation (60). Although deletion of TET2 in $CD4^+$ T cells in mice have intact thymic and peripheral T-cell subpopulations, typical cytokine expression was found decreased, including IL-17, IL-10, and IFN-g (59). The most profound phenotypes have been found in T cells upon codeletion of at least two TET members. For instance, TET2/TET3 DKO mice exhibited a striking increase of iNKT cells with impaired function and enhanced stemness (59, 61, 62). Surprisingly, genome-wide DNA methylation remains unchanged in response to the loss of TET proteins, but the deposition of 5hmC across specific genes, such as Tbx21 and Zbtb7b, is affected, suggesting a focal regulation role of TET members. These TET2/3 DKO iNKT cells can produce large amounts of immune response–related cytokines and drive other immune cell subset expansion and responses. In addition, TET enzymes are also required for the homeostasis of T regulatory (Treg) cells by modulating the expression of the transcriptional factor FOXP3. TET2 and TET3 are able to demethylate two intronic enhancers, termed conserved noncoding sequence (CNS) 2, which is critical for maintenance of FOXP3 expression (63–65). Deleting TET2 and TET3 specifically in Tregs not only results in compromised Treg lineage, but also a gain of aberrant activation and effector function in those cells, which enhances whole-body inflammation and ultimately accelerates death. Double TET1/2 deletion may also result in impaired Treg inactivation and differentiation due to hypermethylation of the CNS locus (66). Given the importance of all kinds of T cells involved in the endometrial cycle and decidualization process, future pharmacological methods specifically targeting TET proteins to modulate T-cell activity may employ a strong biological effect in the endometrial cycle.

B cells

TET-dependent DNA demethylation is essential for B-cell differentiation, maturation, and function. TET protein expression levels are dynamically regulated during B-cell development. TET1 is significantly reduced in pro-B-cells, whereas TET2 and TET3 is increased during B-cell maturation and activation, suggesting a critical role of TET proteins in B-cell biology (67). *In vitro* analysis of TET1 KO cells showed a promoted status of lymphoid bias differentiation with more self-renewing pro-B-cell colonies compared with pre-B-cells (68). Long-term lack of TET1 resulted in lymphocytosis in mice by 18–24 months of age. TET2, one of the most frequently mutated genes in diffuse large B-cell lymphoma, works as a tumor-suppressor gene. Based on previous studies, TET2 was shown to be required for CSR and affinity maturation of antibodies, and disruption of TET2 may result in germinal hyperplasia. Mechanically, TET2 can preferentially strengthen the activity of enhancers (Igk and Aicda) (69). Compared with TET2 deletion, codeletion of TET2 and TET3 may cause more severe B-cell phenotypes during bone marrow development, including halting the pro-B-cell to pre-B-cell transition process and decreasing mature B cells in mice, and it diminishes the rearrangement of the Igk locus by increasing CpG methylation levels at the Igk3′ and distal enhancers (70). Future studies are needed to examine how TET proteins epigenetically affects B-cell biology in the decidual microenvironment.

Myeloidcell

Compared with other TET proteins, TET2 is preferentially abundantly expressed in myeloid cells and further required for the myeloid cell–mediated innate immune response and surely critical in the decidual immune microenvironment (71, 72). TET2 deletion does not dramatically alter alternative macrophage (M2) gene expression levels, but indeed decreases the immunosuppressive function of these cells. TET2-KO macrophages and DCs produce more proinflammatory cytokines, such as IL-6, in response to bacterial activation (43, 73). Compared with wild-type mice, Tet2-KO mice show increased susceptibility to endotoxin-induced shock, DSS induced colitis, and so on, all suggesting the anti-inflammatory function of TET2 (43). Notably, during tumor growth, TET2 expression was found increased in myeloid-derived suppressor cells and tumor-associated macrophages and preserved immunosuppressive gene expression levels. TET2 deficiency in tumor-associated macrophages results in defective immunosuppressive capacity and an altered cytokine expression profile (74, 75). However, the role of TET2 in the myeloid-mediated decidualization process awaits further investigation.

NK cells

NK cells play central roles in boosting inflammation and decidualization, but the evidence is lacking regarding whether and how TET proteins function in NK cells and, thus, have an effect during the endometrial cycle (3, 48). Continued efforts are needed to investigate the possible role of TET proteins in dNK

cells, and this will shed light on the current understanding of the biological role of TET enzymes in decidual immune tolerance.

Macrophages

Macrophages are highly diverse cells and the major antigen-presenting cells at the maternal-fetal interface. In addition to protection of the embryo from the attack of the maternal immune system, decidual macrophages also play a key role in embryo implantation, trophoblastic invasion, spiral artery remodeling, and placentation. Recently, new concepts have emerged to explain how macrophage polarization and function are regulated, including immune metabolism and epigenetics (76, 77). Macrophages are divided into M1-like macrophages and M2-like macrophages. M1 macrophages secrete a variety of cytokines including IL-2, IL-6 and TNF-α, which involved in pro-inflammatory responses, whereas M2 macrophages are mainly involved in anti-inflammatory responses (78). The balance of M1 macrophages and M2 macrophages is critical for various processes in both normal and pathological pregnancy (79). However, the functions of TETs in decidual macrophages are largely unknown. Although only a few studies showed epigenetic regulation in the differentiation and function of decidual macrophages, emerging studies reported the role of epigenetic modulating by TETs in macrophages in other fields (43, 73, 80), which may shed new insights for further studies on decidual macrophages.

Conclusions and future perspectives

Collectively, TET proteins are critical to 5hmC/5Mc/5fC/5caC modification in various decidual immune and stromal cells, which is essential for the decidualization process and early pregnancy. It is expected that using specific compounds modulating TET activity may be useful in pregnancy-related diseases and for modulating immune cell responses during the decidualization process. Thus, it is critical to elucidate the functional role of TET proteins for modulating 5hmC/5mC/5fC/5caC levels in decidual stromal and immune cells, which requires further understanding of the possible underlying molecular mechanism in various cell types. Future work may also be required to explore how to discover and utilize novel TET interactors to modulate immune responses during decidualization and early pregnancy. The elucidation of these aspects will open an exciting field for future work.

Author contributions

MJ conducted the literature search and completed the manuscript of the first draft in collaboration with JJ. XC and YZ helped prepare the manuscript. DW and AL revised and edited the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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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 intestinal immune cells and matrix metalloproteinases in inflammatory bowel disease

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Inflammatory bowel disease (IBD) has become globally intractable. MMPs play a key role in many inflammatory diseases. However, little is known about the role of MMPs in IBD. In this study, IBD expression profiles were screened from public Gene Expression Omnibus datasets. Functional enrichment analysis revealed that IBD-related specific functions were associated with immune pathways. Five MMPS-related disease markers, namely MMP-9, CD160, PTGDS, SLC26A8, and TLR5, were selected by machine learning and the correlation between each marker and immune cells was evaluated. We then induced colitis in C57 mice using sodium dextran sulfate and validated model construction through HE staining of the mouse colon. WB and immunofluorescence experiments confirmed that the expression levels of MMP-9, PTGDS, SLC26A8, and CD160 in colitis were significantly increased, whereas that of TLR5 were decreased. Flow cytometry analysis revealed that MMPs regulate intestinal inflammation and immunity mainly through CD8 in colitis. Our findings reveal that MMPs play a crucial role in the pathogenesis of IBD and are related to the infiltration of immune cells, suggesting that MMPs may promote the development of IBD by activating immune infiltration and the immune response. This study provides insights for further studies on the occurrence and development of IBD.

KEYWORDS

inflammatory bowel disease, matrix metalloproteinases, immune microenvironment, biomarker, gene expression omnibus

1 Introduction

The global incidence of inflammatory bowel disease (IBD) is increasing yearly (1). IBD includes intestinal autoimmune diseases, inflammation, stimulation of inflammatory cells, with the release of inflammatory cytokines, activation of immune cells, and abnormal changes in intestinal microvascular endothelial cell function, which affect immune cell function and the stability of the intestinal environment and lead to cell and tissue damage (2). In recent years, it is generally believed that a part of colorectal cancer can progress from IBD (3, 4). The inflammatory response of the colon is a major factor in the development of colorectal cancer (5). Studies on the treatment of IBD provide insights for the prevention and treatment of colorectal cancer. However, due to the increasing incidence, long course, and delayed healing in IBD, its diagnosis, treatment, and prognosis have become a challenge (6).

Although IBD can be triggered by various factors, the immune response appears critical for the onset of IBD (7, 8). As the largest immune organ of mammals, the gut contains many types of immune cells, including B cells, T cells, dendritic cells, macrophages, eosinophils, and mast cells (7, 9). When the intestinal barrier is damaged, bacterial infection occurs, which affects the process of IBD (10). A focus of our research includes identifying the immune cells that play a role in the pathogenesis of IBD. At present, therapy for IBD mostly involves inhibiting intestinal inflammation (11). Immunotherapy that can regulate the intestinal barrier also provides a new idea for treating IBD.

MMPs are enzymes with specific biological activities (12). MMPs participate in many activities related to maintaining their own stability and play a wide range of roles in the development of disease (13). MMPs can regulate inflammation at all levels. They can regulate the migration of inflammatory cells from the artery to the inflammatory zone and process ECM components, growth factors, cytokines, and chemokines, thus regulating the uptake of inflammatory cells and access to the inflammatory zone (14–16). The inflammatory marker role of metalloproteinases can help in the diagnosis and treatment of some inflammatory diseases (17), especially rheumatoid arthritis (18, 19); however, the role of MMPs in IBD has not been elucidated, and the value of MMPs in the immunotherapy of IBD is rarely demonstrated. Notably, the relationship between MMPs and immune cells may be much more complex than understood (20). Studies have demonstrated high expression of MMP-3 (21), MMP-9 (22, 23), and MMP-13 (24) in damaged colonic mucosa. This gives us confidence to further demonstrate the relationship between other MMPs and IBD.

To evaluate the potential impact of MMPs on IBD, machine learning was used to identify five MMP-related disease markers. The expression of MMP-9, PTGDS, SLC26A8, and CD160 in colitis was increased in the IBD mouse model, whereas the expression of TLR5 was downregulated. In addition, the findings revealed that MMPs regulated the occurrence and development of IBD through CD8.

2 Materials and methods

2.1 Data sources and processing

The GSE94648 and GSE119600 microarray data sets were downloaded from the Gene Expression Omnibus (GEO) datasets. The GSE94648 profile includes samples from 75 patients with IBD and 22 healthy controls, whereas GSE119600 contains samples from 188 patients with IBD and 47 healthy controls. The platforms are GPL19109 and GPL10558, respectively. Finally, the batch effect was eliminated using the "SVA" package in R, and the two datasets were subsequently merged. Metalloproteinase-related genes were retrieved from Gene Cards (https://www.genecards.org/). The cut-offs were set as Relevance Score > 0.2 (Supplementary Table 1).

2.2 Analysis of differentially expressed genes

The "limma" package in R is used to identify various genes. Genes with P-value < 0.05 and absolute log2FC > 0.6 were considered differentially expressed genes (DEGs). Volcano plots and heatmaps were constructed using "heatmap" and "ggplot2" packages in R, respectively.

2.3 Gene set enrichment analysis

Gene ontology (GO) enrichment analysis, KEGG pathway analysis, and DO method combined with "cluster Profiler" in R and the DOSE program were used to study DEGs. The GSEA technique allows for the identification of the most important functional terms in patients with IBD and control groups. "c2.cp.kegg.v7.0.symbols.gm t" is a criterion used for mymbols.gm t. The gene cluster is considered significantly aggregated if $P < 0.05$ or false issue rate < 0.025.

2.4 Candidate diagnostic biomarker screening

Three machine learning methods are used to predict patients' conditions to identify the main prognostic variables. The least absolute compression and selection operation (LASSO) is a new approach that uses regularization methods to improve forecast accuracy. In R, the LASSO regression algorithm uses "glmnet" grouping to identify genetic factors that are significantly associated with IBD and control samples. Support vector machine (SVM) is one of the most widely used supervised machine learning methods. The metadata sequences are optimized using recursive feature elimination (RFE) methods to prevent duplicate screening. SVM-RFE was used to screen for

suitable features to identify the set of gene pools with the highest discrimination power. Then "randomForest" in R was used to implement the random tree algorithm. Finally, the intersection was obtained through the Venn diagram package.

2.5 Discovery of immune cell subtypes

To quantify the rate of invasive immune cells in the gene expression profile of IBD, a bioinformatics algorithm called CIBERSORT (https://cibersortx.stanford.edu/) was used to estimate the invasion rate of the immune system. The number of an immune cell type was estimated using a reference system containing 22 isoforms (LM22) for 1000 permutations. A total of 22 infiltrating immune cell types were correlated using "corrplot" in R. Violin charts were used to represent the infiltration of immune cells in IBD and control samples using "vioplot" in R.

2.6 Correlation analysis between identified genes and infiltrating immune cells

The relationship between identified genetic markers and invading immune cells was evaluated using Spearman's hierarchical correlation in R. The correlations were generated using the graphical technique in the "ggplot2" suite.

2.7 Construction of the mouse model of chronic colitis and experimental design

Male C57 mice (22–24 g), aged ~6 weeks, were purchased from Ltd. in Jiangsu, China. After 1 week of acclimatization, the rats were randomly divided into two groups (n = 6 per group): normal control and dextran sulfate (DSS) groups. For the DSS group, 1.5% (w/v) DSS (36000–50000 Da, China Eason Biochemical Technology Co., Ltd.) was prepared by dissolving in sterilized water, filtered through a 0.22-μm filter, and provided to the rats in the DSS group. The DSS solution was reconfigured every other day. Rats in the control group drank sterilized fresh water and bred in the same facility. A mouse model of chronic colitis lasted for three weeks per period, and after three periods, the mice were euthanized. The mice was weighed every two days, and the blood in the feces and changes in character were evaluated. All mice were euthanized by cervical dislocation. The colon was collected, and its length and thickness were measured.

2.8 HE staining and histological evaluation of colonic damage

Colonic tissue was fixed in 4% methylal solution and left overnight. Then the tissue was fixed and embedded in paraffin and cut into slices with a thickness of ~3 mm. Then after dewaxing, dehydrating, HE staining, dehydrating, and making the samples transparent, the slides were covered with a cover slip coated with neutral gum and sealed. The lesions were observed and recorded under an optical microscope. Lesions and inflammatory cell infiltration were evaluated in colon tissue. Then colon damage was determined histologically according to the scoring criteria of the histological examination provided in Supplementary Table 2.

2.9 Immunofluorescence

Immunofluorescence for MMP-9, PTGDS, the activating NK cell receptor CD160 and TLR5 and SLC26A8 on colonic tissue was performed using standard methods. Colonic sections were deparaffinized and rehydrated. Then antigen retrieval was performed by continuous heating with citrate buffer in a pressure cooker at 98°C for 10 min. The sections were then blocked-in normal serum and labeled with primary antibodies in blocking solution overnight at 4°C. After washing with PBS, the sheet was ligated to Alexa Fluor-488 or Cy-3. The sections were then examined with a fluorescence microscope (Olympus DP72 Microscopic imaging system).

2.10 Flow cytometry

The spleen was cut into pieces with sterilized surgical scissors and put into centrifuge tubes, digested with an appropriate amount of trypsin, and centrifuged at 1000 rpm for 5 min. Then 1×10^6 cells were collected and resuspended with appropriate amount of flow staining buffer, and 5 μL of each antibody was added to the final reaction volume of 100 μL. Anti-CD16/CD32, -CD8, -CD25, and -CD56 antibodies were purchased from Multisciences. The cells were mixed by shaking and incubated for 20 min in the dark at room temperature. Then 1 mL of flow staining buffer was added to each tube. The tube was centrifuged at 300 × *g* for 10 min and the supernatant was discarded. Then 500 μL of flow staining buffer was added to the tube to resuspend the cells and a flow cytometer (Beckman Coulter, Inc.) was used for detection.

2.11 Western blotting

Colonic tissue was cut and placed in EP tubes, and RIPA lysis buffer (Epizyme Biomedical Technology, Shanghai, China) was added at 100 mg/mL (containing 1% phosphatase inhibitor and 1% PMSF). The sample was homogenized with a tissue homogenizer until no visible pieces remained. Then lysis was performed on ice for 30 min. The supernatant was collected and total protein concentration was evaluated using a bicinchoninic

acid kit (TransGen Biotech, Beijing, China). The samples were run using 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Then, the membranes were blocked with QuickBlock Blocking Buffer (Beyotime Biotechnology in Shanghai, China) for 15 min, incubated with diluted primary antibodies overnight at 4°C, washed three times with TBST, and incubated for 1 h with secondary antibodies. TLR5 (19810-1-AP) and SLC26A8 (12776-1-AP) were purchased from Proteintech (Wuhan, China). MMP-9 (TA5228S) was purchased from Abmart Technology (Shanghai, China). PTGDS was purchased from Solarbio Life Science (Beijing, China) and CD160 from Affinity Biosciences (Suzhou, China). Goat anti-rabbit IgG (H+L) HRP (BL003A) was purchased from Biosharp (Hefei, China).

3 Results

3.1 Identification of DEGs in IBD

In this study, data were collected from three GEO data sets, namely GSE94648 and GSE119600, for 263 IBD and 69 control samples, respectively. After correcting for batch processing, metadata DEGs were parsed using the limma software. The results showed that 18 DEGs could be obtained by this method. Furthermore, 11 genes were significantly upregulated and 7 were significantly downregulated (Figures 1A, B). Subsequently, the DEGs were intersected with 3970 metalloproteinase-related genes, and 9 differentially expressed metalloproteinase-related genes (MRDEGs) were finally obtained.

3.2 Functional correlation analysis

The MRDEGs were initially evaluated using GO enrichment, KEGG pathway analysis, and DO pathway enrichment analysis. The results revealed that 205 biological processes, 10 signaling pathways, and 127 diseases were significantly enriched. The biological processes were enriched for positive control of defense response to bacterium, leukocyte mediated immunity, leukocyte degranulation, and myeloid cell activation involved in immune response. In the cellular component, tertiary granule lumen, specific granule, tertiary granule, and anchored component of membrane were enriched. In addition, a significant enrichment of serine-type endopeptidase activity, serine-type peptidase activity, serine hydrolase activity, and oxalate transmembrane transporter activity in molecular function (Figure 2A) was observed. KEGG enrichment analysis showed that thiamine metabolism, folate biosynthesis, bladder cancer, legionellosis, arachidonic acid metabolism, and IBD were enriched (Figure 2B). DO analysis revealed that kidney disease, urinary system disease, arthropathy, central nervous system cancer, infertility, and primary bacterial infectious disease were significantly enriched (Figure 2C). In addition, enrichment differences were further investigated between IBD and control groups using GSEA. In IBD, Galactose metabolism, insulin signaling pathway, leukocyte transendothelial migration, lysosome, the toll-type receptor signaling pathway is an important area of aggregation. In the control group, graft rejection, graft-versus-host disease, Huntington's disease, oxidative phosphorylation, and Parkinson's disease were significantly enriched (Figures 2D, E).

3.3 Identification and validation of diagnostic feature biomarkers

Three methods were used for detecting possible biological markers. The application of MRDEGs was investigated using the LASSO regression method and eight biochemical indicators were identified that could be used for the diagnosis of IBD (Figure 3A). MRDEGs were identified using the SVM-RFE method for four feature points (Figure 3B). In addition, the features of the top 5 were obtained by the random forest algorithm (Figures 3C, D). Finally, the intersection of the three algorithms was used to obtain five related metalloproteinases (Figure 3E). These included CD160, MMP-9, PTGDS, SLC26A8, and TLR5. Subsequently, the accuracy of the relevant genes was evaluated by machine learning as disease diagnostic genes using the ROC curve (Figures 4A–E). The AUC values of the five related genes were 0.732, 0.728, 0.715, 0.748, and 0.751, respectively, showing high sensitivity and specificity.

3.4 Immune cell infiltration

From the results of the analysis, a map was made that showed how immune cells are distributed (Figure 5A) and then a preliminary discussion of the immune cell component of IBD was provided. To investigate the correlation between immune cell expression, correlation analysis was performed. The results showed the relationship between the expression of immune cells in the combined data (Figure 5B). Immune cell differential analysis revealed that in IBD, the expression of plasma cells, T cells CD4 naïve, T cells CD4 memory resting, and neutrophils was higher and B cells memory, T cells CD4 memory activated, macrophages M0, and macrophages M2 was lower than that in the control group (Figure 6).

3.5 Correlation analysis between the five biomarkers and infiltrating immune cells

The correlation of the five biological markers with each immune cell type was investigated (Appendix 5). The five biomarkers were positively correlated with NK cells resting,

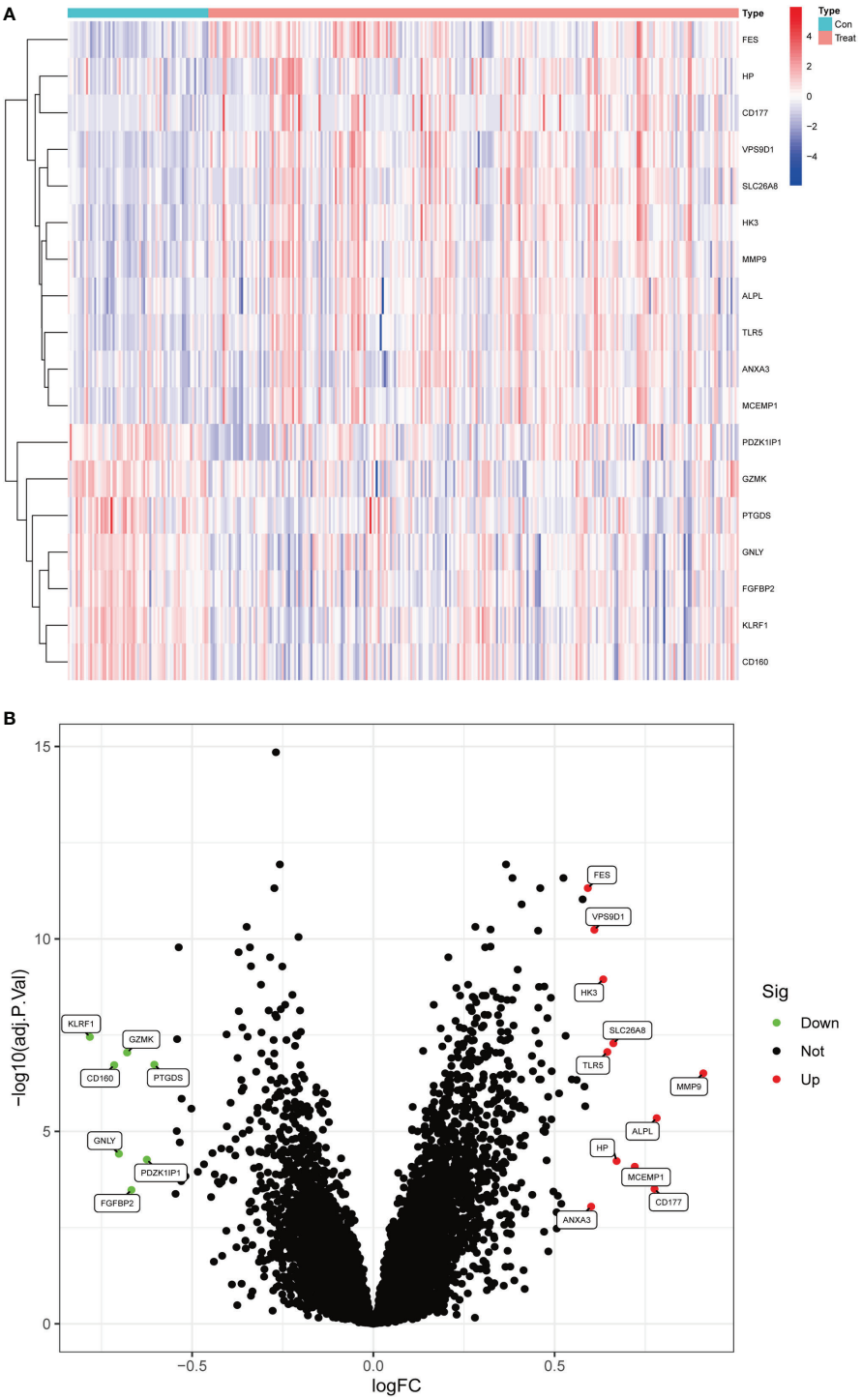

FIGURE 1
Differentially expressed genes in normal and inflammatory bowel disease (IBD). **(A)** Volcano plot of differentially expressed genes. Red dots represent significantly upregulated genes and green dots indicate significantly downregulated genes. **(B)** Heatmap of differentially expressed genes.

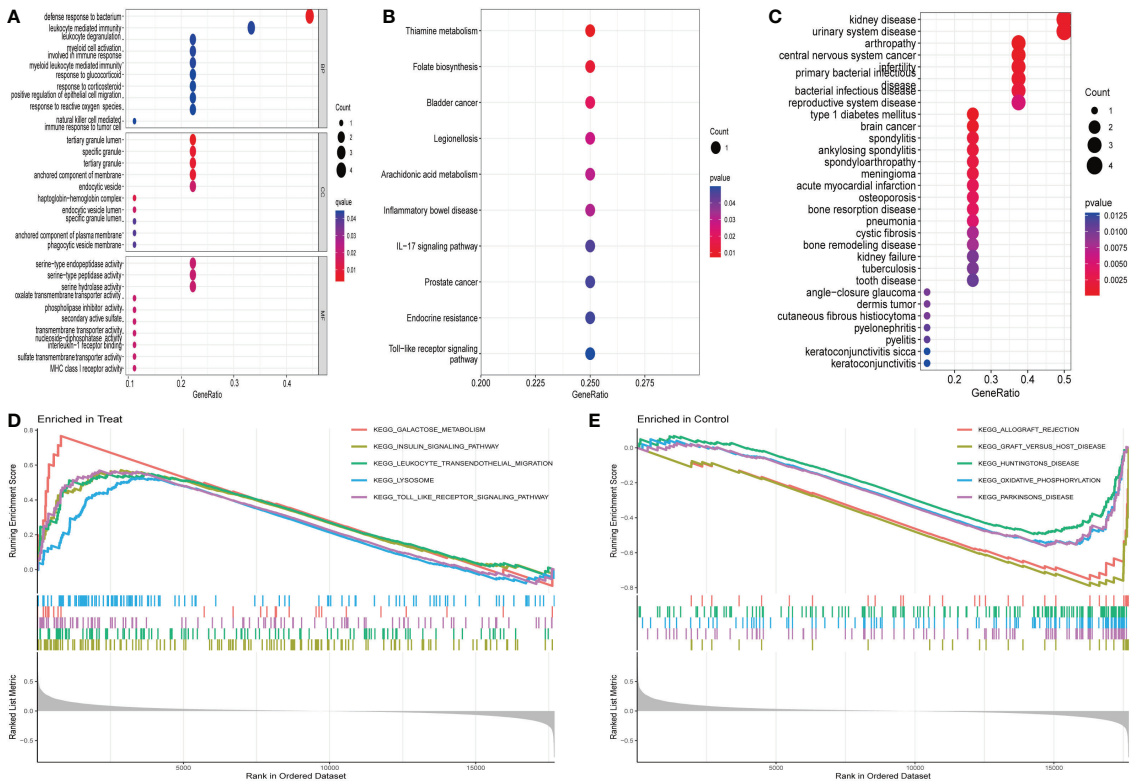

FIGURE 2
Enrichment analysis of differentially expressed genes: **(A)** GO enrichment analysis. **(B)** KEGG enrichment analysis. **(C)** Disease ontology enrichment analysis. **(D, E)** GSEA enrichment analysis.

Neutrophils, and T cells CD4 memory activated, respectively, while negatively correlated with Neutrophils, T cells CD8 and T cells CD4 memory activated (Figures 7A–E).

3.6 Chronic colitis model evaluation and histological identification

The feeding process of mice is shown in Figure 8A. The mice in the DSS group had redness and swelling around the anus. Due to the proliferation and swelling of the perianal mucosa, some mice showed adenomatous changes, with obvious redness and swelling and irregular prolapse of the anus (Figure 8B). Analysis of change in body weight revealed that the body weights of the mice decreased at the initial stage of administration, and in the subsequent modeling, the body weights of the mice in the DSS group increased at a lower rate than that in the control group (Figure 8C). After the mice were euthanized, the cecum was dissected to the anal hilum, stretched and spread spontaneously, and placed flat on A4 paper. When the length of each colon was measured, the intestinal length of the DSS group was found to be shortened (Figure 8D). The control group showed normal colonic structure with smooth mucosal surface and absence of annular folds and villi. The DSS group mainly showed typical manifestations of inflammatory mucosa, including infiltration of a large number of inflammatory cells in the mucosa, submucosa, and muscle layer; atypical gland hyperplasia; structural disorder; submucosal hemorrhage; and edema (Figure 9).

3.7 Expression of immune cells in the spleen

The results of flow cytometry showed that the expression of the T cell CD8 marker (CD8 antibody) was different between the DSS and control groups, whereas that of other neutrophil markers (CD16/32) and T cells CD4 memory activated markers (CD25 and CD65) were not different (Figures 10A–F). CD8 was negatively correlated with MMP-9 and SLC26A8.

3.8 Validation of inflammatory factors and MMPs associated with IBD

We verified the increased expression of inflammatory factors (IL-6 and IL-1β) in DSS group, which indicate that MMPs are closely related to inflammatory response (Figures 11A, B). Changes in the levels of MMP-related genes were evaluated in chronic IBD

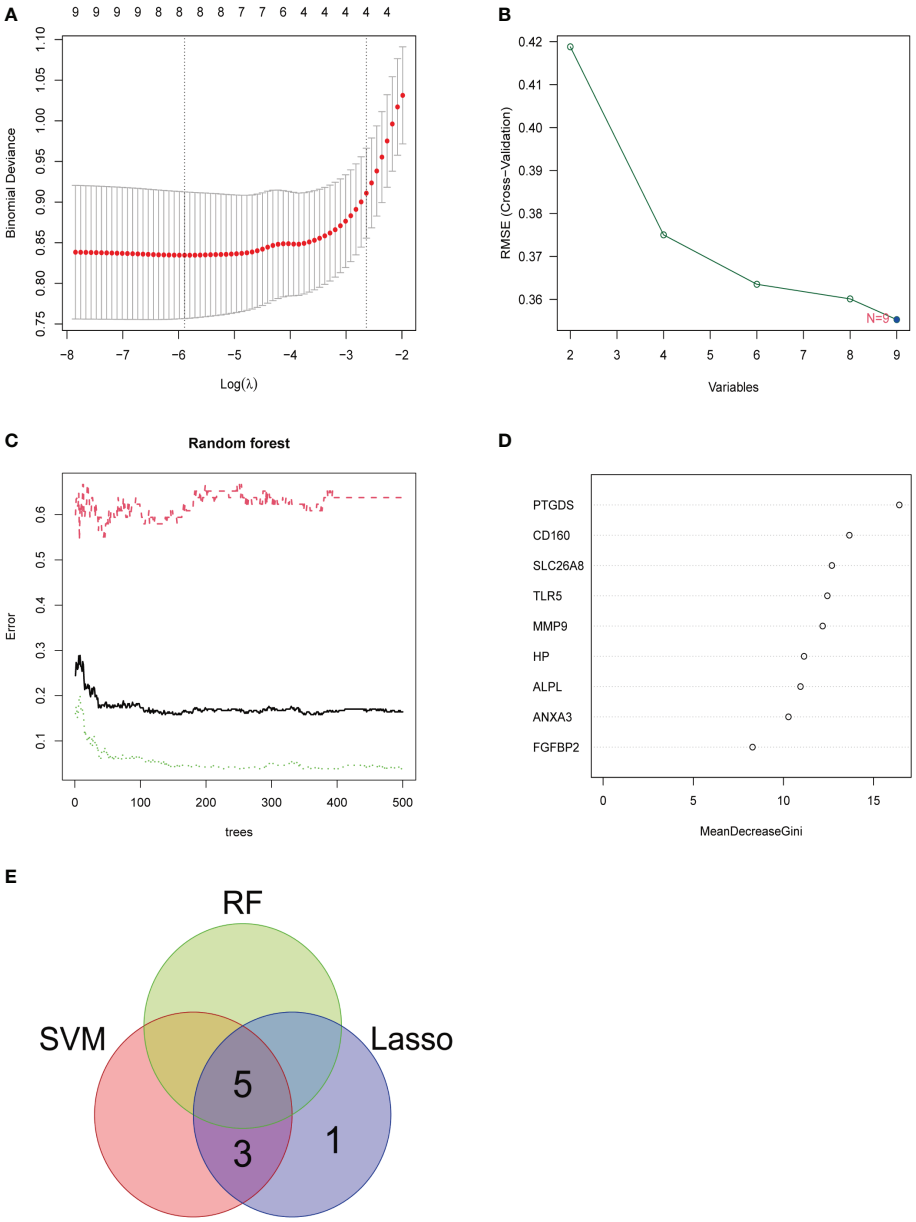

FIGURE 3
LASSO, SVM-RFE, and random forest were used for feature selection. **(A)** LASSO coefficient profiles of eight genes that initially met the prognostic criteria. **(B)** Biomarker selection map based on the support vector machine recursive feature elimination (SVM-RFE) algorithm. **(C)** Random forest model. **(D)** Random forest MeanDecreaseGini assessment. **(E)** Venn diagram shows the five diagnostic markers shared by the three algorithms.

models. Compared with the control group, the expression levels of MMP-9, PTGDS, SLC26A8, and CD160 significantly increased, whereas that of TLR5 decreased in the DSS group, suggesting that the MMPs play a role in the development of the chronic inflammatory intestinal disease mouse model (Figures 11C, D). The results of immunofluorescence assay showed that compared with the control group, the fluorescence intensity of MMP-9, PTGDS, SLC26A8, and CD160 increased, whereas that of TLR5 decreased (Figures 12A–E) in the DSS group, which is consistent with the results of western blotting.

4 Discussion

IBD is an autoimmune disease of the intestine and includes ulcerative colitis and Crohn's disease (25). As an important

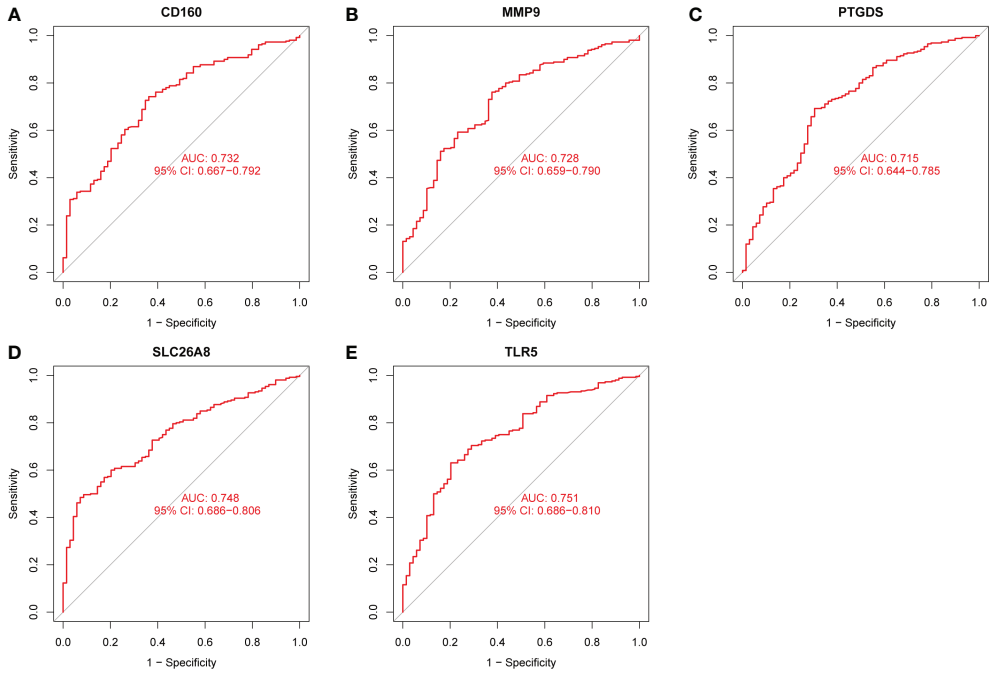

FIGURE 4
The receiver operating characteristic (ROC) curve of the diagnostic effectiveness of the five diagnostic markers. **(A)** ROC curve of CD160. **(B)** ROC curve of MMP9. **(C)** ROC curve of PTGDS. **(D)** ROC curve of SLC26A8. **(E)** ROC curve of TLR5.

causative factor of colorectal cancer, IBD has attracted global attention. However, due to the increasing incidence, long course, and delayed healing in IBD, its diagnosis, treatment, and prognosis have become a challenge. The main feature of IBD is the chronic inflammatory reaction in the intestine (26), which induces immune stimulation of the mucosa and makes the intestinal environment abnormal (27, 28). Therefore, the immune function of the intestinal barrier is crucial for the prevention and resolution of intestinal inflammatory diseases.

