ADVANCED PERSPECTIVES IN CELL THERAPY AND CORRELATED IMMUNOPHARMACOLOGY

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ADVANCED PERSPECTIVES IN CELL THERAPY AND CORRELATED IMMUNOPHARMACOLOGY

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REVIEW
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Necroptosis: A Novel Pathway in Neuroinflammation

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Neuroinflammation is a complex inflammatory process in the nervous system that is expected to play a significant role in neurological diseases. Necroptosis is a kind of necrosis that triggers innate immune responses by rupturing dead cells and releasing intracellular components; it can be caused by Toll-like receptor (TLR)-3 and TLR-4 agonists, tumor necrosis factor (TNF), certain microbial infections, and T cell receptors. Necroptosis signaling is modulated by receptor-interacting protein kinase (RIPK) 1 when the activity of caspase-8 becomes compromised. Activated death receptors (DRs) cause the activation of RIPK1 and the RIPK1 kinase activity-dependent formation of an RIPK1-RIPK3-mixed lineage kinase domain-like protein (MLKL), which is complex II. RIPK3 phosphorylates MLKL, ultimately leading to necrosis through plasma membrane disruption and cell lysis. Current studies suggest that necroptosis is associated with the pathogenesis of neuroinflammatory diseases, such as Alzheimer's disease, Parkinson's disease, and traumatic brain injury. Inhibitors of necroptosis, such as necrostatin-1 (Nec-1) and stable variant of Nec (Nec-1s), have been proven to be effective in many neurological diseases. The purpose of this article is to illuminate the mechanism underlying necroptosis and the important role that necroptosis plays in neuroinflammatory diseases. Overall, this article shows a potential therapeutic strategy in which targeting necroptotic factors may improve the pathological changes and clinical symptoms of neuroinflammatory disorders.

Keywords: neuroinflammation, necroptosis, ripk1, ripk3, mlkl, necrostatin-1

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BACKGROUND

Inflammation, which is usually caused by injury or infection (Andersson and Tracey, 2011), are fundamentally distinguished by pathology (Kearney and Martin, 2017) between acute and chronic forms of inflammation. Acute inflammation responds to irritants in the early stages, which is an essential response that prepares the body to repair damaged areas during acute inflammation and neuroinflammation, including traumatic brain injury (TBI), stroke, and encephalitis (Huang et al., 2018a; Zhang et al., 2018; Venkatesan et al., 2019). Chronic inflammation is caused by persistent stimuli, which leads to injury of the nerve tissue, resulting in neurodegeneration and inducing neuroinflammation into a vicious cycle (Nasef et al., 2017), including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Dong et al., 2018; Ren et al., 2018; Lee et al., 2019). A variety of immunocytes are involved in inflammation (Mack, 2018; Tsai et al., 2018). Acute inflammation is dominated by neutrophil infiltration (Pinho-Ribeiro et al., 2018; Kapur

et al., 2019), whereas chronic inflammation is often accompanied by macrophage and lymphocyte infiltration (Cao et al., 2019; Faissner et al., 2019; Lodygin et al., 2019; Na et al., 2019).

Neuroinflammation is a complex inflammatory response of the nervous system that may be triggered by various pathogens or toxins and induce immunocyte infiltration and activation (Stephenson et al., 2018). Eventually, the effects lead to neuronal and/or axonal degeneration or death (Estes and McAllister, 2016). A feature essential to maintain neuroplasticity is neuroinflammatory homeostasis. Homeostasis is regulated by the interaction between neurons, glial cells and vascular endothelial cells. Homeostatic imbalance caused by different reasons (such as injury, infection or stress) may have similar pathological manifestations (DiSabato et al., 2016).

However, the mechanisms of neuroinflammation remain unclear in different situations. To better understand the inflammatory disease neuritis, acute and chronic neuroinflammation will be discussed separately in the following sections.

NECROPTOSIS

Historically, two forms of cell death, apoptosis and necrosis, have been recognized because of their important roles in homeostasis, development, and pathogenesis (Pasparakis and Vandenabeele, 2015). In the past, necrosis was thought to be accidental death due to excessive cytotoxic damage, carried out *via* conventional molecular events (Vanden Berghe et al., 2014). In contrast, apoptosis is defined as programmed cell death. Under a microscope, apoptosis is characterized by apoptotic bodies, nuclear pyknosis and fragmentation, and an intact cell membrane (Kerr et al., 1972; Choo et al., 2019). However, an increasing number of studies have described another form of necrosis that performs as programmed and regulated cell death, named necroptosis.

Morphologically, necroptosis has the following characteristics: (1) it resembles necrosis–dying cells cluster together, with disrupted membranes, swollen cell bodies and organelles, and fragmented chromatin; (2) a large quantity of inflammasomes; (3) autophagy (Vanden et al., 2013). Compared with apoptosis, necroptotic cells passively pass through the damaged membrane into the extracellular matrix (Zhang et al., 2017a).

At the molecular level, intracellular and extracellular stimuli and corresponding ligands of the death receptor family trigger necrosis (Zhou and Yuan, 2014). The currently known key components of the necroptotic signaling pathway are receptor-interacting protein kinase 3 (RIPK3) and its substrate, mixed lineage kinase domain-like protein (MLKL), which is a pseudokinase (Yuan et al., 2019). Moreover, small molecules, such as necrostatin-1 (Nec-1), Nec-5, and Nec-7, are thought to inhibit the necroptotic signaling pathway (Gonzalez-Juarbe et al., 2015; Strilic et al., 2016; Fuji et al., 2018). Nec-1 is an ATP-competitive allosteric inhibitor of RIPK1. In a mouse stroke model, Nec-1 was able to reduce brain damage caused by reperfusion (Chu et al., 2018). MicroRNA-155 is also considered an inhibitor of RIPK1 (Liu et al., 2011).

Necroptosis is involved in many pathological processes, such as trauma, cerebral ischemia-reperfusion injury, and inflammatory diseases (Wang et al., 2012; Li et al., 2019; Ni et al., 2019; Zhang et al., 2019). Necroptosis has been reported to play a crucial role in the pathogenesis of certain neuroinflammatory disorders (Ito et al., 2016; Chia et al., 2018; Zhang et al., 2019). Because necroptosis shows the potential to be a target for intervention in neuroinflammatory disorders, it has garnered increasing focus from researchers. In our review, the molecular mechanisms of necroptosis and the role of necroptosis in the development and progression of acute and chronic neuroinflammatory disorders will be described, which may be helpful for finding treatments for neuroinflammatory diseases.

NECROPTOSIS PATHWAYS

Mechanisms Regulating TNFR1-Induced Necroptosis

Tumor necrosis factor receptor 1 (TNFR1), which has been reported by studies of TNF signaling in necroptosis, induces the expression of many genes that regulate inflammation (Liu et al., 2014). However, under some conditions, TNF-α also induces cell death (Annibaldi and Meier, 2018; Lafont et al., 2018). TNF-induced cell death requires TNF-α to bind to TNFR1 on the cell membrane and recruit a series of proteins in the cell to form different complexes (Micheau and Tschopp, 2003; Amin et al., 2018). Among them, complex I includes TNFR-associated death domain (TRADD), RIPK1, TNFR-associated factor 2 (TRAF2), the cellular inhibitor of apoptosis protein 1 (cIAP1), cylindromatosis, and the ubiquitin complex (Amin et al., 2018) (**Figure 1**).

An I_kB kinase (IKK) complex consisting of a subunit essential regulator (nuclear factor-kappa B essential modulator, NEMO, also named IKKγ) and two catalytic subunits (IKKα and IKKβ) plays an important role in mediating immunoinflammatory responses and promoting cell survival and oncogenesis (Silke and Brink, 2010). NEMO recruits IKKα/IKKβ, resulting in the rapid and selective IKK-mediated phosphorylation of $I_kB\alpha$. $I_kB\alpha$ activates $NF\text{-}_kB$ and upregulates genes encoding prosurvival and proinflammatory molecules.

In complex I, E3 ligase (e.g., cIAP1) rapidly ubiquitinates RIPK1. The ubiquitination of RIPK1 is important for regulating its kinase activity. Inhibiting RIPK1 ubiquitination by antagonizing E3 ligase leads to an increased sensitivity of cells to TNF-induced necroptosis (Feoktistova et al., 2011). When NF-κB activation is inhibited, deubiquitinated RIPK1, Fas-associated death domain (FADD) and procaspase-8 are assembled into the death-inducing signaling complex, and the complex finally dissociates from the plasma membrane, now referred to as Complex IIa. Complex IIa is involved in apoptosis by affecting the activation of caspase-8 and the subsequent cleavage of RIPK1 (Lin et al., 1999). When RIPK1 is deubiquitinated, the formation

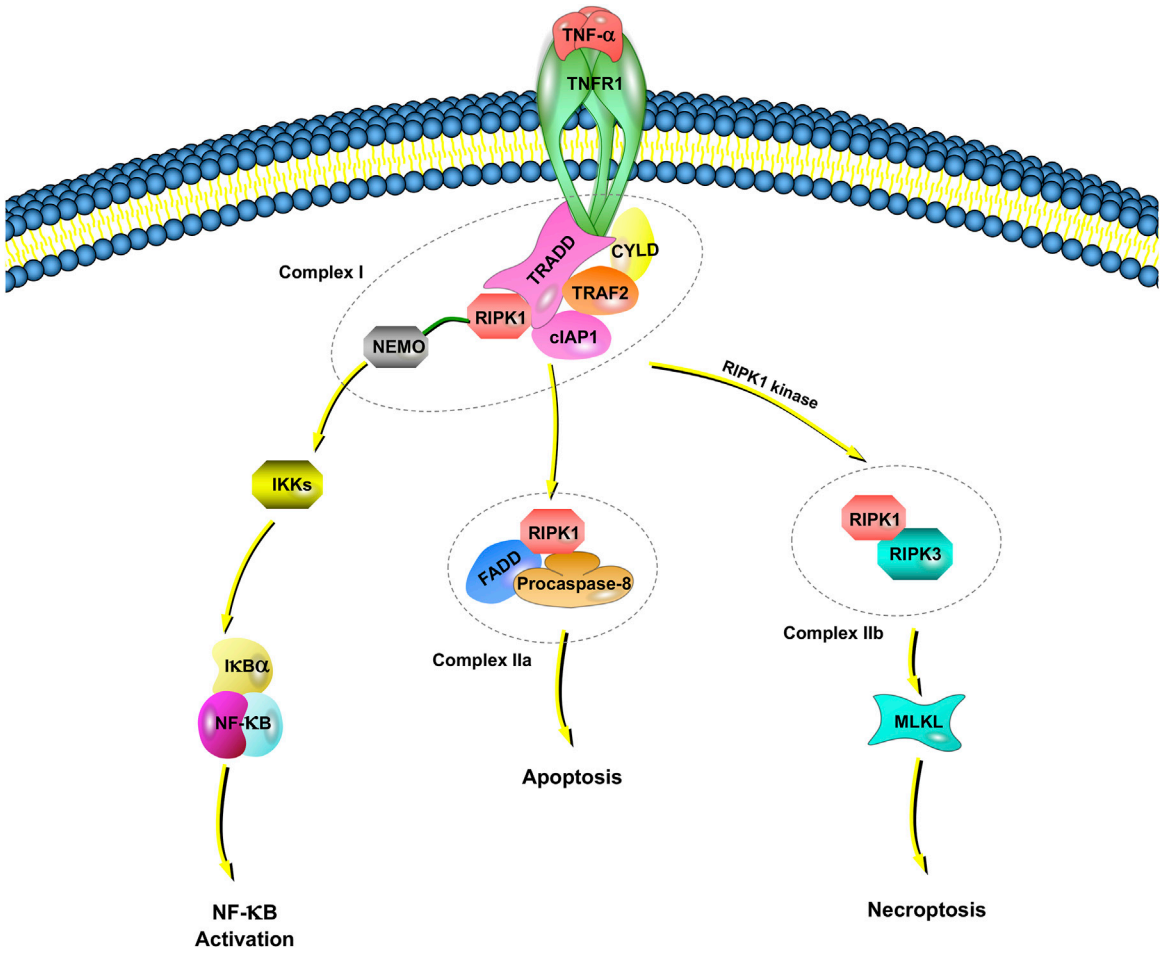

FIGURE 1 | TNFR1-dependent necroptosis pathways. TNF-α binds to TNFR1 and recruits a series of proteins, including TRADD, RIPK1, TRAF2, cIAP1, CYLD, and NEMO, which is called complex I. NEMO recruits IKKα/IKKβ, resulting in the IKK-mediated phosphorylation of IκBα. Once IκBα is phosphorylated, NF-κB signaling pathways are activated. When NF-κB activation is inhibited, deubiquitinated RIPK1, FADD and procaspase-8 are assembled as complex IIa. Complex IIa is involved in apoptosis by activated caspase-8 and cleavage RIPK1. When there is a lack of caspase-8, the cIAP1 in complex I rapidly ubiquitinates RIPK1, leading to the combination of RIPK1 and RIPK3, which is called complex IIb. Complex IIb leads to MLKL-mediated necroptosis.

of complex IIb, which is also called the necrosome, is facilitated by RIPK1, RIPK3, MLKL, FADD and procaspase-8.

Recently, cellular FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP), a noncatalytic inactive homolog of caspase-8, was reported to take part in the regulation of necroptosis (Tsuchiya et al., 2015). If c-FLIP long is involved in the composition of the heterodimer, caspase-8 maintains high proteolytic activity to inhibit the association of RIPK1, RIPK3 and FADD, thus suppressing necroptosis (Tsuchiya et al., 2015). However, caspase-8 has no proteolytic activity if it is formed by c-FLIP short; RIPK1 and RIPK3 can therefore be assembled to promote necroptosis. Inhibitors of the necroptotic signaling pathway, such as Nec-1, Nec-1s (a stable variant of Nec-1) and other small molecules, have been widely applied to elucidate the molecular mechanisms of necroptosis (Takahashi et al., 2012). Necroptosis is also regulated by the pseudokinase MLKL, which is a functional RIP3 substrate. The subsequent conformational change in MLKL causes rapid cell membrane breakage (a morphological sign of necrosis) by the formation of disulfide bond-dependent amyloid-like polymers (Wang et al., 2014).

Necroptosis Regulation by Toll-like Receptors

Unlike TNF-induced necroptosis, the molecular mechanisms of microbial-triggered necroptosis are more elusive (**Figure 2**). Innate immunocytes and macrophages, for example, detect microbial activities and initiate antimicrobial responses though pattern-recognition receptors (Kawai and Akira, 2010). Some of the most well-characterized members of the pattern-recognition receptor family are the TLRs (Fitzgerald and Kagan, 2020). TLRs respond to many pathogen-associated molecular patterns (bacteria, viruses, fungi, parasites, etc.) (Tartey and Takeuchi, 2017). TLR3 detects viral double-stranded RNA or artificial analogs, whereas TLR4 responds to lipopolysaccharide (LPS)

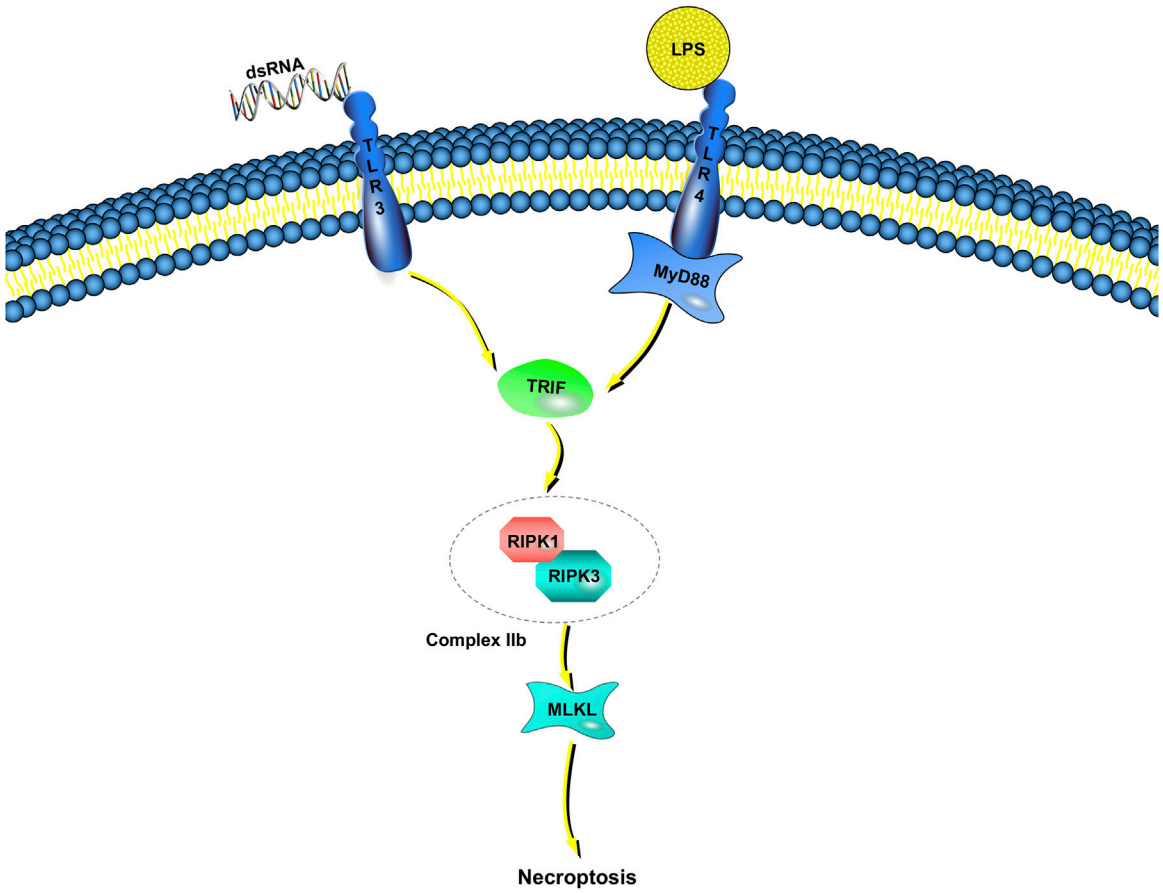

FIGURE 2 | TLR-dependent necroptosis pathways. Engagement of TLR3/TLR4 with dsDNA or LPS induces the interaction between TRIF and complex IIb, which is combined RIPK1 with RIPK3. If caspase-8 catalytic activity is impaired, complex IIb triggers MLKL-dependent necroptosis. TRIF is the only adaptor protein of TLR3, whereas the TLR4 pathway can be activated by either TRIF or myeloid MyD88.

(Alexopoulou et al., 2001; Fitzgerald et al., 2003). Toll/IL-1 receptor domain-containing adaptors are recruited after the binding of TLR3 and TLR4 to their corresponding ligands (Netea et al., 2009). Then, inflammatory cytokines are released, leading to type I interferon (IFN) responses. There is only one adaptor protein for TLR3, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β/TIR domain-containing adaptor molecule 1 (TRIF/TICAM-1), whereas the TLR4 pathway can be activated by either TRIF or myeloid differentiation factor 88 (MyD88) (Fitzgerald et al., 2001; Akira and Hoshino, 2003; Kagan et al., 2008).

In addition to these immune responses, LPS induces necroptosis instead of apoptosis in human macrophages when inhibitors (such as zVAD-fmk) suppress the activation of caspase-8 (Lawlor et al., 2015). Poly (I:C) leads to Jurkat cell (human leukemia T cell) apoptosis when combined with IFN-γ, but the absence of caspase-8 or FADD results in necroptosis rather than apoptosis (Alvarez-Diaz et al., 2016; Boyd-Tressler et al., 2017). Although these results show that necroptosis may be achieved through TLR3 and TLR4 pathways, how to proceed is still unclear (He et al., 2011).

NECROPTOSIS IN ACUTE NEUROINFLAMMATION

Traumatic Brain Injury

TBI is a disease caused by external forces or other pathological changes in the brain, accompanied by changes in brain function (Maas et al., 2008; McDonald et al., 2016). The mortality rate of severe TBI is high and is estimated at 30–40% in unselected populations in observational studies (Rosenfeld et al., 2012). Some TBI patients lose their lives, and even survivors suffer from enormous physical, mental, emotional and cognitive impairments that undermine the lives of patients and their families and cause enormous losses to society (Limb, 2014; Jenkins et al., 2016).

A series of studies in 2012 revealed that multiple cell death pathways participated in the development of TBI and that Nec-1 simultaneously inhibited apoptosis and autophagy (Wang et al., 2012). Pathological and biochemical changes related to necroptosis in a rat model of fluid percussion injury (FPI) were observed by Liu (Liu et al., 2016). In an early phase (6 h) after TBI, RIPK 1 and 3, MLKL, HMGB1 and proinflammatory

TABLE 1 | Necroptosis in acute neurodegenerative diseases.

Disease	Regulatory factors	Synthetic inhibitors	Comment	Reference
Traumatic brain injury	RIPK1	Nec-1	Hypothermia inhibited necroptosis pathway though down-regulation of RIPK1, in moderate TBI models of rats.	The, (2018)
			Necrostatin-1 inhibited apoptosis and autophagy simultaneously.	Wang et al. (2012)
	RIPK3		Oxidative stress, inflammation and apoptosis in astrocytes, which dependent on AMPKa activation, were attenuated by RIPK3-ablation.	Lakhan et al. (2009)
			RIPK3-knockout (KO) attenuated cognitive dysfunction and activation of glia cells in TBI injuryed mice	Lakhan et al. (2009)
	MLKL		RIPK1, MLKL and pro-inflammation cytokines increased in rat FPI models.	Zhang et al. (2017b)
Stroke	NLRP3		NLRP3 inflammasome was found in both immune cells and necroptotic neuron when caspase is inhibited by Q-VD-OPH	Bai et al. (2019)
	RIPK1	Nec-1	Pretreatment with Necrostatin-1 ameliorated cell death by reducing the interact of increased RIPK3 with RIPK1.	Teng et al. (2018)
	RIPK3		Expression level of RIPK3 was increased after ICH.	Teng et al. (2018)
Encephalitis	MLKL		In mice model, the expression of MLKL in neurons was upregulated when JEV infected, while deletion of MLKL mitigated the progression of JE and down-regulated the level of inflammatory factors.	Bian et al. (2017)
	RIPK3		RIPK3 restricts WNV pathogenesis by inhibiting necroptosis in a mouse WNV encephalitis.	Barnett, (2019)
			RIPK3-/- mice was more likely to survive compared to wild-type controls, while lacking the necroptotic effectors (such as MLKL, or both MLKL and caspase-8)	Barnett, (2019)

FBI, Fluid precussion injury; ICH, Intracerebral hemorrhage; JEV, Japanese encephalitis virus; MLKL, Mixed lineage kinase domain-like protein; Nec-1, Necrostatin-1; NLRP3, NLR Family Pyrin Domain Containing 3; RIPK1, Receptor-interacting protein kinase 1; RIPK3, Receptor-interacting protein kinase 3; TBI, Traumatic brain injury; WNV, West Nile Virus.

factors (such as TNF-α, IL-6 and IL-18) were increased in the cortex (Liu et al., 2016). Posttraumatic hypothermia (33 °C) led to decreases in necroptosis regulators, proinflammatory cytokines and brain injury in TBI rats compared to treatment with normal temperature (You et al., 2008). Notably, by targeting necroptosis signaling after TBI, the injured central nervous system (CNS) can be protected from tissue damage and inflammation (You et al., 2008). The following year, hypothermia was reported to significantly reduce RIPK-1 upregulation in moderate TBI rat models, which may inhibit the necroptosis pathway after moderate TBI (Zhang et al., 2017b). Moreover, cognitive dysfunction and glial activation could be observed in TBI mice, and these changes were attenuated in RIPK3-knockout (KO) mice (Liu et al., 2018). Notably, *in vitro* studies have shown that RIPK3-knockdown of astrocytes can reduce oxidative stress, inflammation and apoptosis, which is dependent on the activation of adenosine 5'-monophosphate-activated protein kinase-alpha (AMPKα) (The, 2018). In conclusion, inhibition of RIPK3 may be a therapeutic target against cerebral damage by suppressing immunoinflammatory responses, oxidative stress and apoptosis.

Stroke

Stroke is a broad term that includes diseases caused by blockage or bleeding of blood vessels that supply the brain (Lakhan et al., 2009; The, 2018). Its incidence remains high, while the number of approved treatment methods is low (Ceulemans et al., 2010). As society ages, the number of stroke patients continues to increase and will become an important socioeconomic burden, as 80% of stroke patients remain disabled (Durukan and Tatlisumak, 2007; Candelario-Jalil, 2009).

Ischemia-reperfusion injury (IRI) is a common feature when the blood supply is restored after a period of ischemia (Wu et al., 2018). Several studies have suggested that different mechanisms, including oxidative stress, leukocyte infiltration, platelet adhesion and aggregation, blood-brain barrier disruption, complement activation, and mitochondria-mediated mechanisms, are involved in the pathogenesis of IRI in the nervous system (Bavarsad et al., 2019). In 2005, scientists demonstrated that delayed ischemic brain injury in a mouse model was due to necroptosis, a mechanism different from apoptosis (Degterev et al., 2005). This finding shows a new therapeutic target for stroke with an extended period of time for neuroprotection. The researchers also identified a specific and potent small-molecule inhibitor of necroptosis, Nec-1 (Chu et al., 2018).

Studies in 2018 indicated that necroptosis is probably involved in intracerebral hemorrhage (ICH) (Chu et al., 2018). In an *in vivo* mouse ICH model, pretreatment with Nec-1 protected astrocytes (Zille et al., 2017). Intracerebroventricular treatment with Nec-1 was helpful in reducing cell death, cerebral edema, hematoma volume and neurological score insufficiency (Su et al., 2015; Shen et al., 2017). In addition, the expression level of RIPK3 was increased after ICH, and pretreatment with Nec-1 reduced the interaction between RIPK3 and RIPK1 and promoted cell survival after ICH (Shen et al., 2017). The nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome was reported to participate in necroptosis, accompanied by changes in inflammatory factors such as IL-1β. The study authors also found that NLRP3 is expressed not only in immune cells, such as microglia, but also in necroptotic neurons when caspase is inhibited by Q-VD-OPH (Teng et al., 2018). Furthermore, in an

TABLE 2 | Necroptosis in chronic neurodegenerative diseases.

Disease	Regulatory factors	Synthetic inhibitors	Comment	Reference
AD	RIPK1	Nec-1	Nec-1 reduced A and tau abnormalities in AD animal model.	Ofengeim et al. (2017)
			RIPK1-dependent transcription promoted microglia and lysosomal defects to increase accumulation of amyloid plaques	Hirsch and Hunot, (2009)
	MLKL		MLKL, which was required by necroptosis, was regulated by Flotillin and/or ALI syntenin-1 in AD.	Xu et al. (2017)
PD	RIPK1	Nec-1	Inhibiting th enzyme alleviated the progression of PD by blocking RIPK1 active	Dionísio et al. (2019)
			Nec-1 protected dopaminergic neurons against injury	Chia et al. (2018)
	RIPK3		The level of RIPK3 in the SN were increased in the autopsy of PD patients.	Wu et al. (2015)
	MLKL		The level of MLKL were found upregulated in the body of PD patients.	Wu et al. (2015)
	Parkin		The loss of parkin protected microglia cells from zVAD-induced necroptosis.	Re et al. (2014)
ALS	RIPK1	OPTN	The levels of RIPK1 were elevated in spinal cord extracts from Tg SOD1G93A	Politi and Przedborski, (2016)
			OPTN suppressed RIPK1-dependent necroptosis signaling by regulating its turnover.	Selik et al. (1984)
AIDS	Caspase-8		Upregulation of caspase-8 lead to disorder of HIV-specific CD8(+) T cell proliferation, by promoting necroptosis and cell death.	Gaiha et al. (2014)
Glaucoma and other retinopathy	RIPK1	Nec-1, Cpd27, RIC	Low-levels of RIPK1 and RIPK3 reduced microglia necroptosis when TLR4 def and suppressed retinal inflammation.	Cruz et al. (2018)
			Nec-1, Cpd27 and RIC inhibited downstream pathways following RIPK1 activate including necrosome composition and mitochondrial dysfunctio.	Qin et al. (2018)
	RIPK3		RIPK1-and RIPK3-dependent necroptosis existed in microglis of mice with degenerative, or acute retinal neural injury	Cruz et al. (2018)

Aβ, Amyloid-β; AD, Alzheimer's disease; AIDS, Acquired Immune Deficiency Syndrome; ALS, Amyotrophic lateral sclerosis; HIV-1, Human immunodeficiency virus 1; MLKL, Mixed lineage kinase domain-like protein; Nec-1, Necrostatin-1; OPTN, optineurin; PD, Parkinson's disease; RIC, RIPK1-inhibitory compound; RIPK1, Receptor-interacting protein kinase 1; RIPK3, Receptor-interacting protein kinase 3; SN, Substantia nigra; TLR, Toll-like receptor.

IRI rat brain model, NLRP3 inflammasome deficiency protects cerebral tissue from injury, suggesting that the NLRP3 inflammasome plays an important role in neuronal necroptosis.

Encephalitis

Some acute neuroinflammation is caused by viruses, such as Japanese encephalitis virus (JEV) and West Nile virus (WNV) (Turtle and Solomon, 2018; Bai et al., 2019). In Asia and the Western Pacific, the most common pathogen of viral encephalitis is JEV (Erlanger et al., 2009). JEV infection and inflammation lead to neuronal death, and subsequent cytotoxicity induces deterioration of Japanese encephalitis (JE) (Bai et al., 2019). In 2017, for the first time, necroptosis was discovered to be a reason for neuronal loss in the JE brain (Bian et al., 2017). When JEV infected neurons, the expression of MLKL was upregulated *in vitro* and *in vivo*. The loss of MLKL attenuates the progression of JE and the level of inflammatory factors in a rodent model (Bai et al., 2019).

The same year, RIPK3 was found to restrict WNV pathogenesis independently of cell death in a mouse model of WNV encephalitis (Daniels et al., 2017). Ripk3$^{-/-}$ mice showed higher mortality rates than wild-type (WT) mice, whereas mice with a deficiency of MLKL or MLKL and caspase-8 were little affected (Daniels et al., 2017). The expression of neuronal chemokines was inhibited in Ripk3$^{-/-}$ mice, and recruitment of T cells and other immunocytes in the CNS was reduced, which led to an enhanced susceptibility to death in Ripk3$^{-/-}$ mice. These studies demonstrate the multiple functions of RIPK3 in the pathogenesis of viral diseases. Thus, RIPK3 may be a key regulatory factor during CNS immune processes.

NECROPTOSIS IN CHRONIC NEUROINFLAMMATION

Alzheimer's Disease

AD is recognized by gradual memory worsening, personality disorders and a decline in general cognition (Scheltens et al., 2016; Barnett, 2019). It is the sixth leading cause of death in the United States, causing more than five million deaths (Alzheimer's Association., 2016). Neuropathologically, the main characteristic of AD is severe neuronal loss, paraprotein [tau, amyloid-β (Aβ)] accumulation, and significant neuroinflammation (Parbo et al., 2018; Whitwell et al., 2018). There is growing evidence that the pathogenesis of AD is not limited to the neuronal compartment but is closely related to immune mechanisms in the brain. However, the mechanisms of neuronal death remain unclear (Whitwell et al., 2018; Dionisio-Santos et al., 2019).

In 2013, Zhang et al., observed that Nec-1 prevents neural cells from degenerating in a mouse model of AD (Qinli et al., 2013). Next, Yang SH et al. researched Nec-1, which reduces Aβ and tau abnormalities to mitigate memory loss in an AD animal model (Yang et al., 2017). Caccamo and others observed necroptosis in postmortem brains of AD patients, which was closely related to brain weight and cognitive levels. In addition, they found that RIPK1 plays a crucial role at the transcriptome level in AD.

Moreover, they observed that inhibition of necroptosis reduced cell death in a mouse model of AD (Caccamo et al., 2017).

Etiologically, dysfunction of microglia plays a fundamental role in AD, and in 2017 it was found that RIPK1-dependent transcription promotes disease-associated microglia and lysosomal defects to mediate the accumulation of amyloid plaques in AD. The pseudokinase MLKL is moved from the cytosol to the plasma membrane after it is phosphorylated by the kinase RIPK3, which is required for necroptosis (Ofengeim et al., 2017). Fan et al., also found that phosphorylated MLKL was translocated from membranes through ALIX–syntenin-1–mediated exocytosis or flotillin-mediated endocytic lysosomal degradation (Fan et al., 2019). Thus, targeting RIPK1 and/or RIPK3 may provide an important therapeutic blueprint for the treatment of AD.

Parkinson's Disease

PD is the second most common neurodegenerative disorder. Pathologically, dopaminergic neurons in the substantia nigra (SN) pars compacta degenerate slowly and progressively (Hirsch and Hunot, 2009). The underlying causes of PD remain uncertain, but existing data support the significance of noncellular autonomic pathological mechanisms in PD, most of which are activated by glial cells and/or peripheral immune cells (Xu et al., 2017; Seo et al., 2020). This cell response to neurodegenerative changes can trigger harmful events (such as oxidative stress and cytokine receptor-mediated apoptosis), ultimately resulting in the death of dopamine cells and leading to disease progression (Liu et al., 2019; Trist et al., 2019).

Clinical studies report significant increases in dopaminergic neuron degeneration in the SN of PD patients. In contrast to the control group, RIPK1, RIPK3 and MLKL in the SN were increased at the autopsy of PD patients (Oñate et al., 2020). Downregulation of transforming growth factor β-activated kinase-1 (TAK1) may promote age-dependent PD in the CNS of aging humans. Mitochondrial and lysosomal dysfunction may promote intracellular RIPK1 activation and are prone to necroptosis. Inhibiting the enzyme can slow the progression of PD (Iannielli et al., 2018). Furthermore, scientists have performed some research on PC12 cells. Nec-1, which is associated with the apoptosis signaling pathway in this process, exerted a protective response against injury on dopaminergic neurons (Wu et al., 2015). In 2019, parkin was found to be an E3 ubiquitin ligase involved in PD, suggesting that parkin may alter inflammation and necroptosis by participating in ubiquitination events. The loss of parkin protected microglial cells from zVAD-induced necroptosis, thus accelerating primary neuronal injury caused by inflammation (Dionísio et al., 2019). However, further studies are needed for a detailed understanding of the mechanisms underlying these effects of necroptosis in PD.

Amyotrophic Lateral Sclerosis

ALS is an incurable, adult-onset paralytic disease that manifests as a sporadic disease. It is caused by a decrease in motor neurons in the spinal cord, brainstem, and brain, and 10% of all ALS cases are due to genetic mutations (Chia et al., 2018). For example, mutations in the gene superoxide dismutase 1 (SOD1) lead to familial amyotrophic lateral sclerosis (fALS).

In 2014, scientists used a co-culture model system to study human sporadic astrocyte co-cultured with human embryonic stem cell-derived motor neurons, and necroptosis was found as the central feature of the death of motor neurons (Re et al., 2014). Moreover, RIPK1- and RIPK3-mediated axonal damage has been shown to occur extensively in SOD1 transgenic mice and pathological tissues from ALS patients (Saccon et al., 2013). Mutations of the optineurin (OPTN) gene promoted a marked increase in the secretion of proinflammatory cytokines, as well as neuronal cell death, in both fALS and sporadic ALS (Toth and Atkin, 2018). A lack of OPTN induces the activation of necroptosis in oligodendrocytes, leading to Wallerian-like axonal degeneration (Ito et al., 2016). It was also found that OPTN actively suppressed RIPK1-dependent signaling by regulating its turnover. In contrast to WT oligodendrocytes, $OPTN^{-/-}$ oligodendrocytes are more susceptible to TNF-induced necroptosis, which can be inhibited by Nec-1s (Politi and Przedborski, 2016; Greco et al., 2018). Therefore, the necroptotic pathway is proposed as a novel possible target for the treatment of this incurable disease.

Acquired Immune Deficiency Syndrome

Human immunodeficiency virus 1 (HIV-1) is a 9.7 kb retrovirus found in 1983 that was recognized as the pathogene for an increasing lethal immunodeficiency syndrome named AIDS (Selik et al., 1984). HIV enters and stays in the CNS *via* myelomonocytic cells, such as monocytes, perivascular cells, and microglia (Yadav and Collman, 2009). HIV-1 infection is described as a progressive decrease in CD4+ T lymphocytes and loss of function of the immune system. AIDS manifests in infected individuals years after the initial infection (Doitsh and Greene, 2016).

Recently, necroptosis has been biologically and pathologically researched in HIV-1-infected cells. Unlike almost all infectious diseases, HIV infection precisely targets microglia in the brain and T lymphocytes in the periphery, which are crucial components of the neuroimmune system, resulting in dysfunction of these cells. One report found that necroptosis exists in both the infected primary CD4+ T lymphocytes and CD4+ T cell lines (Pan et al., 2014). Another article suggested that caspase-8 activity was positively correlated with disease severity and programmed cell death-1 (PD-1) expression but negatively correlated with proliferation in HIV-specific CD8+ T cells (Gaiha et al., 2014). NecroX-5 could inhibit defective HIV-specific CD8+ T cell proliferation by blocking necroptosis (Kim et al., 2010; Gaiha et al., 2014). Therefore, chronic stimulation from HIV contributes to caspase-8 activity and increases the cell death of HIV-specific CD8+ T cells through the activation of necroptosis (Gaiha et al., 2014). Drug therapy combined with the inhibitor of necroptosis may improve HIV treatment.

Retinopathy

Oxidative stress, inflammation and neurodegeneration are the main contributors in the most common retinal diseases, such as age-related macular degeneration, glaucoma and diabetic retinopathy (Bapputty et al., 2019; Dieguez et al., 2019). An unbalanced retinal immune reaction involving responses of local microglia and recruited macrophages has been specifically emphasized in retinal degenerative diseases (Akhtar-Schäfer et al., 2018).

Glaucoma is characterized by the loss of retinal ganglion cells and is a leading cause of nonreversible blindness, as well as a deteriorating neurodegenerative disease, with a probable seventy million people suffering worldwide (Tham et al., 2014). Current findings place inflammation and apoptosis as important contributors to retinal cell death under elevated pressure. RIPK1-inhibitory compound (RIC), which performs biochemical functions different from those of previous factors (Nec-1 and Compound 27; Cpd27), inhibits downstream pathways following RIPK1 activation and is mediated by necrosome composition and mitochondrial dysfunction (Do et al., 2017). Microglia play an important role in necroptosis. Necroptotic microglia produce several kinds of proinflammatory cytokines and chemokines, such as TNF-α and chemokine ligand 2 (Chen et al., 2019a)' (Qin et al., 2018). RIPK1- and RIPK3-dependent necroptosis existed in microglia of mouse models with degenerative retina or acute retinal neural injury (Huang et al., 2018b). Necrostatin-1 blocked necroptosis, depressing microglia-mediated inflammation, which protected retinal degeneration or reduced neural injury *in vivo*. In the pathway, knockdown of TLR4 reduces RIPK1 and RIPK3 expression to suppress microglial necroptosis and depress retinal inflammation, which suggests that TLR4 signaling participates in necroptosis-mediated microglial inflammation (Huang et al., 2018b). Thus, microglia in the retina provoke inflammatory activation through TLR4-mediated necroptosis, which aggravates retinal neural damage and retinal degeneration.

In regard to ocular trauma, blast-exposed patients experience subsequent vision loss even after a healthy ophthalmological exam. Increased intraocular RIPK3 suggests that the photoreceptors are depleted because of necroptosis. TNF-a and RIPK3 exacerbate the activation of microglia, indicating that RIPK3 may also result in oxidative stress in the outer retina and lead to progressive cell loss (Thomas et al., 2019). Inhibitors of necroptosis are thought to be a new promising strategy to promote neuroaxonal survival and remyelination, potentially preventing disability in retinal diseases.

NECROPTOSIS INHIBITORS

Several specific necroptosis inhibitors aimed at RIPK1, RIPK3, or MLKL have been found or developed, such as Nec-1, Nec-1s, cpd27, and GSK872. Studies of these specific necroptosis inhibitors have shown therapeutic effects in various neuroinflammatory diseases. In animal models of TBI, Nec-1 (a kind of RIPK1 inhibitor) inhibits apoptosis and autophagy (Wang et al., 2012). Nec-1 also reduces Aβ and tau abnormalities in an AD animal model (Yang et al., 2017). Dabrafenib (an inhibitor of RIPK3 kinase-dependent necroptosis) reduces ischemic brain damage in mice (Cruz et al., 2018). The novel MLKL inhibitors have been proven to be promising tools for studying the biological function of MLKL and as druggable targets of necroptosis. However, the MLKL inhibitors currently known are few (such as TC13172, necrosulfonamide and GW806742X), and no MLKL inhibitor has reached the clinical trial stage (Chen et al., 2017; Yan et al., 2017). However, most studies have yet to conduct clinical trials, and there are still questions about human safety (Chen et al., 2019b).

CONCLUSION

Necroptosis is a new type of programmed necrosis that can be activated by multiple kinds of extracellular and intracellular stimuli. Our understanding of the underlying molecular mechanism and biological function of necroptosis has increased in recent years. In general, necroptosis achieves physiological and pathological effects through the TNF or TLR pathway. Depending on the inhibition of caspase-8 activation, RIPK1, RIPK3, MLKL, FADD and procaspase-8 form complex IIb, which leads to necroptosis. The mechanism by which necroptosis is achieved through the TLR3 and TLR4 pathways is unclear. In the rat model of hydraulic shock brain injury, RIPK1 and 3, MLKL and proinflammatory factors were increased in the cortex (Liu et al., 2016). RIPK1 and 3 and the NLRP3 inflammasome were reported to participate in necroptosis of ICH (Shen et al., 2017). In chronic neuroinflammatory diseases such as AD, RIPK3 impacts necroptosis by phosphorylating MLKL (Ofengeim et al., 2017; Fan et al., 2019). Although the role of necroptosis in inflammatory or apoptotic pathologies has been appreciated, our knowledge of the involvement and impact of necroptosis in neuroinflammatory diseases remains limited. Hence, a deeper understanding of necroptosis in neuroinflammatory diseases, such as AD and stroke, could be beneficial for providing insights into the mechanisms of neuronal death and clinical treatments. The precise mechanism of plasma membrane breakage induced by MLKL in necroptosis remains to be uncovered. Whether MLKL plays a role in necroptosis as a carrier protein of some particular proteins, from cytoplasm to nuclei, also needs further study. While some small-molecule inhibitors of RIPK1 and RIPK3 have made some progress in clinical trials, the efficacy of treatment remains to be confirmed by multicenter experiments.

To conclude, we highlighted the increasing evidence about the role of necroptosis in various neuroinflammatory diseases. In the future, improvement of the application of such signaling inhibitors may remove obstacles for replacement therapies for neurological diseases.

AUTHOR CONTRIBUTIONS

ZY drafted and edited the manuscript and prepared the figures. NJ drafted and edited the manuscript and prepared the table. WS and YZ reviewed and edited the manuscript. All authors conceived the idea of this review together. 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.

GLOSSARY

Aβ Amyloid-β

AD Alzheimer's disease

AIDS Acquired Immune Deficiency Syndrome

ALS Amyotrophic lateral sclerosis

c-FLIP cellular FADD-like interleukin-1β converting enzyme inhibitory protein

cIAP1 Cellular inhibitor of apoptosis protein 1

CNS Central nervous system

DRs Activated death receptors

FADD Fas-associated death domain

fALS Familial amyotrophic lateral sclerosis

FPI Fluid percussion injury

HIV-1 Human immunodeficiency virus 1

ICH Intracerebral hemorrhage

IKK IκB kinase

IRI Ischemia-reperfusion injury

JE Japanese encephalitis

JEV Japanese encephalitis virus

LPS lipopolysaccharide

MLKL Mixed lineage kinase domain-like protein

Nec-1 Necrostatin-1

NEMO Nuclear factor-Kappa B essential modulator

NLRP3 NLR Family Pyrin Domain Containing 3

OPTN optineurin

PD Parkinson's disease

PD-1 programmed cell death-1

RIC RIPK1-inhibitory compound

RIPK1 Receptor-interacting protein kinase 1

RIPK3 Receptor-interacting protein kinase 3

sALS sporadic amyotrophic lateral sclerosis

SN Substantia nigra

SOD1 Superoxide dismutase 1

TAK1 Transforming growth factor β-activated kinase-1

TBI Traumatic brain injury

TICAM-1 TIR domain-containing adaptor molecule 1

TLR Toll-like receptor

TNF Tumor necrosis factor

TNFR1 TNF receptor 1

TRADD TNFR-associated death domain

TRIF Toll/IL-1 receptor domain-containing adaptor inducing IFN-β

WNV West Nile virus

WT wild-type

frontiers in Pharmacology

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Comparison of the Safety and Efficacy of Interferon Alpha-2a and Cyclosporine-A When Combined With Glucocorticoid in the Treatment of Refractory Behçet's Uveitis: A Randomized Controlled Prospective Study

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Purpose: To evaluate and compare the efficacy and safety of interferon alpha-2a (IFN-α2a) and cyclosporine-A (CsA) in patients with refractory Behçet's uveitis (BU).

Methods: In this 12-month randomized, controlled, prospective trial, 26 participants (44 eyes) completed the study. Patients were randomly allocated to the IFN-α2a or CsA groups. All patients in both groups received a standardized prednisone burst and tapering schedule as per protocol. The primary outcome measures were response rate, complete remission rate, and tolerance rate. The secondary outcome measures included time to achieve complete remission, the logarithm of the minimum angle of resolution (logMAR) of best-corrected visual acuity (BCVA), and Behçet's disease ocular attack score 24 (BOS24). T-tests and non-parametric tests were used to compare quantitative variables, and chi-square tests were performed to compare qualitative variables.

Results: The response and complete remission rates were 85.7% (12/14 patients) and 50.0% (7/14 patients) in the IFN-α2a group, compared with 66.7% (8/12 patients) and 25.0% (3/12 patients) in the CsA group, respectively ($p > 0.05$). Complete remission was achieved at 3.3 and 7.0 months after initiation of IFN-α2a and CsA ($p = 0.023$). LogMAR BCVA significantly improved 1 month after IFN-α2a initiation (23 eyes) ($p = 0.002$), and this beneficial effect remained statistically significant during the entire follow-up period ($p < 0.05$); however, this improvement was not observed in the CsA group (21 eyes). At the endpoint, LogMAR BCVA in the IFN-α2a group was significantly better (0.22 vs. 0.31, $p = 0.031$) with a higher improvement rate (60.9 vs. 47.6%, $p > 0.05$). Moreover, compared to the CsA group, more eyes in the IFN-α2a group had a lower BOS24 score (87.0 vs. 57.1%, $p = 0.042$). None of the patients had any side effects that influenced the medication adherence.

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Conclusion: Compared to CsA plus corticosteroid, IFN-α2a plus corticosteroid appears to induce a better treatment response, a significantly greater improvement in visual acuity, and more stable remission of intraocular inflammation in a 12-month study period.

Keywords: Behçet's disease, interferon alpha-2a, cyclosporine-A, uveitis, randomized controlled trial

Clinical Trial Registration: Interferon α2a Versus cyclosporine for refractory Behçet's disease uveitis, NCT03209219.

INTRODUCTION

Behçet's disease (BD) is a multisystemic chronic inflammatory disease of unknown cause characterized by recurrent oral aphthous ulcers, ocular lesions, genital ulcers, gastrointestinal, and central nervous system manifestations (Greco et al., 2018). Uveitis is one of the most common and debilitating organ impairments, affecting 50–70% of BD patients, and may eventually lead to blindness in 25% of patients despite aggressive treatment (Tugal-Tutkun et al., 2004; Greco et al., 2018). Behçet's uveitis (BU) classically manifests as recurrent non-granulomatous uveitis involving the posterior segment of the eye with or without anterior segment inflammation (Paovic et al., 2013), and visual loss is determined by accumulative damage to the intraocular structure caused by repeated episodes of acute uveitis attacks (Tugal-Tutkun et al., 2004; Takeuchi et al., 2005). Therefore, it is of great clinical importance to suppress the inflammation during an acute attack and to prevent recurrence in the quiescent phase.

Current treatments for BU mainly include glucocorticoids, conventional immunosuppressants such as cyclosporine-A (CsA) and azathioprine (AZA), and biological agents such as interferon-alpha (IFN-α) and anti-tumor necrosis factor-alpha (anti-TNF-α) agents (Schwartzman, 2016). While high-dose glucocorticoids are recommended as the mainstay treatment for acute ocular attacks, they are not suitable for long-term use because of their adverse effects (Hatemi et al., 2018). Conventional immunosuppressive agents are usually helpful as add-on treatments for persistent uveitis (Mesquida et al., 2014). Unfortunately, up to 41.3% of refractory BU patients show inadequate responses to conventional immunosuppressives even at optimal therapeutic doses; therefore, switching to biologics could be considered (Celiker et al., 2018).

IFN-α2a has long been reported to be effective in BU patients with different genetic backgrounds (Gueudry et al., 2008; Sobac et al., 2010; Lee et al., 2018; Yang et al., 2019). IFN-α2a has the advantage of rapid onset of action and long-term remission, and accumulating evidence suggests that IFN-α2a may be superior to conventional agents because it is usually effective for BU patients refractory to immunosuppressives (Deuter et al., 2010; Park et al., 2015; Kavandi et al., 2016; Diwo et al., 2017; Hasanreisoglu et al., 2017; Shi et al., 2019; Eser-Ozturk and Sullu, 2020). However, all the above-mentioned studies are retrospective observational studies and uncontrolled case series, and to the best of our knowledge, there is still a lack of prospective studies that provide solid evidence for the effectiveness of IFN-α2a in refractory BU. Therefore, a randomized controlled prospective study was conducted to compare the efficacy and safety of IFN-α2a and CsA in the treatment of refractory BU.

MATERIALS AND METHODS

Study Design and Patient Population

This 12-month randomized controlled prospective study was conducted at the Department of Ophthalmology at Peking Union Medical College Hospital between June 2017 and August 2020. All recruited patients with refractory BU were randomly assigned (1:1) to the IFN-α2a or CsA groups using a random number table. The study protocol was approved by the Institutional Review Board of Peking Union Medical College Hospital (approval number: JS-1342) and conducted according to the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants. This study was registered in ClinicalTrials.gov (NCT03209219).

The study population was adult ($18 \leq$ age ≤ 65) refractory BU patients with acute uveitis attack. BD was diagnosed according to the International Criteria for Behçet's Disease (ICBD) (Davatchi et al., 2014). Uveitis terminology and anatomic classification were described by the Standardization of Uveitis Nomenclature (SUN) (Jabs et al., 2005). Patients were eligible for the study if they had posterior uveitis or panuveitis acute attacks (≥1 + vitreous haze together with the presence of at least one of the following lesions: retinal vasculitis, retinitis, cystoid macular edema, or papillitis) under a medium dose of oral glucocorticoids (prednisone, no less than 15 mg/day or equivalent) and at least one of the following conventional immunosuppressants: CsA (≥100 mg/day), AZA (≥50 mg/day), cyclophosphamide (CTX, ≥100 mg/day), methotrexate (MTX, ≥15 mg/week), mycophenolate mofetil (MMF, ≥1,000 mg/day), thalidomide (THD, ≥2 mg/day), and tacrolimus (TAC, ≥2 mg/day).

Patients with any of the following conditions were excluded: 1) patients who had previously received any biological agent (e.g., IFN-α, anti-TNF-α agents, anti-human IL-6 receptor antibody), had used CsA but did not tolerate, or had any systematic contraindication (e.g., active peptic ulcer, osteoporosis, infection) that prevent using glucocorticoids; 2) patients with malignancy, pregnant, breast-feeding, mental illness, depression, cognitive impairment, poorly controlled hypertension or diabetes mellitus, alcohol abuse or drug abuse, history of acute or chronic inflammatory joint or autoimmune disease, systemic infectious diseases, including hepatitis B virus, hepatitis C virus, HIV, syphilis, or tuberculosis (TB) infection were also excluded; 3) patients with severe extra-ocular involvement; 4) patients who showed a presence of severe pupillary adhesion, cataract and

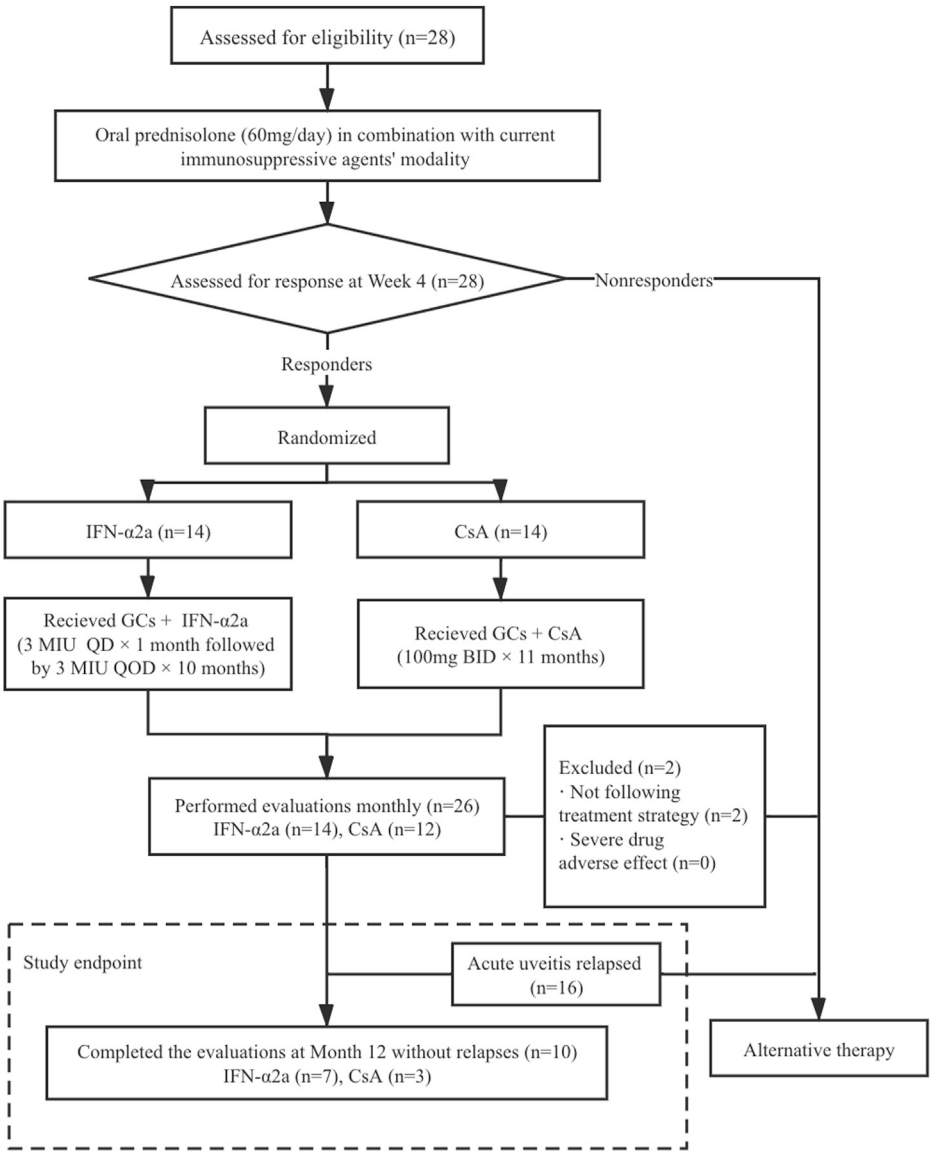

FIGURE 1 | Patient disposition and flow chart of the study. BID, bis in die (twice a day); CsA, cyclosporine-A; GCs, glucocorticoids; IFN-α2a, interferon alpha-2a; MIU, million international unit; QD, quaque die (every day); QOD, quaque omni die (every other day).

posterior capsular opacification that obscured the fundus observation, and/or had other ocular diseases, and intraocular surgery in the previous 3 months; and 5) patients with significant laboratory abnormalities in complete blood counts (e.g., white blood cell count < 3,500/mm^3, platelet count < 100,000/mm^3, Hgb < 8.5 g/dl), urine tests, liver and kidney function (e.g., creatinine > 1.5 mg/dl, alanine transaminase (ALT) or aspartate transaminase (AST) 2× above the normal) were not eligible.

Treatments

As shown in the treatment protocol (**Figure 1**), oral corticosteroid was up-titrated to 60 mg/day of prednisolone with current immunosuppressant modality, which remained unchanged for the first 4 weeks. Responders who showed an improvement in vitreous haze and chorioretinal inflammation were randomly divided into two groups. In the IFN-α2a group, patients received a daily dose of 3 million international units (MIU) of IFN-α2a (Interfon; 3sbio.inc., Shenyang, China) subcutaneously for 4 weeks, followed by 3 MIU every other day as the maintenance dose. In the CsA group, patients received 100 mg of CsA twice per day during the entire study period. Meanwhile, for all patients in both groups, all other immunomodulating agents were discontinued when IFN-α2a or CsA therapy was initiated, and the dose of prednisolone was tapered from 55 mg/day following the same protocol, that is, reduce 5 mg/day every 10 days to 30 mg/day, reduce 2.5 mg/day every 14 days to 15 mg/day, and it remained unchanged thereafter.

In the case of anterior uveitis, corticosteroid and mydriatic eye drops were allowed to prescribe. Gastric mucosal protective agents, vitamin D, calcium, potassium, and hepatoprotectants were administered when necessary.

Follow-Up Schedule, Clinical Assessment, and Endpoints

Patients were recommended to visit our center monthly until 12 months after the initiation of 60 mg/day prednisolone, and whenever symptoms suggestive of disease recurrence were noted.

A detailed ophthalmic examination including best-corrected visual acuity (BCVA), intraocular pressure, slit-lamp biomicroscopy, and fundoscopy, were performed at baseline (before the initiation of 60 mg/day prednisolone) and at each follow-up visit. BCVA was examined using standard logarithmic visual acuity charts and then converted to the logarithm of the minimum angle of resolution (LogMAR) for statistical analysis.

An ocular inflammatory attack was defined as a new-onset of intraocular inflammation and/or worsening of preexisting uveitis, necessitating treatment intensification. The severity of ocular inflammation at baseline and each follow-up visit was evaluated using the BOS24 scoring system, which is a novel and more definite tool for scientific analysis (Kaburaki et al., 2014; Tanaka et al., 2016). The BOS24 consists of 24 points describing six parameters of ocular inflammation manifestations, including anterior chamber cells (maximum 4 points), vitreous opacity (maximum 4 points), peripheral fundus lesions (maximum 8 points), posterior pole lesions (maximum 4 points), subfoveal lesions (maximum 2 points), and optic disc lesions (maximum 2 points). Changes in the BOS24 score before and after IFN-α2a or CsA treatment were recorded and compared.

Bone mineral density and infection screening tests were performed at baseline. Blood pressure was measured at baseline and monthly during the study period. Laboratory tests, including complete blood counts, urine tests, and biochemical tests, were performed monthly or bimonthly.