MMPs are a group of proteolytic enzymes containing active zinc. MMPs can be divided into groups based on the structure of the catalytic region (29). They are classified according to substrate and fragment homology, such as collagenases, gelatinases, stromelysins, elastases, and membrane-type MMPs (30). Dysregulation of MMP expression can cause tissue damage and persistent inflammation. Some studies have demonstrated the antitumor effects of MMPs. Various MMPs are associated with the poor prognosis of tumors (31). Cancer cells can evade the immune system by using MMPs to ensure the survival of metastatic cells (32). In addition to the regulatory role of MMPs in cancer, MMPs are also involved in the inflammatory response of many inflammatory diseases, such as sepsis (33), atherosclerosis (34, 35), and arthritis (36).

In this study, a mouse model of chronic IBD induced by DSS was constructed to evaluate the relationship between immune cells in IBD and MMPs. Histologically, chronic colitis is characterized by shortened colon length and loss of goblet cells and crypts. Additionally, some mice may have adenomatous polyps and tumor-like changes. During the 9 weeks of modeling, mice showed weight loss and mucus and blood in stool. Some mice showed adenomatous changes due to the proliferation and swelling of the perianal mucosa, and obvious redness, swelling, and irregular prolapse in the anus. The histological score was statistically different from that of the control group. Thus, the model of chronic IBD was validated.

In this study, a clustering approach was used to identify the types of differentially expressed metalloproteinase-related genes. Toll-type receptors (TLRs) are an important source of IBD. TLR is a natural immune system receptor that contributes to the pathogenic mechanisms of IBD, including immune response, genetics, and microbiology (37). The TLR signal transduction pathway can induce various factors involved in defense, such as inflammatory factors, chemokines, and antigen presenting factors (38, 39). Inflammatory cytokines play a crucial role in multiple processes of IBD development, when TLR pathway is activated, immune cells will produce a large number of pro-inflammatory factors, such as IL-1β, IL-6.This was also verified in our experiment. According to the results of Western Blot, the expression of inflammatory cytokines in the DSS group was significantly higher than that in the other.

On this basis, CD160, MMP-9, PTGDS, SLC26A8, and TLR5 were selected as indicators of tumor detection. A survey by Marônek et al. (40) showed that reduced MMP-9 in patients with IBD is a risk factor in patients with IBD and infection (41). Liu et al. studied MMP-9 in excreta using nanoparticles (42). A study reported that TLR5 plays a key role in the inflammatory

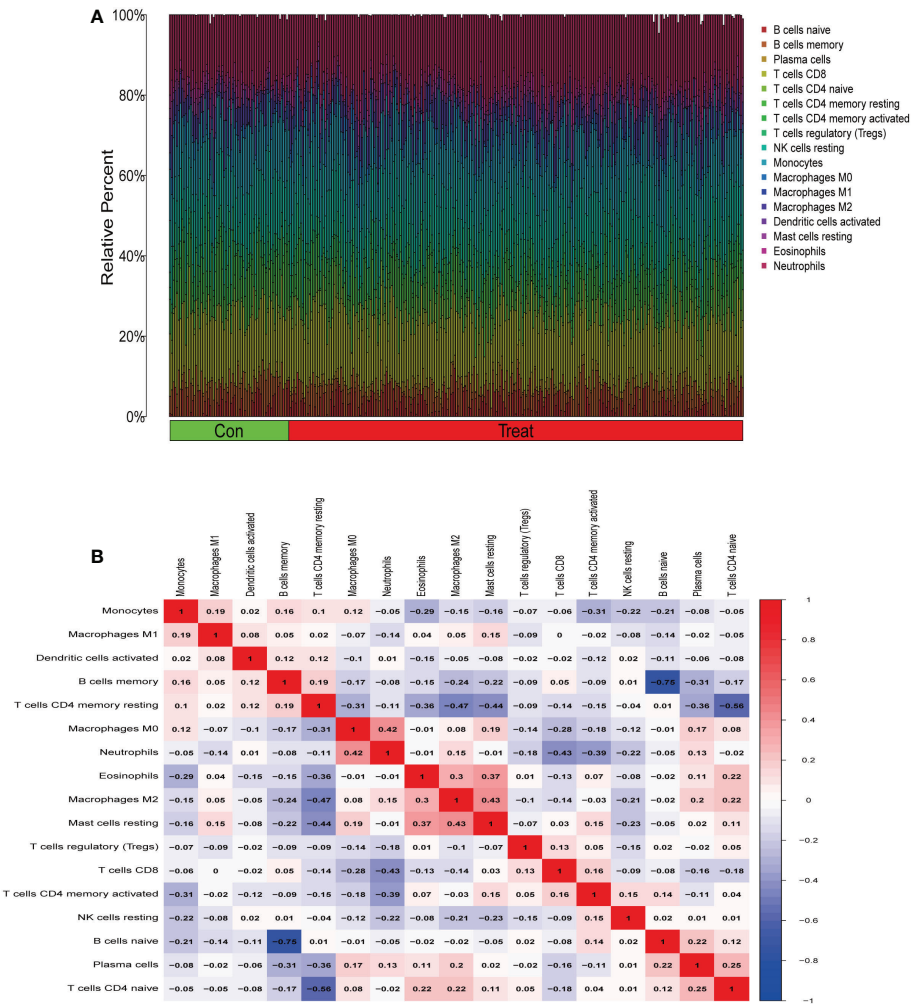

FIGURE 5
Immune infiltration analysis. **(A)** Immune cell distribution map. **(B)** Correlation of immune cells between patients with inflammatory bowel disease (IBD) and normal patients.

response in the intestine through animal tests on TLR5 and that disruption of TLR5 signaling triggers the TNFR2 signaling pathway, which leads to an inflammatory response in the intestine (43). CD160 activates natural killer cells with specific domains, making it a novel therapeutic target in the fight against atherosclerosis, autoimmune diseases, and many cancers (44). In recent years, researchers have shown that CD160 is associated with the recovery of COVID-19 patients (45). PTGDS have been shown to be selectively expressed in cancers, including ovarian cancer (46) and melanoma (47) with overexpression, gastric cancer (48) and lung cancer (49) with low expression. This selective expression also indicates that PTGDS has a more complex mechanism and potential research value. A study has shown that SLC26A8 is a susceptibility gene for hereditary non-polyposis colorectal cancer (50), which also provides reference for our subsequent research on the development of IBD for colorectal cancer. In this study, high levels of MMP-9, PTGDS, SLC26A8, and CD160 and reduced levels of TLR5 protein in patients with IBD (identified using WB and immunofluorescence analysis) suggest that these are clinically significant in IBD.

In the pathogenesis of many chronic diseases, both innate and adaptive immune functions are affected to some extent. A comparison of different cell types identified high levels of plasma cells, T-cell CD4 naive, T-cell CD4 memory, and eosinophils. Mitsialis et al. (51) reported that the abundance of HLA-DR +CD38+ T lymphocytes increased in colonic mucosal samples from patients with IBD, where T-regulatory cells were also present. Based on the relationship of the five antigens different cell types, we suggest that there are differences in the function of CD4 of neutrophils and T cells in the pathogenesis of IBD, which is related to the non-homogeneity of cells. However, all but CD8 were ineffective at elevated expression levels in IBD. Recent data show that CD8+ T lymphocytes (Tc1) and CD8+ (Tc17) play an important role in the development of IBD (52).

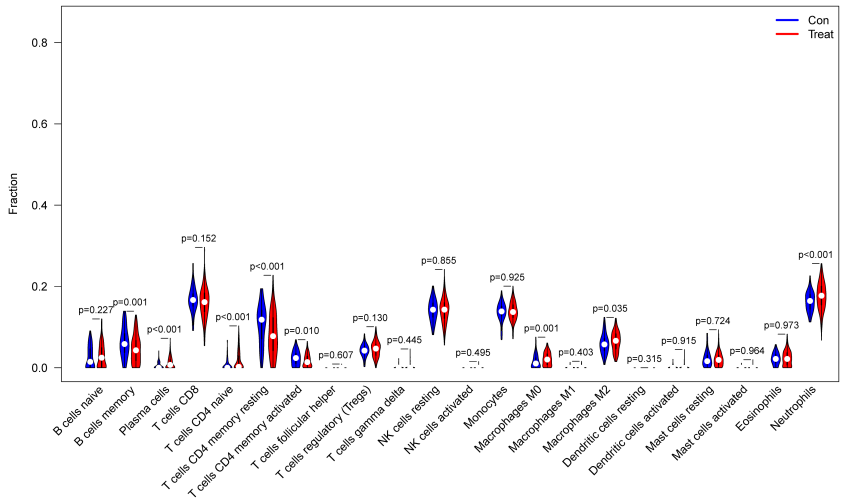

FIGURE 6
Expression of immune cells between patients with inflammatory bowel disease (IBD) and normal patients.

In conclusion, the findings of this study show that MMPS-related genes, namely MMP-9, CD160, PTGDS, SLC26A8, and TLR5, regulate the occurrence and development of IBD. These findings provide insights into future research on the mechanism of IBD. In addition, bioinformatics and mouse model studies revealed that MMP-related genes can participate in the progression of IBD by regulating CD8 cells. However, the detailed mechanism of action of MMPs in IBD is unknown and requires further investigation.

5 Conclusion

The findings of this study show that patients with IBD and healthy controls have significantly different gene expression, identifying the role of metalloproteinases in IBD. Moreover, the results provide insights into immune cell activation through metalloproteinases in IBD. However, more in-depth research is needed in future studies.

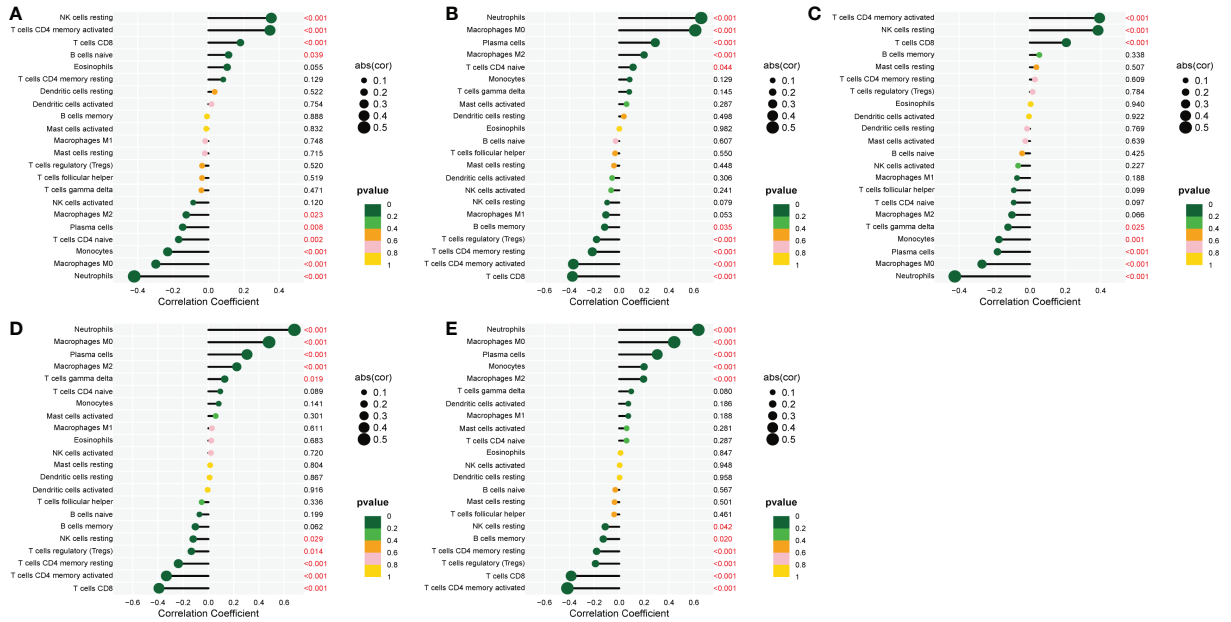

FIGURE 7
Correlation between five metalloproteinase-related genes and infiltrating immune cells. **(A)** Correlation between CD160 and infiltrating immune cells. **(B)** Correlation between MMP-9 and infiltrating immune cells. **(C)** Correlation between PTGDS and infiltrating immune cells. **(D)** Correlation between SLC26A8 and infiltrating immune cells. **(E)** Correlation between TLR5 and infiltrating immune cells.

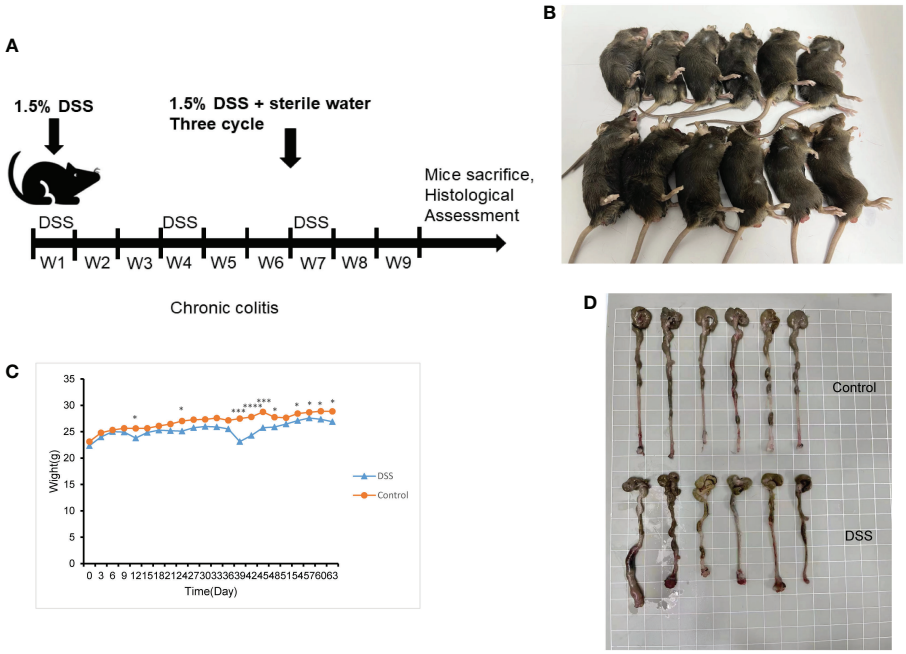

FIGURE 8
The construction of the inflammatory bowel disease (IBD) model. **(A)** Flow chart for mice feeding. **(B)** Irregular perianal prolapse in mice. **(C)** Body weight changes in mice. **(D)** Comparison of the length of colon. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. .

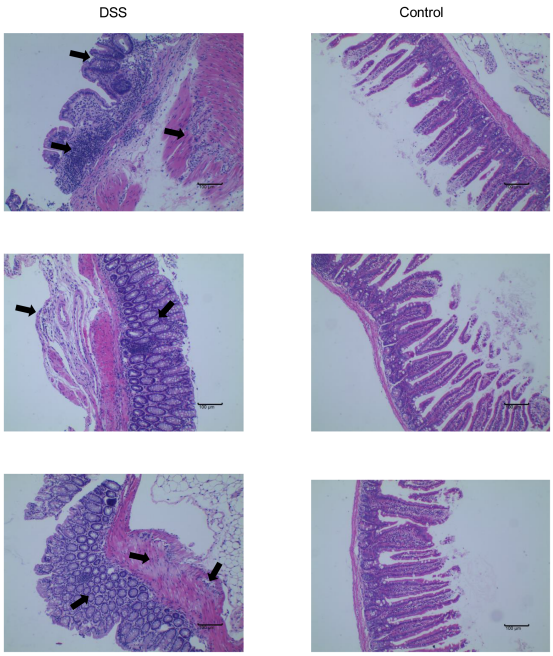

FIGURE 9
Comparison of HE staining between the DSS and control groups.

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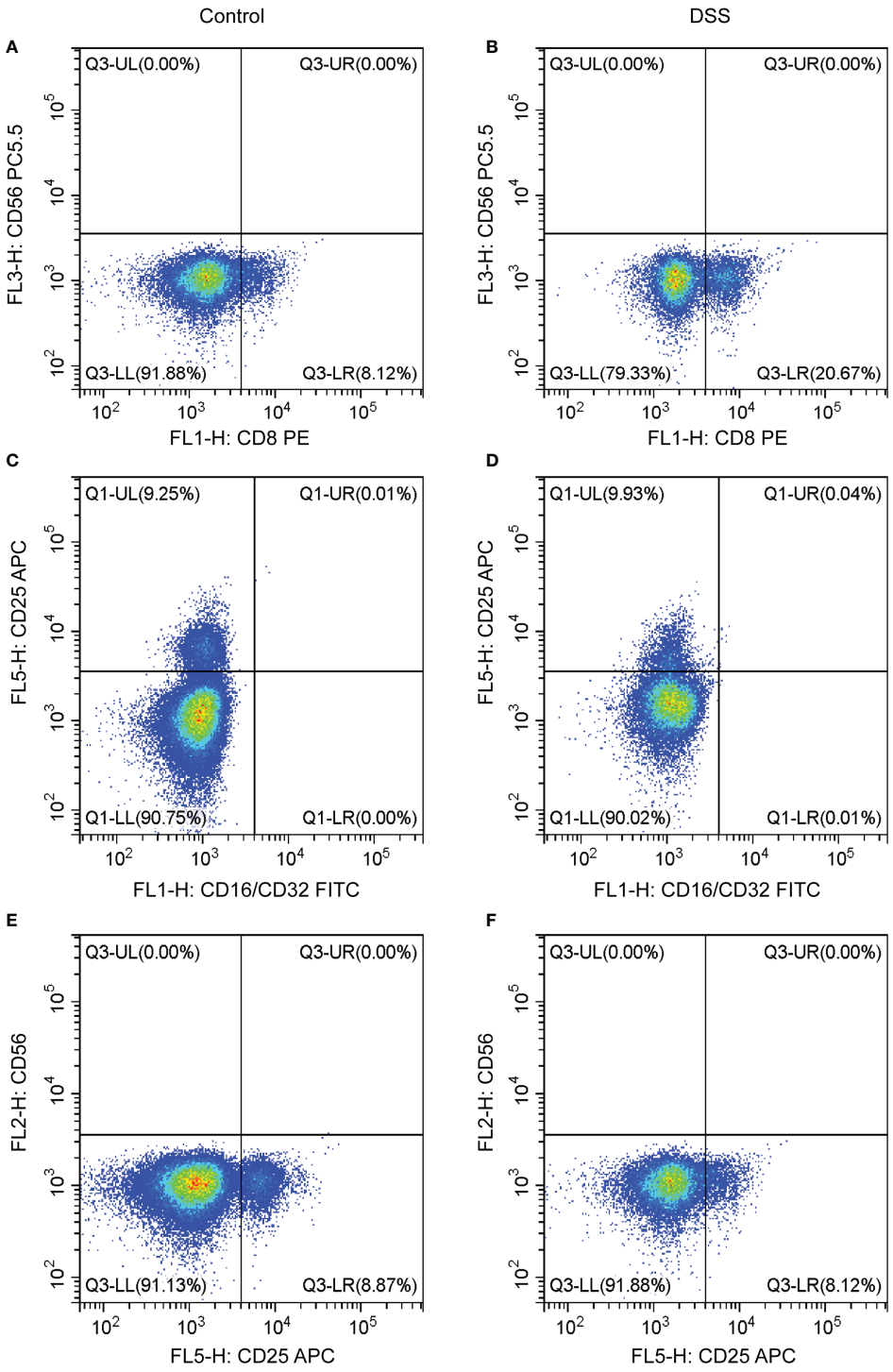

FIGURE 10
Effects of chronic inflammatory bowel disease (IBD) on immune cells in the spleen of mice. **(A–F)** The strategy of CD8, CD56, CD16/CD32, and CD25 in the DSS and control groups.

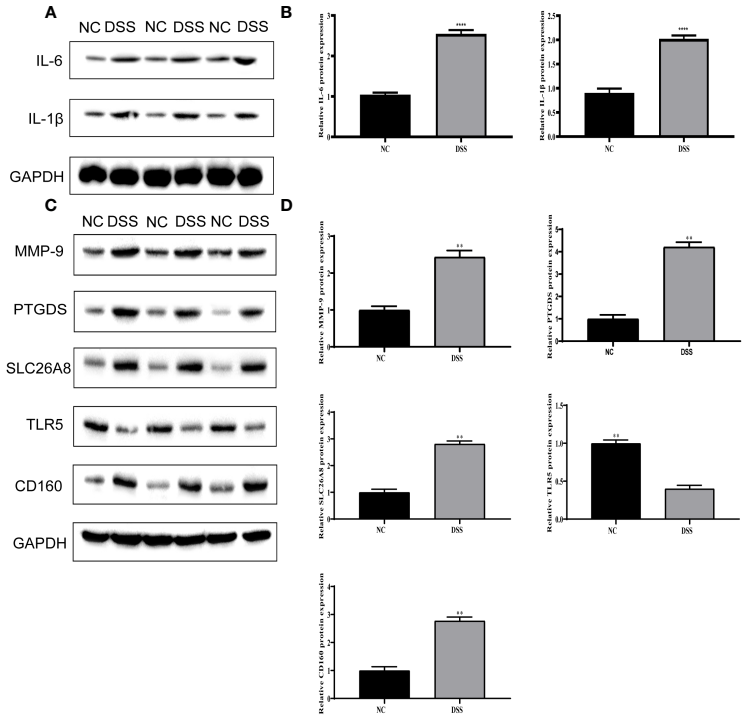

FIGURE 11
Validation of differential expression of inflammatory factors and five biomarkers. **(A)** Protein expression of IL-6 and IL-1β. **(B)** Protein statistics of IL-6 and IL-1β. **(C)** Protein expression of five biomarkers. **(D)** Protein statistics of five biomarkers. $^{**}P < 0.01$, $^{****}P < 0.0001$.

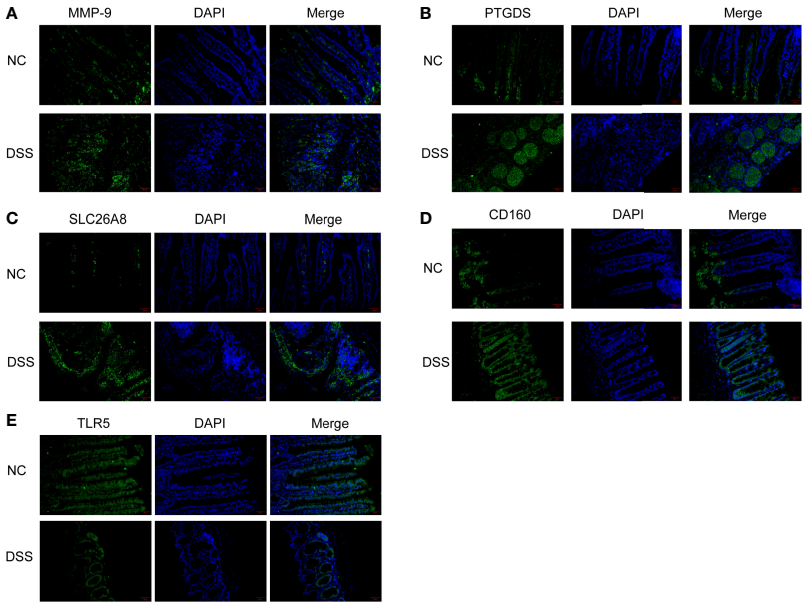

FIGURE 12
Colonic tissue immunofluorescence in mice. **(A–E)** Immunofluorescence of CD160, MMP-9, PTGDS, SLC26A8, and TLR5.

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 authors.

Ethics statement

The studies involving human participants were reviewed and approved by The Committee on the Ethics of Nanjing Normal University, Nanjing Normal University. The ethics committee waived the requirement of written informed consent for participation. The animal study was reviewed and approved by All in vivo animal experiments were approved by the Committee on the Ethics of Animal Experiments of Nanjing Normal University (IRB#2020–0047), Nanjing Normal University.

Author contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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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.2022.1067950/full#supplementary-material

SUPPLEMENTARY TABLE 1
Metalloproteinase-related genes.

SUPPLEMENTARY TABLE 2
Scoring criteria for the histological examination of mice.

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The immunomodulatory role of matrix metalloproteinases in colitis-associated cancer

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Matrix metalloproteinases (MMPs) are an important class of enzymes in the body that function through the extracellular matrix (ECM). They are involved in diverse pathophysiological processes, such as tumor invasion and metastasis, cardiovascular diseases, arthritis, periodontal disease, osteogenesis imperfecta, and diseases of the central nervous system. MMPs participate in the occurrence and development of numerous cancers and are closely related to immunity. In the present study, we review the immunomodulatory role of MMPs in colitis-associated cancer (CAC) and discuss relevant clinical applications. We analyze more than 300 pharmacological studies retrieved from PubMed and the Web of Science, related to MMPs, cancer, colitis, CAC, and immunomodulation. Key MMPs that interfere with pathological processes in CAC such as MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13, as well as their corresponding mechanisms are elaborated. MMPs are involved in cell proliferation, cell differentiation, angiogenesis, ECM remodeling, and the inflammatory response in CAC. They also affect the immune system by modulating differentiation and immune activity of immune cells, recruitment of macrophages, and recruitment of neutrophils. Herein we describe the immunomodulatory role of MMPs in CAC to facilitate treatment of this special type of colon cancer, which is preceded by detectable inflammatory bowel disease in clinical populations.

KEYWORDS

matrix metalloproteinases (MMPs), colitis associated cancer (CAC), immunomodulation, inflammation, extracellular matrix (ECM)

1 Introduction

1.1 Classification and structural characteristics of matrix metalloproteinases

MMPs are a kind of calcium-and zinc-dependent proteolytic enzyme (1), that exist in invertebrates, vertebrates and plants (2). They are produced by multiple cells and tissues, with neutrophils and dermal fibroblasts being the main sources (3). Connective tissue, pro-inflammatory and uteroplacental cells, including endothelial cells, osteoblasts, cytotrophoblasts, lymphocytes, macrophages, and vascular smooth muscle are also capable of secreting MMPs (4). Degrading the ECM is the main function of MMPs (5). The ECM plays an important role in the proliferation, growth, organization, differentiation, migration of cells, and in the exchange among information cells; it also acts as a physical barrier for microorganisms (6, 7). To date, 28 types of MMPs have been found. The homologous domains of these MMPs include the signal peptide domain, propeptide domain, catalytic domain and hinge region or linker peptide along with a hemopexin domain (4, 8). The hinge region connects the catalytic domain to the hemopexin domain (7, 9, 10). MMPs are divided into collagenases (e.g., MMP-1, MMP-8, MMP-13, and MMP-18) (11, 12), gelatinases (e.g., MMP-2 and MMP-9) (13, 14), stromelysins (e.g. MMP-3, MMP-10, and MMP-11) (15, 16), matrilysins (e.g. MMP-7 and MMP-26) (17), membrane-type MMPs (MT-MMPs) (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and others (MMP-12, MMP-19, MMP-21, and MMP-28) based on structural features and substrates (18–24). In particular, gelatinases have a special additional exosome insert in the catalytic domain called the collagen binding domain; matrilysins lack a C-terminal hemopexin-like domain linked by a hinge or linker region, and MT-MMPs have a C-terminal transmembrane domain with a short cytoplasmic tail (9, 25). The classifications and structures of MMPs are shown in Figure 1.

1.2 Regulation of MMPs at multiple levels

MMP activity is regulated in three different ways, through transcriptional regulation, inhibition by specific inhibitors, or activation by the proenzyme (26). At the transcriptional level, the activator protein (AP) -1 and -2 sites, the NF-κB site, the signal transducer and activator of transcription site, the polyomavirus enhancer-A binding protein-3 site, and others are key transcription binding sites for regulation of the *MMP* gene (8, 27–33). The AP-1 site is located close to the most proximal promoter of the typical TATA box and is the major mediator in *MMP* gene regulation. In many *MMP* promoters that contain AP-1 site, the juxtaposed transcription factor binding sites bind multiple erythroblastosis twenty-six factors, which determine the peculiarities among different genes and affect gene expression (8). The polyomavirus enhancer-A

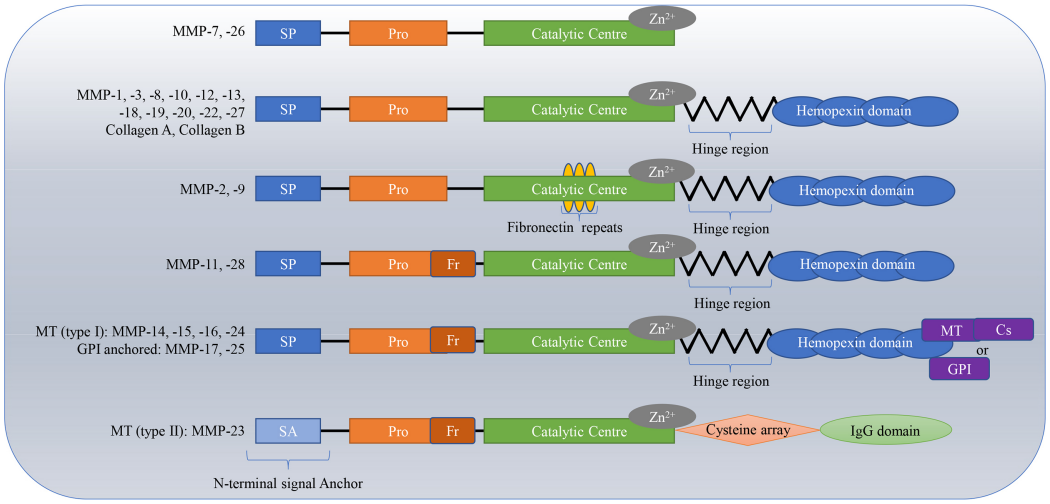

FIGURE 1
Classifications and structures of matrix metalloproteinases (MMPs). The homologous domains of these MMPs include the signal peptide domain (SP), propeptide domain (Pro), catalytic domain, and hinge region or linker peptide along with a hemopexin domain. The hinge region connects the catalytic domain to the hemopexin domain. Matrilysins lack a C-terminal hemopexin-like domain linked by a hinge or linker region; Gelatinases have special additional fibronectin repeats in the catalytic domain; MT-MMPs have a C-terminal transmembrane domain with a short cytoplasmic tail. MT, membrane-type; Cs, cytosolic; GPI, glycosylphosphatidylinositol.

binding protein-3 site, can combine with members of the erythroblastosis twenty-six factors family of oncoproteins and work in synergy with the AP-1 site nearby to promote the production of MMPs among cancer cells for migration and invasiveness (28). MicroRNA is a non-coding single-stranded RNA. In liver cancer cells, mRNA expression of *MMP-2* and *MMP-9* is up-regulated as a result of the inhibition of miR-21, which stimulates the invasion and migration of tumor cells. miR-224 is associated with gene expression of *MMP-1*, which enables breast tumors to metastasize to the bone (34). NEMO-binding domain, a synthetic peptide corresponding to the S100A4-binding domain of methionine aminopeptidase 2 [MetAP2], blocks interaction between the metastasis –enhancing calcium–binding protein (S100A4) and its effector protein (MetAP2). And this blockage inhibits specificity protein 1 (Sp1) and ultimately leads to the downregulation of *MMP-14* gene expression (35). MMP-7 antisense oligonucleotides inhibit gene expression of *MMP-7* and inhibit the metastasis of gastric and colon cancer by interfering with protein translation or promoting mRNA degradation (36). The modulation of gene expression differs in various physiological and pathophysiological events, such as Ets1 enhancing gene expression of *MMP-1* through c-Jun (29, 30). Note that modulation of the *MMP* gene can be affected by several stimuli. Some factors, such as phorbol esters and ultraviolet B radiation, activate expression of the *MMP* gene, whereas others (37–39), such as transforming growth factor β (TGF-β), glucocorticoids and retinoic acid (40, 41), suppress it. In addition, the *MMP* gene may be induced indirectly by several signaling pathways. Inflammatory cytokines, for example, interleukin (IL)-1 and tumor necrosis factor, indirectly influence *MMP* gene expression and activate the ceramide signaling pathway. Three distinct MAP kinase pathways, p38, ERK1/2 and c-Jun N-terminal kinase (JNK) affect ceramide-dependent *MMP-1* gene expression in human skin fibroblasts (42–45). *MMPs* genes are not generally upregulated by gene amplification or mutation like classical oncogenes. Usually transcriptional changes and/or epigenetic modifications result in an upregulation of *MMP* gene expression in colorectal cancer (CRC) (46, 47). Besides transcriptional regulation, MMP activity is related to proMMPs, which are secreted as inactive zymogens. Extracellular activation of proMMPs involves two steps. First, the N-terminal sequence of the propeptide domain is cleaved and releases the Zn^{2+}-binding site stemming from catalytic domain exposition. Second, propeptide cleavage resulting in an active form of enzyme (21, 48, 49). For example, proMMP-9 was activated and then generated MMP-9, thus catalyzing angiogenesis *via* the FGF-2/FGFR-2 pathway (50). In addition, the activity of MMPs is regulated by α2-macroglobulin and tissue inhibitors of MMPs (TIMs), the two main endogenous inhibitors (26, 51). Thiol-modifying reagents, sodium dodecyl sulfate, and oxygen radicals also induce activation of MMPs *in vitro*(52).Variation in temperature or a decrease pH in the physicochemical environment serves the same purpose (25).

1.3 Immunological function of MMPs

The immune system, human beings' main defense against disease, is indispensable. It eliminates foreign invaders through the immune response in a sophisticated and scientific way. Components that participate in immune regulation include innate immune cells, which act as early-responders, and adaptive immune cells, which enhance the response and generate immunological memory and molecules. Cytokines and chemokines control the immunoreaction in time and space. They take part in cell migration to the site of inflammation, proliferation, intercellular communication, and cell death (53, 54). The immune response does not necessarily lead to an inflammatory response, but inflammation accompanies the immune response in most cases (55). Inflammation commonly occurs after infection and damage (56). When antigens enter the body, macrophages or epithelial cells secrete chemokines, causing an increase in the vascular epithelial cell gap and vascular expansion; a large number of neutrophils, mast cells, basophils, and eosinophils infiltrate from the blood vessels into tissue fluid, resulting in localized febrile response, redness, swelling, and pain the cardinal signs of inflammation. An excessive immune response can lead to inflammation, such as pathogenic microbial infection, tumor, autoimmune disease, and tissue damage induced by physical or chemical elements. Immune cells and cytokines play an important role in the occurrence and resolution of inflammation. Numerous immune cells (e.g., macrophages, neutrophils) infiltrate the inflamed area and activated immune cells to release inflammatory factors (e.g., TNF-α, IL-6 and IL-1β), which worsens the inflammation (56, 57). Resolving this inflammatory response requires the release of anti-inflammatory cytokines (e.g., IL-10) by immune cells (58–61).