The endpoints of this study were relapse of posterior or panuveitis, drug (prednisolone, CsA, or IFN-α2a) withdrawal due to intolerance, and completion of the 12-month follow-up since initiation of 60 mg/day prednisolone.

The primary efficacy outcome measures were the response and complete remission rates. Specifically, treatment response was categorized into complete remission, partial remission, and treatment failure. Complete remission was defined as a decrease in vitreous haze to no more than grade 0.5+ and complete disappearance of signs of active fundus inflammation including retinal infiltrates, hemorrhage, and vascular sheathing (Jabs et al., 2005), without any relapses within the 12-month follow-up. Partial remission was defined as improvement in vitreous haze and chorioretinal inflammation, but it did not reach the standard of complete remission. Treatment failure was defined as vitreous haze or chorioretinal inflammation that remained unchanged or even exacerbated during the study period. The secondary efficacy outcome measures included time to reach complete remission, duration of relapse-free, glucocorticoid-sparing effect, and changes in BCVA and BOS24.

The primary safety outcome measure was the tolerance rate to IFN-α2a or CsA treatment. The secondary safety outcome measures included the incidence of adverse effects, significant abnormal changes in vital signs or laboratory test results, and the adverse effects profile.

Statistical Analysis

Statistical analysis was conducted using the Macintosh software (version 25.0; IBM Corp. Released 2017. IBM SPSS Statistics for Macintosh, version 25.0. Armonk, NY: IBM Corp.). The Kolmogorov–Smirnov test was used for normality testing. Normal variables are presented as the mean and standard deviation (SD), and non-normal variables as the median and interquartile range (IQR). T-tests were used to compare the means of normally distributed quantitative variables; otherwise, the Mann-Whitney U test was used. The non-parametric Wilcoxon test was used to compare continuous variables. Chi-square tests were used to compare the qualitative data. Statistical significance was set at p value of <0.05.

Sample Size Analysis

Sample size analysis was conducted using PASS 11.0 software (NCSS, LLC). This randomized controlled prospective study was designed to have a statistical power of 80% and a significance level of 5%. Based on our clinical experience and previous studies, we estimated that the primary endpoint of participants, namely, complete remission rate of IFN-α2a and CsA therapy, was 80 and 30% (Kötter et al., 2004), respectively. Given that 10% of subjects may lost to follow-up or drop out, the minimum number was 14 patients for each group.

RESULTS

Characteristics of Patients

A total of 28 eligible patients were included in the study from June 2017 (enrollment of the first patient) to August 2020 (the date of the last follow-up visit). Two patients who did not follow the treatment protocol were excluded. Therefore, 26 patients with refractory BU (44 eyes) completed the trial and were included in the analysis. As shown in **Table 1**, of the 26 included patients, the mean age was 32.2 ± 9.2 years and 24 patients (92.3%) were men, 14 were in the IFN-α2a group, and 12 were in the CsA group. Eye involvement was bilateral in 18 patients (69.2%). Panuveitis was the most common ocular manifestation, presenting in 26 (58.1%) eyes, and posterior uveitis was present in 18 (41.9%) eyes. The median duration of BD was 25.0 months (range, 1–156 months). Recurrent oral ulcers were present in all patients (100.0%), followed by erythema nodosum in 11 patients (44.0%), genital ulcers in 10 patients (38.5%), pseudo-folliculitis in 7 patients (26.9%), arthritis in 2 patients (8.0%), and thrombophlebitis and perianal abscess each in 1 patient (4.0%). After treatment, no new extraocular manifestations were detected in either group.

Prior to enrollment, all patients were treated with corticosteroids in combination with a median of 1 immunosuppressant (range, 1–3). The median dose of

TABLE 1 | Baseline features of 26 patients with refractory BU.

	Total (n = 26)	IFN-α2a (n = 14)	CsA (n = 12)
Age (years), $\bar{x}$±s	32.2 ± 9.2	32.1 ± 7.8	32.2 ± 10.9
Male, n (%)	24 (92.3)	13 (92.9)	11 (91.7)
Duration of BD (months), M (IQR)	25.0 (20.0–36.0)	24.5 (19.5–30.0)	32.5 (21.0–49.0)
Bilateral involvement, n (%)	18 (69.2)	9 (64.3)	9 (75.0)
Systemic symptoms, n (%)			
Recurrent oral ulcers	26 (100.0)	14 (100.0)	12 (100.0)
Genital ulcers	10 (38.5)	6 (42.9)	4 (33.3)
Skin lesions	17 (65.4)	10 (71.4)	7 (58.3)
Erythema nodosum	11 (42.3)	9 (64.3)	2 (16.7)
Pseudofolliculitis	7 (26.9)	2 (14.3)	5 (41.7)
Arthritis	3 (11.5)	2 (14.3)	1 (8.3)
Perianal abscess	1 (3.8)	0	1 (8.3)
Thrombophlebitis	1 (3.8)	0	1 (8.3)
Uveitis type (44 eyes), n (%)			
Posterior uveitis	18 (40.9)	9 (39.1)	9 (42.9)
Panuveitis	26 (59.1)	14 (60.9)	12 (57.1)
Number of immunosupressants, M (IQR)	1 (1–2)	1 (1–2)	2 (1–3)
Concomitant immunosupressants, n (%)			
Cyclosporine-A	19 (73.1)	10 (71.4)	9 (75.0)
Azathioprine	10 (38.5)	7 (50.0)	3 (25.0)
Cyclophosphamide	5 (19.2)	1 (7.1)	4 (33.3)
Thalidomide	3 (11.5)	0	3 (25.0)
Mycophenolate mofetil	2 (7.7)	0	2 (16.7)
Tacrolimus	1 (3.8)	1 (3.8)	0
Methotrexate	1 (3.8)	0	1 (8.3)

BD, Behçet's disease; BU, Behçet's uveitis; IFN-α2a, interferon alpha-2a; CsA, cyclosporine-A.

TABLE 2 | Efficacy outcomes of the 26 refractory BU patients treated with IFN-α2a and CsA.

	Total (n = 26)	IFN-α2a (n = 14)	CsA (n = 12)	*p*
Treatment response, n (%)				
Complete remission	10 (38.5)	7 (50.0)	3 (25.0)	0.248
Partial remission	10 (38.5)	5 (35.7)	5 (41.7)	1.000
Treatment failure	6 (23.1)	2 (14.3)	4 (33.3)	0.365
Time to achieve complete remission (months) (10 eyes), $\bar{x}$± s	4.4 ± 2.5	3.3 ± 1.4	7.0 ± 3.0	**0.023**
Duration of relapse-free (months) (16 eyes), $\bar{x}$± s	4.8 ± 2.8	4.7 ± 3.7	4.8 ± 2.2	0.966
Baseline prednisone dose (mg/day), M (IQR)	20.0 (19.4–30.0)	20.0 (16.9–24.4)	20.0 (20.0–30.0)	0.207
Endpoint prednisone dose (mg/day), M (IQR)	15.0 (15.0–30.0)	15.0 (15.0–32.5)	25.0 (15.0–37.5)	0.432
Baseline LogMAR BCVA (44 eyes), M (IQR)	0.96 (0.17–1.40)	0.52 (0.10–1.00)	1.00 (0.61–1.52)	0.147
Endpoint LogMAR BCVA (44 eyes), M (IQR)	0.56 (0.00–1.20)	0.22 (0.00–0.92)	0.92 (0.31–1.70)	**0.031**
Distribution of low BCVA in baseline (44 eyes), n (%)				
20/50 or worse	33 (75.0)	16 (69.6)	17 (81.0)	0.494
20/200 or worse	22 (50.0)	9 (39.1)	13 (61.9)	0.227
LogMAR BCVA change rate (44 eyes), n (%)				
Improved ≥0.2LogMAR	12 (25.0)	8 (34.8)	4 (19.0)	0.318
Improved <0.2LogMAR	10 (20.5)	6 (26.1)	4 (19.0)	0.724
Stability	9 (25.0)	3 (13.0)	6 (28.6)	0.272
Deteriorated	13 (29.5)	6 (26.1)	7 (33.3)	0.744
Baseline BOS24 score (44 eyes), M (IQR)	5 (3–7)	5 (3–7)	5 (3.5–6.5)	0.803
Endpoint BOS24 score (44 eyes), M (IQR)	1 (0–4.75)	1 (0–3)	2 (0–6)	0.124
BOS24 score change rate, n (%)				
Improved	32 (72.7)	20 (87.0)	12 (57.1)	**0.042**
Stability	5 (11.4)	1 (4.3)	4 (19.0)	0.176
Deteriorated	7 (15.9)	2 (8.7)	5 (23.8)	0.232

LogMAR, logarithm of the minimum angle of resolution; BCVA, best-corrected visual acuity; BOS24: Behçet's disease ocular attack score 24; IFN-α2a: interferon alpha-2a; CsA: cyclosporine-A. Bold values: $p < 0.05$.

prednisolone was 20.0 mg/day (range, 15.0–40.0 mg/day). The baseline immunosuppressive agents taken by patients included CsA (19 patients, 73.1%, median dose 125 mg/day), AZA (10 patients, 38.5%, median dose 100 mg/day), CTX (5 patients, 19.2%, median dose 100 mg/day), THD (3 patients, 11.5%, median dose 2 mg/day), MMF (2 patients, 7.7%, median dose

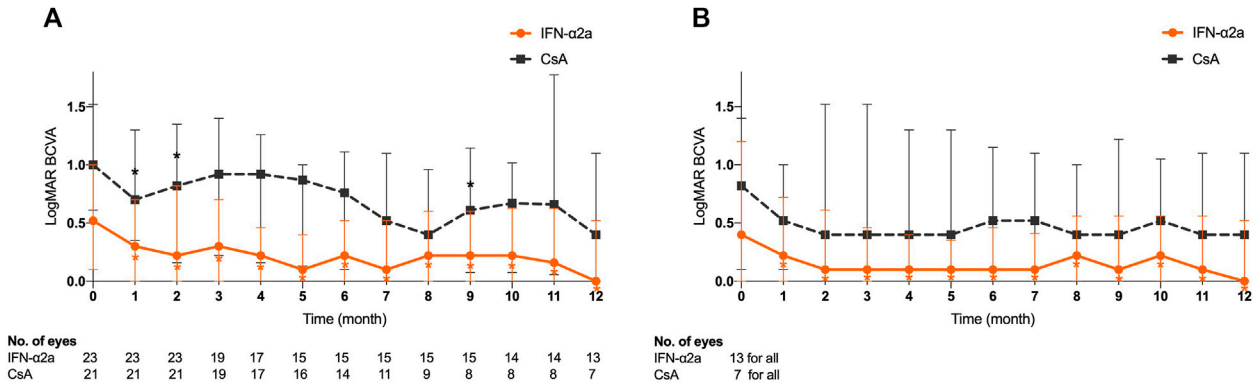

FIGURE 2 | Changes in logarithm of the minimum angle of resolution (LogMAR) best-corrected visual acuity (BCVA) for eyes treated with interferon alpha-2a (IFN-α2a) and cyclosporine-A (CsA). **(A)** Changes in median LogMAR BCVA for all eyes treated with IFN-α2a (n = 23) and CsA (n = 21) during the 12-month follow-up. **(B)** Changes in LogMAR BCVA for eyes with complete remission treated with IFN-α2a (n = 13) and CsA (n = 7) during the 12-month follow-up. Data are shown as the median and IQR. *: $p < 0.05$.

125 mg/day), TAC (1 patient, 3.8%, dose 2 mg/day), and MTX (1 patient, 3.8%, dose 15 mg/week). The IFN-α2a and CsA groups were not significantly different in basic demographic data, baseline clinical features, and treatments.

Treatment Response

Of the 26 patients, 20 (76.9%) responded (complete and partial remission) to IFN or CsA treatment (**Table 2**). Specifically, 12/14 (85.7%) patients responded to IFN-α2a treatment, while 8/12 patients (66.7%) responded to CsA treatment ($p = 0.365$). Notably, complete remission (no relapse within the 12-month follow-up period) was achieved in 7 (50.0%) patients in the IFN-α2a group, compared to only 3 (25.0%) patients in the CsA group ($p = 0.248$). Of those patients who completely responded to the therapy, the duration between the therapy initiation to a complete absence of ocular inflammation was 3.3 and 7.0 months in IFN-α2a and CsA group, respectively ($p = 0.023$). On the other hand, for incomplete responders and nonresponders who suffered further uveitis attacks during the study period, the relapses occurred on average 4.7 ± 3.7 and 4.8 ± 2.2 months after IFN-α2a and CsA initiation, respectively ($p = 0.966$).

Effect on Visual Acuity

The analysis included 23 eyes in the IFN-α2a group and 21 eyes in the CsA group with refractory BU.

The baseline LogMAR BCVA was 0.52 (0.10–1.00) in the IFN-α2a group and 1.00 (0.61–1.52) in the CsA group ($p = 0.147$). BCVA equal or below 20/50 and 20/200 were found in 16 eyes (69.6%) and 9 eyes (39.1%) in the IFN-α2a group, compared to 17 eyes (81.0%) and 13 eyes (61.9%) in the CsA group ($p > 0.05$), respectively.

Of the 23 enrolled eyes in the IFN-α2a group, the improvement in LogMAR BCVA started at the first month's visit after treatment initiation ($p < 0.001$), and this beneficial effect sustained to the endpoint visit ($p = 0.026$) (**Figure 2A**). In contrast, compared with the baseline level, LogMAR BCVA of 21 eyes in the CsA group did not show either continuous improvement during the follow-up period or at the endpoint visit ($p > 0.05$). Notably, at the end of the study, the median LogMAR BCVA increased to 0.22 (0.00–0.92) and 0.92 (0.31–1.70) in the IFN-α2a group and CsA group, respectively ($p = 0.031$).

Furthermore, of all eyes in the IFN-α2a group, BCVA improved ≥0.2 LogMAR from study initiation in 8 eyes (34.8%), improved but less than 0.2 LogMAR in 6 eyes (26.1%), remained stable in 3 eyes (13.0%), and worsened in 6 eyes (26.1%). On the other hand, in the CsA group, BCVA improved by ≥0.2 logMAR in only 4 eyes (19.0%), but it stabilized and deteriorated in 6 eyes (28.6%) and 7 eyes (33.3%), respectively.

Among 20 eyes that responded (complete and partial remission) to the IFN-α2a therapy, LogMAR BCVA was 0.60 (0.17–1.30) at baseline, and significantly increased 1 month after treatment initiation ($p = 0.001$), remained statistically significant at every follow-up visit, and eventually improved to 0.31 (0.00–0.98) at study endpoint ($p = 0.020$). Meanwhile, in the complete remission subgroup, a total of 13 eyes showed similar VA progression (**Figure 2B**). However, no such improvement was observed in either 7 complete remission or 7 partial remission eyes in the CsA group ($p > 0.05$).

BOS24 Score in Patients With BU

The median baseline BOS24 scores were 5 (3–7) and 5 (3.5–6.5) in the IFN-α2a and CsA groups, respectively ($p = 0.803$). Of all eyes in the IFN-α2a group, the BOS24 score showed a significant decrease 1 month after treatment initiation ($p < 0.001$) and remained low during the entire study period ($p = 0.001$) (**Figure 3A**). However, in the CsA group, statistically significant reductions in BOS24 scores were not observed in a few follow-up visits and the endpoint visit, as compared to the baseline. At the end of this study, the BOS24 score fell to 1 (0–3) and 2 (0–6) in the IFN-α2a and CsA groups ($p = 0.124$), respectively.

Moreover, at the endpoint visit, a decreased BOS24 score was obtained in 20 out of 23 eyes (87.0%) and 12 out of 21 eyes (57.1%) in the IFN-α2a and CsA groups, respectively ($p = 0.042$).

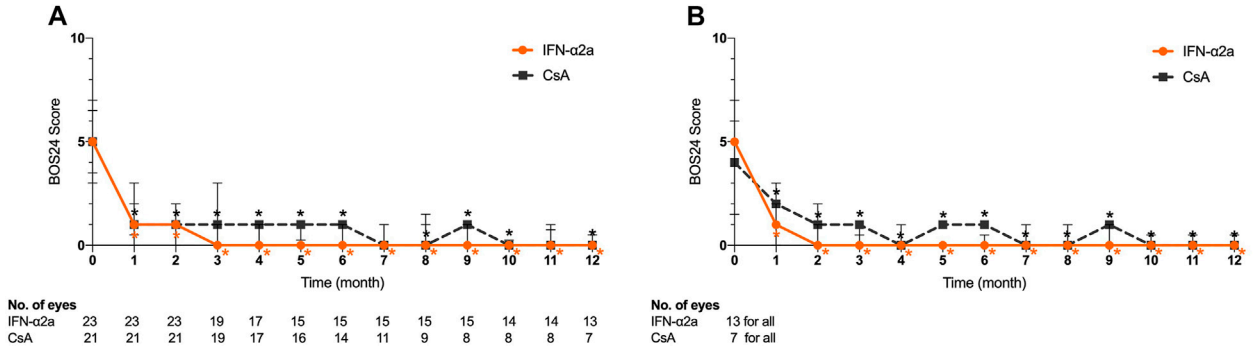

FIGURE 3 | Changes in Behçet's disease ocular attack score 24 (BOS24) for eyes treated with interferon alpha-2a (IFN-α2a) and cyclosporine-A (CsA). **(A)** Changes in median BOS24 score for all eyes treated with IFN-α2a (n = 23) and CsA (n = 21) during the 12-month follow-up. **(B)** Changes in BOS24 score for eyes with complete remission treated with IFN-α2a (n = 13) and CsA (n = 7) during the 12-month follow-up. Data are shown as the median and IQR. *: $p < 0.05$.

In contrast, only 2 eyes (8.7%) showed a higher BOS24 score when relapse occurred during the IFN-α2a treatment period, while 5 eyes (23.8%) in the CsA group had an elevated score at the endpoint, indicating a more severe ocular inflammation status ($p = 0.232$).

Among eyes with complete and partial remission, the BOS24 score decreased over time in both the IFN-α2a and CsA groups (**Figure 3B**). Compared with the baseline BOS24 score, a significant BOS24 reduction was observed at monthly follow-up visits and was preserved at the final visit of the study ($p < 0.05$).

Corticosteroid-Sparing Effect

After IFN-α2a or CsA treatment, the prednisolone dose was reduced in 8 (57.1%) and 5 (41.7%) patients at the end of the study, respectively ($p = 0.695$). The median corticosteroid dosage before enrollment was 20.0 mg (16.9–24.4) and 20.0 mg (20.0–30.0) per day in the IFN-α2a and CsA groups, respectively ($p = 0.207$). At the endpoint, the average dosage of corticosteroid was significantly decreased to 15.0 mg per day in complete remission patients treated with IFN-α2a ($p = 0.024$). Nevertheless, no obvious corticosteroid-sparing effects were observed in patients in the other subgroups, including partial remission and treatment failure patients in the IFN-α2a group, and all CsA subgroups ($p > 0.05$).

Safety

The tolerance rate of both the IFN-α2a and CsA groups was 100% in this study. No treatment discontinuation was required because of the side effects. No serious adverse drug effects were observed. The incidence of adverse events in patients treated with IFN-α2a and CsA was 78.6% (11/14) and 66.7% (8/12), respectively ($p = 0.665$). Compliance with IFN-α2a was satisfactory. IFN-associated side effects, which were mild and reversible, included flu-like syndrome associated with fever, myalgia, and headache (at the initiation phase of the treatment) (71.4%; n = 10), mild elevation of serum liver enzymes (ALT and/or AST, 28.6%; n = 4), hair loss (28.6%; n = 4), skin disorders (erythema at injection site, reddish rash; 28.6%; n = 4), minor leukopenia (14.3%; n = 2), dryness of mouth (14.3%; n = 2), and mild depression (14.3%; n = 2). The side effects related to the CsA treatment were as follows: increased ALT/AST (33.3%; n = 4), increased uric acid (25.0%; n = 3), hyperlipidemia (25.0%; n = 3), hypertension (16.7%; n = 2), hematuria (16.7%; n = 2), and increased bilirubin (16.7%; n = 2). Hirsutism was observed in one female patient in the CsA group.

DISCUSSION

CsA has been one of the best-validated immunosuppressants for refractory eye disease in patients with BD (Chighizola et al., 2017). However, the beneficial effect of CsA was not sustained in the long term, with a high rate of side effects (BenEzra et al., 1988). On the other hand, accumulating evidence indicates that IFN-α is noticeably effective for refractory BU patients with a high tolerance rate (Kötter et al., 2003; Gueudry et al., 2008). Therefore, in the most recent EULAR recommendations (Hatemi et al., 2018), IFN-α is one of the recommended agents for patients with recurrent episodes of acute sight-threatening uveitis based on its efficacy in inducing rapid ocular inflammation remission, preventing recurrences, and maintaining useful vision in medium to long terms. To the best of our knowledge, this is one of the first clinical trials to address head-to-head comparisons between IFN-α and CsA. Another advantage of this study was the application of the BOS24 scoring system for disease activity of BU (Kaburaki et al., 2014), which has a low level of variability among different examined ophthalmologists and has been successfully applied in previous studies (Kaburaki et al., 2014; Tanaka et al., 2016).

In the literature, the dosage regimens of IFN and CsA vary among different clinical centers and study protocols. IFNα-2a is usually subcutaneously injected at doses ranging from 3 to 9 MIU, 3 to 7 times a week (Kötter et al., 2004), and CsA is orally administered at dosages ranging from 2 to 16 mg/kg/day (Whitcup et al., 1994; Evereklioglu, 2005). In our current study, the initial dose of IFNα-2a was 3 MIU daily for the first month, followed by 3 MIU every other day as the maintenance dose, based on experiences gained from our retrospective study (Shi et al., 2019). CsA was administered at a dosage of 200 mg/day

(with an average of 2.7 m g/kg/day) during the entire study period, which was commonly prescribed for patients with refractory BU in our clinical practice.

A review of previous studies has revealed invariably high (78% to over 90%) response rates of IFN-α2a for treatment of BU (Krause et al., 2008; Hazirolan et al., 2013). The rate of patients who achieved complete remission, however, was quite different among investigations, ranging from 36.4% to 85.0% (Tugal-Tutkun et al., 2006; De Simone et al., 2020). The reported response rates of CsA are generally lower, ranging from 50% to 85% (Masuda et al., 1989; Murphy et al., 2005). In accordance with the literature, in our current 12-month study, the IFN-α2a group showed both higher response rates and complete remission rates than the CsA group (85.7% vs. 66.7% and 50% vs. 25.0%, respectively), indicating the superiority of IFN-α2a over CsA for long-term control of refractory BU.

The advantage of IFN-α2a over CsA was also reflected by the time to reach complete remission, and the improvements in visual function and disease severity, as indicated by LogMAR BCVA and BOS24 score, respectively. Our current study showed that the use of IFN-α2a treatment led to a significantly earlier complete remission in refractory BU patients than CsA treatment. Additionally, during the entire 12-month period, treatment with IFN-α2a can effectively achieve sustained disease control by markedly increasing visual acuity and reducing BOS24 score, regardless of whether the patients achieved complete remission. Consequently, at the endpoint of the study, more patients in the IFN-α2a group achieved a prominent visual acuity improvement with amelioration of intraocular inflammation, as compared to the CsA group. Therefore, this randomized prospective comparative clinical trial provides multiple lines of evidence suggesting that in the treatment of refractory BU, IFN-α2a treatment can not only reduce the dosage of glucocorticoids but also display superiority in inducing rapid disease remission and maintaining disease quiescence in 12 months.

Our study also revealed generally favorable safety profiles for both IFN-α2a and CsA regimens. Although adverse effects were recorded in 78.6 and 66.7% of the patients in the IFN-α2a and CsA groups, respectively, they were all reversible and well tolerated. The most frequent side effects of IFN-α2a and CsA were flu-like symptoms (71.4%) and renal toxicity (33.3%), respectively, which are in accordance with previous studies (Chighizola et al., 2017; Shi et al., 2019). We also calculated the 1-year costs of our IFN-α2a and CsA regimens, which were approximately $1,050 and $1,600, respectively. Therefore, IFN-α2a treatment is more cost-effective than CsA treatment for patients with refractory BU in China.

This study has some limitations. First and most importantly, the sample size was relatively small and inadequate for more detailed analyses and comparisons. The approval of adalimumab for refractory non-infectious uveitis in March 2020 in China and the COVID-19 pandemic have made it difficult to recruit participants further. Second, we noticed that there was a difference in the baseline BCVA between the IFN-α2a and CsA groups, although this disparity was not statistically significant. Third, the current study period was not long enough to evaluate the long-term efficacy of IFN-α2a and CsA treatment. It would be of higher clinical qualifications to conduct the study over a longer time span.

In conclusion, this randomized, controlled, prospective clinical trial provides multiple lines of evidence suggesting that IFN-α2a is superior to CsA when combined with glucocorticoid for refractory BU during a study period of 12 months. Compared to CsA, IFN-α2a induces a higher rate of treatment response, a significantly better improvement in visual acuity, and a more stable disease remission in 12 months for refractory BU.

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 studies involving human participants were reviewed and approved by the institutional review board of the Peking Union Medical College Hospital (approval number: JS-1342). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design of this study. YuQ, YiQ, MP, AL, and JX acquired the data. YuQ and YiQ performed the data analysis and interpretation. YuQ wrote the manuscript. MZ, CZ, and FG designed the therapeutic protocol and recruited the patients. MZ and CZ critically reviewed the manuscript and provided valuable revisions to the manuscript. All authors read and approved the final manuscript.

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Functions and Diseases of the Retinal Pigment Epithelium

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The retinal pigment epithelium is a fundamental component of the retina that plays essential roles in visual functions. Damage to the structure and function of the retinal pigment epithelium leads to a variety of retinopathies, and there is currently no curative therapy for these disorders. Therefore, studying the relationship between the development, function, and pathobiology of the retinal pigment epithelium is important for the prevention and treatment of retinopathies. Here we review the function of the retinal pigment epithelium and its relevance to the pathobiology, and discuss potential strategies for the treatment of retinopathies. In doing so, we provide new viewpoints outlining new ideas for the future study and treatment of retinopathies.

Keywords: retina, retinal pigment epithelium, development, function, disease, retinopathy, therapy

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INTRODUCTION

Retinal pigment epithelium (RPE) is formed from a single layer of regular polygonal cells arranged at the outermost layer of the retina. The outer side of the RPE is connected to Bruch's membrane and the choroid, while the inner side is connected to the outer segment of photoreceptor cells. The outer side exhibits basal infolding, which increases cell surface area and facilitates substance exchange. The basement membrane is closely connected to the basal folds by half desmosomes located in the innermost layer of Bruch's membrane. The inside of RPE cells harbors microvillous structures extending between photoreceptor outer segments (POS), which participate in the phagocytic function of the RPE (Song and Zhou, 2020; Zhou and Zhou, 2020; Yang et al., 2021). The tight junction formed between the single-layer RPE and the gap junction control the movement of substances and at the same time forms the choroid-blood-retinal barrier with Bruch's membrane and choroid at the lateral retina (Xie et al., 2020). The RPE appears dark brown due to its melanin content, which reduces damage to the retina and internal nerves from ultraviolet light (Tian et al., 2021). The RPE also harbors a complex metabolic system that reduces excessive accumulation of reactive oxygen species (ROS) and consequent oxidative damage.

Therefore, RPE structure and function are essential to normal vision, and alterations in the RPE can impair function and lead to retinopathy. For example, retinitis pigmentosa (RP), age-related macular degeneration (AMD), and Stargardt disease (SD) are degenerative retinal diseases in which RPE dysfunction has been implicated in their pathogenesis [for an excellent review, see Zarbin (Zarbin, 2016)]. RP afflicts 100,000 people in the United States and usually causes visual loss in childhood or young adulthood. AMD afflicts 1.75 million people in the United States alone, is the leading cause of blindness in individuals over 55 years of age in the United States and Europe, and was estimated to affect ~196 million people worldwide in 2020. SD is the most common form of inherited juvenile macular degeneration, with a

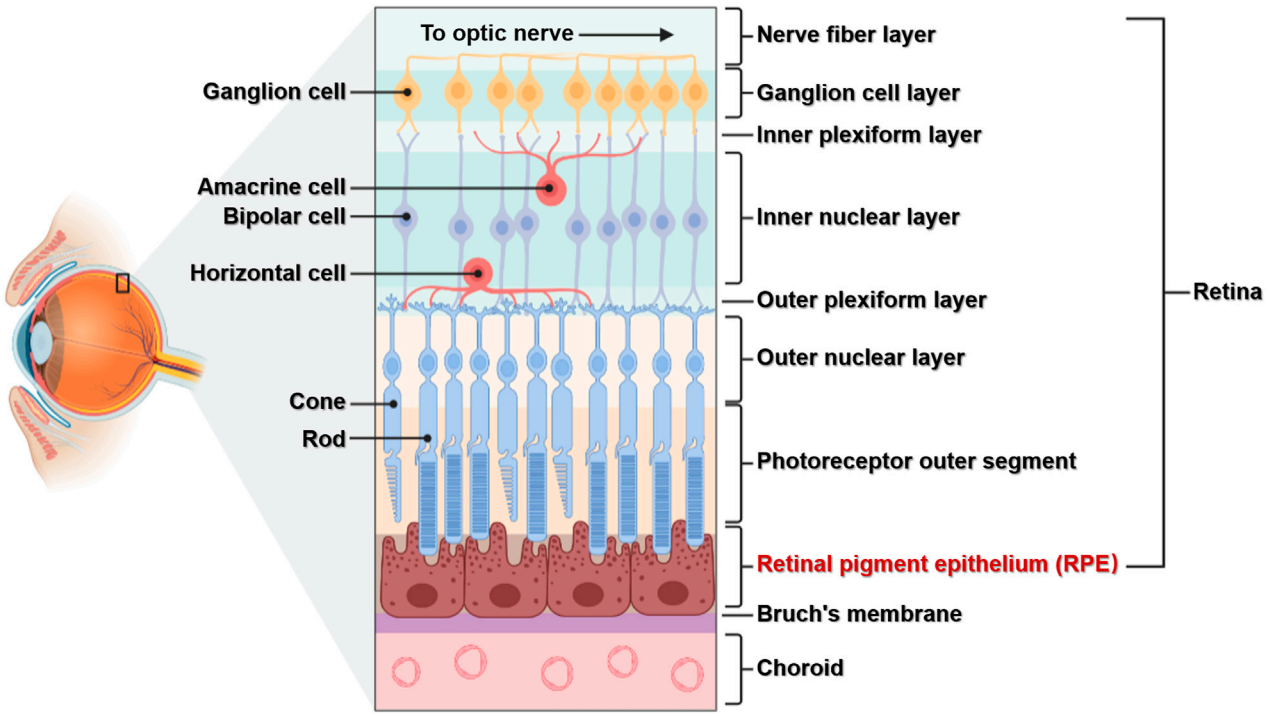

FIGURE 1 | The structure of the retina. The retina is composed of multiple layers and different cell types. The RPE is composed of a single layer of RPE cells, which are connected to the choroid membrane through Bruch's membrane.

prevalence of 1 in 10,000 births. There are currently no cures for these degenerative diseases, so understanding the role of RPE in their pathogenesis is important for the development of new approaches to manage these common and debilitating disorders. Here we review the functions and diseases of the RPE to provide a theoretical basis for the treatment and prevention of associated diseases.

RETINAL DEVELOPMENT AND STRUCTURE

The human eye begins to develop at embryonic day (E)18. The visual groove is formed at E22 before continuing to sag to form the visual vesicle, which expands to form the inner and outer layers of the optic cup. The RPE layer begins to differentiate around E30, with pigment particles found in RPE cells at E35. A set of genes (including *PAX6*, *LHX2*, *RAX*, and *SIX3*) expressed in the neural plate before E8 are involved in eye determination and eventually form the optic cup (Hoon et al., 2014). In general, vertebrate RPE cells develop and differentiate from optic vesicles. During embryonic development, early optic vesicle cells have bidirectional potential and can develop into the retinal neurocortical layer or the RPE layer. The cell fate decision and differentiation of RPE precursor cells is not spontaneous but rather influenced by a variety of microenvironmental factors. Under the influence of extracellular signals, differentiation is guided in strict temporal and spatial order through the regulation of transcription factors and intracellular signaling pathways. In particular, the transcription factor MITF (microphthalmia-associated transcription factor) has been confirmed to be involved in the normal RPE development, and *Mitf* knockout results in abnormal retinal development in mice (Bharti et al., 2008; Ma et al., 2019).

Light entering the eye is focused on the retina, which converts light signals into electrical signals that travel through the optic nerve to the visual center of the brain (Grossniklaus et al., 2015). The retina is located in the fundus of the eye and, as an important tissue forming vision, has a complex structure. The retina has multiple layers containing various cell types: the RPE lies at the boundary, while the retinal nerve layer contains five main neuronal cells including photoreceptor cells (rods and cones), horizontal cells, bipolar cells, amacrine cells, and ganglion cells. RPE cells are located between photoreceptor cells and the choroid membrane, with the basal side connected to Bruch's membrane and tip microvilli connected to the outer segment of photoreceptor cells (**Figure 1**). The RPE is located in a specific position and has important functions, and the cells have no regenerative potential (Masland, 2012; Silverman and Wong, 2018). Therefore, studying the relationships between its structure, function, and associated diseases is important for the prevention and treatment of RPE lesions.

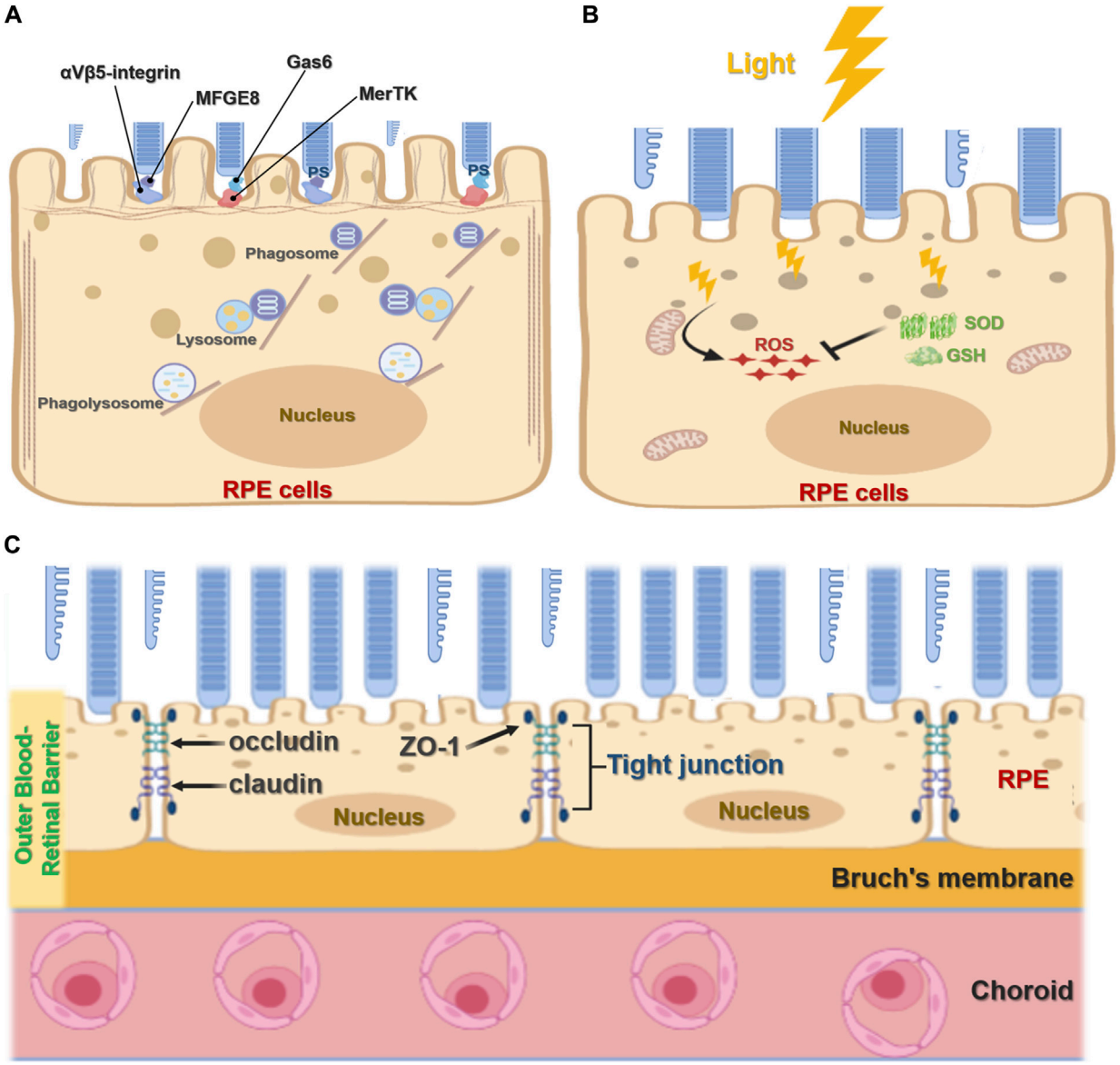

FIGURE 2 | Function of retinal pigment epithelial cells. (A), The phagocytic function of RPE cells. RPE cells recognize and bind phosphatidylserine (PS) exposed by POS through MerTK/Gas6 and αVβ5-integrin/MFGE8 pathways to initiate phagocytosis. It further forms phagosomes and binds with lysosomes to form the phagolysosome, which digests POS. **(B)**, Antioxidant function of RPE cells. Light stress produces ROS. RPE cells absorb light through melanin or eliminate ROS accumulation through antioxidants such as superoxide dismutase (SOD) and glutathione (GSH). **(C)**, RPE cell barrier function. The RPE forms an outer blood-retinal barrier between the interior of the retina and the choroid. The RPE cells form tight junctions, including ZO-1, occludin, and claudin, which act as barriers.

FUNCTION OF THE RETINAL PIGMENT EPITHELIUM

Maintaining the Visual Cycle and Phagocytosis

The RPE plays an important role in maintaining visual function and the visual cycle. RPE cells are phagocytic, with the ability to engulf and eliminate exfoliated POS and maintain the normal renewal of visual cells (Ran et al., 2020; Ran and Zhou, 2020). In mammals, each RPE cell is responsible for about 30 photoreceptors, and of all cell types RPE cells consume the most material in a mammal's lifetime (Young, 1967; Young, 1971; Penberthy et al., 2018). RPE cell phagocytosis is divided into three stages: binding, endocytosis, and elimination. During binding, the inner microvilli cell membranes of RPE cells bind to the shed outer segment of the visual cell before being endocytosed into the cell and finally being transported by the cytoskeleton and vesicles to lysosomes for elimination. The TAM receptor tyrosine kinase MerTK is expressed by RPE cells and is crucial for RPE function, mediating the recognition and endocytosis of the POS by RPE cells. RPE cells without MerTK cannot engulf the POS, causing complete degeneration of the photoreceptor and blindness after birth

(Prasad et al., 2006; Burstyn-Cohen et al., 2012). Other studies have shown that mice lacking αvβ5 integrin have gradually reduced retinal phagocytic capacity with age (Nandrot et al., 2004; C. Yu et al., 2019a) *via* a mechanism by which αvβ5 integrin acts as a receiver for the POS (**Figure 2A**). In addition, the *RPE65* gene encodes all-trans retinol ester isomerase, which is essential for the retinoid cycle. Mutation of the *RPE65* allele has been found to destroy optic cells and cause clinical manifestations of Leber congenital amaurosis type 2 (LCA2) and early-onset retinal dystrophy, eventually leading to complete blindness (Gu et al., 1997; Marlhens et al., 1997; Aguirre et al., 1998).

Protection and Anti-Oxidative Functions

Located in the outermost layer of the retina, the RPE is rich in pigment particles including melanin and lipofuscin, which prevent light damage. These pigment particles are formed *in utero* and are no longer synthesized after birth. RPE melanin absorbs and filters natural light and protects the neural parts of the retina. Since the eyes are exposed to various light stimuli, they exist in a physiological state of photooxidation, accumulating high levels of oxygen free radicals that threaten oxidative damage. As a result, RPE cells contain many antioxidants such as superoxide dismutase and glutathione (**Figure 2B**). Melanosomes also participate in the antioxidant process, scavenging oxygen free radicals. Several mechanisms have been shown to underpin the antioxidant capacity and regulation of RPE cells including the ERK signaling pathway (Chong and Zheng, 2016; Chen et al., 2021); MMP-14 and TIMP-2 (Alcazar et al., 2007); micro(mi)RNA-23 (Lin et al., 2011); and toll-like receptor 3 (TLR3) (Patel and Hackam, 2013).

Barrier and Substance Transport Functions

RPE cells are terminally differentiated in a mitotic quiescent phase. As a typical barrier cells, they guard both the inside and outside of the retina and strictly control substance entry and exit. RPE cells form tight junctions through ZO-1, occludin, and claudin, acting as the outer blood barrier between choroidal pore capillaries and the retinal photosensitive layer (**Figure 2C**). RPE cells use membrane pumps, endocytosis, passive diffusion, and other mechanisms to complete transport and play a key role in nutrient, water, and electrolyte transport between the choroid and retinal cells (Danesh-Meyer et al., 2016; Sun and Zhou, 2020). To achieve epithelium-specific functions, tight junction permeability and selectivity must match the epithelium-specific extracellular transport mechanism. At the same time, the rich membrane pump system in the retina, including the Na-K-ATPase system, enables ion concentrations on both sides to reach a dynamic balance and maintain normal retinal function. In addition, the rich RPE cell transporter system facilitates the transport of substances inside and outside the retina (Sugasawa et al., 1994).

RETINAL PIGMENT EPITHELIAL DISEASES AND PATHOGENESIS

Oxidative Stress and Inflammation

The cornea has a transparent structure and the RPE is exposed to light for long periods of time, has a rich oxygen supply, and consequently large amounts of reactive oxygen are easily generated. In addition, increased systemic glucose levels, such as in diabetics, can facilitate excessive ROS accumulation (F. Yu et al., 2019b; Yu et al., 2020). In degenerative retinopathy, antioxidant levels decrease in cells; that is, the capacity of RPE cells to remove ROS variably reduces, resulting in a large accumulation of POS (Mitter et al., 2014; Campello et al., 2020). Many studies have been conducted on retinal epithelial damage caused by oxidative stress and inflammation, and recent studies have shown that the redox-sensitive microRNA (miR)-144 plays an important role in the regulation of antioxidant signaling pathways in human and mouse RPE. Oxidative stress enhanced the expression of miR-144-3p and mir-144-5p, decreased expression of *Nrf2* and downstream antioxidant target genes *Nqo1* and *Gclc*, decreased glutathione levels, and increased RPE cell death (Yam et al., 2019; Jadeja and Martin, 2020). In summary, oxidative stress and inflammation cause RPE cell damage, which in turn causes retinal dysfunction and even blindness.

Apoptosis and Autophagy

AMD is a serious neurodegenerative disease and a major cause of blindness in developed countries. Transcriptional profiling has shown that diseased RPE exhibits increased apoptosis, autophagy, and endoplasmic reticulum stress levels than normal cells. Other studies have shown that retinopathy is associated with disrupted cellular homeostasis and increased apoptosis, endoplasmic reticulum stress, and autophagy. Moreover, RPE cell death *via* apoptosis and endoplasmic reticulum stress has been observed in AMD and other retinal degenerative diseases (Dunaief et al., 2002; Feher et al., 2006; Bazan, 2007). In a study of RPE cells cultured *ex vivo* from AMD and normal donors, RPE cells from AMD donors accumulated lipid droplets and glycogen particles, harbored disintegrated mitochondria, and had increased numbers of autophagosomes. In addition, compared to RPE cells cultured from normal donors, RPE cells from AMD donors showed increased sensitivity to oxidative stress and decreased mitochondrial activity. The impaired autophagy function of AMD donor RPE was also demonstrated through measurement of the ratio of autophagy markers LC3-II/LC3-I (Golestaneh et al., 2017). These findings indicate a potential pathological mechanism for AMD through abnormal apoptosis and autophagy, thereby providing new targets for novel therapeutic strategies.

Cell Polarity and Interactions

The polarity and cell junctions of the RPE play critical roles in the blood-retinal barrier, maintaining the stability of the internal photoreceptor microenvironment and supporting the choroidal system. Disrupted cell polarity and cell junctions significantly increase the risk of retinal degenerative disease (Caceres and Rodriguez-Boulan, 2020). RPE polarity and cell junction stability are related to the unique basal and apical structures of the retina, which affect phagocytosis and material exchange. For example, cholesterol efflux is mediated by the ABCA1 transport protein at the top and basolateral aspects of the cell (Storti et al., 2017). RPE phagocytic defects are related to photoreceptor degeneration, so

further study of the RPE endocytic pathway may help establish new mechanisms of retinal diseases (Anderson et al., 2017; Kaur et al., 2018; Ran et al., 2021). In addition, patients with RP have been shown to have RPE polarity and functional defects, and the cilial mRNA splicing factor *PRPF* was mutated in these RPE cells (Buskin et al., 2018). In summary, abnormalities in RPE polarity, barrier destruction, and retinal stability may contribute to the pathogenesis of blinding retinal diseases.

TREATMENT OF RETINAL PIGMENT EPITHELIAL DISEASES

Cell Therapy

Stem cell-derived RPE and photoreceptors have restored vision in pre-clinical models of human retinal degenerative diseases. Stem cell transplantation may therefore be an effective future approach to treat RPE diseases. The sources of cells used for retinal cell therapy include stem cells such as embryonic stem cells (ESCs), adult stem cells, and induced pluripotent stem cells (iPSCs). Currently, ESCs, and iPSCs are mainly used for differentiation into RPE, but these cell types still have some limitations including allogeneic rejection and carrying donor pathogenic genes. Stem cell transplantation is feasible for the treatment of retinal epithelial lesions (Zamiri et al., 2006). Indeed, allogeneic fetal retina-RPE transplants under the retina were not rejected in people with RP and advanced AMD (Enzmann et al., 1999). However, the immune privilege of the RPE is not absolute (Zamiri et al., 2004). In another study, patients with AMD undergoing CNV resection received subretinal allogeneic RPE transplantation, and there was immune rejection after immunosuppressive therapy was stopped (Tezel et al., 2007). The clinical application of cell therapy for retinal degenerative diseases faces some important challenges including cell manufacturing, delivery, survival, and physiological behavior; immune responses; and a risk of cancer development.

Gene and Drug Therapy

Thanks to many years of research into retinal diseases, many genes and signal transduction pathways have been identified as potential targets for gene therapy or other therapeutics. For example, ITH12674 is a melatonin and sulforaphane hybrid drug that induces expression of the transcription factor *Nrf2*, which can alleviate retinal degeneration leading to blindness (Campello et al., 2020). The lipid molecule ELV blocks the CB1 receptor and PLD2 in the eye to delay the development of degenerative and inflammatory retinal pathology (Bermudez et al., 2019). Emixustat is a non-retinal small molecule hydrochloride that acts as a highly efficient and selective visual cycle modulator targeting visual cycle isomerase. In the AMD animal model, emixustat can reduce A2E levels, protect the retina from light-mediated damage, and reduce neovascularization in premature retinopathy models (Kubota et al., 2020). Humanin (HNG), a 2.7 kDa 24 amino acid polypeptide, was discovered in a cDNA library derived from the brains of patients with familial Alzheimer's disease. HNG protects primary RPE cells from oxidative damage (Nashine et al., 2017). Retinal cell mitochondria are severely damaged in AMD patients, and HNG is an important cell survival factor that can protect ARPE-19 RPE cell line mitochondria, making it an exciting target in AMD (Gong et al., 2018). In addition, as the first approved target for ophthalmological treatment, recombinant adeno-associated viruses (AAVs) have been used to deliver the *RPE65* gene into RPE cells with mutations or absence of RPE65 to prevent and treat inherited retinal diseases, such as LCA2 and inherited retinal dystrophy (Acland et al., 2001; Russell et al., 2017).

OUTLOOK

The RPE promotes normal retinal function and is an integral part of the retinal system, arising at the earliest stage of retinal development. As specialized phagocytes, RPE cells undertake the most intensive phagocytic task in the body through POS phagocytosis and maintaining the normal renewal and function of rod and cone cells. Its function in the blood-retinal barrier plays an important role in nutrient transport, maintenance of ion homeostasis, and the steady state of the microenvironment inside and outside the retina. The excessive accumulation of ROS and consequent retinopathy due to RPE dysfunction mean that these cells remain an important research focus. The visual circulation maintenance, barrier, substance transport, protection, and antioxidant functions of the RPE are all impaired to varying degrees in retinal degenerative diseases, highlighting the importance and necessity to protect the RPE and maintain or restore its function. Multiple mutations associated with AMD have been identified using sequencing techniques of different strategies. The genes in the complement system, such as complement C3 (C3), complement C9 (C9), complement factor I (CFI), and complement factor H (CFH), are associated with the development of AMD. When the membrane cofactor protein CD46 was knocked out, the mice developed lesions consistent with human dry AMD. In addition, mutations in the promoter of the *HTRA1* (high-temperature requirement protein A1) gene lead to increased HTRA1 expression, resulting in the occurrence of AMD. *FGD6* (FYVE, Rho GEF and PH domain-containing 6) gene mutation leads to increased expression of HTRA1 protein, which increases the risk of wet AMD (Lyzogubov et al., 2016; de Breuk et al., 2020).

At present, there is no effective and feasible method for the treatment of retinal degenerative diseases, although our increased understanding of the cellular biology and molecular genetics of retinal diseases might provide new avenues for prevention and treatment. Given the importance of the RPE in normal retinal function, this cell type provides an excellent focus for the development of treatments for retinopathy. In that regard, stem cell transplantation and gene therapy are currently hot research topics. While directing stem cell differentiation into RPE cells to restore function is an exciting and promising research

direction, application to humans might be hampered by poor efficacy or immune reactions; further research is necessary. Encouragingly, as the study of the genes involved in the development of retinopathy and their signaling pathways increases, various inhibitors designed to target pathogenic genes and mutations are emerging, particularly for the treatment of RP. However, it still remains to be determined whether these targets are applicable to humans and whether modulation of these signaling pathways has off-target effects that might result in adverse reactions.

AUTHOR CONTRIBUTIONS

SY wrote the manuscript and drew the figures. JZ revised the manuscript. DL conceived the study and edited the manuscript.

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Immune Cell Landscape of Patients With Diabetic Macular Edema by Single-Cell RNA Analysis

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Purpose: We performed single-cell RNA sequencing (scRNA-seq), an unbiased and high-throughput single cell technology, to determine phenotype and function of peripheral immune cells in patients with diabetic macular edema (DME).

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from DME patients and healthy controls (HC). The single-cell samples were loaded on the Chromium platform (10x Genomics) for sequencing. R package Seurat v3 was used for data normalizing, clustering, dimensionality reduction, differential expression analysis, and visualization.

Results: We constructed a single-cell RNA atlas comprising 57,650 PBMCs (24,919 HC, 32,731 DME). We divided all immune cells into five major immune cell lineages, including monocytes (MC), T cells (TC), NK cells (NK), B cells (BC), and dendritic cells (DC). Our differential expression gene (DEG) analysis showed that MC was enriched of genes participating in the cytokine pathway and inflammation activation. We further subdivided MC into five subsets: resting $CD14^{++}$ MC, proinflammatory $CD14^{++}$ MC, intermediate MC, resting $CD16^{++}$ MC and pro-inflammatory $CD16^{++}$ MC. Remarkably, we revealed that the proinflammatory $CD14^{++}$ monocytes predominated in promoting inflammation, mainly by increasingly production of inflammatory cytokines (*TNF*, *IL1B*, and *NFKBIA*) and chemokines (*CCL3*, *CCL3L1*, *CCL4L2*, *CXCL2*, and *CXCL8*). Gene Ontology (GO) and pathway analysis of the DEGs demonstrated that the proinflammatory $CD14^{++}$ monocytes, especially in DME patients, upregulated inflammatory pathways including tumor necrosis factor-mediated signaling pathway, I-kappaB kinase/NF-kappaB signaling, and toll-like receptor signaling pathway.

Conclusion: In this study, we construct the first immune landscape of DME patients with T2D and confirmed innate immune dysregulation in peripheral blood based on an unbiased scRNA-seq approach. And these results demonstrate potential target cell population for anti-inflammation treatments.

Keywords: single-cell sequencing, peripheral blood mononuclear cells, monocytes, DME, chronic vascular inflammation

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INTRODUCTION

Diabetic retinopathy (DR) is a significant microvascular complication of diabetes, dividing into nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) clinically based on the proliferative status of the retinal vasculature (Cheung et al., 2010). Diabetic macular edema (DME) is a significant complication of DR, mainly caused by the breakdown of the blood-retinal barrier (BRB) and leaking microaneurysms (Xu and Le, 2011). DME can occur at any stage in the pathogenesis of DR and lead to severe vision loss in diabetic patients (Das et al., 2015).

Recent studies suggest that DR is a chronic low-grade inflammatory disease (Donath and Shoelson, 2011; Tang and Kern, 2011). Inflammation plays a critical role in diabetic retinopathy and contributes to vascular permeability and edema by releasing inflammatory cytokines, leukocyte activation, and leukostasis (Miyamoto et al., 1999; Chibber et al., 2000; Noda et al., 2012; Mesquida et al., 2019). Increasing evidence indicates that cytokines such as interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), IL-6, and IL-8 were significantly upregulated in vitreous and serum of DR patients, resulting in a persistent chronic inflammation state in the retina (Ben-Mahmud et al., 2004; Demircan et al., 2006; Boss et al., 2017; Feng et al., 2018; Khaloo et al., 2020). Innate immune cells, especially monocytes, are reported to play a pivotal role in promoting the pathogenesis of DR (Van Hove et al., 2020; Wan et al., 2020). In peripheral blood of diabetic patients with microvascular or macrovascular complications, $CD45^{+}CD14^{+}$ classical monocytes were increased, but $CD16^{+}$ nonclassical monocytes were decreased, compared with patients without complications (Min et al., 2012). Serra et al. showed that circulating CD11b monocytes from diabetic mice were preferentially trapped in retinal microvascular bed and may lead to diabetic retinal vasculopathy by expressing higher levels of chemokine receptor CCR5(Serra et al., 2012). Recently, neutrophils were identified to promote microvascular occlusions and small-vessel vasculitis by producing neutrophil extracellular traps in PDR (Binet et al., 2020). These studies indicate that immune cells may play essential roles via attaching to vascular endothelium and cause retinal vasculopathy. One single-cell RNA sequencing study has established a high-resolution transcriptome landscape of blood immune cell subsets in T1D children and revealed a high level of IL-32 produced mainly by activated T cells and NK cells could be an early indicator for T1D (Kallionpaa et al., 2019).

Despite these studies, it is still unclear whether chronic vascular inflammation accelerates BRB breakdown and fluid accumulation in DME patients with T2D. Furthermore, little is known regarding the phenotypic and functional diversity of different immune cell types in DME. In addition, their contribution to vascular inflammation remains to be fully elucidated. Thus, defining key cell subsets and their states in DME is crucial in acquiring critical insights into the immune mechanisms and developing new therapeutic strategies for DME.

Here, to clarify the phenotype and function of peripheral immune cells in DME, we utilized single-cell RNA sequencing (scRNA-seq) to comprehensively characterize the transcriptional heterogeneity of PBMCs from healthy individuals and DME patients. Our study depicted a landscape of blood immune cell subsets, including monocytes, dendritic cells, NK, T, and B cells, and characterized their gene expression programs.

MATERIALS AND METHODS

Human Subjects

Four DME patients and four healthy individuals were enrolled at the Zhongshan Ophthalmic Center, Guangzhou, China. All patients were diagnosed with type 2 diabetes with diabetic retinopathy determined by fluorescein angiography and comprehensive ophthalmologic examinations. The clinical-stage of diabetic retinopathy was classified according to the International Clinical Diabetic Retinopathy (Wilkinson et al., 2003). The characteristic of the patients is shown in **Supplemental Table S1**. We selected DME patients with a central macular thickness of 300 μm or more evaluated by optical coherence tomography (OCT). Individuals with autoimmune disease, cancer, cardiovascular diseases, and other eye diseases (such as age-related macular degeneration, cystoid macular edema of other origins, uveitis) were excluded to avoid confusion with other systemic diseases. Written informed consent was obtained from all patients after explaining the purpose and procedures to be used. The study was approved by the Ethics Committee of Zhongshan Ophthalmic Center, China.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) for scRNA-Seq

Blood samples from healthy individuals and patients were processed within 2 h after collection and diluted 1:1 with phosphate-buffered saline (PBS, Gibco, C10010500BT). Then, the diluted samples were layered onto the Ficoll-Paque PLUS (GE Healthcare Life Sciences, 17-1440-03) in the centrifuge tubes and centrifuged at 400 g for 30 min at 18–20°C. The PBMCs layer was collected and washed twice in PBS and identified the viability and quantity of single cells using Trypan blue. If the cell survival rate exceeded 90%, PBMC samples were used for the following scRNA-seq experiment.

scRNA-Seq

The single-cell samples were loaded on the Chromium platform (10x Genomics) for library preparation, and the barcoded scRNA-seq libraries were constructed using the Chromium Single Cell 5′ Reagent kit (10x Genomics) and following the manufacturer's instructions. In brief, single-cell gel beads in emulsions (GEMs) were generated, and reverse transcription (RT) was performed to produce 10x barcoded, full-length cDNA from polyadenylated mRNA. Then, the 10x barcoded cDNA was amplified via PCR, followed by enzymatic fragmentation, end repair, A-tailing, adaptor ligation, and sample index PCR. After the library preparation was completed, the next-generation sequencing was performed on

the 10x Genomics Chromium Illumina NovaSeq6000 platform according to Illumina standard procedures. The quality of the libraries was checked using the FastQC software.

ScRNA-Seq Data Alignment and Quality Control

The raw sequencing data of patients and healthy controls were demultiplexed by CellRanger Software (version 3.1.0) and aligned to the GRCh38 human reference genome with default parameters. The CellRanger count function was used to generate single-cell feature counts for a single library, and the CellRanger aggr function was used to aggregate gene counts of all patients and healthy controls. The single-cell expression matrix was further analyzed by Seurat (V3) according to the tutorial at https://satijalab.org/seurat/site. For quality control, high-quality cells were retained following the criteria: 1) gene number was between 200 and 3,000; 2) the percentage of mitochondrial RNA was <8% per cell. Low-quality cells with high HBB and HBA1 expression levels were also filtered, which identified as the RBC-contaminated cell population. After quality control, 57,650 cells (24,919 HC and 32,731 DME) were left for the following analysis. Mitochondria (M.T.) and ribosomes (RPL and RPS) genes were also eliminated in downstream analysis.

Dimensionality Reduction and Clustering Analysis

Data normalization, scaling, clustering, dimensionality reduction, differential expression analysis, and visualization were processed using the R package Seurat. The global-scaling normalization method "LogNormalize" was employed to normalize the feature expression measurements for each cell. Highly variable features were identified by the FindVariableFeatures function, and data was scaled by the ScaleData function. Moreover, the R package harmony was used to remove batch effects to create a corrected expression matrix for further analysis. Next, dimensionality reduction was performed using principal component analysis (PCA), and cell clusters were visualized with the t-distributed stochastic neighbor embedding (t-SNE) algorithm.