The composition of the tumor microenvironment is very complex. The tumor stroma is composed of the ECM, immune cells, fibroblasts, endothelial cells and other non-neoplastic cells (62). MMPs produced in immune cells take part in innate and acquired immunity (63). Many immune cells express low levels of MMPs in the resting state. In mouse splenic CD4+ T cells, membrane-anchored *disintegrin metalloproteinase-10 (ADAM-10)* and *ADAM-17* mRNAs are expressed highly, whereas the mRNA expressions of *MMPs*, such as *MMP-2*, *MMP-9* and *MMP-14* are low (64). Under normal conditions, when the expression of inflammatory cytokines and chemokines increases, MMPs of immune cells are secreted and activated. When stimulated by IL-8, TNF, or chemo-attractive formyl-Met-Leu-Phe peptide, MMP-9 in neutrophils is immediately released from gelatinase granules (also called tertiary granules)

(65). In addition, TIMP1 protein is not expressed in neutrophils (66). However, B cells, T cells, and unstimulated human peripheral blood monocytes are able to express TIMPs in the steady state. The transcript levels of TIMP1, TIMP2 and TIMP4 are expressed more highly in monocytes than in B cells or T cells. In contrast, *TIMP3* mRNA is highly expressed in B cells (67). ADAM17 and MMP-8 cleave the lymphotoxin (LT)-α1β2 heterotrimer, causing the release of heterotrimers from polarized T helper 1 (TH1) and TH17 cells (68). The combination of LTα1β2 heterotrimers and LTβ receptor activates primary synovial fibroblasts, eventually leading to synovial inflammation (68). The OX40 (a member of the TNF superfamily)–OX40 ligand axis is involved in numerous inflammatory diseases, anti-tumor immune responses and metabolic syndromes (69). MMP-2-specific CD4(+) T cells exist in tumor-infiltrating lymphocytes from melanoma patients, and they have an inflammatory T(H)2 (Type 2 helper T cells) profile. Dendritic cells (DCs) with MMP-2 initiates TH2 responses against several melanoma-associated antigens. As a reaction to exogenous melanoma antigens, active MMP-2 promotes TH2 cell differentiation, degrades the IF-α/β receptor in immature DCs and increases the protein expression of OX40 ligand in mature DCs. Therefore, researchers speculate that the mechanisms by which activated MMP-2 promotes tumor development is as follows: MMP-2 polarizes tumor-infiltrating lymphocytes toward a TH2 cell phenotype, which restrains the tumoricidal TH1-type response. Moreover, MMP-2 inhibits the powerful promoter of TH1 cell polarization-IL-12 subunit p35 (IL-12α) (70).

Important factors in building an immune response are efficient migration of neutrophils along a chemotactic gradient and extravasation through blood vessels and tissues to sites of infection. MMPs play a role in these processes by modifying chemotactic agents. A cleavage mediated by MMP-7 releases the heparan sulfate proteoglycan syndecan 1, and its associated CXC-chemokine ligand 3 (CXCL3), which attracts neutrophils to the site of infection (71). MMP-8 which is mainly produced by neutrophils can be detected in the inflammatory response and some malignant tumors. In one study, there was a persistent inflammatory response after MMP-8-deficient mice were injected with methylcholanthrene. The incidence of skin tumors in male mice of this type increased significantly; female mice that were treated with tamoxifen or had their ovaries removed were more likely than wild-type mice to develop tumors. These results indicate that MMP-8 has a tumor suppressor function to some extent (72). This function is also supported by the finding that MMP-8 inhibits melanoma growth *in vitro* and *in vivo* (73). MMP-8 is necessary for recruiting chemokine CXCL6 to activate neutrophils; neutrophils are not able to migrate to sites of LPS administration without MMP-8 (74). IL-8 is a prototype chemokine that activates neutrophils. There is positive feedback between MMP-9 and IL-8. Stimulated by IL-8, neutrophils secrete gelatinase granules whose main component is MMP-9 (65). MMP-9 truncates an amino-terminal fragment of IL-8 for large increases in IL-8 potency (75). MMP-2 cooperates with MMP-9 to promote neutrophil infiltration (76, 77). MMP-2 and MMP-9 have synergistic effects on cleaving CXCL5 to increase neutrophil migration to the peritoneum during IL-1β-induced peritonitis (78). Meprins are members of the metzincin superfamily of zinc metalloproteinases, the cleaved substrate involved in many pathological processes, such as inflammation, cancer and fibrosis. Meprins participate in activating MMP-9 in the immune response. MMP-3 is an efficient activator of proMMP-9. The cleavage mediated by meprins improves the activation kinetics of proMMP-9 by MMP-3 (79). In contrast, MMP-2 may suppress the inflammatory response inactivating monocyte chemotactic protein 3 or Chemokine (C-C motif) ligand 7 (80).

Monocyte precursors are capable of differentiating into local macrophages in tissues (81). In different microenvironments, the cell surface phenotypes and functions of macrophage populations are heterogeneous (82). Macrophages play a prominent role in anti-infection and, anti-tumor activity and immune regulation (83). Similar to what happens in neutrophils, macrophages move directionally along the concentration gradient of certain chemicals and accumulate at the site of the lesion, where these substances are released (56). Metalloproteinases are able to affect macrophage recruitment (84–86). In mice with TIMP3-null mammary glands, the inflammatory response is exacerbated, the number of CD3+ T-cells increases, and macrophage infiltration is more pronounced than in wild-type mice glands (87). A classic means of activating macrophages (classically activated macrophages or M1 macrophages) is through Toll-like receptor ligands and pro-inflammatory mediators, such as TNF-α, interferon-γ (IFN-γ), and IL-1. Additionally, alternatively-activated macrophages, or M2 macrophages, can also be alternatively activated by distinct mediators, like IL-4 and IL-13 (88, 89). In inflammation, M1 macrophages effectively dispose of infectious organisms, and orchestrate angiogenesis and the ingress of connective tissue cells to form a granuloma. The function of MMPs ECM remodeling is vital in that process (90). In healing, M2 macrophages may promote connective tissue cells to remodel the ECM (88, 89). *In vitro*-differentiated M1 macrophages, mRNA expression of *MMP-1, MMP-3, MMP-7, MMP-10, MMP-14 and MMP-25* are increased, and mRNA expression of *TIMP-3* is decreased. mRNA expression of *MMP-11, MMP-12, MMP-25* and *TIMP-3* are upregulated, whereas MMP-2, MMP-8 and MMP -19 were reduced in M2 macrophages (91). Researchers speculate that the function of macrophages is related to the profile of MMP expression profile. The upregulation of MMP-12 in M2 macrophages is a major influence on the formation of aneurysms (92). Higher levels of MMP-1 collagenase may might be linked to higher collagenolytic activity of M1 macrophages (91). Macrophages also participate in specific immune response by releasing either pro- or anti-inflammatory cytokines (93, 94).

MMP-14 (MT1-MMP) can control inflammatory gene responses. MMP-14-deficient macrophages produce excessive chemokine and cytokine responses to immune stimulation both *in vitro* and *in vivo*: they increase the gene and protein expression levels of the pro-inflammatory *IL-12p40* (also called *IL-12β*) and *IL-6*, along with decrease the gene and protein levels of the anti-inflammatory *IL-10*. Phosphoinositide 3-kinase δ (PI3Kδ), a key regulator of macrophage immune responses, is the downstream transcriptional target of MMP-14 (95–97). Protein expression of MMP-14-dependent PI3Kδ evokes the expression and activation of a PI3Kδ/Akt/GSK3β signaling axis, thus mediating the immunoregulatory Mi-2/nucleosome remodeling and nucleosome remodeling and deacetylase to limit the expression of proinflammatory mediators in macrophage (97–100). MMP-12 originating from macrophages participated in abrogating the acute immune response. In MMP-12-deficient mice, leukocytes accumulated at the site of infection. MMP-12 cleaves and inactivates numerous CXC-chemokines and CC-chemokines which are implicated in the influx of leukocytes at the site of inflammation (101).

MMP-7 is involved in the immune activity of macrophages and neutrophils. One immunological function of MMP-7 is proteolytically activating α-defensins (cryptdins), which are a group of six cationic anti-bacterial peptides that work by disrupting bacterial membranes (102, 103). When stimulated by bacterial products, such as LPS and lipoteichoic acid, α-defensins are secreted from neutrophils, monocyte/macrophages and Paneth cells at the base of the crypts in the small intestine (104). α-defensins also act as the chemo-attractants for monocytes, T-cells and DCs to connect innate immunity to adaptive immunity (104). α-defensins are also mitogenic for epithelial cells and fibroblasts to aid in wound healing (105).

The cDNA sequence of MMP-25 from Japanese sea bass (Lateolabrax japonicus) (LjMMP25) regulates the production of inflammatory cytokines and promotes phagocytosis and bactericidal activity in monocytes/macrophages. Moreover, LjMMP25 regulates the inflammatory response by modulating NF-κB activity during innate immunity (106). Macrophages have a negative impact on cancer treatment (107–109). They create an inflammatory environment to promote tumorigenesis and tumor progression, such as angiogenesis, migration and invasion, and immunosuppression (109). For example, the penetration of cancer cells and leukocytes into the cerebral vessels is a complex multi-step process. The activity of macrophage-derived MMP-2 and MMP-9 is pivotal to leukocyte's ability to penetrate the parenchymal basement membrane in mice with the ability autoimmune encephalomyelitis. These MMPs can be inhibited to protect the brain parenchyma from damage by preventing the infiltration of leukocytes (110). Moreover, the activation of TNF-α by MMPs contributes to tumor progression (63, 111). The membrane-bound precursor, proTNF-α, is mainly expressed in macrophages. ADAM17 (a TNF-converting enzyme) and MMPs, such as MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, MMP-14, MMP-15 and MMP-17, convert proTNF-α into TNF-α (112).

2 MMPs in pathological processes

2.1 Multifaceted role of MMPs in biological and pathological processes

Under normal physiological conditions, the activities of MMPs are controlled by various stimuli at multiple levels. However, under pathological conditions, this dynamic balance is broken. Over-degradation of the ECM due to overactivation of MMPs, is associated with a great many diseases, such as cardiovascular disease (4, 113–115), arthritis (116), periodontal diseases (117–119), osteogenesis imperfecta (120), disorder of the central nervous system (121), tumor invasion and metastasis (34, 122), age-related macular degeneration (123) and many other pathological states (7). Moreover, a decrease in MMPs can give rise to hypertensive pregnancy, preeclampsia (124), and inflammatory damage (125, 126).

As mentioned above, there are several main types of MMPs including collagenases, gelatinases, stromelysins, matrilysins, and MT-MMPs. Different MMPs have different three-dimensional structures, along with corresponding specific inhibitors or drugs. A typical MMPs consists of a prodomain, a propeptide, a catalytic domain and a hemophosphate domain. Approximately 80 amino acids make up the propeptide domain, and about 170 amino acids make up the catalytic metalloproteinase domain. The polypeptide folding of the MMP catalytic domain is basically superposition. The domain consists of a five-stranded β fold sheet, three α helices, and connecting rings. It contains two zinc ions and up to three stable calcium ions. The joint peptide contained in MMPs consists of a hinge region of variable length and a hemophosphate domain of about 200 amino acids. Exceptions include MMP-7, MMP-26, and MMP-23, which lack the hinge region and heme domain; MMP-23 has unique cysteine-rich domains and immunoglobulin domains (127–129). MMP-1, MMP-8, MMP-13, and MMP-18 are collagenases, and their key feature is their ability to cleave interstitial collagen I, II, and III at specific sites three-fourths away from the N-terminus. MMP-2 and MMP-9 are gelatin enzymes, which have three type II fiber-fiber-domain repeats in the catalytic domain and can bind to gelatin, collagen and laminin to digest denatured collagen. MMP-3 and MMP-10 are matrix lysins that digest ECM components (21). The classification of common MMPs implied in pathological processes is shown in Table 1. Collagenases recognize the substrate *via* a hemopexin-like domain, degrade fibrillar collagen and affect the ECM environment (19). Collagenases are closely related to the occurrence and development of diseases characterized by the degradation or change in the ECM are

TABLE 1 The classification of common matrix metalloproteinases (MMPs) implied in pathological processes.

Category	Structural feature	MMPs	The related diseases	Effects and Mechanisms	Refs
Collagenases	Made by a pro-peptide, a highly Conservative N-terminal catalytic domain and a C-terminal hemopexin-like domain; The catalytic domain and the hemopexin-like domain are connected by an intermediate hinge region;	MMP-1	Atherosclerosis	Inhibit atherosclerosis development by degrading major constituents of vascular ECM and contributing to remodeling of ECM and destruction of plaque;	(130)
			Cardiac dysfunction	Produce loss of interstitial cardiac collagen in conjunction with a marked worsening of cardiac systolic and diastolic function;	(131)
			Osteoarthritis	Participate in matrix degradation in human articular cartilage, a phenomenon commonly observed in osteoarthritis;	(132)
			Liver fibrosis	Attenuate established fibrosis and induces hepatocyte proliferation;	(133)
			Hypertrophic cardiomyopathy	Promote degradation of collagen I and reduce passive diastolic dysfunction;	(134)
		MMP-8	Arthritis	Play a protective role in arthritis mice joints by reducing interleukin-1β, prokineticin receptor 2 and pentraxin-3 expression;	(135)
			Atherosclerosis	Increase degradation of interstitial collagen I, and contributes a “stable” fibrotic phenotype by causing serious intra-plaque hemorrhages;	(136)
		MMP-13	Atherosclerosis	Increase degradation of interstitial collagen I, and contributes a “stable” fibrotic phenotype by causing serious intra-plaque hemorrhages;	(136, 137)
			Osteoarthritis	Participate in matrix deterioration in joint cartilage in humans, commonly observed phenomenon in osteoarthritis;	(132)
			Liver fibrosis	Reduce collagen deposition and ameliorate liver fibrosis;	(138)
Gelatinases	Consists of a pre-peptide, a strongly reserved N-terminal catalytic domain and a C-terminal hemopexin-like domain; A curious additional lateral insertion in the catalytic domain, that is known as Collagen Binding Domain;	MMP-2	Colon adenocarcinoma	Enhance tumor invasion by discontinuing basement membrane deposition;	(139)
			Malignant gliomas	Involve in glial invasion and angiogenesis;	(140)
			Hypertrophic cardiomyopathy	Promote degradation of collagen I and reduce passive diastolic dysfunction;	(134)
		MMP-9	Colon adenocarcinoma	Enhance tumor invasion by discontinuing basement membrane deposition;	(139)
			Guillain-Barré syndrome	Promote to peripheral nerve dysfunction leading to demyelination in Guillain-Barré syndrome;	(141)
			Acute ischemic stroke	Associate with higher risk of death and severe disability;	(142)
			Malignant gliomas	Lie in remodeling associated with neovascularization;	(140)
			COVID-19 disease among obese-diabetic patients	Be associated with ARDS;	(143)

(Continued)

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TABLE 1 Continued

Category	Structural feature	MMPs	The related diseases	Effects and Mechanisms	Refs
Stromelysins	Consists of a pre-peptide, a strongly reserved N-terminal catalytic domain and a C-terminal hemopexin-like domain;	MMP-3	Osteoarthritis	Participate in matrix deterioration in joint cartilage in humans, commonly observed phenomenon in osteoarthritis;	(132)
			Prostate cancer	Contribute to growth of prostate cancer in bone through intrinsic cell growth and extrinsic angiogenesis;	(144)
			Primary Biliary Cholangitis	Associate with liver function disorders and may play a role in the physiology of liver fibrosis in primary biliary cholangitis (PBC);	(145)
		MMP-10	Aortic stenosis	Play a core role in aortic stenosis calcification through phosphorylation of Akt;	(146)
			Non-small cell lung cancer	PKCiota-Par6alpha-Rac1 signaling axes actuates anchorage-independent proliferation and aggression of non-small cell lung cancer (NSCLC) cells through induction of MMP-10 production;	(147)
		MMP-11	Breast cancer	Enhance tumor cells migration and relate to poorly differentiated tumors;	(148)
			Colorectal cancer	Enhance tumor cells migration;	(149)
Matrilysins	Consists of a pre-peptide, a strongly reserved N-terminal catalytic domain; Lack a C-terminal hemopexin-like domain that is connected by a hinge or linker region;	MMP-7	Recurrent tonsillitis	Participate in recurrent tonsillitis progrssion by degrading ECM in response to inflammatory changes in tonsil tissue;	(150)
			Lung cancer	Cleave nucleolin which augment oncogenesis; Cleave the extracellular matrix and contributes to tumor aggression;	(151)
			Permanent hearing loss	Participate in modulating the cochlear reaction to sound hyperstimulation;	(152)
			Duchenne muscular dystrophy	Be related to duchenne muscular dystrophy cardiac dysfunction and myocardial fibrosis, possibly through remodeling of the extracellular matrix;	(153)
			COVID-19 disease among obese-diabetic patients	Be associated with ARDS;	(143)
			Multiple sclerosis	Promote the entry or restimulation of immune cells into the perivascular area, a key event in multiple sclerosis;	(154)
		MMP-26	Colon adenocarcinoma	Enhance tumor invasion by discontinuing basement membrane deposition;	(139)
MT-MMPs	Made by all protein domains specific to MMPs, from the N-terminal to the C-terminal (a propeptide, a catalytic domain, a hinge region and a C-terminal hemopexin domain); Have a C-terminal transmembrane domain that possesses a short cytoplasmic tail;	MMP-14 (MT1-MMP)	Cancer	Enhance tumor invasion by degrading ECM;	(155–158)
			Malignant gliomas	Involve in both glial invasion and angiogenesis;	(140)
			Osteoarthritis	Participate in matrix deterioration in joint cartilage in humans, commonly observed phenomenon in osteoarthritis;	(132)
		MMP-15 (MT2-MMP)	Gastric cancer	Enhance tumor invasion by degrading ECM;	(159)
		MMP-16 (MT3-MMP)	Meningiomas	Enhance tumor invasion;	(160)

(Continued)

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TABLE 1 Continued

Category	Structural feature	MMPs	The related diseases	Effects and Mechanisms	Refs
		MMP-17 (MT4-MMP)	Atherosclerosis	Reduce atherosclerosis by inhibiting patrolling monocyte recruitment to early lesions;	(85)
			Breast cancer	Accelerates tumor proliferation, induces vascular enlargement and is associated with an increase in lung metastases;	(161)
		MMP-24 (MT5-MMP)	Alzheimer's disease	Promote amyloid pathology associated with the ability of the protease to facilitate trafficking to one of the subcellular sites of amyloid production, cognitive decline, neuroinflammation and neuronal excitability;	(162–164)
			Breast cancer	Modulating cancer cell aggressiveness in a rigid ECM environment during tumor development;	(165)
		MMP-25 (MT6-MMP)	Colon carcinoma and brain tumor	Facilitate tumor development through its capacity to activate progressive proteinase A in the cell membrane;	(166)

closely related to collagenase, including heart failure, atherosclerosis, cancer, arthritis, abdominal aortic aneurysm (130–132, 136, 137, 167, 168). In addition, they play a protective role in some diseases, for instance hypertrophic cardiomyopathy (134). Furthermore, collagenases improve liver fibrosis (133, 138). MMP-1 inhibits the development of atherosclerosis (130). MMP-8 plays a protective role in arthritis (135). Another category of MMPs is gelatinase. These MMPs act as digestive agents for components of the ECM, such as type I and IV collagen (19). They are induced or inhibited by a diverse range of resolvable factors, including growth factors, cytokines and hormones, and are acted on by cellular contacts through specific signaling pathways (169). MMP-9 has pro-inflammatory properties, whereas MMP-2 has pro-homeostatic properties (169, 170). Gelatinases have a profound influence in inflammatory process and tumor progression and have therefore long been considered one of the most significant anti-tumor targets (139, 140, 171). In terms of non-neoplasticity, gelatinases are mainly involved in cardiovascular pathology and auto-immune diseases (20). Moreover, MMP-9 is associated with many respiratory diseases (143). A reduction in vascular MMP-2 and MMP-9 gives rise to hypertensive pregnancy and preeclampsia (124). Stromelysins, another class of MMPs, have a structural domain arranged similarly to that of collagenases. However, these MMPs do not cleave fibrillar collagen type I (19). An important physiological function of stromelysins is to activate other members of the MMP family (21, 129). The most widely described pathological role of stromelysins is in cancer progression (144, 147–149). In addition, they function in the progression of cardiovascular, degenerative, and auto-immune diseases (145, 146, 172). Matrilysins, yet another category of MMPs, do not contain a hemopexin-like domain and are able to decompose collagen type IV but not type I (19). Matrilysins are associated with a number of pathological conditions in humans, mainly cancer and, respiratory, cardiovascular, and neurological diseases (20, 143, 151, 153, 154). A large number of studies have demonstrated that MMP-7 acts in the development and migration of cancer (151, 173). Moreover, MMP-7 also has a critical role in pathogenesis of tonsillitis and permanent hearing loss (150, 152). A study confirmed the early role of MMP-26 in the invasion and angiogenesis of malignant tumors (139). The final member of the MMPs family discussed here is MT-MMPs, which are an important mediator of infiltration. The influence of MT-MMPs on pathological process is mainly reflected in their promotion of tumor invasion (140, 155–161, 165, 166). The ability to activate MMP-2 is one of the reasons why most MT-MMPs play these roles (173). A different example is MMP-17, which has no regulatory effect on MMP-2 although it still affects tumor invasion (174). In addition, studies have demonstrated that MT-MMPs are also implicated in the pathological process of osteoarthritis, atherosclerosis and Alzheimer's disease (85, 132, 162–164). MMPs are medicinal

targets highly relevant to the treatment of a variety of diseases. As understanding of the role of MMPs in biology and pathology increases, greater understanding of the structural similarities and differences among MMP families makes it possible to discover highly selective MMP inhibitors.

In the tumor microenvironment, the activity of a variety of MMPs, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11 and MMP-14, are up-regulated. These MMPs participate in tumor proliferation, survival, and angiogenesis, enabling replication immortality, invasion/migration, immunity evasion and other processes (175, 176). These MMPs control tumor cell growth by the releasing of ectodomains of growth factor, regulating the bioavailability of growth factors and regulating signaling pathways related to cell proliferation (177). MMP-3 and MMP-7 expression in tumor cells may contribute to an apoptosis resistant phenotype (178, 179). In addition, the MMP family is necessary for tumor angiogenesis *via* a two-way action, that is promoting or inhibiting angiogenesis. MMP-1, MMP-2, MMP-7, MMP-9 and MMP-14 regulate this process, and the first three of them play critical roles (180–182). Another key process in which MMPs are the migration of tumor cells. MMP-14 is among the key contributors to cancer invasion and promotes cancer development by activating proMMP-2 and degrading the ECM to promote cancer migration (176). MMP-7 acts in tumor cell metastasis by activating the ERK 1/2 and JNK 1/2 signaling pathways (183). MMP-1, MMP-2, MMP-8, MMP-11, and MMP-13 are implicated in the regulation of tumor cell migration (184–188). Finally, MMPs, such as MMP-14, also participate in tumor immune monitoring (176, 189, 190). Increasing attention has been paid to the role of MMPs in tumor immune regulation, such as their effects on inflammatory and immune responses, the tumor immune microenvironment and their diagnostic or prognostic potential (191–196).

2.2 Relationship between MMPs and immune-related diseases

MMPs affect the process of colitis. MMP-2 is causative for inflammatory bowel disease (IBD), which is derived from weak mRNA expression of pro-inflammatory cytokines including *IFN-*γ and *TNF-*α, and weak protein expression of IL-6 and less overgrowth of the colonic lumen by potentially pro-inflammatory enterobacteria from the commensal gut microbiota (197). MMP-9 plays a potentially key role in the progress of ulcerative colitis (UC) by regulating the immune system (198). MMP-19 coordinates the appropriate innate immune response in colitis, which is critical to balancing the host response to colon pathogens (126). MMP-9 is a member of MMPs closely related to cancer. MMP-9 is related to immune infiltration in pan-cancer and can be used as a biomarker of cancer prognosis and metastasis (199). It is overexpressed in peripheral blood NK cells of prostate cancer (86). And MMP-9 also effectively reduces the tumor killing-effect of T cells *via* cutting the MHC class I molecule, cell surface antigen-presenting complex molecules expressed in melanoma cells (200). In addition, high expression of MMP-11 is associated with worse survival rate in breast cancer, which is related to a low immune response, such as the reduction in the number of CD8+T cells, CD4+T cells, B cells and activated DCs (201).

2.3 Relationship between MMPs and inflammatory diseases

Inflammation is a fundamental pathological process that occurs when biological tissue is stimulated by certain kinds of injury, such as trauma and infection. Topical presentations of inflammation include redness, swelling, heat, pain and functional impairment. Systemic reactions include fever and changes in peripheral blood levels. MMPs are vital elements implicated in the manifold regulation of inflammation (202, 203). In one study, levels of some MMPs, such as MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13, were significantly elevated in ulcer biopsies from patients with inflammatory disease (204–208). MMPs have not only a negative influence (125), but they also have an impact on vascular permeability (209–211), ECM remodeling, epithelial proliferation, and angiogenesis in different stages of inflammation (4, 173). In a model of colonic injury induced by sodium dextran sulfate, MMP-10 had a positive effect on disease (172). Because the progression of damage due to lack of MMP-10 is accelerated with viciousness-potential, enhancing expression of MMP-10 is helpful (125). A similar observation can be found for MMP-19 (126).

3 Key MMPs in CAC and their immunomodulatory aspects

3.1 Important role of MMPs in colitis

Chronic inflammatory disease is often associated with the occurrence and development of various cancers. A classic example is the increased risk for CAC in patients with IBD. In chronic environments marked by chronic inflammation, the ECM is a major factor in maintaining and promoting tumor growth, and MMPs are the major protease involved in the pathogenesis of IBD. Although both sporadic CRC and CAC are malignancies of the colon, CAC differs from sporadic colon cancer in several respects. CRC is produced through three main pathways: the adenomato-carcinoma sequence, the serrated pathway, and the inflammatory pathway. In contrast, the

development of CAC is associated with the inflammatory-dysplasia-carcinoma pathway. MMPs counteract ECM proteins expressed in the gastrointestinal tract during inflammation (212, 213). Therefore, this study was conducted to evaluate the role of MMPs in CAC and its related mechanisms (214). The essential role of these enzymes in the remodeling and destruction of tissue in IBD has been well documented (205, 215–220). Pathological results of IBD, progressive mucosal disintegration (e.g., ulcers and fistulas) and fibrosis due to excessive deposition of collagen (the main component of ECM), is related to a disruption in the balance between composition and breakdown of the ECM (221). As an important molecule in mucosa and submucosa, ECM is the substrate of MMPs, which is why MMPs play such an essential role in the development of IBD. In Crohn's disease (CD), TNF-α and activated T cells stimulate mesenchymal cells to increase the secretion of MMPs, and then MMPs causes tissue damage by degrading the lamina propria matrix (222, 223). MMP-3 and MMP-9 participate in the formation of fistula in CD by degrading the ECM (224). Moreover, MMPs are the key element in wound healing in the late stage of IBD through their effects on degradation of the ECM (225). MMP-1, MMP-7, and MMP-10 are expressed in migratory enterocytes in this process (226), which is important for epithelial regeneration and wound granulation (225, 227, 228). Furthermore, MMP-3 is crucial in scar contraction and ECM remodeling (229–231).

Regarding the ECM, MMPs have roles in a diverse array of substrates (232), including cytokines (90, 233), chemokines (234–237), TNF-α (238), α1-antitrypsin/α1-antichymotrypsin (239), IL-1β (240, 241), stromal cell-derived factor-1 (234), growth factors (239) and so forth. Some factors, such as TNF-α and IL-1β, in turn, stimulate the production of MMPs (242–244). Injury to the intestinal barrier is also responsible for IBD. When the intestinal barrier is disrupted, gene and protein expression of *MMP-1*, *MMP-2*, *MMP-3*, *MMP-7*, *MMP-9*, *MMP-10* and *MMP-13* increase, and leukocytes are summoned to inflamed areas (219, 245–247). MMP-8 and MMP-9 are released from neutrophils to regulate proinflammatory cytokines and chemokines to increase the number of leukocytes and eliminate bacteria (204, 224, 245). Macrophages phagocytose bacteria, along with MMP-9 released externally and MMP-12 entering into the phagosome (248). MMP-12 has a direct bactericidal effect. Briefly, when bacterial pathogens invade, MMP-12 is mobilized to macrophage phagolysosomes and adhere to bacterial cell walls, destroying cell membranes and causing bacterial death (249). MMP-7 plays an indirect bactericidal role by activating and releasing bactericidal alpha defensins into the gut lumen (250, 251). MMP-10 from infiltrating myeloid cells participates in the recovery of DSS-induced damage to the colon (125). Research has also shown that the susceptibility to colitis, including significant disease progression, increased mortality, severe tissue destruction, increases level of pro-inflammatory regulators in the colon and plasma, and a significant delay in neutrophil infiltration and persistent inflammation, increased markedly in MMP-19-null mice. In IBD, MMP-14 in endothelial cells promotes angiogenesis, which is achieved by combining the C-terminal fragment of MMP-14 substrate thrombospondin-1 with CD47/αvβ3 integrin to produce nitric oxide (252). Moreover, the migration of macrophages that lack MMP-19 is reduced *in vivo* and *in vitro* and the mucosal barrier is damaged (126). Chemokine fractalkine (CX3CL1), a substrate of MMP-19, is an essential component of the response to DSS in acute colitis. Because CX3CL1 receptors exist on innate immune cells (e.g., macrophages, neutrophils), impaired immune cell trafficking may be associated with a lack of the soluble CX3CL1 in MMP-19-deficient mice. Mice without the receptor CX3CR1 have more serious symptoms of DSS-induced colitis (126, 253–255). The application value of MMPs as biomarkers in IBD has also been recognized. A number of studies have demonstrated the high sensitivity of MMP-9 in evaluation of active UC (256–258). In addition, through an analysis of emerging BiomARKers (EMBARK), the researcher not only proposed that the combination of fecal calprotectin and serum MMP-9 can be used as a biomarker of UC, but also confirmed the value of MMP-9 as a biomarker of CD, indicating the combination of fecal calprotectin, serum MMP-9 and serum IL-22 can be used as a biomarker of CD (259).

In conclusion, MMPs participate in the host immune defense, would healing, and epithelial regeneration and they have bidirectional effects in IBD. On the one hand, they are involved in the development of IBD through the process of inflammation. MMPs are indirectly associated with progressive organ damage, ulceration or over accumulation of collagen, the persistence of inflammation, and fibrosis because of their substrate ECM. On the other hand, some members of MMP family have an inhibitory effect on inflammation (215, 260).

3.2 Key MMPs in CAC

CAC is a very common fatal complication of IBD (261–264). The pathogenesis of CAC is multifactorial, although a key driver of colitis is neoplastic progression (265–267). The lifetime risk for CAC in IBD patients is 15-40%, and CAC accounts for about 15% of mortality in these patients (268). Chronic inflammation generates oxidative stress that induces DNA damage that might activate some oncogenes and inactivate some anti-oncogenes (267). Related mechanisms include oxidative base lesions, replication stress, DNA crosslinking, and strand breaks, which eventually lead to genomic destabilization and tumorigenesis (269). MMPs play a roles in both promoting and inhibiting regulation of CAC development and progression, as shown in Table 2. The main role of MMPs in colitis and CAC is shown in

TABLE 2 The key matrix metalloproteinases (MMPs) in colitis associated cancer.

MMPs	Protein Expression	Molecular Mechanisms	Effects	Refs
MMP-2	↑	Histone demethylase (JMJD2D) and β-Catenin interacts physically (JMJD2D demethylates H3K9me3 on the promoter of β-Catenin target genes), hence this interaction increases promoter activity of target genes (including *MMP-2*) of β-Catenin, activates transcription of *MMP-2* and others; Macrophages infiltrate and express MT1-MMP, causing MMP-2 activation;	Promote CRC cell to proliferate, migrate and invade and form colorectal tumors in mice; Promote submucosal invasion of transforming growth factor (TGFB) signaling-repressed epithelial cells;	(270, 271)
MMP-3	↑	TNF-α and bradykinin enhance the expression of MMP-3 at a transcriptional level through protein kinase C /protein kinase D1 /mitogen-activated protein signal 20 pathway;	Promote tumor invasion;	(272–274)
MMP-7	↑	Lack of adenomatosis polyposis coli lead to deregulation of WNT signaling pathway, and binding accumulation of β-catenin and T-cell factor-4; Stat-3 signaling is activated by FGFR, thereby inducing MMP-7 expression;	Relate to the occurrence and development of CAC;	(275–278)
MMP-9	↑	Histone demethylase (JMJD2D) and β-Catenin interacts physically (JMJD2D demethylates H3K9me3 on the promoter of β-Catenin target genes), hence this interaction increases promoter activity of target genes (including *MMP-9*) of β-Catenin, activates transcription of *MMP-9* and others;	Activate $p21^{WAF1/Cip1}$ by regulating notch activity, a key transcription factor in epithelial cell lineage, resulting in β-catenin inhibition and cell cycle arrest; Acts tumor suppressive effect by activating MMP-9-Notch1-ARF-p53 axis, which lead to apoptosis and DNA damage in colonic epithelium; Reduce reactive oxygen species accumulation and DNA destruction; Inhibit metastasis and adhesion of colorectal cancer cells; Reduce tumor angiogenesis; Act on EGFR-nuclear transcription factor-specificity protein 1 (Sp1) signaling pathway to sustain the epithelial mucosal and function as well as immune homeostasis; Maintain epithelial and mucosal integrity by increasing mucin and intestinal trefoil factor (ITF) and downregulating STAT3 pathway; Maintain the balance of microbiota;	(212, 213, 270, 276, 279–282)
MMP-10	↑	Activate proTNF-α turning into TNF-α, then promote NF-κB signaling pathway activation;	Destroy intestinal barrier function; Facilitate the resolution of inflammation;	(283)
MMP-11	↑	Associate with the increase of β-catenin accumulated crypts number;	Reduce apoptosis of cancer cells;	(276, 284, 285)
MMP-13	↑	Activate proTNF-α turning into TNF-α, then promote NF-κB signaling pathway activation;	Destroy intestinal barrier function; Facilitate the resolution of inflammation;	(283, 286)

Figure 2, and the network of MMPs that interfere with CAC is shown in Figure 3. A list of all genes mentioned here could be found in Table S1.

MMPs degrade the protein components of the ECM and basement membranes, which provides a channel for cancer cells to invade to the vascular and lymphatic systems as well as promotes metastasis (276). In the process of tumor growth, MMPs are up-regulated, which strengthens the permeability of vascular endothelial cells, thereby increasing cell proliferation, migration and angiogenesis (287). Histone demethylase (JMJD2D) and β-catenin interacts physically (JMJD2D demethylates H3K9me3 on the promoter of β-catenin target genes), which increases the promoter activity of target genes (including *MMP-2* and *MMP-9*) of β-catenin; activates transcription of *MMP-2*, *MMP-9*, and others; and ultimately cause CRC cells to proliferate, migrate and invade, and form colorectal tumors in mice (270). In a mouse model of tumor invasion, macrophages infiltrate and express MT1-MMP, resulting in activation of MMP-2 and consequent inhibition of TGF-β. This process leads to submucosal invasion of epithelial cells when it occurs in conjunction with KRAS or phosphatase and tensin homolog deleted on chromosome 10 (271). Specifically, when the inhibition of TGF-β is accompanied by the expression of KRAS, activation of the epidermal growth factor receptor (EGFR) signaling pathway is increased as a result of increased protein

expression of epiregulin and mRNA expression of *Errb1*. When the inhibition of TGF-β is accompanied by phosphatase and tensin homolog deleted on chromosome 10 deletion, mRNA expressions of *cyclin-dependent kinase (CDK) inhibitors (Cdkn2b/p15^{Ink4b}*, *Cdkn1a/p21^{Cip1}* and *Cdkn1b/p27^{Kip1})* is down-regulated (288, 289). The level of MMP-3 secreted from myofibroblasts is up-regulated in IBD and tumorigenesis (272, 273). TNF-α and bradykinin enhance expression of MMP-3 at a transcriptional level through the protein kinase C/protein kinase D1/mitogen-activated protein signaling pathway, and thus mediate CAC (272). This mediating effect is related to the promotion of tumor invasion by MMP-3 (274). MMP-7 is connected to the occurrence and progression of CAC, and is expressed intensely at crypt bases of epithelial cells and in dysplastic CAC biopsy, as observed in CRC (275). Because of the lack of adenomatosis polyposis coli, the WNT signaling pathway is deregulated and β-catenin and T-cell factor-4 accumulate. Hence, the expression of MMP-7 up-regulated (276, 277). In addition, fibroblast growth factor receptors in cancer-related fibroblasts activate Stat-3 signaling, thereby inducing MMP-7 expression (278). In contrast, the highly expressed MMP-9 in CAC inhibits the tumor by affecting the Notch signaling pathway. Specifically, MMP-9 activates p21$^{WAF1/Cip1}$ by regulating notch activity, a key transcription factor in epithelial cell lineage, resulting in β-catenin inhibition and cell cycle arrest (213). MMP-9 from the colonic epithelium also acts as a tumor suppressor by activating the MMP-9-Notch1-ARF-p53 axis, which leads to apoptosis and DNA damage (279). Previous study claimed that epithelium-derived MMP-9 is beneficial for chronic inflammation, regardless of tissue origin, in contrast to neutrophil-derived MMP-9. They also proposed that MMP-9 (stemming from epithelium or neutrophils) is a pivotal regulator of acute IBD and sporadic cancers (279). MMP-9 reduces reactive oxygen species and DNA destruction in CAC as well (212). Some researchers have also found that the hemopexin domain of MMP-9 has an inhibitory effect on the metastasis and adhesion of CRC (280). The decrease in MMP-9 in plasma causes down-regulation of angiostatin synthesis, which results in tumor growth and vascularization (280, 281). MMP-9 expressed in the colonic epithelium maintains the microbiota balance. Antimicrobial peptides including REG3 and S100A families, are effective agents of the innate immune system (290–292). In transgenic mice constitutively expressing MMP-9 in the colonic epithelium mRNA levels of *TNF-α, IL-6, IL-1β* and *IFN-γ* increased, but mRNA levels of *IL-22, REG3γ* and *S100A8* decreased. MMP-9 maintains epithelial and mucosal integrity by increasing mucin and intestinal trefoil factor protein levels and down-regulating the STAT3 pathway *in vivo*. Moreover, MMP-9 acts on the EGFR-nuclear transcription factor-Sp1 signaling pathway to sustain epithelial mucosa and functioning as well as immune homeostasis (282).