ScRNA-Seq Differential Expression Analysis

Seurat package FindMarkers function with default parameters was used to perform differential gene expression analysis between the control and disease groups of the same cell type. The Wilcoxon rank-sum test within the FindAllMarkers function was used to analyze all single-cell differential gene expression for identified cell subsets. The marker genes for each cluster were detected by comparing them against all other cells in the experiment. The upregulated and downregulated differentiated expressed genes among different comparisons were shown by the volcano plots. In addition, the Venn diagram was used to show the overlap of differentiated expressed genes among different cell clusters.

GO and Pathway Enrichment Analysis

The detected differentiated expressed genes were further used to perform Gene Ontology, gene-set enrichment analysis, and KEGG pathway analysis using Metascape webtool (www.metascape.org) (Zhou et al., 2019).

Transcription Factor Module Analysis

The gene regulatory network of monocytes was constructed by SCENIC, a computational method to predict critical regulators and identify cell state from single-cell RNA -seq data. The R package GENIE3 was first used to generate co-expression gene regulatory networks (GRN), and the co-expression data was then subjected to *cis*-regulatory motif analysis using the R package RcisTarget. Furthermore, the AUCell algorithm was used to score the activity of significant regulons enriched in different clusters.

Cell-Cell Communication Analysis

The cell-cell communication networks between monocytes and other cell clusters were performed using CellphoneDB statistical analysis, a computational approach predicting cell-cell interactions by ligand-receptor interactions analysis. The ligand-receptor interactions calculated by CellphoneDB were based on the expression of a ligand by one cell cluster and a receptor by another cell cluster. Using this method, we compared the enriched ligand-receptor interactions in DME with HC and NPDR. Furthermore, the dot plots generated by R package ggplot2 were used to visualize the top significant interactions in DME.

Statistical Analysis

The Wilcoxon rank-sum test was used to identify the DEGs and compare the differences in the expression of genes of interest between the HC and DME groups. The hypergeometric test and the Benjamini- Hochberg p value were used in Metascape to identify the ontology terms. Furthermore, we used Wilcoxon rank-sum test to assess the significance of the number differences of MC subsets between the HC and DME group.

RESULTS

Study Design and Analysis for Single-Cell Immunophenotyping in DME Patients

To profile the peripheral immune microenvironment of DME, we performed single-cell RNA sequencing (scRNA-seq) to investigate PBMCs from four prospectively enrolled DME patients with T2D and four healthy donors as controls (**Figure 1A**). Single-cell suspensions of PBMCs were collected and converted to barcoded scRNA-seq libraries using 10X Genomics. CellRanger software was used for the initial processing of the sequencing data. Quality metrics included the number of unique molecular identifiers (UMI), genes detected per cell, and reads aligned to the human genome. Miscellaneous cells with high HBB and HBA1 expression levels were filtered, which identified as the RBC-contaminated cell population. After quality control, a total of 57,650 cells (24,919 HC and 32,731 DME) were used for downstream analysis.

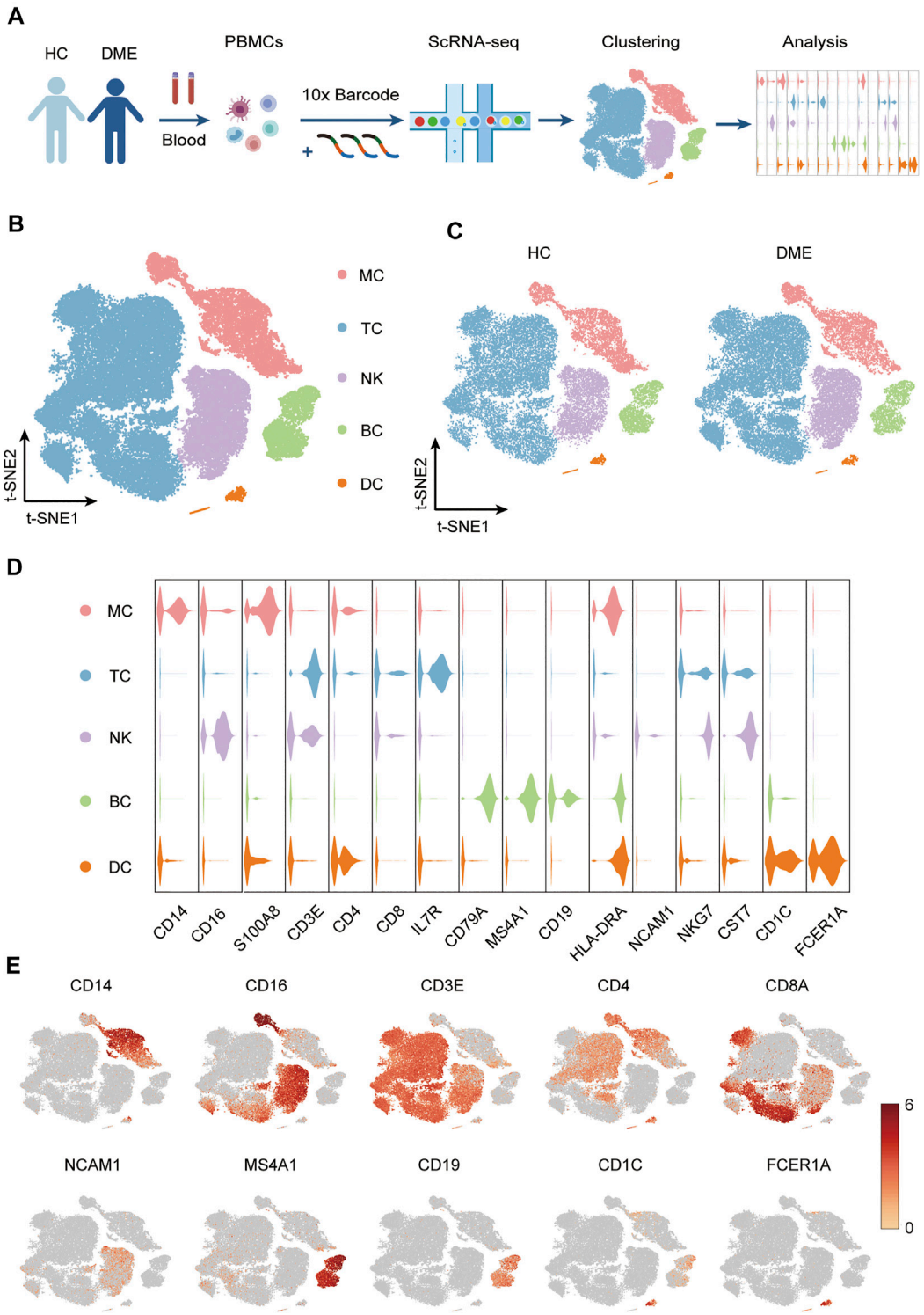

FIGURE 1 | Experimental approach and characterization immune cell clusters from scRNA-seq data. **(A)** Experimental outline showing PBMC collection and scRNA-seq data analysis. **(B)** T-sne plot of major immune cell clusters in PBMCs. Cell types are labeled with colors as indicated. Monocytes (MC); T cells (TC); NK cells (NK); B cells (BC) and dendritic cells (DC). **(C)** T-sne plot of cell clusters in HC and DME respectively. Cell types are labeled with colors as indicated. HC: health control; DME: diabetic macular edema. **(D)** Violin plot of major immune cell clusters in PBMCs, clustered by their relative expression of the cell type-specific markers. Monocytes (MC); T cells (TC); NK cells (NK); B cells (BC) and dendritic cells (DC). **(E)** The feature plot showing the expression of representative markers in each cluster.

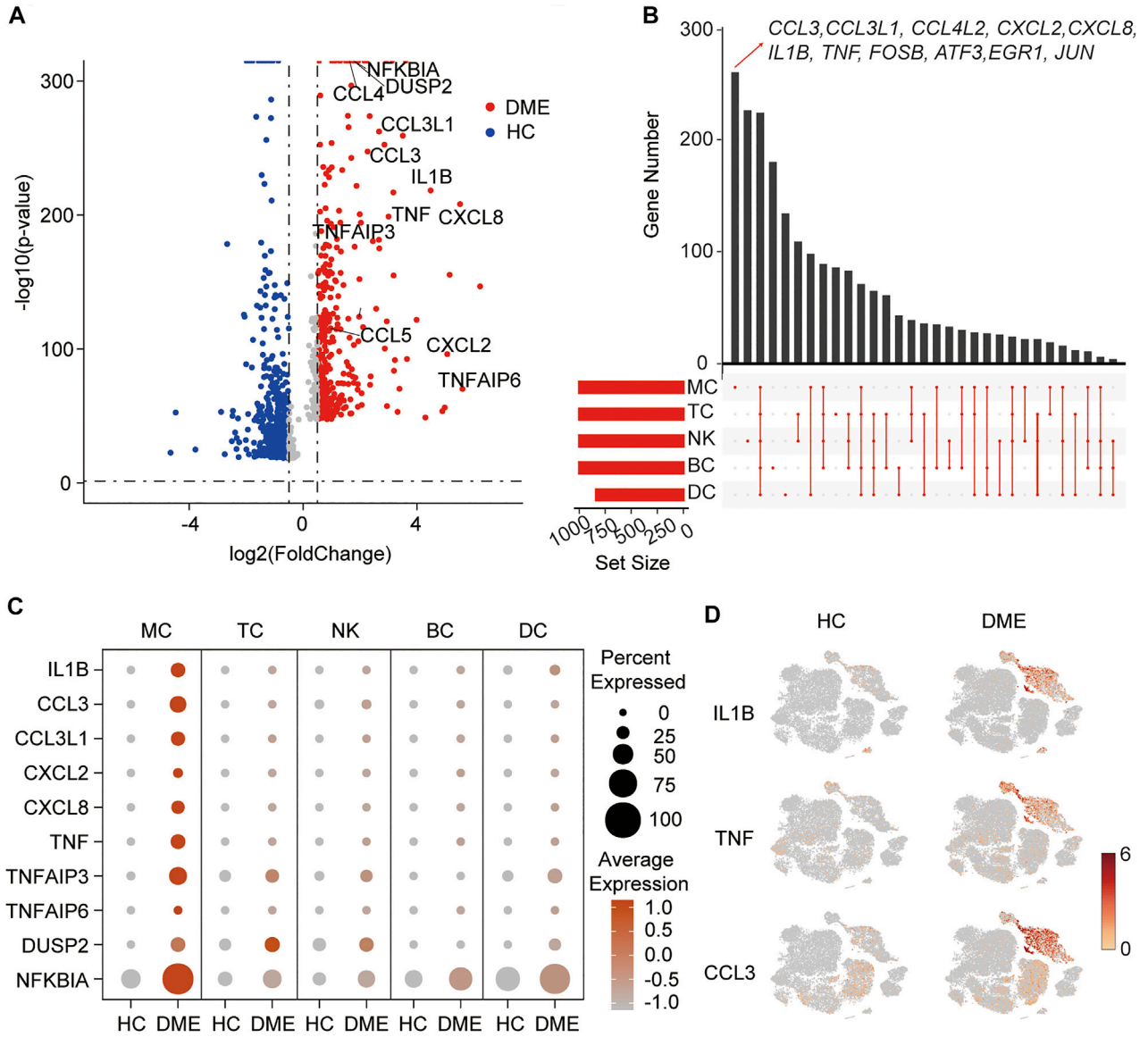

FIGURE 2 | A proinflammatory phenotype mediated by MC in DME patients. **(A)** Volcano plot showing up-regulated and down-regulated DEGs of all immune cells in DME compared to in HC (DME: red; HC: blue). DEG: differential expression gene. **(B)** Venn plot showing unique upregulated genes expressed in MC, DC, TC, NK and BC from DME compared to the ones from HC. **(C)** The dot plot showing the expression of inflammatory genes in the five major cell clusters from the HC and DME group. **(D)** The feature plot showing the expression of representative inflammatory genes, *ILB*, *TNF*, and *CCL3*, in each cluster from the HC and DME group.

Based on the expression of canonical markers in each cluster, we divided all immune cells into five major immune cell lineages, including monocytes (MC), T cells (TC), NK cells (NK), B cells (BC), and dendritic cells (DC). Then we generated two-dimensional visualization of the high-throughput sequencing data using t-distributed stochastic neighbor embedding (t-SNE), an unbiased dimensionality reduction algorithm (**Figure 1B**). We demonstrated that the residual batch effect was removed, and the scRNA-seq data across different groups showed consistent repeatability after gene expression normalization (**Figure 1C**). The violin plots indicated expression levels, and the t-SNE maps confirmed the relative distribution of cell type-specific marker genes across all clusters (**Figures 1D,E**). These plots showed that each cluster was identified by their unique signature genes: *CD14* and *CD16* (MC marker), *CD3E*, *CD4* and *CD8A* (TC marker), *NCAM1* (NK marker), *MS4A1* and *CD19* (BC marker), *CD1C* and *FCER1A* (DC marker) (**Figures 1D,E**).

Proinflammatory Phenotype Mediated by Monocytes in DME Patients

It has been demonstrated that immune cells, such as T cells, NK cells, and monocytes, play different roles in neovascularization

and vascular permeability in diabetic retinopathy (Kallionpaa et al., 2019; Wan et al., 2020). However, the predominant immune cell populations contributing to macular edema in DME remain unknown. Firstly, to understand the transcriptional changes in the immune cells, we conducted a comparative analysis of differential expression genes (DEGs) between HC and DME patients. The volcano plot revealed that inflammatory-related genes (*TNF, TNFAIP6, IL1B, NFKBIA,* and *DUSP2*), chemokines (*CCL3, CXCL2,* and *CXCL8*) were all expressed at high levels in DME patients compared to HC (**Figure 2A**). These highly expressed inflammation-associated genes implied that the immune cells in the blood of DME patients were in a proinflammatory state, which may contribute to vascular endothelial cell damage and retinopathy.

To further investigate transcriptional heterogeneity of immune cell signatures, we analyzed DEGs of each cluster in DME patients compared to HC, and we showed unique and shared upregulated expression genes of MC, TC, NK, BC, and DC in DME using the venn plot (**Figure 2B**). Specific upregulated DEGs of the five immune cell subsets were different in numbers and presented distinct biological functions. Remarkably, we found MC contained the highest number of specific upregulated genes, followed by NK (**Figure 2B**). Interestingly, upregulated DEGs of DME, including inflammatory genes (for example, *CCL3*, *IL1B*, and *TNF*) and transcriptional factors (for example, *FOSB* and *JUN*) found in the volcano plot were significantly differentially expressed by MC only (**Figures 2A,B**), suggesting that MC may mediate the expression changes to activate the inflammatory response. Except for MC, other clusters displayed a resting state with low numbers of proinflammatory genes (**Figure 2B**).

In order to systematically show the differences in the DEGs of immune cell subsets, we compared the expression level of the top inflammatory genes among the five clusters in HC and DME, respectively. The MC had the most robust inflammatory signature with high levels of cytokine genes expression among five immune cell subsets (**Figure 2C**). Our comparison between the two groups also suggested the considerable accumulation of increased cytokine activity in DME patients (**Figure 2C**). The t-SNE maps further confirmed that distribution of upregulated inflammatory genes, such as *IL1B*, *TNF*, and *CCL3*, concentrated in MC of DME patients especially (**Figure 2D**).

A Proinflammatory Monocyte Subset Predominated in the Pathological Process of DME

The analysis above demonstrated that MC was the main proinflammatory cell in DME. Based on the relative expression of *CD14* and *CD16*, monocytes can be traditionally subclassified as classical ($CD14^{++}$ $CD16^{-}$), nonclassical ($CD14^{dim}$ $CD16^{++}$), and intermediate monocytes ($CD14^{++}$ $CD16^{+}$) (Ziegler-Heitbrock et al., 2010). To further understand the heterogeneity of MC in DME patients, we re-clustered all the MC and conducted precise cell classification (**Figure 3A**). We observed distinct distributions of MC subsets in HC and DME on the t-SNE maps (**Figure 3B**). Based on the expression level of canonical lineage markers (*CD14* and *CD16*) and inflammatory-related markers (*IL1B* and *TNF*) (**Figure 3C**), we classified five monocyte subsets and described each subset by the top 10 markers (**Figure 3D**). Here, we discovered that the $CD14^{++}$ MC consisted of two clusters (**Figure 3D**). One presented high *CD14* gene expression and low inflammatory gene expression signature, namely resting $CD14^{++}$ MC. Another cluster called proinflammatory $CD14^{++}$ MC exhibited high *CD14* and inflammatory gene expression (**Figure 3D**), indicating that this subpopulation may be associated with pathogenic processes through inflammation activation. Furthermore, the $CD16^{++}$ MC can also be re-clustered into two subsets. So, we subdivided MC into five subsets: resting $CD14^{++}$ MC, proinflammatory $CD14^{++}$ MC, intermediate MC, resting $CD16^{++}$ MC and proinflammatory $CD16^{++}$ MC, based on the expression of canonical monocyte marker genes and proinflammatory genes (**Figure 3D**). From the heatmap and dot-plot of the five MC subsets, we found that both the $CD14^{++}$ MCs expressed not only recognized markers (*S100A9*, *LYZ*, *S100A8*, *VCAN*, and *S100A12*) but also newly identified markers (*MS4A6A*, *CAPG*, *MGST1*, and *RBP7*) (**Figures 3D,E**). The proinflammatory $CD14^{++}$ MC highly expressed distinguishing biomarkers, including inflammatory markers (*CCL3*, *CCL4*, *CCL4L2*, *CCL3L1*, *IL1B*, *NFKBIA*, and *TNF*) and typical transcription factors (*IER2* and *EGR1*) (**Figures 3D,E**). The intermediate MC with high CD14 and moderate CD16 expression was at the connection of the $CD14^{++}$ MCs, and $CD16^{++}$ MCs showed in the t-SNE plot (**Figure 3A**). HLA-related genes, including *HLA-DPB1, HLA-DRA,* and *HLA-DQA1,* were upregulated in this subset, suggesting an increased antigen processing and presentation (**Figures 3D,E**). The two $CD16^{++}$ MCs represented nonclassical monocytes with high expression of *CD16* and other unique signature makers identified by our scRNA-seq data, such as *LYPD2, VM O 1, CDKN1C, MS4A7,* and *HMOX1* (**Figures 3D,E**). Inflammatory markers (*TNF*, *IL1B,* and *NFKBIA*) were highly expressed in the proinflammatory $CD16^{++}$ MC compared to the resting $CD16^{++}$ MC (**Figures 3D,E**). Among all the MC subsets, the proinflammatory $CD14^{++}$ MC expressed the highest level of inflammatory genes (**Figures 3D,E**). We also observed that the composition of cell subsets in MC differed largely between the HC and DME groups, and the fraction of proinflammatory $CD14^{++}$ MC was remarkably elevated in DME patients (**Figure 4A**). Furthermore, the frequency of proinflammatory $CD14^{++}$ MC in all PBMCs was significantly increased in DME compared to HC (HC vs DME; $p < 0.05$; **Figure 4C**), while resting $CD14^{++}$ MC was significantly decreased (HC vs DME; $p < 0.05$; **Figure 4B**). These results suggested that $CD14^{++}$ MC in DME patients turned from a resting state into a pro-inflammatory state.

The Inflammatory Genes and Signaling Pathways Were Enriched in the Proinflammatory $CD14^{++}$ MC

In order to delineate how MC changed between HC and DME, we first compared the unique DEGs of each group. The MC in DME was uniquely characterized by the upregulation of inflammatory

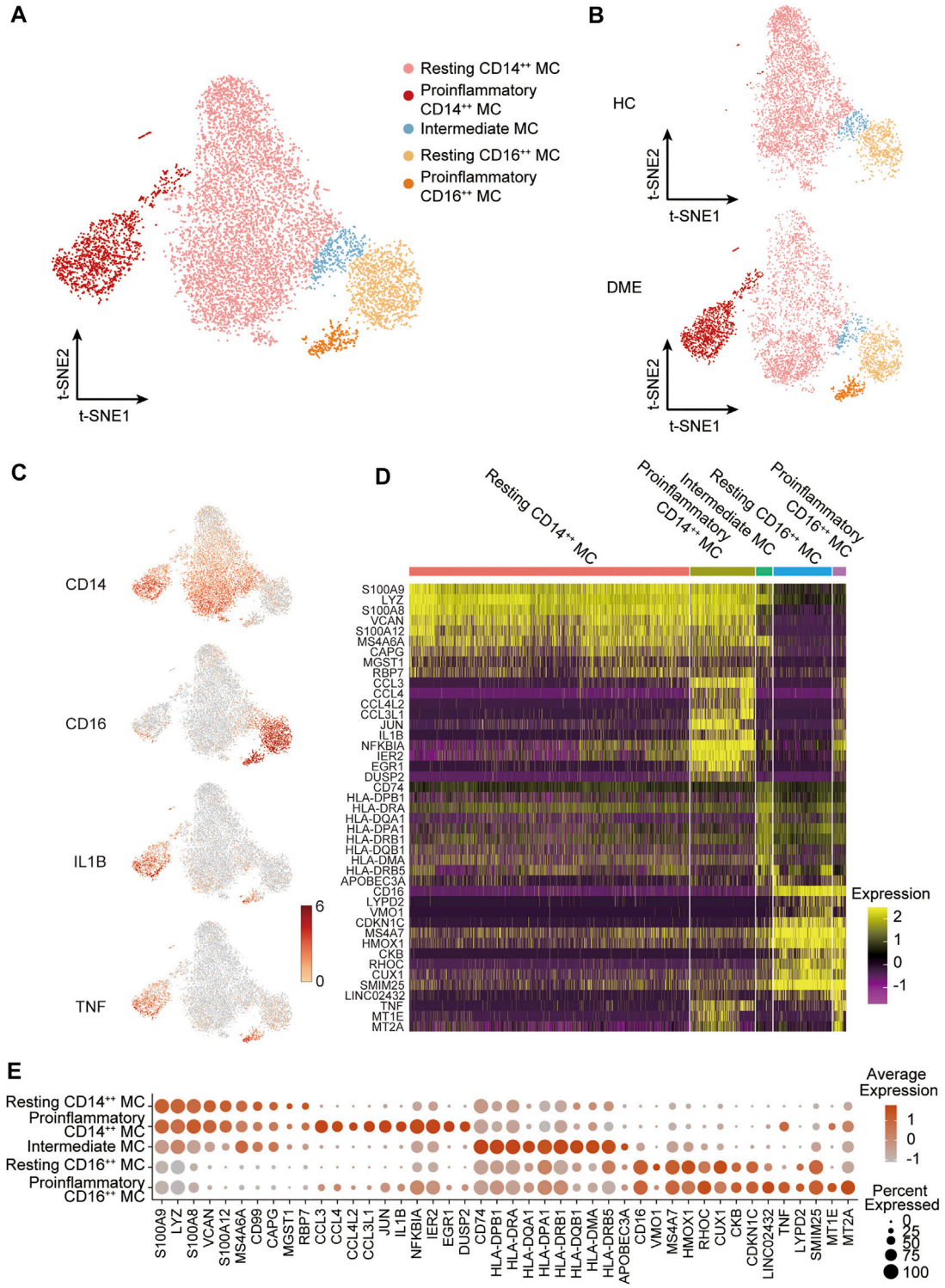

FIGURE 3 | MC subset classification and characterization in DME patients. **(A)** T-sne plot of major MC subsets. Cell subtypes are labeled with colors as indicated. **(B)** T-sne plot of major MC subsets in HC and DME, respectively. Cell subtypes are labeled with colors as indicated. **(C)** The feature plot showing the expression of canonical lineage markers in all clusters. **(D)** The heatmap showing expression of top 10 marker genes in all MC subsets. **(E)** The dot plot showing expression of top 10 marker genes in all MC subsets.

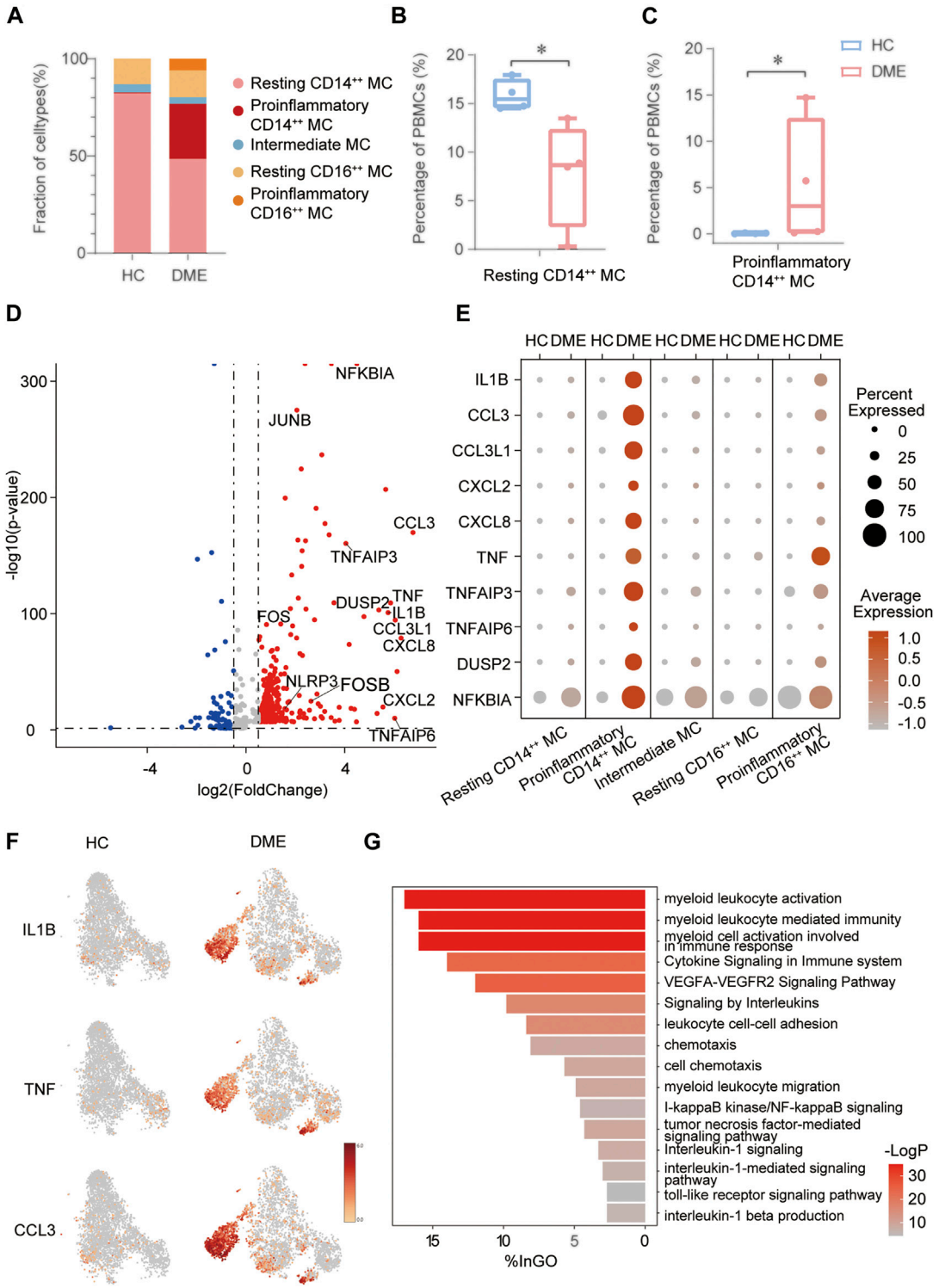

FIGURE 4 | The inflammatory genes and signaling pathways enriched in proinflammatory CD14^{++} MC. **(A)** Comparation of relative fractions of MC subsets from the HC and DME group. **(B)** Quantification of resting CD14^{++} MC in PBMCs from the DME group compared to the HC group. (n = 4, *: *p* value <0.05). **(C)** Quantification of proinflammatory CD14^{++} MC in PBMCs from the DME group compared to the HC group. (n = 4, *: *p* value <0.05). **(D)** Volcano plot showing up-regulated and down-regulated DEGs of MC in DME compared to in HC (DME: red; HC: blue). **(E)** The dot plot showing the expression of inflammatory genes in the five MC subsets from the HC and DME group. **(F)** The feature plot showing the expression of inflammatory markers from the HC and DME group. **(G)** The bar plot showing the signaling pathways enriched in proinflammatory CD14^{++} MC by Gene Ontology (GO) analysis.

genes, including inflammatory cytokines (*TNF*, *IL1B*, *NFKBIA*, *DUSP2*, *NLRP3*, and *TNFAIP6*), chemokines (*CCL3*, *CCL3L1*, *CCL4L2*, *CXCL2*, and *CXCL8*), and transcriptional factors (*FOS*, *FOSB*, and *JUNB*) (**Figure 4D**). The most distinct transcriptional differences of the proinflammatory $CD14^{++}$ MC compared to other MC subsets were the higher levels of inflammatory genes, consistent with a proinflammatory phenotype (**Figure 4E**). Furthermore, the dot plot revealed DME patients owned the most activated MC, while MC in H.C. presented a resting state (**Figure 4E**). We further identified that distribution of upregulated inflammatory genes, such as *IL1B*, *TNF*, and *CCL3*, concentrated in the proinflammatory $CD14^{++}$ MC of DME patients especially confirmed by the t-SNE maps (**Figure 4F**). These analysis data further confirmed that MC, especially the proinflammatory $CD14^{++}$ MC, played an essential role in the pathogenesis of DME by inflammation activation.

We identified five distinct MC subsets and demonstrated the most activated and proinflammatory MC by transcriptional analysis. To further investigate functional heterogeneity of the proinflammatory $CD14^{++}$ MC in DME, we analyzed signaling pathways of this subset by Gene Ontology (G.O.) and pathway enrichment analysis using the upregulated DEGs in DME compared to HC (**Figure 4G**). Consistent with the high levels of inflammatory genes, signaling pathways related to monocytes activation and inflammatory response were enriched in the proinflammatory $CD14^{++}$ MC of DME (**Figure 4G**). When activated, circulating monocytes were recruited to the sites of inflammation and initiated immune responses in the pathology of many diseases (Shi and Pamer, 2011). As for DME, the proinflammatory $CD14^{++}$ MC was characterized by highly upregulated myeloid leukocyte activation, migration, and chemotaxis pathways (**Figure 4G**). We found the VEGFA-VEGFR2 pathway, known to promote neovascularization and BRB breakdown, was also significantly up-regulated (**Figure 4G**). The abundance of inflammatory cytokine-related pathways, such as cytokine signaling in the immune system, signaling by interleukins, and interleukin-1 beta production, revealed that the proinflammatory $CD14^{++}$ MC initiated inflammatory responses mainly by cytokine production, especially *IL1B* (**Figure 4G**). This MC subset also enhanced cell adhesion pathways (**Figure 4G**), indicating that monocytes may interact with other immune cells and retinal vascular endothelial cells. Common upregulated inflammatory pathways included tumor necrosis factor-mediated signaling pathway, I-kappaB kinase/NF-kappaB signaling, and toll-like receptor signaling pathway (**Figure 4G**). These signaling pathway analyses highlighted the activation and inflammatory functions of the proinflammatory $CD14^{++}$ MC.

Specific Transcription Factors Predicted by SCENIC Regulated Activation of the Proinflammatory $CD14^{++}$ MC

We conducted single-cell regulatory network inference and clustering (SCENIC) analysis to evaluate the expression levels of transcription factors (TFs) in the four distinct monocyte subsets and explore potential TFs involved in the inflammatory responses. This computational method can predict critical regulators and their direct target genes (Van de Sande et al., 2020). We observed different SCENIC-predicted TFs expressed exclusively in specific monocyte clusters, including new and canonical transcription factors (**Figure 5A**). The proinflammatory $CD14^{++}$ MC was enriched in inflammation-relevant TFs, such as FOS, JUN, JUNB, JUND, NF-κB1, NF-κB2, REL, and XBP1, compared to the other four subsets (**Figures 5A,B**). The abundance of these TFs may promote proinflammatory $CD14^{++}$ MC in DME (Wagner and Eferl, 2005; Martinon et al., 2010; Sun, 2017). The motif enrichment of these TFs is mainly localized in the regions of the proinflammatory $CD14^{++}$ MC (**Figure 5B**), consistent with the activity of inflammatory gene expression (**Figures 4E,F**). Through SCENIC analysis, we predicted candidate TFs of the proinflammatory $CD14^{++}$ MC participating in the inflammatory process. Taken together, these results further demonstrated that the proinflammatory $CD14^{++}$ MC was predominated in the pathological process of DME.

Cell-Cell Communication Was Enhanced Among Monocytes and Other Immune Cells in DME

The immune system is a complex network, and blood circulating immune cells can contact and influence each other by cell-cell interactions, which can be identified via scRNA-seq data analysis (Armingol et al., 2021). To understand how monocytes communicated with other four immune cell clusters in the DME patients, we applied CellPhoneDB (Efremova et al., 2020), a computational approach predicting cell-cell interactions by ligand-receptor partners analysis, to explore cellular behavior alterations of DME compared to HC (**Figure 6A**). We discovered that the interactions of eight chemokine, seven cytokine, and five adhesion molecule ligand-receptor pairs were significantly elevated in DME patients (**Figure 6A**). Notably, the interaction patterns of these ligand-receptor pairs were mostly from MC to other immune cell subsets, consistent with the high expression levels of inflammatory genes (**Figure 2C**, **Figure 6A**). Compared to the two groups, the CCL3-CCR1 and CCL3L3-CCR1 pair were limited to the MC-MC and MC-DC interaction, and the CCL3L3-DPP4 pair was only found in MC-TC (**Figures 6A,B**).

CCL4-CNR2 pair occurred only in MC-BC interaction (**Figures 6A,B**). In contrast, the CCL4L2-VSIR pair contributed to interactions of MC and other subsets except for BC (**Figures 6A,B**). MC can also recruit TC and NK through CXCL2 secretion. However, CXCL8 interacted uniquely with its receptor CXCR2 in NK (**Figures 6A,B**). The proinflammatory cytokine IL1B and receptor ADRB2 specially communicated MC and TC and NK (**Figures 6A,B**). The cytokine TNF contributed to a broad spectrum of cell communication through increased ligand-receptor pairs such as TNF-VSIR, TNF-FAS, TNF-TNFRSF1A, and TNF-TNFRSF1B (**Figures 6A,B**). The adhesion molecule ICAM1 played an extensive role in cell-cell interactions with its receptor such as SPN, ITGAL, aMb2, aXb2, and aLb2 complex (**Figures 6A,B**). Specifically, we found that

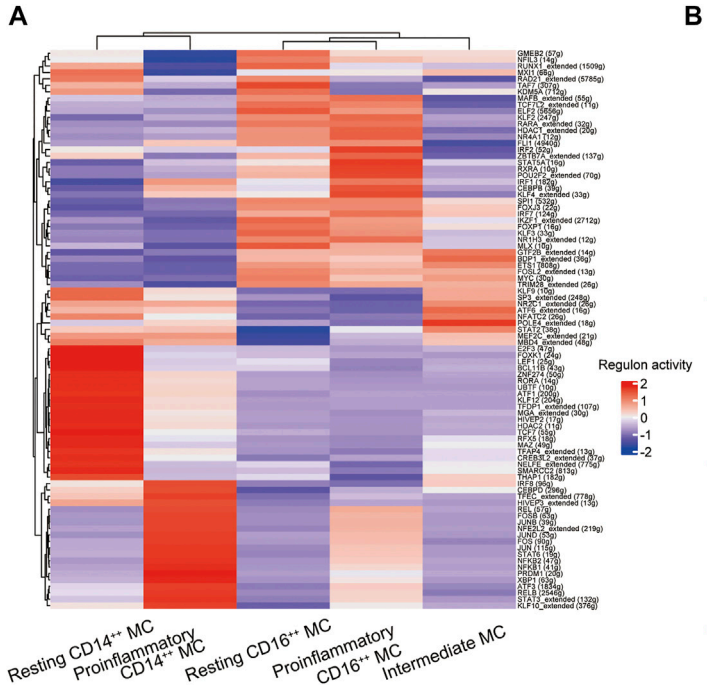

FIGURE 5 | A gene regulatory network among the TFs predicted by SCENIC. **(A)** Heatmap of the AUC scores of transcription factors (TFs) in monocyte subsets, as estimated using SCENIC. **(B)** T-sne plots showing the regulon activity of the corresponding TFs (FOS, JUN, JUNB, JUND, NFKB1, NFKB2, REL, and XBP1).

MC required self-recruitment and activation through significantly increased cell interactions in DME patients (**Figure 6A**). These results confirmed that monocytes had a considerably enhanced propensity to initiate inflammation responses by secreting chemokines and cytokines. Taken together, these results predicted the possible molecular mechanisms underlying cell-cell communication in DME patients, further demonstrating the activation and proinflammatory signatures of MC.

DISCUSSION

It is increasingly recognized that chronic low-grade and sterile inflammation contributes to the pathogenesis of DR, from early phases to the vision-threatening advanced stages (Tang and Kern, 2011; Mesquida et al., 2019; Semeraro et al., 2019). Some studies had reported an increased adherence of leukocytes that contributes to physically capillary occlusion and microvascular damage by producing cytokines (Schroder et al., 1991; Ulbrich et al., 2003; Joussen et al., 2004). Despite decades of research, the immune mechanisms contributing to these processes in DME remain largely unresolved, and the identification of specific immune dysregulation is needed to develop new therapeutic strategies for DME. This study depicted the first single-cell immune atlas of peripheral blood in DME patients, allowing a precise understanding of inflammatory immune mechanisms. Compared to HC, a hyper-inflammatory response in DME was observed, which may explain why some DME patients had severe vision loss. In addition, we identified five major cell clusters with unique gene expression patterns and discovered that monocytes are the domain proinflammatory cells in DME patients. The monocytes were subdivided into four subsets, and their activation status, function signatures, and immune mechanisms were comprehensively described.

It has been demonstrated that monocytes are crucial participants in mediating chronic inflammatory diseases such as diabetes, atherosclerosis, and rheumatoid arthritis (Weber et al., 2008; Grossmann et al., 2015; Tabas and Lichtman, 2017; Cecchinato et al., 2018; Jordan et al., 2019; Zhang et al., 2019). Furthermore, accumulating evidence suggests that monocytes are involved in the pathogenesis of diabetic complications, including diabetic nephropathy and diabetic retinopathy (Nakajima et al., 2002; Theocharidis et al., 2020; Torres et al., 2020; Wan et al., 2020). In our study, monocytes in DME displayed unique differences and highly specialized functions compared to other immune cell subsets (**Figures 2A,C,D**). With DEG analysis of our transcriptional data, we observed that the inflammatory cytokines (*TNF*, *IL1B*, and *NFKBIA*) and chemokines (*CCL3*, *CCL4L2*, *CXCL2*, and *CXCL8*) are highly expressed in monocytes (**Figures 2B,C**). Compared to HC, monocytes showed higher expression of inflammatory genes in DME patients (**Figures 2C,D**), consistent with the high protein levels in vitreous and serum of DME patients reported in the previous studies (Ben-Mahmud et al., 2004; Demircan et al., 2006; Boss et al., 2017; Feng et al., 2018; Khaloo et al., 2020). Thus, it is reasonable to conclude that monocytes in DME exhibited an activated and proinflammatory

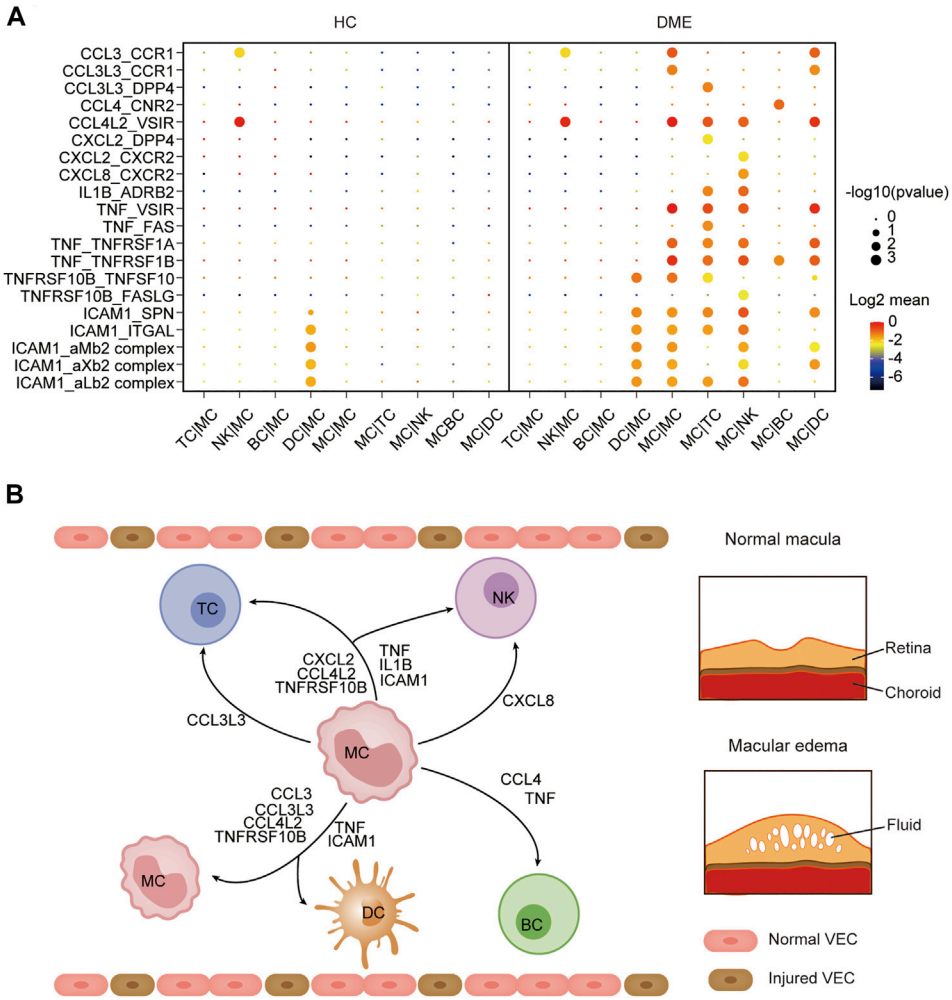

FIGURE 6 | Cell-cell communication was enhanced among MC and other immune cells in DME. **(A)** Dot plot of the predicted interactions between monocytes and the indicated immune cell types in the HC and DME group. **(B)** Summary illustration depicting the potential cytokine-receptor interactions between MC and other types of peripheral immune cells in the DME.

status, thus enhancing the generation of chronic low-grade inflammation in the diabetic retina by releasing inflammatory cytokines and chemokines.

Human monocytes are traditionally subdivided into classical ($CD14^{++}$ $CD16^{-}$), nonclassical ($CD14^{dim}$ $CD16^{++}$), and intermediate monocytes ($CD14^{++}$ $CD16^{+}$), according to the relative expression of CD14 and CD16 (Ziegler-Heitbrock et al., 2010; Wong et al., 2011). Here, we discovered that $CD14^{++}$ MC showed two distinct states based on the expression of inflammatory genes (**Figures 3A,C–E**). Therefore, we classified $CD14^{++}$ MC into resting $CD14^{++}$ MC and pro-inflammatory $CD14^{++}$ MC (**Figure 3A**). We found that the upregulation of inflammatory genes expression, including inflammatory cytokines and chemokines, was largely focused on the proinflammatory $CD14^{++}$ MC (**Figures 4E,F**). Moreover, our GO and pathway analysis indicated the proinflammatory $CD14^{++}$ MC in DME was enriched with proangiogenic and proinflammatory pathways (**Figure 4G**). Previous studies have reported that VEGFA-VEGFR2 pathway contributed to neovascularization through regulating proliferation and sprouting of vascular endothelial cells, and also by increasing the permeability (Goel and Mercurio, 2013; Stitt et al., 2016). Hence, it is likely that the proinflammatory $CD14^{++}$ MC may promote angiogenesis and macular edema with up-regulated VEGFA-VEGFR2 pathway. Corresponded to the high levels of inflammatory cytokines, the proinflammatory $CD14^{++}$ MC was enriched in inflammatory cytokine-related pathways, such as signaling by interleukins, tumor necrosis factor-mediated signaling pathway, I-kappaB kinase/NF-kappaB signaling, toll-like receptor signaling pathway, and so on (**Figure 4G**). Consistent with the upregulation of chemokines, the proinflammatory $CD14^{++}$ MC was characterized by highly upregulated myeloid leukocyte migration, chemotaxis, and differentiation pathways (**Figure 4G**). These data suggested the proinflammatory $CD14^{++}$ MC was the predominant activated cell population in peripheral blood of DME patients.

Previous studies had reported AP-1 family TFs (FOS, FOSB, JUN, JUNB, and JUND), NF-κB family TFs (NF-κB1, NF-κB2, and REL), and XBP1 that participated in immune response, including immune cell activation, differentiation, and proinflammatory cytokines production (Wagner and Eferl, 2005; Martinon et al., 2010; Sun, 2017). Our DEG analysis indicated the proinflammatory $CD14^{++}$ MC was uniquely characterized by higher gene expression of transcriptional factors (*FOS*, *FOSB,* and *JUNB*) (**Figure 4D**). Through SCENIC analysis, we predicted FOS, JUN, JUNB, JUND, NF-κB1, NF-κB2, REL, and XBP1 as key regulators directing inflammatory gene expression proinflammatory $CD14^{++}$ MC (**Figures 5A,B**). These results may explain the transition from resting $CD14^{++}$ MC to proinflammatory $CD14^{++}$ MC in DME patients.

Interaction between monocytes and other immune cells may help understand the functional states of monocytes in DME. CellPhoneDB analysis predicted monocyte-centric ligand-receptor pairs and constructed interaction networks (**Figures 6A,B**). As a result, multiple inflammation-related ligand-receptor pairs' expression was significantly increased in DME patients compared to HC (**Figure 6A**). Previous studies have noted that monocytes can sense environmental changes, migrate into lesions and differentiate into macrophages, playing a significant role in chronic inflammatory disease (Jakubzick et al., 2017). In our study, monocytes predominated in producing chemokines, cytokines, and adhesion molecules, which promoted interaction with other immune cells by different ligand-receptor pairs (**Figure 6A**). Early reports suggest increasing chemokines and cytokines results in immune cells recruitment to the diabetic retina and lead to immune dysregulation and retinal tissue damage (Rubsam et al., 2018).

Intravitreal anti-VEGF drugs are first-line treatments for DME, but a large fraction of patients didn't show complete response (Antonetti et al., 2012). There is need to explore new therapeutics targeting VEGF-independent mechanisms, such as anti-inflammation drugs. Due to technical and ethical issues, we could only collect immune cells from patients' peripheral blood samples but not directly from their retinal tissues. Nevertheless, we believe that a single-cell analysis on peripheral immune cells could provide new insight into the systemic control of diabetes that may reduce DME, possibly aided by anti-inflammatory agents targeting monocytes in peripheral blood. We revealed that the proinflammatory $CD14^{++}$ MC in DME was uniquely characterized by the upregulation of inflammatory genes, including inflammatory cytokines (*TNF*, *IL1B*, *NFKBIA*, *DUSP2*, *NLRP3*, and *TNFAIP6*), chemokines (*CCL3*, *CCL3L1*, *CCL4L2*, *CXCL2*, and *CXCL8*), and transcriptional factors (*FOS*, *FOSB*, and *JUNB*). GO and pathway analysis of the DEGs demonstrated that the proinflammatory $CD14^{++}$ monocytes upregulated inflammatory pathways including tumor necrosis factor-mediated signaling pathway, I-kappaB kinase/NF-kappaB signaling, and toll-like receptor signaling pathway. Further studies are needed to investigate the roles of these up-regulated inflammatory cytokines and chemokines and signaling pathways in the proinflammatory $CD14^{++}$ MC for new drug development.

Our study was limited by the fact that we did not examine the entire immune landscape of DME patients, given that we only included PBMCs in our study, without including granulocytes and other immune cells. Further studies should be undertaken to investigate other cell populations associated with DME that were not fully addressed in the current paper, such as the NK cell sub-populations.

In conclusion, this study constructed the first immune landscape of DME patients with T2D and confirmed innate immune dysregulation in peripheral blood based on an unbiased scRNA-seq approach. With this high-resolution technology, one particular cell subset, the proinflammatory $CD14^{++}$ MC, was identified to predominate in the pathogenesis of DME, providing a more comprehensive understanding of monocytes in human peripheral blood. Notably, we discovered that this subset in DME required a discriminative inflammatory gene expression signature, indicating its activated status and proinflammatory functions. Anti-inflammation treatments targeting this proinflammatory monocyte subset would be helpful for DME patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://bigd.big.ac.cn/gsa-human/browse/HRA001139.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Zhongshan Ophthalmic Center, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL and YZ had full access to all the data in the study and took responsibility for the data's integrity and the accuracy of the data analysis. Concept and design: YL, PM, YZ. Acquisition, analysis, or interpretation of data: All authors. Drafting of the article: PM, YZ. Critical revision of the article for important intellectual content: All authors. Statistical analysis: PM, YZ. Administrative, technical, or material support: PZ, BL. Supervision: YL, YZ.

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Comparative Study of a Modified Sub-Tenon's Capsule Injection of Triamcinolone Acetonide and the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Retinitis Pigmentosa Combined With Macular Edema

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Retinitis pigmentosa (RP) is a hereditary retinal degenerative disease leading to eventual blindness. When RP is combined with macular edema (ME), the visual impairment further worsens. We compared a modified sub-Tenon's capsule injection of triamcinolone acetonide (TA) and the intravenous infusion of umbilical cord mesenchymal stem cells (UCMSCs) in the treatment of RP combined with ME (RP-ME) to assess their safety and efficacy in eliminating ME and restoring visual function. A phase I/II clinical trial enrolled 20 patients was conducted. All patients were followed up for 6 months. There were no severe adverse effects in both groups. In retinal morphological tests, the central macular thickness (CMT) in TA group significantly decreased at first week, first and second month after injection ($p < 0.05$). The CMT in UCMSCs group significantly decreased at first month after infusion. The rate of reduction of CMT in TA group was significantly greater than that in UCMSCs group at second month ($p < 0.05$). Reversely, the rate of reduction of CMT in UCMSCs group was significantly greater than that in TA group at sixth month ($p < 0.05$). In visual functional test, although there were no significant differences in visual acuity or visual fields within each group or between groups, but the amplitude of P2 wave of flash visual evoked potential (FVEP) showed significant increasing in TA group at second month in UCMSCs group at sixth month ($p < 0.05$). At 6th month, the rate of growth in the amplitude of P2 wave in USMCSs group was significantly greater than that in TA group ($p < 0.05$). This study suggests both modified sub-Tenon's capsule injection of TA and intravenous infusion of UCMSCs are safe for RP-ME patients. TA injection is more effective at alleviating ME while improving visual function in a short term. UCMSC intravenous infusion shows slow but persistent action in alleviating ME, and can improve the visual function for a longer time. These

approaches can be applied separately or jointly depending on the disease condition for patients to benefit maximumly.

Clinical Trial Registration: http://www.chictr.org.cn, identifier ChiCTR-ONC-16008839

Keywords: umbilical cord mesenchymal stem cells, triamcinolone acetonide, retinitis pigmentosa, macular edema, sub-Tenon's capsule injection

INTRODUCTION

Retinitis pigmentosa (RP) is a group of hereditary retinal degenerative diseases characterized by progressive RPE dysfunction and photoreceptor loss. The clinical symptoms include poor night vision, visual field constriction and eventual blindness (Anasagasti et al., 2012). To date, there are no effective interventions to prevent this disease from advancing. When RP is combined with macular edema (ME), the condition is more difficult to treat, and the visual impairment becomes worse (Strong et al., 2017; Tan et al., 2021). The prevalence of ME in RP ranges from 11 to 49% depending on different approaches used for examination (Hajali et al., 2008; Huckfeldt and Comander, 2017; Salvatore et al., 2013). Thus, for patients who have RP combined with ME (RP-ME), effective control of the ME is crucial in order to rescue visual function. The mechanisms involved in RP-ME may include breakdown of the blood-retinal barrier, retinal pigment epithelial and Müller cell dysfunction, production of antiretinal antibodies, etc (Strong et al., 2017). Inflammation and auto-immune processes were found to be the major underlying pathogenesis (Yoshida et al., 2013; Narayan et al., 2016; Strong et al., 2017). Given the complicated mechanisms RP-ME has, a therapeutic regime covering multiple perspectives of action is needed.

Cortical steroids are important therapeutic agents for tissue inflammation and edema, among which, triamcinolone acetonide (TA) is widely used because of its long-acting anti-inflammation and immune modulation effects (Saraiva et al., 2003). Local administrations including intravitreal injection and sub-Tenon's capsule injection of TA have been applied in many retinal pathological conditions combining with ME (Ip et al., 2004). Compared with intravitreal injection, the sub-Tenon's capsule injection has less risks and complications. For RP-ME, a short-term study has demonstrated a beneficial effect of sub-Tenon's capsule injection of TA (Karasu, 2020). To our knowledge, there are still no studies focusing on the long-term observation and visual functional assessments following sub-Tenon's capsule injection of TA in RP-ME.

On the other hand, the attempt of stem cell therapy in RP has been long explored. As a major kind of mesenchymal stem cells, umbilical cord mesenchymal stem cells (UCMSCs) have a wide range of biological effects, such as anti-inflammation, immune modulation, paracrine and neurotrophy (Nagamura-Inoue & He, 2014; Xiuying et al., 2014; Zou et al., 2012). Many clinical trials have demonstrated beneficial effects of intravenous infusion of UCMSCs in the treatment of different systemic diseases including neurological, cardiac and osteoarticular disorders (Bartolucci et al., 2017; Mukai et al., 2018; Riordan et al., 2018). In our previous study, UCMSCs were intravenously administered in RP patients. During the 12 months of follow up, most patients improved their visual acuities in the first 3 months, and maintained their visual function for the whole duration of follow up. Besides, the visual acuity related life quality, which was assessed by the relevant questionnaire scores, was significantly increased during first 3 months (Zhao et al., 2020). However, it is still unknown if UCMSCs can help alleviate the RP-ME. Although UCMSCs and TA have some common functions, their effectiveness may vary in onset time, maintaining duration or magnitude of improvement. To know the differences between these two agents and make single or combined treatment regime accordingly will help patients achieve maximum benefit. In this study, we compared the safety and efficacy of an intravenous infusion of UCMSCs and a modified sub-Tenon's capsule administration of TA in RP-ME. Here, we report the results of our study.

MATERIALS AND METHODS

Study Design

This is a prospective, open label, randomized, phase I/II clinical trial. This study was approved by the Medical Ethics Committee of Southwest Hospital, the Army Medical University, and conducted between July 2016 and March 2018. The subjects in the UCMSCs infusion group received a single intravenous infusion of 3×10^6 UCMSCs, and the subjects in the TA injection group received a single injection of 20 mg of TA. All of the subjects were followed for 6 months. Systemic and ophthalmological examinations were performed to assess the safety and efficacy (**Figure 1**). The study adhered to the principles of the Declaration of Helsinki and the International Ethical Guidelines for Biomedical Research Involving Human Subjects and was registered in the Chinese Clinical Trial Registry (Primary Registry of the International Clinical Trials Registry Platform of the World Health Organization) (ChiCTR-ONC-16008,839). Every patient that was recruited for the study signed a written informed consent form.

Umbilical Cord Mesenchymal Stem Cells Preparation

The UCMSCs used for this study were derived from neonatal umbilical cord tissue according to the standard protocol and met the criteria approved by the International Society for Cellular Therapy (Dominici et al., 2006; Majore et al., 2011; Mushahary et al., 2018; Salehinejad et al., 2012). The preparation of the cells

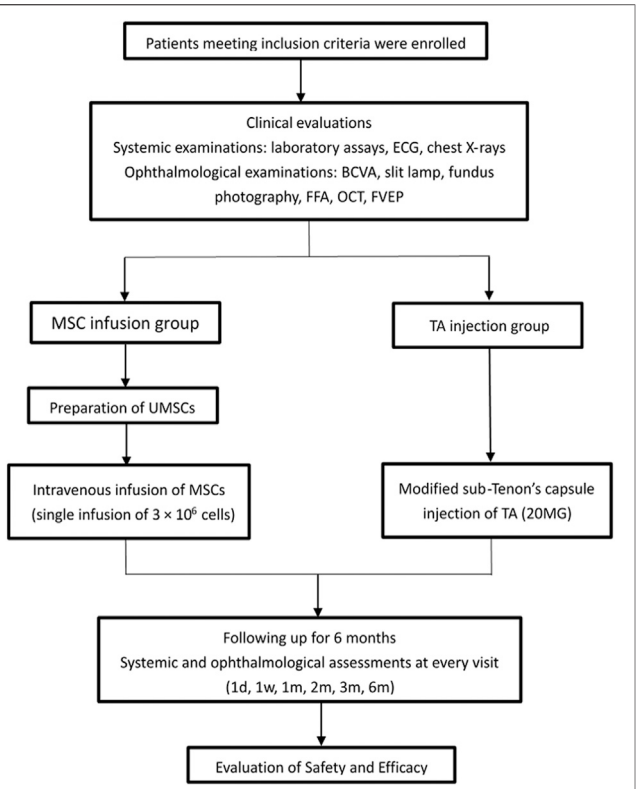

FIGURE 1 | Flow chart of the study.

was performed by the Biotherapy Centre of the Army Medical University. Briefly, the Wharton's Jelly tissue was aseptically cut into a homogenate of 2–3 mm^3 tissue blocks, then the blocks were seeded into T75 flasks in Mesenchymal Stem Cell Basal Medium (DAKEWE, Beijing, China) supplemented with 5% UltraGROTM (HPCFDCRL50, Helios). The tissue blocks were cultured at 37°C, 5% CO2 for about 10 days for UCMSCs to reach confluence. Then cells were digested with 0.125% Trypsin and passaged at 1:3 ratio. Each enzymatic digestion step was considered to be a passage. Cells at P3-P5 were used for infusion. All infused UCMSCs were prepared based on the criteria approved by the International Society for Cellular Therapy. The final products met all of the following criteria: cell viability was no less than 95%; the cells were sterile; the cells did not have endotoxins, *mycoplasma*, hepatitis B, hepatitis C, or syphilis; and the cells exhibited expression of the appropriate surface markers (the positive rate of CD34 and CD45 was less than 0.5%, the positive rate of CD29 was more than 95%, the positive rate of CD90 was more than 95%, the positive rate of CD105 was more than 95%, and the positive rates of CD71 and CD73 were more than 95%). (**Supplementary Figures S1–S3**).

Patient Screening

The inclusion criteria were as follows:

1) Patients aged 18–65 years (including 18 and 65 years) who had signed an informed consent form.
2) Patients with at least one eye or both eyes suffering from impaired vision caused by retinitis pigmentosa combined with macula edema.
3) Patients who voluntarily selected UCMSCs infusion or TA injection for the treatment of retinitis pigmentosa combined with macula edema.
4) Using the Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity checklist at a distance of 4 m, the best corrected visual acuity scores was ≥19 letters and ≤73 letters (or the equivalent of about Snellen eyesight from 20/400 to 20/40).
5) Patients who had the ability to adhere to the study follow-up and protocol requirements.

The exclusion criteria were as follows:

1) Patients with any active intraocular inflammation, infection, or concomitant diseases in their eyes that may affect the interpretation of the results of the study or may lead to visual impairment, including severe cataracts, glaucoma, retinal vascular obstruction, retinal detachments, macular holes, vitreous macular traction, and choroidal neovascularization.
2) Patients with a history of intraocular surgery.
3) Patients with a stroke, coronary heart disease, renal insufficiency requiring dialysis or kidney transplantation, or other systemic chronic diseases.
4) Patients with hypertension (systolic pressure >140 mmHg or diastolic pressure >90 mmHg) or diabetes that cannot be controlled by drugs.
5) Females who planned to become pregnant within the next 6 months, were pregnant or were lactating.

Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells

The vital signs of all of the patients involved in this study, including their temperature, respiration rate, pulse, blood pressure, oxygen saturation, electrocardiogram signals and pain severity, were continuously monitored before, during and up to 2 h after the infusion. The patients underwent treatment only when all of their vital signs were normal. Every patient received a sequential intravenous infusion of UCMSCs (3 × 106 cells, 250 ml per person) through the dorsal hand vein within 60 min. The infusion was stopped immediately and treated in a timely manner when immune rejection, anaphylaxis, and infusion reactions, such as headache, dizziness, nausea and vomiting, occurred.

Modified Sub-tenon's Capsule Injection of Triamcinolone Acetonide

A system of modified sub-Tenon's capsule injection was developed (Patent No. ZL 2013 20740,202.0, China). Briefly, a 2 mm incision was made 10 mm to the limbus at the superotemporal bulbar conjunctiva. The conjunctiva, bulbar fascia and Tenon's capsule were bluntly dissected exposing the

TABLE 1 | Baseline assessments of the subjects.

	TA	MSC	*p* Value
Subjects (eyes)	10 (20)	10 (20)	
Sex (Male/Female)	8/2	6/4	
Age (years)	45.6 ± 12.2	38.3 ± 13.5	>0.05[a]
Central Macular Thickness (μm)	330 ± 42.63	294.13 ± 29.11	>0.05[b]
BCVA (ET[a]S numbers)	56.0 ± 13.8	57.4 ± 20.3	>0.05[a]
visual field sensitivity (dB)	494.80 ± 114.53	538.56 ± 176.62	>0.05[b]
Amplitude of P2 in [a]EP (μv)	8.38 ± 4.49	12.18 ± 7.69	>0.05[b]
Latency of P2 in FV[a] (ms)	111.33 ± 12.17	109.88 ± 11.16	>0.05[b]

[a]*independent* t *test*[a]
[b]*Mann–Whitney* U *test.*

sclera. A specially developed curved needle with a blunt tip was inserted through the incision and posteriorly run along the surface of the sclera until it reached the posterior pole that corresponded to the macular area. Then, 20 mg of TA (LISAPHARMA, Italy) was injected into the sub-Tenon's capsule space. The needle was slowly withdrawn to avoid any leaking of the drug, and pressure was applied to the conjunctiva incision for a few seconds. No sutures were needed. An antibiotic ointment was applied in the injected eye, and the eye was patched for 1 day. Antibiotic eye drops were used for a few days following the injection.

Clinical Evaluation

The safety and efficacy parameters were evaluated at baseline and at first day, first week, and first, second, third, sixth month after the intravenous UCMSCs infusion or the TA injection. Relevant blood biochemical indexes were measured before and after treatment. The best corrected visual acuity (BCVA) was used as the standard for visual acuity evaluation and was determined by the Early Treatment Diabetic Retinopathy Study (ETDRS) alphabet (Topcon CC 100 XP, Japan). Optical computed tomography (OCT) scans were performed to evaluate the central macular thickness (CMT) (OCT-1000, Topcon, Japan). The rate of change (ROC) of CMT at every visit was calculated as (%): (value measured - baseline value)*100/baseline value. The visual fields were tested using a Humphrey Visual Field Analyzer. The sum of visual field sensitivity (dB) was calculated. The flash visual evoked potential (FVEP) was tested according to the standardized procedures developed by the International Society for Clinical Electrophysiology of Vision (ISCEV) (Espion E2 Diagnosis, U.S.A.). The ROC of amplitude of P2 wave was calculated as (%): (value measured - baseline value)*100/baseline value.

Statistical Analysis

The IBM SPSS Statistics 26 (IBM, Corp, Armonk, NY, United States) software was used to describe and analyze the data. Continuous variables were described by mean ± SD. The independent-samples *t*-test or the Mann–Whitney U test was used for comparing continuous variables between two groups. The Wilcoxon signed-rank test was used for detecting differences between variables before and after intervention. $p < 0.05$ was considered statistically significant.

RESULTS

Safety Assessments of Triamcinolone Acetonide Injection and Umbilical Cord Mesenchymal Stem Cells Infusion

There were 20 patients (40 eyes) enrolled in the study, and they were randomized into TA injection group and UCMSCs infusion group (**Table 1**). All of the patients were clinically diagnosed with RP-ME. In the TA injection group, there were no local or systemic adverse effects for all of the patients before or after the injection. In the UCMSCs group, the vital signs of all patients were stable during the infusion process. There were no adverse effects such as fever, infection, headache, vertigo, nausea, vomiting, allergic reactions or immune rejection reactions that happened during or after the UCMSCs infusion. There were no hemorrhage, exudation or inflammatory signs found during all follow ups in both groups (**Figure 2**). There were three patients who had a substantial increase of interleukin-6 (IL-6) in UCMSCs group at first week, and then the IL-6 decreased to normal level subsequently. No definite IL-6 change was found in TA group. There were no significant changes in white blood cell, liver and renal function, blood glucose, C-reactive protein (CRP), procalcitonin (PCT) throughout the follow-up period in both groups (**Supplementary Tables S1, S2**).

Assessments of Central Macular Thickness

Central Macular Thickness Analysis in Each Group

There was no significant difference between the baselines of the two groups ($p > 0.05$, $n = 20$, Mann–Whitney U test). Compared with baseline, CMT in TA group significantly decreased at first week, first and second month ($p = 0.04$,0.028,0.005 respectively, $n = 20$, Wilcoxon signed-rank test). There was a rebound at third and sixth month when the CMT increased again, and was not statistically different from baseline ($p > 0.05$, $n = 20$, Wilcoxon signed-rank test) (**Figures 3A,B**). In the UCMSCs infusion group, CMT at first month was significantly lower than baseline ($p = 0.018$, $n = 20$, Wilcoxon signed-rank test). The CMT at third and sixth were also lower than baseline, but there was no significant difference ($p > 0.05$, $n = 20$, Wilcoxon signed-rank test) (**Figures 3A,C**).

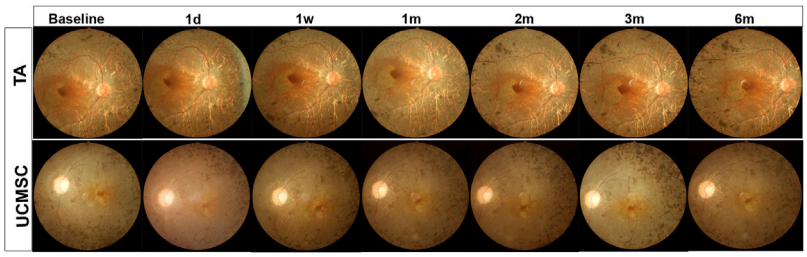

FIGURE 2 | Fundus photograph of representative RP patient in both groups. The fundus photograph shows typical fundus appearance of RP, with yellowish color of optic disc, thin blood vessels, gray retinal color and osteocyte like pigment deposits in peripheral retina. There were no adverse effects such as hemorrhage, exudation or inflammatory signs found at all follow ups in both groups.

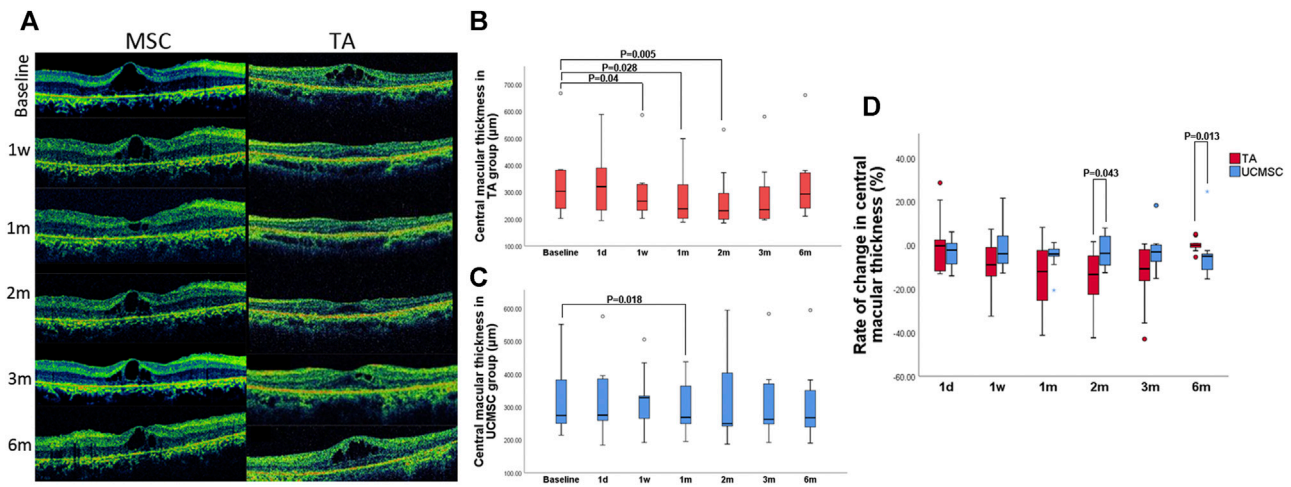

FIGURE 3 | Assessment of central macular thickness. **(A)** Morphological changes in the UCMSCs infusion and TA injection groups **(B)** In the TA group, the CMT significantly decreased at first week, first and second month (p = 0.04,0.028,0.005 respectively, n = 20, Wilcoxon signed-rank test). At 3rd and sixth month, the CMT gradually increased to baseline level (p > 0.05, n = 20, Wilcoxon signed-rank test). **(C)** In the UCMSCs group, the CMT at first month was significantly lower than baseline (p = 0.018, n = 20, Wilcoxon signed-rank test). The CMT at third and sixth month were also lower than baseline, but there was no significant difference (p > 0.05, n = 20, Wilcoxon signed-rank test). **(D)** Comparison of the rate of change in CMT between two groups: At 2nd month, the rate of reduction of CMT in TA group was significantly greater than that in UCMSCs group (p = 0.043, n = 20, Mann–Whitney U test). At 6th month, the rate of reduction of CMT in UCMSCs group was significantly greater than that in TA group (p = 0.013, n = 20, Mann–Whitney U test).

Comparison of Rate of Change in Central Macular Thickness

To further evaluate the effect of both agents on reducing macular edema, the rate of change (ROC) in CMT was calculated. In TA group, the ROC was more negative at first and second month, then it was gradually close to 0 at third and sixth month. In MSC group, the ROC showed a relatively small amplitude of variation. When compared with UCMSCs group, the ROC of TA group was significant different (more negative) at second month indicating a significant decrease in CMT (p = 0.043, n = 20, Mann–Whitney U test). Whereas, a reverse change was observed at sixth month, when the ROC of UCMSCs group was significantly lower (more negative) than that in TA group (p = 0.013, n = 20, Mann–Whitney U test), indicating a stronger act of UCMSCs in reducing CMT (**Figure 3D**).

Assessments of Visual Functions

Comparison of Visual Field Sensitivity and BCVA

The total value of visual field sensitivity was calculated. In the TA injection group, this value peaked at the second month without a significant difference (p > 0.05, n = 20, Wilcoxon signed-rank test). In the UCMSCs group, the overall sensitivity value increased at first, second and third month, but was not statistically different (p > 0.05, n = 20, Wilcoxon signed-rank test). When comparing the visual field sensitivities of the two groups, there were no significant difference found between any follow ups (p > 0.05, n = 20, Mann–Whitney U test) (**Figures 4A,B**). Compared with baseline, the BCVA showed no significant differences at any follow up in each group (p > 0.05, n = 20, Wilcoxon signed-rank test). The intergroup comparison found no significant difference neither (p > 0.05, n = 20, Mann–Whitney U test) (**Figure 4C**).

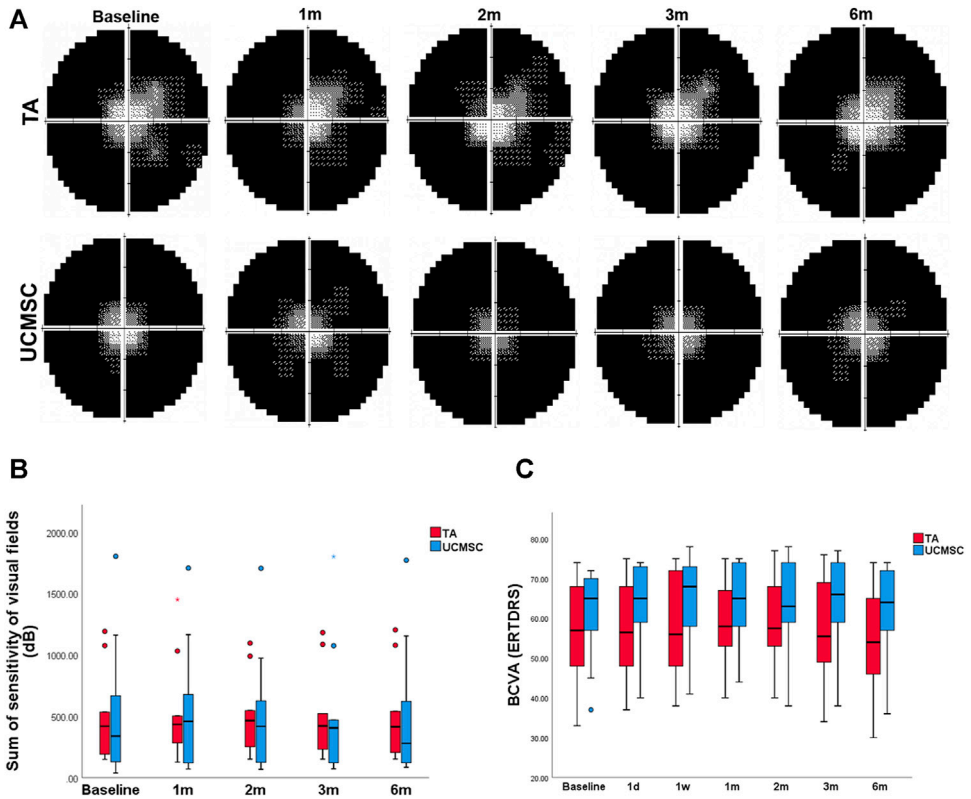

FIGURE 4 | Assessments of BCVA and visual field sensitivity. **(A, B)** The sum of visual field sensitivity in TA group peaked at the second month without a significant difference ($p > 0.05$, $n = 20$, Wilcoxon signed-rank test), and then decreased to the baseline level at the sixth month. In the UCMSCs infusion group, this value increased at first, second and third month without significant differences ($p > 0.05$, $n = 20$, Wilcoxon signed-rank test). Then it decreased to baseline value at sixth month. There were no significant differences found within each group or between the two groups ($p > 0.05$, $n = 20$, Mann–Whitney U test). **(C)** There were no significant differences of BCVA found within each group or between the two groups ($p > 0.05$, $n = 20$, Wilcoxon signed-rank test and Mann–Whitney U test).

Comparison of Flash Visual Evoked Potential

To obtain stable parameters, each waveform of FVEP was the result of overlay of 64 times of detections, and two paralleling waveforms were generated for each eye (**Figure 5A**). In the TA injection group, the amplitude of P2 wave in FVEP significantly increased at second month ($p = 0.028$, $n = 20$, Wilcoxon signed-rank test). At 3rd and sixth month, it dropped down and had no statistical differences with baseline level ($p > 0.05$) (**Figures 5A,B**). In UCMSCs infusion group, the amplitude of P2 wave demonstrated a trend of increase at third and sixth month, but not until sixth month did it show a significant rise comparing with baseline ($p = 0.008$, $n = 20$, Wilcoxon signed-rank test) (**Figures 5A,C**). For the latency of P2 wave, there were no significant differences among follow ups within each group or between the two groups ($p > 0.05$) (**Figure 5D**). To compare the two group more precisely, the ROC of amplitude of P2 wave was also calculated. The ROC in amplitude of P2 wave in UCMSCs group was significantly higher than that of TA group at sixth month ($p = 0.000$, $n = 20$, Mann–Whitney U test) (**Figure 5E**).

DISCUSSION

As a major worldwide retinal degenerative disease that causes blindness, the hereditary modes of RP can be autosomal dominant (30–40%), autosomal recessive (50–60%), or a X-linked trait (5–15%) (Hartong et al., 2006). The inherited nature of RP leads to progressive photoreceptor apoptosis and irreversible visual loss. However, when RP is combined with ME, the impairment of visual function becomes worse. The impairment of the blood retinal barrier (BRB) is thought to be the main cause of ME in RP (Vinores et al., 1995; Larsen et al., 1997). With the progression of RP, both retinal vascular endothelium and RPE lose their normal intercellular junctions, which gives rise to increased retinal vascular permeability and a flow of interstitial fluid from the choroid to the retinal tissue (Larsen et al., 1997; Marmor, 1999; Vinores et al., 1999). Inflammation and auto-immune processes play an important role in the pathogenesis of vascular endothelium and RPE dysfunction and the subsequent breakdown of the BRB(Yoshida et al., 2013; Narayan et al., 2016; Strong et al., 2017).

Different methods have been used to treat RP-ME, such as systemic administration of carbonic anhydrase inhibitors or steroids, intravitreal injection of steroids or anti-VEGF agents, laser photocoagulation and surgery (Bakthavatchalam et al., 2018; Liew et al., 2015). Triamcinolone acetonide (TA) is a kind of synthetic long-acting steroid and has been widely used in the treatment of ME because of its pharmacological actions of anti-inflammation, immune modulation, BRB stabilization and VEGF

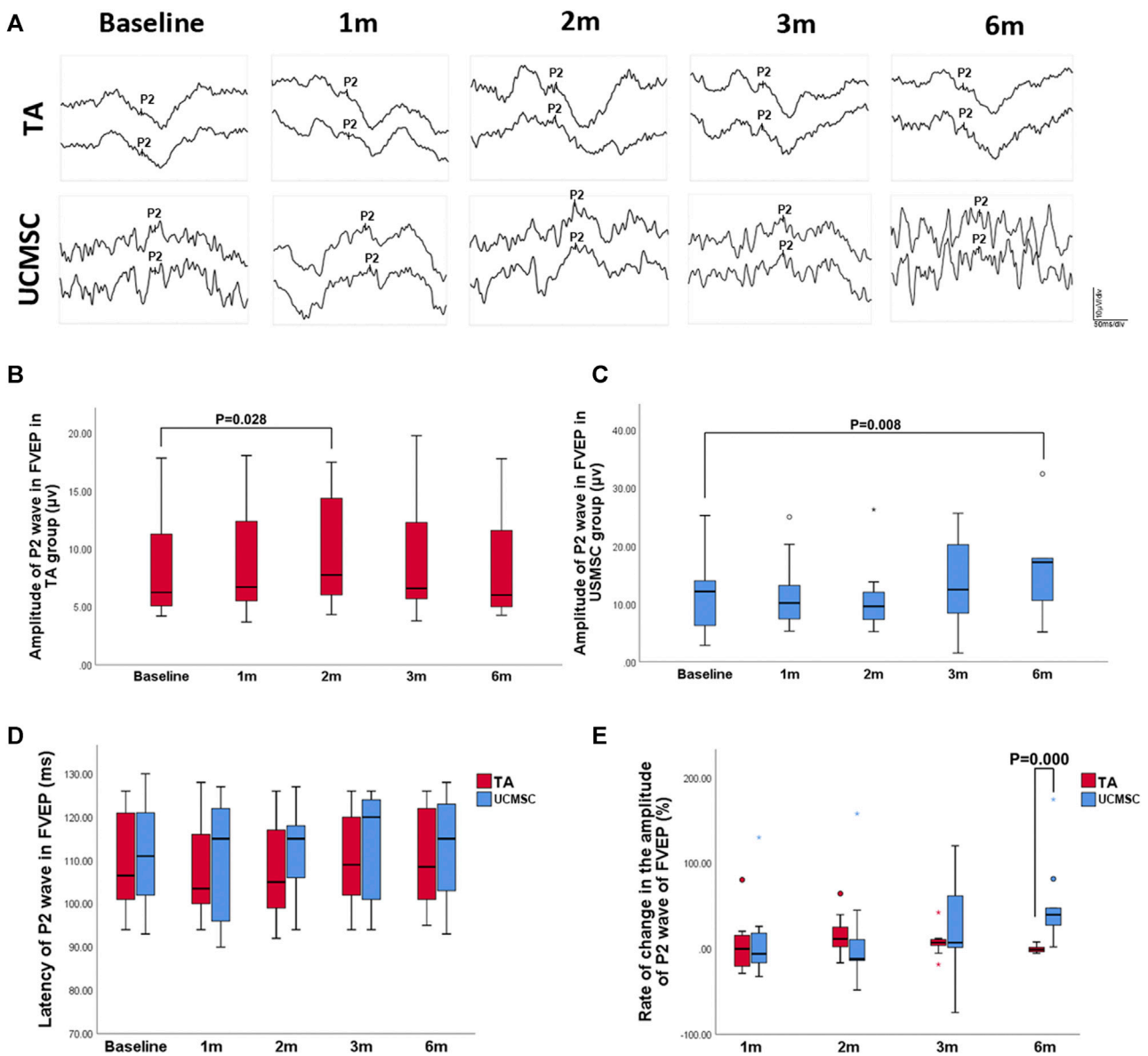

FIGURE 5 | Assessments of FVEP. **(A, B)** In the TA injection group, the amplitude of P2 wave in FVEP significantly increased at second month ($p = 0.028$, $n = 20$, Wilcoxon signed-rank test). **(A, C)** In UCMSCs infusion group, the amplitude of P2 wave showed a significantly increase at sixth month ($p = 0.008$, $n = 20$, Wilcoxon signed-rank test). **(D)** There were no significant differences of latency of P2 wave among follow ups within each group or between the two groups ($p > 0.05$). **(E)** The ROC in amplitude of P2 wave in UCMSCs group was significantly higher than that of TA group at sixth month ($p = 0.000$, $n = 20$, Mann–Whitney U test).

downregulation (Ip et al., 2004; Moldow et al., 1998). Intravitreal injection and sub-Tenon's capsule injection are two major approaches administrating TA in ocular tissue. Although intravitreal injection of TA has the advantage of directly loading drug to the target position, multiple secondary complications including elevated intraocular pressure, cataract, vitreous hemorrhage, endophthalmitis have been reported. Delivering TA by a sub-Tenon's capsule injection is a relatively safe approach for treating ME and has been applied in different retinal diseases (Koga et al., 2005). More recently, Sub-Tenon's capsule administration of TA has been reported to alleviate RP-ME in a short term (Karasu, 2020). But the long-term effect and visual function outcomes are still unknown. The sub-Tenon's capsule injection is able to keep and restrict drugs in the sub-Tenon's capsule space for a relatively long time without allowing diffusion into the orbit tissue, which makes more drugs permeate into the choroid and retina. To treat ME more precisely, we modified the sub-Tenon's capsule injection technique by replacing the traditional short, sharp needle with a long, curved, blunt needle that is able to run along the surface of the sclera and reach the posterior pole of the eyeball, where the placement of the needle is accurately correspond to the ME lesion.

Delivery of TA by a modified sub-Tenon's capsule injection can facilitate the drug diffusion while avoiding risks and complications secondary to intraocular administration.

Our results suggest administration of TA by a modified sub-Tenon's capsule injection significantly reduces CMT from 1 week to 2 months following delivery. This sustaining therapeutic effect of TA can be explained by its pharmacokinetics. Former studies have found the vitreous concentration of TA peaked at 12–24 h after sub-Tenon's capsule injection, and then the drug can be intraocularly present for 8 weeks and longer which is possibly attributed to the binding of TA to retinal pigment epitheliums (Kovacs et al., 2012). The serum concentration of TA has also been tested, and is much lower (tens of nanogram per ml) than that of vitreous body (Kovacs et al., 2012). In spite of the minimal concentration of TA in serum, it can still disrupt the metabolic equilibrium of specific patients such as patients with diabetes (Zaka-ur-Rab et al., 2009; Kovacs et al., 2012). In our study, there were neither definite metabolic abnormalities nor any ocular local side effects observed during all follow ups indicating this modified sub-Tenon's capsule injection of TA has good systemic and local tolerance.

Given the wide range of biological effects, such as anti-inflammation, immune modulation and neurotrophy, UCMSCs have been applied in many systemic diseases and have been shown to have promising therapeutic effects (Bartolucci et al., 2017; Mukai et al., 2018; Riordan et al., 2018). As to retinal pigmentosa, biological therapy has been long explored (Satarian et al., 2017; Smith, 2004; Zhang, 2016). Recently, the sub-Tenon's capsule administration of UCMSC has been applied in RP patients. After 12 months follow up, the outcome is significantly beneficial with increased outer retinal thickness and improved retinal function (Ozmert and Arslan, 2020). In our previous study, we found intravenous administration of USMSCs can also stabilize or enhance the overall visual function of RP patents and more notably, can significantly improve their vision related life quality (Zhao et al., 2020). However, it has not been reported if UCMSCs can help relieve ME, a major pathological condition secondary to RP.

In this study, we found intravenous infusion of UCMSCs exerted significant effect on reducing CMT at first month. At 3rd and sixth month, the CMT was still controlled lower than baseline. The long-term acting of USMSCs may be due to their multiple biological effects. In animal models of RP, intravenously administered UCMSCs were found to produce large amounts of neurotrophic factors, and therefore, the photoreceptors were partially protected from apoptosis (Ding et al., 2017; Ng et al., 2014; Wang et al., 2010). Additionally, the infused UCMSCs can directionally migrate to retinal lesions and exert their biological effects to promote the growth of blood vessels, improve the function of BRB and help reconstruct normal retinal structure (Hou et al., 2010; Shibata et al., 2008). The high potential of proliferation and differentiation may make USMSCs keep functioning in a long term when administrated intravenously, which also has been proved by applications in other systemic disease (Bartolucci et al., 2017; Wang et al., 2019). Due to the low immunogenicity of UCMSCs, few adverse effects have been reported after intravenous infusion. A major concern is the change of relevant inflammatory markers. In our study, there were several patients whose IL-6 level were remarkably increased at 1 week after infusion, and dropped down to baseline level subsequently. But the all patients showed no definite clinical symptoms and other severe systemic adverse effects. The increase of IL-6 has also been found in animal experiments reported formerly. Significantly increased IL-6 level was found in cynomolgus monkeys shortly after intravenous infusion of UCMSCs, but recovered in approximately 1 month, and there were no obvious pathological changes associated with the infusion of cells in the general and microscopic examinations (He et al., 2017).

We compared the effects of these two agents in alleviating ME. In TA injection group, patients showed a more rapid reduction of the CMT in the first 2 months and then the macular thickness rebounded to the baseline level, which implied the quick but relatively short-term effect of the TA injection in relieving ME. Whereas in UCMSCs infusion group, the onset of CMT reduction was slower than TA group, but the ME can be continuously controlled till sixth month when the rate of reduction is significantly higher than TA group, which indicated a slow but more persistent action of the UCMSCs. The BCVA and visual field sensitivity of all of the patients in the two groups did not change significantly, and there was no significant difference between the two groups, implying a limitedly improved but stabilized photoreceptor function occurred following the relief of ME. FVEP is used for evaluating the visual function from retinal ganglion cells to the visual cortex. For advanced RP patients, FVEP can still be recordable when the electroretinogram is extinguished. The change of FVEP may be difficult to interpret when visual acuity changes subtly. But, considering the detection is objective and independent of patient's cooperation, FVEP can still be an important reference for assessing visual function. We performed multiple detections (up to 64 times) to obtain a stable overlayed waveform of FVEP. In our previous study, we found FVEP can be used to evaluate the residual visual function of advanced RP patients (M. Zhang et al., 2021). In this study, the comparison of FVEP between the two groups is similar with that of ME reduction. With relieving of ME, patients in TA groups showed a relatively rapid improvement of P2 wave amplitude (second month), then it dropped down to baseline level. In UCMSCs group, the amplitude of P2 wave was gradually increased to a significant high level at sixth month, at which point it was significantly higher than that in the TA group. This result implied that the UCMSCs infusion may be more beneficial than the TA injection in terms of improving the overall visual function in a long term, which may be due to the wide range of biological effects and more accessible delivery approach from which more retinal neurons such as ganglion cells, bipolar cells can benefit.

In summary, the modified sub-Tenon's capsule injection of TA and intravenous infusion of UCMSCs were both safe for RP patients with ME. Our results suggested that TA injection can reduce macular edema more quickly and effectively than UCMSCs infusion in the short term, but the effect of the UCMSCs infusion may be more persistent. UCMSCs infusion is more beneficial for improving the overall visual function in a long term. This study demonstrated that both modified sub-Tenon's capsule injection of TA and intravenous infusion of UCMSCs are promising therapeutic approaches for patients who have RP-ME. Because of the different acting characteristics, these approaches can be applied separately or jointly depending on the

disease condition for patients to achieve maximum benefits. Nevertheless, more controlled cohorts and a larger number of subjects are needed to confirm the results.

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 Medical Ethics Committee of Southwest Hospital, the Army Medical University, Chongqing, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TZ: patient recruitment, data acquisition, analysis, manuscript writing; HL: patient recruitment, data acquisition, analysis; FW: patient recruitment, data acquisition; YL: conception and design, revision of manuscript; XM: data analysis and interpretation, revision of manuscript; ZY: conception and design, revision of manuscript; SL: data analysis and interpretation, revision of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.694225/full#supplementary-material

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The Future of Gene Therapy for Transfusion-Dependent Beta-Thalassemia: The Power of the Lentiviral Vector for Genetically Modified Hematopoietic Stem Cells

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β-thalassemia, a disease that results from defects in β-globin synthesis, leads to an imbalance of β- and α-globin chains and an excess of α chains. Defective erythroid maturation, ineffective erythropoiesis, and shortened red blood cell survival are commonly observed in most β-thalassemia patients. In severe cases, blood transfusion is considered as a mainstay therapy; however, regular blood transfusions result in chronic iron overload with life-threatening complications, e.g., endocrine dysfunction, cardiomyopathy, liver disease, and ultimately premature death. Therefore, transplantation of healthy hematopoietic stem cells (HSCs) is considered an alternative treatment. Patients with a compatible human leukocyte antigen (HLA) matched donor can be cured by allogeneic HSC transplantation. However, some recipients faced a high risk of morbidity/mortality due to graft versus host disease or graft failure, while a majority of patients do not have such HLA match-related donors. Currently, the infusion of autologous HSCs modified with a lentiviral vector expressing the β-globin gene into the erythroid progenitors of the patient is a promising approach to completely cure β-thalassemia. Here, we discuss a history of β-thalassemia treatments and limitations, in particular the development of β-globin lentiviral vectors, with emphasis on clinical applications and future perspectives in a new era of medicine.

Keywords: β-thalassemia, hematopoietic stem cells (HSCs), gene therapy, lentiviral vector, transfusion-dependent, allogeneic HSC transplantation

INTRODUCTION

β-thalassemia belongs to a family of inherited hemoglobin disorders and is characterized by a quantitative reduction in β-globin chains. β-thalassemia has a wide range of clinical severity, from severe transfusion-dependent thalassemia major to the highly variable non-transfusion-dependent thalassemia intermedia. More than 200 mutations in the β-globin gene have been reported (Olivieri, 1999; Giardine et al., 2007). The mutations include the following: mutations affecting transcription, RNA processing, or RNA translation; small insertions or deletions within the gene; single base substitutions; mutations affecting translation initiation, elongation, termination, and more rarely, deletions of a substantial proportion of the regulatory elements in the locus control region (LCR) or

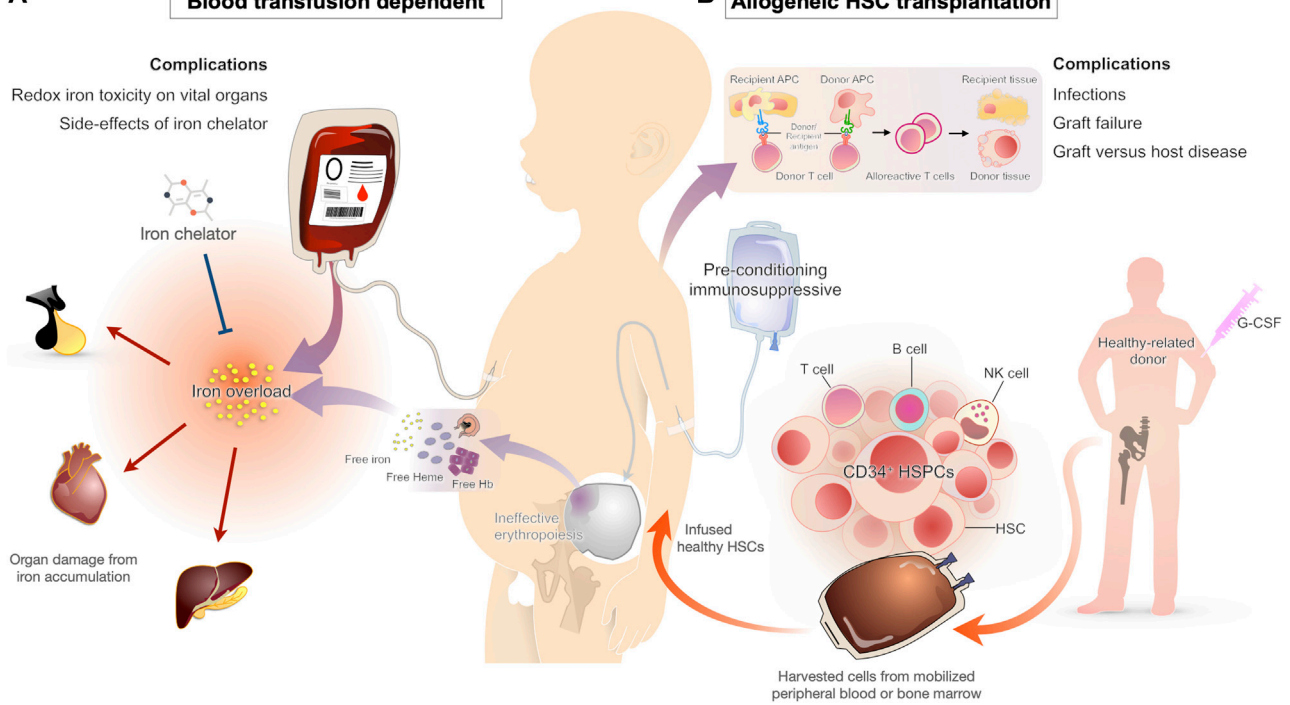

FIGURE 1 | Conventional therapy approaches for β-thalassemia patients and their complications **(A)** Chronic blood transfusion is the standard of care for β-thalassemia patients to maintain adequate hemoglobin levels for effective cardiovascular status. One of the major complications from a blood transfusion is iron overload. As the transfused blood contains iron and the human body lacks a functional iron excretion mechanism, all individuals treated with chronic transfusion develop iron overload, which leads to organ damage from oxidative injury. Iron chelators are typically started early in transfusion-dependent β-thalassemia to prevent the complications of transfusional iron overload **(B)** HSC transplantation has been used for decades as the curative approach for β-thalassemia patients. The HSC replacement substitutes ineffective erythropoietic stem cells with effective cells from a healthy donor. Even with the improvement of transplant technologies during the last decade, severe complications such as infections, graft versus host disease, and graft failure are occasionally seen.

deletions of the open reading frame (Thein, 1998), resulting in either a complete absence (β^0-thalassemia), or a partial deficiency (β+-thalassemia) of β chains.

In normal individuals, there is a balance between α- and β-globin chain synthesis. In individuals with β-thalassemia, mutations lead to imbalanced globin chain synthesis and an excess of α chains. Unbound α-globin chains precipitate in red blood cell precursors and their progeny causing cellular damage, a process that leads to defective erythroid maturation, ineffective erythropoiesis, and shortened red blood cell (RBC) survival (Weatherall, 1998). Ineffective erythropoiesis combined with shortened RBC survival leads to anemia. If left untreated, the disease could induce an expansion of marrow cavities and massive extramedullary cell proliferation resulting in skeletal deformities, hepatosplenomegaly, and extramedullary pseudotumors (Haidar et al., 2010). Erythroid hyperplasia and ineffective erythropoiesis are responsible for increased intestine iron absorption, which, in addition to regular blood transfusion, results in chronic iron overload with life-threatening complications such as endocrine dysfunction, cardiomyopathy, liver disease, and ultimately, premature death.

Generally, the treatment approaches in thalassemia include anemia correction, suppression of ineffective erythropoiesis, and iron management. The available treatments for β-thalassemia consist of several therapeutic modalities ranging from conventional treatments such as blood transfusion combined with iron chelation therapy, splenectomy, and hematopoietic stem cell (HSC) transplantation (**Figure 1**) to recently approved novel treatments such as luspatercept, an activin receptor fusion protein that improves erythropoiesis, and cell and gene therapy (Taher et al., 2018; Motta et al., 2020).

CURRENT AND FUTURE THERAPIES FOR TRANSFUSION-DEPENDENT β-THALASSEMIA

Transfusion Therapy for β-thalassemia

Transfusion therapy is considered a mainstay treatment in thalassemia patients (**Figure 1**). The blood transfusion serves to provide normal RBCs and suppress the ineffective erythroid proliferation, which prevents the downstream pathophysiological consequences (Cazzola et al., 1995). In the absence of blood transfusions, most patients with β-thalassemia major die within the first 5 years of birth (Rachmilewitz and Giardina, 2011). For this reason, regular blood transfusions are recommended in early childhood as soon as the clinical manifestations develop. As there is no controlled mechanism for iron excretion in the human body,

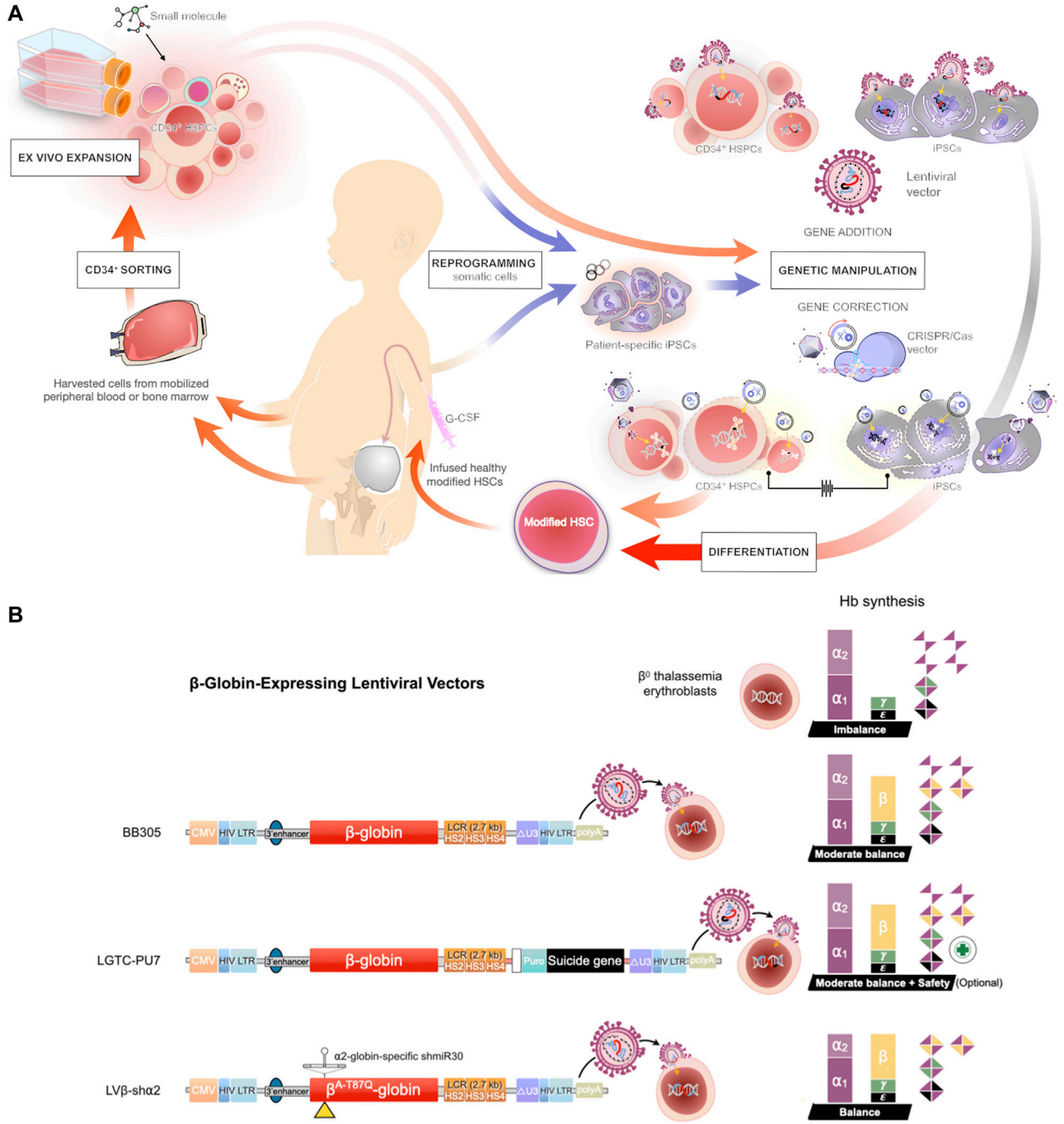

FIGURE 2 | Gene therapy approaches for transfusion-dependent β-thalassemia patients **(A)** Schematic representation of the protocols for gene therapy in β-thalassemia. Induced pluripotent stem cells (iPSCs) are reprogrammed from somatic cells and differentiated into HSCs, or the HSCs are directly harvested from the mobilized peripheral blood or bone marrow of a patient and further manipulated by *ex vivo* maintenance or expanded by co-culture with a cocktail of cytokines and small molecules. The harvested HSCs are subjected to gene transfer (addition) by lentiviral vector or gene editing using CRISPR/Cas9 technology. The engineered HSCs are then applied to replace the inherited defective β-globin gene of a patient and restore function of the erythroid lineage in β-thalassemia **(B)** Prospective of modified lentiviral vector expressing the β-globin gene. The three indicated lentiviral vectors encode the β-globin chain under the control of the human β-globin promoter and hypersensitive sites (HS) of the β-globin locus control region (LCR) (Top) A LentiGlobin™ BB305 vector, the current gene therapy drug product for the treatment of non-$\beta 0/\beta^0$ thalassemia **(Middle)** A modified LentiGlobin™ BB305 vector, LTGC-PU7, encodes the β-globin chain and the puromycin N-acetyltransferase (PAC) cassette with an optional suicide gene **(Bottom)** LentiGlobin™ BB305 is modified to express the miR-30-based short hairpin RNA (shRNA) selectively targeting the α2-globin mRNA, the therapeutic option for β0/β0 thalassemia patients.

chelation therapy is typically needed within 1 year after starting the transfusion regimen (Franchini et al., 2017; Shah et al., 2019).

With the advancements in transfusion and iron chelation therapy in the recent decades, the life expectancy of transfusion-dependent β-thalassemia patients has improved significantly in high-income countries (Borgna-Pignatti et al., 2004; Ladis et al., 2011). Nonetheless, despite the improvements in survival, the quality of life in thalassemia patients undergoing conventional non-curative management remains limited compared with curative therapies (Caocci et al., 2016; Badawy et al., 2021). As regular transfusion regimens require missing school or work for 1 day every 3–4 weeks, in addition to the risk of complications from transfusions and iron chelation therapies, the non-curative treatments may greatly affect patient's daily activities.

Cell and Gene Therapy for the Treatment of Transfusion-dependent β-thalassemia

Hematopoietic Stem Cell Transplantation

Unlike supportive blood transfusions, allogeneic bone marrow transplantation (BMT) or HSC transplantation offers the hope of a definitive cure for patients with transfusion-dependent β-thalassemia. Transplantation with hematopoietic cells from matched related donors has an 80–87% probability of curing young patients (Lucarelli and Gaziev, 2008), suggesting that high resolution human leukocyte antigen (HLA) typing selection is realized. BMT with unrelated donors yields success rates similar to those obtained with the use of matched sibling donors, but with more severe graft versus host disease (GVHD) (La Nasa et al., 2005; Li et al., 2012). Hence, umbilical cord blood (CB) is an alternative cell source for transplantation that requires less stringent HLA matching (Boncimino et al., 2010); however, graft failure due to a reduced number of stem cells during infusion is a major cause of treatment failure (Ruggeri et al., 2011). Overall, graft failure, and GVHD remain significant causes of transplant failure and complications, especially for adult patients (**Figure 1**). Although stem cell transplantation is an exciting development for young patients with β-thalassemia, precise clinical judgment needs to be made to balance a potential cure with a risk of mortality and morbidity against life-long treatment with blood transfusions and the long-term complication of iron overload (Higgs et al., 2012). BMT drawbacks, such as GVHD and graft failure, may be avoided by the use of autologous HSC transplantation after transduction with therapeutic genes or gene therapy.

β-globin Vector Development and Preclinical Evaluation

Gene therapy refers to a technique involving the introduction of exogenous DNA sequences or therapeutic genes into an appropriate host genome (**Figure 2**). These therapeutic sequences could have the ability to modify a specific mutation and correct or complement unusual function of the cells in order to overcome a disease (Smith, 2003). Stem cells, particularly HSCs or $CD34^+$ cells, are highly attractive target cells for gene therapy because of their ability to reconstitute tissues throughout life. Consequently, most gene therapy approaches for the treatment of hematological disorders focus on targeting the therapeutic gene to repopulating HSCs.

β-thalassemia gene therapy is based on the transfer of a human β-globin gene into autologous HSCs, which resolves the absence of compatible donors and eliminates the risk of GVHD and graft failure associated with allogeneic BMT. The aim of autologous HSC gene therapy in β-thalassemia is to provide normal β-globin protein expression. In the past, gamma-retroviral vectors were used to transfer the β-globin gene and its regulatory elements. However, this technique is problematic, as oncoretroviral vectors containing LCR sequences, together with the β-globin regulatory elements, were difficult to produce at high viral vector titers and were very unstable (Novak et al., 1990; Gelinas et al., 1992). Identifying and removing the DNA sequences responsible for vector instability and low titers was intended to improve transduction efficiency (Leboulch et al., 1994; Sadelain et al., 1995); however, condensing the LCR sequence to less than one kilobase (kb) was responsible for high clonal variation in β-globin gene expression both *in vitro* (Sadelain et al., 1995) and *in vivo* (Raftopoulos et al., 1997). Overall, oncoretroviral vectors with these modifications remained sub-optimal at transducing mouse HSCs and had limited capacity for expression of the therapeutic β-globin protein.

Lentiviral vectors, a family of complex retroviruses characterized by stable insertion of their viral genome into the host chromosomes of differentiated lymphocytes and macrophages. The prototype of this virus family is the human immunodeficiency virus-type 1 (HIV-1), a pathogen of the immune system with cytopathic effects, which can be transduced into non-dividing cells arrested at the G1–S boundary of the cell cycle (Naldini et al., 1996a; Naldini et al., 1996b). In addition, lentiviral vectors have the capacity to accept the insertion of large and complex DNA sequences (Kumar et al., 2001) due to the presence of a strong RNA export element (RRE) that binds the RNA-binding viral protein (Cullen, 1998). Side-by-side comparison of lentiviral vectors containing LCR genomic regions of 1 kb versus lentiviral vectors containing longer genomic fractions (3 kb) confirmed that the vector insert size limitation was a major issue for β-globin expression levels (May et al., 2000). Similar lentiviral vectors from two independent groups carrying the β-globin gene (including introns) under the control of the β-globin promoter and LCR elements (2.7–3 kb) enabled efficient transfer and stable integration of the human therapeutic gene in a mouse model of β-thalassemia intermedia (May et al., 2000; Imren et al., 2002). The transduction was sustained in both primary and secondary transplant in immunodeficient mice. Ninety-five percent of the RBCs in all immunodeficient mice who received transplantation contained human β-globin, contributing to one-third of all β-like globin chains. The β-thalassemia phenotype, as assessed by hematological parameters (hemoglobin levels, reticulocytes, and red blood cell counts), was clearly improved. In addition, free α-globin chains were completely cleared from the membranes of RBCs, extramedullary hematopoiesis was ablated, and iron deposits were almost eliminated in liver sections (Imren et al., 2002). In the complete absence of endogenous mouse β-globin gene

expression, which is the most severe context of mouse β-thalassemia, the expression level of β-globin per vector copy in transduced RBCs was shown to be approximately half that of the hemizygous endogenous hemoglobin (Hb) production (Rivella et al., 2003). Interestingly, a corrected phenotype in mice with β-thalassemia intermedia was obtained at a transduction rate of 30–50% with cells harboring an average vector copy number (VCN) of 1 (Miccio et al., 2008). In another mouse model of β-thalassemia, a minority proportion of genetically modified HSCs as low as 10–20% of the proportion of normal donor cells resulted in significant improvement of the phenotypes (Persons et al., 2001). These observations are consistent with the preferential survival of normal erythroid cells against a high apoptotic rate of erythroid precursors and RBC hemolysis in β-thalassemia (Centis et al., 2000).

The ability of lentiviral vectors to transduce HSCs from human origin was initially assayed in human umbilical CB cells transplanted into NOD-scid IL2Rγ null immunodeficient mice (NSG mice). Six months after β-globin lentiviral vector transduction and transplantation, around 50% of the human progenitors were genetically modified (Imren et al., 2004), indicating a high transduction efficacy of CB-HSCs by the lentiviral β-globin vectors. The capacity of lentiviral vectors carrying the β-globin gene to correct the β-thalassemia major phenotype was studied in cultured erythroid cells derived from β-thalassemia patients (Centis et al., 2000). Normalized β-globin levels were achieved, leading to effective cell expansion, normal erythroid cell differentiation, and reduction of apoptosis (Puthenveetil et al., 2004; Malik et al., 2005). The gene-corrected human thalassemia $CD34^+$ cells were transplanted into NSG mice. Normal levels of human β-globin and effective erythropoiesis were observed in the erythroid progenitor cells derived from human HSCs. Importantly, the expression of β-globin protein was similar to that measured in erythroid colonies derived from normal control subjects (Puthenveetil et al., 2004). Moreover, a number of samples from β-thalassemia patients of different geographic and ethnic origins and from several genotypes (β^0-thalassemia homozygous for β^+ mutations or compound heterozygous for β^0 and β^+ mutations) were shown to be transduced *in vitro*. Rescue from apoptosis and correction of ineffective erythropoiesis were potent in most samples (Roselli et al., 2010). As expected, lentiviral β-globin vectors targeted transcriptionally active regions but without bias for cancer-related genes in normal CB stem cells (Imren et al., 2004) as well as in HSCs derived from β-thalassemia patients (Roselli et al., 2010). Preclinical studies further provided the proof of efficacy and safety of those vectors *in vivo* (Ronen et al., 2011; Negre et al., 2015). These studies provided solid preclinical data for the inclusion of patients in clinical trials, with an acceptable risk/benefit ratio.

Clinical Trial in β-thalassemia Patients

The first successful use of gene therapy for the treatment of β-thalassemia patients was reported in 2010. In this trial, HSCs were purified and modified to express a β-like globin protein in the erythroid precursors and were then re-infused into patients. Theoretically, the modified HSCs reconstitute the hematopoietic system, thereby producing normal gene-corrected RBCs (Sankaran and Weiss, 2015). The trial, in which lentiviral vector was used to transfer a globin gene into patient-derived HSCs, was planned and announced in 2005 (Bank et al., 2005) and began in 2006 in Paris, France (Cavazzana-Calvo et al., 2010). The first patient in this trial failed to engraft because the purified HSCs had been compromised technically, without relation to the gene therapy vector. The second participant has been carefully followed after the gene transfer procedure. The patient, a male who was 18 years old at the time of treatment, had severe βE/β0-thalassemia and began regular blood transfusion at age 3. His hemoglobin level decreased to as low as 4 g/dl several times, and he did not have a related HLA-matched donor. The patient was conditioned with intravenous busulfan (3.2 mg/ kg per day for 4 days) before transplantation with autologous gene-modified $CD34^+$ cells. The transduced $CD34^+$ cells contained 0.6 vector copies per cell. One year after transplantation, the patient became transfusion-independent with clear biological and clinical improvement. His hemoglobin level remained stable, between 9 and 10 g/ dl, of which approximately one-third of the total hemoglobin was composed of the therapeutic hemoglobin (β^{A-T87Q}). Although correction of the anemia was partial, there was a concurrent decrease in blood reticulocyte and erythroblast counts; however, the hyper-erythroid state remained. The percentage of vector-bearing nucleated blood cells after transplant progressively increased and stabilized to approximately 11%. Chromosomal integration site (IS) analysis of the β^{A-T87Q}-globin vector detected a dominant clone in which the vector was inserted in the third intron of the high-mobility group AT-Hook2 (HMGA2) gene. The clone was found in granulocytes, monocytes, and erythroblasts (Cavazzana-Calvo et al., 2010) but not in B and T lymphocytes. The proportion of HMGA2 in peripheral blood remained stable at approximately 2–3% of the circulating nucleated cells (Payen and Leboulch, 2012). A cryptic splice site present in the vector led to the production of an HMAG2 mRNA containing only exons 1, 2, and 3 of the five exons, and the removal of a target site for let-7 microRNA (normally present in exon 5) resulted in increased production of a functional truncated HMGA2 protein. As overexpression of truncated HMGA2 has been involved in benign neoplasia (Cleynen and Van de Ven, 2008), careful and regular follow-up of the patient has been pursued. Currently, the patient is transfusion-independent, with no signs of clonal overgrowth or toxicity.

A recent comprehensive study reported the results of phase 1/2 studies to evaluate the safety and efficacy of gene therapy for β-thalassemia with the use of the LentiGlobin BB305 vector (Thompson et al., 2018), which was modified from a previous LentiGlobin vector (Cavazzana-Calvo et al., 2010). In this study, mobilized autologous $CD34^+$ cells were obtained from 22 patents (12–35 years old) and the cells were transduced *ex vivo* with the BB305 vector, which encoded the β^{A-T87Q}-globin gene. After the cells were re-infused in the patients, who had undergone myeloblative busulfan conditioning, the efficacy and adverse effects of the vector were observed. The 22

patients were monitored up to 3 years (15–42 months) after transplantation, and no serious adverse events or unexpected safety issues related to the transduced cells have been detected. However, nine of the patients had a severity of the disease that results in microcytic, hypochromic anemia, specifically a β0/β0 genotype. The patients with a β0/β0 genotype showed a reduction in transfusion volume along with decreased annual number of transfusions; indeed, 3 patients stopped RBC transfusion. Interestingly, all of the patients with a βE/β0 genotype, which is the prevalent genotype of β-thalassemia, were able to discontinue transfusions. Of note, treatment-related adverse effects and clonal dominance related to vector integration have not been observed to date (Thompson et al., 2018). In summary, autologous gene therapy with the LentiGlobin BB305 vector transduced in $CD34^+$ cells and infused back into patients has been demonstrated as a potential curative treatment in patients with severe β-thalassemia without any adverse events (Biffi, 2018). The clinical data led to the conditional approval of the LentiGlobin BB305 gene therapy vector by the European Commission for transfusion-dependent non-β0/β0 thalassemia in patients 12 years and older. This drug product, owned by bluebird bio, Inc., is the first approved gene therapy for transfusion-dependent β-thalassemia (Harrison, 2019).

In the TIGET-BTHAL study, a phase I/II clinical trial conducted in Italy, 9 patients with β^0 or severe β^+ mutations were treated with intrabone autologous genetically modified HSCs using the GLOBE lentiviral vector. Transfusion reduction was observed in adults, and 3 of the 4 children were transfusion independent at the last follow-up. Superior treatment outcomes were observed in younger patients. Higher HSC repopulating capacity and bone marrow function in children could contribute to better clinical benefits (Marktel et al., 2019).

Current Potentials and Limitations of β-Thalassemia Gene Therapy

Despite their potential for curative outcomes in β-thalassemia, gene therapies contain related concerns, especially the theoretical risks of genotoxicity due to genome manipulation. Post-treatment myelodysplastic syndrome (MDS) was reported 36 months following Lentiglobin infusion in a patient with sickle cell disease. The disease etiology was investigated by multiple molecular- and cytogenetic- assays, which demonstrated no vector integration of $CD34^+$ blast cells. Vector-mediated insertional oncogenesis was excluded. In this case, the MDS was likely associated with myeloablative conditioning (Hsieh et al., 2020). Of note, treatment-related adverse effects and clonal dominance related to vector integration and generation of replication have not been observed to date (Cavazzana et al., 2019; Thompson et al., 2019). One case report demonstrated a patient successfully treated with LentiGlobin BB305 drug product and was later diagnosed as wild-type HIV infection. Mostly, the differentiation between wild-type HIV and the lentivirus is difficult to distinguish via polymerase chain reaction (PCR) test as the lentiviral vectors also contain partial HIV-derived gene sequences, which can be false positive for a screening HIV PCR assay in gene therapy patients. In this case, the wild-type HIV infection was confirmed by western blotting and next-generation sequencing. Even though the lentiviral vector is derived from HIV-1, the vector contains a low risk of generating replication-competent virus due to safety modifications in the vector design (Hongeng et al., 2021).

In summary, autologous gene therapy with the lentiviral vector transduced in $CD34^+$ cells and infused back into patients has been demonstrated as a potentially curative treatment in patients with severe β-thalassemia. Still, long-term follow-up is critically necessary (Biffi, 2018). In addition to lentiviral vector-based gene therapy, a few ongoing phase I/II clinical trials are currently evaluating the gene-editing approaches using CRISPR/Cas9 and zinc finger nucleases methods, but only in a small number of participants. Indeed, positive preliminary results were observed (Smith et al., 2019; Frangoul et al., 2020), however, more patients and longer follow-up are essential to determine the clinical significance.