MMP-9 and MMP-10 are only significantly expressed in inflamed tissue, not normal colon tissue, and they start to peter out when healing begins (276). MMP-10 is mostly expressed by macrophages. In UC, it is found in enterocytes at the margins of ulcers and in the cells of granulation tissue (276). Researchers believe that MMP-10 from infiltrating bone marrow cells plays a role in resolving the inflammation. With a lack of MMP-10, susceptibility to DSS-induced colitis increases, and prolonged IBD may eventually lead to dysplasia (276). In miR-148/152-deficient mice, expressions of MMP-10 and MMP-13 increases, thus activating pro-TNF-α turning into TNF-α and promotes activation of the NF-κB signaling pathway. Damaged functioning of the intestinal barrier accelerates colitis and CAC (283). Similarly,

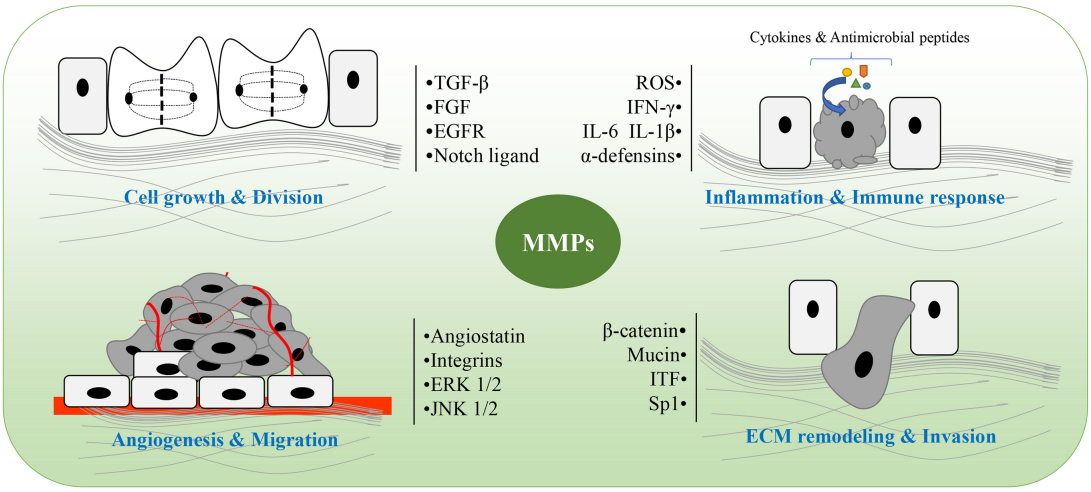

FIGURE 2
The main role of matrix metalloproteinases (MMPs) in colitis and colitis-associated cancers (CAC). MMPs are involved in pathological processes of colitis and CAC, including cell growth and division, angiogenesis and migration, ECM remodeling and invasion, as well as inflammation and immune response. ROS, reactive oxygen species; ITF, intestinal trefoil factor; Sp1, specificity protein 1; ECM, extracellular matrix.

MMP-11 is virtually absent in regular tissues (276). The mRNA level of *MMP-11* is related to CAC in some way (284, 285). The mRNA level of *MMP-11* is up-regulated in CAC, and is associated with the increase in the number of β-catenin accumulated crypts (284). The proton pump inhibitor-omerprazole and TNF-α blocker-infliximab reduce the mRNA level of *MMP-11* and induces cells apoptosis in CAC (285). MMP-13 is highly increased in CAC colonic tissues, but do not change as the CAC progression (286). Compared to other MMPs, MMP-14 (MT1-MMP) does not increase markedly in CAC. Researchers have also found that Omerprazole and Infliximab were able to down-regulate the mRNA levels of *MMP-14* (284). In a mouse model of CAC, miR-128, miR-134 and miR-330 are influenced by Dicer1. These microRNAs inhibit tumor growth *in vitro* and *in vivo* and modulate expression of MMP-3, MMP-10, and MMP-13 (285, 293).

3.3 Mechanisms underlying of typical MMPs in CAC

In CAC, typical MMPs affecting the organism's immune function and their expressions are regulated by the immune system, as shown in Figure 4 (10). MMP-7 decreases the sensitivity of mice to intestinal bacteria. Specifically, MMP-7 knockout mice do not activate pro-a-defensins in the gut to their mature active forms, with the result that these mice are highly susceptible to intestinal bacterial infection (250). MMP-8 affects the immune response to tumor and helps to resolve necrosis, which is positively related to the degree of primary tumor necrosis and blood neutrophil count, as well as negatively correlated with destructive inflammatory infiltration and Crohn's-like lymphoid reaction (294). MMP-8, which is involved in resolving acute and chronic inflammation and helps to recruit neutrophils during acute inflammation, is mainly produced by neutrophils (295, 296). It plays a role in the recruitment of neutrophils to necrotic areas and in tissue remodeling, including collagen breakdown (294). MMP-9 is associated with the onset of lymphadenitis in patients with CAC, and is significantly up-regulated before the onset of lymphadenitis in these patients (297). In addition, MMP-9 maintains the integrity of epithelial mucosa and acts as a tumor suppressor in CAC, which is inseparable from its function of mediating the level of proinflammatory cytokines (282). The linings of gastrointestinal epithelial mucosa act as an external physical barrier and a functional immune barrier for an immune monitoring system (298). The imbalance in immune cells is crucial to the development of cancer (299). The inflammatory cytokines released by immune cells function in immune defense, and promote the development of cancer in specific circumstances (300, 301). MMP-9 increases mRNA

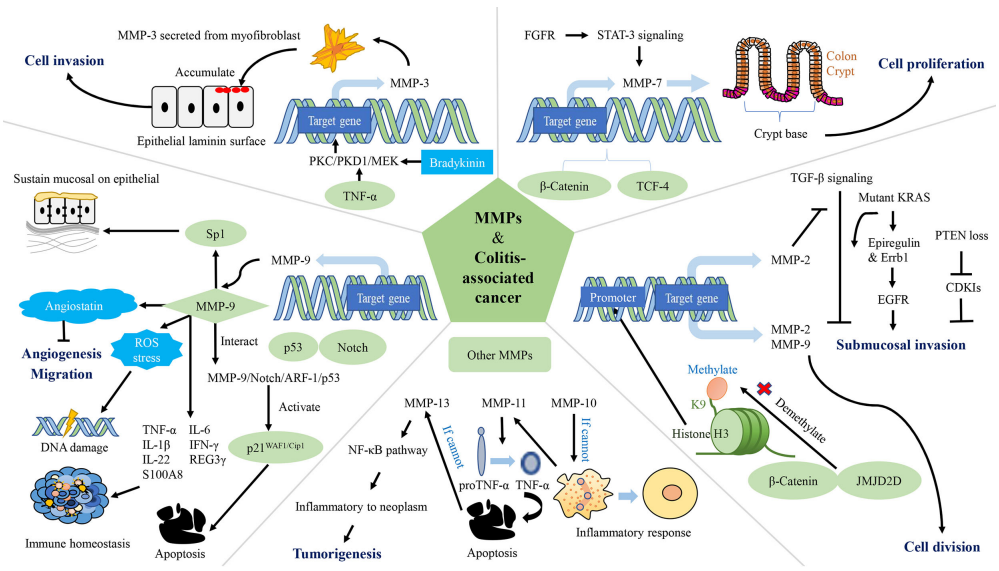

FIGURE 3
The network of matrix metalloproteinases (MMPs) interfering with colitis-associated cancers (CAC). MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, and MMP-13 are involved in the regulation of CAC. MMP-2 and MMP-9 promote CRC cell proliferation. MMP-2 and MMP-3 contribute to tumor invasion. Expression of MMP-9 leads to apoptosis, reduced accumulation of ROS, DNA damage and inhibition of tumor vascularization, while maintaining epithelial mucosa, microbiota homeostasis and immune homeostasis. MMP-10 plays a role in inflammation regression. MMP-11 reduces apoptosis in cancer cells. PKC/PKD1/MEK: protein kinase C/protein kinase D1/mitogen-activated protein; TCF-4: T-cell factor-4; PTEN: phosphatase and tensin homolog deleted on chromosome 10; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptors; CDK, cyclin dependent kinase; Sp1, specificity protein 1; ROS, reactive oxygen species.

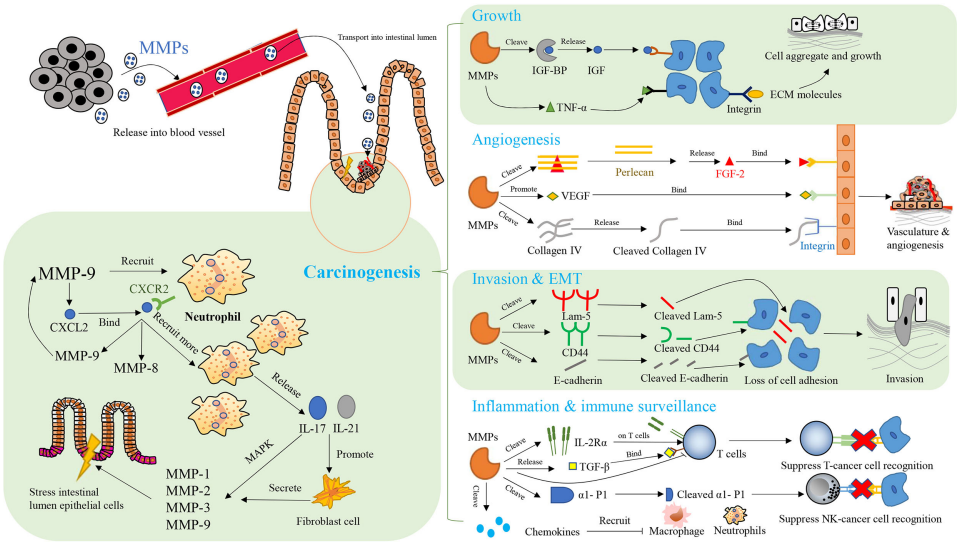

FIGURE 4
The network of MMPs and therapeutic targets in colitis-associated cancers (CAC). Key aspects of carcinogenesis are mediated by MMPs, including cell growth, angiogenesis, invasion, epithelial-mesenchymal transition (EMT), inflammation, and immune surveillance in immunomodulatory manner. CXCL2: CXC-chemokine ligand 2; CXCR 2: CXC chemokine receptor 2; MAPK, mitogen-activated protein kinases; FGF-2, fibroblast growth factor 2; IGF, insulin-like growth factors; IGF-BP, insulin-like growth factors binding protein; TNF-β, tumor necrosis factor-β; TNF-α, tumor necrosis factor-α; ECM, extracellular matrix; Lam-5, laminin-5; CD44, cluster of differentiation-44; VEGF, vascular endothelial-derived growth factor; α1-P1, alpha 1-proteinase inhibitor;IL-17, interleukin-17; IL-21, interleukin-21; IL-2Rα, interleukin-2 receptor alpha.

levels of *IL-6*, *IL-1β*, *TNF-α* and *IFN-γ*, but decreases the mRNA level of *IL-22* (282). Regarding the regulation of MMPs by the immune system, elevated MMP-8 is associated with systemic inflammation and increased secretion of various cytokines, including IL-1ra, IL-7 and IL-8, and is negatively associated with the number of tumors infiltrating mast cells (302). In a mouse model of colitis-associated CRC, the NF-κB mediated inflammatory reaction promotes protein expression of cyclin D1, phosphorylated ribosomal protein S6 and MMP-9 in the colon tissues of these mice, which plays a beneficial role in CRC progression (303). In the mouse model of CAC, MMP-9 expression is associated with excessive angiogenesis and cell proliferation, which is related to CXCL2 and neutrophil recruitment (304). CXCR2 is present in neutrophils and interacts with CXCL2 (305). This interaction promotes the recruitment of neutrophil and the synthesis of MMP-8 and MMP-9 (304, 306, 307). The proinflammatory factors IL-17 and IL-21 increase the MMPs secreted by human intestinal fibroblasts, including MMP-1, MMP-2, MMP-3 and MMP-9 (308–310). Among them, the inducing effect of IL-17 on MMP-1 and MMP-3 depends on the rapid activation of mitogen-activated protein kinase (308, 311). The regulation of MMPs by IL-21 does not occur at the level of transcription and translation and stimulating fibroblast with IL-21 does not increase the intracellular level of MMP RNA transcripts and proteins. The up-regulation of MMPs by IL-21 may depend on preferentially increasing the secretion of preconstituted or newly synthesized MMPs (309). In addition, expression of MMPs is regulated by TNF-α and IFN-γ (312).

3.4 Potential clinical applications of MMP inhibitors

Clinical trials of MMPs mainly focus on three factors. They are respectively the changes in clinical levels of MMPs in different disease states, the clinical use of MMP inhibitors in colitis and colorectal cancer, and combining MMPs and some regulatory factors with other drugs to control inflammation and tumors. MMP-2, MMP-3, MMP-7 and MMP-9 are the key MMPS in this process. However, current clinical trials have shown that inhibiting MMPs has no obvious effect on tumor responses, although it has a certain role in stabilizing the condition of diseases.

Many MMPs are upregulated in IBD. These MMPs remodel tissue and release several small protein fragments. In a clinical trial with 164 volunteers, these protein fragments could be used to distinguish between CD and UC. For example, measuring segments of vimentin (MMP-2 and MMP-8 decomposed and citrullinated-vimentin [VICM]) and type III (MMP-9 decomposed collagen type III [C3M]) can distinguish between CD and UC (313). A total of 138 participants took part in an IBD-related study, including different types of disease. Fecal MMP-9 can be used to diagnose and differentiate between UC

and pouchitis, because it is strongly associated with clinical, histological, and endoscopic activities of different forms of IBD (257). A clinical trial evaluated the relationship between MMP and prognosis in CRC. This study enrolled 198 consecutive patients who had undergone operation for CRC, (85 females and 113 males). Of the patient, 67% were older than 65 years old, and their Tumor-Node-Metastasis classification ranged from 1 to 4. Expression of MMPs was higher in tumor tissue than in normal mucosa. This result indicates that high expression of MMP-2 and MMP-9 in the mucosa of CRC patients is related to poorer 5-year survival rates (314). MMP-7 is implicated in multiple processes of tumor development. To estimate the contribution of serum MMP-7 to the prognosis of resected CRC, researchers have conducted several clinical trials. In a study with 303 CRC patients (87 healthy controls, 96 nonmetastatic patients and 120 advanced patients), high serum MMP-7 was associated with a higher risk of death in terminal CRC patients (315). Included in another study were 175 curatively resected CRC patients. In two Cox proportional hazard models (overall survival and disease-free survival), higher MMP-7 was associated with higher recurrence and faster progression (316). Given the role of MMP-3 in cancer progression and metastasis, a study with 73 CRC patients who underwent minimally invasive colorectal resection investigated the relationship between increased plasma MMP-3 and residual metastases after surgery. Minimally invasive surgery directly up-regulated MMP-3 levels owing to surgery or subsequent wound healing or indirectly up-regulated MMP-3 by increasing TNF-α and IL-1 in the acute inflammatory response after surgery (317).

BAY 12-9566 inhibits MMP-2, MMP-3, and MMP-9. A phase I clinical enrolled 13 patients with colorectal, renal, gastroesophageal junction, duodenum, lung, and sarcoma cancer. Subjects were given BAY 12-9566 at four dosages. No tumor responses were found, but two patients had stable disease after 1.1 and 1.5 years of treatment (318). In another phase I clinical trial, 27 patients with advanced solid tumors took BAY 12-9566 100 to 1,600 mg/day. These patients had colorectal, lung, breast, ovarian and cervical cancers. The condition of 48% patients was stable. BAY 12-9566 did not reduce the size of the tumor, but slowed their growth (319). BMS-275291 is another wide-spectrum inhibitor of MMPs. In an open-label, phase I trial, 40 late-stage or metastatic cancer patients were given BMS-275291, most of them had CRC or non-small cell lung cancer. Although the researchers found no objective tumor responses, the condition of some patients stabilized (320).

Two clinical trials have been conducted on drug combinations. In a randomized, double-blind, clinical trial of rectal cancer, 34 patients receiving chemoradiotherapy were divided into a placebo group and a conjugated linoleic acid group. Supplementing conjugated linoleic acid decreased the levels of TNF-α, IL-1β, hsCRP, MMP-2 and MMP-9, which are biomarkers of tumor aggression and angiogenesis (321). A trial that included 37 patients with CRC lasted for 7 weeks. These patients who underwent chemotherapy, were separated into two groups: a fisetin group (n=18) and a placebo group (n=19). Flavonoid fisetin reduced levels of MMP-7, and significantly lowered levels of high-sensitivity C-reactive protein and IL-8 by the end of the study (322).

Despite the important role of MMPs in many human diseases, no broad-spectrum synthetic MMP inhibitor has successfully passed the clinical trial stage because of the bilateral pro-tumor and anti-tumorigenic effects of MMPs in cancer (323). A variety of MMPs, including MMP-2, MMP-9 and MMP-14, can degrade the basal layer of capillaries and promote exosmosis of tumor cells. MMP-9 also down-regulates the IL receptor on the surface of T cells, further inhibiting immunity and promoting cancer tolerance (324–326). By eliminating cell apoptosis, MMP-7 reduces the effect of chemotherapy even promoting tumor growth. However, MMP-8 may directly inhibit tumor metastasis in tumor cells. One of the side effects of broad-spectrum MMP inhibitors is that they interfere with the tumor-inhibiting function of MMP-8 (76). With more specific MMPs inhibitors now available, MMPs targeting can be reconsidered for cancer therapy (326).

4 Conclusion

Given their role in degrading the ECM, MMPs are associated with the occurrence and development of many diseases, especially inflammatory diseases. Most MMPs, such as MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13 are increased in colitis and CAC. Therefore, reducing levels of these MMPs could effectively prevent the development of inflammation and CAC, as well as the progression of colitis- the eventual cause of CAC- from acute inflammation to chronic. However, the effect of some MMPs, like MMP-9, on CAC is bidirectional, which means they are involved in the pathogenesis of IBD and promote the metastasis and spread of malignant tumors, but also play a role in tumor suppression as well. Therefore, how to balance the bidirectional role of MMPs in clinical applications is a vital question. In specific diseases, it might be advisable to clarify the therapeutic target, especially the definitive role and efficacy of a certain MMP. Given their multifaceted role in colitis and CAC, more in-depth research is needed. In addition, MMPs participate in the host immune defense, wound healing, and epithelial regeneration. Normally MMPs are secreted and activated in immune cells when the expression of inflammatory cytokines and chemokines increase. MMPs modulate immune system activity by interfering with the differentiation and immune activity of immune cells, recruitment of macrophages, and migration of neutrophils. In clinical trials, the condition of CRC patients could be stabilized to a certain extent by inhibiting levels of MMPs. Therefore, levels of MMPs could be used to predict the condition and development of inflammatory diseases and CAC. Furthermore,

MMPs have very broad prospects in the treatment of CAC through immunoregulation, which is also a promising direction in future research.

Author contributions

LH and QK wrote and revised this manuscript. KC constructed figures in manuscript and helped to revise it. YZ helped to revise the manuscript and provided valuable feedback to this conception. ZZ revised the entire manuscript and edited the language for scientific presentation. The corresponding authors, ZZ and WT conceived and organized this study. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1093990/full#supplementary-material

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Targeting matrix metalloproteases in diabetic wound healing

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Chronic inflammation participates in the progression of multiple chronic diseases, including obesity, diabetes mellitus (DM), and DM related complications. Diabetic ulcer, characterized by chronic wounds that are recalcitrant to healing, is a serious complication of DM tremendously affecting the quality of life of patients and imposing a costly medical burden on society. Matrix metalloproteases (MMPs) are a family of zinc endopeptidases with the capacity of degrading all the components of the extracellular matrix, which play a pivotal part in healing process under various conditions including DM. During diabetic wound healing, the dynamic changes of MMPs in the serum, skin tissues, and wound fluid of patients are in connection with the degree of wound recovery, suggesting that MMPs can function as essential biomarkers for the diagnosis of diabetic ulcer. MMPs participate in various biological processes relevant to diabetic ulcer, such as ECM secretion, granulation tissue configuration, angiogenesis, collagen growth, re-epithelization, inflammatory response, as well as oxidative stress, thus, seeking and developing agents targeting MMPs has emerged as a potential way to treat diabetic ulcer. Natural products especially flavonoids, polysaccharides, alkaloids, polypeptides, and estrogens extracted from herbs, vegetables, as well as animals that have been extensively illustrated to treat diabetic ulcer through targeting MMPs-mediated signaling pathways, are discussed in this review and may contribute to the development of functional foods or drug candidates for diabetic ulcer therapy. This review highlights the regulation of MMPs in diabetic wound healing, and the potential therapeutic ability of natural products for diabetic wound healing by targeting MMPs.

KEYWORDS

matrix metalloproteases, diabetic wound healing, natural products, chronic inflammation, clinical research studies

1 Introduction

Diabetes mellitus (DM) is a chronic metabolic noncommunicable disease principally characterized by consistently high blood glucose levels that may affect more than 783 million individuals in 2045 worldwide (1, 2). Sustained exposure to high levels of glucose induces neurological, microvascular and macrovascular lesions and low immune response in the body, which thereby contributes to the impaired wound healing, a crucial matter in diabetic patients (3). Such chronic wound ulcer is often exacerbated by tissue ischemia or constant stress, especially in the foot, which can eventually lead to amputation if no appropriate therapeutic strategy are applied (4). Besides, high glucose (HG) also affects the functions of cornea, leading to several diabetic corneal complications especially delayed epithelial wound healing (5). Unlike wound healing in healthy individuals, the healing process in DM is retarded in the inflammatory phase, as manifested by the elevation of pro-inflammatory cytokines, proteases, and reactive oxygen species (ROS), and the dysfunctions of numerous cell types (6). In addition, wounds under diabetic condition are more susceptible to bacterial infection on account of damaged immune responses, which brings about substantial recruitment of inflammatory cells that produce various ROS and destroy structural elements of the extracellular matrix (ECM) (7). Noticeably, ROS together with pro-inflammatory cytokines can further impairs the wound by inducing matrix metalloproteinases (MMPs) expression, which results in degradation of the ECM and growth factors (8).

MMPs, a set of zinc-dependent proteolytic enzymes, participate in kinds of wound healing events by degrading almost all protein components of ECM (9). According to the structure of substrates and domains, MMPs can be primarily classified as collagenases (like MMP-1, MMP-8, and MMP-13), matrilysins (such as MMP-7), stromelysins (such as MMP-3, MMP-10, and MMP-11), gelatinases (such as MMP-2 and MMP-9), membrane type metalloproteinases (such as MMP-14), as well as others (10, 11). Usually, MMPs are inactive and exist as latent precursors "zymogens" *in vivo*, but turn into active status when stimulated by external stimuli like cytokines, growth factors, as well as cell-matrix interactions (12). A critical mechanism for the modulation of MMPs is through conjugation with endogenous inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), α2-macroglobulin, and small molecules with TIMP-like domains (13–15). During the progress of diabetic ulcer, MMPs show higher level of protease activity, they degrade protein, undermine the temporary ECM, remodel the granulation tissue, modulate angiogenesis, and administrate the activity of growth factors (16–18). Intriguingly, multiple signaling pathways associated with inflammatory response, oxidative stress, and apoptosis, as well as epigenetic modification are illustrated to be implicated in the regulation of MMPs during diabetic wound healing (19–21), which indicate that targeting these signals-mediated MMPs is a promising strategy for diabetic ulcer.

Natural products are a dominating resource for developing drug candidates with specific bioactivities to prevent diseases and can function as dietary supplements to provide benefits for human health. Over the past decade, increasing evidence has shown that naturally occurring flavonoids, polysaccharides, saponins, and alkaloids exhibit outstanding therapeutic activities on wound healing under a variety of pathological conditions (22). In addition, animal-derived peptides and hormones have also been reported to possess promising ability to improve diabetic wound healing (23, 24). These compounds mainly improve diabetic wound healing through accelerating re-epithelization and ECM formation, alleviating oxidative stress, relieving inflammation, promoting angiogenesis, and suppressing apoptosis *via* MMPs-relevant signaling pathways (25–29). As a result of the favorable ability of natural products in regulating aberrantly altered MMPs during diabetic wound healing, developing the novel agents based on the structures of these natural products may contribute to the treatment or mitigation of diabetic ulcers.

This review highlights the physiological and pathological regulation of MMPs in diabetic ulcers, and the potential therapeutic ability as well as mechanisms of natural products for diabetic wound healing by targeting MMPs. In addition, clinical research studies focused on MMPs in the process of diabetic wound healing are also addressed. At the same time, the application prospect of natural products targeting MMP in the prevention and treatment of diabetic ulcers is pointed.

2 MMPs in diabetic ulcer

2.1 Stem cells and MMPs

Mesenchymal stem cells (MSCs) are illustrated to accelerate wound healing under diabetic condition *via* regulating ECM proteolysis. MSCs reverses the reduction of COL I and COL II, down-regulates MMP-9 expression, and suppresses the levels of activated MMP-9 in diabetic wounds. In addition, MSCs administration up-regulates the expression of miR-29b in diabetic wounds and HG-treated dermal fibroblasts (30), indicating that MSCs exert therapeutic effect on diabetic wound healing through repressing proteolysis and improving COL levels in ECM *via* targeting miR-29/MMP-9 axis. Interestingly, adipose derived mesenchymal stem cells (ADSCs) are demonstrated to improve diabetic ulcer through modulating the expression of ECM remodeling-associated genes. Ghaneialvar et al. reported that ADSCs administration down-regulates the gene expressions of MMP-9 and up-regulates the expressions of MMP-2 as well as TIMP-1 in STZ-induced mice. Besides, the mRNA expression of urokinase-type plasminogen activator (uPA) is elevated at the early stages of wound healing process, which may promote the activity of MMP in the inflammatory phase, while ADSCs intervention reduces the expression of uPA in diabetic group (31). Additionally, Wang et al. reported that extracellular vesicles derived from adipose-derived stem cells (ADSC-EVs) play a significant role in diabetic wound healing by promoting collagen (COL) deposition through down-regulating MMP-9 expression. *In vitro* studies demonstrated that ADSC-EVs promotes the proliferation of AGEs-BSA-treated HaCaT cells, while inhibits the secretion of MMP-9. Besides, ADSC-EVs improves the wound healing rate of diabetic mice through accelerating the re-epithelialisation, facilitating COL deposition, and reducing MMP-9 levels in the wound fluids (32).

Bone-marrow-derived mesenchymal stem cells (BM-MSCs) has been utilized as effective therapeutic strategy for wound healing due to their abilities to modulated inflammation, ECM production, as well as angiogenesis. Kamiya et al. reported that BM-MSCs transplantation accelerates wound healing in STZ-induced rats and HG-induced keratinocytes through improving re-epithelialization, elevating the suppressed viability of HKCs, and increasing the expressions of MMP-2, epidermal growth factor (EGF), human insulin-like growth factor 1 (IGF-1) as well as p-FAK (33). In addition, mouse bone marrow (BM)-derived allogeneic MSCs (allo-mBM-MSCs) facilitate wound healing in STZ-induced diabetic mice through secreting the growth factors and proteins such as MMP-1. Allo-mBM-MSCs administration improves the wound healing rate through promoting the re-epithelialization, granulation tissue formation, COL deposition and vascular proliferation. Notably, Allo-mBM-MSCs accelerates the wound repair through secreting the factors and proteins such as COL-1, keratinocyte growth factor, MMP-1, Ang-2, IGF-1, hepatocyte growth factor, prostaglandin E2 and vascular endothelial growth factor (VEGF) (34). Altogether, these results suggest that modulating the secretion of MMP-1 by allo-mBM-MSCs may be a potential therapy for diabetic wound repair.

Stem cells from human exfoliated deciduous teeth (SHED) possess strong differentiation capacity that display outstanding therapeutic effect in wound repair. Lv et al. found that SHED treatment up-regulates MMP-9 and MMP-2 in rats with DFU, resulting in the improvement of wound healing, enhancement of angiogenesis, reduction of inflammation, as evidenced by the up-regulation of VEGF and eNOS, as well as the down-regulation of interleukin (IL)-10, Tumor necrosis factor (TNF)-1α as well as IL-1β. However, SHED transplantation is not as effective as MSCs in wound healing (35). Even so, SHED is a potential treatment for diabetic ulcer healing and may address the surgical invasiveness associated with MSCs transplantation.

Considerable studies have illustrated that endothelial progenitor cells (EPCs) play a pivotal part in vasculo-genesis, which thereby gives rise to the reconstitution of microcirculation and healing. Impeded neovascularization and impaired EPCs are major features of diabetic wound healing. Angiopoietin (Ang)-1is a potent mobilizer of EPCs from the BM, which improves re-epithelialization and EPC recruitment in the wounds of diabetic mice though up-regulating the expression of MMP-9 and stem cell factor (SCF). Interestingly, SCF treatment can reverses the decreased mobilization of EPCs in MMP-9$^{-/-}$ mice, and Ang-1 overexpression elevates the re-epithelialization of wounds in MMP-9$^{-/-}$ mice (36), which suggest that the protective effects of Ang-1 on diabetic wound healing are involved in the EPC recruitment and MMP-9 regulation.

2.2 Inflammation-associated signals pathways and MMPs

During the development of diabetic wounds, the excessive inflammation coupled with advanced glycation end products (AGEs) accumulation cause down-regulation of growth factors, rapid degradation of matrix, as well as reduction of COL, ultimately leading to impeded wound healing in patients (37). Numerous studies have reported that nuclear transcription factor-κB (NF-κB) signaling pathways contribute to the expression of MMPs in diabetic foot ulcers (DFUs). Chang et al. reported that DFUs infection impairs the wound healing by increasing inflammation and inhibiting angiogenesis, which gives rise to the up-regulation of MMP-9 through activating the NF-κB signaling pathway *via* increasing ROS, whereas it does not affect the level of MMP-8. Remarkably, the inhibitor of MMP-8 delays the diabetic wound healing, but (R)-ND-336, the inhibitor of MMP-9, promotes the wound repair, which suggest that MMP-8 facilitates wound healing in DM, whereas MMP-9 does not (18, 38). In addition, Notch1/NF-κB/MMP-9 axis also participates in diabetic wound closure. Zhu et al. reported that Notch1 signaling pathway is activated in skin of diabetic rats and AGEs-BSA-treated primary human keratinocytes, as evidenced by the up-regulation of Notch intracellular domain (NICD), Delta-like 4 (Dll4), as well as Hes1, which contributes to the elevation of MMP-9 activation. Remarkably, the regulatory effect of Notch1 on MMP-9 relies on NF-κB activation, and suppression of Notch1 significantly prevents the nuclear translocation of NF-κB induced by AGEs-BSA in keratinocytes. Interestingly, inhibiting Notch1 signal with DAPT represses NICD and MMP-9, resulting in the improvement of COL accumulation and diabetic wound healing (39). Moreover, MMP-9 in diabetic wound healing is also mediated by receptor for AGE (RAGE), MAPK as well as NF-κB signaling pathways. In AGEs-BSA-treated HaCaT cells, MMP-9 expressions are significantly up-regulated, while such effect is reversed by the intervention of inhibitors of extracellular regulated protein kinases (ERK)1/2, p38, as well as NF-κB. In addition, AGEs-BSA also elevates the expression of RAGE in HaCaT cells and promotes NF-κB p65 translocation. Remarkably, silence of RAGE abrogates MMP-9 activation and the phosphorylation of ERK1/2 as well as p38 (21).

Indeed, long-term HG exposure is elucidated to impair keratinocyte migration and obstacle wound healing through stimulating M1 macrophage polarization *via* TNF-α TIMP-1/MMP-1 axis. Huang et al. reported that pro-inflammatory M1 macrophages and TNF-α levels are obviously increased in the perilesional area of diabetic rats, as evidenced by the up-regulated ratio of C-C chemokine receptor type 7 (CCR7)/CD68. Besides, TNF-α from M1 type macrophage suppresses the migration of keratinocytes by down-regulating MMP-1 and up-regulating TIMP-1, while TNF-α antibody addition or gene-silencing of TIMP-1 restore the impaired function of keratinocytes. Further *in vivo* studies demonstrated that TNF-α antagonist promotes wound healing process in diabetic rats (40). In addition to high systemic blood glucose concentration, local hyperglycemia can also inhibit wound healing. Kruse et al. found that the migration of keratinocyte and fibroblast is suppressed by 5.6 mM glucose intervention, which results in the delayed close of scratch wounds. In addition, local hyperglycemia inhibits the wound healing and re-epithelialization in rats through increasing the levels MMP-1 (41). Interestingly, Feng et al. reported that MMP-9 blocks the wound healing process in DM mice through attenuating EPCs recruitment *via* suppressing CXCL12 activation. The expressions of phagocyte-derived MMP-9

and pro-inflammation factors (such as TNF-α and IL-6) are augmented, whereas the numbers of EPC and levels of CXCL12 are decreased in STZ-induced diabetic mouse. However, the inhibitor of MMP significantly facilities the diabetic wound healing compared with TNFα-treated group (42). Therefore, these results suggest that the inhibitor of MMPs may be a potential agent for impaired wound closure in diabetic patients. Furthermore, IL-1β is demonstrated to impede fibroblasts functions from diabetic wound tissues by modulating the expression of MMPs *via* a p38-mediated pathway. A recent study reported that IL-1β levels are up-regulated in the wounds and serum of diabetic individuals as well as that of *db/db* mice, which suppresses the proliferation and migration of fibroblasts, enhances MMP-2 and MMP-9 expression, and down-regulates TIMP-1 and TIMP-2. Additionally, IL-1β intervention dose-dependently promotes the phosphorylation of p38 in cultured fibroblasts, while SB203580 (a p38 inhibitor) countervails the effects of IL-1β on collagenase, MMPs, and TIMPs (43), suggesting that IL-1β takes part in delayed wound healing in DM by altering levels of ECM remodeling proteins through activating p38 signal.

Altogether, these studies indicate that targeting inflammatory response-relevant signaling pathways involving NF-κB, MAPK, TNF-α, as well as IL-1β is a promising therapeutic strategy to modulate MMP-9, MMP-8, and MMP-1 expression during diabetic ulceration (Figure 1).