Prospective Approaches for β-thalassemia Treatment

Induced Pluripotent Stem Cells: A Promising Prospect for Cell and Gene Therapy

The concept of gene therapy with genetically modified HSCs for the treatment of β-thalassemia has been successfully demonstrated in a new era of medicine. Many researchers have attempted to develop and modify novel strategies to improve future directions of HSC gene therapy, such as manipulation of induced pluripotent stem cells (iPSCs) derived from patient HSCs/somatic cells. iPSCs are autologous somatic cells that are reprogrammed into an embryonic-like stage in vitro. Two key features of human iPSCs are the capacity for self-renewal and pluripotency, which is the ability to differentiate into all cell types (Takahashi and Yamanaka, 2006; Yu et al., 2007), including HSCs (Bisogno et al., 2020; Demirci et al., 2020). Therefore, human iPSCs derived from patients are an attractive cell source for the development of novel strategies for the treatment of hematological disorders (Donada et al., 2020; Kennedy et al., 2012) (**Figure 2A**). Since the generation of human iPSCs was successfully demonstrated by using different types of cells (e.g., skin or blood) from transfusion-dependent β-thalassemia patients (Papapetrou et al., 2011; Wongkummool et al., 2017). Editing of the endogenous β-globin locus is an attractive strategy; indeed, the β-globin genes mutations can be corrected with various approaches such as CRISPR/Cas9 technology (Song et al., 2015; Niu et al., 2016). However, most of the studies assessed only the potential of hematopoietic differentiation in vitro. In vivo transplantation of HSCs derived from corrected iPSC-derived from β-thalassemia patients into immunodeficient mice has also been demonstrated. They found that the corrected cells could produce hemoglobin, but a low level of hematopoietic cell reconstitution was still observed (Wang et al., 2012; Ou et al., 2016). A recent study used a thalassemic mouse model to mimic thalassemic patients as an implantation model. After

transplantation with genetically corrected iPSCs derived from β-thalassemia patients, the corrected cells can differentiate into erythrocytes, nonetheless, an anemia symptom was not effectively recovered (Xian et al., 2020). As genomic instability in the iPSCs, including β-thalassemia patient-derived cells was observed (Ma et al., 2015; Yoshihara et al., 2019). Therefore, a key caution of the iPSCs transplantation is the potential of tumor development, e.g., the induction of p53-mediated DNA damage and cell cycle arrest (Haapaniemi et al., 2018). Estimation of DNA mutation and tumorigenesis of the manipulated-iPSCs are therefore obligatory to test before clinical implication. Moreover, the development of transfusion products from iPSCs would provide autologous RBC transfusion for β-thalassemia patients, despite only allowed a shorter-term treatment. Additionally, in vitro culturing with good manufacturing practice (GMP) platform is always associated with cost raising (Chang et al., 2010; Hirose et al., 2013). Indeed, transfusions of iPSCs-derived RBCs are now representing one of the most promising strategies from iPSC-based therapies (Hansen et al., 2019). However, the expression of many surface antigens of RBCs (blood group system) is still a challenge for clinical application using iPSCs-derived RBCs. To date, although human iPSCs provided a continuous generation of HSCs, the stemness properties of iPSC-derived cells were not completely functional (Tan et al., 2018). Consequently, the engraftment ability of human iPSC-derived HSCs was very low in the animal transplantation model (Li et al., 2017), indicating a long way off to implantation in patients. Thus, it is essential to develop an efficient procedure for human iPSC-derived HSCs to expand and maintain their stemness properties.

Ex vivo Expansion of HSCs for Cell and Gene Therapy Manipulation

The clinical requirement of HSCs for transplantation is more than 2 million cells/ kg body weight of patients (Hequet, 2015). Thus, to reach therapeutic demand, increasing the number of HSCs by *ex vivo* cell culture would improve transplantation outcomes and permit the use of samples in which the number of HSCs is initially low (Lee et al., 2013). The ability to expand the HSCs and also preserve their functions would be potentially useful in the clinical setting (Psatha et al., 2016). *Ex vivo* expansion using a cocktail of recombinant cytokines has been shown to increase the HSC fraction (Sauvageau et al., 2004). However, some studies have reported that the combination of several cytokines can cause a loss of the ability for self-renewal and can induce HSC differentiation and/or exhaustion (Goff et al., 1998; Ueda et al., 2000). Co-culture of HSCs with stromal cells to simulate the internal hematopoietic niche is another strategy to improve the expansion of HSCs (Jing et al., 2010), but such procedures were also found to produce negative regulators of hematopoiesis (Larsson et al., 2003). A recent study revealed that several small molecules could enhance HSC *ex vivo* expansion by promoting self-renewal, delaying differentiation, increasing homing, and inhibiting apoptosis of HSCs (Zhang and Gao, 2016). For example, a pyrimidoindole derivative, UM171, clearly increased *ex vivo* expansion of long-term HSCs derived from human CB and mobilized peripheral blood (Fares et al., 2014). Therefore, identifying novel small molecules to enhance *ex vivo* HSC self-renewal and preserve HSC pluripotency may be beneficial for HSC expansion, specifically expansion of corrected HSCs (**Figure 2A**).

Ex vivo Selection and Optional Suicide Gene of the Genetically Modified HSCs

Some cases of β-thalassemia gene therapy have experienced massive loss of transduced HSCs by contamination with non-transduced cells; thus, the population of genetically modified cells is out of reach for treatment application. Moreover, dominant clonal overexpression of proto-oncogene HMGA2 in erythroid cells was also observed in the first β-thalassemia gene therapy clinical trial (Cavazzana-Calvo et al., 2010). Therefore, various strategies have been considered to improve transduction efficiency to achieve a high level of HSC modification without increasing the concurrent risk of insertional mutagenesis and oncogene activation. We recently demonstrated the possibility of fusing therapeutic genes with selection and suicide genes, including the puromycin *N*-acytyltransferase (PAC) and the herpes simplex virus thymidine kinase (TK) (**Figure 2B**). We revealed that the puromycin resistant gene allowed optimal *ex vivo* selection of genetically modified HSCs. After selection, transduced HSCs survived and were able to reconstitute human hematopoiesis in immunodeficient mice. Furthermore, the vector was able to express the β-globin gene and produced the suicide protein *in vivo* for elimination of transduced stem cells if necessary (Bhukhai et al., 2018). Thus, expression of PAC and TK cassettes could maintain effective levels of the therapeutic gene, suggesting the procedure for human clinical application with affording the additional safety of conditional suicide gene.

Therapeutic Option for β0/β0 Thalassemia

The currently available gene therapy, LentiGlobin BB305, is not approved for the treatment of β0/β0-thalassemia, the severest form of β-thalassemia. Although the treatment could reduce the annual number of RBC transfusions, most of the β0/β0 individuals in clinical trials fail to reach transfusion independence. Moreover, compared with individuals with non-β0/β0 thalassemia, β0/β0 individuals generally require higher VCN of the therapeutic vector to achieve curative levels of Hb, which increases the risk of oncogenicity due to insertional mutagenesis (Thompson et al., 2018; U.S. National Library of Medicine, ; Breda et al., 2012). In many studies, the concept of reducing α-globin synthesis is proposed as an optional modality in gene therapy for β-thalassemia, as the excess α-globin leads to toxic aggregation in RBCs, resulting in immature apoptosis (Mettananda et al., 2016; Sachith et al., 2017). Based on this information, one study has investigated a multiplex lentiviral gene therapy vector with coordinated β-globin expression and α2-globin reduction (LVβ-shα2) modified from LentiGlobin BB305 as an optional strategy for the β0/β0 genotype. LVβ-shα2 demonstrates reduction of α2-globin expression while maintaining expression of the therapeutic β^{A-T87Q}-globin gene, which improves α/β-globin balance and decreases cellular damage from unbound α-globin chains in erythroid cells (**Figure 2B**). Compared with LentiGlobin

BB305, LVβ-shα2 also requires a lower VCN of the viral vector for equivalent efficacy, which could improve safety (Nualkaew et al., 2021). This gene therapy approach is promising for curative treatment of the β0/β0 disease, but further studies are required to explore efficacy and reliability in a clinical setting.

CONCLUSION

Although the use of lentiviral vectors carrying the β-globin gene has been successfully validated for β-thalassemia treatment, this type of treatment faces challenges involving outcome effectiveness and cost sustainability. Because the long-term consequences of HSC genome editing mechanisms are not completely known, the gene editing strategy may not be considered safer than that of lentiviral-mediated gene transfer. The typical gene addition method remains the most effective therapy to date. Accordingly, challenges the framework of treatment including development of novel directions in gene therapy and fair public access, to get better outcome with effectiveness are urgently required to be available in a large number of β-thalassemia patients.

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Dynamics of Adaptive Immune Cell and NK Cell Subsets in Patients With Ankylosing Spondylitis After IL-17A Inhibition by Secukinumab

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Background: Anti-IL-17A therapy is generally effectively applied in patients with Ankylosing Spondylitis (AS) to achieve and maintain remission. However, the influence of anti-IL-17A on the composition of the immune system is not apparent. Our prospective study was to explore the changes in immune imbalance regarding T cell, B cell and natural killer (NK) cell subsets after secukinumab treatment in AS patients.

Methods: Immune cell distribution of 43 AS patients treated with secukinumab for 12 weeks and 47 healthy controls (HC) were evaluated. Flow cytometry using monoclonal antibodies against 25 surface markers was accomplished to explore the frequencies of lineage subsets. The differences between HC, AS pre-treatment, and post-treatment were compared using the paired Wilcoxon test, Mann-Whitney *U* test, and ANOVA.

Results: AS patients had altered immune cell distribution regarding T cell and B cell subsets. Apart from activated differentiation of $CD4^+$ T cell, $CD8^+$ T cell and B cell, higher levels of cytotoxic T (Tc) two cells and Tc17 cells were noted in AS patients. We confirmed that helper T (Th) one cell became decreased; however, Th17 cells and T follicular helper (Tfh) 17 cells went increased in AS. After 12 weeks of secukinumab therapy, CRP and ASDAS became significantly decreased, and meanwhile, the proportions of Th1 cells, Tfh17 cells and classic switched B cells were changed towards those of HC. A decreased CRP was positively correlated with a decrease in the frequency of naïve $CD8^+$ T cells (p = 0.039) and B cells (p = 0.007) after secukinumab treatment. An elevated level of T cells at baseline was detected in patients who had a good response to secukinumab (p = 0.005).

Conclusion: Our study confirmed that AS patients had significant multiple immune cell dysregulation. Anti-IL-17A therapy (Secukinumab) could reverse partial immune cell imbalance.

Keywords: ankylosing spondylitis, T cell, B cell, IL-17A, Th17 cell

BACKGROUND

Ankylosing Spondylitis (AS) is a chronic inflammatory disease with complex etiology. Other than the genetic contribution of HLA-27 and other genes (Fiorillo et al., 2019), the innate and the adaptive immune system, driven primarily by Th1 and Th17 cells, contribute to pathogenic processes of AS. Human genetics and animal model studies strongly support the notion that the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-17 exert a substantial influence on AS pathogenesis (Sieper and Poddubnyy, 2017; Watad et al., 2018). TNF-α inhibitor (TNFi) has become a widely used medication for AS. According to the ASAS/EULAR recommendations, biologic DMARD therapy, typically TNFi therapy, should be considered in patients with persistently high disease activity despite conventional treatments (Noureldin and Barkham, 2018). We have reported AS patients have altered immune cell frequencies, including $CD4^+$ T cells, $CD8^+$ T cells and B cell, and found that anti-TNF-α therapy could improve the frequency of immune dysregulation of $CD4^+$ T cells and negative regulatory cells (Yang et al., 2020). Although most patients had improvement with TNFi therapy, there is an unmet need that not all patients respond well to or can tolerate TNFi treatment (Noureldin and Barkham, 2018).

IL-17, produced predominately by $CD4^+$ T helper (Th) 17 cells, is also a significant proinflammatory cytokine linked to pathogenic processes in autoimmune and inflammatory diseases, especially in AS. Blockade of IL-17 has become a promising treatment option (Sieper and Poddubnyy, 2017). As a fully human monoclonal antibody against IL-17A, secukinumab (Cosentyx®) received FDA approval to treat AS and psoriasis arthritis (PsA) in 2016. Sustained, long-time efficacy of secukinumab in patients with AS have also been reported (Pavelka et al., 2017; Baraliakos et al., 2018). However, these clinical trials revealed that some patients did not respond well to anti-IL-17A therapy, the reason of which remains unclear. Given side-effects and expenses of biological agents, it is required to identify biomarkers to predict treatment effects.

Limited literature explored the function of natural killer (NK) cells in AS. RNA sequencing of blood cells from AS patients and controls identified downregulated genes were enriched in $CD8^+$ T cells and NK cells, revealing NK cells might take part in the pathogenesis of AS (Li et al., 2017).

Downstream effects of IL-17 blockade are worthy of exploration for a better understanding of this biological agent. Here, we conducted a prospective study 1) to verify the immune imbalance in AS patients; 2) to analyze clinical improvement and the alteration in immune cell frequency after IL-17A inhibition by secukinumab; and 3) to explore the predictors of good responses to secukinumab in AS patients.

MATERIALS AND METHODS

Study Population

A prospective study was designed to observe lymphocyte alteration and short-term efficacy after 12 weeks of secukinumab treatment in patients with AS. We included AS patients from Department of Rheumatology and Immunology at Third Affiliated Hospital of Sun Yat-sen University. All the patients fulfilled 1984 Modified New York Criteria (van der Linden et al., 1984) and had high disease activity (ASDAS ≥ 1.3). Patients with severe infection, had a vaccination, biologic agents, unstable use of non-steroid anti-inflammatory drugs (NSAIDs) or disease modifying anti-rheumatic drugs (DMARDs) within 3 months prior to the study and pregnant women were excluded from the study. Demographic and clinical variables, including age, sex, disease duration, clinical manifestation, medication, C-reactive protein (CRP) and ASDAS were recorded at baseline and after secukinumab treatment. All the patients received a subcutaneous injection of secukinumab (Cosentyx®) 150 mg at week 0, 1, 2, 3, 4, 8, and 12. Physician's overall assessment (good response, no response) was acquired to evaluate the treatment effect of secukinumab.

Healthy controls (HC) were recruited from healthy volunteers at our hospital. HC with diagnosed chronic diseases, a complaint of back pain, skin rash, and other possible symptoms of AS, medication intake and positive family history of AS were excluded from the study. Blood samples (Heparin sodium tube 5 ml and blood collection tube without anticoagulant 3 ml) were acquired from the participants. The entire study was conducted from March 2020 to February 2021. All the participants gave written consent forms.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were acquired and then were stained for surface markers for 20 min in PBS containing fluorescent antibodies. Fluorescently labeled antibodies included CD3-PerCP-Cy5.5, CD25-PE, CD45RA-FITC, CD8-PerCP-Cy5.5, CD19-PerCP-Cy5.5 and CD56-PE-Cy7 (Tianjin Three arrows, China); CD4-APC-H7, CD8-BV510, CD127-BV421, CCR7-AF647, CD28-PE-Cy7, CD3-APC-H7, CD4-PE-Cy7, CXCR3-Alexa488, CXCR6-BV510, CXCR5-Alexa647, CCR4- BV421, PD-1-PE, CD45-APC-H7, CD27-BV421, IgD-BB515, IgM-BV510, CD38-APC, CD24-PE, and CD21-PE-Cy7 (BD, United States). The instrument settings and gating strategies were adopted from previous works (**Supplementary Figure S1**) (Yang et al., 2020; Zhu et al., 2020). All experiments, including cell separation and sample preparation, were performed according to standardized experimental manuals. Samples were analyzed using CytoFLEX flow cytometer (Beckman, United States). Results are expressed as the proportion of cells expressing particular markers. T cell subsets, including cytotoxic T (Tc) cells, helper T (Th) cells, T follicular helper (Tfh) cells, regulatory T (Treg) cells, B cells and NK subsets were identified (**Supplementary Table S1**).

Statistical Analyses

First, we performed a descriptive analysis of the participants. Data with normal distribution were stated as mean ± standard deviation, and those with non-normal distribution were recorded as median (interquartile range). Comparisons between subgroups were completed using paired Wilcoxon test, Mann-Whitney *U* test, and ANOVA. We applied the Pearson correlation or Spearman's rank correlation analysis to examine the relationship between clinical parameters and the frequency of immune cell subtypes. Cluster analyses of immunophenotypic variables were performed using R package.

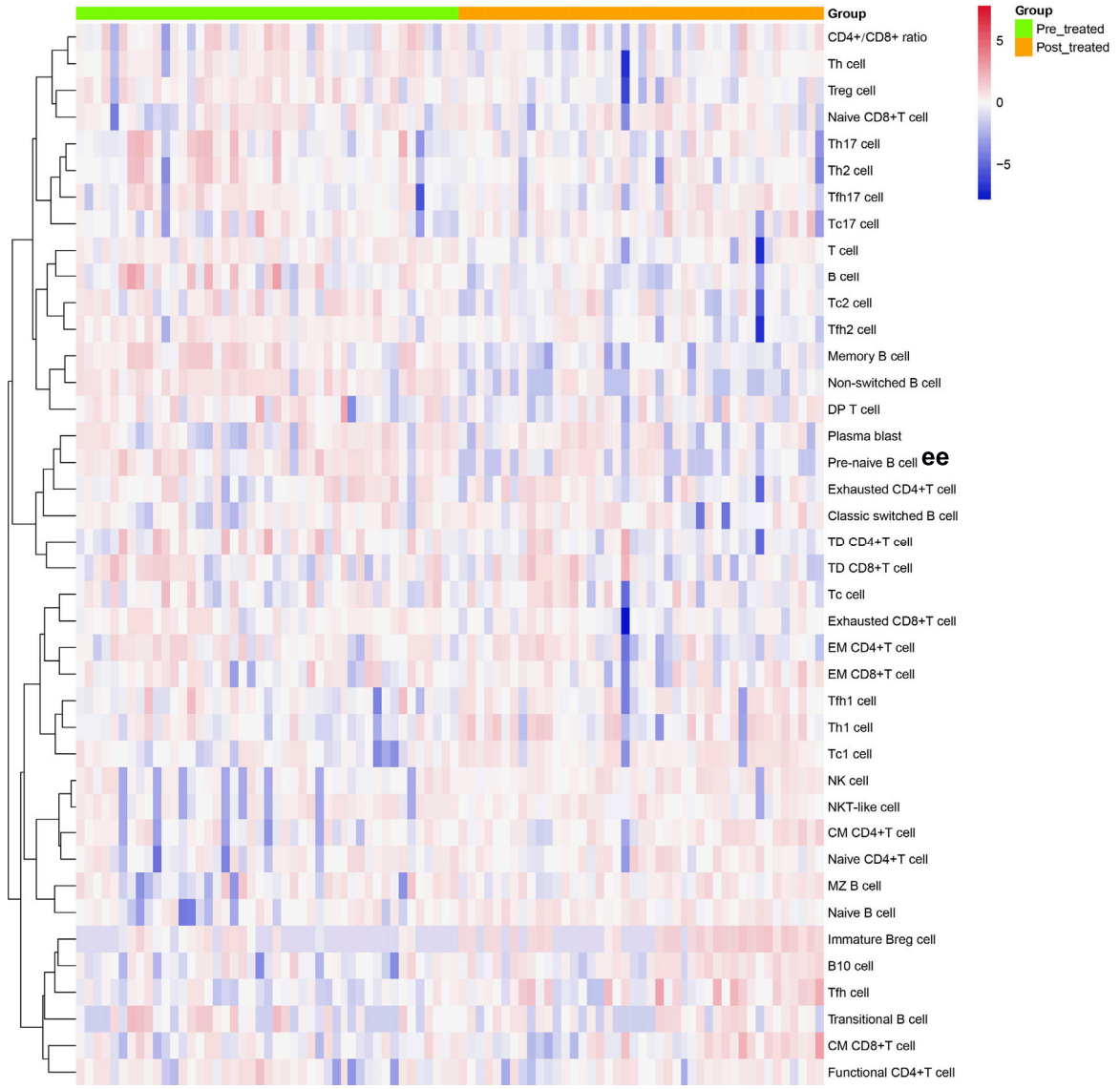

FIGURE 1 | Cluster analyses of immune cell frequency in the patients with AS before and after treated with secukinumab. Each column represented individual patients with AS. Pre-treatment group was marked as green and post-treatment group was marked as orange. The rows represented immune cell subsets that are differentially expressed. The magnitude of parameter expression was color-coded with red for an increase in expression and blue for a decrease in expression. Th cell, helper T cell; Treg cell, regulatory T cell; Tc cell, cytotoxic T cell; Tfh cell, follicular helper T cell; DP T cell, double positive T cell; TD, terminally differentiated; EM, effector memory; CM, central memory; NK cell, natural killer cell; Breg cell, regulatory B cell.

A p value less than 0.05 was considered significance. All the analyses were completed using SPSS, release 20.0 (IBM, Armonk, NY, United States), and graphs were made using Prism (GraphPad Software, Inc., La Jolla, CA, United States).

RESULTS

General Characteristics of the Participants

Totally 45 AS patients and 47 HC were included in the current study. Two patients who did not receive secukinumab at the specific time-point were excluded for further analysis of immune cell frequency. The mean age of 43 patients was 28.3 ± 9.9 years in AS group. The median disease duration was 6.5 (3.1–9.6) years. The median C-reactive protein (CRP) was 17.7 (1.5–23.8) mg/L at baseline (**Supplementary Table S2**). Age- and sex-matched HC had a mean age of 30.6 ± 5.1 years.

Changes in T Cell Subsets Between Ankylosing Spondylitis and Healthy Controls

We analyzed different differentiation stages of immune cells and found significant changes between AS patients and HC

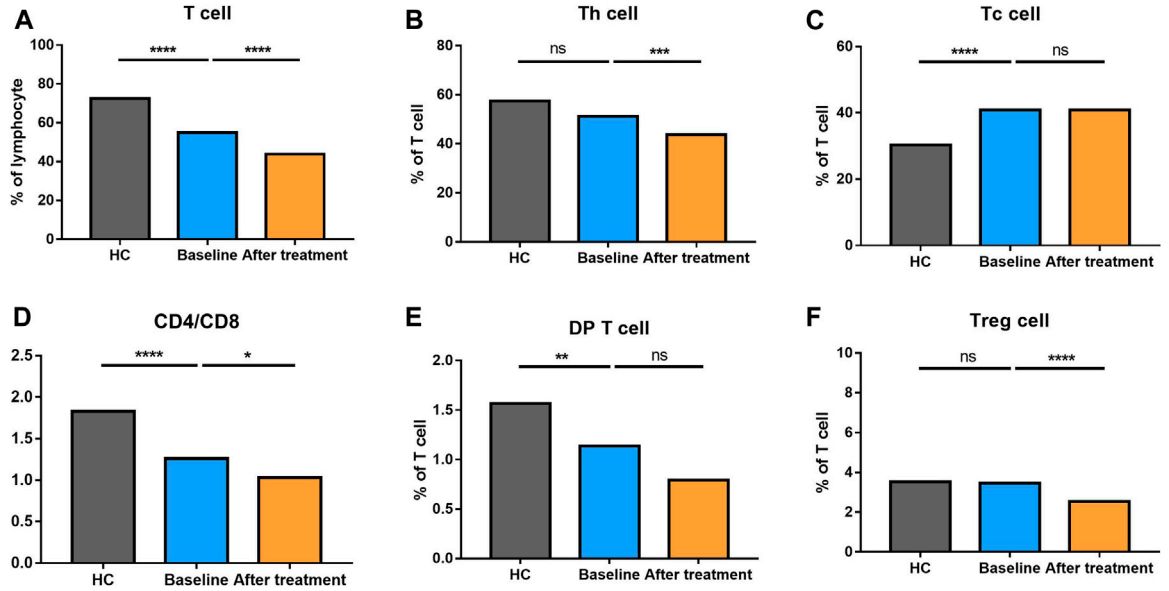

FIGURE 2 | Altered expression of T cell subsets in AS patients after treated with secukinumab. The proportion of T cell subsets measured by flow cytometry at baseline and after receiving secukinumab in AS patients. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$; ****, $p < 0.0001$.

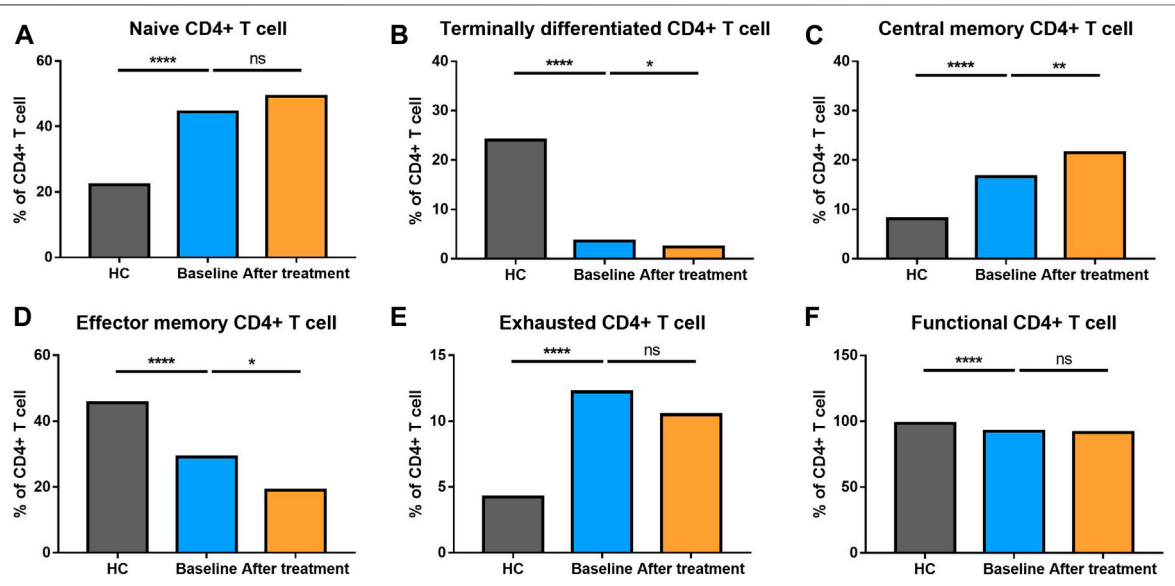

FIGURE 3 | Altered expression of $CD4^+$ T cell subsets in AS patients after treated with secukinumab. The proportion of $CD4^+$ T cell subsets measured by flow cytometry at baseline and after receiving secukinumab in AS patients. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$; ****, $p < 0.0001$.

(**Figure 1**). AS patients had a lower proportion of total T cells, CD4/CD8 ratio, and double-positive (DP) T cells (**Figure 2**). In $CD4^+$ T cell subsets, the proportions of naïve $CD4^+$ T cells, central memory $CD4^+$ T cells and exhausted $CD4^+$ T cells became significantly increased, but the levels of terminally differentiated $CD4^+$ T cells, together with effector memory $CD4^+$ T cells, were reduced significantly in AS (**Figure 3**).

The proportion of $CD8^+$ T cells at different stages of differentiation was also determined (**Figure 4**). AS patients had elevated levels of naïve $CD8^+$ T cells, central memory $CD8^+$ T cells, effector memory $CD8^+$ T cells and exhausted $CD8^+$ T cells, but comparatively, a reduced level of terminally differentiated $CD8^+$ T cells.

We also explored the proportion of Th ($CD3^+CD4^+$) cell subsets (Th1 cells, Th2 cells and Th17 cells), Tfh ($CD3^+CD4^+CXCR5^+$) cell subsets (Tfh1 cells, Tfh2 cells, Tfh17 cells), and Tc ($CD3^+CD8^+$) cell subsets (Tc1 cells, Tc2 cells, Tc17 cells) between AS and HC (**Figure 5**). There were no significant

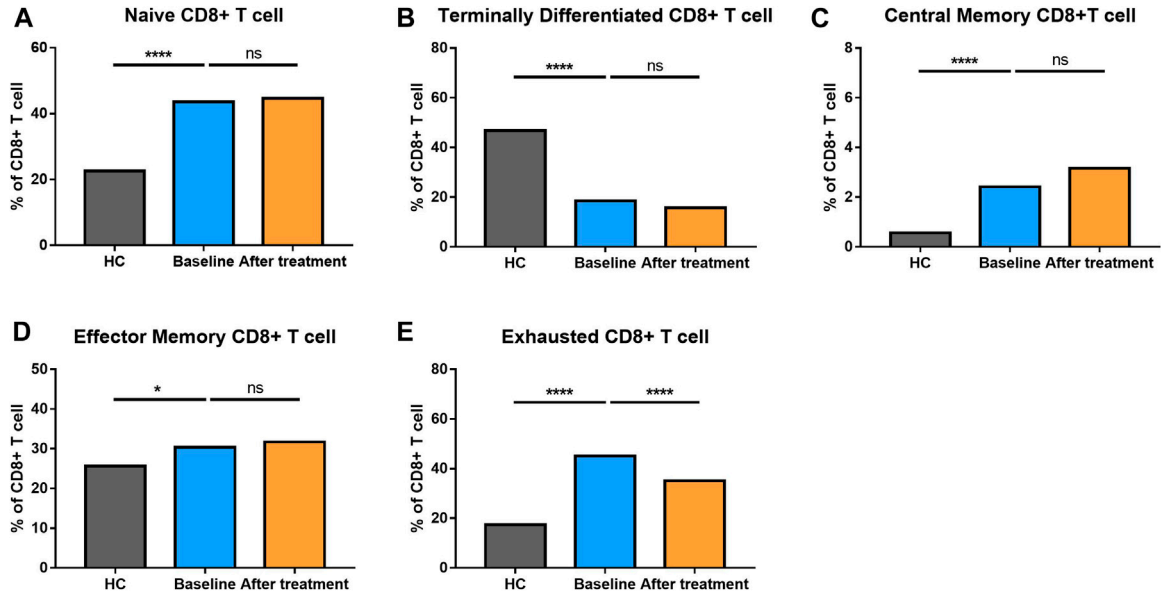

FIGURE 4 | Altered expression of $CD8^+$ T cell subsets in AS patients after treated with secukinumab. The proportion of $CD8^+$ T cell subsets measured by flow cytometry at baseline and after receiving secukinumab in AS patients. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$; ****, $p < 0.0001$.

changes in total Th cells and Tfh cells between the two groups. However, there was a significantly higher proportion of Tc cells in AS patients, together with a higher level of Tc2 cells. Comparatively, lower levels of Tc1and Tc17 cells were found in AS group. Th1 cells became decreased; however, Th17 cells went up in AS patients. The proportion of Tfh17 cells were increased in the patients' group, while there were no differences in the proportion of Th2 cells, Tfh1 cells and Tfh2 cells. The results reflected that AS patients had a significantly altered proportion of T cell subsets.

Changes in B Cell Subsets Between Ankylosing Spondylitis and Healthy Controls

The percentage of B cells at different stages of differentiation was compared in **Figure 6**. In B cell subtypes, the proportions of B cells, naïve B cells, marginal zone (MZ) B cells, and transitional B cells were not altered in AS group. However, pre-naive B cell, plasma cells, and classic switched B cells were decreased. Nevertheless, memory B cells, non-switched B cells and B10 cells became increased in AS patients compared to HC.

Changes in Regulatory Lymphocytes Between Ankylosing Spondylitis and Healthy Controls

As shown in **Figures 1**, **5**, regulatory lymphocytes including Treg cells and Breg cells were evaluated. We found that Breg cells were significantly lower in AS. Treg also became decreased in AS patients; however, no significant difference was detected between the groups ($p > 0.05$).

Altered Distribution of T Cell Subsets After Secukinumab Therapy

None of AS patients underwent safety issues during the treatment or quitted the study for personal reasons. After 12 weeks of secukinumab therapy, CRP and ASDAS became significantly decreased (**Supplementary Table S3**). There were significant differences in immune cell frequency at baseline and after treatment (**Figures 1–6**). Both T cells and B cells were decreased after treated with secukinumab. The proportion of $CD4^+$ T cells was reduced, leading to a lower CD4/CD8 ratio. In $CD4^+$ T cell subsets, central memory $CD4^+$ T cells were increased, while terminally differentiated $CD4^+$ T cells and effector memory $CD4^+$ T cells became decreased after anti-IL-17A therapy.

Th2 cells, Th17 cells and Tc17 cells remained stable after IL-17A inhibition. Tfh1 cells turned elevated while Tfh2 cells became reduced after secukinumab treatment. An increased level of Th1 cells and Tc1 cells, together with a decreased number of Tfh17 cells and Tc2 cells, were noticed after anti-IL-17A therapy. Moreover, the levels of Th1 cells and Tfh17 cells were changed towards those of HC after secukinumab treatment.

Altered Distribution of B Cell Subsets After Secukinumab Therapy

Whereas the total number of B cells were reduced, the proportion of naïve B cells and classic switched B cells became elevated after anti-IL-17A therapy. Comparatively, the proportion of pre-naïve B cells, memory B cells and non-switched B cells went down significantly. Plasma cells did not alter after the treatment. These results revealed a complex alteration of T cell and B cell subsets in AS patients who underwent secukinumab treatment.

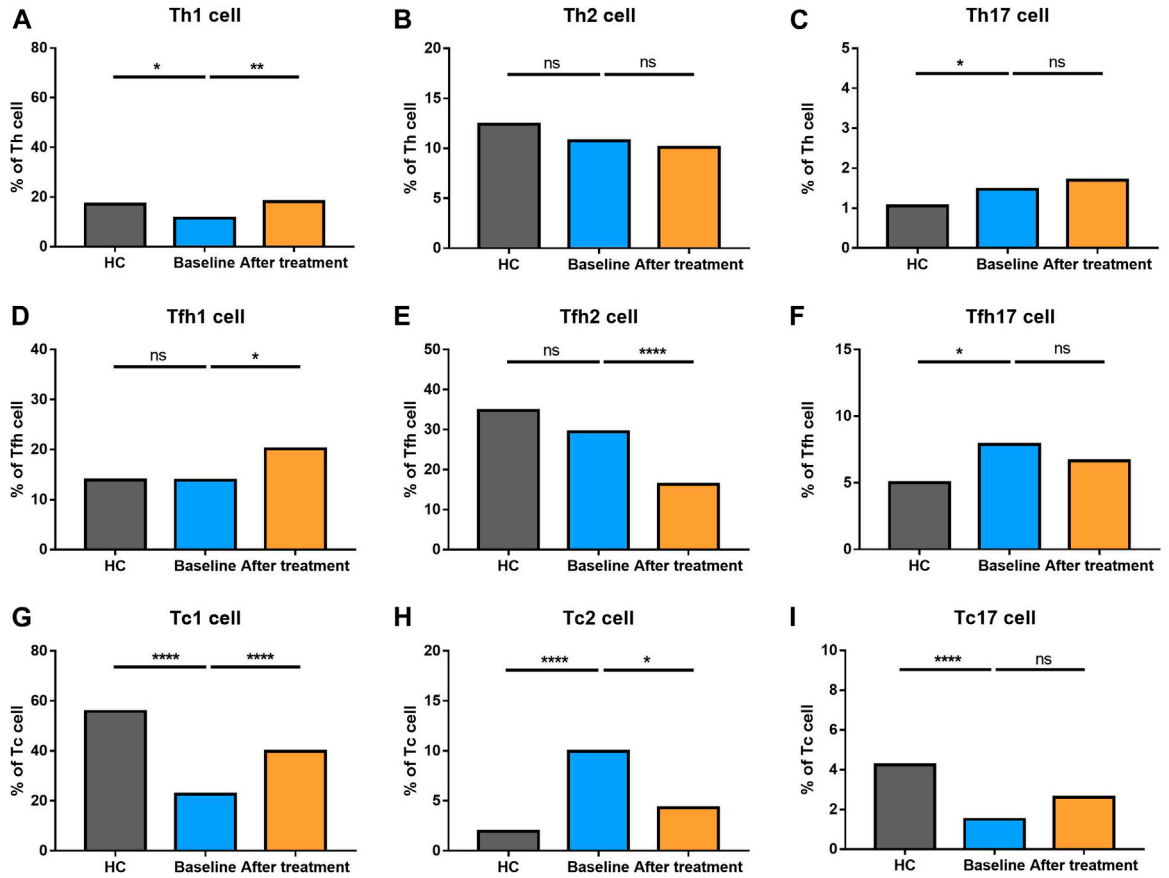

FIGURE 5 | Altered expression of Th, Tfh, and Tc cell subsets in AS patients after treated with secukinumab. The proportion of Th, Tfh, and Tc subsets measured by flow cytometry at baseline and after receiving secukinumab in AS patients. Th cell, helper T cell; Tfh cell, follicular helper T cell; Tc cell, cytotoxic T cell; ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$; ****, $p < 0.0001$.

Altered Distribution of Regulatory Cells, NK Cells and NKT-Like Cells After Secukinumab Therapy

Both the circulating regulatory T cells and B cells were changed significantly after secukinumab treatment. Treg cells were reduced, while Breg cells were increased after anti-IL-17A treatment. We also tested the proportions of NK cells ($CD3^{-}CD56^{+}$) and NKT-like cells ($CD3^{+}CD56^{+}$) and found that NKT-like cells were significantly reduced, while NK cells remain stable after treatment with secukinumab in AS patients.

Correlation Between Clinical Variables and Immune Cell Frequency

To explore the correlation between changes of CRP values and variations in immune cell frequency after secukinumab therapy, we performed Spearman's rank correlation analyses and found that the decrease in CRP was positively correlated with the decrease in the frequency of naïve $CD8^{+}$ T cells (rho = 0.331, $p = 0.039$) and B cells (rho = 0.423, $p = 0.007$).

To further explore the predictors of good responses of secukinumab, we compared immune cell frequency between the subgroups who had a great response to secukinumab ($n = 33$) and those who failed to have satisfying improvement ($n = 10$) according to physicians' overall assessment. There was no difference in age, CRP and ASDAS between the two subgroups. The only difference was an elevated level of T cells at baseline in those secukinumab responders (60.4 (51.9–66.4)) compared to non-responders (36.4 (25.5–55.7)) ($p = 0.005$).

DISCUSSION

Apart from the contribution of the HLA-B27 and the interaction with Tc cells, the differentiation of $CD4^{+}$ T cells and the cytokines secreted are proved to participate in the pathogenesis of AS. Recent studies have highlighted the role of Th17/Treg imbalance and IL-17A/IL-23 cytokine dysregulation in AS (Lee, 2018). Anti-IL-17A (secukinumab) is recommended in active AS patients according to previous guidelines (Noureldin and Barkham, 2018). How this biological agent regulates immune cells remains unclear. Our study first explored the proportions of various

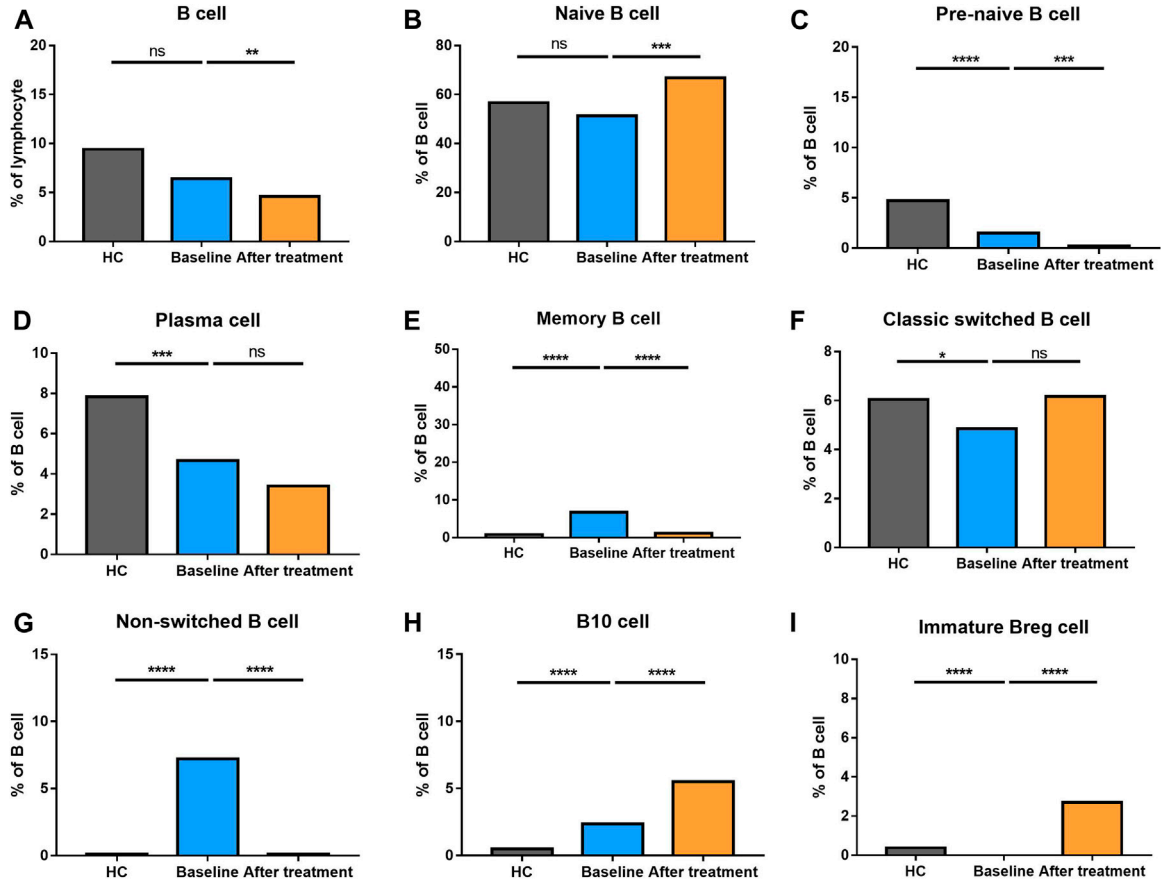

FIGURE 6 | Altered expression of B cell subsets in AS patients after treated with secukinumab. The proportion of B cell subsets measured by flow cytometry at baseline and after receiving secukinumab in AS patients. Breg, regulatory B cell. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$; ****, $p < 0.0001$.

subsets of peripheral T cells and B cells, together with NK cells, after anti-IL-17A treatment, and described that anti-IL-17A treatment could lead to the alteration of immune cells, which may be related to therapeutic functions. In these lymphocytes, Th1 cells, Tfh17 cells and classic switched B cells were changed towards those of HC after secukinumab treatment. Moreover, the baseline level of T cells might be an indicator of the excellent treatment effect of secukinumab.

Recent studies have reported immune cell dysregulation related to inflammatory disorders play a significant role in the pathogenesis of AS (Fiorillo et al., 2019). We proved again that AS patients have altered $CD4^+$ T cell and $CD8^+$ T cell subsets at different stages of differentiation, revealing that both $CD4^+$ T cells and $CD8^+$ T cells were activated in disease processes (Yang et al., 2020). $CD4^+$ T cell subgroups can transform to each other and play diverse roles under appropriate environments. Naïve $CD4^+$ T cells differentiate into Treg cells under the induction of TGF-β and differentiate into Th17 cells under the combined action of TGF-β and IL-6 or IL-21. Th17 cells produce cytokines including IL-17A, IL-17F, IL-21 and IL-22, thus participating in the inflammatory response, while Treg cells play a negative immunomodulatory role after being activated by homologous antigens. Th17 and Treg cells can undergo phenotypic transformation under certain conditions. In recent years, immunopathological studies on autoimmune diseases and inflammatory diseases have found that Th17/Treg imbalance is an essential factor in the occurrence and development of such diseases (Miossec and Kolls, 2012). Our findings also proved that Th1 cells became decreased, while Th17 cells were increased with a disturbed Th17/Treg balance in AS patients, which was consistent with previous reports (Xueyi et al., 2013; Fasching et al., 2017; Hajialilo et al., 2019).

IL-17A, expressed by Th17 cells and multiple lineages of the innate immune system, can act as a chemokine directly on immune cells, thus bridging innate and adaptive immunity (Gaffen, 2009; Miossec and Kolls, 2012). The inhibition of IL-17A would exert various physiological effects more than the suppression of Th17 cell activity (Patel et al., 2013). As a highly selective, fully human immunoglobulin G1k (IgG1k) monoclonal antibody directed against the IL-17A cytokine, secukinumab has been assessed in the treatment of some autoimmune diseases (Patel et al., 2013). Anti-IL-17A has become a second-line therapy for treating AS according to international guidelines (Kiltz et al., 2012). Like other biologicals, agents targeting IL-17A have the theoretical

potentials to influence the immune system (Patel et al., 2013). Our study also demonstrated that secukinumab could lead to multiple variations of innate and adaptive immune responses. Th1 cells went back to normal after 12 weeks' secukinumab treatment, and meanwhile, Th17 cells and Th17/Treg were also reduced but still higher than healthy participants. The total of $CD8^+$ T cells remained stable; however, the proportion of Tc1, Tc2 and Tc17 cells were changed toward those of HC. A more extended observation is needed to explore when Th17 cells and Th17/Treg could reduce to a normal range after secukinumab treatment.

As is known, Tfh cells can support effector B cells and boost autoimmunity. Tfh17 cells help induce naïve B cells to produce immunoglobulins *via* IL-21, which is essential for B cell proliferation and differentiation (Bautista-Caro et al., 2014). The presence of antibodies targeting anti-CD74 autoantibodies (Riechers et al., 2019), protein phosphatase magnesium-dependent 1A (Kim et al., 2014), a variety of microbial components (Wen et al., 2017) in patients with AS are suggestive of B cells' contribution. We also found that the proportion of Tfh17 cells and classic switched B cells was increased significantly, indicating B cells differentiation may also take part in the pathogenesis of AS. After IL-17A inhibition, the levels of Tfh17 cells and classic switched B cells were changed towards those of HC, reflecting the effect of anti-IL-17A therapy on B cell differentiation. Noticeably, total B cells were decreased significantly after secukinumab treatment, which was opposed to the changes brought by anti-TNF therapy (Lin et al., 2009; Yang et al., 2020).

NK cells play a vital role in innate immune responses. $CD56^+$ T cells are a set of pro-inflammatory lymphocytes with some characteristics of NK cells (Krijgsman et al., 2019), which are also called NKT-like cells. This group of cells possess both cytotoxic capabilities and regulatory function of the immune response via the secretion of pro- or anti-inflammatory cytokines upon activation (Krijgsman et al., 2018). Our study showed no significant change in NK cells but a reduced level of NKT-like cells after IL-17A inhibition. Further studies involving cytokines secretion were needed to confirm the findings.

The current study had some limitations which should be carefully considered. First, AS has different phenotypes regarding axial and peripheral involvement and different HLA-B27 status. It remains questionable how various clinical manifestations are related to immune cell imbalance. Second, longitudinal data with prolonged secukinumab treatment would be helpful to provide more knowledge about the long-lasting alterations of these immune cells. Third, the peripheral proportion of immune cell subsets could partially reflect of how immune system works after the use of IL17A inhibitor. A subsequent function investigation would better explain relevant mechanisms.

CONCLUSION

Our prospective study confirmed AS patients had significant alteration of immune cell frequency. Anti-IL-17A therapy (Secukinumab) could reduce inflammation and reverse partial immune cell imbalance. The baseline level of total T cells might be an indicator of good response to secukinumab.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Third Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation was performed by YJ, JW, and QW. Data collection was performed by QW, YX, MY, and YH. Data analysis were performed by YJ, JW, YZ, and ZL. The first draft of the manuscript was written by YJ and MY, with the assistance from ZL. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.738316/full#supplementary-material

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Treatment With Melatonin After Corneal Graft Attenuates Rejection

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Background: Immunologic graft rejection is the main complication of corneal transplants. This study aimed to investigate the effect of melatonin (MT) on the rejection of corneal transplantation.

Methods: Corneal allografts were performed by grafting corneas from BALB/C mice to C57BL/6 hosts. MT (50 mg/kg) was intraperitoneally injected into the hosts every day from the day of transplantation. The survival of grafts was observed by slit lamp biomicroscopy, and inflammatory cell infiltration was detected by hematoxylin and eosin staining and immunohistochemistry. The balance of Teff and Treg immune cells in draining lymph nodes (DLNs) was detected by flow cytometry. The levels of cytokines related to the grafts and DLNs were detected using real-time fluorescence quantitative PCR. Additionally, we used the mouse macrophage line RAW264.7 to study the effect of MT on the activation of NLRP3 inflammatory body.

Results: MT treatment improved the graft survival rate, reduced inflammatory cell infiltration in the graft, decreased the percentage of Th1/Th17 cells in the DLNs, and increased the percentage of Treg cells. Melatonin inhibited the activation of the NLRP3 inflammasome, thereby reducing the expression of IL-1β and other related proinflammatory cytokines such as MCP-1, MIP-1, NLRP3, ASC, TNF-a and VEGF-A (all $p < 0.05$).

Conclusion: Our study demonstrates that MT promotes the survival of mouse corneal grafts by inhibiting NLRP3-mediated immune regulation, reducing immune cell activation and cell migration, and inhibiting the production of inflammatory-related cytokines. Treatment with MT might provide a potential clinical therapeutic target for corneal transplantation.

Keywords: corneal transplant, NLRP3 inflammasome, macrophages, CD4$^+$ T cells, melatonin

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INTRODUCTION

Currently, more than 10 million people worldwide suffer from corneal blindness (Amouzegar et al., 2016). Corneal trauma and disease can cause irreversible damage to the normal structure and physiological function of the cornea, and patients usually require corneal transplantation. Corneal allograft rejection is a major complication leading to decreased vision after transplantation. The incidence of postoperative immune rejection of inflammation and vascularized cornea can reach as high as 50% (Armitage et al., 2019). Although the application of corticosteroids and other immunosuppressive agents has improved the survival rate after corneal transplantation, the

long-term use of these drugs can lead to serious complications such as glaucoma, cataract and weight gain (Nguyen et al., 2007). Therefore, it is necessary to determine new therapeutic targets according to the mechanism of corneal transplantation rejection.

Allograft failure is mainly caused by immune-mediated graft destruction, a complex and highly coordinated process involving interactions between innate and adaptive immune cells (Ingulli, 2010). The migration of immune cells to the graft site and local lymphoid tissue plays a considerable role in the pathogenesis of post-transplant rejection (Murooka and Mempel, 2012). The innate immune cells involved in the immune rejection reaction in the early stage can induce the subsequent activation of adaptive immune cells. Macrophages are an indispensable antigen presenting cell (APC). After activation, these cells can regulate the clearance of apoptotic neutrophils and promote the activation and proliferation of T cells (Yamada et al., 2005). Activated T cells, especially CD4+T cells, which are important adaptive immune cell, can enhance the migration of innate immune cells (macrophages and neutrophils, etc.), promote the explosion of inflammatory cytokines, and trigger transplant rejection. This reaction indicates that once the adaptive immune response is activated, it can amplify the influx of APCs, triggering a strong hypersensitivity reaction and exacerbating rejection (Slegers et al., 2004).

The NOD-like receptor protein (NLRP3) inflammasome is a protein complex composed of NLRP3, apoptosis-related speckle-like protein (ASC) and caspase-1. It promotes interleukin (IL-1β) secretion and release of IL-18, which in turn triggers an inflammatory response and induces pyroptotic cell death (Weigt et al., 2017). Cytokines derived from macrophages and treated with caspase 1 can activate the adaptive immune response in mice. The activation of these cytokines that cause a series of triggering responses requires NLRP3 (also confirmed in serum samples of patients with pancreatitis) (Sendler et al., 2019). NLRP3 promotes CD4+T cell activation and macrophage differentiation during transplant rejection, induces inflammatory chemokine secretion, and exacerbates immune-mediated rejection injury (Wei et al.; Tian et al., 2021). Therefore, NLRP3, as an important regulator of adaptive immunity, may be a potential therapeutic target.

Melatonin (MT), also known as N-acetyl-1-5-methoxychromatamine, is a neural hormone that mainly acts on circadian rhythms; however, it also has immunomodulatory, antioxidant and anti-inflammatory properties (Fildes et al., 2010; Esteban-Zubero et al., 2016). Many *in vitro* and *in vivo* studies have shown that MT inhibits NLRP3 inflammasome activity *via* various intracellular signaling pathways (Ehirli et al., 2021). Melatonin exerts a protective effect against acute lung injury by negatively regulating the NLRP3 inflammasome in macrophages (Ma et al., 2018). Furthermore, MT suppresses T cell cytokine production and NLRP3 inflammasome activation and ameliorates airway inflammation (Wu et al., 2019). However, it is not clear whether MT plays a role in regulating the innate and adaptive immune responses during corneal graft rejection.

Our study aimed to further demonstrate that MT inhibits NLRP3 in a corneal transplantation model, thereby affecting the migration of both innate and adaptive immune cells to the corneal and cervical lymph nodes, as well as the secretion of inflammatory factors and chemokines, thereby modulating the immune response to promote rejection after transplantation.

MATERIALS AND METHODS

Animals

Female C57BL/6 and BALB/c mice (6–8 weeks of age) were used in this study. The animals were placed in a pathogen-free sterile animal room. All experiments were carried out by closely observing the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the approved protocols by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center at Sun Yat-sen University (Ethics approval number: 2021–057).

Mouse Model of Corneal Transplantation and Pharmaceutical Treatments

According to the standard protocol, penetrating allograft corneal transplantation was performed between BALB/C (donor) and C57BL/6 (recipient) mice that were fully mismatched (Tomoyuki et al., 2019). For all animals, 1% pentobarbital sodium (40–50 mg/kg) was intraperitoneally administered as general anesthesia. Briefly, the bilateral central corneas (2 mm diameter) of BALB/C mice were excised and fixed in the central graft beds of the left eyes of C57BL/6 mice, which were prepared using a 1.5 mm trephine (Suzhou Mingren Medical Equipment Co., Ltd., China) with eight interrupted sutures (11–0 nylon, Alcon, United States). Air was used to restore the anterior chamber of the eye, and antibiotic ointment was applied locally after the operation. Recipient mice were randomly assigned to experimental groups, and intraperitoneally administered with 50 mg/kg/day MT (Selleckchem, Houston, TX, United States); 0.15 ml injections were given at 09:00 h (Del Sole et al., 2011) once a day for 9 days. Meanwhile, other recipient mice were administered with physiologic sterile saline (7.5 ml/kg, IP) as a vehicle control.

Clinical Evaluation

Corneal grafts were examined with a slit-lamp microscope every day (n = 8 per group). According to the previously mentioned corneal transplant rejection evaluation criteria, the main analysis aimed to evaluate the degree of corneal opacity, edema, and neovascularization (Reuer et al., 2018). When the opacity score is ≥ 3 and the total score of the initial transparent graft is ≥ 5, it is defined as transplant rejection. Cases of postoperative transplant failure due to technical reasons, such as anterior chamber bleeding or endophthalmitis, were excluded. To improve the accuracy of assessing corneal graft rejection, each animal was observed and evaluated by two experienced inspectors.

Quantitative Real-Time PCR

After grinding the corneas and DLNs tissue (Including submandibular lymph nodes, surperficial cervical lymph nodes, and internal jugular lymph nodes) on an ice box, we

TABLE 1 | Primer sequences of mouse genes for real-time RT-PCR.

MCP-1	
forward primer	TTAAAAACCTGGATCGGAACCAA
reverse primer	GCATTAGCTTCAGATTTACGGGT
MIP-1	
forward primer	TTCTCTGTACCATGACACTCTGC
reverse primer	CGTGGAATCTTCCGGCTGTAG
NLRP3	
forward primer	ATCAACAGGCGAGACCTCTG
reverse primer	GTCCTCCTGGCATACCATAGA
ASC	
forward primer	GACAGTGCAACTGCGAGAAG
reverse primer	CGACTCCAGATAGTAGCTGACAA
IL-1β	
forward primer	TTCAGGCAGGCAGTATCACTC
reverse primer	GAAGGTCCACGGGAAAGACAC
TNF-α	
forward primer	CAGGCGGTGCCTATGTCTC
reverse primer	CGATCACCCCGAAGTTCAGTAG
VEGF-A	
forward primer	GCACATAGAGAGAATGAGCTTCC
reverse primer	CTCCGCTCTGAACAAGGCT
IFN-γ	
forward primer	AGTTCTGGGCTTCTCCTCCT
reverse primer	GGCTTTCAATGACTGTGCCG
IL-17	
forward primer	CCCTCAGACTACCTCAACCG
reverse primer	CATGTGGTGGTCCAGCTTTC
TNF-α	
forward primer	CTTGTTGCCTCCTCTTTTGCTTA
Reverse primer	CTTTATTTCTCTCAATGACCCGTAG
FoxP3	
forward primer	CTCTAGCAGTCCACTTCACCAA
reverse primer	CACCCACCCTCAATACCTCTCT
GAPDH	
forward primer	TGACCTCAACTACATGGTCTACA
reverse primer	CTTCCCATTCTCGGCCTTG

Melatonin attenuates corneal graft rejection.

extracted total RNA from the samples using TRIzol reagent (Invitrogen, Waltham, Massachusetts, United States), and measured the concentration using a spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, Texas, United States). Next, the RNA was reverse-transcribed into cDNA using Reverse transcription reaction with PrimeScript™ RT Master Mix (Perfect Real Time, TaKaRa Bio. Co., Japan), the Kit reverse transcription kit was used to synthesize the first strand of cDNA. Subsequently, we used SYBR Premix Ex TaqII (TaKaRa Bio. Co.) dye method fluorescent quantification reagent to quantify mRNA levels according to the manufacturer's instructions. Finally, the results were analyzed by the 2-ΔΔCt method, and the expression of each gene was normalized based on the expression of GAPDH. The primers were purchased from Invitrogen (Carlsbad, California, United States), and the relevant sequences are shown in **Table 1**.

Assessment of Lymphangiogenesis and Angiogenesis in Flat-Mounted Corneas

We quantified the formation of corneal neovessels with corneal whole mounts. To produce these mounts, mice were sacrificed at a fixed time. The eyeballs were removed, the corneas were blocked with 3% bovine serum albumin in phosphate-buffered saline with 0.3% Triton X-100 for 1 h at room temperature, and then incubated overnight at 4°C with rabbit anti-mouse LYVE-1 monoclonal antibody (1:100; Abcam) or rabbit anti-CD31 (1:50; Abcam) polyclonal antibody. Next, the samples were incubated with Alexa Fluor 488-coupled donkey anti-rabbit antibody (1:800; Abcam) or anti-rabbit IgG Alexa Fluor 555 (cell signaling technology, 1:1,000) for 2 h at room temperature. The cornea was laid flat under a microscope (DMI3000 B; Leica, Wetzlar, Germany). Photos were automatically taken to reconstruct the entire image of the cornea. ImageJ software was then used (NIH, Bethesda, Maryland, United States) to outline the innermost lymph nodes of the limbus to calculate the total area of the cornea, and the area of the newborn lymphatic vessels in each mouse were subsequently calculated (Ren et al., 2020).

Corneal Immunostainings

For the corneal inflammatory cell recruitment test, mouse eyes were embedded in a compound at the optimal cutting temperature, sequentially sectioned (8 μm) and stored at −80°C. Subsequently, the frozen sections were blocked with 3% bovine serum albumin at 37°C for 1 h. Then at 4°C, the frozen sections were inducible rat anti-F4/80 (Abcam, ab66440, 1:100) and rat anti-CD11b (Abcam, ab8878, 1:100) overnight. Sections were then washed with phosphate buffered saline and incubated with anti-rat IgG Alexa Fluor 555 (cell signaling technology, 1:1,000) and anti-rat IgG Alexa Fluor 488 (Cell signaling Technology, Germany, 1:1,000) for 1 h at room temperature, followed by DAPI (Abcam, Ab228549) solution staining. Photographs were taken with a fluorescence microscope (DM 4000B). The number of positive cells was quantified using Adobe Photoshop CC (Adobe Systems, Inc., San Jose, CA, United States) with the method described by et al. (Dai et al., 2018). All histological assessments were performed as blinded studies by the same two observers.