2.3 Oxidative stress-associated signals and MMPs

ERK1/2 signal is demonstrated to participate the development of diabetic wound healing by modulating MMPs and activator protein-1 (AP-1). AP-1 comprises c-Jun and c-Fos proteins and has been illustrated to function as the modulator of MMPs transcriptions under the diabetic condition. The protein levels of AP-1 and MMP-9 are enhanced in the epithelium of diabetic skin tissues. Besides, the protein stability of c-FOS/c-Jun, the subunits of AP-1, as well as the activation of ERK1/2 are elevated in HG-treated HaCaT cells, while ERK1/2 inhibitor reverses the phosphorylation of c-FOS and c-Jun, and down-regulates MMP-9 expression, suggesting that ERK1/2 activated by HG can stabilize AP-1, which leads to the transcription and expression of MMP-9 and subsequently the delayed wound healing (44). In addition, increasing studies proved that the transcriptions of MMP-2 as well as MMP-9 are regulated by AP-1, and c-Jun is a suppresser of p53 in immortalized fibroblasts. Tombulturk et al. reported that c-Jun, MMP-2, as well as MMP-9 are up-regulated in diabetic rats during wound healing process concomitant with the increase of p53 (20). Moreover, several up-stream signals have been considered as important targets for ERK1/2-mediated MMPs regulation in diabetic ulcer. CXCL16-CXCR6 axis promotes the diabetic wound healing in diabetic mice and *db/db* mice through improving MSC migration by targeting the expression of MMP-2 *via* FAK-Src-ERK1/2-MMP2 signaling pathway. Dhoke et al. observed that CXCR6 gene therapy facilitates the wound healing in mice with T1DM or T2DM through accelerating the re-epithelialization and neovascularization. Meanwhile, CXCR6 overexpression up-regulates the expression of MMP-2 through increasing the levels of FAK, Src and ERK1/2 *in vitro* experiment. Interestingly, the inhibitor of CXCL16 or the lack of CXCR6 gene attenuates wound repair through inhibiting the MSC migration and the increase of MMP-2, which suggests that CXCL16-CXCR6 axis play a critical role in diabetic wound recovery (45). In a nutshell, these findings reveal that interfering with ERK1/2-centered signaling pathways is paramount for regulating the expression of MMPs in diabetic wound healing.

Nuclear factor erythroid 2-related factor 2 (Nrf-2) regulates the adaptive response to exogenous and endogenous oxidative stresses. Previous studies have shown that severe oxidative stress can be observed in the wound tissue of DM patients, as revealed by activation of Nrf-2 as well as the downstream genes such as HO-1 and NQO1. Long et al. reported that $Nrf2^{-/-}$ diabetic mice exhibits poor wound healing, which is due to oxidative DNA damage, up-regulation of MMP-9, and decrees of transforming growth factor (TGF)-β1. Nevertheless, Nrf-2 activation contributes to wound healing in HaCaT cells, which is conferred through elevating TGF-β1 and suppressing MMP-9 (46). Thus, it suggests that targeting NRf-2/MMP-9 is a promising axis for treating diabetic wound healing. Furthermore, elevated secretion of ROS in diabetic wounds is regarded as a hazardous factor that may contribute to delayed wound healing under the diabetic condition through prolonging infiltration of M1 macrophages and impairing dermal fibroblast and keratinocyte function (47). Seraphim et al. found that higher M1/M2 macrophage ratio and basal ROS levels, as well as decreased antioxidant defenses and angiogenesis are observed in Rag-2 and IL-2Rγ double knockout (KO) diabetic mice that lack T, B, as well as innate lymphoid cells cell function. However, the increased expression of MMP-9 in diabetic conditions is not observed in KO mice, which demonstrated that lymphocyte may mediate the up-regulation of MMP-9 in diabetic wounds to a certain degree (48). However, such mechanism is not clear at present, and further experiments are needed to verify how lymphocyte affects the expression of MMP-9 under diabetic condition.

2.4 Apoptosis-related signals and MMPs

Apoptosis has been illustrated to plays a pivotal part in diabetic ulcer, and recent studies reported that MMP-9 contributes to delayed wound healing under diabetic condition through regulating fibroblasts apoptosis, while TIMP-1 is demonstrated to promote cells growth and prevent apoptosis. Down-regulated expression of TIMP-1 can be observed in diabetic skin tissues as well as in AGEs-intervened fibroblasts, whereas active protein of TIMP-1 prevents apoptosis triggered by AGEs or DM (49). MMP-9 is significantly up-regulated in HG and hyper-homocysteine medium-treated skin fibroblasts, which results in the decrease of cell proliferation, viability, COL secretion, and migration. However, these inhibitory effects of MMP-9 on fibroblasts are abrogated by TIMP-1 (50). Moreover, AGEs-BSA suppresses the migration of keratinocyte through increasing MMP-9 expression, while reducing TIMP-1 levels. Besides, AGEs-BSA application also down-regulates the expression of phospho-focal adhesion kinase- Tyr397 (p-FAK) as well as α2β1 in keratinocytes (51), suggesting that in the context

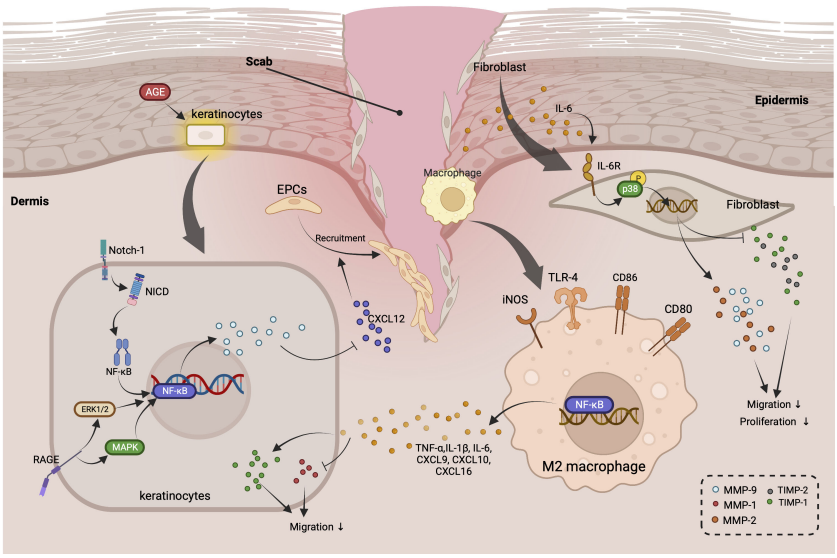

FIGURE 1
Roles of MMPs in regulating inflammation-related cross-talks among keratinocytes, macrophage, and fibroblast during diabetic wound healing. (AGEs stimulates the activation of Notch-1/NICD/NF-κB, RAGE/ERK1/2, and MAPK signaling pathways in keratinocytes, which gives rise to the up-regulation of MMP-9. MMP-9 released by keratinocytes suppresses the recruitment of EPCs through down-regulating the expression of CXCL-12. In addition, TNF-α, IL-6, and other pro-inflammatory cytokines secretes by M2 macrophage elevate TIMP-1 and reduce of MMP-1, resulting in the suppressed migration of keratinocytes. Intriguingly, IL-6 also inhibits the migration as well as proliferation of fibroblast *via* promoting the expression of MMP-2 and MMP-9, while decreasing the levels of TIMP-2 through activating IL-6R/p38 pathway.).

of the chronic hyperglycemia, the influences of AGEs-BSA on keratinocyte are conferred through regulating MMP-9/TIMP-1, p-FAK, and α2β1 integrin. Besides, Yang et al. observed that MMP-9 and TIMP-1 levels in diabetic rats changed dynamically with the alteration of wound. Specifically, the mRNA and protein levels of MMP-9 are obviously elevated in DM rats compared with control group, which reach the peak on day 3. On contrary, the mRNA and protein levels of TIMP-1 are obviously lowered, leading to the increase of MMP-9/TIMP-1 ratio. Intriguingly, changes in MMP-9 and TIMP-1 levels occur long before the skin is traumatized, suggesting the presence of hidden damage to diabetic skin that may cause ulcers (52). Thus, it suggests that targeting MMP-9/TIMP-1 axis is a promising strategy to alleviate apoptosis during diabetic wound healing.

Additionally, a recent study manifested that FasL/Fas signal is also implicated in the regulation of MMP in diabetic wound ulcer. Elevated MMP-9 in AGEs-treated keratinocytes promotes the apoptosis of keratin-forming cells through up-regulating the expression levels of cleaved caspases-3 as well as FasL, which suggests that MMP-9 may exert pro-apoptotic effect to suppress diabetic wound healing *via* a FasL/Fas-mediated pathway (53). Thus, targeting MMP-9/FasL/Fas axis may be a feasible strategy for diabetic wound healing therapy.

2.5 Non-coding RNAs and MMPs

Epigenetic modification especially the regulation of non-coding RNAs like microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circ_ RNAs), as well as small hairpin RNA (shRNA) on the secretion and expression of MMPs, has been considered as another integral mechanism associated with the pathogenesis of diabetic ulcer. Wang et al. observed that excessive miR-129 and miR-335 down-regulate the expression of MMP-9 *via* directly targeting Sp1 in AGEs-treated HaCaT cells. *In vivo* studies illustrated that miR-129 and miR-335 agomir accelerates wound healing through improving re-epithelialization and COL deposition by decreasing Sp1 and MMP-9 in diabetic rats, which suggest that miR-129 and miR-335 regulate MMP-9 levels *via* Sp1-mediated axis (54). In addition, miR-21 is demonstrated to promote wound healing in DM rats through improving fibroblast functions *via* targeting MMPs. Human keratinocyte-derived microvesicle miRNA-21 increases the migration as well as fibroblast-mediated angiogenesis, and accelerates diabetic cutaneous wound healing in rats through up-regulating IL-6, IL-8, MMP-1, as well as MMP-3, down-regulating TIMP-3 and TIMP-4, suppressing the expression of PTEN and RECK, and activating MAPK/ERK signaling pathway (55). Besides, miR-217 also participates in diabetic wound healing *via* modulating hypoxia inducible factor-1α (HIF-1α)/VEGF pathway and the down-stream proteins such as MMP-2 and MMP-9. The serum levels of miR-217 are enhanced in DFU individuals and rats, which causes the down-regulation of VEGF by repressing the target gene HIF-1α. Noticeable, inhibition of miR-217 reduces foot ulcer area, improves ulcer healing, and elevates the micro-vessel density through suppressing the levels of inflammatory factors, while up-regulating MMP-2, MMP-9, VEGF, VEGFR-2, and eNOS in DFU rats (56).

Moreover, ten-eleven translocation-2 (TET2)-interacting lncRNA (TETILA) can facilitate active DNA demethylation of the

MMP-9 promoter in wound healing under diabetic condition. TETILA is obviously elevated in HaCaT cells and diabetic skin tissues induced by AGEs, which enhances the protein levels of TET2 as well as its nuclear translocation, thus activating MMP-9 promoter demethylation. Besides, as a molecular scaffold, TETILA provides a binding surface for the assemble of TET2 and thymine-DNA glycosylase (TDG), contributing to the base excision repair-mediated MMP-9 promoter demethylation and the transcriptional activation of MMP-9 (19). Thus, it suggests that TETILA may function as a genomic homing signal for TET2-mediated demethylation specific loci in MMP-9 promoter, which ultimately disrupts the progress of wound healing in DM. Additionally, Circ_PRKDC is illustrated to hamper wound healing in DFUs by modulating the proliferation and migration of keratinocyte. Circ_PRKDC overexpression down-regulates MMP-2 and MMP-9 in human epidermal keratinocytes through targeting miR-31/FBN1 axis, which results in the suppression of cell migration (57). Notably, the slow wound healing of diabetic cornea is related to MMP-10 overexpression. Studies show that recombinant adenovire-driven shRNA promotes wound healing in diabetic corneas by inhibiting MMP-10 and cathepsin F, which activates the phosphorylation of epidermal growth factor receptor (EGFR) and Akt. Also, the combination of shRNA and c-Met overexpression can activate p38 and thus the downstream EGFR-Akt pathway, showing a more significant wound healing effect (58), suggesting that targeting MMP-10/EGFR/Akt is a promising axis for the treatment of diabetic keratopathy. Taken together, these studies manifest that targeting non-coding RNAs-mediated signaling pathways is a feasible option to modulate the expression of MMPs in wound healing under the diabetic condition.

2.6 FOXO-1 and MMPs

Recently, increasing evidences illustrated that forkhead box protein O1(FOXO1) is a critical regulator in wound healing, whose up-regulation may lead to the deterioration of diabetic ulcer. $Foxo1^{L/L}$ diabetic mice exhibits better wound healing, which is related to down-regulation of MMP-9 and decrees of FOXO1. Remarkably, elevation of FOXO1 in HG-treated keratinocytes enhances the transcriptional activity as well as expression of MMP-9 through binding to its promoter, whereas FOXO1 depletion prevents HG-induced keratinocyte migration, through up-regulating the expression of the TIMP1 while inhibiting the expression of MMP-9 (59). Thus, it suggests that targeting FOXO1/MMP-9 axis is a possible way for the treatment of diabetic wound healing.

2.7 uPA/uPAR and MMPs

Recent studies found that corneal wounds of diabetic mice heals more slowly than those of normal mice, which may be related to the inhibition of Serpine1(PAI-1), uPA, and uPA receptor (uPAR) expression by hyperglycemia. Interestingly, epithelial wound healing is accelerated by the addition of Serpine1 to the corneal conjunctiva of diabetic mice. Further experiments show that increasing Serpine1 up-regrates the expression of Plau, Plaur and MMP-3 in the cornea of DM mice (60), which indicates that targeting uPA proteolytic pathway is a promising option for regulating MMP-3 in treating diabetic keratopathy. Transcription factor homeobox A3 (HOXA3) plays a principal part in wound repair and angiogenesis, which is increased during wound healing and leads to the elevation of endothelial cell migration, promotion of angiogenesis, and up-regulation the levels of MMP-14 as well as uPAR in endothelial cells. Nevertheless, the expression of HOXA3 is blocked in the wounds of diabetic mice, contributing to the delayed wound repair and angiogenesis. Exogenous HOXA3 application reverses these adverse phenomena in diabetic mice, and facilitates migration of endothelial cells and keratinocytes *via* a uPAR-dependent mechanism (61). In berif, targeting uPA/uPAR is a possible way to modulate MMPs expression in diabetic wound healing.

2.8 DNA methylation and MMPs

Site-specific DNA demethylation of the MMP-9 promoter is demonstrated to be a paramount mechanism for MMP-9 regulation during diabetic wound healing. Ling et al. reported that TNF-α intervention augments MMP-9 expression and decrease the demethylation trend at the -36 bp promoter site in HaCaT cells. Besides, the alteration at the -36 bp site is the most significant among the CpG sites that distinctively demethylated in the MMP-9 promoter region, and higher transcriptional activity can be detected in the promoter with only the -36 bp site demethylated, which suggests that the -36 bp site is required in MMP-9 expression, while other CpG sites might play synergistic effects in TNF-α-stimulated keratinocytes (62). In addition, the activation of MMP-9 in AGEs-BAS-induced HaCaT cells is accompanied by the elevation of RhoA, GTP-RhoA and ROCK1, suggesting that mevalonate pathway participates in the expression of MMP-9 in AGEs-treated HaCaT keratinocytes. Moreover, AGEs-BSA stimulation promotes the activation of ERK1/2 and RAS through mevalonate pathway, thereby demethylating the -562bp site of MMP-9 promoter and upregulating MMP-9 level. Interestingly, the HMG-CoA reductase inhibitor simvastatin blocks demethylation at the -562bp site (63). Furter studies illustrated that TET2, a DNA demethylation enzyme, is elevated in AGEs-BSA-stimulated human primary keratinocytes, while the methylation of the MMP-9 promoter is decreased. TET2 can directly bind to a segment around the transcriptional start site in the MMP-9 promoter domain and regulate its expression, thus affecting the migration and proliferation of skin keratinocytes (64). Therefore, it suggests that the MMP-9 promoter DNA demethylation would pass though the mevalonate pathway to TET-2. Interestingly, another study proved that GADD45a plays an important role in demethylation of the MMP-9 promoter, which is augmented in diabetic wound and AGEs-induced HaCaT cell.

Nevertheless, GADD45a knockout suppresses AGEs-induced increase of MMP-9 and demethylation of the MMP-9 promoter, and enhances the HaCaT cells migration without influencing the apoptosis and proliferation of HaCaT cells, whereas overexpression of GADD45a improves the MMP-9 promoter demethylation. Remarkably, HG induces the binding of GADD45a to MMP-9 promoter and promotes GADD45a to thymine-DNA glycosylase recruitment for base excision repair-mediated demethylation (65). Altogether, these findings reveal that targeting the site-specific demethylation of MMP-9 promoter through interfering with mevalonate pathway, ERK1/2 and RAS signals, TET-2, as well as GADD45a is a promising therapeutic strategy to modulate the aberrant expression of MMP-9 during diabetic ulcer.

2.9 Endogenous substance and MMPs

Substance P (SP) is a type of neuropeptide consisting of 10-amino acid, which has been demonstrated to accelerate the skin wound repair under diabetic condition. Compared with non-diabetic acute wounds, impaired re-epithelization, decreased formation of granulation tissue, and suppressed re-vascularization can be observed in diabetic wounds in mice, accompanied by the reduction of SP, up-regulation of MMP-9, as well as elevation of cytokines associated with inflammation in wound fluids (66). SP facilities the wound healing in DM rats through relieving inflammation, promoting fibroblast proliferation, promoting COL deposition and improving angiogenesis, which up-regulates the expressions of IL-10 and HO-1, down-regulates TNF-α, IL-1β, as well as MMP-9, and enhances neovascularization *via* elevating VEGF, TGF-β1, and eNOS (67). In short, these findings indicate that SP may possess great potential in treating diabetic cutaneous wounds.

Leucine-rich α-2-glycoprotein-1 (LRG1), as a crucial factor that participates in angiogenesis as well as cutaneous wound repair, has been proved to be down-regulated in the corneal epithelium of DM mice and in HG-treated TKE2 cells. Nevertheless, exogenous administration of LRG1 improves corneal re-epithelialization, nerve regeneration, and wound healing through up-regulating MMP-3 and MMP-13, which is accompanied by the activation of JAK2/STAT3, AKT, EGFR as well as TGF-β3 signaling. Remarkably, these protective effects of LRG1 are abrogated by MMP-3 and MMP-13 inhibitors, indicating that LRG1 accelerates wound repair in diabetic corneal epithelium *via* modulating MMPs (68). Thus, targeting LRG1/MMPs axis is a promising strategy for wound healing in diabetic keratopathy.

Angiotensin II is a type of fibrogenic factor that modulates COL metabolism and capillary formation of skin cells by regulating Ang II type 1 (AT1) and AT2 receptors. Ren et al. reported that Angiotensin II up-regulates the expression levels of TIMP-1, TGF-β, COL I, and COL III in diabetic skin fibroblasts without influencing the expression of MMP-1, thus leading to the imbalance of MMP-1 and TIMP-1, as well as the improvement of COL synthesis. However, losartan, an AT1 receptor blocker, suppresses the effects of Angiotensin II (69), which demonstrates that targeting Angiotensin II/AT1 receptor and TGF-β-associated axis may contribute to the balance of MMP-1 and TIMP-1 in diabetic skin.

Cytochrome P450 (CYP) epoxygenases play a critical part in diabetic wound healing, which catalyzes arachidonic acid to produce epoxyeicosatrienoic acids (EETs). Zhao et al. found that CYP2C65 and CYP2J6 are obviously decreased in the granulation tissues in *ob/ob* mice, leading to the down-regulation of EETs, the aggravation of inflammation, and the inhibition of angiogenesis. However, exogenous EETs administration down-regulates the levels of TNF-α, IL-6, as well as IL-1β, the expressions of MMP-9, and the infiltration of neutrophil and macrophage, resulting in the improvement of wound healing, angiogenesis and COL deposition (70). Therefore, these findings reveal that targeting CYP epoxygenases-mediated MMP-9 expression is a potential option for diabetic wound healing.

Aldose reductase is the first enzyme present in the polyol pathway, whose inhibition plays a paramount part in the progression of diabetic keratopathy in humans. Aldose reductase inhibitor treatment facilitates the corneal wound healing in galactose-induced diabetic rats by inhibiting sorbitol accumulation, down-regulating the gene and protein expressions of MMP-10, and up-regulating the protein expression of integrin α3. Notably, topical treatment with the recombinant MMP-10 impairs the wound healing in DM rats (71), which reveal that targeting MMP-10 is a promising option to improve wound healing in diabetic retinopathy.

In addition to TIMPs, neutrophil gelatinase-associated lipocalin (NGAL) is another significant regulator of MMP-9, which forms a complex with MMP-9, stabilizing it and preventing its degradation. NGAL/MMP-9 complex attenuates the diabetic wound healing in DM rats by facilitating the inflammation *via* up-regulating MMP-9 expression. Abdollahi et al. observed that the wound healing rate is decreased, whereas the number of neutrophils in tissue and circulating, as well as the expression of NGAL, MMP-8, as well as MMP-9 are elevated in diabetic group. However, insulin reverses these phenomena induced by HG. Besides, insulin also down-regulates the pro-inflammation factors TLR4, TLR2 and TNF-α in diabetic skin wound granulation tissue (72). Thus, these findings suggest that targeting NGAL/MMP-9 complex may be a potential therapy for diabetic wound repair.

3 Clinical research studies of MMPs in regulating diabetic ulcer

High levels of MMP-9 in serum, wound fluid, as well as skin tissue of diabetic individuals is a signal that may indicate the poor healing process and connected with failed dermal grafting of DFUs (73). Jindatanmanusan et al. reported that MMP-9 contents in wound fluid from poor healers are dynamic and obviously higher than those of good healers, while the MMP-9 remained at a lower level throughout the treatment period in the good healer group (74). Notably, the original MMP-9 level at week 0 proved to be a

predictor of good/poor healing during the 12-week follow-up. Besides, compared with patients with non-healing DFUs, the levels of pro-MMP-9 and active-MMP-9 in wound fluid of patients with healing DFUs were significantly reduced, while TIMP-1 and TGF-β1 were significantly increased (75). These results suggest that the elevated MMP-9/TIMP-1 ratio affects the healing of DFUs. Interestingly, single nucleotide polymorphism (SNP -1562C>T) (rs3918242) in the promoter region of MMP-9 gene that modifies the transcriptional activity of MMP-9 is relevant to the development of DFUs. Singh et al. reported that Increased frequency and expression of T allele of SNP -1562C>T in MMP-9 gene are related to up-regulation of MMP-9 in wound fluids of T2DM patients, which results in degradation of matrix and the development of chronic wound (76). Trøstrup et al. found that there are no significant differences between MMP-9 levels in the wound fluid from patients with venous leg ulcers (VLUs) and patients with DFUs, but are both higher than that of healing wounds. Notably, serum levels of MMP-9 in patients with DFUs are higher than that of patients with VLUs (77).

Furthermore, increased ratio of serum MMP-9/TIMP-1 has been proved to predict poor wound healing in DFUs. The level of MMP-9 in the serum of good healers is lower than poor healers at first visit, and it reduces about 5-fold after 4-week therapy, while the serum level of MMP-9 in the poor healer shows little change. Remarkably, the MMP-9/TIMP-1 ratio can better reflect the healing before therapy and after 4-week therapy compared with MMP-9 (78). Dinh et al. reported that higher levels of TNF-α, monocyte chemoattractant protein-1 (MCP-1), MMP-9, as well as fibroblast growth factor (FGF)-2 can be detected in the serum of individuals whose ulcers are unable to heal. In addition, the results of skin biopsy analysis demonstrated that diabetic individuals have elevated immune cell infiltration, as well as increased MMP-9 expression, which adversely modulates the signals related to insulin, leptin, as well as growth factors (79). Moreover, MMP-9 is up-regulated in the skin tissue of diabetic wounds with bacterial infection compared with nondiabetic patients with wounds, while that of TIMP-1 as well as VEGF is down-regulated, which indicate that an excessively high ratio of MMP-9/TIMP-1 contributes to delayed wound healing in infected DFUs through reducing VEGF levels (80). The activity of MMP-9 as well as A Disintegrin and A MetalloProtease Domain 17 (ADAM17)/TNF-Alpha Converting Enzyme (TACE) is proved to be enhanced in ischemic diabetic wound biopsies compared with neuropathic biopsies, while the mRNA levels of MMP-9 and ADAM17/TACE are comparable between the two groups. Importantly, TIMP-3 is significantly lower in ischemic samples, which indicates that increased protein hydrolysis milieu may be a trigger for diabetic ulcer development (81).

In addition to MMP-9, MMPs contains MMP-1, MMP-2, MMP-3, as well as MMP-8 are illustrated to play indispensable roles in individuals with diabetic ulcer. The expressions of MMP-1, MMP-9, and TIMP-1 in diabetic individuals are in dynamic change during the wound healing process. The initial levels of MMP-1 as well as the MMP-1/TIMP-1 ratio are obviously higher in wound fluid from DFU patients with better wound healing, while that of MMP-9 is significantly lower in these patients. Besides, the MMP-1 level starts to elevate at week 4 in patients with better wound healing and is followed by a reduction at week 8, whereas that of MMP-1 is stable in patients with poor wound healing (82). Thus, MMP-9, MMP-1, as well as TIMP-1 may be useful biomarkers for DFU therapy at the first patient visit. Min et al. reported that faster wound closure rate is relevant to lower plasma MMP-2 and MMP-9 at week-4 and week-8 visits. In addition, the percentage of $CD16^{++}$ monocytes is negatively correlate with plasma MMP-2 and pro-MMP-9, but is positively related to the percentage of CD163 monocytes. Remarkably, MMP-9 and percentage of $CCR2^{+}$ are significantly decreased, while non-classical percentage of $CD16^{++}$ and MMP-3 are increased in the DFUs healing group after 8 weeks compared with the DFUs non-healing group (83). Therefore, MMPs and non-classical percentage of $CD16^{++}$ may be biomarkers for detecting the degree of healing of DFUs. Besides, Kupczyk et al. found that the serum levels of MMP-2 and MMP-3 in diabetic individuals with ulcer are obviously higher than those in the control group, which may serve for the delayed healing of chronic wounds and the aggravation of vascular complications (84). Another study illustrated that MMP-9 levels in the wounds of diabetic individuals are parallel to NF-κB p65. When skin injury occurs, a mass of neutrophils will be mobilized to the site of injury, which release cytokines like MMP-8, MMP-9, and ROS to resist bacterial infection as well as modulate thrombus formation. Interestingly, excessive ROS activates NF-κB signal, which subsequently triggers the up-regulation of MMP-9 and eventually the delayed wound healing. On the contrary, MMP-8 contributes to the COL deposition and ECM formation (85). The changes of MMPs levels in individuals with diabetic ulcers are shown in Figure 2.

4 Regulation of MMPs in diabetic ulcer by natural products

4.1 Flavonoids

Luteolin (Figure 3) is a critical flavonoid extracted from numerous of plants, such as leaves of M. annua Linn., which is reported to reduce blood glucose levels, enhance cutaneous wound healing process, and accelerate skin wounds re-epithelization in diabetic rats through attenuating inflammation and oxidative stress. Luteolin represses the infiltration of inflammatory cell, reduces the levels of TNF-α, IL-6, IL1-β and MMP-9 *via* down-regulating the NF-κB signaling pathway. Meanwhile, luteolin brings down the expressions of SOD1 and glutathione peroxidase (GSH-Px), as well as p-Nrf2 to modulate oxidative stress (86). Therefore, these phenomena indicate that luteolin may be a possible agent to treat diabetic wound injury by targeting NF-κB/MMP-9 axis and Nrf2-meidated anti-oxidant system.

Myricetin (Figure 3), a bioflavonoid widely presents in a variety of plants, tea, fruits as well as vegetables, is demonstrated to exert numerous biological activities, especially preventing cellular oxidative stress through regulating antioxidant enzymes. Recent

study found that myricetin intervention obviously mitigates the damages on dermal fibroblasts stimulated by DM through modulating MMPs. Myricetin inhibits the formation of MMP-1, MMP-2, and MMP-9 in diabetic fibroblasts, and suppresses catalase (CAT) and SOD. In contrast, myricetin increases the mRNA expression of TIMP-1, resulting in the increase of the ratio of TIMP1/MMPs in diabetic fibroblasts. Additionally, pro-COL I and III levels in diabetic fibroblasts are enhanced after myricetin intervention, which is conducive to diabetic wound repair (26). All in all, myricetin can relive DM-induced damages on dermal fibroblasts, which implies that myricetin may function as a drug candidate to accelerate wound healing under in patients with DM.

Quercetin (Figure 3) is a naturally occurring flavonoid compound with a variety of bioactivities, covering anti-ulcer, anti-inflammation, and cardiovascular protection. Recently, several studies reveal that quercetin displays promising effect on diabetic ulcer (87), which accelerates the wounds closure and reduces wound diameter through down-regulating the pro-inflammatory cytokines and enzymes, including TNF-α, IL-1β, as well as MMP-9, while up-regulating IL-10, VEGF, and TGF-β. In addition, topical application of quercetin improves the formation of thick granulation tissue with more new blood vessels, accelerates re-epithelialization and fibroblast conversion into the phenotypic of myofibroblast, promotes the COL synthesis, and deposition and orientation in wounds of diabetic rats (88). Thus, its suggests that quercetin exhibits great potential to mitigate diabetic ulcer.

Rutin (quercetin-3-O-rutoside, Figure 3) is a common flavonoid that can be found in the seeds, stems, leaves, as well as flowers of buckwheat, which improves wound healing in hyperglycemic rats *via* preventing oxidative stress and inflammatory response. Specifically, rutin intervention obviously relieves the body weight loss and metabolic dysfunctions in rats induced by DM, decreases the number of inflammatory cells, promotes the activity of Nrf-2 as well as the expression of related antioxidant enzymes such as SOD1 and GSH-Px, down-regulates the levels of TGF-β1, MMP-2, MMP-9, NF-κB, IL-1β, IL-6, TNF-α, and VEGF, and elevates the expression of neurogenic-related protein (89). Thus, these results reveal that targeting NF-κB-mediated MMPs axis is one of the crucial mechanisms of rutin in regulating diabetic wound healing, and rutin could function as a potential drug for DFUs.

Vicenin-2 (VCN-2, Figure 3) is a flavonoid glycoside separated from numerous natural plants, which attenuates oxidant stress and inflammatory, and improves epithelialization as well as cell remodeling. More recently, studies illustrated that VCN-2 intervention facilities wound healing in STZ-induced DM rats through improving cells proliferation as well as reducing the inflammatory cells, which down-regulates the expressions of pro-inflammatory cytokines *via* NF-κB signal pathway. Meanwhile, VCN-2 augments the number of fibroblast cells and neoangiogenesis *via* down-regulating the levels of MMP-9 and anti-HIF-1α *via* VEGF and TGF-1β signal pathway. Compared with diabetic group, VCN-2 treatment decreases the levels of blood glucose, reduces food and fluid intakes, while increases insulin levels, body weight, as well as the percentage of wound closure (90). Altogether, it suggests that VCN-2 may be an available agent for diabetic wound repair *via* modulating NF-κB, VEGF, and TGF-1β signal pathways.

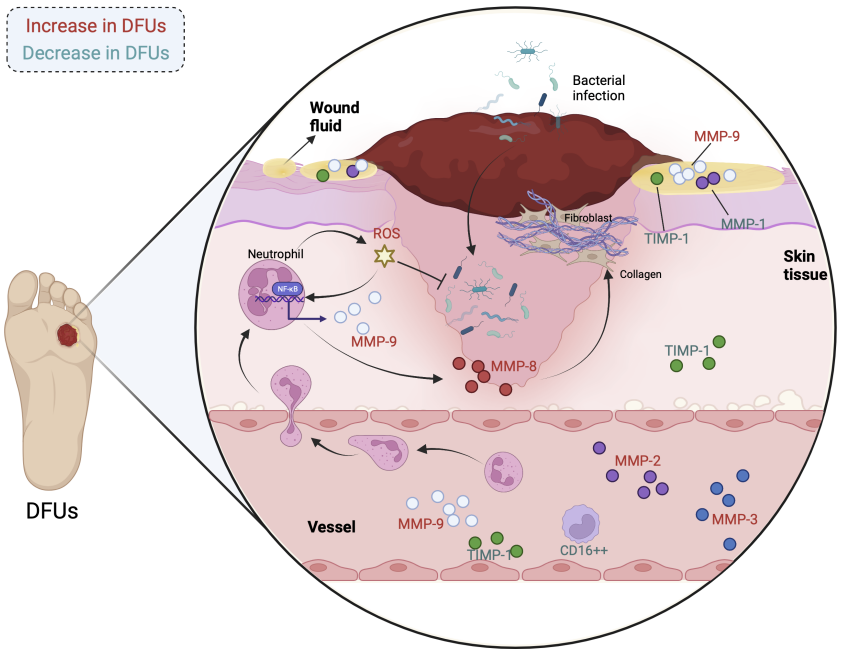

FIGURE 2
The changes of MMPs levels in individuals with diabetic ulcers. (MMPs are influential regulators in DFUs, whose expressions are diverse in the wound fluid, skin tissue, and blood of patients. The correlations have been observed between MMPs and physiological as well as pathological processes of DFUs, involving COL deposition, inflammatory response, oxidative stress, and bacterial infection. The MMPs in red are increased in individuals with DFUs, while the MMPs in green are decreased in individuals with DFUs.).

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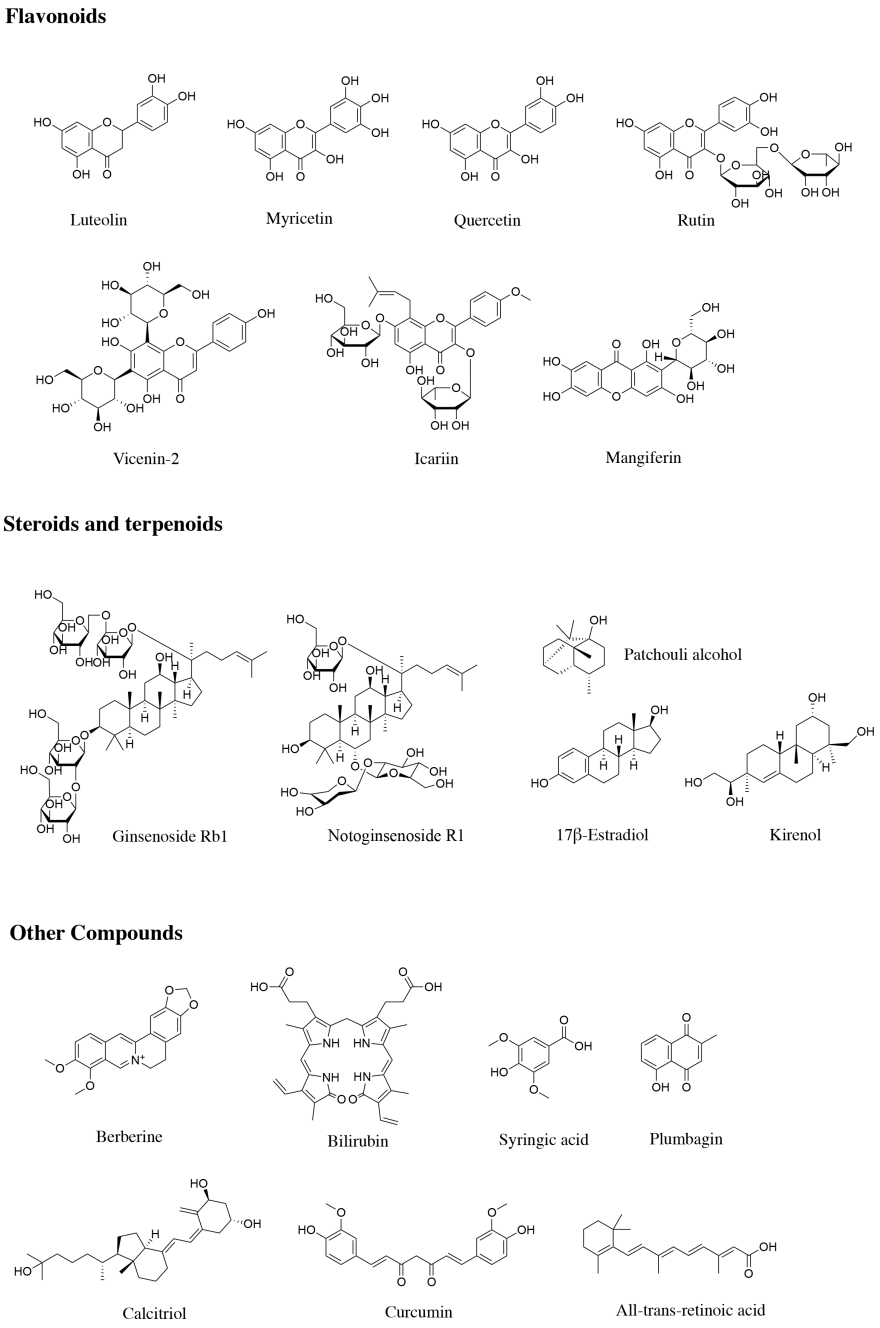

FIGURE 3
Structures of natural products regulating MMPs in diabetic wound healing.