Histology

On the 9th day after corneal transplantation, eyeballs were removed, embedded in paraffin and cut into 5 μm thick sections which were then stained with hematoxylin eosin.

Flow Cytometry

For Treg/Teff cell staining (Yang, et al., 2018), live/dead cells were first labeled with antibodies to distinguish living cells (Waltham, Ma, United States). The antibodies used included; anti mouse CD4 percp-cy5.5, anti-mouse cd45-bv510, anti-mouse IL-17A APC and anti-mouse IFN-γ PE, anti-mouse TNF-α Bv421, anti-mouse Foxp3 FITC (all from BioLegend). For Teff cells, cytokine staining analysis mainly containing Th17 and Th1, the cells mixed with acetate (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich) and brefidine (1 μg/ml; Sigma-Aldrich) were cultured together and placed in 96 well plates with cytokine secretion blockers for 4 h. Stained cells were

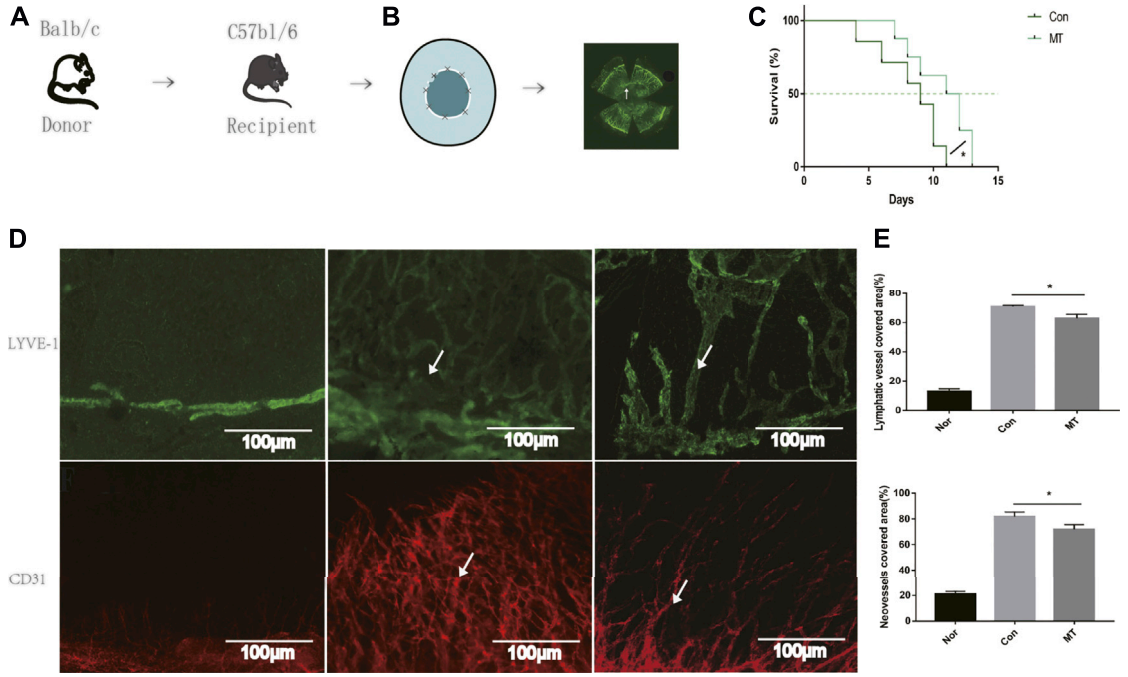

FIGURE 1 | MT therapy alleviates corneal graft rejection and corneal lymphangiogenesis and angiogenesis. **(A)** Establishment of the corneal transplantation model. **(B)** Combined image of all lymphatic vessels reaching the interface 9 days after corneal transplantation (white arrows). **(C)** Postoperative corneal graft survival curve of mice (CT; MT:50 mg/kg). MT injection delayed corneal graft rejection compared to the saline group ($n = 8$ per group). **(D)** On day 9 of transplantation, LYVE-1 and CD31$^+$ staining (white arrows) were performed on the entire cornea of mice. In the normal mouse eye, lymphatics and neovessles are located only at the margin of the cornea and the corneal lymphangiogenesis and angiogenesis developed after transplantation (Scale: 100 μm). **(E)** MT inhibited corneal lymphangiogenesis and angiogenesis ($n = 3$ per group) (Nor: Normal group; Con: control group; MT: treated with MT). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

measured using BD LSR Fortessa flow cytometer (BD Biosciences) and data were obtained and were analyzed with Flowjo 10.0 (Flowjo company, United States).

To Use CCK-8 Kit to Detect Cell Viability

RAW264.7 mouse macrophages were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbacco modified Eagle medium (DMEM). Cells were stored in a 0.2 ml suspension of 2×10^5 cells/well in a 96-well plate, supplemented with 10% FBS and 1% penicillin-streptomycin and treated with melatonin (0.8, 4, 20, 100, 500, and 2500 μM) in a CO2 incubator at 37°C (Kos et al., 2006). After 48 h, 10 μl CCK-8 reagent (Japanese Dojindo) was added to each well and cells were further cultured at 37°C for 1–2 h. The absorbance at 450 nm in each well was measured with a microplate reader (Swedish Famasia). The experiment was repeated three times for each group.

Enzyme-Linked Immunosorbent Assay

To measure the effect of melatonin on macrophages *in vitro*, RAW264.7 cells were stimulated with 100 ng/ml LPS for 4 h and then incubated with different concentrations of melatonin (0.8, 4, 20, 100, 500, and 2,500 μM). In addition, cell supernatants were collected at specific time points and stored at −80°C. The ELISA kit (Invitrogen) was used to measure the concentration of inflammation-related cytokines. All samples were analyzed in triplicate and measured at 450 nm wavelength.

Statistical Analysis

The data in this study were expressed as mean ± SEM using GraphPad Prism software (GraphPad Software, La Jolla, CA, United States). The survival probability was estimated using Kaplan-Meier survival curve and evaluated using logarithmic rank test. Student's t-test, Mann–Whitney test, and one-way analysis of variance (ANOVA) were used to compare differences between groups. Statistical significance was set as $p < 0.05$.

RESULTS

MT Treatment Prolonged Corneal Allograft Survival

Two of the eight allografts remained non-rejected in the MT group, whereas none of the eight allografts survived in mice treated with physiological saline. The average time of transplant rejection in the control group was 8.28 ± 0.94 days, while that in the MT-treated group was 10.63 ± 0.82 days. Compared with the control group, intraperitoneal injection of MT delayed immune rejection ($p < 0.05$, **Figures 1A–C**, $n = 8$ per group).

Given that lymphangiogenesis and angiogenesis are critical factors aggravating graft rejection (Hos et al., 2014), in our experiments, we used LYVE-1 (a specific antibody used to label lymphatic endothelial cells) and CD31 (a vascular endothelial cell marker) for immunofluorescence staining of corneal grafts. Lymphatic and

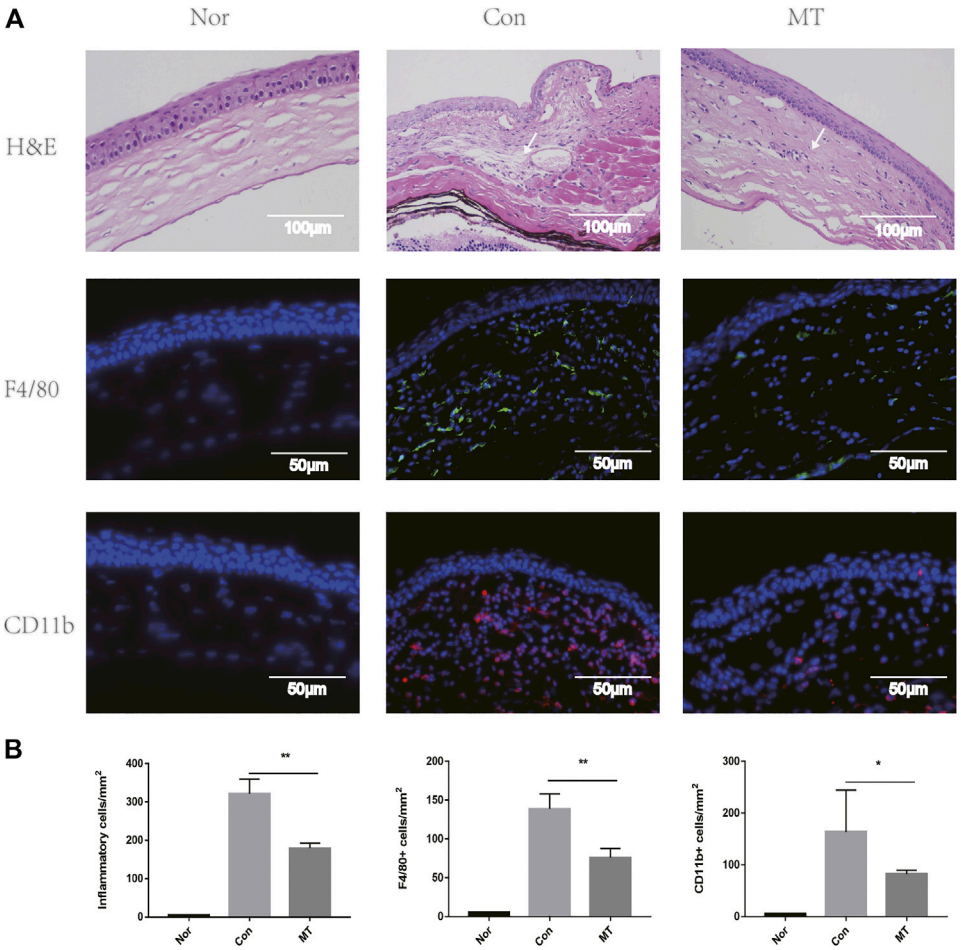

FIGURE 2 | MT inhibits inflammatory cells, F4/80, and CD11b recruitment. **(A)** At day 9 of transplantation, histopathology revealed less inflammatory cell (white arrows) infiltration in corneal grafts in the MT group (representative images for each group). Fluorescence images showing infiltration of F4/80 and CD11b in the grafts of each group at day 9. Corneal sections were stained with immunofluorescent antibodies for positive cells, as shown (Scale: 50 μm). **(B)** Count of positive cells in corneal grafts of each group on day 9 (n = 3 per group). (Nor: Normal group; Con: control group; MT: treated with MT). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Blood vessels have been shown to be located only at the limbus of the cornea in normal mice (Maruyama et al., 2005). On the 9th day after transplantation, immunofluorescence of the whole cornea revealed new lymphatic and blood vessels were present in the cornea beds. Compared with the saline group, the area of corneal lymphatic and corneal neovessels in the MT treated group was reduced ($p < 0.05$, **Figures 1D,E**). The results showed that MT inhibited lymphangiogenesis and angiogenesis after corneal transplantation.

MT Application Inhibited Inflammatory Cells, F4/80+, and CD11b + Positive Cells in Corneas

The corneal grafts were examined and scored using a slit lamp biometric microscope (Zhong et al., 2018). On the 9th day after transplantation, mouse corneal grafts treated with saline showed turbidity and edema. HE staining showed substantial infiltration of immune cells, collagenous fiber disorder, and stromal edema in the saline group, in which grafts were rejected. In contrast, grafts that were not rejected in the MT group showed mild turbidity, few vessels, and edema, while HE staining showed a significant reduction in inflammatory cell infiltration. It has previously been demonstrated that macrophages play an important role in inducing lymphangiogenesis and angiogenesis in a mouse transplantation model (Ji and Sciences, 2012). To further understand the effect of MT on lymphangiogenesis and angiogenesis, we used F4/80, CD11b immunofluorescence staining in the cornea to compare the groups. The number of CD11b [which controls monocyte migration (Zheng et al., 2015)] and F4/80 positive cells increased in corneal graft rejection, and MT treatment attenuated inflammatory cells (**Figure 2**).

MT Inhibits the Expression of Chemokines and Proinflammatory Factors During Allokeratoplasty

Inflammatory cells, such as macrophages, regulate the formation of new blood vessels by releasing large amounts of chemotactic

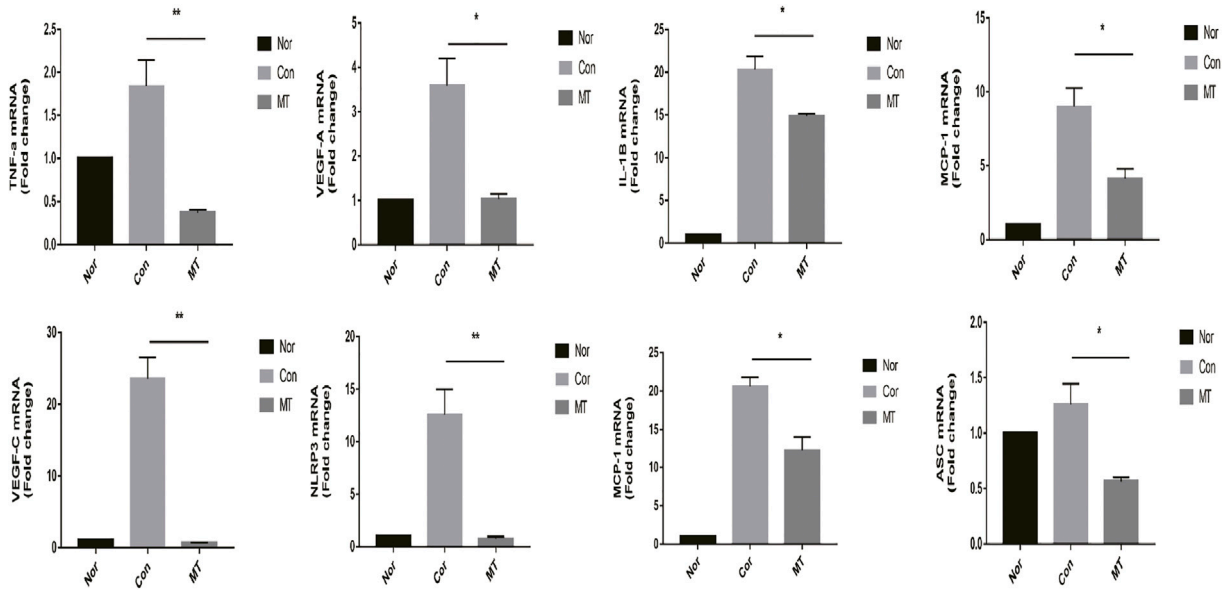

FIGURE 3 | MT inhibits the expression of chemokines and proinflammatory factors in the cornea. On the 9th day after surgery, the mRNA expression levels of relevant cytokines in the grafts were determined by quantitative RT-PCR ($n = 4$ per group). (Nor: Normal group; Con: control group; MT: treated with MT). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and pro-inflammatory cytokines (Wu et al., 2012). Melatonin displays anti-inflammatory and antioxidant properties that can influence tissue growth and apoptosis. Studies have shown that MT reduces organ and species dependence and decreases graft rejection. Therefore, we studied the effect of MT on the expression of chemokines and pro-inflammatory factors in transplant rejection, and further explored the mechanism of MT in transplant rejection. Our results showed the expression levels of chemokines, including MIP-1 and MCP-1, pro-inflammatory cytokines (IL-1β and TNF-α), NLRP3, VEGF-A, and ASC, were up-regulated in corneal transplants (**Figure 3**).

MT Regulates Treg/Teff Balance in the Body

Effector T cells, including Th1 and Th17 cells, play a major role in corneal graft rejection. Therefore, we evaluated the effect of MT treatment on Treg/Teff immune cell balance *in vivo*. In animal models of postoperative graft rejection, flow cytometry showed that MT increased the frequency and number of Tregs in DLNs (**Figures 4A–G**). Meanwhile, MT reduced the frequency and number of Th1/Th17 cells compared with the control group, as shown in (**Figures 4B–H**).

MT Inhibits the Expression of Chemokines and Pro-Inflammatory Factors in RAW264.7 Cells *in vitro*

We measured the viability of RAW264.7 cells after MT intervention with the CCK8 assay. The cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and were treated with a gradient concentration of MT for 4 h, and then subsequently treated with 100 ng/ml LPS for 24 h. High concentrations of MT affected the viability of RAW264.7 cells ($p < 0.05$, **Figure 5B**); However, most macrophages, present at low concentrations, survived.

RAW264.7 cells were incubated with MT for 6 h and subsequently treated with LPS for 4 h; the expression of chemokines and proinflammatory factors was then measured. The qPCR and ELISA results showed that melatonin (500 mM) could inhibit the expression of inflammatory cytokine mRNA and protein ($p < 0.05$, **Figures 5A,C**).

DISCUSSION

In order to explore a more effective and safe treatment for transplant rejection, we used MT to treat corneal transplant mice to study its potential therapeutic effects and underlying mechanisms. During these experiments, the mice were intraperitoneally injected with 50 mg/kg MT, as described previously (Chang et al., 2016). Our results are the first to demonstrate that MT promotes corneal allograft survival. This conclusion was based on the following observations. First, MT treatment decreased the number of macrophages *in vivo and in vitro*, and reduced the transcription level of inflammatory cytokines. Second, MT could prolong the survival time of experimental mouse corneal transplant rejection, reduce lymphangiogenesis and corneal edema, and inhibit inflammatory cell infiltration. Finally, MT could regulate the balance of Treg/Teff in the draining lymph nodes of mice.

The inflammation is considered to be a key participant in acute and chronic allograft rejection. In the completely mismatched heart transplant model, compared with the

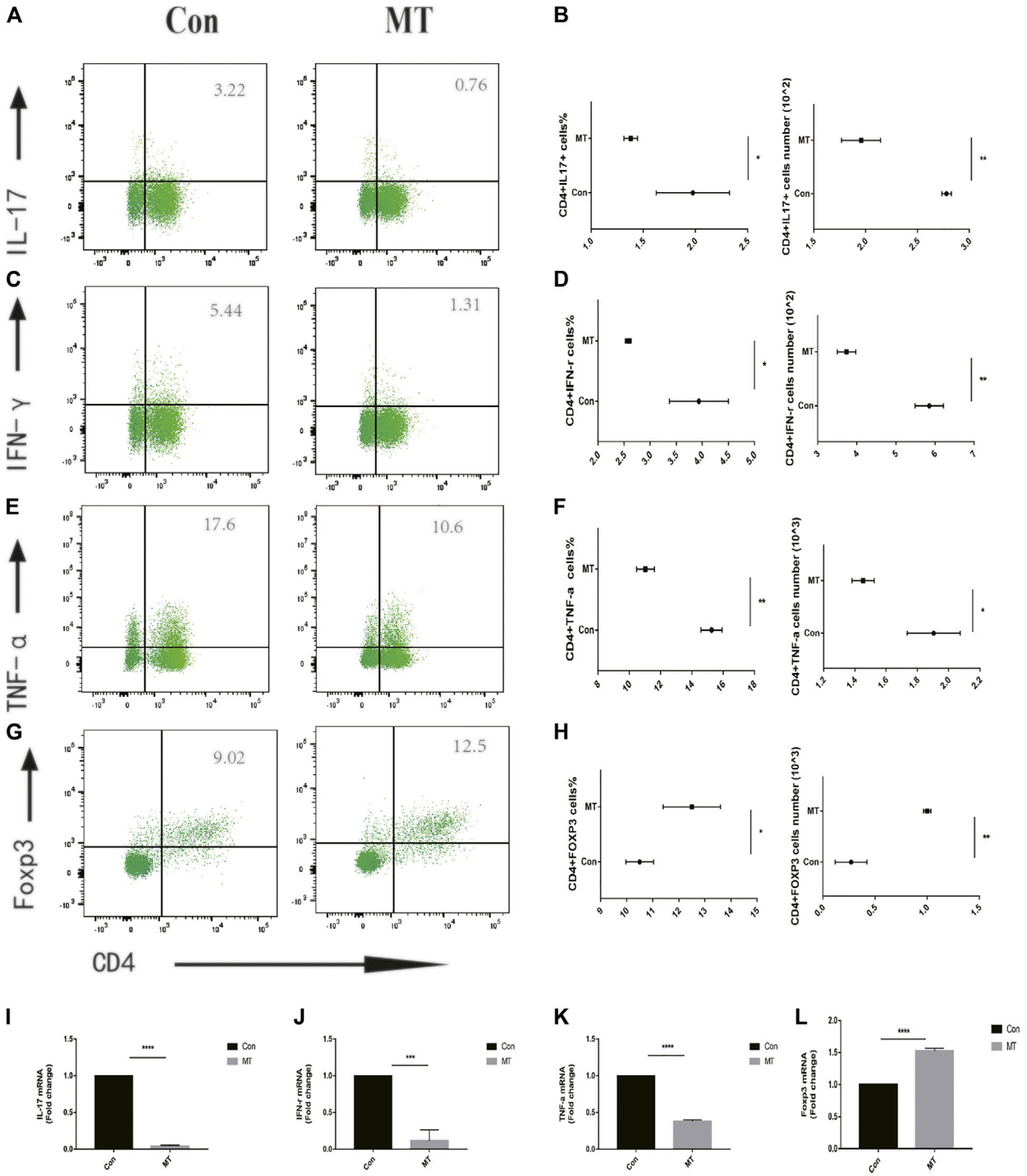

FIGURE 4 | MT regulates Teff/Treg balance in corneal graft model mice. **(A–F)** On the 9th day after operation, the levels of Teff/Treg cells in DLNs were measured by flow cytometry. MT was found to inhibit the proportion of Th1/Th17 cells in DLNs ($n = 6$ per group). **(G–H)**, while treatment increased the proportion of Treg cells in DLNs ($n = 6$ per group). **(I–L)** Real-time quantitative PCR was used to measure the expression level of inflammatory related mRNA. MT inhibited the mRNA expression of IL-17, IFN-γ, TNF-α, and Foxp3 ($n = 4$ per group). (Con: control group; MT: treated with MT). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

allograft control group, the heart allograft with rejection in the grafts, the expression of ASC increased significantly, and this expression pattern mimicked the expression of IL-1β (Seto et al., 2014). In a retrospective study of 1,271 kidney transplant patients, NLRP3 inflammasomes were significantly positively correlated with the risk of acute rejection (Dessing et al., 2016). These studies

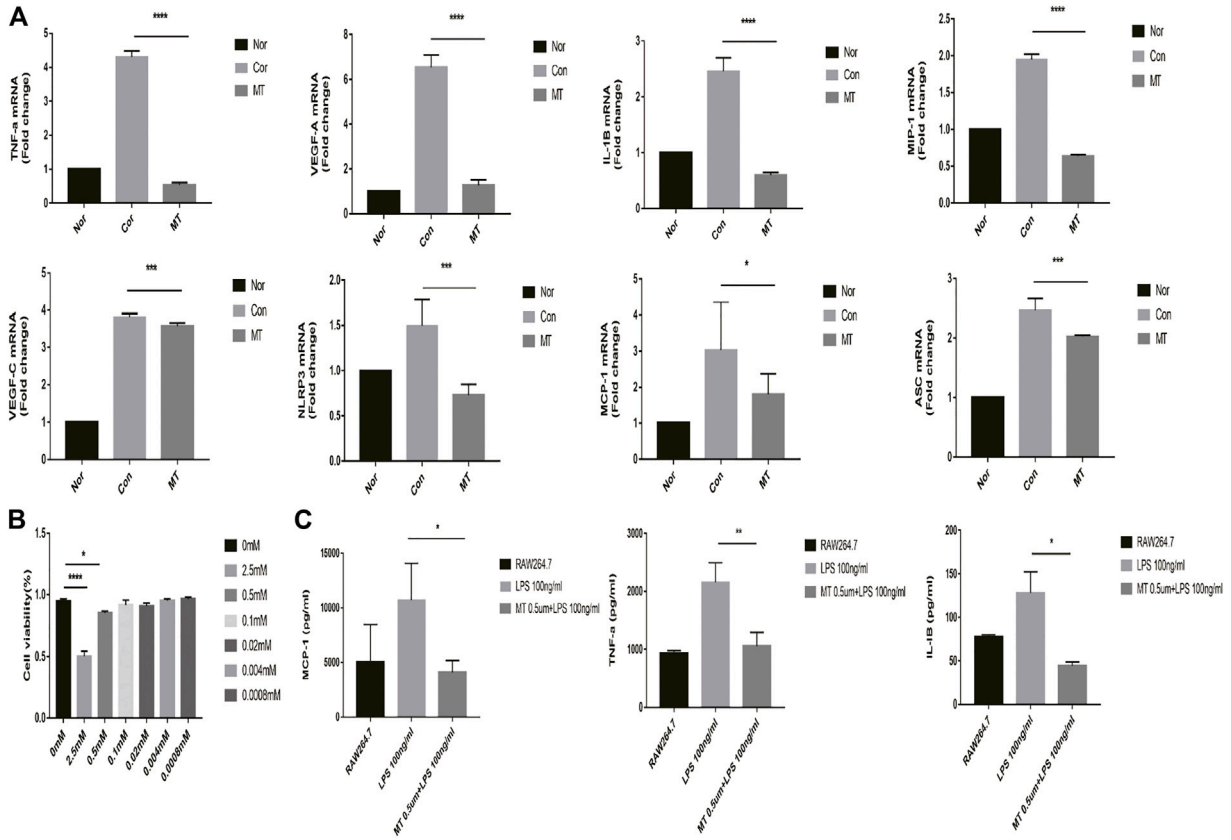

FIGURE 5 | MT inhibits the expression of inflammatory cytokines in RAW264.7 macrophages. **(A)** mRNA expression of inflammatory cytokines was measured by real-time quantitative PCR (n = 4 per group). **(B)** The cell viability was determined by CCK8 assay (n = 4 per group). **(C)** Protein expression of inflammatory cytokines in culture supernatants was measured by ELISA (n = 4 per group). (Nor: Normal group; Con: control group; MT: treated with MT). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

indicate that inflammasomes may represent a key target to reduce the incidence of acute rejection. NLRP3 plays a key role as a possible target for transplant rejection intervention and reduces the rate of dysfunction of chronic allografts, and thus provides a potential clinical treatment strategy for corneal transplantation. In this study, MT was found to inhibit NLRP3 inflammasome activation. MT acts as an effective anti-inflammatory agent to treat whole joint replacement by inhibition of NLRP3 (Wu et al., 2021).

The innate immune system is the first barrier to the immune defense (Ploeger et al., 2013). Activated macrophages are inflammatory cells with antigen presentation and costimulatory functions, and the role of the APC, expressed by macrophages in corneal graft immunity, mainly includes interactions with adaptive immune cells such as T cells, triggering a cascade and postcorneal graft rejection (Oh et al., 2013). In addition, recent studies have shown that immune-rejected corneal grafts show a high number of inflammatory macrophages, which are involved in the initial stages of transplantation rejection, and which are scarce in non-rejected grafts (Oh et al., 2013). It has been demonstrated that removal of rat macrophages can completely prevent corneal transplant rejection during a 3-month follow-up period (Veen et al., 1994). A previous study showed that targeting small molecules to inhibit the NLRP3 inflammasome secreted by macrophages can significantly improve the rejection of mouse skin allografts (Amouzegar et al., 2016). In this study, we investigated the possible regulatory effect of MT on the NOD-like receptor protein 3 (NLRP3) inflammasome-mediated release of IL-1β from macrophages. We found that macrophage activity was down-regulated in the MT-administered group. It is generally believed that the autophagy mechanism induced by LPS is closely related to the activation of NLRP3 inflammasomes (Tian et al., 2021). In addition, we showed in the inflammatory macrophage model that different concentrations of MT can have the same inhibitory effect on macrophage activation in cells *in vitro*. Our *in vitro* study showed that MT upregulated IL-1 secretion by blocking the NLRP3 inflammasome activation.

T cells, located in the DLNs of the transplant recipient, are activated by antigen-presenting cells, a mechanism which is characteristic of organ rejection (Wieland and Shipkova, 2015). Immune imbalance between Tregs and Teffs (Tahvildari et al., 2016) has also been shown to aggravate transplant rejection (Zhang et al., 2016). Teffs are a type of pro-inflammatory T cell, predominantly encapsulating Th1/Th17 cells. The increase in the number of Teff cells in corneal transplant patients

exacerbates the progression of immune rejection (Yin et al., 2015). Treg cells exhibit protective and anti-inflammatory effects, and transplant rejection in corneal transplant patients has been suggested to be related to the decrease in the frequency and abundance of Tregs (Bian et al., 2021). A recent study found that activation of the NLRP3 inflammasome exacerbates corneal graft rejection through subsequent T cell imbalance (Wei et al., 2020). NLRP3 expression promotes monocyte differentiation, and activated macrophages further secrete MCP-1, recruiting more monocytes and/or macrophages to the site of wound and further activating T cells. In our study, MT inhibited T cell differentiation, promoted Treg polarization, weakened Teff cell polarization, and reduced NLRP3 expression of mouse transplanted corneas. Our results suggest that MT regulates the transition from innate to adaptive immune responses in transplant rejection.

Lymphangiogenesis plays a key role in the immune response, and newly formed lymphatic vessels increase the risk of graft rejection after subsequent corneal transplantation (Jiang et al., 2004). In previous studies, we discovered a parallel development of corneal angiogenesis and lymphangiogenesis after keratoplasty and corneal lymphatic vessels might play a more important role than blood vessels in allograft rejection. On the one hand, corneal lymphangiogenesis enables the exit of antigenic material, antigen-presenting cells (APCs) and so on from the graft to the regional lymph node, and accelerate the rejection, which result in corneal edema. On the other hand, corneal lymphatic vessels are conducive to the reabsorption of tissue fluid and reduce corneal edema. In our experiment, we found that corneal lymphatics decreased and corneal edema also decreased. We concluded that this was because the effect of corneal lymphatics mediated immune rejection was much greater than its function of absorbing tissue fluid.

In the current study, we observed decreased corneal lymphangiogenesis in MT-treated mice, which could have resulted in an increase in allograft survival. Recently, it has been demonstrated that corneal lymphangiogenesis is induced by macrophages infiltrating the cornea. Depletion of macrophages inhibits corneal lymphangiogenesis and corneal graft rejection (Maruyama et al., 2005). Moreover, studies have shown that among tumor-infiltrating immune cells, macrophages promote lymphangiogenesis via NLRP3-dependent IL-1β secretion (Benjamin Weichand et al., 2017). Our work showed that MT inhibits NLRP3-dependent IL-1β secretion by macrophages, which might be partially responsible for the decrease in corneal lymphangiogenesis in MT treatment mice.

In this experiment, BALB/C mice were selected as the donor and C57BL/6 as the recipient. We observed more serious inflammation and anterior adhesion and higher rejection rate of the recipient than in previous experiments. In the study, two of the eight allografts remained non-rejected in the MT group, whereas none of the eight allografts survived in mice treated with physiological saline, which means that the graft rejection rate of this corneal transplantation model is 100%. Relevant studies have shown that the number of spontaneously formed lymphatic vessels and activated macrophages is significantly higher in C57BL/6 cornea than in the BALB/C cornea, both of which are risk factors for rejection (Wei et al., 2020). In our study, we found both corneal lymphangiogenesis and angiogenesis occurred in normal C57BL/6 mice corneas (**Figure 1D**). Therefore, corneal transplantation performed on C57BL/6 "cornea bed" is regarded as a "high-risk" model of corneal transplantation.

Our research is limited by the following facts: due to the lack of experimental samples, we lack the control group of autologous syngeneic corneal transplantation. In addition, we need to further explore the optimal therapeutic concentration and treatment mode of MT, as well as toxic and side effects. Further studies await to elucidate it.

Based on the above experimental data, we conclude that MT inhibits the activation of NLRP3 inflammasomes to reduce corneal transplant rejection, and that this effect is related to macrophage suppression, T cell regulation, and the reduction in corneal lymphangiogenesis. In summary, MT may be a promising treatment for individuals who undergo corneal transplants.

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 All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China (Ethics approval number: 2021–057).

AUTHOR CONTRIBUTIONS

SL defined the research theme. TW and RP designed the methods. TW and ZZ interpreted the results. ZZ, HS, SS, SL, LZ, and RP analyzed the data.

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Anaphylaxis Associated With Allergen Specific Immunotherapy, Omalizumab, and Dupilumab: A Real World Study Based on the US Food and Drug Administration Adverse Event Reporting System

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Background: Real-world studies on the allergen specific immunotherapy (AIT), omalizumab, and dupilumab associated anaphylactic events are limited. We aimed to analyze the characteristics of drug associated anaphylaxis, and to compare the differences among different drugs.

Methods: A disproportionality analysis and Bayesian analysis were used in data mining to identify suspected anaphylaxis associated with AIT, omalizumab, and dupilumab based on the Food and Drug Administration (FDA) Adverse Event Reporting System (FAERS) from January 2004 to March 2021. Demographic information, time interval to onset, and death rates of AIT, omalizumab, and dupilumab associated anaphylaxis were also analyzed.

Results: Totally 9,969 anaphylactic events were identified. Reports of AIT, omalizumab, and dupilumab associated anaphylactic events were 64, 7,784, and 2,121, respectively. AIT had a high reporting odds ratio (ROR) of 5.03 [95%confidental interval (CI) 3.69–6.85], followed by omalizumab (ROR 2.24, 95% CI 2.18–2.29), and dupilumab had a negative signal for anaphylaxis. In children, most anaphylactic reactions (68%) were reported in the 12–17-year-old group. More reports of anaphylaxis related to AIT were in boys (73%), while more reports of anaphylaxis related to omalizumab (63%) and dupilumab (58%) were in girls. Most symptoms occurred on the day of drug initiation. The death rate of AIT related anaphylaxis was the lowest (0%), the death rate of omalizumab was 0.87%, while the death rate of dupilumab was 4.76%. No significant differences were observed among these drugs.

Conclusion: AIT and omalizumab had a positive signal for anaphylaxis, while dupilumab had a negative signal for anaphylaxis. Patients should be strictly monitored after

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administration of AIT and also biologics. It also gives us a suggestion for choosing a combined biologics with AIT when the risk of anaphylaxis was considered.

Keywords: anaphylaxis, allergen specific immunotherapy, omalizumab (xolair), dupilumab, FDA adverse event reporting system (FAERS)

INTRODUCTION

Allergic diseases including allergic rhinitis (AR) and asthma are becoming a worldwide chronic health problem in recent years (Brożek et al., 2017). Treatment of AR and asthma includes avoidance of allergens, drugs to control symptoms, allergen immunotherapy (AIT), and recently biologics (Roberts et al., 2018; Global Initiative for Asthma, 2020). AIT is an effective method for allergic diseases, with a history of 110 years since the first use in 1911. AIT can alleviate symptoms, change the allergic march, and still has a long-term effect when the treatment finished (Roberts et al., 2018). The emergence of biologics has provided a promising targeted therapy for asthma patients. These therapies have been shown to reduce asthma exacerbations and improve quality of life in appropriate patients (McGregor et al., 2019). With the approval of biologics such as omalizumab, dupilumab, benralizumab, mepolizumab, and reslizumab, they are more and more commonly used in patients with asthma or other allergic diseases. And the combination of biologics and AIT has been already explored in clinical practice.

However, although very rare, adverse effects especially anaphylaxis of AIT is still the problem that we face (Ryan et al., 2018). Bernstein et al. showed that the estimated frequency of very severe allergic reactions of SCIT was 1 in 2.5 million injection visits (Bernstein et al., 2004). Anaphylaxis in patients receiving omalizumab and reslizumab is also reported by post-marketing surveillance, which ranges from 0.1 to 0.3% (Harrison et al., 2015; Cazzola et al., 2018).

Studies about anaphylaxis related to AIT and biologics in the real world are insufficient. As only omalizumab and dupilumab are available in China, we aimed in this study to analyze the anaphylactic reactions related to AIT, omalizumab, and dupilumab based on the US Food and Drug Administration Adverse Event Reporting System (FAERS).

MATERIALS AND METHODS

Data Source

Using the FAERS database, a retrospective pharmacovigilance study was conducted from January 2004 to March 2021. The FAERS database is a public, voluntary, spontaneous reporting system (SRS) which contains adverse drug events and medication error reports submitted by health professionals, patients, and manufacturers from the United States and other countries. Seven types of datasets are included in the FAERS data files. It comprises patient demographic and administrative information (DEMO), drug information (DRUG), adverse events (REAC), patient outcomes (OUTC), report sources (RPSR), therapy start dates and end dates for reported drugs (THER), and indications for drug administration (INDI).

A total of 15,870,538 reports were got from the FAERS database. Then duplicated records were excluded according to the FDA recommendations. If the CASEIDs (number for identifying a FAERS case) were the same, the latest FDA_DT (date FDA received case) was selected. If the CASEID and FDA_DT were the same, the higher PRIMARYID (unique number for identifying a FAERS report) was selected. The final number was 9,969 (**Figure 1**). This study was approved by the institutional review board (IRB) of our hospital.

Adverse Event and Drug Identification

According to Medical Dictionary for Regulatory Activities (MedDRA, version 22.1) at the Preferred Term level, anaphylactic symptoms were chosen from the REAC files. We considered the following preferred terms as related to anaphylactic symptom, especially in the scenario when AIT, omalizumab, and dupilumab were administered: "anaphylactic reaction (10002198)", "anaphylaxis (10002218)", "wheezing (10047924)", "dyspnea (10013963)", "cough (10011224)", "respiratory distress (10038687)", "hypoxemia (10021142)", "stridor (10042241)", "dysphonia (10013952)", "throat tightness (10043528)", "pharyngeal swelling (10082270)", "abdominal pain (10000081)", "vomiting (10047700)", "diarrhea (10012727)", "hypotension (10021097)", "syncope (10042772)", "loss of consciousness (10024855)", "incontinence (10021639)", "blood pressure decreased (10005734)", with/without urticaria.

The AIT (including both subcutaneous immunotherapy and sublingual immunotherapy), omalizumab, and dupilumab's generic and brand names were selected using IBM Micromedex as the dictionary during the data mining process.

Data Mining

Depended on the primary principles of the Bayesian analysis and non-proportional analysis, the reporting odds ratio (ROR), proportional reporting ratio (PRR), Bayesian confidence propagation neural network, and multi-item gamma Poisson shrinker algorithms was adopted to identify the relation between the drug and the selected adverse events. The equations and criteria for each of the four algorithms are shown in **Table 1**(Evans et al., 2001; Szarfman et al., 2002; van Puijenbroek et al., 2002; Hauben, 2003; Hauben et al., 2005; Norén et al., 2006; Ooba and Kubota, 2010; Szumilas, 2010). We compared the association between anaphylactic reactions and different drugs. The given drug was considered as "primary suspect" in the ROLE_COD (code for the drug's reported role in event) field of the DRUG files.

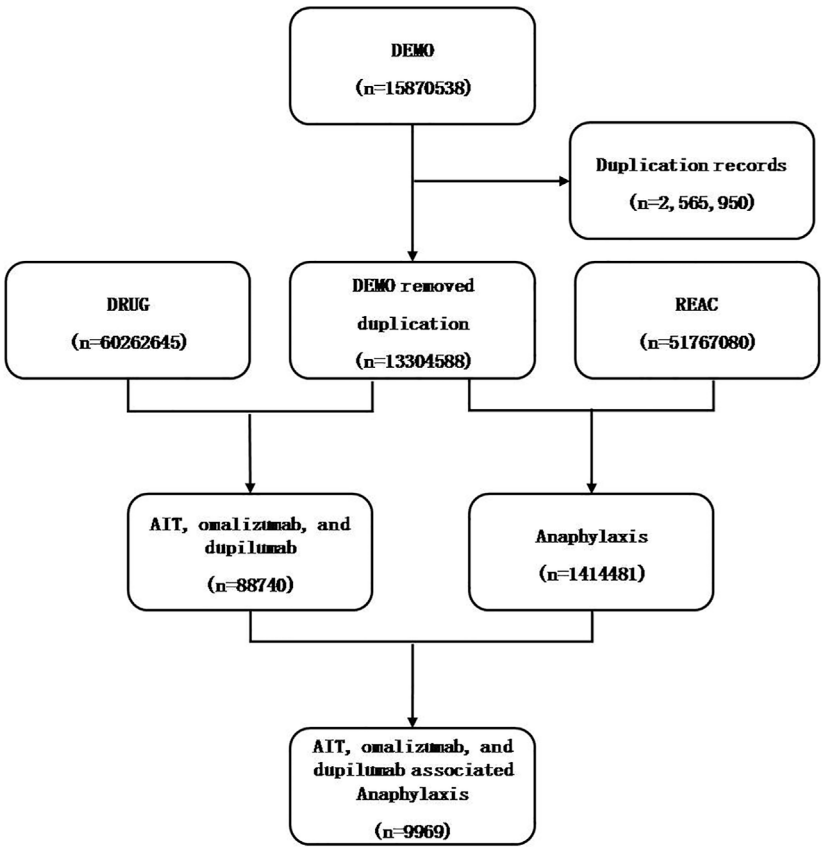

FIGURE 1 | Flowchart of data mining process of anaphylaxis related to AIT, omalizumab, and dupilumab.

TABLE 1 | Summary of major algorithms used for signal detection.

Algorithms	Equation[a]	Criteria
ROR	ROR = (a/b)/(c/d) 95% CI = $e^{\ln(ROR)\pm1.96(1/a+1/b+1/c+1/d)0.5}$	95% CI > 1, N ≥ 2
PRR	PRR = [a/(a + c)]/[b/(b + d)] $\chi^2 = \Sigma$ [(O-E)2/E]; [O = a, E = (a + b) (a + c)/(a + b + c + d)]	PRR ≥ 2, $\chi^2 \geq 4$, N ≥ 3
BCPNN	IC = $\log_2$a (a + b + c + d)/[(a + c) (a + b)] IC025 = $e^{\ln(IC)-1.96(1/a\ +\ 1/b\ +\ 1/c\ +\ 1/d)0.5}$	IC025 > 0
MGPS	EBGM = a (a + b + c + d)/[(a + c) (a + b)] EBGM05 = $e^{\ln(EBGM)-1.64\ (1/a+1/b+1/c+1/d)0.5}$	EBGM05 > 2, N > 0

[a]*a: number of reports containing both the suspect drug and the suspect adverse drug reaction. b: number of reports containing the suspect adverse drug reaction with other medications (except the drug of interest). c: number of reports containing the suspect drug with other adverse drug reactions (except the event of interest). d: number of reports containing other medications and other adverse drug reactions. Abbreviations: ROR, reporting odds ratio; CI, confidence interval; N, the number of co-occurrences; PRR, proportional reporting ratio; χ^2, chi-squared; BCPNN, Bayesian confidence propagation neural network; IC, information component; IC025, the lower limit of the 95% two-sided CI of the IC; MGPS, multi-item gamma Poisson shrinker; EBGM, empirical Bayesian geometric mean; EBGM05, the lower 90% one-sided CI of EBGM.*

The time to onset of the anaphylactic reaction for the different kinds of drugs was also estimated. It was defined as the interval between the EVENT_DT (adverse event onset date) and the START_DT (start date of the drugs administration). Records with wrong entry or incorrect inputs (EVETN_DT earlier than START_DT) were removed.

Furthermore, reports with fatal events due to anaphylactic drug reactions and the mortality rate were analyzed.

Statistical Analysis

A descriptive analysis was used to describe the clinical characteristics of the cases with anaphylactic events due to AIT, omalizumab, and dupilumab from the FAERS database. The onset times of drug-associated anaphylactic symptoms among different drugs were compared using non-parametric tests (the Mann-Whitney U-test for dichotomous variables and the Kruskal-Wallis test when there were more than two

TABLE 2 | Demographic characteristics of cases with AIT, omalizumab, and dupilumab associated anaphylaxis.

Characteristics	Reports (n)			
	AIT	Omalizumab	Dupilumab	Total
Age (years)				
<18	15	507	134	656
≥18	49	7,273	1,987	9,309
Unknown	0	4	0	4
Gender				
Female	34	5,338	881	6,253
Male	26	2,005	474	2,505
Unknown	4	441	766	1,211
Reporter				
Consumer	14	2,959	1,328	4,301
Lawyer	0	3	0	3
Other health-professional	19	1,036	111	1,166
Pharmacist	2	147	33	182
Physician	22	3,326	488	3,836
Unknown	7	313	161	481
Report year				
2004–2009	7	655	0	662
2010–2015	42	2,003	0	2,045
2016–2021	13	5,123	2,121	7,257
Unknown	2	3	0	5
Total	64	7,784	2,121	9,969

AIT: Allergen immunotherapy.

subgroups of respondents). A pearson's chi-squared test or Fisher's exact test was used to compare the outcomes among different kinds of drugs. The statistical significance was set at $p < 0.05$ with 95% confidence intervals. All data mining and statistical analyses were conducted using SPSS, version 16.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

General Characteristics of all Reported Cases With Anaphylaxis Related to AIT, Omalizumab, and Dupilumab

In total, 9,969 reports of anaphylaxis related to AIT, omalizumab, and dupilumab were identified in the FAERS database from January 2004 to March 2021 (Flowchart is shown in **Figure 1**). The number of anaphylactic events related to AIT, omalizumab, and dupilumab were 64, 7,784, and 2,121, respectively. Omalizumab accounted for most of the reports (78%). Omalizumab associated anaphylaxis were more commonly reported in the recent 5 years (66%) than in the previous years. Anaphylactic events were more common in female (71 vs. 29%, $p < 0.01$), and children accounted for 7% of all reports. The demographic characteristics are shown in **Table 2**.

Anaphylactic Events Associated With Different Drugs

Anaphylactic events were screened for AIT, omalizumab, and dupilumab depended on the criteria for the four algorithms (**Table 3**). Among these drugs, AIT had the highest ROR, PRR, information component (IC), and empirical Bayes geometric mean (EBGM), which was considered to be more related to anaphylaxis. Omalizumab showed a relatively lower ROR, while dupilumab had a negative signal for anaphylactic reaction.

General Characteristics of Cases Reporting Anaphylaxis Related to AIT, Omalizumab, and Dupilumab in Children

Among the 9,969 reports of anaphylaxis related to AIT, omalizumab, and dupilumab, 656 (7%) were children (under 18 years old). They were further divided into three age groups as 0–5, 6–11, and 12–17 years. Most anaphylactic reactions were reported in the 12–17 year-old group ($n = 446$, 68%). More reports of anaphylaxis related to AIT were in boys (73%), while more reports of anaphylaxis related to omalizumab (63%) and dupilumab (58%) were in girls (**Table 4**). Asthma was the most common indication for use of omalizumab (69%), followed by chronic spontaneous urticaria (16%). Atopic dermatitis was the most common indication for use of dupilumab (57%), followed by asthma (30%) (**Table 5**).

Time Interval Between Drug Initiation and Anaphylactic Symptoms in Children

Most symptoms occurred on the day of drug initiation, the percentage of anaphylaxis was small at seven and more days after drug initiation (**Figure 2**). The median day from drug initiation to onset of symptoms was 0 [interquartile range (IQR) 0–246] day, 17.5 (IQR 0–156.8) days, and 14 (IQR 0–142) days for AIT, omalizumab, and dupilumab, respectively (**Figure 3**). There was no significant difference among the three drugs ($p > 0.05$).

Prognosis of Cases With AIT, Omalizumab, and Dupilumab Related Anaphylaxis in Children

We further analyzed the prognosis of cases with AIT, omalizumab, and dupilumab related anaphylaxis in children

TABLE 3 | Comparison of anaphylaxis signals associated with AIT, omalizumab, and dupilumab.

	N	ROR (95%CI)	PRR (χ^2)	IC (IC025)	EBGM (EBGM05)
AIT	64	5.03 (3.69, 6.85)	3.52 (129.22)	1.82 (1.33)	3.52 (2.72)
Omalizumab	7,784	2.24 (2.18, 2.29)	1.98 (4,179.78)	0.98 (0.95)	1.97 (1.93)
Dupilumab	2,121	0.36 (0.35, 0.38)	0.38 (2,300.84)	−1.37 (-)	0.39 (0.37)

ROR: reporting odds ratio; CI: confidence interval; PRR: proportional reporting ratio; χ2: chi-squared; IC: information component; IC025: the lower limit of the 95% two-sided CI of the IC; EBGM: empirical Bayesian geometric mean; EBGM05: the lower 90% one-sided CI of EBGM; AIT: allergen immunotherapy.

TABLE 4 | Demographic characteristics of cases with AIT, omalizumab, and dupilumab associated anaphylaxis in children.

Characteristics	Reports (n)			
	AIT	Omalizumab	Dupilumab	Total
Age (years)				
0–5 y	0	22	4	26
6–11 y	6	145	33	184
12–17 y	9	340	97	446
Total	15	507	134	656
Gender				
Girl	4	310	57	371
Boy	11	184	42	237
Unknown	0	13	35	48
Total	15	507	134	656

AIT: Allergen immunotherapy.

TABLE 5 | Indications for the application of AIT, omalizumab, and dupilumab in children.

Indications	Reports (n)			
	AIT	Omalizumab	Dupilumab	Total
Anaphylactic reaction	0	2	0	2
Anti-allergic therapy	1	0	0	1
Asthma	0	347	35	382
Bronchospasm	0	1	0	1
Chronic spontaneous urticaria	0	79	0	79
Atopic Dermatitis	0	3	66	69
Food allergy	0	3	0	3
Hypersensitivity	3	1	0	4
Immune system disorder	1	1	0	2
Immune tolerance induction	3	0	0	3
Mast cell activation syndrome	0	1	0	1
Nasal polyps	0	0	11	11
Obstructive airways disorder	0	1	0	1
Allergic rhinitis	1	1	0	2
Seasonal allergy	2	0	0	2
Sinusitis	0	1	0	1
Skin test	0	1	0	1
Unknown	0	62	4	66
Total	11	504	116	631

AIT: Allergen immunotherapy.

(**Table 6**). Death rate of AIT was the lowest (0), while death rate of dupilumab was the highest (4.76%). However, no differences were observed among the three drugs ($p > 0.05$). Hospitalization (initial or prolonged) rate and life-threatening rate of AIT were higer than omalizumab and dupilumab, differences among the three drugs was not significant ($p > 0.05$).

DISCUSSION

In this study, we conducted a real-world study of anaphylactic events associated with AIT, omalizumab, and dupilumab based on FAERS. The clinical characteristics and outcome of reported cases particularly in children with anaphylaxis were described and ROR of anaphylactic reaction was analyzed. It may reflect the real-world condition in clinical practice. This study will give us more experience for application of these drugs which are usually prescribed in allergic patients.

Among all the reports of anaphylaxis related to AIT, omalizumab, and dupilumab, omalizumab associated anaphylaxis accounted for the most. Omalizumab was approved by FDA in 2003 and was widely used for nearly 20 years. While dupilumab was approved by FDA in March 2017, the using time was shorter than omalizumab. This might be one of the reasons why anaphylactic reports of omalizumab were more than dupilumab.

The anaphylactic symptoms of omalizumab and dupilumab were more common in female than in male. In another study of biologics related anaphylaxis based on FAERS, females made up a large part of reported cases (Li et al., 2021). A report of anaphylaxis associated with omalizumab also revealed a preponderance of female subjects (84%) (Lieberman et al., 2017). This reminded us that female might be a potential risk factor of biologics associated anaphylactic reaction. In children, AIT related anaphylaxis was more common in boys, omalizumab and dupilumab related anaphylaxis was more common in girls. We should be more cautious when boys were

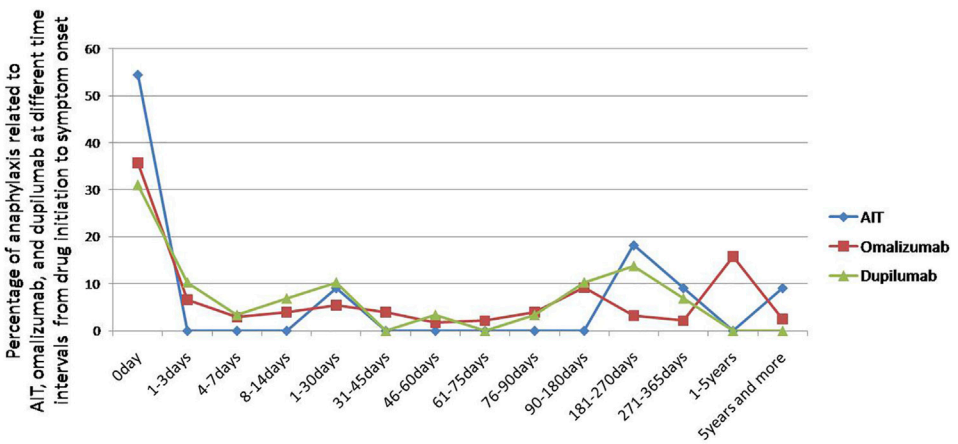

FIGURE 2 | Percentage of anaphylaxis related to AIT, omalizumab, and dupilumab at different time intervals from drug initiation to symptom onset.

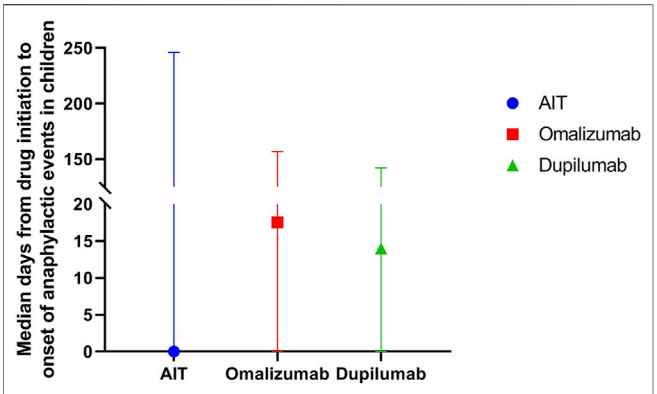

FIGURE 3 | Median days (IQR) from drug initiation to onset of anaphylactic symptoms of AIT, omalizumab, and dupilumab in children.

prescribed of AIT, and when girls were prescribed of omalizumab and dupilumab. However, as the number of anaphylactic reactions of AIT was small, this will be further analyzed in future studies.

The Bayesian analysis and non-proportional analysis showed that AIT had the highest ROR, which was considered to be highly related to anaphylaxis. In a survey of near-fatal immunotherapy reactions from 1990 to 2001 conducted among member practices of the American Academy of Allergy, Asthma and Immunology, fatal reactions was estimated to occur every 1 per 2.5 million injections, with an average of 3.4 deaths per year. Among the fatal deaths, most were asthmatic patients who were not optimally controlled (Bernstein et al., 2004). Then from 2008 to 2012, among 23.3 million injection visits, subcutaneous allergen immunotherapy (SCIT)-related systemic allergic reactions (SRs) remained stable at 0.1% (Epstein et al., 2014). Later, data of 2013 was added and totally 28.9 million injection visits were gathered from 2008 to 2013. The rate of SRs from SCIT was 1.9%, with 0.08 and 0.02% of grade 3 and grade 4 SRs, respectively (Epstein et al., 2016). The newly updated data was from 2008 to 2018, with 64.5 million injection visits gathered. One fatal reaction occurred per 7.2 million injection visits. Ten confirmed fatalities occurred since 2008, including three new fatalities since 2017. SCIT-related fatalities have declined since 2008, with a slight increase in recent years (Epstein et al., 2021). Risk management should focus mainly on patients with uncontrolled asthma, with recent worsening in asthma symptoms and peak expiratory flow rate (Bernstein and Epstein, 2020). According to the above studies, the number of anaphylaxis was more than the number of this study, with the reason that some events might not be reported by the FAERS database. And this was also a limitation of the database.

Omalizumab had a positive signal for anaphylactic reaction and dupilumab had a negative signal for anaphylactic reaction. Omalizumab was reported of an anaphylactic rate of 0.1–0.2% (Cox et al., 2011), and FDA issued a black boxing warning for this drug. In the clinical trial of dupilumab in moderate-to-severe uncontrolled asthma, the most frequent adverse events were injection-site reaction and eosinophilia, and no anaphylaxis was reported (Castro et al., 2018). Reactions are less common with fully human biologics due to their lack of mouse antibody parts. However, immunogencity persists likely due to the use of transgenic mouse cell lines. Humanized biologics has 90% of human component with the generic suffix as "-zumab", and fully human biologics has 99% of human component with the generic suffix as "-umab". The more composition of human component, the lower potential of immunogenicity (Isabwe et al., 2018). Therefore, we should also be careful for these biologics. This study suggested the risk of anaphylaxis might be lower when AIT was combined with dupilumab than with omalizumab. Although biologics-AIT combination therapy is a valuable option treatment to improve both AIT safety and efficacy in widely variable scenarios of clinical risk, it seems that the use of biologics as add-on should be limited to those patients in whom a build-up escalation or maintenance dose can't be tolerated, and where the use of AIT remains mandatory. Clinical trials are also needed to identify target patients, as well as optimal dosing schedules and duration of treatment, and better define cost-effectiveness (Malipiero et al., 2021).

In this study, most anaphylactic symptoms occurred on the day of drug initiation. In another national surveillance study of adverse reactions to allergen immunotherapy, nearly all fatal anaphylactic reactions and SRs occur within 30 min of injections. Delayed-onset SRs beginning over 30 min after injections accounted for 15% of all SRs. Therefore, a 30-min observation period is recommended (Bernstein and Epstein, 2020).

Death rate of AIT, omalizumab, and dupilumab associated anaphylaxis in children was low. Other studies also showed that the fatal reactions to AIT were low (Epstein et al., 2021). Death rate of omalizumab associated anaphylaxis was a little lower than that of dupilumab associated anaphylaxis, which was consistent with another real-world study about anaphylaxis related to biologis (Li et al., 2021). In a systematic review for the EAACI guidelines of recommendations on the use of biologics in severe asthma, omalizumab might increase serious adverse events in

TABLE 6 | Clinical outcome of cases with AIT, omalizumab, and dupilumab related anaphylaxis in children.

Outcome	Reports (n, %)		
	AIT	Omalizumab	Dupilumab
Death	0 (0.00)	4 (0.87)	2 (4.76)
Disability	0 (0.00)	4 (0.87)	1 (2.38)
Hospitalization-initial or prolonged	4 (33.3)	114 (24.68)	7 (16.67)
Life-threatening	1 (8.33)	22 (4.76)	0 (0.00)
Other serious (important medical event)	6 (50.00)	316 (68.40)	32 (76.19)
Required intervention to prevent permanent impairment/damage	1 (8.33)	2 (0.43)	0 (0.00)

AIT: Allergen immunotherapy.

adolescent/adults (Risk ratio 1.62, 95%CI 0.76–3.45) with low certainty of evidence. No drug-related serious adverse events were reported for children of 6–11 years old (Agache et al., 2020).

This study also had some limitations. First, the total number of patients who received the treatments was unknown, therefore the rate of anaphylactic events for suspected drug couldn't be analyzed. Second, the information of the cases reported was incomplete. Types of anaphylaxis couldn't be analyzed. And the underlying diseases of the patients were unknown, which might impact on the outcome results. Third, this database was voluntarily reported by physicians, pharmacist, consumer, etc. Reporting behaviors might be influenced by recent publication of adverse events or FDA warning. These might lead to overestimate or underestimate of the results.

In conclusion, in this real-world study based on FAERS, AIT had the highest ROR for anaphylactic events, followed by omalizumab, and dupilumab had a negative signal for anaphylactic events. As well as AIT, patient should also be strictly monitored after administration of biologics. It also gives us a suggestion for choosing a combined biologics with AIT when the risk of anaphylaxis was considered.

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.

AUTHOR CONTRIBUTIONS

KG and ZC designed and revised the manuscript. SB analyzed data and drafted the manuscript. PZ drafted and revised the manuscript. LL, ZW, LC, and YX revised the manuscript. BZ directed the data mining in the FAERS database and revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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The Trend of Targeted Therapies in Chinese Patients With Ankylosing Spondylitis: Results From a Real-Life Survey

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Introduction: Targeted medication, including mostly biologics and small-molecule chemical drugs, is an important therapy for ankylosing spondylitis (AS). There are still limited data on the preference of different targeted drugs in Chinese AS patients.

Methods: A questionnaire-based cross-sectional study was performed on AS patients from six hospitals in three provinces in South China. Anti-rheumatic diseases' medication history includes the recent and previous usage of biologics or Janus kinase inhibitors (JAKi) in the last complete course of treatment, disease severity, and reasons for targeted-treatment change or preference.

Results: 354 of 366 participants responded to the online survey. The participants' median age was 32 years, with a median of 7.3 years of disease duration; 79.7% were male. 63.6% of them were in the course of biologics or JAKi. Generic ETN is the most widely used and willing-to-use biologic though the proportion of its usage shrunk in the present compared with the past. The choice of original-branded ADA demonstrated an increase in usage. The preference of secukinumab and tofacitinib depicted a quick ascending trend.

Conclusion: TNF-α inhibitors (TNFi) are still the most popular targeted medication for AS in China. Their price influences patients' preferences mostly. The doctor's recommendation is also part of the equation. Rheumatologists should pay more attention to patients' education to formulate targeted therapeutic plans.

Keywords: ankylosing spondylitis, biologics, preference, Chinese, cross-sectional study

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory disease, primarily affecting the spine and sacroiliac joints, eventually leading to the loss of spinal mobility (Haroon et al., 2015; Masters et al., 2009). Targeted medication, including mostly biologics and small-molecule chemical drugs, can inhibit the action of specific types of immune-mediated cells or the binding of proteins which play a

key role in AS development, including pathways such as tumor necrosis factor (TNF)-α, interleukin (IL)-17A, or Janus kinase (JAK) (van der Heijde et al., 2017).

The anti-TNF treatment has been considered a significant advance in managing patients with AS for many years (Braun et al., 2011). Various sources of antibodies have been applied in routine treatment. The different structures of TNF-α inhibitors (TNFi) have presumably similar mechanisms of action (Ritchlin et al., 2009). There are some kinds of TNFi used in clinical practice worldwide; the most classic components that are also becoming more common currently (Moorkens et al., 2020) are recombinant human TNF receptor–IgG Fc fusion protein, for example, etanercept [ETN (Tracey et al., 2008)], human/mouse chimeric antibodies [infliximab (IFX; Tracey et al., 2008)], and fully humanized antibodies [adalimumab (ADA; Burmester et al., 2013) and golimumab (GLB; Tahir and Kavanaugh, 2018)], which are aimed at the TNFR. The market entry of numerous new generics has brought a significant influence on the usage of biologics because of the reduction in costs, along with interchangeability with the originators in quality, safety, and clinical efficacy (Huang et al., 2017); these might move the preference from the original-branded products toward their generic ones (Glintborg et al., 2018).

Currently, the trend of TNFi in Chinese AS patients is still uncertain, let alone how the emerging secukinumab (Blair 2019) and tofacitinib (van der Heijde et al., 2017) aim to block IL-17A and JAK3 and/or JAK1 (van der Heijde et al., 2017), respectively, and influence the market share of TNFi. There are no reported studies yet on the evolving trends in targeted medication use among AS patients in China. Therefore, the present study would provide some information on patients' preferences for different targeted drugs from six representative health centers in South China.

METHODS

Study Design and Study Population

This study was conducted between July and September 2020 in six hospitals from three provinces located in South China, including The Third Affiliated Hospital of Sun Yat-sen University (the principal center); The Third Affiliated Hospital of Sun Yat-sen University, Yuedong Hospital; Dongguan People's Hospital from Guangdong Province; Fujian Provincial Hospital from Fujian Province; Panyu Hospital of Chinese Medicine; and Wuzhou GongRen Hospital from Guangxi Province. The population of interest aged 18 years or older and with a previous diagnosis of AS, who had been recorded in the database from each center, were invited to answer a 10 min online survey. As no treatment (either active or placebo) was administered to the participants in this study, no ethical committee approval was sought.

Questionnaire for Patients

Individuals accepting to participate in the online survey were requested to answer an anonymous questionnaire under rheumatologists' instructions. The questionnaire gathered information on demographic data, time of diagnosis, medication history of anti-rheumatic diseases, disease severity [Bath Ankylosing Spondylitis Disease Activity Index (BASDAI; Garrett et al., 1994)], and reasons for treatment change (see the **Supplementary Material**).

TABLE 1 | Characteristics of the participants.

Characteristics	*N* = 354
Age, median (IQR) (years)	32.0 (27.0; 38.0)
Gender, yes, n (%)	
Male	282 (79.7)
Income	
No salary	125 (35.3)
≤5,000	140 (39.6)
5,000–10,000	58 (16.4)
10,000–20,000	23 (6.5)
≥20,000	8 (2.3)
Disease duration, median (IQR) (years)	7.30 (2.76; 12.51)
Diagnostic delay, median (IQR) (years)	1.09 (0.30; 4.08)
BASDAI, median (IQR)	3.3 (2.3; 4.9)
State of the disease, n (%)	
Inactive, BASDAI < 4	213 (60.2)
Active, BASDAI ≥ 4	141 (39.8)
Extraarticular symptoms, n (%)	
Uveitis	52 (14.7)
Inflammatory bowel disease	54 (15.3)
Sausage-like fingers or toe	8 (2.3)
Enthesitis	86 (24.3)
Biologics' history, n (%)	
Never	39 (11.0)
Withdrawn or changed	90 (25.4)
Being under treatment	225 (63.6)
Recent non-biologic medication, n (%)	
None of any medication	15 (4.2)
NSAIDs	193 (54.5)
cDMARDs	72 (20.3)
Traditional Chinese medication	24 (6.8)

Objectives

The objectives of the present study were targeted medication history of patients and their preference for and reasons for the usage of the above-mentioned drugs. The relevant medication history includes the recent and previous usage of biologics or JAKi in the last complete course of treatment.

Statistical Analysis

The presentation of the data is descriptive. Continuous data are presented as mean ± standard deviation (SD) or median with interquartile range (IQR) as appropriate. Categorical variables are presented as frequency counts with percentages. R (version 3.6.1) was utilized, and statistical significance was assumed at the $p < 0.05$ level. Power analysis will be performed with the "pwr" package. The significance level (1-α) and power (β) will be set as 0.05 and 0.9, respectively.

RESULTS

Baseline Characteristics

The questionnaire recorded the data of 366 patients with AS. The response rate was 96.7% (354/366). The baseline data are

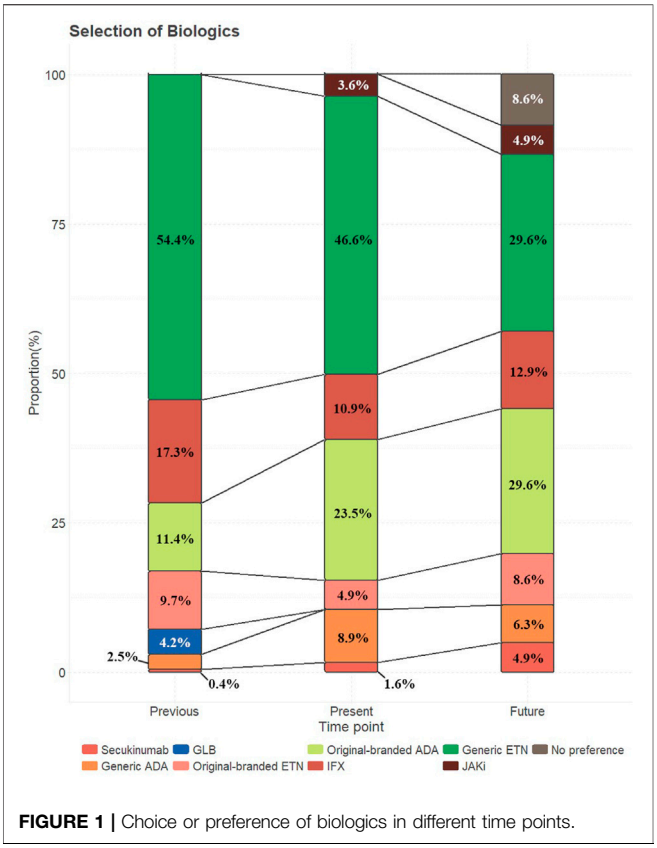

FIGURE 1 | Choice or preference of biologics in different time points.

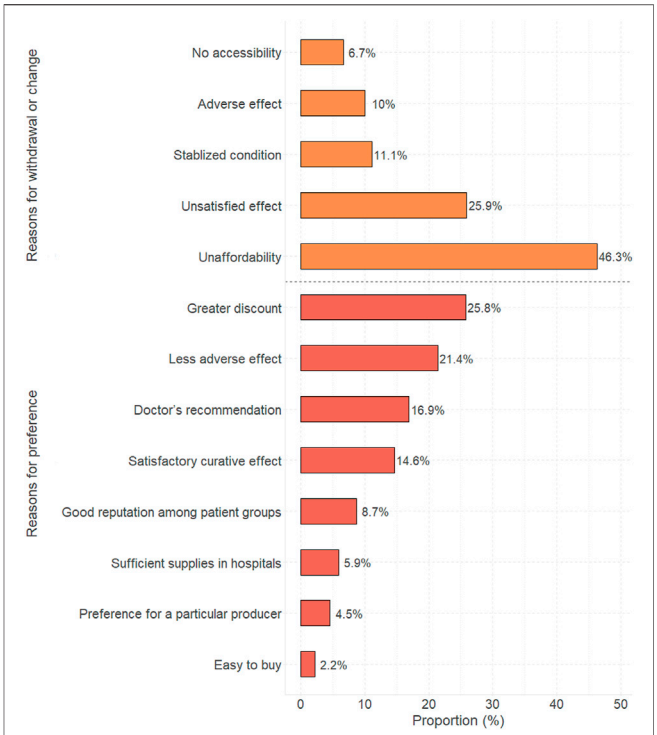

FIGURE 2 | Proportion of reasons for withdrawal/change of or preference for biologics.

shown in **Table 1**. The participants' median age was 32 years, with a median of 7.3 years of disease duration; 79.7% were male. 60.2% of patients were under inactive conditions (BASDAI<4). 63.6% of them were in the course of biologic or JAKi treatment. 39 (11%) reported that they had never used biologics. After power analysis with the effect size (0.298), the PASS software calculated that a total sample size of at least 143 would suffice. Non-steroidal anti-inflammatory drugs (NSAIDs) were the most common non-biologic medication (54.5%). Missing data did not occur since questions in our questionnaire were compulsory.

The Adherence of Biologics' Usage

As shown in **Figure 1**, after excluding patients who never had biologics, generic ETN is the most used and willing-to-use biologic though the proportion of its usage shrunk, from 54.4% of participants in the past to 46.6% in the present and then to 29.6% still preferring to choose it if AS recurred in the future. 8.6% of participants declared that they did not have a specific preference for biologics.

Original-branded ADA demonstrated more than a double increase in the presence and growth stably when it comes to the future. The preference of secukinumab (Blair, 2019) and tofacitinib depicted a similar, quick ascending trend.

Contrary to generic ADA, which had an inverted "U-shape" trend, ETN and IFX showed a "U-shape" one. GLB was used in 10 (4.2%) of participants previously, but none of them continue to use it currently or would choose GLB in the future.

The Reasons for Biologics' Change/ Withdrawal and Preference

After excluding those who had never used biologics, as shown in **Figure 2**, the most mentioned reason for withdrawal or change is "unaffordability" (46.3%). Unsatisfied effect (25.9%) or adverse effect (10%) was counted as the third of all reasons. "Not accessible to the biologics in the primary health center" was chosen by 6.7% of participants who underwent biologics' change/withdrawal. It was shown that some novel biologics are difficult to get in the primary health center so that these patients have to choose another one.

Followed by "less adverse effect" (21.4%), more than a quarter of participants (25.8%) recognized that greater discount (namely, being more affordable) is an important, influential factor when choosing biologics. The doctor's recommendation (16.9%) and "satisfactory curative effect" (14.6%) are also frequently mentioned reasons for preference. The reasons for withdrawal of or preference for these drugs are given in **Supplementary Figures S1, S2**.

DISCUSSION

This study found that most patients with AS in South China preferred to use TNFi over time, whether they were domestically produced generic or original-branded and even if other novel biologics partially occupied their dominance. Among all sorts of TNFi, ETN, IFX, and their domestically generic drugs were still the first choice for most AS patients.

Original-branded ADA demonstrated more than a double increase in the presence and growth stably when it comes to future preference. It has been becoming popular since it was first marketed in China for AS in 2010; however, although domestically generic ADA witnessed a 3.6-fold increase after its marketing in the home in 2019, the patients' present preference for it was still tepid.

Secukinumab (Blair, 2019) and tofacitinib showed a preemptive trend in patients' preference. Secukinumab, which demonstrated a favorable safety profile over long-term treatment in patients with AS and psoriatic arthritis (PsA) (Baraliakos et al., 2020; Deodhar et al., 2019), was approved by the Chinese National Medical Products Administration (NMPA) for treating refractory AS in April 2020 (https://www.novartis.com.cn/news/nuo-hua-ke-shan-ting-si-ku-qi-you-dan-kang-qiang-zhi-xing-ji-zhu-yan-gua-ying-zheng-huo-pi, in Chinese). This biologic has been noted in some participants in our cohort in a short time. Tofacitinib entered the Chinese market in 2017 (http://list.cde.org.cn/index/detail/id/522, in Chinese) as a medication for rheumatoid arthritis (RA) (Blair, 2019). Although there has been a phase II clinical trial that revealed tofacitinib has a satisfactory clinical efficacy for AS (van der Heijde et al., 2017), AS is still not an indication of tofacitinib in China. However, in our study, nine participants reported that they have used tofacitinib in "the present."

Affordability was the primary associated factor for patients' preference. That is the reason why generic ETN is in our study since it was included in the Chinese National Reimbursement Drug List (part B) in 2017, but ETN was not until March 2021 (http://bmfw.www.gov.cn/ybypmlcx/index.html). Contrarily, though ETN and IFX have reduced prices due to the fierce market competition, they are still much more expensive than domestic generics. Therefore, an evident decrease in the presence and ascending preference were observed for original-branded ETN. These U-shape trends might be partly owing to the ongoing, nationwide inclusion of health coverage after 2020. Due to a substantial discount before entering the above-mentioned list in 2020, the preference for original-branded ADA keeps increasing, but not for generic ADA. The unsatisfactory effect could count on the decline (see **Supplementary Figure S1**).

Notably, except for prices and efficiency, the doctor's recommendation and reputation among patients also impact patients' choice, which reflects the inadequate literacy of patients using the targeted medication. Accessibility is becoming less important, presumably due to the rapid development of the domestic logistics industry (http://www.chinawuliu.com.cn/lhhzq/202102/23/541764.shtml, in Chinese).

Overall, our study showed that TNFi are still the most popular targeted medication for AS in China, especially the domestically produced, generic drugs of ETN. The price influences patients' preference mostly, followed by curative efficiency and adverse effects. With more generic biologics rushing in the market and with the original-branded drugs lowering their prices or entering the health coverage, patients would have more active choices. The doctor's recommendation is also part of the equation. Therefore, in clinical practice, rheumatologists should pay more attention to patients' education to formulate targeted therapeutic plans.

There are several limitations of our study. First, we cannot exclude the possibility of patient selection bias because centers that participated in this study were tertiary referrals in South China; thus, results from this study cannot represent the actual situation in China. More large, nationwide surveys assessing the AS patient's preference for targeted medication and reasons may provide us a new perspective for patient-oriented treatment.

Key Messages

What Is Already Known About This Subject?

Targeted medications are an important therapy for ankylosing spondylitis. There are numerous choices of these drugs.

What Does This Study Add?

In this study, we provided some information on the evolving trend of some representative biologics from the perspective of Chinese patients. Moreover, we dug in the reasons for and influential factors of patients' preference for targeted medications.

How Might This Impact on Clinical Practice or Future Developments?

The price influences patients' preference mostly. Also, the doctor's recommendation is also an impact factor. Therefore, in clinical practice, rheumatologists should pay more attention to patients' education to formulate targeted therapeutic plans.

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 studies involving human participants were reviewed and approved by The Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QC and QJ contributed to the conception and design of the study. YW, BX, FY, ZX, and YJ contacted and introduced patients to do online survey and collected data. ZH and YW contributed to the analysis of the data. All authors were involved in the interpretation of the data and reviewed and approved the content before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.763707/full#supplementary-material

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ORIGINAL RESEARCH
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Human Retinal Progenitor Cells Derived Small Extracellular Vesicles Delay Retinal Degeneration: A Paradigm for Cell-free Therapy

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Retinal degeneration is a leading cause of irreversible vision impairment and blindness worldwide. Previous studies indicate that subretinal injection of human retinal progenitor cells (hRPCs) can delay the progression of retinal degeneration, preserve retinal function, and protect photoreceptor cells from death, albeit the mechanism is not well understood. In this study, small extracellular vesicles derived from hRPCs (hRPC-sEVs) were injected into the subretinal space of retinal dystrophic RCS rats. We find that hRPC-sEVs significantly preserve the function of retina and thickness of the outer nuclear layer (ONL), reduce the apoptosis of photoreceptors in the ONL, and suppress the inflammatory response in the retina of RCS rats. *In vitro*, we have shown that hRPC-sEV treatment could significantly reserve the low-glucose preconditioned apoptosis of photoreceptors and reduce the expression of pro-inflammatory cytokines in microglia. Pathway analysis predicted the target genes of hRPC-sEV microRNAs involved in inflammation related biological processes and significantly enriched in processes autophagy, signal release, regulation of neuron death, and cell cycle. Collectively, our study suggests that hRPC-sEVs might be a favorable agent to delay retinal degeneration and highlights as a new paradigm for cell-free therapy.

Keywords: human retinal progenitor cells, photoreceptor cells, microglia, apoptosis, inflammation, retinal degeneration, cell-free therapy, small extracellular vesicles (sEVs)

INTRODUCTION

Retinal degeneration (RD), including retinitis pigmentosa (RP) and age-related macular degeneration (AMD), is characterized by the progressive degeneration of rod and cone photoreceptors, resulting in the gradual loss of vision and eventually blindness (Ferrari et al., 2011; Zhang et al., 2012). This group of retinal disorders shares the same pathological events including apoptosis, autophagy, and necrosis of photoreceptors (Boya et al., 2016). There are no therapies available to neither prevent the gradual loss of vision nor restore the damaged retina. Treatment of RP and AMD is largely an unmet need. Stem cell replacement therapy is a future direction that might provide a cure for RD. It has been reported that human retinal progenitor cells (hRPCs) transplanted into the subretinal space (SRS) of RD rats can maintain visual function (Wang

et al., 2008). Our previous study demonstrates that hRPCs significantly improve the visual acuity of eight RD patients in a 6-month follow-up period after transplantation (Liu et al., 2017). hRPCs could promote the survival of photoreceptors in retinal neurodegenerative diseases that are largely due to the beneficial properties of hRPCs themselves including their ability to migrate and integrate into the damaged host retina and thus provide neuroprotection (Cuenca et al., 2014; Mollick et al., 2016). Although the underlying mechanism is unclear, recent studies have attributed the benefits to the paracrine effect of hRPCs, which mediate the therapeutic function of the mother cells before dividing to daughter cells (Ratajczak et al., 2006; Doorn et al., 2012; Luo et al., 2014; Ma'ayan Semo et al., 2016). Therefore, we speculate that small extracellular vesicles derived from hRPCs (hRPC-sEVs) may be the one that exerts neuroprotective effects in treating RD.

Extracellular vesicles (EVs) are the collective term for the vesicles secreted from eukaryotic cells (Xie et al., 2020). Exosomes/sEVs are phospholipid bilayer-enclosed nanoscale vesicles with size ranging from 30 to 150 nm and carry cell type–specific RNAs, microRNAs (miRNAs), biologically active proteins, and genetic material that play a major role in cell-cell communication (Kalluri and Lebleu, 2020; Ma et al., 2020; Priglinger et al., 2021). A study suggests that human neural progenitor cells protect retinal neurons from dystrophies by producing a multitude of neurotrophic factors (Mohlin et al., 2011). Our latest study demonstrates that the exosomes derived from grafted mouse neural progenitor cells could inhibit microglia activation and reduce inflammation in RD process and thus protect photoreceptors from apoptosis (Bian et al., 2020). The recent analysis reveals that human EVs could be internalized by mouse RPCs and transferred to the nucleus (Zhou et al., 2018). On the basis of the evidence mentioned above, we believe that SEVs derived from RPCs could be used to treat retinal degenerative diseases.

In our study, we inject hRPC-sEVs (20 μg/eye) once into the SRS of RCS rats to investigate the effect on the degenerating retina during the early 4 weeks after injection, especially on photoreceptors and microglia. We have shown that hRPC-sEVs could not only suppress microglia activation but also promote the survival of photoreceptors both *in vivo* and *in vitro*. Moreover, we have analyzed the miRNAs of hRPC-sEVs and predicted target genes that are related to inflammation, processes autophagy, signal release, regulation of neuron death, and cell cycle, which suggest that hRPC-sEVs contain abundant miRNAs to support photoreceptor survival and alleviate neuroinflammation. This study supports a paradigm that a cell-free sEVs-mediated therapy is effective in treating RD diseases.

MATERIALS AND METHODS

Animals

The RCS rats used in this study were raised in the animal facility of the Southwest Eye Hospital, Third Military Medical University. The rats were reared according to a standard 12-h/12-h light/dark cycle. All animal experiments were approved by the Third Military Medical University Animal Care and Use Committee (no. AMUWEC20210132).

Cell Isolation and Culture

Isolation and culture of hRPCs were performed as described in the study (Ma'ayan Semo et al., 2016). Primary hRPCs were isolated from human fetal neuroretina at 12–16 weeks of gestation. The retinas were provided from the embryonic tissue bank in the Department of Obstetrics at the Southwest Hospital, Army Medical University. All experiments involving human cells and tissues are conducted in accordance with the principles of the Declaration of Helsinki; the experiments of human tissues/cells were approved by the Ethics Committee of Southwest Hospital, Army Medical University (no. KY2019109). The neuroretina was enzymatically digested into a cell suspension with TrypLE Express (Gibco, 12604021). The RPCs were seeded in UltraCULTURE medium, supplemented with human epidermal growth factor (20 ng/ml), human basic fibroblast growth factor (10 ng/ml), and 1% penicillin-streptomycin. All cells were incubated in a 37°C 5% CO_2 saturation humidity incubator. At passage 4, hRPCs were plated in T75 flasks for 6 h. The GFP virus was added to the complete medium and poured into hRPC-cultured well. After 24 h of incubation, the medium was replaced with fresh complete medium. GFP-labeled cells were detected with fluorescent microscopy.

For the glucose deprivation experiment, the 661W cells were cultured in six-well plates for 24 h with High-Glucose DMEM (HyClone) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (ScienCell) and were washed with phosphate-buffered saline (PBS) three times, and then, 661W cells were cultured with low-glucose serum-free medium treated with sEVs (60 μg/ml) or vehicle (PBS) as control for 48 h. Cells and supernatant are collected to apoptosis assays or lactate dehydrogenase (LDH) release assay (Emery et al., 2011).

BV2 and HMC3 were both cultured with High-Glucose DMEM (HyClone) with 10% FBS and 1% penicillin-streptomycin. Lipopolysaccharides (LPSs; Sigma-Aldrich, L4516) were supplemented to the medium (ultimate concentration, 1 μg/ml) for 4 h to stimulate microglia (Awada et al., 2014). Microglia were washed with PBS three times and then incubated with or without hRPC-sEVs (60 μg/ml) for 48 h. Afterward, the mRNA and culture media were collected for reverse transcription quantitative PCR (RT-qPCR) and ELISA assays.

Flow Cytometry (FCM)

hRPCs were isolated as single-cell suspensions in TrypLE Express at a concentration of 1×10^6/100 μl and were incubated with conjugated antibodies including PAX6 (BD Biosciences), Nestin (Invitrogen), SOX2 (BD Biosciences), and isotype antibody, as previously described (Schmitt et al., 2009). hRPCs were washed with Wash Buffer and resuspended with 300 μl of PBS and then transferred to a flow cytometry tube for FCM analysis. FlowJo software was used to analyze the data.

Preparation and Characterization of Small EVs and PKH26 Labeling

Cell culture from proliferating hRPCs (passages 3–5) was collected and used for the isolation of sEVs. The culture

supernatant was collected and centrifuged at 1,500 rpm for 10 min and at 2,500 rpm for 15 min at 4°C to remove cell debris. After centrifugation, the cell supernatant was filtered through 0.22-μm pores to eliminate large cellular debris and was ultracentrifuged at 110,000 g for 70 min, and the microspheres were washed with PBS and ultracentrifuged at 110,000 g for 70 min (Clotilde Théry, 2006; Thery et al., 2018). Finally, sEVs were resuspended in PBS for use. The sEVs were observed by transmission electron microscopy (TEM). To perform the nanoparticle tracking analysis (NTA) of sEVs, a NanoSight instrument was used according to the instructions of the manufacturer (NanoSight NS300), as previously described (Noble et al., 2020). Western blot (WB) was used to examine the EVs surface markers of CD9, CD63, and CD81. According to the recommendations of the manufacturer, the sEVs were labeled with PKH26 dye and tracked by fluorescence microscopy (Sigma-Aldrich). Total protein of EVs amount is measured by bicinchoninic acid assay (BCA) (Beyotime). The dose of sEV is 20 μg/eye *in vivo* and 60 μg/ml *in vitro*. To preserve the consistency of the experiment, fresh sEVs are used in our study.

Subretinal Transplantation

To better observe the transplantation, we transfected hRPCs with GFP-lentiviral vectors and labeled hRPC-sEVs with PKH26. Then, 2 μl of concentrated hRPC-sEVs PBS solution (containing 20 μg of protein) were injected into the SRS per eye or 2×10^5 hRPCs both in the temporal direction of the eye of postnatal week 3 RCS rats. The control group was injected with the same amount of PBS solution (n = 5 eye per group). All RCS rats, including vehicle group, received oral cyclosporine A (210 mg/L) (Sandoz, United Kingdom) dissolved in drinking water from day 7 before transplantation to day 14 after transplantation (Park et al., 2019).

Scotopic Electroretinogram Recording

Electroretinogram (ERG) was recorded 1–4 weeks after the operation. RCS rats were dark-adapted for at least 16 h before the ERG test. RCS rats were abdominal general anesthesia. Then, the animals were anesthetized on the ocular surface and pupil dilation. The corneal electrodes were attached to the cornea as recording electrodes. The reference and ground electrode were placed under the skin of the cheek and tail. A strobe white stimulus was presented to the dark adaptation eyes; light stimuli were rendered at intensities of −2.5, −0.5, −0.02, 0.5, and 1 log(cd*s/m2); and the responses were recorded and stored using the RETI-scan system.

Tissue Processing and Immunohistochemistry

To prepare the tissue, the eyecups of RCS rats were collected 1–4 weeks after operation, then fixed in 4% PFA for 2 h, and then stored overnight in 30% sucrose for dehydration. The tissue was frozen with optimal cutting temperature compound and stored at −80°C. Each group of tissues used the same horizontal angle to cross cut the optic disc; the frozen tissues were sectioned along the transplantation area of the eye through the optic nerve head (ONH), as previously described (Inoue et al., 2007). The tissue was sectioned with thickness of 16 μm, air-dried at room temperature, and stored at −20°C. For immunofluorescence assay, the sections were washed with PBS and blocked in PBS supplemented with 3% goat serum and 0.3% Triton X-100. Then, the primary antibodies were added to the sections overnight at 4°C. The next day, the sections were washed with PBS and incubated with secondary antibodies for 2 h at 37°C. Finally, the nuclei were counterstained with DAPI.

Outer Nuclear Layer Thickness Analysis

The morphological changes of retina were observed after injection. The degree of retinal damage was assessed by measuring the thickness of ONL. The thickness quantification of the outer nuclear layer (ONL) was measured at six regions of retina: near the limbal on both sides recorded as 3 and −3; the graft region and the opposite region recorded as 2 and −2; the optic nerve area and the opposite region recorded as 2 and −2. The ONH was defined as the original location (recorded as 0). The thickness of ONL was measured by ImageJ.

Lactate Dehydrogenase Release Assay

The experiment is to detect the release of LDH (Beyotime, C0016) in the supernatant of 661W cells. The experiment is divided into three groups: 661W cells cultured with High-Glucose DMEM with 10% FBS medium, 661W cells alone cultured with low-glucose medium, and 661W cells cultured with low-glucose medium within hRPC-sEVs (60 μg/ml) for 48 h. All supernatant was collected for detection and tested according to the protocol of the manufacturer.

ELISA Assays

The enzyme-linked immunosorbent assays (ELISAs) were utilized to quantitate cytokines in cell culture medium. After LPS stimulation of microglia, the supernatant of microglia cell culture with or without hRPC-sEVs (60 μg/ml) treatment for 48 h was harvested for the assay. ELISAs, including levels of tumors necrosis factor–α (TNF-α) (DAKEWE, 1117202; BioLegend, 430,907), interleukin-1β (IL-1β) (DAKEWE, 1110122; DAKEWE, 1210122), and IL-6 (DAKEWE,1110602; DAKEWE, 1210602), were performed according to the instructions of the manufacturer. The results of ELISA were detected at 450 nm by using a microplate reader.

Apoptosis Assays

We used AnnexinV/PI fluorescein isothiocyanate (FITC) staining to detect cell apoptosis. 661W cells treated with hRPC-sEVs were collected, incubated with Annexin V–FITC and PI to the cells according to the instructions of the manufacturer (BD Biosciences, 556547). Then, flow cytometry was used to detect the number of apoptotic cells, and the ratio of apoptotic cells to non-apoptotic cells was analyzed.

TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) (Beyotime, C1088) is a method for detecting apoptotic DNA

fragmentation. The slides were incubated with DAPI (Solarbio, C0065) and TUNEL label solution using an *in situ* cell death detection kit. Slides were then observed under a fluorescence microscope and count positive cells.

Total RNA Isolation and Real-Time Quantitative PCR

Total RNA was extracted from cells (661W, BV2, and HMC3 cells) or retinal issues using RNAiso Plus followed by chloroform, as per the instructions of the manufacturer. The cDNA was generated using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, RR047A). Real-time qPCR was performed on CFX96 system (Bio-Rad) by an SYBR Premix Ex TaqTM II kit (Takara, RR820A) to measure the expression of the primer sequences (**Supplementary Table S1**) of each gene.

Western Blot

hRPC-sEV pellets were lysed in 1× RIPA buffer with protease inhibitor cocktail. Protein concentration was measured with a BCA Protein Assay Kit. The sample was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, and the proteins were electroblotted to polyvinylidene difluoride membranes. The membranes were incubated with anti-rabbit CD9, CD63, and CD81 and anti-rabbit horseradish peroxidase (HRP) conjugated for secondary antibodies (SBI System Biosciences, EXOAB-KIT-1). Chemiluminescent detection was performed using a kit (Thermo Fisher Scientific, catalog no. 32106).

Functional Enrichment Analysis

miRNA sequencing data are from a previous study (Bian et al., 2020). miRNA abundance analysis by small RNA sequencing. Gene Ontology (GO) enrichment analysis of the biological process (BP) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were obtained to predict the potential functions of hRPC-sEV miRNA target genes.

Statistics

Each experiment is repeated in at least three biological samples (individually indicated in the figure legends). Data are presented as means ± SD. SPSS V22.0 software is used to perform statistical analysis on the data. The statistical differences are measured with unpaired two-tailed Student's t-test for comparison between two groups or analysis of variance among multiple groups by Dunnett's T3 multiple comparison tests or Turkey's t-test. p-value < 0.05 was considered statistically significant.

RESULT

Transplantation of hRPC-sEVs Preserves Visual Function in RCS Rats

Retina progenitor cells obtained from the neural retina of human fetal eyes, which are identified as positive for expression of PAX6, SOX2, and Nestin and weakly expression of GFAP (**Supplementary Figure S1A,B**), as previously described (Schmitt et al., 2009). The TEM examination shows that the hRPC-sEVs are cup-shaped vesicles, with the diameter about 130 nm (**Supplementary Figure S2A**). WB analysis demonstrates that the hRPC-sEVs positively express characteristic markers CD9, CD63, and CD81 (**Supplementary Figure S2B**). NTA shows that these nanoparticles have a particle size distribution between 20 and 150 nm (**Supplementary Figure S2C**).

To demonstrate the therapeutic effects of hRPCs and their sEVs, we then give one injection of hRPCs and hRPC-sEVs, with PBS as the negative control to the RCS rats, and observe until 2 weeks after injection (**Figure1A**). Scotopic fERG is performed to determine vision outcome of RCS rats. The visual function assessed by ERG indicate a progressive deterioration of the a- and b-wave amplitudes 4 weeks after transplantation in all three groups caused by RD. We find that, compared with the PBS group, both hRPC group and hRPC-sEV group significantly preserve the amplitudes of b-waves at 2 weeks after injection, and this effect has maintained to 4 weeks after injection (**Figure 1B**). Although both hRPC-sEVs and hRPCs transplanted into RCS rats could significantly increase the

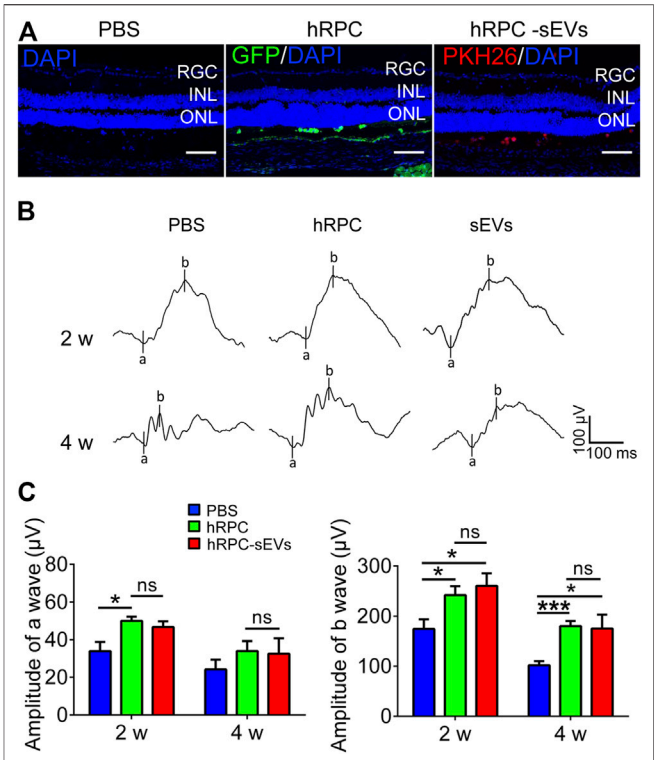

FIGURE 1 | Effects of hRPCs and hRPC-sEV treatment on visual function in RCS rats. **(A)** Confocal images showing RCS retina injected with PBS (L), GFP-labeled (green) hRPC (M), or PKH26-labeled (red) hRPC-sEVs (R) in subretinal space. Scale bars, 50 μm. **(B)** Representative scotopic fERG waveforms elicited at 1 log(cd*s/m^2) light intensity in PBS (untreated groups), hRPCs, and hRPC-Exos groups at 2 and 4weeks after injection (n = 5 eyes). **(C)** Statistical analysis of scotopic fERG a-wave amplitudes **(left panel)** and b-wave amplitudes **(right panel)** elicited at 1 log(cd*s/m^2) light intensity in PBS (untreated groups), hRPCs, and hRPC-sEVs groups at 2 and 4 weeks after injection (n = 5). Data are shown as means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

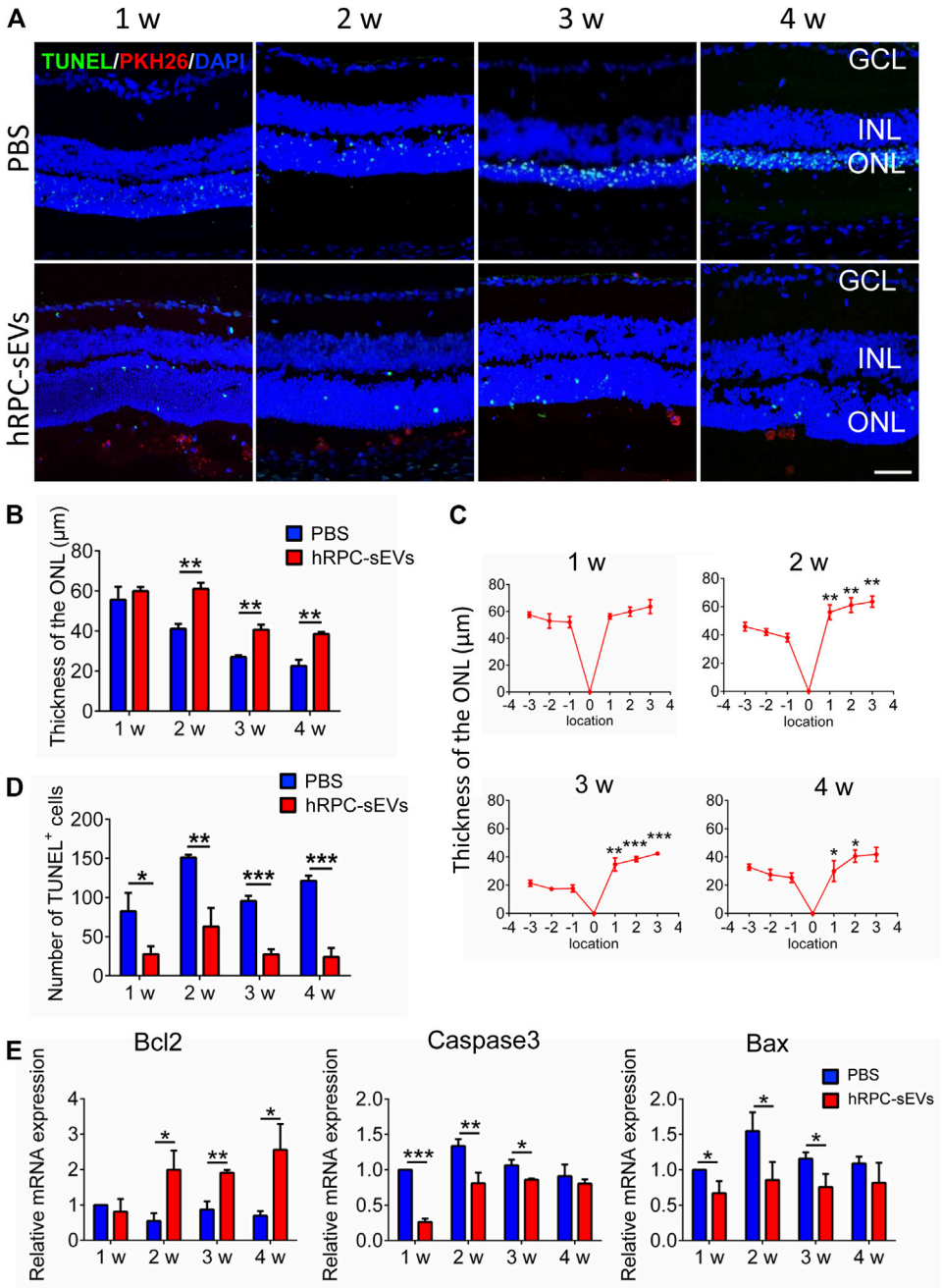

FIGURE 2 | hRPC-sEVs protect photoreceptors from apoptosis in RCS rats. **(A)** Apoptosis detection of TUNEL and DAPI staining in hRPC-sEV– and PBS-treated RCS retinas at 1 to 4 weeks after injection. hRPC-sEVs are prelabeled with PKH26 (red). Scale bar, 50 μm. **(B)** Thickness of the ONL at the injected sites in hRPC-sEV and PBS groups at each time point. The thickness of ONL around the injected area is significantly preserved in the hRPC-sEV group 2 weeks after injection compared to the PBS group, and this effect has maintained to 4 weeks after injection (n = 3). **(C)** The thickness of ONL is compared in hRPC-sEVs at different injected bitemporal locations, distance from optic nerve head (ONH), from 1, 2, 3, and 4 weeks after transplantation (n = 3). The thickness of the ONL in the transplanted area (temporal field) is significantly preserved compared to that of the distal area (nasal field). **(D)** The numbers of TUNEL-positive cells in the retina of RCS rats are analyzed in hRPC-sEV and PBS groups at each time point (n = 3). hRPC-sEV treatment significantly reduces the number of TUNEL-positive cells around the injected area of eyes in RCS rats. **(E)** Real-time qPCR analysis showing relative mRNA expression of apoptotic factors Bcl2, Caspase3, and Bax in the retinas of the hRPC-sEV and PBS groups. hRPC-sEVs significantly reduce the apoptotic factors Bax and Caspase3 and markedly increase the antiapoptotic factor Bcl2 in the retina compared to the PBS group (n = 3). Data are shown as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

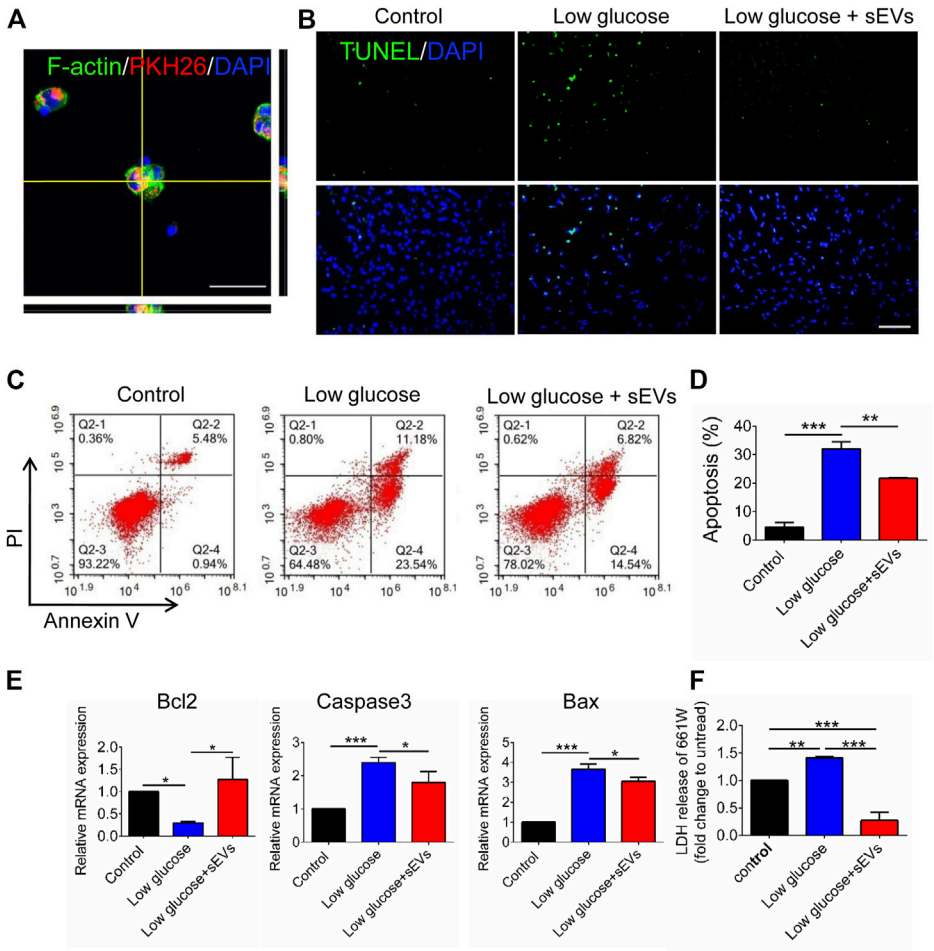

FIGURE 3 | hRPC-sEVs protect 661W cells from apoptosis in low-glucose preconditioned culture. **(A)** Representative confocal images showing the hRPC-sEVs (PKH26-labeled, red) are localized with Factin-labeled mouse 661W cells (green). **(B)** Confocal images of TUNEL (green, **top panel**) and DAPI (blue, **bottom panel**) staining in low-glucose cultured 661W cells. hRPC-sEV–treated groups show low TUNEL-positive labeling compared with the PBS groups (n = 3). Scale bar, 50 μm. **(C)** Flow cytometry representative images showing Annexin V/PI staining in control (normal glucose), low glucose without treatment, and low glucose with treatment of hRPC-sEVs (n = 3 per group). The double-positive cells are end-stage necrotic cells. A lower percentage of Q2-2 cells (6.82%) in hRPC-sEV–treated group compared to non-treated group (11.18%), suggesting that hRPC-sEVs are protecting 661W cells from death. **(D)** Statistical analysis of the apoptosis assays from flow cytometry testing (n =3). **(E)** Real-time qPCR analysis showing relative mRNA expression of apoptotic factors Bax, Caspase3, and Bcl2 in the 661W cells. hRPC-sEV–treated 661W cells significantly reverse the high levels of Bax and caspase3 and the low level of Bcl2 (n = 3). **(F)** LDH release of 661W cells (n = 3). hRPC-sEV treatment dramatically reduces the LDH release in 661W cells compared to the untreated group (n = 3 per group). Data are shown as means ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

amplitude of b-waves at 2 and 4 weeks after injection, the effect of two groups shows no significant difference (**Figure 1C**). These results demonstrate that, in the early stage of RD, the direct administration of hRPC-sEVs could preserve visual function, and the effect of RCS rats is as good as hRPCs.

hRPC-sEVs Protect Photoreceptors From Apoptosis *in vivo*

It has been reported that RCS rats suffer from photoreceptor loss (Lew et al., 2020). Therefore, the ONL thickness is analyzed to investigate the protective function of the grafted hRPC-sEVs (**Figure 2A**). We find that, compared with the PBS group, the ONL thickness around the injected area is significantly retained in the hRPC-sEV group 2 weeks after injection, and this effect has maintained to 4 weeks after injection (**Figure 2B**). The thickness of ONL has been measured, and we find that the thickness of the ONL in the transplanted area (temporal field) is significantly preserved compared to that of the distal area (nasal field) (**Figure 2C**) at 4 weeks after injection. It is proved that hRPC-sEVs have a protective effect on the ONL of retina, and this effect is limited to the local areas.

Moreover, TUNEL staining reveals that hRPC-sEV treatment significantly reduce the number of TUNEL-positive cells around the injected area of eyes at 4 weeks after injection in RCS rats (**Figure 2D**). Furthermore, RT-qPCR shows that hRPC-sEVs significantly reduce the apoptotic factors Bax and Caspase3 and markedly increase the antiapoptotic factor Bcl2 in the retina compared to the PBS group (**Figure 2E**). These

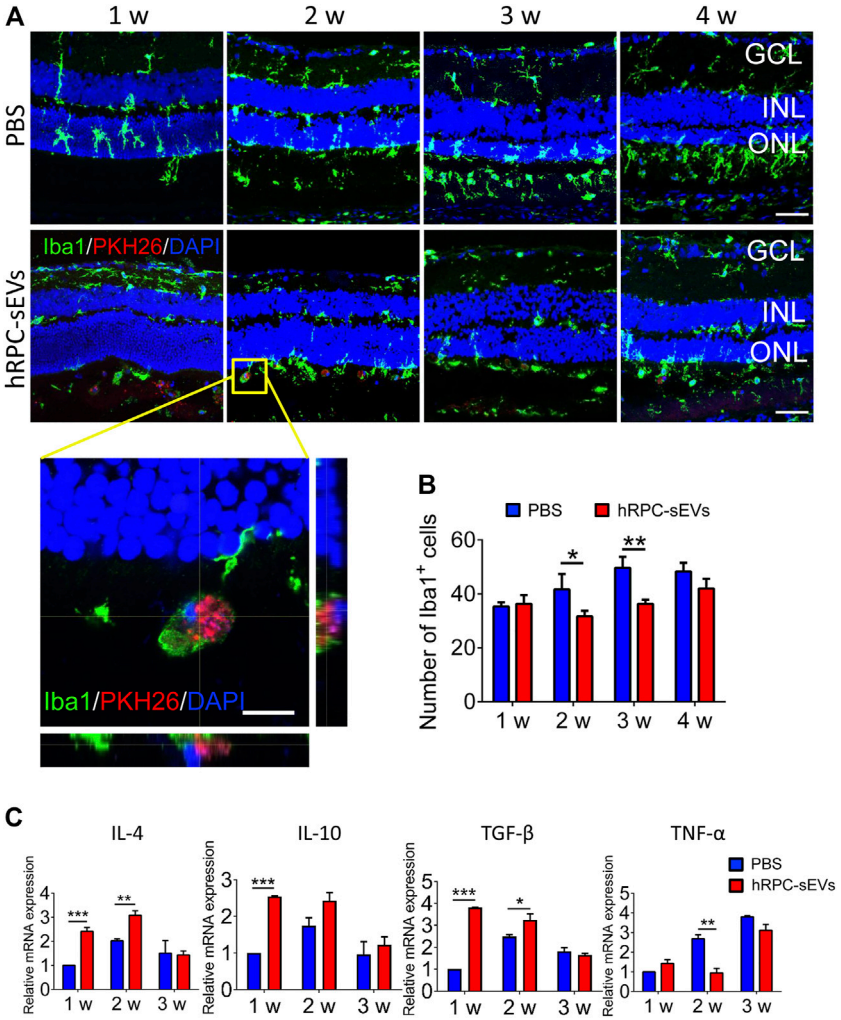

FIGURE 4 | hRPC-sEVs suppress the activation of microglia and regulate cytokines in the retina of RCS rats. **(A)** Iba1 (green) staining of PBS-treated **(top panel)** and hRPC-sEV–treated **(bottom panel)** RCS retinas at different time after transplantation. hRPC-sEVs are labeled by PKH26 (red). Enlarged orthogonal view of hRPC-sEVs at the injected site shows that the EVs are co-located with microglia in subretinal space (SRS). **(B)** The number of Iba1-positive cells in the injected area of the retina is analyzed and compared in hRPC-sEV– and PBS-treated groups at different time points (n = 3 per group). **(C)** Relative mRNA levels of cytokines IL-4, IL-10, TGF-β, and TNF-α in the retinas (n = 3 per group). Data are shown as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

observations demonstrate that the grafted hRPC-sEVs protect photoreceptors from apoptosis and delay the progressivity of RD.

hRPC-sEVs Inhibit Low-Glucose–Induced Photoreceptor Apoptosis *in vitro*

To investigate the protective on degenerating photoreceptor cells, we establish a low-glucose–induced apoptosis model of 661W cells. The 661W cells, a mouse photoreceptor cell line, as previously described (Emery et al., 2011). After incubation with hRPC-Exos for 6 h, the PKH26-labeled hRPC-sEVs are shown toco-locate with 661W cells, which suggest that the photoreceptors are interacting directly with hRPC-sEVs (**Figure3A**). TUNEL assay shows that an exposure to low glucose significantly increases apoptosis compared to the untreated group, whereas the administration of hRPC-sEVs significantly attenuates the apoptosis in low-glucose–treated 661W cells (**Figure 3B**). Flow cytometry also shows a higher cell death percentage at 24 h low-glucose preconditioning compared to the control group, whereas treatment with hRPC-sEVs significantly mitigates low-glucose–induced cell death in photoreceptors compared to the untreated group. This result is consistent with TUNEL assay (**Figure 3C,D**). RT-qPCR shows that Bax and Caspase3 are upregulated and Bcl-2 is downregulated compared to the control in 661W cells after 24 h of low-glucose preconditioning, whereas hRPC-sEV–treated 661W cells significantly reverse the high levels of Bax and Caspase3 and the low level of Bcl2 (**Figure 3E**). 661W cells preconditioned with low glucose for 24 h lead to

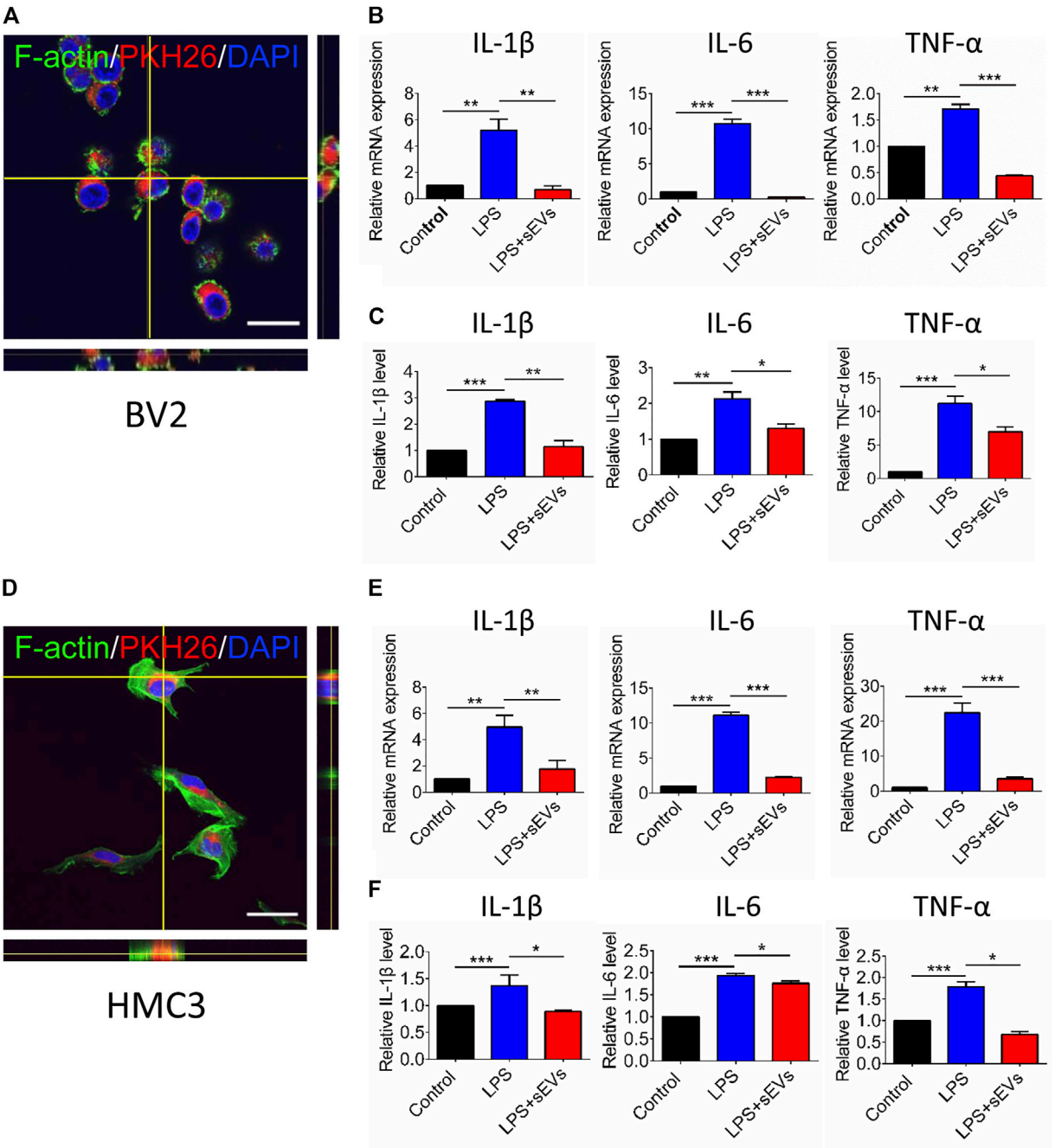

FIGURE 5 | Microglia phagocytosis assay of hRPC-sEVs and cytokines changes after LPS stimulation of microglia. Representative confocal images showing the hRPC-sEVs (red) in the medium are located with Factin-labeled mouse **(A)** or human **(D)** microglia cells (green). Enlarged orthogonal view shows that the cytoplasm of microglia contains a large number of hRPC-sEV particles after LPS stimulation. Scale bar, 50 μm. Real-time qPCR analysis showing relative mRNA expression of cytokines IL-1β, IL-6, and TNF-α in cultured mouse microglia **(B)** and human microglia **(E)**. Analysis of cytokine concentrations such as TNF-α, IL-6, and IL-1β in the supernatant of mouse microglia **(C)** and human microglia. **(F)** Culture detected by ELISA. Data are shown as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

dramatic release of LDH to the medium. hRPC-sEV treatment dramatically reduces the LDH release in 661W cells compared to the untreated group (**Figure 3F**). The results indicate that hRPC-sEVs could be taken up and internalized by 661W cells and protect photoreceptor from low-glucose–induced apoptosis *in vitro*.

hRPC-sEVs Inhibit Microglia Activation and Mitigate Ocular Inflammation

To investigate the effect of sEVs on microglia in RD, microglia is detected with Iba-1 immunofluorescence staining. Microglia is observed to migrate from the inner nuclear layer to SRS and co-locate with hRPC-sEVs (**Figure 4A**). Compared to the PBS group, the number of migrated microglia in hRPC-sEV–treated group is markedly decreased at 4 weeks after injection, in both ONL and SRS. Retinal treatment with small EVs has markedly reduced the number of microglia in the ONL of the retina (**Figure 4B**). Microglial cells migrate from the inner to the outer retina in RD progress, which symbolizes their activation in response (Cuenca et al., 2014). The proliferation of microglia is often corresponding with excessive production of inflammatory cytokines. After the small EV treatment, we find that gene expressions of IL-4, IL-10, and transforming growth factor–β (TGF-β) were significantly increased, and TNF-α is reduced in the retina compared to PBS-treated RCS rat (**Figure 4C**). The results suggest that hRPC-sEVs could inhibit microglia activation and mitigate the inflammatory response in RCS rats.

hRPC-sEVs Reduce the Expression of Pro-inflammatory Cytokines in Microglia *in vitro*

Because the production and release of pro-inflammatory cytokines are essential in microglia-mediated inflammation. we next determine the gene and protein expression of pro-inflammatory mediators produced by activated microglia *in vitro*. First, PKH26-labeled small EVs are co-cultured with BV2 and HMC3 cells for 6 h. The labeled sEVs co-localized with BV2 cells are mainly located in the perinuclear region within the BV2 cell margins (**Figure 5A**). We find that LPS stimulation significantly increases the production of TNF-α, IL-1β, and IL-6. However, these pro-inflammatory effects are significantly attenuated by co-culture with hRPC-sEVs, in both gene and protein levels. We concluded that hRPC-sEVs inhibit neuroinflammation by stabilizing microglia not to release cytokines (**Figures 5B,C**). The labeled sEVs also co-localized with HMC3 cells (**Figure 5D**). A consistent trend was found in HMC3 group that both gene and protein levels of pro-inflammatory cytokines significantly decreased in hRPC-sEV–treated HMC3 cells (**Figures 5E,F**). These results show that, both in mouse and human cell lines, hRPC-sEVs inhibit neuroinflammation by inhibiting microglia activation and suppress secreting cytokines.