Icariin (Figure 3) is a pivotal flavonoid derived from *Epimedium* genus with various bioactivities, including anti-cancer, anti-inflammation, and pro-angiogenesis. Icariin administration improves wound repair in diabetic rats through down-regulating the protein expressions of NF-κB, TNF-α, MMP-2, as well as MMP-9, elevating the levels of IL-10, up-regulating the expression of CD31, and increasing the relative COL deposition in the healing tissue. In short, icariin contributes to the progression of diabetic wound healing *via* alleviating inflammation, improving angiogenesis, and promoting normal ECM formation as well as remodeling in the healing tissue (27), which suggest that icariin may serve as a promising agent for diabetic ulcer.

Mangiferin (Figure 3) a well-known naturally occurring polyphenol widely distributed in various plant species, is demonstrated to exert numerous bioactivities, particularly preventing cancer and DM. Lwin et al. reported that mangiferin application attenuates the ROS-induced oxidative stress, lessens the wound area, and increases the skin thickness of around the wound. In addition, mangiferin elevates EGF, FGF, TGF-β, VEGF, PI3K, and Nrf-2 protein expression in diabetic wound, while reduces the

expression of MMP-2, TNF-α and NF-κB p65, suggesting that mangiferin can shorten the inflammatory phase of wound tissue under hyperglycemia (91). Therefore, mangiferin is a potential agent for promoting wound repair in individuals with DM, and targeting MMP-2 is an underlying mechanism of mangiferin in treating diabetic ulcer.

4.2 Steroids and terpenoids

Ginsenoside Rb1 (G-Rb1, Figure 3), an active substance widely existed in *Panax ginseng*, has been demonstrated to possesses numerous pharmacological activities, including anti-inflammation, antioxidant, and antimicrobial effects. Recent studies revealed that G-Rb1 displays promising effect on diabetic chronic wounds healing *in vitro*, which significantly increases cell proliferation and COL synthesis. Specifically, G-Rb1 up-regulates VEGF, TGF-β1, TIMP-1 in cultured fibroblasts from patients with DFUs. Interestingly, TGF-β1 and TIMP-1 may increase COL synthesis, and VEGF improves the formation of thick granulation tissue with more new blood vessels in G-Rb1-treated diabetic (92). Taken together, it suggests that G-Rb1 is a candidate agent for the wound-healing activity of diabetic fibroblasts. Nevertheless, further *in vivo* studies are required to investigate the activity of G-Rb1 on diabetic would healing.

Notoginsenoside R1 (NR1, Figure 3), a dominating bioactive ingredient separated from Panax notoginseng, is demonstrated to facilitate wound healing in diabetic rats by obviously accelerating the wound closure rate, increasing ECM secretion, elevating COL growth, up-regulating the expression of CD31, and down-regulating the expression of cleaved caspase-3. More importantly, NR1 administration gives rise to the down-regulation of MMP-9, MMP-3, IL-1β, and IL-6, while up-regulation of TIMP1 as well as TGF-β1. The results of RNA-Seq technology illustrate that NR1 mainly influence ECM related processes and inflammation in diabetic wound healing by targeting TIMP-1 and MMP-3 (93). Thus, these phenomena indicate that NR1 may be a feasible candidate agent for diabetic ulcer *via* regulating MMP-mediated signaling pathway.

Patchouli alcohol (PA, Figure 3) is a bioactive ingredient separated from patchouli, with anti-inflammatory and anti-influenza effects. Recently, several studies reveal that PA administration improves wound healing in HFD-fed mice by up-regulating TGF-β1, MMP-2, MMP-9, COL1A1, down-regulating the levels of NF-κB, p-IκB, TNF-α as well as MCP-1, promoting adenosine monophosphate activated protein kinase (AMPK) phosphorylation and relieving inflammation. Simultaneously, PA intervention significantly reverses the decreased viability of LPS-induced HaCaT cells, impaired cell migration and proliferation, increases AMPK phosphorylation and activates TGF-β1 pathway in a dose-dependent manner. Notably, TGF-β1 siRNA blocks the effect of PA on LPS-induced HaCaT cells (94). Therefore, it suggests that PA is a candidate agent for obesity or insulin resistance, which facilitates diabetic wound healing through relieving inflammatory response.

17β-Estradiol (E2, Figure 3) is one of the most essential forms of estrogen, which is demonstrated to control the expressions of COL and MMP, and regulate cytokines, growth factors, as well as ECM turnover in diabetic wound healing. Recently, Pincus et al. investigates the regulatory effects of E2 in *db/db* mice, and they found that topical E2 treatment accelerates cutaneous wound healing by modulating the expressions of MMP. E2 can not only directly reduces the levels of MMP-13 and MMP-2, but also indirectly declines MMP-13 and MMP-2 *via* decreasing the MMP-14, pro-MMP-2. Besides, uterine weight and COL fibers deposition augmented in E2-treatment group compared with placebo treated mice. In addition, E2 treated up-regulates the expressions of estrogen receptor-α (ER-α) (95). Therefore, these findings illustrate that E2 may act as a therapeutic agent for diabetic wound injury by targeting ER-α-mediated signaling pathway.

Kirenol (Figure 3) is an important diterpenoid separated from the medicinal plant *Siegesbeckia orientalis*, which possesses various bioactivities, including anti-inflammation, anti-rheumatism, and wound healing. Kirenol administration reverses the up-regulation of angiogenesis-associated genes MMP-2 and MMP-9 in wounds of STZ-induced DM rats, decreases inflammation-related genes NF-κB, cyclooxygenase-2 (COX-2), as well as iNOS, reduces the contents of malonaldehyde (MDA), while increases the activities of antioxidant enzymes, which result in the alleviation of oxidative trauma. In addition, the results of histopathological examination demonstrate that kirenol intervention gives rise to the decline of inflammatory cell infiltration, elevation of fibroblasts, new blood vessels, as well as granulation tissue configuration (96). In short, these evidences illustrate that kirenol is promising for improving wound curing in diabetic ulcer.

4.3 Polysaccharide and glucoside

Hsian-tsao polysaccharides (WEP) are the major functional component in *Mesona procumbens* Hemsl., which has potent anti-oxidant and anti-inflammatory effects. Recently, several studies revealed that Hsian-tsao extracts (EE) and WEP displays promising effect on wound healing in diabetic, which decrease crust and improve the formation of thick granulation tissue with more new blood vessels, and re-epithelialization. Specifically, EE and WEP up-regulate IL-8, MIP-2, MCP-1, TIMP-1, as well as VEGF, down-regulate MMP-2 and MMP-9, and suppress MG-triggered protein glycation and ROS accumulation. Furthermore, both EE and WEP enhance the methylglyoxal (MG)-impeded phagocytosis of *Staphylococcus aureus* and *Pseudomonas aeruginosa* driven by macrophages, which maybe improve impaired wound healing. Interestingly, WEP is more effective on regulating the factors associated with diabetic wound repair than EE (97). Therefore, it suggests that EE and WEP are the candidate agent for chronic diabetic wounds.

Dendrobium Polysaccharides (PDC) is the main bioactive substance of Dendrobium candidum, which has anti-tumor and anti-aging effects. Recently, these studies revealed that PDC at the concentrations of 100, 200, as well as 400 μg/mL elevates the level of COL, improves the viability of human skin fibroblasts, suppresses the cell apoptosis induced by HG, accompanied by the elevation of TIMP-2 and reduction of MMP-2. Therefore, these results confirmed that PDC can display protective effects on diabetic ulcer *in vitro*, and the mechanism may be related to the modulation of TIMP-2 and MMP-2, which provides a new idea for the prevention and treatment of diabetic skin ulcer or wound (98). However, further *in vivo* researches are required to clarify the activity of PDC against diabetic wound healing.

4.4 Other compounds

Berberine (Figure 3) is a naturally occurring alkaloids separated from *Coptis chinensis* Franch. and has been demonstrated to possesses various pharmacological activities, including anti-microorganisms, anti-obesity, and improving insulin resistance (99). Recent studies found that berberine displays promising therapeutic effect on diabetic ulcer, which alleviates HG-induced HaCaT cell damage and enhances cell proliferation by activating thioredoxin reductase 1 (TrxR1)/c-Jun N-terminal kinase (JNK) pathway, and relieves oxidative stress and apoptosis through increasing GSH, SOD, and total antioxidant capacity (T-AOC), while down-regulating ROS, MDA, TUNEL-positive rate as well as caspase-3 activity. Notably, topical berberine application promotes the wound healing and elevates ECM synthesis in T2DM rats stimulated by HFD and STZ *via* decreasing MMP-9 and elevating TGF-β1 and TIMP-1 (28). Therefore, these findings illustrate that the wound healing effect of berberine against diabetic ulcer might be conferred through modulating TrxR1/MMP9 signaling pathway.

Bilirubin (Figure 3) is final metabolite of heme in mammals, which promotes wound healing by ameliorating oxidant stress, inflammation and angiogenesis. Recent study illustrated that bilirubin accelerates wound repair by facilitating COL fibers deposition, granulation tissue formation and contraction, induce neoangiogenesis and anti-inflammatory in STZ-induced diabetic rats. Bilirubin intervention alleviates inflammation through augmenting the expressions of IL-10 and decreasing IL-1β, which improves the angiogenesis and wound closure *via* up-regulating the levels of TGF-β1, HIF-1α, VEGF, IL-10 and SDF-1α and down-regulating TNF-α and MMP-9. Meanwhile, the results of histopathological assay indicate that re-epithelization of skin wound in bilirubin-treated group better than the control group (100). Thus, these results display bilirubin enhances skin wound healing in DM rats through balancing the levels of factors-associated with the process of wound closure.

Syringic acid (Figure 3), a critical phenolic compound synthesized *via* shikimic acid pathway in plants, is widely distributed in numerous edible plants like olives, pumpkin, and grapes. Syringic acid administration accelerates the wound closure rate and epithelization of diabetic wounds in rats, accompanied by the increase of hydroxyproline content and total protein levels. In addition, 14 days after syringic acid intervention, the inflammation and oxidative stress in diabetic wounds are alleviated, as evidenced by down-regulation of p65, IL-8, TNF-α, IL-2, IL-1β, MDA, and elevation of IL-10, Nrf-2, Keap1, as well as antioxidant enzyme activities. Intriguingly, syringic acid significantly reduces MMP-2, MMP-8, and MMP-9, up-regulates TIMP-1 and TIMP-2, elevates the contents of TGF-β1, COL I, α-SMA, CD31, CD68, as well as VEGF in diabetic wounds (29). Thus, its suggests that syringic acid exhibits great potential to mitigate diabetic ulcer.

Plumbagin (Figure 3), one of the bioactive constituents separated from the roots of *Plumbago zeylanica*, has emerged as a promising agent for diabetic wound healing. Plumbagin administration significantly promotes the wound closure as well as contraction of diabetic rats through accelerating epithelialization and the deposition of COL, promoting the secretion of insulin, improving the antioxidant status,and lowering lipid peroxides and lipid levels while elevating the HDL level Specifically plumbagin up-regulates the expression of Nrf-2, COL I, TGF-β as well as a-SMA down-regulates the expression of Keap1and rescues the decreased activities of the antioxidant enzymes in diabetic rats. Interestingly, plumbagin also increases EGF, VEGF and FGF, decreases MMP-2, COX-2, iNOS, CD8, CD163, as well as NF-κB p65, and suppresses IL-6 and IL-1β (101). Thus, alleviating inflammation and oxidation-induced injury is a possible mechanism of plumbagin in diabetic ulcer therapy.

Calcitriol (Figure 3) is the active form of vitamin D, which regulates the proliferation and differentiation of keratinocytes. Recent studies demonstrate that Calcitriol exerts promising protective effect on diabetic wound healing by targeting MMPs. The slower wound healing during DM is in connection with the up-regulation of MMP-1, MMP-9, and TIMP-1, as well as the down-regulation of MMP-8 and MMP-10 in wound tissue. Calcitriol intervention leads to the decrease of MMP-1 and MMP-10 levels, and contributes to wound healing in primary keratinocytes from the patients with DFUs (102). Therefore, Calcitriol may serve as a feasible modulator of MMP expression to accelerate wound healing in DM.

Curcumin (Figure 3) is a naturally occurring diketone compound principally extracted from the rhizomes of some plants in Zingiberaceae and Araceae, with potent anti-inflammatory and anti-cancer properties. Recently, several studies revealed that curcumin displays promising effect on diabetic ulcer, which accelerates the wounds closure through down-regulating the pro-inflammatory cytokines and enzymes, including TNF-α, IL-1β, and MMP-9, up-regulating IL-10 levels, and elevating the activities of SOD, CAT, as well as GSH-Px. Besides, topical administration of curcumin improves thick granulation tissue formation with more new blood vessels and fibroblasts, and promotes the COL synthesis, deposition and orientation in diabetic wounds (103). In addition, another study reported that combination of substance P (SP) and curcumin is a potential strategy for diabetic wound healing. SP is derived from the body and has the function of regulating angiogenic factors. Combination of SP and curcumin intervention promotes the

formation of thick granulation tissue, reduces wound diameter, increases fibroblasts, and accelerates COL synthesis, deposition, as well as orientation in wounds of diabetic rats. In addition, combination of SP and curcumin facilitates new blood vessels formation through the levels of VEGF, TGF-β1, HIF-1α, SDF-1α, HO-1 as well as eNOS. Simultaneously, after combination of SP and curcumin administration, the inflammation and oxidative stress in diabetic wounds are alleviated, as evidenced by the down-regulation of TNF-α, IL-1β and MMP-9, and the elevation of IL-10, SOD, GSH-Px, growth associated protein-43 (GAP-43) and CAT activities (104). Therefore, it suggests that curcumin is a candidate agent for diabetic ulcer, which promotes wound healing through relieving inflammatory response and oxidative stress.

All-*trans*-retinoic acid (RA, Figure 3), an intermediate product of vitamin A metabolism in animals, exerts a broad spectrum of bioactivities. RA can moderate the skin of chronological aging process by declining the levels of COL-degrading MMPs and augmenting the COL. Interestingly, RA treatment was established that accelerate the diabetic wound healing in organ culture through improving epidermal hyperplasia, elevating soluble COL and pro-COL production, as well as down-regulating the expressions of active MMP-9 and active MMP-1 and up-regulating TIMP-1. Meanwhile, Lateef et al. also reported the results of TIMP-1 inhibiting MMP function are similar to RA intervention. It is likely that RA attenuates the function of MMP *via* increasing the level of TIMP-1 (105). Taken together, although these findings indicate RA possibly be an agent to treat diabetic wound injury, further experiments are needed to clarify the specific mechanisms.

Relaxin, a peptide hormone with the molecular weight of 6 kDa, can improve wound healing under diabetic condition. Relaxin intervention elevates the mRNA and protein contents of VEGF in wounds from diabetic mice on postoperative day 3 and 6. Daily treatment of relaxin improves the levels of SDF1-α, accelerates healing process in the wounds of diabetic mice, shortens the time of complete wound closure through mediating VEGF and SDF1-α. Further studies demonstrated that treatment of relaxin markedly increases the level of microvessel density, augment levels of VEGFR-2, vascular endothelial cadherin, MMP-11, and enhances immunostaining of CD34 and VEGFR-1 in both non-diabetic and diabetic mice. More importantly, the results from clinical observation displayed that relaxin administration represents an alternative therapeutic regimen without any side effects (106). Thus, relaxin may possess a promising application in diabetic wound healing.

Exendin-4 is a polypeptide hormone isolated from the saliva of the *Heloderma suspectum*, which is proved to improve the transcription level of insulin gene, stimulate the release of insulin, and control blood glucose concentration. Recently, Exendin-4 was reported to possesses potent wound healing activity in DM, which facilities the wound healing in spontaneously diabetic ZDF rats *via* attenuating inflammation, promoting fibroblast/myofibroblast activities, and augmenting total COL content *via* decreasing the CRP concentration and the level of MMP-9, as well as elevating the level of TIMP-1. However, exendin-4 at the concentration of 100 nM suppresses fibroblast/myofibroblast metabolic activity and reduces COL production, which suggests that high exendin-4 doses display a cytotoxic effect (107). Another study showed that Glucagon-like peptide (Glp)-1 analogue exendin (Ex)-4 improves chronic gastric ulcer through suppressing inflammation and promoting angiogenesis in STZ-induced diabetic rats, which ameliorates the polymorphonuclear leukocytes (PMN) infiltration, up-regulates the levels of MCP-1, IL-10, eNOS, and cAMP, while down-regulates the levels of MMP-2, myeloperoxidase, superoxide anions, and IL-1β (108). Thus, these results demonstrate that exendin-4 is a potential therapeutic option for diabetic wound repair, but the safety doses need further investigation.

5 Conclusions and future directions

MMP family plays an indispensable role in numerous biological processes, involving tissue remodeling and growth, wound repair, tissue defense mechanisms, as well as immune responses. Under diabetic condition, tissues are trapped in inflammatory phase; continuous intensive stimulation of inflammatory cytokines leads to the dysregulation of MMPs, which subsequently degrades growth factors and matrix proteins necessary for wound repair, resulting in delayed wound healing. Notably, the expression of MMPs in diabetic ulcers is influenced by various internal and external factors, including DNA methylation, miRNAs, lncRNAs, AGEs, TIMPs, SP, LRG1, CYP, NGAL, etc. Additionally, signaling pathways such as Notch1/NF-κB, ERK1/2, p38, CXCL16-CXCR6, NRf-2, uPA/uPAR, FOXO1, as well as FasL/Fas are demonstrated to be concerned with the expression of MMPs during diabetic wound healing, which mainly improve the processes associated with inflammation, oxidative stress, apoptosis, angiogenesis, ECM formation, and re-epithelization. Furthermore, stem cells-mediated MMPs expression is another significant mechanism of diabetic wound healing (Figure 4). In short, these evidences indicate that developing agents targeting MMPs and the related signals or pathways has important implications for diabetic ulcers therapy.

Natural products are essential modifiable factors that affect human health and disease. In the last decade, more and more researchers have focused on bioactive natural ingredients to address the emergency situation of diabetic ulcers. Natural products exhibit a lot of beneficial effects in diabetic ulcers via targeting MMPs, such as alleviating inflammatory infiltration and oxidative stress of the wound, promoting angiogenesis, as well as enhancing granulation tissue configuration, ECM secretion, and COL growth (Table 1). Interestingly, natural products have been regarded as outstanding regulators that target miRNAs and DNA methylation in DM and related complications including diabetic ulcer. However, whether natural products mediate the expression of MMPs by regulating DNA methylation or non-coding RNAs and thus facilitate the diabetic wound repair process needs further investigation. In addition, these compounds possess different structures, but some of them are able to regulate the same MMPs or signaling pathways, suggesting that studying the structure-activity relationships of these compounds is important for elucidating the potential mechanisms by which they regulate MMPs to act as anti-diabetic ulcer agents. More importantly, not all natural products mentioned above are free from toxicity or side effects, a small percentage of them, such as

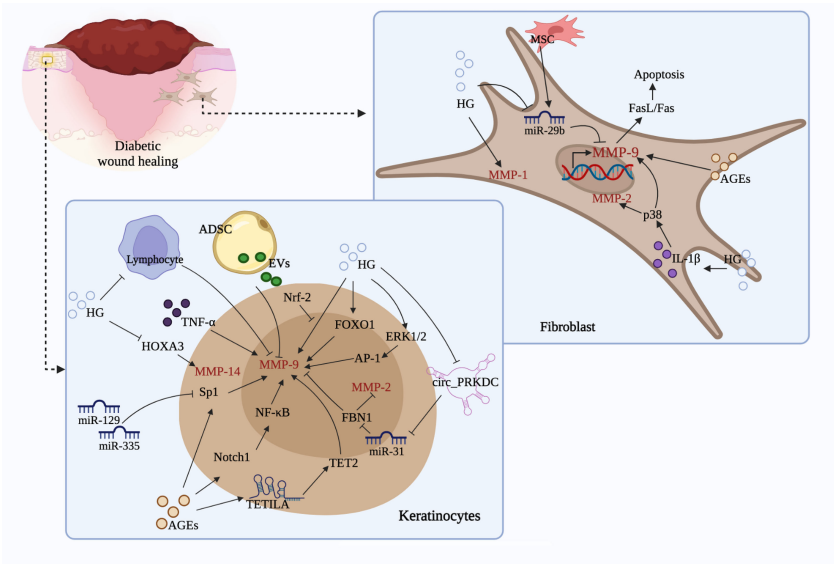

FIGURE 4
Regulation of MMPs by different cell types and related mechanisms. (Stimulated by HG and AGEs, keratinocytes and fibroblasts could produce a large amount of MMP-9, MMP-2, MMP-1 and MMP-14, which leads to decreased activity and migration, and ultimately causes impaired wound healing. During this process, signals such as miRNAs, lncRNAs, circRNAs, ERK1/2, FOXO1, HOXA3, Sp1, Notch, NF-κB, p38, FasL/Fas, and cells like ADSC, MSC, as well as lymphocyte are involved, which may promote or slow down the production of the above mentioned MMPs and thus affect diabetic wound healing.).

TABLE 1 Natural products in diabetic wound healing targeting MMPs.

Compound	Dosage	Administration route	Model/Cells	Targets	Ref
Luteolin	100 mg/kg (14 days)	Intraperitoneal.	STZ-induced diabetic rats with incision in the skin on the dorsal thorax	TNF-α↓, IL-6↓, IL1-β↓, **MMP-9↓**, NF-κB↓, SOD1↓, GSH-Px↓, Nrf2↓, VEGF↓, UCH-L1↑	(86)
Myricetin	3 μM	–	Fibroblasts from female T2DM patient	**MMP-9↓, MMP-2↓, MMP-1↓**, TIMP1↑,	(26)
Quercetin	0.3% quercetin ointment (21 days)	Topically.	STZ-induced diabetic rats with incision on the back	TNF-α↓, IL-1b↓, **MMP-9↓**, IL-10↑, VEGF↑, TGF-β1↑	(88)
Rutin	100 mg/kg	Intraperitoneal.	STZ-induced diabetic rats with incision on the back	Nrf2↑, SOD1↑, GPx↑, TGF-β1↓, **MMP-2↓**, **MMP-9↓**, NF-κB↓, IL-1β↓, IL-6↓, TNF-α↓, VEGF↓	(89)
Vicenin-2	12.5, 25, and 50 μM (14 days)	Topically.	STZ-induced diabetic rats with incision on the dorsal	IL-1β↓, IL-6↓, TNF-α↓, VEGF↑, TGF-1β↑, NO↓, iNOS↓, COX-2↓, NF-κB↓, **MMP-9↓**, anti-HIF1α↓	(90)
Icariin	0.04, 0.2, 1, and 5 ng/μg (14 days)	Topically.	STZ-induced diabetic rats with excisional wound on the back	IL-10↑, NF-κB↓, TNF-α↓, **MMP-2↓**, **MMP-9↓**	(27)
Mangiferin	1 and 2%	Topically.	STZ-induced type-2 diabetic male rat	EGF↑, FGF↑, TGF-β↑, PI3K↑, VEGF↑, TNF-α↓, Nrf2↑, **MMP-2↓**, NF-κBp65↓	(91)
Ginsenoside Rb1	10 ng/mL	–	Diabetic fibroblasts	VEGF↑, TGF-β1↑, TIMP-1↑	(92)
Notoginsenoside R1	0.038 mg/cm^2 (15 days)	Topically.	HFD/STZ-induced diabetes rats with incision on the dorsum	Caspase-3↓, ECM↑, CXCL1↑, FOS↑, TGF-β1↑, **MMP-9↓**, IL-1β↓, IL-6↓, **MMP3↓**, TIMP1↑	(93)
Patchouli alcohol	20 mg/kg	Intraperitoneal.	HFD-fed mice	TGFb1↑, **MMP-2↑**, **MMP-9↑**, COL1A1↑, p-AMPK↑, NF-κB↓, p-IκB↓, TNFα↓, MCP-1↓,	(94)
	0~30 mg/mL	–			

(Continued)

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TABLE 1 Continued

Compound	Dosage	Administration route	Model/Cells	Targets	Ref
			LPS-induced HaCaT cells		
17β-Estradiol	50 mg Estrogel 0.06% (7days)	Topically.	Female *db/db* mice with full-thickness wounds on the back	**MMP-13↓, MMP-2↓, MMP-14↓**, ER-α ↑	(95)
Kirenol	15% and 30% (14 days)	Topically.	STZ-induced diabetic rats with incision on the backside	NF-κB↓, COX-2↓, iNOS↓, **MMP-2↓, MMP-9↓**,	(96)
Hsian-tsao polysaccharides	100 μL	Topically.	STZ-induced diabetic mice	IL-8↑, MIP-2↑, MCP-1↑, TIMP-1↑, VEGF↑, **MMP-2↓, MMP-9↓,** ROS↓	(97)
	0~200 μg/mL	–	MG-induced RAW 264.7 cells and 3T3-L1 fibroblasts		
Dendrobium Polysaccharides	100, 200, and 400 μg/mL	–	HG-induced HSF cells	TIMP-2↑, **MMP-2↓**	(98)
Berberine	0.06 mg/ml (12 days)	Topically.	HFD/STZ-induced diabetes rats with incision on the dorsum	GSH↑, SOD↑, T-AOC↑, ROS↓, MDA↓, caspase-3↓, **MMP-9↓**, TGF-β1↑, TIMP1↑, ECM↑	(28)
	1.5625, 3.125, and 6.25 μM	–	HG-induced in HaCaT cells		
Bilirubin	0.3% bilirubin ointment (twice/ days)	Topically.	STZ-induced diabetic rats with incision on the dorsal thoracic region	HIF-1α↑, VEGF↑, SDF-1α↑, TGF-β1↑, IL-10↑, TNF-α↓, IL-1β↓, **MMP-9↓,** MVD↑	(100)
Syringic acid	2.5% and 5% (14 days)	Topically.	STZ-induced diabetic rats with incision on the dorsal midline	Nrf2↑, Keap 1↑, MDA↓, SOD↑, CAT↑, GPx↑, GST↑, GR↑, collagen-1↑, α-SMA ↑, TGF-β↑, NF-κB↓, p65↓, IL-1β↓, IL-8↓, **MMP-2↓**, **MMP-9↓**, TNF-α↓, TIMP-1↓, TIMP-2↓, **MMP-8↓**, VEGF↑, IL-2↓	(29)
Plumbagin	10% and 20%	Topically.	STZ-induced diabetic rats with full thickness wounds on the back	Nrf2↑, TGF-β↑, α-SMA↑, Keap1↓, SOD↑, CAT↑, GPx↑, GR↑, GST↑, EGF↑, VEGF↑, FGF↑, **MMP-2↓**, COX-2↓, iNOS↓, CD68↓, CD163↓, NF-κBp65↓, NF-α↓, IL-6↓, IL-1β↓	(101)
Calcitriol	0.001 μM	–	Primary epidermal keratinocyte from DFUs	**MMP-1↓**, **MMP-10↓**	(102)
Curcumin	400 μl of curcumin 0.15% (19 days)	Topically.	STZ-induced diabetic rats with full thickness excisional wounds	TNF-α↓, IL-1β↓, **MMP-9↓**, IL-10↑, VEGF↑, TGF-β1↑, HIF-1α↑, SDF-1α↑, HO-1↑, eNOS↑, SOD↑, GPx↑, GAP-43↑	(103, 104)
	400 μl of curcumin 0.3% (19 days)				
All-*trans*-retinoic acid	0.75 g/ml providing at 2- to 3-day intervals, incubating 9 days	–	2-mm punch biopsies were obtained from hip skin of 16 diabetic patients	active **MMP-9↓**, active **MMP-1↓**, TIMP-1↑	(105)
Relaxin	25 μg/d (12 days)	Subcutaneously.	*db/db* mice with incisional wound on the back	VEGF↑, SDF1-α↑, p-eNOS↑, VEGFR-1↑, VEGFR-2↑, VE-cadherin↑, **MMP-11↑**	(106)
Exendin-4	week1: 3 μg/kg; week2: 6 μg/kg; week3: 10 μg/kg	Intraperitoneal.	HFD-induced diabetic rats with subcutaneous implantation of foreign material	CRP concentrations↓, **MMP-9↓**, TIMP-1↑,	(107)
	0~100 nmol/l	–	Fibroblasts/ myofibroblasts obtained from rat wounds *in vivo* experiment		
		Intraperitoneal.		IL-10↑, eNOS↑, peNOS↑, **MMP-2↓**, cAMP↑	(108)

(Continued)

TABLE 1 Continued

Compound	Dosage	Administration route	Model/Cells	Targets	Ref
	0.5 μg/kg/d (7 days)		STZ-induced diabetic rats with Acetic acid-induced chronic peptic ulcer		

Arrow up denotes increase, arrow down denotes decrease.
The members of the MMPs family are written in bold text.

bilirubin, calcitriol, and all-*trans*-retinoic acid have been proved to cause damage to brain and liver or produce teratogenic effects in overdose (109–111). Thus, it suggests that the long-term toxicity of natural products needs further consideration due to the long healing time of diabetic wounds requiring prolonged administration.

Author contributions

JC, SQ, KZ, YJ, and SL collected literatures. JC, XW, and FP analyzed literatures and summarized results. JC, SQ, and KZ drafted the manuscript. DL and CP revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Glossary

ADAM17	A Disintegrin and A MetalloProtease Domain 17
ADSC-EVs	Adipose-derived stem cells
ADSCs	Adipose Derived Mesenchymal Stem Cells
AGEs	Advanced glycation end products
allo-mBM-MSCs	Mouse bone marrow-derived allogeneic MSCs
AMPK	Adenosine monophosphate activated protein kinase
Ang	Angiopoietin
BM	bone marrow
BM-MSCs	Bone-marrow-derived mesenchymal stem cells
CAT	Catalase
CCR7	C-C chemokine receptor type 7
circ-RNAs	circular RNAs
COL	Collagen
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450
DFUs	Diabetic foot ulcers
Dll4	Delta-like 4
DM	Diabetes mellitus
E2	17β-Estradiol
ECM	Extracellular matrix
EE	Hsian-tsao extracts
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPCs	endothelial progenitor cells
ERK	Extracellular regulated protein kinases
ER-α	estrogen receptor-α
FGF	fibroblast growth factor
FOXO1	Forkhead box protein O1
GAP-43	Growth associated protein-43
G-Rb1	Ginsenoside Rb1
GSH-Px	Glutathione peroxidase
HG	High glucose
HIF-1α	hypoxia inducible factor-1α
HOXA3	Homeobox A3
IGF-1	Human insulin-like growth factor 1
IL	Interleukin
JNK	c-Jun N-terminal kinase
lncRNAs	long non-coding RNAs
LRG1	Leucine-rich α-2-glycoprotein-1
MCP-1	Monocyte chemoattractant protein-1
MDA	Malonaldehyde
MG	methylglyoxal
miRNAs	microRNAs
MMPs	Matrix metalloproteases
MSCs	Mesenchymal stem cells
NF-κB	Nuclear transcription factor-κB
NGAL	neutrophil gelatinase-associated lipocalin
NICD	Notch intracellular domain
NR1	Notoginsenoside R1
Nrf-2	Nuclear factor erythroid 2-related factor 2
PA	Patchouli alcohol
PAI-1	inhibition of Serpine1
PDC	Dendrobium Polysaccharides
RA	All-*trans*-retinoic acid
RAGE	Receptor for AGE
ROS	Reactive oxygen species
SCF	Stem cell factor
SHED	Stem cells from human exfoliated deciduous teeth
shRNA	small hairpin RNA
TACE	TNF-Alpha Converting Enzyme
T-AOC	Total antioxidant capacity
TDG	thymine-DNA glycosylase
TET2	ten-eleven translocation-2
TETILA	TET2-interacting lncRNA
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of metalloproteinases
TNF	Tumor necrosis factor
TrxR1	Thioredoxin reductase 1
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
VCN-2	Vicenin-2
VEGF	Vascular endothelial growth factor
VLUs	venous leg ulcers
WEP	Hsian-tsao polysaccharides

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Anti-cyclooxygenase, anti-glycation, and anti-skin aging effect of *Dendrobium officinale* flowers' aqueous extract and its phytochemical validation in aging

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Introduction: *Dendrobium officinale* Kimura et Migo (*D. officinale*) , widely called as "life-saving immortal grass" by Chinese folk, is a scarce and endangered species. The edible stems of *D. officinale* have been extensively studied for active chemical components and various bioactivities. However, few studies have reported the well-being beneficial effects of *D. officinale* flowers (DOF). Therefore, the present study aimed to investigate the in vitro biological potency of its aqueous extract and screen its active components.

Methods: Antioxidant tests, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), the ferric reducing ability of plasma (FRAP), and intracellular reactive oxygen species (ROS) level analyses in primary human epidermal keratinocytes, anti-cyclooxygenase2 (COX-2) assay, anti-glycation assay (both fluorescent AGEs formation in a BSA fructose/glucose system and glycation cell assay), and anti-aging assay (quantification of collagen types I and III, and SA-β-gal staining assay) were conducted to determine the potential biological effects of DOF extracts and its major compounds. Ultra-performance liquid chromatography-electrospray ionisation-quadrupole-time-of-flight-mass spectrometry (UPLC-ESI-QTOF-MS/MS) was performed to investigate the composition of DOF extracts. Online antioxidant post-column bioassay tests were applied to rapidly screen major antioxidants in DOF extracts.

Results and discussion: The aqueous extract of *D. officinale* flowers was found to have potential antioxidant capacity, anti-cyclooxygenase2 (COX-2) effect, anti-glycation potency, and anti-aging effects. A total of 34 compounds were identified using UPLC-ESI-QTOF-MS/MS. Online ABTS radical analysis demonstrated that 1-O-caffeoyl-β-D-glucoside, vicenin-2, luteolin-6-C-β-D-xyloside-8-C-β–D-glucoside, quercetin-3-O-sophoroside, rutin, isoquercitrin, and quercetin 3-O-(6″-O-malonyl)-β-D-glucoside are the major potential antioxidants. In addition, all selected 16 compounds exerted significant ABTS radical scavenging ability and effective AGE suppressive activities. However, only

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certain compounds, such as rutin and isoquercitrin, displayed selective and significant antioxidant abilities, as shown by DPPH and FRAP, as well as potent COX-2 inhibitory capacity, whereas the remaining compounds displayed relatively weak or no effects. This indicates that specific components contributed to different functionalities. Our findings justified that DOF and its active compound targeted related enzymes and highlighted their potential application in anti-aging.

KEYWORDS

Dendrobium officinale, flavone di-C-glycosides, UPLC-ESI-qTOF-MS/MS, anti-aging, antioxidant

1 Introduction

Skin is mainly composed of several structures, including the epidermis and dermis, and protects the body against sunlight radiation. The epidermis, the outmost layer of the skin, mainly consists of keratinocytes and absorbs most of the ultraviolet B (UVB) irradiation (1). UVB irradiation stimulates the overproduction of various reactive oxygen species (ROS), which can trigger complex inflammatory signal cascades, such as aberrant cyclooxygenase-2 (COX-2) expression (2), and ultimately lead to various adverse symptoms, including wrinkle formation, skin sagging, and dryness. The dermis, a support tissue maintaining skin resistance and elasticity, harbours critical skin cells referred to as fibroblasts, which are responsible for the synthesis and secretion of the extracellular matrix (ECM) (3). Collagen, mainly types I and III, is the most abundant fibrous protein found within the ECM. However, collagen degradation occurs with age, and its content decreases at a rate of approximately 1% annually after the age of 20, leading to wrinkles, sagging skin, and premature aging (4). Additionally, increased cellular senescence has been found to promote the aging process. Senescent cells exhibit various distinctive molecular properties; senescence-associated β-galactosidase (SA-β-gal) is one of the best-characterised and frequently used biomarkers to identify senescent cells (5, 6). Normal cells have a limited capacity to replicate and eventually enter the senescent state, in which SA-β-gal activity increases rapidly (7, 8).

Furthermore, superfluous reactive free radicals produced during the oxidation process induce abnormal protein modification, destroy the secondary structure, and ultimately accelerate the formation of advanced glycation end products (AGEs), which stimulate ROS production. AGEs are a series of compounds gradually formed as a result of a non-enzymatic glycation reaction with fluorescent and non-fluorescent entities, such as argpyrimidine and *N*-carboxymethyl lysine (CML) (9). Accumulation of AGEs occurs throughout life, and they are found at significantly higher levels among the elderly population (10). Growing evidence has shown that AGEs are a major etiologic factor in age-related disorders, especially skin aging, as it is the most direct manifestation of body aging. A few studies also have shown that external stimuli such as excessive free radicals and spontaneous AGE generation *in vivo*, which are irreversible once formed in the body (11), are associated with skin fibroblast damage, destruction of collagen and elastic fibres, a yellow complexion without splendour, and deterioration with aging (12). Therefore, antioxidant, anti-inflammatory, and anti-glycation effects, as well as collagen protection and SA-β-gal inhibition, are important for the development of anti-aging products.

D. officinale Kimura et Migo, widely referred to as "life-saving immortal grass" by the Chinese, is a scarce and endangered species due to its unique environmental requirements, low fertility, slow growth, and sparse distribution (13). The edible stems of *D. officinale*, known as "TiepiShihu", have been extensively studied in recent years for active chemical components (14) and used as a precious medicinal herb to improve immune function, nourish the stomach, alleviate the symptoms of diabetes, and postpone senility (15). However, as a medicinal by-product, the flowers of *D. officinale* are typically discarded and rarely studied. Recently, the flowers of *D. officinale* have been reported to be rich in nutrients and other chemical ingredients, including anthocyanins, flavonoids, and polysaccharides, and specific bioactivities have been elucidated, such as liver protection as well as hypoglycaemic, antioxidant, and antihypertensive effects (16–18). Therefore, it is necessary to establish reliable methods for component analysis of *D. officinale* flowers and expand its use for health-related products through comparison with stems.

The *D. officinale* flower is popular in China due to its anti-aging properties and beneficial effects on yin deficiency syndromes. However, scientific data to confirm the pharmacological effects listed above is limited in the literature. Therefore, the purpose of this study was to tentatively conduct more systematic experiments on the aqueous extracts of *D. officinale*, including its biological effects on skin aging, such as antioxidant capacity (through 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS), the ferric reducing ability of plasma (FRAP)), anti-inflammatory effect (through COX-2), anti-glycation potency (through inhibition of non-enzymatic glycation reaction and inhibition of CML expression in fibroblasts), and anti-aging evaluation (through the SA-β-gal staining test and collagen expression). The phytochemical composition of *D. officinale* was

also analysed using ultra-performance liquid chromatography-electrospray ionisation-quadrupole-time-of-flight-mass spectrometry (UPLC-ESI-QTOF-MS/MS). Antioxidants in *D. officinale* flower aqueous extract were identified using the online ultra-performance liquid chromatography-photodiode array detection-mass spectrometry-2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (UPLC-PDA-MS-ABTS+·) method and the biological capacities of specific isolated constituents were explored (through DPPH, ABTS, FRAP, anti-COX-2, and AGE inhibitory activities). Previous research has shown that *D. officinale* flowers can alleviate brain aging and improve spatial learning abilities in senescent rats (17). However, previous literature contains few reports on the anti-glycation effect of *D. officinale* flower aqueous extract. Online antioxidant investigation of *D. officinale* flower aqueous extract and various biological activities of its identified compounds that are described in the work have not been reported previously.

2 Materials and methods

2.1 Plant material, solvents, and chemicals

The flowers of *D. officinale* were purchased by Amway (Shanghai) Technology Co. and authenticated as *D. officinale* Kimura et Migo flowers by doctor Gangqiang Dong, Amway (China) Botanical Research Centre. Analytical-grade methanol was purchased from Honeywell Co. (Charlotte, USA). Acetic acid and liquid LS-MS-HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) lyophilised powder, ABTS (98%), DPPH· (98%), 6-hydroxyl-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), tyrosinase, ascorbic acid, NS-398 (COX-2 inhibitor, CAS: 123653-11-2), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Darmstadt, Germany). High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin stock solution (PS, 10,000 U/ml), phosphate-buffered saline (PBS), and pancreatin solution were purchased from Gibco (Carlsbad, CA, USA). The reference standards vicenin-2, luteolin 6-*C*-*β*-D-xyloside-8-*C*-*β*-D-glucoside, vicenin-1, luteolin-6-*C*-*β*-D-glucoside-8-*C*-*β*-D-xyloside, quercetin-3-*O*-sophoroside, schaftoside, luteolin-6-*C*-*β*-D-glucopyranoside, vicenin-3, apigenin-6-*C*-*β*-D-glucoside-8-*C*-*α*-arabinoside, rutin, isoquercitrin, apigenin-6-*C*-*α*-L-arabinoside-8-*C*-*β*-D-xyloside, quercetin 3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside, kaempferol-3-*O*-rutinoside, astragaline, and isorhamnetin-3-*O*-glucoside were purchased from Nature Standard Biotech Co. (Shanghai, China). Aminoguanidine hydrochloride (AG) and glucose were purchased from Aladdin (Shanghai, China), and methylglyoxal (MGO) was purchased from Adamas (Delaware, USA). The Cell Counting Kit-8 (CCK-8), FRAP kit, and SA-*β*-gal staining kit were purchased from Beyotime Biotechnology Co. (Shanghai, China). Alexa Fluor®488 Donkey anti-mouse IgG was purchased from Thermo Fisher (Waltham, Massachusetts, USA). Carboxymethyl lysine antibody, Alexa Fluor® 488 donkey anti-mouse IgG, Alexa Fluor® 488 donkey anti-rabbit IgG, Alexa Fluor® 568 goat anti-rabbit IgG, type I collagen primary antibody, type III collagen primary antibody, and mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Abcam (Cambridge, UK). The types I and III collagen enzyme-linked immunosorbent assay (ELISA) kits were purchased from BIO-SWAMP Co. (Wuhan, China). The human dermal fibroblasts (HDFs) were purchased from Archgene Biotechnology Co. (Shanghai, China). Zebrafish were purchased from Hunter Biotechnology Co. (Hangzhou, China). All other chemicals were purchased from Titan Co. (Shanghai, China). Water was purified using a Milli-Q purification system (Barnstead, USA).

2.2 Extraction procedure

Aqueous extracts of *D. officinale* flower (DOF) were prepared using slightly modified methods [3]. Briefly, the pulverised flower powder was reflux-extracted twice with distilled water at a solid-to-solvent ratio of 1:12 (w/v) for 1 h at 100°C. The extract was separated through centrifugation at 19,000×*g* for 15 min at 4°C (RWB3220CY-2, Eppendorf, Germany). The supernatant was evaporated at 65°C using a scale rotary evaporator (Hei-VAP Expert, Heidolph; Schwabach, Germany) until a small volume remained, then lyophilised using a freeze dryer. The DOF hot-water extract (DOF-W) was then stored at −18°C until further analysis.

2.3 Antioxidant capacity (DPPH·, ABTS·+, and FRAP assays)

Fast colourimetric methods were slightly modified for the *in vitro* assessment of DPPH· scavenging (19), ABTS·+ decolourisation capacity (20), and total antioxidant capacity of FRAP (21). The stock solutions of derivatisation reagents were diluted as follows before measurement: DPPH was diluted with absolute ethanol until the absorbance was 0.8 ± 0.05 at λ = 517 nm. ABTS working solution was prepared with phosphate buffer (0.2 M, pH 7.4) to display absorbance of 0.8 ± 0.05 at λ = 729 nm, based on a previous study (22). The FRAP solution was prepared according to the instructions of the FRAP kit. Absorbances were measured using an automatic microplate reader (Molecular Group Ltd., USA), and all analyses were performed using 96-well plates. All measurements were performed in triplicate, and Trolox was used as a positive control.

For DPPH· scavenging, 100 µl sample solution was mixed with 100 µl fresh DPPH ethanolic solution, and the absorbance of the mixture was measured after 10 min at 517 nm. Ethanol was used as a negative control.

For ABTS·+ decolourisation capacity, 100 µl of ABTS working solution was mixed with 200 µl of sample solution, and the absorbance was recorded after 10 min at 729 nm. Phosphate buffer (0.2 M, pH 7.4) was used as a negative control.

DPPH· and ABTS·+ radical scavenging activities of the tested sample were both calculated using the following formula:

% inhibition = (*A* control − *A* sample)/A control × 100

Where *A* control and *A* sample represent the absorbance of the control and test samples, respectively. The IC_{50} was calculated graphically from the dose-inhibition curves.

For total antioxidant capacity, 180 µl of FRAP solution was mixed with 5 µl of sample solution, and the absorbance was measured after 5 min at 539 nm with PBS as the negative control. $FeSO_4$ solutions (0.15, 0.3, 0.6, 0.9, 1.2, and 1.5 mM) were used for the calibration curve. The FRAP value represents the corresponding concentration of $FeSO_4$ solutions (mM $FeSO_4$). The regression equation from the standard curve was used to calculate the equivalent concentration 1 (EC_1) of each sample. EC_1 is defined as the concentration of the test sample with an absorbance equivalent to that of 1.0 mmol/L of $FeSO_4$ solution, determined by its calibration curve (23).

2.4 Cellular antioxidant activity assay

2.4.1 Cell culture and UVB treatment

Primary human epidermal keratinocytes (NHEKs) were purchased from Lifeline® Cell Technology (Frederick, MD, USA) and cultured in DermaLife K Keratinocyte Calcium-Free Medium (Cat. LL-0029). NHEKs were incubated in a humidified incubator with 5% CO_2 at 37°C. NHEKs were exposed to a spectral peak at 312 nm of the UVB irradiation by using an UVB lamp (Spectroline Model EB-160C, New York, NY, USA) at doses of 10 mJ/cm^2. After UVB irradiation, the cells were washed with warm PBS, and then fresh medium with and without different concentrations of DOF-W (10 and 40 µg/ml) was added and incubated for 24 h.

2.4.2 Cell viability assay and measurement of ROS generation

Cell viability was determined using the CCK-8. After 24 h of incubation, the optical density (OD) was recorded using a microplate reader at 450 nm, according to the instructions of the kit. Based on the methods reported in a previous study [4], the relative levels of ROS were detected using CellROX® Reagent (Life Technologies, Waltham, MA USA). Briefly, NHEKs were seeded in 96-well microplates at 2×10^4 cells/well for 48 h, followed by the UVB treatment and incubation periods specified above. The cells were then incubated with 5 µM CellROX® for 1 h and washed thrice with PBS. ROS production was measured through the fluorescent intensity with the excitation and emission wavelengths set at 485 and 520 nm, respectively.

2.5 Anti-COX-2 assay

Anti-inflammatory activity was assessed by measuring COX-2 enzymatic inhibition on the basis of a described method by using the COX-2 Inhibitor Screening Kit (Beyotime, No. S0168) [5]. Celecoxib, a COX-2 inhibitor, was used as a positive control. The results of anti-COX-2 activities are presented as IC_{50} values (µg/ml or µM), a measurement of the inhibition of enzyme activity by each sample by 50%.

2.6 Anti-glycation capacity

2.6.1 Fluorescent AGEs formation in a BSA-fructose/glucose system

The formation of total fluorescent AGEs in glycated samples was assessed by determining their fluorescent intensities at an excitation/emission wavelength of 350/450 nm, as previously described (24). Glycated protein was prepared *in vitro* by incubating BSA in the presence of D-glucose (GLC) and D-fructose (FRC). The DOF extract and specific standards were dissolved in DMSO:water (1:4). BSA (4 mg/ml, 100 µl) was preliminary mixed with 50 µl of GLC (0.5 M) and 50 µl of FRC (0.5 M) in 20 mM sodium phosphate buffer (pH 7.4), and then 100 µl samples were added in the 96-well plate and incubated at 37.5°C for 7 days. All solutions were prepared under sterile conditions and filtered using a 0.22-µm syringe before incubation. The assay was performed in triplicate, and AG was used as a positive control. The percentage inhibition of fluorescent AGE formation was calculated using the following equation:

$$Inhibition\,(\%) = 1 - [(IF\ sample - IF\ sample\ control)/(IF\ control - IF\ blank\ control)] \times 100\,\%.$$

Where IF sample is fluorescence intensity in the presence of samples and BSA, IF sample control is fluorescence intensity in the presence of samples without BSA, IF control is fluorescence intensity without samples, and IF blank control is fluorescence intensity without samples and BSA. The results of anti-glycation activities are presented as IC_{50} values (µg/ml or µM), a measurement of the 50% inhibition of enzyme activity by each sample.

2.6.2 Glycation cell assay

Based on previously reported methods (25), human primary dermal fibroblasts (HDFs) were induced by MGO to establish a cell model of high CML expression.

HDFs were cultured in high-glucose DMEM supplemented with 10% FBS containing 100 g/ml of PS at 37°C in a humidified 5% CO_2 incubator. Cell viability was assessed using the CCK-8 kit. HDFs were cultured in 12-well plates to approximately 70% confluence and then treated with 0.5 mmol/L of MGO for 48 h to induce glycation and the formation of CML. Subsequently, different concentrations of samples were added and co-cultured with MGO for an additional 48 h. Cells were then washed once with PBS and fixed in polyformaldehyde overnight at 4°C. Triton-X-100 solution (0.1%) was assigned to fixed cells for 5 min at room temperature to improve cell membrane permeability. After washing twice with PBS, cells were blocked in 1% BSA for 1 h, followed by incubation with the primary CML antibody (1:50) overnight at 4°C. The secondary antibody donkey-anti-Mouse Alexa Fluor® 488

(1:1,000) was then added for 2 h at room temperature. Counterstaining was performed by adopting anti-fluorescence quenching and sealing liquid containing DAPI, followed by visualisation and fluorescence image capture using a microscope (Leica, Germany). ImageJ software was used to quantify CML expression from different images randomly selected in each group.

2.7 Anti-aging assay on HDFs

2.7.1 Cell culture

HDFs were cultured in DMEM with 10% (v/v) FBS and 1% PS at 37°C in a humidified 5% CO_2 incubator. Cells were subcultured using pancreatin solution after reaching confluence. All experiments were performed between the third and eighth passages.

2.7.2 Quantification of collagen types I and III using immunofluorescence

The synthesis of collagen types I and III was detected using IF (26) and ELISA (27). HDFs were digested and seeded in a 24-well culture plate on slides at a density of 50,000 cells/well in a complete medium. After reaching 80% confluency, cells were starved with the medium in the absence of serum for 16 h. Cells were then treated with positive controls or active samples (prepared in medium without serum) for an additional 48 h. For analysis and visualisation of types I and III collagens, the supernatants and remaining cell slides were both collected and analysed using ELISA kits and the IF staining method, respectively.

Collected supernatants were centrifuged at 10,000 rpm for 10 min and used for types I and III collagen ELISA, according to the manufacturer's protocol. The remaining cell slides were washed once with PBS and fixed in polyformaldehyde overnight at 4°C. Subsequently, the slides were treated with 0.5% Triton-X100 for 5 min. After washing, cells were blocked with 1% BSA at room temperature for 1 h. Cells were then incubated with types I and type III collagen primary antibodies (1:100) for 2 h at room temperature. After washing, cells were stained with secondary antibody (1:1,000) for 1 h (Alexa Fluor® 568 goat anti-rabbit IgG and Alexa Fluor® 488 donkey anti-rabbit IgG were used to detecting fluorescence of types I and type III collagen, respectively). Finally, the nuclei were counter-stained with DAPI, and relevant fluorescent images were captured using a microscope. ImageJ software was used to quantify collagen expression from different images randomly selected in each group.

2.7.3 SA-β-gal staining assay

Senescent human skin fibroblasts induced by D-galactose (D-gal) were established based on a previously reported method (28, 29). HDFs were divided into the control, aging model, and sample (aging model with DOF extract) groups. Except for the control group, HDFs were cultured in six-well plates to approximately 70% confluence and then treated with 20 mg/ml D-gal for 72 h to induce cell senescence. Subsequently, different concentrations of DOF extract were added to the sample group and co-cultured with D-gal for an additional 72 h. The media was then aspirated from cells, and the wells were washed once with PBS. SA-β-gal staining was performed using an SA-β-gal staining kit (Beyotime Co.). Fixative was added and incubated for 10 min at room temperature. Cells were then washed twice with PBS and incubated in 1 ml of SA-β-gal staining solution (freshly prepared according to the protocol of the kit). Plates were maintained in the dark, overnight, in a humidified incubator at 37°C without CO_2. The following day, the staining solution was removed and cells were maintained in the final solution of PBS. The staining of SA-β-gal was observed, and relevant images were captured using a microscope. ImageJ software was used to determine the average value of the proportion of blue-stained cells of five randomly selected microscopic images in each group.

2.8 UPLC-PDA-QTOF-ESI-MS/MS analysis

DOF extract (1 mg) was diluted with 1 ml of distilled water, sonicated for 20 min, and filtered using a 0.45-μm syringe filter before analysis.

The UPLC analysis was performed using an Agilent 1290 UPLC system (California, USA) combined with an Agilent Q-TOF 6545 LC/MS system, a sample manager, a PDA detector, and a binary solvent manager, and was controlled using MassHunter Workstation Software. The Acquity HSS T3 reverse phase column (2.1 × 100 mm, 1.8 μm; Waters, Milford, MA, USA) at a separation temperature of 30°C was used to perform the chromatographic separation of 2 μl of each sample with a wavelength scanning range of 190–400 nm. Gradient elution at a flow rate of 0.2 ml/min was completed with the mobile phase consisting of solvent A (0.2% acetic acid in ultrapure water) and solvent B (acetonitrile) in the following order: 0–5 min, 2% B; 5–8 min, 2%–10% B; 8–12 min, 10% B; 12–20 min, 10%–15% B; 20–28 min, 15%–20% B; 28–31 min, 20% B; 31–38 min, 20–80% B; and 38–40 min, 80% B. Finally, the initial conditions were reintroduced over the course of 2 min. Before each run, the column was equilibrated for an additional 2 min. The MS was operated in both positive and negative ion modes. The optimised MS conditions were as follows: TOF mass range, m/z 50–1,700; ion source gas, 50 psi; curtain gas, 35 psi; ion spray voltage, 5 kV; ion source temperature, 500°C; and collision energy, 10 eV. The following MS/MS parameters were applied: MS/MS mass range, 50–1,250 m/z; collision energy, 40 eV; declustering potential, 100 V; and collision energy spread, 20 eV. Compounds were identified and analysed by comparing their retention times, fragment ions, and formulas using corresponding standards and commercial databases.

2.9 Antioxidant online profiling using UPLC-PDA-QDa coupled with postcolumn derivatisation with ABTS

Online identification of antioxidant components of DOF extract was performed using a UPLC system (Milford, MA, USA) consisting of a Waters photodiode array detector and a Waters

postcolumn derivatisation system supplying fresh ABTS solution (UPLC-PDA-QDa-ABTS). Gradient elution at a flow rate of 0.8 ml/min was completed with the mobile phase consisting of solvent A (0.2% acetic acid in ultrapure water) and solvent B (acetonitrile) in the following order: 0–5 min, 2% B; 5–8 min, 2%–10% B; 8–12 min, 10% B; 12–20 min, 10%–15% B; 20–28 min, 15%–20% B; 28–31 min, 20% B; 31–38 min, 20%–80% B; and 38–40 min, 80% B. Separation of compounds was performed at 30°C using an Acquity reversed-phase column (4.6 × 250 mm, 5 µm; Waters, Milford, MA, USA). The detection wavelengths were set at 280 and 734 nm, and the injection volume of the sample was 10 µl.

The technical route and relevant device installation are shown in Figure 1. To detect radical scavengers, the UPLC system was coupled with a Waters pump, which supplied freshly prepared ABTS·+ solution into a reaction coil (15 m, 0.25 mm ID) with a flow rate of 0.2 ml/min at 37°C. Negative peaks were recorded based on a decrease of absorbance at 729 nm after the reaction of individual compounds with the ABTS·+ radical. For preliminary identification of compounds, a Waters Acquity QDa mass detector in negative ionisation mode was connected in series to PDA with the following parameters: electrospray ion source; cone voltage, 15 V; atomiser, N2; and scanning range, 100–800 m/z.

2.10 Statistical analysis

The data were statistically processed using GraphPad software. The test data were expressed as mean ± standard deviation (SD), and significance was evaluated by one-way analysis of variance (ANOVA) and Tukey's test. Statistical significance was indicated as follows: $^{*}p< 0.05$ and $^{**}p< 0.01$.

3 Results

3.1 *In vitro* antioxidant potency of DOF extracts

The growing awareness of herbs with antioxidant properties has been noted over the last few decades, mainly due to the discovery of ROS closely involved in chronic non-infectious diseases. Using several antioxidation assays and various models is vital for a more comprehensive assessment of natural products. Therefore, in the present study, DPPH, ABTS, and FRAP assays, as well as intracellular ROS levels induced by UVB radiation in NHEKs cells, were used for measuring the antioxidant potential of the *D. officinale* flower aqueous extract (DOF-W).

For the concentration range of 15.6–1,000 µg/ml, DOF-W showed antioxidant activity in a dosage-dependent manner, as shown in Figure 2. For the DPPH and ABTS assays, the IC_{50} value of DOF-W was 669.7 ± 20.59 µg/ml and 224.57 ± 0.65 µg/ml, respectively, whereas the IC_{50} values of Trolox were 24.7 ± 1.0 µg/ml and 27.68 ± 1.04 µg/ml. For the FRAP assay, the EC1 value of DOF-W was 4,580 ± 260 µg/ml, whereas the EC1 value of Trolox was 55.35 ± 5.22 µg/ml. Though DOF-W showed a weaker antioxidant ability compared to a classical antioxidant standard (Trolox), it still exhibited potential antioxidant activity, particularly at high concentrations, which were consistent with various previous studies (30, 31).

Safety and non-toxicity are essential for functional foods, as well as skin-care cosmetics. The DOF-W had no significant damage to NHEK cell viability in the range of 1–40 µg/ml (Supplementary Data). After being irradiated by UVB (10 mJ/cm^2), cell survival was significantly reduced. However, DOF-W did not further aggravate UVB-induced NHEK cell mortality (Supplementary Data). As Figure 2D shows, UVB irradiation leads to an increasing ROS level of NHEK cells. Treatment with 40 µg/ml of DOF-W notably reduced intracellular ROS levels.

3.2 Anti-COX-2 capacity of DOF extracts

As COX-2 is an inducible enzyme that produces prostaglandins (PGs) and is responsible for generating ROS, it is always regarded as a pathologic enzyme chiefly responsible for inflammation (32), and COX-2 inhibitors, which can cause a sharp drop in the amount of ROS, are also found to be highly associated with potential antioxidant effects (33). In this study, the COX-2 inhibition assay was used to evaluate the anti-inflammatory effect of DOF-W.

The COX-2 inhibitory activity of DOF-W was compared with a well-known selective COX-2 inhibitor (Celecoxib). For the

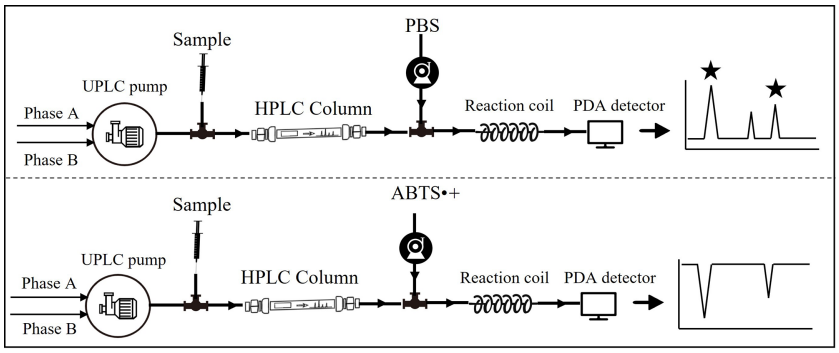

FIGURE 1
Flow chart of online detection of antioxidants.

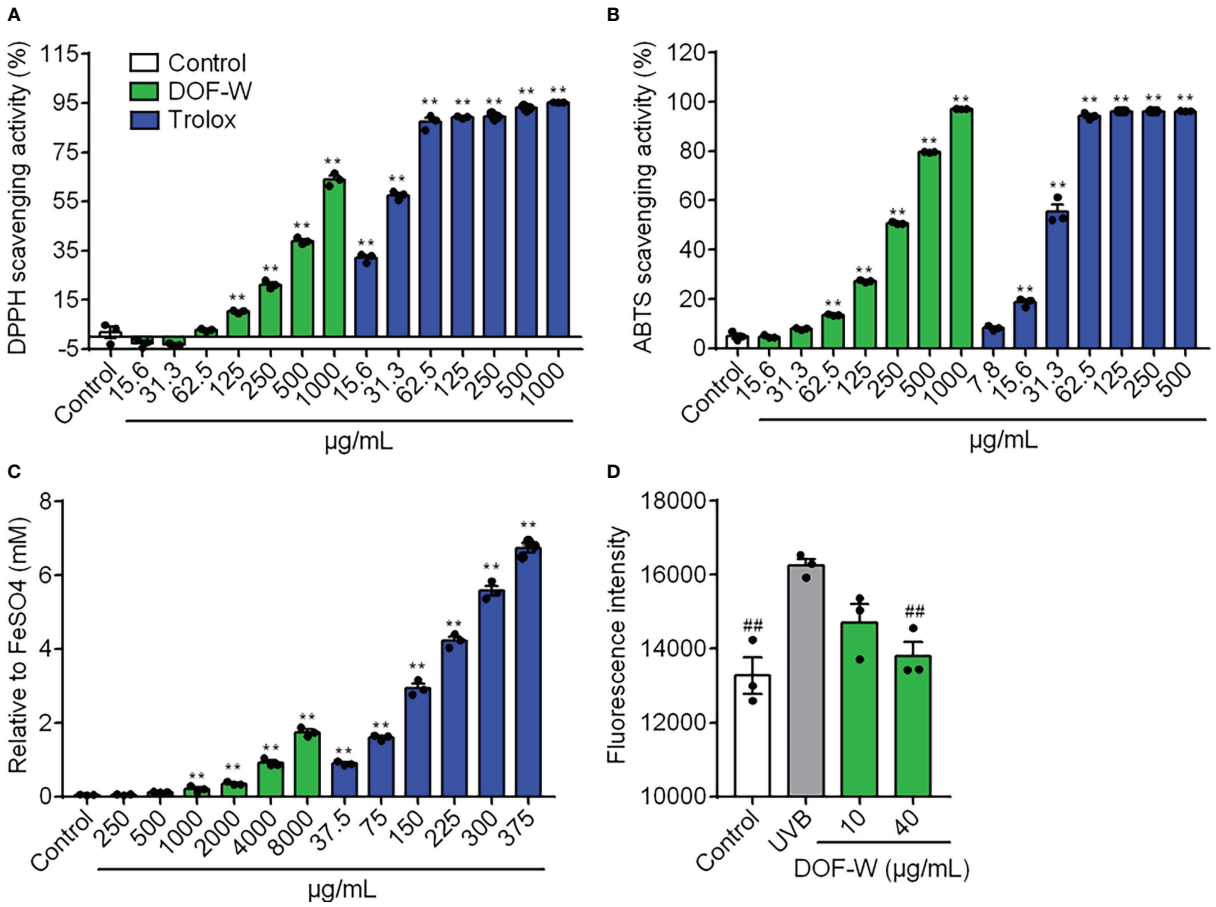

FIGURE 2
Antioxidant potency of DOF extracts. DPPH scavenging activity **(A)**, ABTS scavenging activity **(B)**, FRAP value **(C)**, and intracellular ROS level **(D)** of DOF-W. Compared with the control, $^{**}p< 0.01$. Compared with the UVB model, $^{\#\#}p< 0.01$.

concentration range of 31.3–2,000 μg/ml, DOF-W showed great concentration-dependent COX-2 inhibitory activity, as shown in Figure 3. The DOF-W had an IC_{50} value of 133.5 ± 27.4 μg/ml, whereas Celecoxib had an IC_{50} value of 0.53 ± 0.30 μg/ml. Although DOF-W may show lower capacities due to the dilution of the active constituents with neutral ones, it still could be used as a promising COX-2 inhibitor.

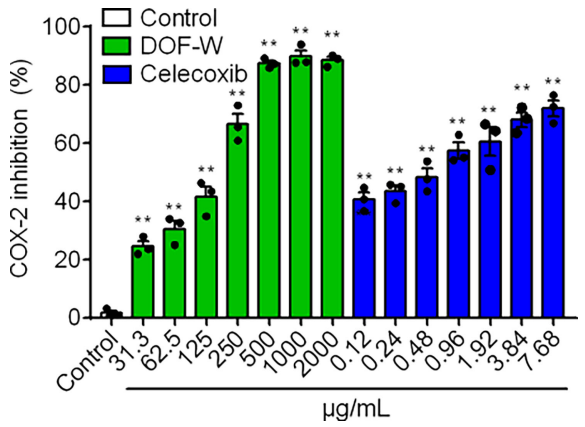

FIGURE 3
COX-2 inhibition of DOF-W. Compared with the control, $^{**}p< 0.01$.

3.3 Anti-glycation activity of DOF-W

Glycosylation within tissues is a slow and complicated process. Excessive generation and accumulation of AGEs in the process will cause irreversible damage to the body. Various AGEs in skin collagen, including CML, can damage skin fibroblasts, resulting in skin aging and the formation of dark spots (10). In the present study, the determination of anti-glycation activities was evaluated by multimodal methods, including routine chemical tests, cell experiments, and a zebrafish assay.

3.3.1 Inhibition of DOF-W on total fluorescent AGEs of non-enzymatic glycation

Incubation of reducing sugars and BSA induced the production of fluorescent AGEs (34). In this study, the BSA-fructose/glucose as a model reaction system was first applied to preliminarily evaluate the effect of DOF aqueous extracts on the inhibition of AGEs. As shown

in Figure 4A, DOF-W extracts can reduce levels of fluorescent AGEs in a concentration-dependent relationship with IC_{50} values of 428.4 ± 75.1 μg/ml. AG, a synthetic AGE inhibitor, expectedly displayed an effective inhibition with IC_{50} values of 177.3 ± 11.6 μg/ml.

3.3.2 Anti-glycation effect of DOF-W in human primary fibroblasts

Anti-glycation primary assay of BSA/reducing sugar system as the above showed that DOF extract was a potential anti-glycans. The DOF extract was then further tested in an *in vitro* glycation assay to confirm its activities on cells. MGO is a critical and potent precursor in the formation of AGEs, reacting with proteins to produce *N*ε-carboxymethyl lysine (CML), which is one of the principal AGEs in the skin without fluorescence properties and cannot be detected by conventional assay (35). Fibroblasts were treated with MGO to induce glycation, which was then visualised and quantified using IF. No obvious CML green fluorescence staining in the blank control group was observed, which indicated that normal cells hardly secrete CML, whereas a large amount of CML was expressed in MGO-induced cells after CML IF, as shown in Figure 4D. The treatment of the cells with the positive compound AG resulted in significant inhibition of glycation by 88.11%. Since no cytotoxicity was found under concentrations of 40 μg/ml, as shown in Figure 4B, treatment with 10 and 40 μg/ml of DOF extract in HDFs both resulted in significant inhibition of glycation by 77.88% and 69.70%, respectively, without a distinct dose-dependent manner (Figures 4C, D).

3.4 Effect of DOF extracts on collagen synthesis in human skin fibroblast cells

Dermal fibroblasts are thought to be responsible for synthesizing various dermal ECM proteins, including fibrous collagens. Type I collagen accounted for approximately 80% of total collagen, whereas type III collagen is more prevalent in young skin than aged skin and is particularly involved in wound healing (36). Since skin aging is characterized by the degradation of ECM components such as types I and III collagen breakdown, we investigated whether DOF could enhance the expression of types I and III collagen in HDFs.

As shown in Figures 5A, B, IF assay revealed that DOF-treated cells synthesized more amount of type I collagen than untreated control cells after 48 h. DOF increased the extent of collagen type I staining by 111.88% and 154.59% of control at a concentration of 10 and 40 μg/ml, respectively. However, the level of expression of type III collagen was not statistically altered or even decreased by DOF extract, as shown in Figures 5C, D. Collagen types I and III expressions were significantly increased by vitamin C by 190.20% and 125.90% of control as a positive drug, respectively.

We also tested the extracellular content of collagen types I and III for 48 h on HDFs in the culture medium. As shown in Figures 5E, F, treatment with vitamin C (VC), 10 μg/ml of DOF, and 40 μg/ml of DOF increased secreted collagen type I levels by 21.94%, 42.6%, and 57.65%, respectively, whereas DOF had no

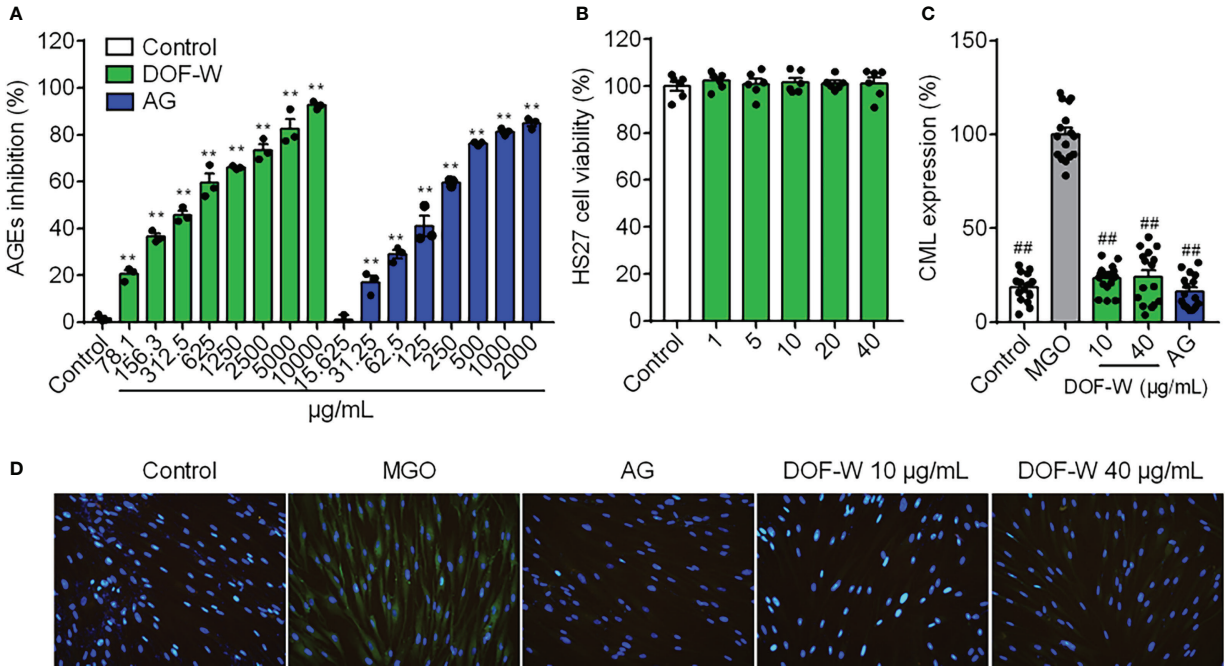

FIGURE 4
Anti-glycation activity of DOF-W. **(A)** Inhibition of fluorescent AGEs by DOF-W extract. **(B)** Cytotoxicity of DOF-W extract to fibroblasts. **(C)** Effect of CML expression in glyoxal-induced HDFs by DOF-W extract. **(D)** Representative images of HDFs by fluorescence microscope and statistics. $^{\#\#}p<0.01$ compared with the MGO model. $^{**}p<0.01$ compared with control. AG, aminoguanidine hydrochloride; MGO, methylglyoxal; CML, *N*-carboxymethyl lysine.

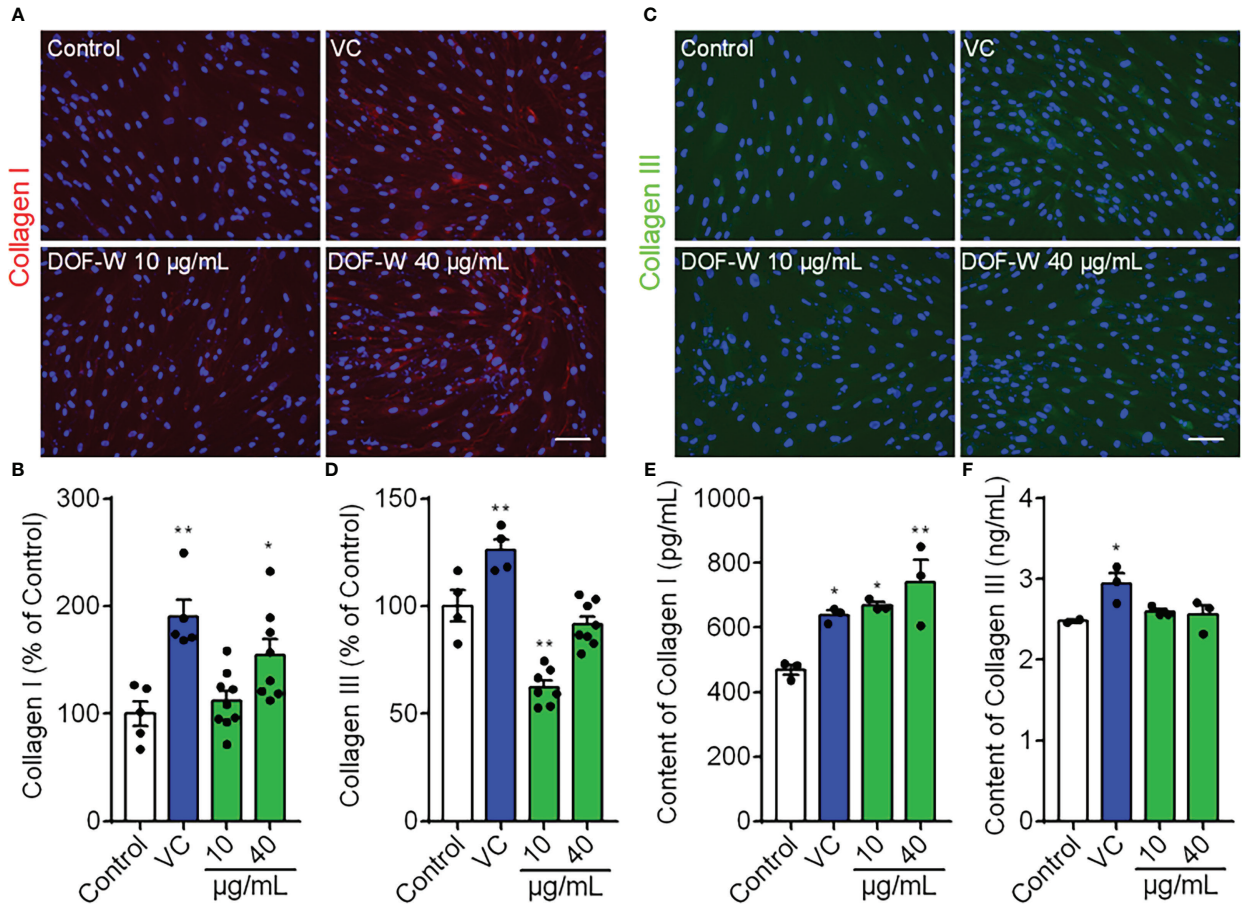

FIGURE 5
Effects of DOF extract on the expression of collagens by IF staining. HDFs were treated with VC (200 μg/ml, positive control) and DOF extract (10 and 40 μg/ml), whereas HDFs were incubated without any treatment as the control group. **(A)** Images of collagen I staining (red color) and quantification **(B)**. **(C)** Images of collagen III staining (green color) and quantification **(D)**. Extracellular content of types I **(E)** and III **(F)** collagen on HDFs using ELISA. Bar scale, 100 μm. Compared with the control group, *$p< 0.05$, **$p< 0.01$.

obvious effect on type III collagen secretion, further confirming the corresponding significantly stronger effect on collagen type I. Therefore, we conclude that DOF extracts can upregulate collagen type I level in HDFs, but they have no positive effect on type III collagen.

3.5 SA-β-Gal staining assay on DOF-W

Cellular senescence is an irreversible physiological phenomenon in which normal cells have lost their proliferative potential but are still alive and maintaining their metabolic activity (37). Cell senescence can be measured using SA-β-gal staining assay, which is widely used to locate SA-β-gal-positive cells (blue-stained cells) by optical microscope. Among several existing types of aging models, such as X-ray, H_2O_2, and D-Gal-induced aging models, D-gal induced aging models to resemble natural aging but take a shorter time than the latter (38).

As shown in Figure 6, few blue-stained cells were observed in the control group (3.0% ± 1.2%). SA-β-gal-positive cells significantly increased after treatment with 20 mg/ml of D-gal for 6 days, consecutively, in the model group (7.9% ± 2.0%). When senescent cells coincubated with 10 and 40 μg/ml of DOF extracts, the proportion of SA-β-gal-positive cells were 4.0% ± 0.9% and 5.6% ± 1.2%, respectively, which were conspicuously lower than those of the model group.

3.6 Determination of phytochemicals using UPLC-Q/TOF-MS/MS

The 25 compounds were further characterised by structural analysis using UPLC-PDA-ESI-Q/TOF-MS/MS (Table 1; Supplementary Materials). The total ion chromatogram of the aqueous extract of the sample under both negative and positive ion mode is shown in Figure 7. The retention time (Rt), molecular formula, and ion and ions after fragmentation are shown in Table 1. The Rt and fragmentation information of compounds 13, 14, 16, 17, 19, 20, 23, 24, and 25 were compared with those of standards. The major constituents of *DOF-W* were flavonoids (Figure 8). A typical MS spectrogram fragmentation mechanism for flavonoid disaccharide C-glycoside such as schaftoside is shown in Supplementary Figure S2.

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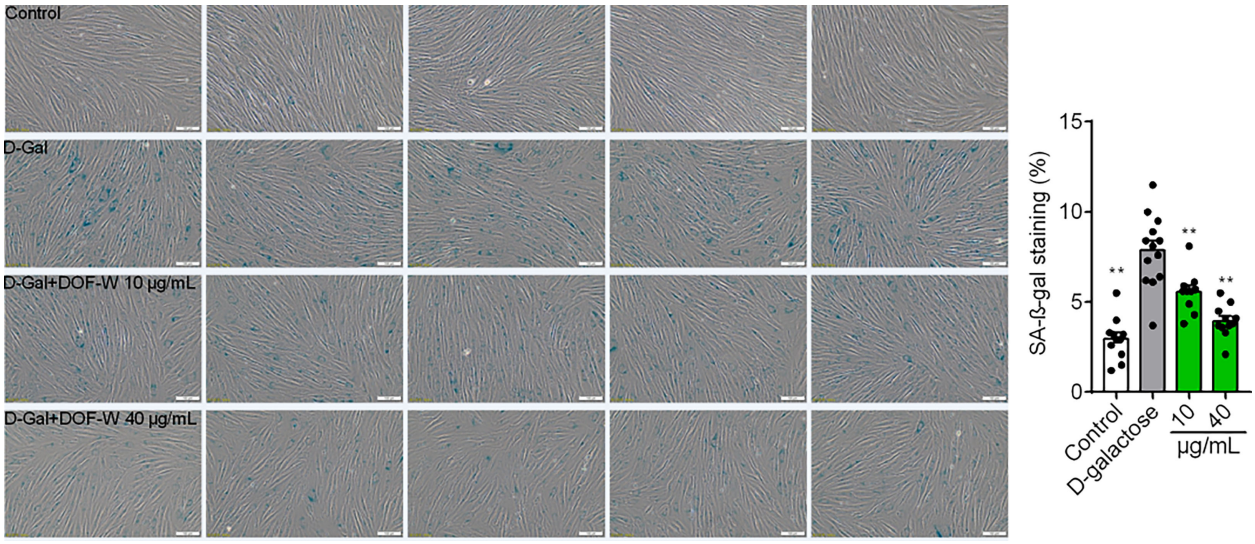

FIGURE 6
Images of fibroblasts with SA-β-gal staining. Statistics results of fibroblasts with SA-β-gal staining. Bar scale, 100 μm. Compared with the model group, $^{**}p < 0.01$.

TABLE 1 The physical properties of the identified compounds from the flowers of *Dendrobium officinale*.

No.	$T_{(min)}$	Ion	*m/z*	ppm	Formula	Mol. wt.	Name	MS/MS data
1	3.08	$[M+H]^+$	294.1547	−2.0	$C_{12}H_{23}NO_7$	293.15	Fructoseleucine	276.1445; 258.1295; 230.1362; 212.1264; 182.1158
2	5.78	$[M-H]^-$	282.0842	1.3	$C_{10}H_{13}N_5O_5$	283.09	Guanosine	150.0397; 133.0150; 108.0204; 78.9594
3	6.83	$[M+H]^+$	268.1047	0.5	$C_{10}H_{13}N_5O_4$	267.10	Adenosine	136.0618; 119.0350; 94.0394; 57.0330
4	8.04	$[M-H]^-$	299.0776	3.0	$C_{13}H_{16}O_8$	300.08	Salicylic acid 2-*O*-*β*-D-glucoside	137.0238; 123.0080; 93.0346
5	11.49	$[M-H]^-$	341.0889	4.8	$C_{15}H_{18}O_9$	342.10	1-*O*-caffeoyl-*β*-D-glucoside	179.0347; 161.0246; 135.0448; 133.0296
6	14.41	$[M-H]^-$	325.0935	3.6	$C_{15}H_{18}O_8$	326.10	1-*O*-(4-coumaroyl)-*β*-D-glucose or isomer	163.0392; 145.0293; 117.0345; 59.0135
7	15.45	$[M-H]^-$	325.0935	3.6	$C_{15}H_{18}O_8$	326.10	1-*O*-(4-coumaroyl)-*β*-D-glucose or isomer	163.0376; 145.0296; 117.0348; 59.0139
8	16.53	$[M-H]^-$	771.1987	0.4	$C_{33}H_{40}O_{21}$	772.21	Quercetin 3-*O*-glucosyl-rutinoside	771.1974; 609.1456; 463.0794; 301.0343; 178.9978
9	17.57	$[M-H]^-$	609.1461	0.2	$C_{27}H_{30}O_{16}$	610.15	Luteolin-6-*C*-*β*-D-glucoside-8-*C*-*β*-D-galactoside	489.1025; 429.0796; 399.0700; 369.0604
10	19.86	$[M-H]^-$	593.1519	2.1	$C_{27}H_{30}O_{15}$	594.16	Vicenin-2	593.1533; 473.1087; 383.0768; 353.0659; 297.0749
11	19.86	$[M-H]^-$	579.1352	1.3	$C_{26}H_{28}O_{15}$	580.14	Luteolin- 6-*C*-*β*-D-xyloside-8-*C*-*β*-D-glucoside	429.0776; 399.0709; 369.0602; 339.0487
12	20.82	$[M-H]^-$	579.1355	−0.2	$C_{26}H_{28}O_{15}$	580.14	Luteolin-6-*C*-xyloside-8-*C*-glucoside isomer	459.0916; 429.0811; 399.0711; 369.0605; 339.0518
13	21.06	$[M-H]^-$	623.1620	0.0	$C_{28}H_{32}O_{16}$	624.16	Isorhamnetin-3-*O*-neohesperidoside	503.1182; 413.0874; 383.0774; 357.0621; 315.0635
14	22.03	$[M-H]^-$	563.1398	−0.5	$C_{26}H_{28}O_{14}$	564.15	Vicenin-1	563.1490; 503.1179; 473.1081; 383.0680; 353.0667
15	22.03	$[M-H]^-$	579.1353	−0.2	$C_{26}H_{28}O_{15}$	580.14	Luteolin-6-C-β-D-glucoside-8-*C*-*β*-D-xyloside	429.0799; 399.0710; 369.0616
16	22.36	$[M-H]^-$	625.1396	−1.4	$C_{27}H_{30}O_{17}$	626.15	Quercetin-3-*O*-sophoroside	625.1370; 463.0864; 301.0338

(Continued)

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TABLE 1 Continued

No.	$T_{(min)}$	Ion	*m/z*	ppm	Formula	Mol. wt.	Name	MS/MS data
17	22.93	$[M-H]^-$	563.1404	0.6	$C_{26}H_{28}O_{14}$	564.15	Schaftoside	563.1398; 503.1182; 473.1082; 383.0770; 353.0664
18	23.07	$[M-H]^-$	447.0932	−0.2	$C_{21}H_{20}O_{11}$	448.10	Luteolin-6-*C*-*β*-D-glucoside	411.0716; 357.0613; 327.0495; 298.0475; 285.0412
19	24.91	$[M-H]^-$	563.1426	4.5	$C_{26}H_{28}O_{14}$	564.15	Vicenin-3	563.1391; 473.1082; 443.0974; 383.0768; 353.0663
20	25.14	$[M-H]^-$	593.1521	1.0	$C_{27}H_{30}O_{15}$	594.16	Glucosyl-vitexin	431.0946; 311.0549; 293.0463; 59.0139
21	25.56	$[M-H]^-$	563.1404	−0.2	$C_{26}H_{28}O_{14}$	564.15	Neoschaftoside	473.1073; 444.1005; 413.0873; 383.0770; 353.0649; 311.0548
22	25.87	$[M-H]^-$	533.1309	1.1	$C_{25}H_{26}O_{13}$	534.14	Apigenin-6-*C*-*β*-D-xyloside-8-*C*-*α*-L-arabinoside-	413.0855; 383.0770; 353.0660
23	26.07	$[M-H]^-$	609.1469	2.2	$C_{27}H_{30}O_{16}$	610.15	Quercetin-7-*O*-rutinoside	609.1433; 463.0865; 301.0333; 300.0277; 271.0242
24	26.52	$[M-H]^-$	609.1484	4.7	$C_{27}H_{30}O_{16}$	610.15	Rutin	609.1455; 301.0346; 300.0283; 178.9980
25	27.46	$[M-H]^-$	463.0897	4.4	$C_{21}H_{20}O_{12}$	464.10	Isoquercitrin	301.0281; 300.0281; 271.0254; 255.0302; 178.9985
26	29.05	$[M+H]^+$	535.1457	1.0	$C_{25}H_{26}O_{13}$	534.14	Apigenin-6-*C*-*α*-L-arabinoside-8-*C*-*β*-D-xyloside	463.0998; 433.0909; 403.0807; 391.0804; 379.0806; 325.0697; 307.0595
27	29.38	$[M-H]^-$	549.0882	0.2	$C_{24}H_{22}O_{15}$	550.10	Quercetin 3-*O*-(6″-malonyl-glucoside)	549.0832; 505.0937; 463.1016; 301.0437; 300.0274; 271.0244; 255.0295
28	29.76	$[M-H]^-$	593.1517	1.7	$C_{27}H_{30}O_{15}$	594.16	Kaempferol-3-*O*-rutinoside	593.1506; 285.0398; 284.0323; 255.0293; 151.0030
29	30.45	$[M+H]^+$	565.1561	0.7	$C_{26}H_{28}O_{14}$	564.15	Apigenin-6-*C*-arabinosyl-(1→2)-*O*-*β*-D-glucoside	367.0808; 349.0704; 337.0704; 313.0702; 283.0598
30	30.82	$[M+H]^+$	565.1538	−3.4	$C_{26}H_{28}O_{14}$	564.15	Apigenin-8-*C*-glucosyl-(1→2)-*α*-L-arabinoside	367.0810; 349.0697; 337.0704; 313.0702; 283.0601
31	30.83	$[M-H]^-$	447.0942	3.3	$C_{21}H_{20}O_{11}$	448.10	Astragalin	284.0320; 255.0293; 227.0343; 151.0034
32	31.60	$[M-H]^-$	477.1038	−0.3	$C_{22}H_{22}O_{12}$	478.11	Isorhamnetin-3-*O*-glucoside	314.0426; 299.0176; 285.0401; 271.0237; 257.0448; 243.0291
33	31.86	$[M-H]^-$	505.0987	−0.1	$C_{23}H_{22}O_{13}$	506.11	Quercetin 3-*O*-(6″-acetyl-glucoside)	300.0273; 271.0243; 225.0307
34	33.76	$[M-H]^-$	533.0935	−0.3	$C_{24}H_{22}O_{14}$	534.10	Kaempferol 3-*O*-(6″-malonylglucoside)	284.0326; 255.0299; 229.051

3.7 Determination of antioxidants by the online UPLC-PDA-MS-ABTS·+ scavenging

To preliminarily screen out antioxidant phytochemicals in DOF-W, in the present study, UPLC coupled with PDA-Qda and ABTS-based assay was performed. The constituents, for which MS data and reference compounds were available, can be identified from positive peaks in Supplementary Figure S2. The area of reordered negative peaks on the lower chromatogram at 734 nm conforms to the ABTS radical scavenging activity of individual compounds of the DOF extract. As it may be judged from the size of the negative peaks in the chromatogram, 1-*O*-caffeoyl-*β*-D-glucoside (compound 5), vicenin-2 (compound 10), luteolin-6-*C*-*β*-D-xyloside-8-*C*-*β*-D-glucoside (compound 11), quercetin-3-*O*-sophoroside (compound 16, rutin (compound 24), isoquercitrin (compound 25), and quercetin 3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside (compound 27) were the strongest ABTS radical scavengers in the investigated extract. Since components 10 and 11 are not well separated, it is a challenge to distinguish which component is mainly responsible for the negative peak and needs further confirmation by extra experiments. In a previous report (30), by employing precolumn DPPH and ABTS assay followed by HPLC-DAD analysis, 1-*O*-caffeoyl-*β*-D-glucoside, rutin, and isoquercitrin were identified as major components with obvious scavenging free radical abilities in methanol extract of DOF.

3.8 *In vitro* pro-health potency of specific compounds from DOF extracts

In consideration of the time-consuming process of isolating components in flowers and to further identify and confirm the

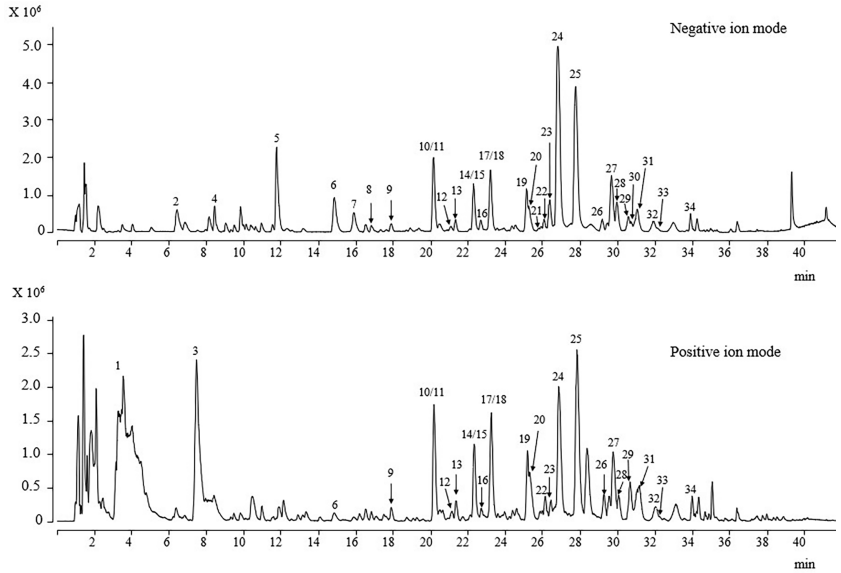

FIGURE 7
UPLC-ESI/MS/MS total ion chromatogram of DOF-W.

biological activities of components that may contribute to the antioxidative, anti-inflammatory, and anti-glycation properties of the extract, specific available compounds were selected for more detailed investigation on the assays of DPPH, ABTS, FRAP, COX-2, and BSA/reducing sugar glycation. The results of these isolated compounds are shown in Table 2.

In view of the limitations of different antioxidant methods, the use of at least two or more assays with different mechanisms of oxidation is strongly recommended. Three *in vitro* assays (DPPH, ABTS, and FRAP) were performed to comprehensively analyse the antioxidant capacities of selected compounds (39). These examined compounds demonstrated various influences on these functional activities. All compounds were observed to display significant scavenging capacities against the ABTS radical, with IC_{50} values ranging from 6.63 ± 0.41 μM (compound 16) to 96.39 ± 2.11 μM (compound 14). However, as for the DPPH radical and FRAP assay, some compounds (compounds 10, 14, 17, 19, 21, 26, 28, and 31) showed a low level of inhibition with IC_{50} values that were even higher than 5,000 μM, whereas other compounds (compounds 11, 15, 16, 18, 24, 25, and 27) exhibited extraordinary inhibition, with IC_{50} of DPPH varying from 24.70 ± 0.96 to 226.93 ± 47.26 μM and EC_1 of FRAP ranging from 205.0 ± 12.2 to 1,100.3 ± 138.4 μM, respectively. The remaining compound 32 had a relatively weak inhibition, with an IC_{50} of DPPH of 2,366.67 ± 87.61 and an EC_1 of FRAP of 2,757.3 ± 103.8. Based on previous reports, flavonoids appear to exhibit anti-inflammatory properties through the modulation of ROS (40). According to our findings, our data from the anti-COX-2 assay indeed revealed a similar tendency, which was in line with the results observed in the DPPH radical and FRAP assays. Compounds 11, 15, 16, 18, 24, 25, and 27 showed great COX-2 inhibition, with the lowest IC_{50} being 7.9 ± 2.4 μM, as opposed to no inhibitory effects of compounds 10, 14, 17, 19, 21, 26, 28 and 31. Similar to the antioxidant results (DPPH and FRAP) of compound 32, its inhibitory effect on COX-2 was weak with an IC_{50} of 535.3 ± 150.0 μM.

Analysis of the influence on AGE formation illustrated that all compounds were observed to display excellent anti-AGE effects, with IC_{50} ranging from 209.2 ± 3.1 to 374.4 ± 18.1 μM. All the results are very close to that of rutin (IC_{50}, 222.03± 13.49 μM), which is not only the main component of DOF-W extracts but also a well-known AGE inhibitor, and the inhibitory effects of all compounds are even better than aminoguanidine hydrochloride (1,604.1 ± 104.5 μM), another frequently used synthetic AGE inhibitor.

4 Discussion

In the current study, the results demonstrated that DOF-W exhibited promising antioxidant capacity (DPPH, ABTS, FRAP, intracellular ROS level in NHEK cells), anti-COX-2 effect, anti-glycation potency (inhibition of non-enzymatic glycation reaction and inhibition of CML expression in fibroblasts), and anti-aging effect (SA-β-gal staining test and collagen expression in fibroblasts). In addition, chemical and cellular anti-glycation activity as well as the anti-COX-2 effect on DOF-W were reported for the first time.

Oxidative stress and inflammation caused by unstable free radicals, which are highly deleterious to cells and skin, are both major contributors to the aging process (Wang, 2021). Compared with younger skin, elderly skin is more susceptible to environmental stimuli and needs external support such as antioxidants. It is demonstrated that DOF-W had obvious antioxidant and anti-inflammatory effects based on chemical, enzymatic, and cellular methods. Previous studies indicated that DOF-W could increase the antioxidant status and inhibit the inflammatory response in alcohol-impaired mice (Wu et al., 2020), which is consistent with our experimental results. Online UPLC-PDA-MS-ABTS·+

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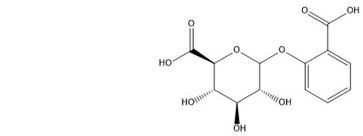
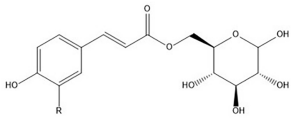
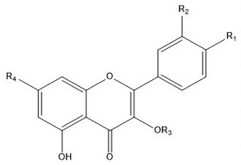


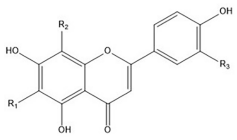
Salicylic Acid 2-*O*-*β*-D-Glucoside **(4)**



	R
1-*O*-caffeoyl-*β*-D-glucoside (**5**)	OH
1-*O*-(4-Coumaroyl)-*β*-D-Glucose or isomer (**6 or 7**)	H



	R_1	R_2	R_3	R_4
Quercetin 3-*O*-glucosyl-rutinoside (**8**)	OH	OH	glu-rha-(1-6)-glu	OH
Isorhamnetin-3-*O*-neohesperidoside (**13**)	OH	OCH_3	glu-rha	OH
Quercetin-3-*O*-sophoroside (**16**)	OH	OH	glu-(1-2)-glu	OH
Quercetin-7-*O*-rutinoside (**23**)	OH	OH	OH	rha-(1-6)-glu
Rutin (**24**)	OH	OH	rha-(1-6)-glu	OH
Isoquercitrin (**25**)	OH	OH	glu	OH
Quercetin-3-*O*-(6''-malonyl-glucoside) (**27**)	OH	OH	malonyl-glu	OH
Kaempferol-3-*O*-rutinoside (**28**)	OH	H	rha-(1-6)-glu	OH
Astragalin (**31**)	OH	H	glu	OH
Isorhamnetin-3-*O*-glucoside (**32**)	OH	OCH_3	glu	OH
Quercetin 3-*O*-(6''-acetyl-glucoside) (**33**)	OH	OH	acetyl-glu	OH
Kaempferol 3-*O*-(6''-malonylglucoside) (**34**)	OH	H	malonyl-glu	OH



	R_1	R_2	R_3
Luteolin-6-C-β-D-glucoside -8-C-β-D-galactoside (**9**)	glu	gal	OH
Vicenin-2 (**10**)	glu	glu	H
Luteolin-6-C-xyloside-8-C-glucoside (**11 or 12**)	xyl	glu	OH
Vicenin-1 (**14**)	xyl	glu	H
Luteolin-6-C-glucoside-8-C-xyloside (**15**)	glu	xyl	OH
Schaftoside (**17**)	glu	*α*-L-ara	H
Luteolin-6-C-β-D-glucoside (**18**)	glu	H	OH
Vicenin-3 (**19**)	glu	xyl	H
Glucosyl-vitexin (**20**)	H	glu-glu	H
Neoschaftoside (**21**)	glu	*β*-L-ara	H
Apigenin-6-C-*β*-D-xyloside-8-C-*α*-L- arabinoside (**22**)	xyl	ara	H
Apigenin-6-*C*-*α*-L-arabinoside-8-*C*-*β*-D-xyloside (**26**)	ara	xyl	H
Apigenin-6-*C*-arabinosyl-(1→2)-*O*-*β*-D-glucoside (**29**)	ara-(1-2)-glc	H	H
Apigenin-8-*C*-glucosyl-(1→2)-*α*-L-arabinoside (**30**)	H	glu-(1-2)-ara	H

FIGURE 8
The chemical structures of main herb markers in the DOF-W.

scavenging results indicated that seven compounds (5, 10 or 11, 16, 24, 25, and 27) are most likely to be major contributors to the overall antioxidant potential of DOF-W extracts. However, although all selected compounds displayed an excellent capacity to scavenge ABTS radicals, only compounds 11, 15, 16, 18, 21, 24, 25, and 27 showed great antioxidant potency with respect to DPPH and FRAP assays. Due to the absence of standard 5, its authentic antioxidant capacity cannot be verified in this paper. Combining the results of online and traditional chemical methods, compounds 11, 16, 24, 25, and 27 were predicted to be the main antioxidants of DOF-W extract. Based on previous reports, flavonoids appear to exhibit anti-inflammatory properties *via* the modulation of ROS (40). The anti-cyclooxygenase-2 assay was indeed observed to reveal a similar tendency as the results of the DPPH radical and FRAP assays. Only compounds 11, 15, 16, 18, 21, 24, 25, and 27 showed outstanding COX-2 inhibitory effects. According to our study, the antioxidant and anti-inflammatory effects of DOF-W extract are more likely to be attributed to compounds 11, 15, 16, 18, 24, 25, and 27. Luteolin *C*-glycosylflavones such as compounds 11, 15, and 18 demonstrated great antioxidant and COX-2 inhibitory effects. On the other hand, though apigenin, as one of the most widely distributed flavonoids in the plant kingdom and most frequently studied by researchers, is characterized as a fantastic free-radical scavenger and a remarkable anti-inflammatory agent (41), apigenin *C*-glycosylflavones such as compounds 10, 14, 17, 19, 21, and 26 exhibited very weak biological effects. Among these compounds, compounds 11 and 15 possess the same glycosidic bond as compounds 14 and 19, respectively. Therefore, it looks like that the type of aglycone rather than *C*- or *O*-glycosides of flavonoids, had a great effect on antioxidant and COX-2 inhibitory potency. It has also been reported that both luteolin and apigenin *C*-glycosylflavones had much lower inflammatory effects than those observed with their corresponding aglycones and *O*-glycosides in LPS-induced RAW264.7 (42). Our results may be explained by the speculation that *C*-glycosylation of apigenin leads to a reduction of antioxidant and anti-inflammatory potential. Since most of the

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TABLE 2 Antioxidant (DPPH, ABTS, FRAP), anti-cyclooxygenase (COX-2), and AGE inhibition activity (BSA/reducing sugar reaction) (% of inhibition) of various compounds from *Dendrobium officinale* flower-aqueous extract.

No.	Compound name	DPPH	ABTS	FRAP	COX-2	BSA-glycation
		IC_{50} (μM)	IC_{50} (μM)	EC_1 (μM)	IC_{50} (μM)	IC_{50} (μM)
10	Vicenin-2 (apigenin 6,8-C-diglucoside)	>5,000	74.41 ± 0.69	>5,000	nd	257.67 ± 7.56
11	Luteolin 6-*C*-*β*-D-xyloside-8-*C*-*β*-D-glucoside	226.93 ± 47.26	15.90 ± 0.19	1,009.1 ± 143.3	14.2 ± 3.4	251.6 ± 5.1
14	Vicenin-1 (apigenin 6-*C*-*β*-D-xyloside-8-*C*-glucoside)	>5,000	96.39 ± 2.11	>5,000	nd	255.63 ± 5.44
15	Luteolin-6-*C*-*β*-D-glucoside-8-*C*-*β*-D-xyloside	145.13 ± 51.37	13.80 ± 0.82	1,100.3 ± 138.4	13.9 ± 2.4	293.6 ± 13.3
16	Quercetin-3-*O*-sophoroside	172.13 ± 76.03	6.63 ± 0.41	949.6 ± 41.9	11.0 ± 0.7	295.7 ± 8.5
17	Schaftoside (apigenin 6-*C*-*β*-D-xyloside-8-*C*-arabinoside)	>5,000	75.59 ± 12.12	>5,000	nd	250.30 ± 4.16
18	Luteolin-6-*C*-*β*-D-glucopyranoside	102.28 ± 6.20	7.10 ± 0.10	506.6 ± 78.3	7.9 ± 2.4	316.6 ± 15.6
19	Vicenin-3 (apigenin 6-*C*-*β*-D-glucoside-8-*C*-xyloside)	>5,000	74.76 ± 0.92	>5,000	nd	242.90 ± 11.97
21	Neoschaftoside	>5,000	21.45 ± 0.24	>5,000	nd	430.3 ± 8.0
24	Rutin	43.33 ± 1.75	33.09 ± 0.37	205.0 ± 12.2	10.3 ± 2.8	222.03 ± 13.49
25	Isoquercitrin	197.30 ± 42.07	10.67 ± 0.03	520.2 ± 17.9	15.4 ± 3.7	212.7 ± 10.4
26	Apigenin-6-*C*-*α*-L-arabinoside-8-*C*-*β*-D-xyloside	>5,000	80.36 ± 0.77	>5,000	nd	272.7 ± 4.7
27	Quercetin 3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside	24.70 ± 0.96	28.36 ± 0.47	826.7 ± 98.5	12.5 ± 2.9	288.2 ± 17.9
28	Kaempferol-3-*O*-rutinoside	>5,000	35.01 ± 0.22	>5,000	nd	374.4 ± 18.1
31	Astragaline (kaempferol 3-*β*-D-glucopyranoside)	>5,000	34.31 ± 0.10	>5,000	nd	275.7 ± 33.0
32	Isorhamnetin-3-*O*-glucoside	2,366.67 ± 87.61	44.71 ± 1.52	2,757.3 ± 103.8	535.3 ± 150.0	209.2 ± 3.1
Positive	Trolox	158.87 ± 4.47	145.67 ± 1.22	559.8 ± 19.4		
	Celecoxib				89.7 ± 6.4	
	Aminoguanidine hydrochloride					1,604.1 ± 104.5

nd, not detected; values represented as mean ± standard deviation (n = 3); values in the same columns followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test.

flavonoids in plants exist primarily as *O*-glycosides, *C*-glycoside flavonoids received relatively less attention than flavonoid *O*-glycosides, especially in the absence of comprehensive studies on their biological benefits. It is more purposeful to explore the pharmacokinetic properties of flavonoid *C*-glycosides and their bioactivities.

Numerous studies have also shown that external stimuli such as excessive free radicals and spontaneous AGE generation *in vivo*, which are irreversible once formed in the body (11), are associated with skin fibroblast damage, destruction of collagen and elastic fibres, a yellow complexion without splendour, and aging deterioration (12). DOF-W extract displayed excellent capacity to

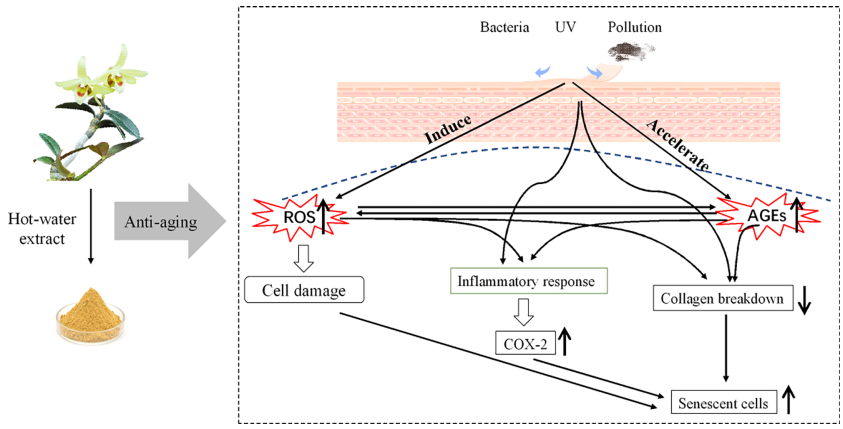

FIGURE 9
Graphic review of the anti-aging effect of *D. officinale* flower aqueous extract.

inhibit AGE formation with low IC_{50} values and decreased CML expression obviously in MGO-induced fibroblasts. In addition, all selected compounds were observed to have a potent capacity to inhibit AGE formation. Therefore, anti-glycation ability of DOF-W may be justified by the synergistic action of most polyphenolic compounds present in the extract. So far, several studies have reported that various flavonoids, both *O*- and *C*-glycosides, can cause a marked decrease on AGE production in several *in vitro* and *in vivo* experimental models (43, 44).

Human skin fibroblasts, which primarily exist in the dermis, can form a large amount of collagen, which is a key factor in maintaining skin elasticity. Studies have reported that skin aging may be related to the accumulation of aging fibroblasts within our skin (Wlaschek et al., 2021). Both immunofluorescence assay and extracellular ELISA assay showed that DOF-W extracts can increase the expression of collagen type I, but they have no obvious effect on collagen type III. Moreover, DOF-W can also significantly decrease the proportion of D-Gal-induced senescent cells. In previous research, it had been reported that the *Dendrobium officinale* flower can alleviate brain aging and improve spatial learning abilities in senescent rats (23). Continuous efforts should be made for the detailed identification of representative bioactive constituents in DOF, which may be followed by a systematic clinical study on suitable animal models and humans.

5 Conclusion

Based on the current research regarding *in vitro* studies and phytochemistry analysis, it is suggested that DOF-W is potent with antioxidation, anti-glycation, and anti-aging effects (Figure 9) and deserves further research and development. Both DOF-W and its specific compounds might be promising agents for skin anti-aging.

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 below: MTBLS6933 (MetaboLights).

Author contributions

Data curation: HZ and RY. Funding aquisition: RY. Project administration: LZ. Supervision: BL and RY. Validation: HZ. Writing original draft: HZ. Writing review and editing: HZ and RY. All authors contributed to the article and approved the submitted version.

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Conflict of interest

HZ and BL are employed by Amway (Shanghai) Innovation and Science Co., Ltd. LZ is employed by Shanghai Archgene Biotechnology Co. Ltd. BL is employed by company Amway (China) Botanical R&D Center.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1095848/full#supplementary-material

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