GO and KEGG Analysis of hRPC-Exos/sEVs miRNAs

Small EVs contain cell-specific proteins or mRNA/miRNA that can interfere host tissue homeostasis and function (Geis-Asteggiante et al., 2018). Through analysis of small RNA sequencing, we are able to show the abundant expressed miRNAs in enriched hRPC-sEVs (**Supplementary Table S2**). The pie chart shows the top five miRNAs expressed in the hRPC-sEVs. It is found that the miRNAs mainly contained in hRPC-derived small EVs are miR-21-5p, let-7i-5p, miR-100-5p, miR-148a-5p, and miR-151a-3p, which take proportion of over 50% (**Figure 6A**). Subsequently, GO and KEGG enrichment pathway analyses are used to predict the target genes of top 20 expressed miRNAs in hRPC-sEVs (listed in **Supplementary Table S2**). The functions of the miRNAs (highly expressed in hRPC-sEVs) are predicted to be involved several signaling pathways (listed in **Table 1**) (Ouyang et al., 2019; Fu et al., 2020; Wei et al., 2020; Wen et al., 2020; Zhang H. et al., 2020; Zhang Y. et al., 2020; Li et al., 2021). Some of the enriched GO BPs are related to inflammation, such as neutrophil mediated immunity, neutrophil activation in immune response, and neutrophil activation (Nabavi et al., 2017; Liang et al., 2020). Moreover, processes autophagy, signal release, regulation of neuron death, and cell cycle are also significantly enriched (**Figure 6B**). KEGG analysis of the top 20 enriched pathways suggests that they are related to miRNAs targets in MAPK signaling pathway and pathway of neurodegeneration-multiple disease (**Figure 6C**).

DISCUSSION

In this study, we demonstrate that the hRPC-sEVs significantly preserve the function of retina and thickness of the ONL, reduce the apoptosis of photoreceptors, inhibit microglia activation and mitigate ocular inflammation, and delay the degeneration of the retina in RCS rats. *In vitro*, we have shown that hRPC-Exos treatment could significantly reserve the low-glucose preconditioned apoptosis of photoreceptors and reduce the expression of proinflammatory cytokines in microglia.

Microglia cells, as the reaction cells of retinal cell damage, participate in injury repair and inflammatory status (Xie et al., 2019). A persistent chronic pro-inflammatory environment is an important common feature of retinal degenerative diseases and neurological diseases that affect vision (Chen and Xu, 2015; Zhao et al., 2015; Ramirez et al., 2017). Therefore, regulating microglial reactivity has become a promising therapeutic approach (Simons and Raposo, 2009; Fruhbeis et al., 2013). In recent studies, exosomes are one of therapeutic approaches to show huge potential for neurological diseases (Alvarez-Erviti et al., 2011; Doeppner et al., 2015; Tatullo et al., 2020). Furthermore, neuronal EV as an endogenous protective factor inhibits microglial phagocytosis by targeting platelet-activating factor receptor, thereby reducing ischemia-induced neuronal death (Yang et al., 2021). Our recent study has found that RPCs from human embryonic stem cell–derived retinal organoids inhibit the activation of microglial, gliosis, and the production of inflammatory mediators (Zou et al., 2019). In addition, exosomes/sEVs derived from neural stem cells could significantly inhibit the activation of microglia and protect photoreceptors from apoptosis (Bian et al., 2020). Therefore, we are interested in the effect of hRPC-sEVs on microglia in RCS rats. In our study, we have observed that small EVs can be internalized by microglia in the retina. Moreover, we have compared the number of microglia and the expression of

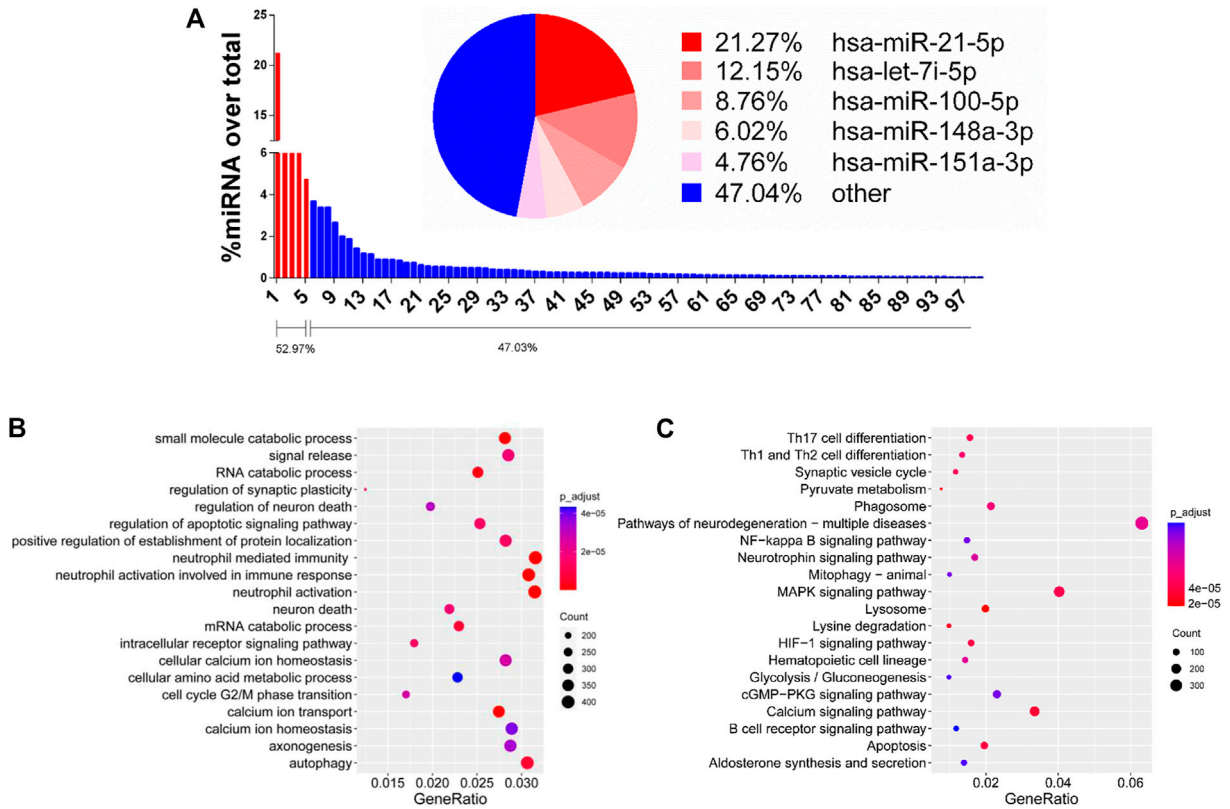

FIGURE 6 | Functional analysis of EVs/Exo-miRNAs. **(A)** Bar diagram showing miRNAs expressed in theen riched hRPC-sEVs. Left to right is showing high to low percentages. The pie chart shows the top five miRNAs in hRPC-sEVs. Selected enriched GO biological processes **(B)** and KEGG pathways **(C)** for the top expressed miRNAs in hRPC-sEVs. The coloring of the q-values indicates the significance of the rich factor. The circle indicates the target genes that are involved, and the size is proportional to the gene numbers. The *X*-axis indicates the rich factor, which refers to the ratio of the number of genes located in the differentially expressed gene to the total number of the annotated genes located in the pathway. The *Y*-axis indicates the names of enriched pathways.

TABLE 1 | List of direct targets of selected miRNAs highly expressed in hRPC-sEVs and their functional role.

miRNA	Functions	Study
Hsa-miR-21-5p	inhibit cardiac microvascular endothelial cell apoptosis	Liao et al. (2021)
	regulated the proliferation and apoptosis	Zhang et al. (2020b)
	anti-inflammatory effect	Ouyang et al. (2019)
Hsa-let-7i-5p	attenuate hypoxia-induced apoptosis	Zhang et al. (2020a)
Hsa-miR-100-5p	protected cell from pyroptosis and injury	Liang et al. (2020)
		Nabavi et al. (2017)
Hsa-miR-26a-5p	reduce the apoptosis of myocardial cells and the expression of inflammatory factors	Li et al. (2021)
		Wen et al. (2020)
Hsa-miR-151a-3p	inhibited cell viability and promoted lactate dehydrogenase release	Fuet al. (2020)

inflammatory factors in the retina with or without sEVs treatment. The results confirm that hRPC-sEVs can protect the degenerating photoreceptors from death in the RD model by suppressing microglia activation. Our work further supports the idea that exosomes inhibit neuroinflammation by inhibiting the activation of microglia in neurodegenerative diseases (Bian et al., 2020).

We have observed that hRPC-sEVs exerts a good anti-apoptosis effect on damaged photoreceptors. It is reported that the transplanted stem cells protect retina from damage via multiple mechanisms, and the exchange of therapeutic cargoes to host cells via extracellular vehicles is one of the most important mechanism. The miRNA of small EVs is one of the therapeutic mechanisms of acellular therapy in the retina (Wang et al., 2020; Peruzzotti-Jametti et al., 2021). Exosomes can be used to deliver interfering miRNA, siRNA, or drug active substances (Alvarez-Erviti et al., 2011; Zhuang et al., 2011; Jiang et al., 2020). Therefore, it is speculated that small EVs can reduce cell

apoptosis by transferring their functional substances miRNAs. Our result shows that the main components (>50%) of miRNAs in hRPC-Exos are miR-21-5p, let-7i-5p, miR-100-5p, miR-148a-5p, and miR-151a-3p. Previous studies have reported that miR-21-5p of cardiac telocyte–derived exosomes can inhibit apoptosis in cardiac microvascular endothelial cells (Liao et al., 2021). Enriched miR-100-5p in human umbilical cord mesenchymal stem cell–derived exosomes protects against H/R-induced cardiomyocyte pyroptosis and injury through suppressing FOXO3 expression (Liang et al., 2020). Overexpressing let-7i-5p could attenuate hypoxia-induced apoptosis and mitochondrial energy metabolism dysfunction in AC16 cells (Zhang et al., 2020b). MiR-26a-5p could target and regulate ADAM17 and reduce the apoptosis of myocardial cells and the expression of inflammatory factors in acute myocardial infarction (Wen et al., 2020). These miRNAs are known to be associated with cell proliferation, anti-inflammatory effect, and apoptosis and play a significant role in regulation of inflammation and cell apoptosis. These findings are consistent with prediction of the target genes, which may be the mechanism to inhibit microglial activation and alleviate ocular inflammation. Further studies are therefore essential to confirm the function of these miRNAs and their targets in neurodegenerative diseases.

Considerable benefit of EVs is that it does not contain cell nucleus as a treatment agent and is storable (Kusuma et al., 2018; Mead and Tomarev, 2020). EV therapy can keep treatment effect of the stem cells using non-living cell products (Luga et al., 2012) and is free of concerning viable cell risk such as tumor genesis or complications associated with the stem cell transplant (Kuriyan et al., 2017; Huang et al., 2019). As previously described, EVs showed minimal toxicity and immunogenicity about systemic administration or repeated dosing in mice (Zhu et al., 2017; Saleh et al., 2019; Rodrigues et al., 2021). The advantage of EVs is that the lipid bilayer composition protects encapsulated cargo from degradation and has been widely studied as drug delivery vectors exploiting natural properties (Ge et al., 2014; Li et al., 2015; Kooijmans et al., 2016; Sunkara et al., 2016). It has been reported that small EVs/exosomes are storable at −80°C for more than 6 months while maintaining functions (Clotilde Théry, 2006; Aryani and Denecke, 2016; Mead et al., 2018). For these reasons, small EV approach is valuable to be translated into clinical application.

Although hRPC-sEVs have a good application prospect in neurodegenerative diseases, exosomes as cell derivatives have some limitations waiting to be solved (Kooijmans et al., 2016; Shao et al., 2020). Quantification of EVs; timing, dose, and duration of treatment; underlying mechanisms, etc., remain to be optimized and elucidated (Buschmann et al., 2021). hRPC-sEVs are locally injected in the SRS of RCS rats in this study, and the therapeutic effects remained 4 weeks after injection. However, the pharmacokinetics of the grafted sEVs in SRS remains unclear. Lai et al. and Sung et al. have reported the system to track the EV biogenesis, uptake, and intracellular transport via a live cell reporter system (Lai et al., 2015; Sung et al., 2020). EVs are also reported to be administered intravenously and peritoneally, and with the latest imaging technologies, we can review the biodistribution of grafted EVs (Takahashi et al., 2013; Gupta et al., 2020). Thus, improving the route of administration, studying pharmacokinetics, developing slow-release reagent for hRPC-sEVs application, and so on, may be beneficial for application in diseases in RD.

In summary, we demonstrate that the hRPC-sEVs is a favorable cell-free therapy to treat retinal degenerative diseases in the short term, as it could significantly preserve the function of retina and thickness of the ONL, reduce the apoptosis of photoreceptors, and inhibit microglia activation and mitigate ocular inflammation, although it is a short-term treatment. Microglia internalization of therapeutic miRNAs cargoes from small EVs is one of the mechanisms that exert neuroprotective effect. Our study highlights the potential of hRPC-sEVs as cell derivatives therapeutic for neuroprotective and regenerative therapy in retinal degenerative disease and provides a new paradigm for cell-free therapy.

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 Southwest Hospital, Army Medical University (Third Military Medical University). A written informed consent to participate in this study was provided by the legal guardian/next of kin of the participants. The animal study was reviewed and approved by the Third Military Medical University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

YoL and BB conceived and designed the experiments. MC and CR performed the experiments, analyses, and interpretation of the data and drafted this manuscript. BR, FC, YF, QL, YZ, and YiL helped the other authors to develop the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.748956/full#supplementary-material

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REVIEW
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Pyroptosis: A New Insight Into Eye Disease Therapy

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Pyroptosis is a lytic form of programmed cell death mediated by gasdermins (GSDMs) with pore-forming activity in response to certain exogenous and endogenous stimuli. The inflammasomes are intracellular multiprotein complexes consisting of pattern recognition receptors, an adaptor protein ASC (apoptosis speck-like protein), and caspase-1 and cause autocatalytic activation of caspase-1, which cleaves gasdermin D (GSDMD), inducing pyroptosis accompanied by cytokine release. In recent years, the pathogenic roles of inflammasomes and pyroptosis in multiple eye diseases, including keratitis, dry eyes, cataracts, glaucoma, uveitis, age-related macular degeneration, and diabetic retinopathy, have been continuously confirmed. Inhibiting inflammasome activation and abnormal pyroptosis in eyes generally attenuates inflammation and benefits prognosis. Therefore, insight into the pathogenesis underlying pyroptosis and inflammasome development in various types of eye diseases may provide new therapeutic strategies for ocular disorders. Inhibitors of pyroptosis, such as NLRP3, caspase-1, and GSDMD inhibitors, have been proven to be effective in many eye diseases. The purpose of this article is to illuminate the mechanism underlying inflammasome activation and pyroptosis and emphasize its crucial role in various ocular disorders. In addition, we review the application of pyroptosis modulators in eye diseases.

Keywords: pyroptosis, eye disease, inflammasome, NLRP3, pyroptosis inhibitors

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INTRODUCTION

Pyroptosis, a programmed cell death dependent on the pore-forming activity of the gasdermin protein family with an inflammatory response, plays an essential part of the body's intrinsic immune response in antagonizing pathogen infection and sensing endogenous risk signals (Man et al., 2017; Shi et al., 2017). Cells are stimulated to form a multiprotein complex called the inflammasome that can convert inactive pro-caspase-1 to active caspase-1, which can cleave gasdermin D (GSDMD) at its central linker domain and release the N-terminal GSDMD domain, causing N-terminal GSDMD domain fragments to perforate the plasma membrane and form membrane pores, further leading to cell swelling and lytic cell death. Meanwhile, active caspase-1 processes inflammatory factors (IL-1β, IL-18, etc.) to the mature form and releases them to the extracellular matrix through ruptured membranes, conferring the proinflammatory nature of pyroptosis. Mature IL-1β acts as a potent proinflammatory mediator to recruit innate immune cells to sites of infection and regulate adaptive immune cells. Mature IL-18 can promote the secretion of interferon (IFN-γ) and enhance the cytolytic activity of natural killer cells and T cells, contributing to the clearance of pathogenic

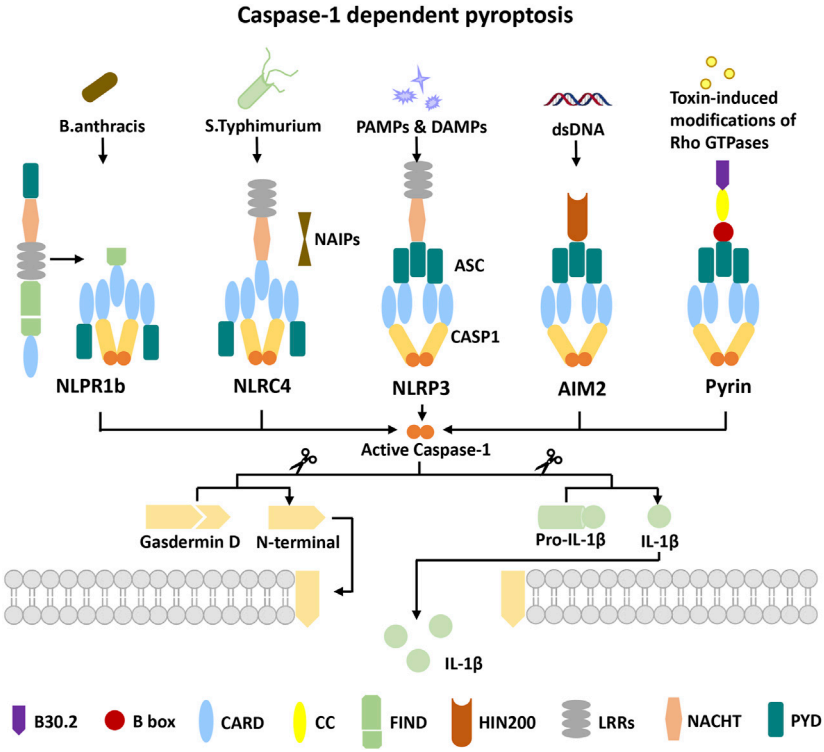


FIGURE 1 | Overview of five inflammasome complexes. NLRP1 undergoes autocatalytic processing in its FIIND followed by proteasomal degradation of its autoinhibitory N-terminus to engage caspase-1 in the C-terminal CARD for its activation. NAIPs are necessary for NLRC4 inflammasome activation to recognize *S. typhimurium*. In NLRP1 and NLRC4 activation events, caspase-1 can be directly recruited independently of the adapter ASC. The NLRP3 inflammasome can be activated by a broad spectrum of exogenous and endogenous stimuli. AIM2 activation requires dsDNA of microbial or host origin in the cytosol. Toxin-induced modifications of Rho GTPases reduce the phosphorylation of pyrin, which promotes the assembly of the pyrin inflammasome. NLRP3, AIM2, and pyrin activation events all require the adaptor ASC to activate caspase-1. Finally, activated caspase-1 drives gasdermin D cleavage to release the N-terminus, which forms the gasdermin D pore and drives pyroptosis. At the same time, activated caspase-1 cleaves pro-IL-1β to mature IL-1β into the extracellular matrix.

microbial infections or aberrant cells *in vivo* (Dinarello, 2009). Apoptosis, the first programmed cell death to be described, can be initiated through extrinsic and intrinsic pathways and characterized by cell contraction, nuclear condensation and division, and dynamic membrane blistering and loss. It activates the execution phase of cell death and allows the clearance of apoptotic cells without eliciting an inflammatory response, which is critical to physiological homeostasis in almost every organ system (Galluzzi et al., 2018). Indeed, a growing amount of evidence demonstrates that abnormal pyroptosis is closely related to autoimmune diseases, metabolic diseases, infectious diseases, cardiovascular diseases, neurological-related diseases, and ocular diseases (Man et al., 2017; Van Gorp et al., 2019; Zeng et al., 2019; McKenzie et al., 2020; Sharma and Kanneganti, 2021). The goal of this review is to discuss the role of pyroptosis and inflammasomes in the pathogenesis of ocular disorders and the application of pyroptosis inhibitors in eye diseases.

THE CLASSIFICATION OF INFLAMMASOMES

The inflammasome is defined as a complex formed by pattern recognition receptors (PRRs), apoptosis speck-like protein (ASC), and pro-caspase-1 protein when cells are stimulated by a danger signal, functioning as cleaving pro-caspase-1 to active caspase-1. In most cases, it is a critical process of pyroptosis.

Pattern recognition receptors (PRRs), a class of immune receptors mainly expressed in immune cells capable of identifying multiple pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) of invading microorganisms, are the first link of the innate immune system against infection. According to the homology of the protein domain, most PRRs can be classified into five families: Toll-like receptor (TLR), C-type (carbohydrate-binding lectin domain) lectin receptor (CLR), NOD-like receptor (NLR), retinoic acid–inducible gene (RIG)-I–like receptor (RLR), and AIM2-like receptor (ALR). According to protein localization, PRRs can be separated into unbound intracellular receptors and membrane-bound receptors. The former class includes NLRs, RLRs, and ALRs, which recognize the presence of intracellular pathogens in the cytoplasm. TLRs and CLRs are located on cell members or endocytic compartments, which recognize the presence of microbial ligands in the extracellular space or endosomes (Dostert et al., 2008; Brubaker et al., 2015; Plato et al., 2015). Multiple proteins of the NLR family and ALRs families have been found to form inflammasomes that mediate

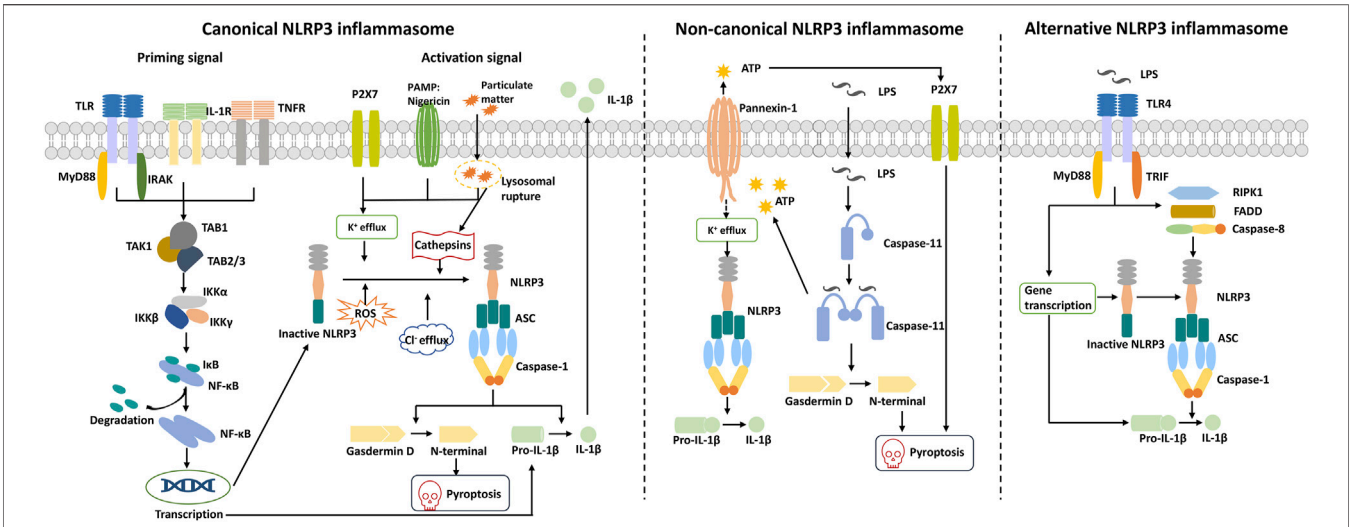


FIGURE 2 | Mechanisms of NLRP3 inflammasome activation. Canonical NLRP3 inflammasome activation requires two signals: a priming signal and an activation signal. The priming signal is triggered by endogenous cytokines or microbial molecules and causes the upregulation of NLRP3 and pro-IL-1β through the activation of the transcription factor NF-κB signaling pathway. The activation signal is triggered by various stimuli, such as pore-forming toxins, particular matter, and viral RNA, which can induce K^+ efflux, Cl^- efflux, mitochondrial dysfunction (ROS release), and lysosomal disruption (cathepsins). These events promote NLRP3 oligomerization to recruit ASC and pro-caspase-1 and form the activated NLRP3 inflammasome. Activated caspase-1 cleaves gasdermin D to release the N-terminal domain and induce pyroptosis. Meanwhile, caspase-1 cleaves pro-IL-1β to IL-1β and is released to the extracellular matrix. Non-canonical NLRP3 inflammasome activation is induced by cytosolic LPS released from gram-negative bacteria. LPS is delivered into the cytosol and activates caspase-11, whose activated state can trigger the pannexin-1 channel to induce K^+ efflux and ATP release. Subsequently, the NLRP3 inflammasome is activated, and mature IL-1β is released. Activated caspase-11 also induces pyroptosis via cleavage of gasdermin D and formation of pores on the cell membrane. Alternative NLRP3 inflammasome activation occurs when human monocytes are exposed to LPS, which requires RIPK1, FADD, and caspase 8 to activate the NLRP3 inflammasome rather than K^+ efflux. ASC speck formation and pyroptosis are also absent in this pathway.

the occurrence of pyroptosis (**Figure 1**). PRRs have been found to form inflammasomes, including NLRP1, NLRP3, NLRC4, NLRP6, NLRP9, NLRP12, pyrin, AIM2, IFI16, and CARD8. NLRP3 is the most widely studied and the best characterized inflammasome.

NLRP3

NLRP3 is composed of three domains, the PYD and NACHT and LRR domains. It can recruit and activate caspase-1 indirectly with the involvement of the adaptor protein ASC. NLRP3 can be activated by a wide variety of factors, including not only pathogen-related molecular patterns from invading pathogens such as bacterial RNA and toxin ion channel proteins but also damage-related molecular patterns such as ATP and oxidative mitochondrial DNA and multiple molecules closely associated with diseases such as β-amyloid in Alzheimer's disease and cholesterol crystallization in atherosclerosis (Swanson et al., 2019). Therefore, the NLRP3 inflammasome plays a very important role in various human diseases, such as pathogen infection, autoimmune diseases, neurodegenerative diseases, and cancer.

Canonical NLRP3 inflammasome activation generally requires two signals: a priming signal and an activation signal (**Figure 2**). The priming signals are provided by microbial components such as lipopolysaccharide (LPS), which can be recognized by the Toll-like receptor TLR4 and can also be endogenous molecules, including TNF-α or IL-1β (Bauernfeind et al., 2009; Franchi et al., 2009). The priming signals induce NLRP3 and pro-IL-1β expressions through activation of the NF-κB signaling pathway (Gritsenko et al., 2020). The activation signal is triggered by ATP, Nigericin, silica, particulate matter, viral RNA, etc. These activation factors can induce cellular stress and activate the NLRP3 inflammasome, which is indirectly sensed by NLRP3 instead of being directly recognized by NLRP3.

Non-canonical inflammasome pathway activation is mediated through humanized caspase-4/5 or murine caspase-11, which directly identifies LPS and can be activated (**Figure 2**). This effect is not dependent on the conventional LPS extracellular receptor TLR4 and does not require the involvement of other receptor proteins, such as NLRP3 and the adaptor protein ASC (Kayagaki et al., 2011; Hagar et al., 2013; Kayagaki et al., 2013; Shi et al., 2014). Activated caspase-4/5/11 cleaves the pyroptosis effector protein GSDMD, which triggers pyroptosis. Then, it causes pannexin-1–mediated ATP release, triggers K^+ efflux, and further activates the NLRP3 inflammasome (Baker et al., 2015; Kayagaki et al., 2015; Rühl and Broz, 2015; Yang et al., 2015).

Unlike macrophages, human monocytes can activate caspase-1 and secrete mature IL-1β without activation signals after LPS stimulus (Netea et al., 2009). This pathway is defined as the alternative NLRP3 inflammasome pathway independent of K^+ efflux, and there is no evidence for ASC speck formation or pyroptosis (Gaidt et al., 2016). Studies have shown that TLR4-TRIF-RIPK1/FADD/caspase-8 signaling is involved in NLRP3-mediated inflammatory factor release and that activated caspase-8 can cleave GSDMD to lead to pyroptosis (He et al., 2013; Orning et al., 2018) (**Figure 2**).

To date, there is no consensus model of NLRP3 activation, and multifarious upstream signals are involved in the regulation of

NLRP3 inflammasome activation, including potassium ion (K^+) efflux, chloride ion (Cl^-) efflux, flux of calcium ions (Ca^{2+}), lysosomal disruption, mitochondrial dysfunction, metabolic changes, and trans-Golgi disassembly. These upstream signals might act in tandem or independently on NLRP3 inflammasome activation. With few exceptions, low intracellular concentrations of K^+ are a necessary upstream event in NLRP3 activation (Muñoz-Planillo et al., 2013). Nigericin, as a perforated toxin, can directly cause K^+ efflux. After ATP stimulation, P2X7 family receptors (non-selective cation channels) promote Ca^+ and Na^+ influx and coordinate with the K+ channel TWIK2 to mediate K^+ efflux, which enhances NLRP3 inflammasome activation (Di et al., 2018). The lower intracellular concentration of Cl^- is also an essential signal for NLRP3 inflammasome activation by several stimuli (Nigericin, ATP, etc.), and the chloride intracellular channel (CLIC) is involved in the process (Tang et al., 2017). Nigericin-induced mitochondrial damage and ROS production can promote the plasma membrane translocation of CLICs and mediate Cl^- efflux to regulate NLRP3 inflammasome assembly (Domingo-Fernández et al., 2017). Mitochondrial dysfunction and ROS-generating mitochondrial regulation are the other key upstream signals in NLRP3 activation (Zhou et al., 2011). Under normal physiological conditions, thioredoxin-interacting protein (TXNIP), an NLRP3-binding protein, interacts with thioredoxin (TRX) and blocks its activation. With the accumulation of ROS-generating mitochondria and ROS, TXNIP is released from the TRX complex and in turn binds NLRP3 to activate the NLRP3 inflammasome (Zhou et al., 2010). Lysosome rupture and Ca^{2+} release activate the Ca^{2+}-CAMKII-TAK1-JNK pathway, which regulates NLRP3 inflammasome activation through the oligomerization of ASC (Okada et al., 2014). However, the role of Ca^{2+} signaling in NLRP3 activation remains controversial. Various NLRP3 stimuli lead to the disassembly of the intracellular trans-Golgi network (TGN) into various dispersed structures, forming the dTGN. NLRP3 is recruited to the dTGN through negatively charged phosphatidylinositol-4-phosphate (PtdIns4P) on its ionic membrane. It serves as a scaffold for oligomerization of the adaptor protein ASC to aggregate into multiple puncta, thereby activating caspase-1 and downstream signals (Chen and Chen, 2018).

NLRP3 inflammasome activation contributes to the host's defense against microbial infection. However, NLRP3 dysfunction leads to inflammatory disease. Thus, it is critical to precisely regulate NLRP3 inflammasome activation to provide adequate immune protection without damaging the host. NLRP3 inflammasome activation is precisely regulated by posttranslational modifications such as phosphorylation and ubiquitination (Kelley et al., 2019), as well as modification of NLRP3 by certain pathogen proteins such as S-nitrosylation and ADP-ribosylation (Mishra et al., 2013; Bose et al., 2014).

AIM2

AIM2 is a major cytosolic dsDNA sensor consisting of the C-terminal HIN-200 domain and the N-terminal PYD (Jin et al., 2012). In the normal state, the HIN-200 domain and PYD interaction maintains the AIM2 receptor in an autoinhibited state in the absence of dsDNA (Jin et al., 2013). Cytoplasmic DNA derived from damaged host tissue, microbial or viral pathogens, can be sensed directly and combined with the HIN-200 domain of AIM2 to expose the AIM2 PYD and recruit the adaptor protein ASC, leading to activation of the ASC pyroptosome and caspase-1 (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). The AIM2 inflammasome is essential for host defense against various pathogens, including bacteria, viruses, fungi, and parasites. However, several studies have shown that AIM2 is involved in the occurrence and development of aseptic inflammatory diseases such as atherosclerosis, chronic kidney disease, skin diseases, liver disease, and neuroinflammation (Sharma et al., 2019).

NLRC4

NLRC4 belongs to the NLR family and consists of an N-terminal CARD, a C-terminal LRR domain, and an NACHT domain activated by bacterial flagellin and components of flagella-associated secretion systems (Poyet et al., 2001). However, NLRC4 recognizes these ligands indirectly, and NLRC4 inflammasome activation depends on NLR family apoptosis inhibitory protein (NAIP) (Kofoed and Vance, 2011). The binding of the ligand (microbial flagellin) to NAIP induces a conformational change leading to NLRC4 assembly, and exposure of its oligomerization interface is initiated to recruit and activate caspase-1 (Haloupek et al., 2019).

NLRP1 and CARD8

NLRP1 was the first discovered member of the NLR family and is activated by a variety of stimuli, such as anthrax lethal toxin, *Shigella flexneri*, *Toxoplasma gondii*, and the small-molecule DPP8/9 inhibitor Val-boroPro (VbP) (Martinon et al., 2002; Ewald et al., 2014; Okondo et al., 2018; Sandstrom et al., 2019). Human NLRP1 consists of the PYD, NACHT domain, LRR domain, FIIND, and CARD, wherein the FIIND is a specific domain different from other NLR family proteins that can be autolytically cleaved and key for NLRP1 activity (D'Osualdo et al., 2011; Finger et al., 2012). CARD8 has an FIIND similar to NLRP1 but lacks the N-terminal domain in NLRP1. In general, the activation of NLRP1 and CARD8 is dependent on autolytic cleavage of the N-terminal peptide fragment, and activated NLRP1 recruits pro-caspase-1 to construct the NLRP1 inflammasome that cleaves pro-caspase-1 into active caspase-1 (Chavarría-Smith et al., 2016).

Pyrin

Pyrin, a member of the TRIM protein family, can bind to the inflammasome adaptor protein ASC to form a caspase-1–activating inflammasome complex by sensing the pathogen, inactivating Rho GTPases, and decreasing the RhoA-dependent phosphorylation of pyrin, leading to pyroptosis (Xu et al., 2014; Park et al., 2016).

OCULAR DISEASES, INFLAMMASOMES, AND PYROPTOSIS

Ocular diseases involve in excessive or chronic inflammation. During the development of diseases, several cellular processes are

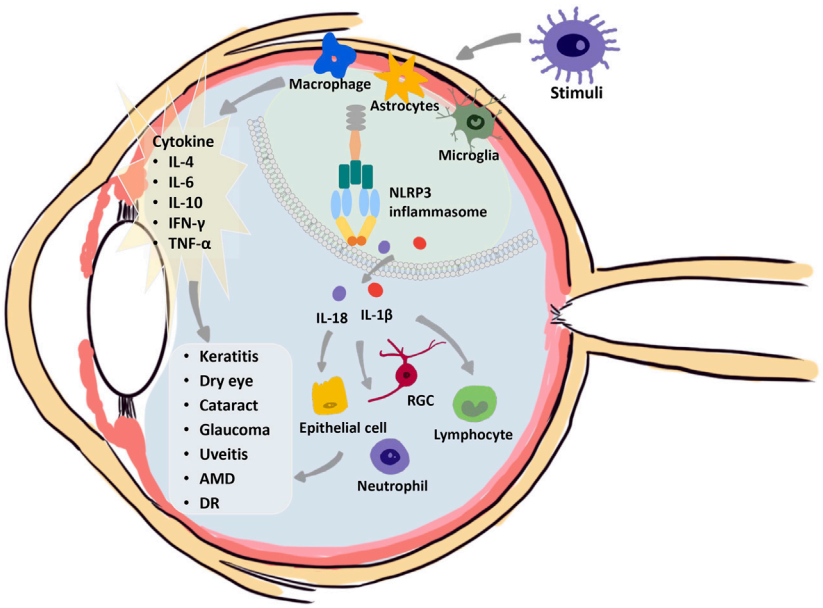


FIGURE 3 | Crucial role of the NLRP3 inflammasome in the pathogenesis of eye diseases. The NLRP3 inflammasome mediates eye inflammation occurrence under various stimuli, such as bacterial and viral infections, desiccating stress, autoimmune factors, drusen, and complement proteins. Activation of immune cells results in a cascade of massive inflammatory cytokines, including IL-4, IL-6, IL-10, IFN-γ, and TNF-α. Meanwhile, NLRP3 inflammasome activation occurs in activated macrophages, astrocytes, and microglia, which drives the secretion of IL-1β and IL-18 to recruit inflammatory cells and induce the damage or cell death of different cells. Multiple damage factors induce various injuries in the different ocular tissues, leading to keratitis, dry eye, cataracts, glaucoma, uveitis, age-related macular degeneration, or diabetic retinopathy.

activated, involving different cell deaths, including necroptosis, pyroptosis, ferroptosis, and autophagy, which can cause ocular tissue damage. DAMPs and PAMPs seem to be important connections between inflammation and cell death (Murakami et al., 2020). Although current studies advance the knowledge of the cell death mechanisms involved in ocular disorders, the diversity of consequences shows the complexity of various cell deaths that may be involved in disease pathogenesis. Further exploration of the value of different cell deaths in the pathogenesis of ocular disorders is warranted. It is now clear that inflammasomes have a central role in the pathogenesis of basically all types of chronic inflammation in metabolic, hereditary, systemic, and eye diseases (**Figure 3**). It acts as the processing unit to integrate the signals of pathogens, cell or tissue damage, and foreignness (Di Virgilio, 2013). The human eye has a well-developed immune surveillance system that can help fight against various pathogens, involving the contribution of NLRP1, NLRP3, NLRC4, NLRP12, and AIM2 inflammasomes (Gu et al., 2019; Pronin et al., 2019; Chen et al., 2020b; Lyon et al., 2020; Jassim et al., 2021; Yao et al., 2021). Unlike the protective effect of activated inflammasomes in combating pathogens in other tissue infections, such as infections of the lung with *Mycobacterium tuberculosis*, the activated inflammasomes in ocular diseases lead to increased severity of inflammation and more serious tissue destruction (Master et al., 2008; Akhtar-Schäfer et al., 2018). Aberrant inflammasome activation and IL-1β abundance upregulation have been shown to have no protective role in eye disease. Thus, in the eye, therapies that inhibit inflammatory body activation or IL-1β production may help to improve disease outcomes (Mesquida et al., 2019). Here, we emphasize the crucial role of pyroptosis and inflammatory inhibition in several ocular disorders, including keratitis, dry eyes, cataracts, glaucoma, uveitis, age-related macular degeneration, and diabetic retinopathy.

Keratitis

Keratitis can easily cause corneal ulcers and even blindness. Various inflammasomes are assembled in response to bacterial, viral, fungal, and parasitic infections of the eye through DAMP/TLR signaling. A moderate inflammatory response contributes to resistance to infection by enabling leukocyte migration, but a severe and uncontrolled inflammatory response leads to corneal damage and ultimately corneal ulceration and perforation (Hazlett, 2004). Mouse macrophages infected with *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* trigger NLRP3- and NLRC4-mediated inflammatory responses (Sutterwala et al., 2007; Karthikeyan et al., 2013). Pneumococcal hemolysin is a TLR4 ligand that mediates the activation of NLRP3 via TLR4 signaling (Malley et al., 2003). During inflammation, the assembly of inflammasomes and activated caspase-1 promote IL-1β release and attract inflammatory mediators such as cytokines and chemokines (Khan et al., 2007). In the course of pathogen elimination, cleaved GSDMD triggers cell pyroptosis, and corneal tissue may be damaged, resulting in partial or permanent blindness (Zhao et al., 2021).

The study indicated the involvement of non-canonical pyroptosis in *P. aeruginosa* keratitis, and the expression of caspase-4/5/11 and cleaved GSDMD in LPS-induced cell models, keratitis rat models, and *P. aeruginosa* keratitis patients was increased (Xu et al., 2021). Thus, targeting the activation of caspase-4/5/11 could inhibit the development of *P. aeruginosa* keratitis and suppress the release of proinflammatory cytokines. The protein GSDMD, as a universal substrate for inflammatory caspases, is an important performer of pyroptosis. Pretreatment with GSDMD siRNA attenuated the corneal inflammatory response significantly in mouse corneas infected with *Aspergillus fumigatus*, accompanied by decreased IL-1β secretion and attenuated recruitment of neutrophils and macrophages (Zhao et al., 2021). Inhibitors of IFNR, JAK/STAT, and caspase-1 can inhibit the expression of GSDMD, which may emerge as a potential therapeutic target (Zhao et al., 2021). In fungal keratitis, pretreatment with thymic stromal lymphopoietin (TSLP), a kind of inflammatory factor similar to IL-7 that is mainly produced by epithelial cells, promoted the expression of NLRP3, ASC, caspase-1, GSDMD, IL-1β, and IL-18 in THP-1 macrophages, which was abolished by NLRP3 knockdown. TSLP induces caspase-1–dependent pyroptosis through activation of the NLRP3 inflammasome, suggesting that it may be a potential target for fungal keratitis (Ji et al., 2021).

Dry Eye

Dry eye is a common ocular surface disease characterized by a loss of homeostasis of the tear film, causing eye irritation and even visual disturbance (Craig et al., 2017). Damage to the ocular surface and hyperosmolarity-triggered inflammation play important roles in dry eye pathogenesis, which impacts the ocular surface epithelium and resident immune cells (Pflugfelder and de Paiva, 2017). Multiple activated inflammatory pathways trigger the secretion of innate inflammatory molecules, which provoke goblet cell loss, reduce mucus secretion, and trigger apoptosis of epithelial cells, initiating a vicious self-perpetuation cycle to destroy the tear film (Wei and Asbell, 2014). The NLRP3 inflammasome interacts with the ASC protein to trigger caspase-1 activation and IL-1β and IL-18 maturity; in addition, activated caspase-1 induces GSDMD-driven cell pyroptosis (Swanson et al., 2019). GSDMD-driven pyroptosis in desiccating stress–induced dry eye mice has been demonstrated. Desiccating stress–induced TLR4 activation promotes NLRP12, and NLRC4 inflammasome–mediated GSDMD-dependent pyroptosis is responsible for processing bioactive IL-33, which exacerbates inflammation in dry eye via caspase-8 signaling (Chen et al., 2020b). Inhibition of NLRP12 or NLRC4, deletion of GSDMD, and neutralization of mature IL-33 significantly attenuated desiccating stress–induced corneal epithelial damage, suggesting that these components are potential therapeutic targets for dry eye disease (Chen et al., 2020b). A recent study examined the increased expression of the pyroptosis executor GSDMD N-terminal domain in tears from dry eye patients and demonstrated direct evidence of the involvement of pyroptosis in dry eye patients. Calcitriol can effectively alleviate hyperosmotic stress–induced corneal epithelial cell damage by inhibiting the NLRP3–ASC–caspase-1–GSDMD pyroptosis signaling pathway (Zhang et al., 2021).

Cataract

Cataracts, the loss of lens transparency, are the major cause of blindness in the world, accounting for half of all blindness (Flaxman et al., 2017). Although it can be treated by surgically removing the opaque lens and implanting the artificial lens, it is essential to develop new therapeutic targets to prevent cataract progression. Several studies have demonstrated that pyroptosis might have a role in cataract formation and that the levels of pyroptosis markers, such as NLRP3, pro-caspase-1, active caspase-1, GSDMD-N, IL-1β, and IL-18, are significantly increased in the capsule tissues or cells of cataract patients (Sun et al., 2020). Furthermore, active caspase-1 expression was increased in lens epithelium cells treated with H_2O_2, which was downregulated by using caspase-1 inhibitors, followed by pyroptosis inhibition. H_2O_2-induced oxidative stress could activate NF-κB signaling and increase NLRP3 expression significantly in human lens epithelial cells via caspase-1 activation and maturation of IL-1β, which contributes to pyroptosis and the development of cataracts (Jin et al., 2018). Human lens epithelial cells (LECs) irradiated with UVB induced pyroptosis, in which the expression of NLRP3, active caspase-1, pro-caspase-1, and GSDMD-N was increased significantly (Sun et al., 2020). Moreover, a high level of CRTAC1 plays an important role in pyroptosis in cataracts, while the downregulation of CRTAC1 significantly attenuates UVB-induced pyroptosis (Sun et al., 2020). In addition to the canonical inflammasome pathway, non-canonical inflammasomes are involved in caspase-11–triggered and GSDMD-triggered cataracts in a pyroptosis-dependent manner in short-wavelength blue light–exposed rat lens cells (Wang X. et al., 2021). A recent study demonstrated that the expression of the pyroptosis markers caspase-1, caspase-11, and GSDMD in rat LECs was increased after short-wavelength blue light exposure (Wang et al., 2020).

Glaucoma

Glaucoma is a neurodegenerative disease characterized by the loss of retinal ganglion cells (RGCs), thinning of the retinal nerve fiber layer, and cupping of the optic disc (Weinreb et al., 2014). Several recent studies have supported the crucial roles of inflammation in the pathogenesis of acute glaucoma (Baudouin et al., 2021). Inflammation activation primarily occurs in the optic nerve head (ONH) and retina, in which activated astrocytes, activated microglia, and proinflammatory cytokines (IL-1β) are detected (Sun et al., 2013; Bordone et al., 2017; Williams et al., 2017). Activated inflammation interacts with oxidative stress, such as reactive oxygen species (ROS) and nitric oxide (NO), and promotes an increase in the amount of each other, creating a chronically activated state of inflammation (Adornetto et al., 2019; Fernández-Albarral et al., 2021). The increased inflammatory cytokines IL-1β, IL-4, IL-6, IL-10, and IFN-γ, in turn, led to a reduction in RGCs in the glaucoma model, which might be associated with microglial activation (Bosco et al., 2015).

Dysregulation of the NLRP3 inflammasome has been associated with various neurodegenerative diseases. One study has shown that the NLRP3 inflammasome, caspase-1, and caspase-8 are increased in human glaucoma compared to normal eyes (Yang et al., 2011). Clinical studies have observed an upregulation level of IL-1β mRNA and protein expression in the blood of glaucoma patients, suggesting activation of the NLRP3 inflammasome in glaucoma (Markiewicz et al., 2015). In an inducible mouse model of acute glaucoma, microglia-induced pyroptosis-mediated RGC death was associated with glaucomatous vision loss (Chen et al., 2020a). Caspase-8 plays a critical role in IOP-induced cell death. TLR4 signaling, mediated by caspase-8, is essential to activate NLRP3 inflammasomes and process pro-IL-1β (Chi et al., 2014). Targeted inhibition of TLR4 and caspase-8 signaling significantly blocked the production of IL-1β and attenuated RGC death and retinal ischemic damage (Chi et al., 2014). NLRP12 collaborates with NLRP3 and NLRC4, downstream of the caspase-8–HIF-1α axis, to trigger pyroptosis by GSDMD cleavage and IL-1β maturation through caspase-1 activation (Chen et al., 2020a). High-mobility group box 1 (HMGB1) protein is released, mediated by rapid IOP elevation, triggering caspase-1–dependent NLRP3 inflammasome activation and IL-1β production via caspase-8 (Chi et al., 2015).

Uveitis

Uveitis is a complex ocular inflammatory disease usually caused by underlying autoimmune diseases (non-infectious/autoimmune uveitis) or bacterial or viral infections (infectious uveitis) (Shome et al., 2021). Current therapies concentrate on topical corticosteroids to achieve rapid remission of initial inflammation, followed by treatment with immunosuppressants to control long-term chronic inflammation (Lee K. et al., 2014). The inflammatory pathway, especially the role of the inflammasome, is highlighted as a responsibility for the development of uveitis. Most cells express one or more NLRs and contain the machinery to form inflammasomes, including NLRP1–4 (Lim et al., 2020). Activated inflammasomes, especially NLRP3, as well as the proinflammatory cytokines IL-1β and IL-18, have been implicated in uveitis. The imbalance of the adaptive immune system, especially macrophages, myeloid cells, and activated T helper cells, is credited for chronic uveitis. Th1, Th2, Th17, and T regulatory cells (Tregs) are diverse subsets of naïve T cells after stimulation by cytokines and transcription, which can lead to cytokine release in turn and upregulate lymphocyte infiltration to exacerbate inflammation (Lee RW. et al., 2014). Recent studies have indicated that the inflammasome might play an important role in the differentiation, expansion, and survival of Th17 cells (Mills et al., 2013). IL-1β produced by activated inflammation with IL-23 induces the secretion of IL-17 from Th17 cells (Sutton et al., 2006). Treatment of mice with IL-1β along with retinoid-binding protein injection enhanced experimental autoimmune uveitis development, whereas treatment with an anti-IL-1β antibody attenuated the inflammatory response (Zhao et al., 2014). IL-1 receptor–deficient mice showed better tolerance to the experimental autoimmune uveitis animal model, further confirming the importance of IL-1β in autoimmune uveitis (Wan et al., 2016). As a novel target for autoimmune uveitis, IL-6 impacts the differentiation and stimulation of Th17 cells with TGF-β and IL-23, while considering that IL-1β is necessary for the production and regulation of IL-6, the inflammasome might be highly involved in the development of uveitis (Rose-John, 2012). These studies reveal major molecular mechanisms of inflammasome assembly during inflammatory processes. However, there is no clinical evidence for the role of inflammasomes and pyroptosis in human uveitis. Most of the evidence for inflammasome assembly and pyroptosis comes from cellular and animal models. Therefore, further exploration of the role of the inflammasome and pyroptosis in uveitis is required.

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a degenerative disease leading to progressive photoreceptor loss and retinal pigment epithelium (RPE) damage. Inflammation has a role in AMD pathogenesis (Ambati et al., 2013). The NLRP3 inflammasome can be activated by drusen, RPE, complement protein, nucleic acid, oxidative stress, and its products beyond homeostasis-maintaining parainflammation (Tseng et al., 2013). Drusen, as a hallmark of AMD progression, has a rich proteinaceous and potentially damaging composition that triggers potential interactions with the NLRP3 inflammasome, including lipids, lipoproteins, RPE-derived cellular debris, e.g., organelles, melanin granules, lipofuscin, amyloid-β (Aβ), apolipoprotein E, and oxidation byproducts, as well as numerous inflammation-related factors, such as complement components, immunoglobulins, HLA molecules, and acute phase proteins (Hageman et al., 1999; Hageman and Mullins, 1999; Crabb et al., 2002; Sakaguchi et al., 2002; Johnson et al., 2011).

The chronic, sustained pathological simulated stage of the complement system could generate activated complement factors, promoting the formation of a terminal membrane attack complex to intensify the AMD pathological course (Khandhadia et al., 2012). Drusen extracts isolated from AMD donor eye tissues are able to activate the NLRP3 inflammasome, wherein complement factor 1q, as an activated signal, indirectly induces caspase-1 cleavage and increases IL-1β secretion in human monocytic THP-1 cells (Doyle et al., 2012).

Amyloid-β, a component of drusen in AMD eyes, contributes to inflammasome activation and AMD pathological progression. Aβ was discovered to be produced by cleaving the intramembranous proteolysis of amyloid precursor protein as a pathologically neurotoxic peptide, showing specific deposition within drusen in AMD eyes (Haass and Selkoe, 2007). In the RPE cell culture model, Aβ promotes RPE gene expression changes in pathways associated with inflammation and the immune response (Kurji et al., 2010). It also stimulates upregulation of the IL-1β, IL-6, IL-18, caspase-1, and NLRP3 genes in the retina using intravitreal Aβ injection in rat models, which demonstrates NLRP3 inflammasome activation (Liu et al., 2013). Mice with caspase-1 knockout showed increased photoreceptor survival and better protected retinal function with an attenuated inflammatory response (Hanus et al., 2015). With prolonged inflammation of

the retina, RPE cells became enlarged or swollen and exhibited a significant increase in proteolysis of full-length GSDMD in RPE choroidal tissue, and the expression of GSDMD-N, caspase-1, IL-1β, and IL-18 was also significantly increased in intravitreal Aβ-induced AMD models (Gao et al., 2018; Sun et al., 2018). Earlier studies have reported that the cytolytic effect of pyroptosis is mediated by the oligomerization of the N-terminal fragment (N-GSDMD) of GSDMD on the cellular membrane, leading to the formation of cellular rupture pores (Liu X. et al., 2016; Sborgi et al., 2016). Hence, this confirms the activation of the pyroptotic pathway in the Aβ-injected models. These studies illustrated the existence of Aβ-induced AMD models with secretion of mature proinflammatory cytokines, including inflammatory factors (IL-18 and IL-1β), and evidence supporting GSDMD-mediated activation of the pyroptosis pathway in RPE cells, suggesting the importance of pyroptosis in AMD pathogenesis, while inhibiting the target of the pyroptosis pathway may benefit the prognosis of AMD.

Another trigger of inflammasomes is oxidation byproducts. In a normal physiological state, RPE cells phagocytose the deciduous outer segments of photoreceptors. However, the ability of RPE cells to recycle "waste" from photoreceptors significantly decreased with age, resulting in the accumulation of the lipid peroxidation byproduct lipofuscin in the RPE (Schütt et al., 2002). Deposition of lipofuscin in RPE causes lysosomal damage and directly triggers the activation of the NLRP3 inflammasome, as well as other lipid peroxidation end products, such as 4-hydroxynonenal and carboxyethylpyrrole (Doyle et al., 2012; Kauppinen et al., 2012; Tseng et al., 2013).

Diabetic Retinopathy

Chronic inflammation plays a crucial role in diabetic retinopathy (DR) pathology (Tang and Kern, 2011). NLR-mediated inflammatory pathways function via NF-κB signaling, which is a transcription factor that regulates proliferation, angiogenesis, apoptosis, and the immune response (Xie et al., 2010; Motta et al., 2015). Moreover, NF-κB is a known regulator of IL-1β expression and can modulate the activation of the NLRP3 inflammasome (Bauernfeind et al., 2009). High expression of NLRP3, caspase-1, and IL-1β is observed in retinal proliferative membranes and vitreous samples obtained from DR patients with vitreoretinal surgery (Loukovaara et al., 2017; Zhang et al., 2017). STZ-induced diabetic mice with DR features exhibit higher levels of NLRP1 and NLRP3. Consistently, NLRP1$^{-/-}$ DR mice attenuated the expression of ASC and caspase-1 and further markedly decreased retinal NF-κB, IL-1β, and IL-18 levels compared to WT/DR mice (Li et al., 2018). In spontaneously hypertensive rats fed a high-fat diet to form proinflammatory state models, the interaction of TXNIP–NLRP3 and the expression of cleaved caspase-1 and cleaved IL-1β were markedly increased, indicating that TXNIP is required for NLRP3 inflammasome activation and IL-1β release (Mohamed et al., 2014). High glucose–induced TXNIP activation in retinal endothelial and Müller glial cells was also shown to promote oxidative stress (Devi et al., 2013). The TXNIP$^{-/-}$ DR mouse model showed attenuated NLRP3 activation and retinal inflammation (Perrone et al., 2010). In short, NLRP3 plays a significant role in perpetuating inflammation in DR.

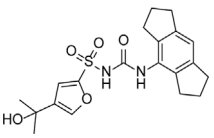
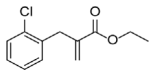
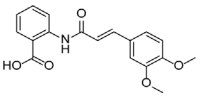
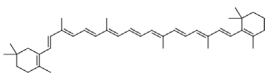
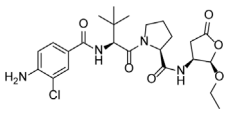
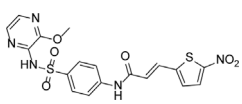
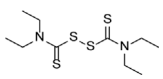
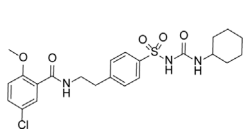
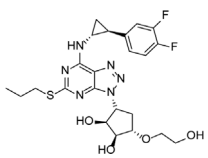
Retinal microglial and Müller cell activation contributes a major role in the onset of inflammatory processes in the early stages of DR. *In vitro*, primary or immortalized Müller cells treated with high glucose all have elevated NLRP3 protein expression (Li et al., 2019). Mouse RGCs treated with high glucose also upregulated NLRP1 and NLRP3 expressions, which were attenuated with the TLR4 inhibitor TAK-242 (Hu et al., 2017; Li et al., 2018). Mitochondria play a significant role in DR, in which damaged mitochondrial DNA (mtDNA) and impaired transcription of mtDNA genes are released into the cytosol, directly activating the NLRP3 inflammasome (Shimada et al., 2012; Kowluru, 2019). Augmented mitophagy/autophagy could lead to mitochondrial dysfunction and excessive ROS production, which is associated extensively with NLRP3 activation (Zhang W. et al., 2019).

Recent studies have demonstrated that a chronic hyperglycemic state induces GSDMD-mediated pyroptosis, which plays an important role in DR development. A high glucose state can stimulate the NLRP3–caspase-1–GSDMD signaling axis, which further promotes plasma membrane pore generation and inflammatory factor (IL-1β and IL-8) secretion in human retinal progenitor cells (HRPs) (Gan et al., 2020b). In addition, activated NLRP3–caspase-1–GSDMD–dependent pyroptosis induces retinal pericyte loss in a concentration-dependent manner in high-glucose surroundings (Gan et al., 2020b). GSDMD gene silencing markedly prevented HRP pyroptosis by inhibiting the NLRP3/caspase-1/GSDMD signaling axis. Another study showed that inhibition of caspase-1 can decrease the GSDMD protein and attenuate the release of IL-1β, IL-18, and LDH in a serum albumin–induced DR environment, illustrating the appearance of caspase-1–dependent pyroptosis in HRPs (Yu et al., 2021). P2X7R, as an oxidative stress and metabolic sensor, contributes to NLRP3 inflammasome activation and is distributed in the retinal microvascular epithelium, neural cells, and macrophages. P2X7R indirectly affected advanced glycation end product–induced retinal microvascular endothelial cells via the NLRP3 inflammasome. Meanwhile, the inhibition of P2X7R significantly reduced the expression and activation of the NLRP3 inflammasome, indicating that the initiation of NLRP3 inflammasome activation is dependent on the activation of P2X7R mediated by AGE–BSA–induced diabetic retinopathy (Yang K. et al., 2020).

THE PYROPTOSIS INHIBITORS IN OCULAR DISORDERS

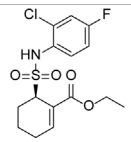
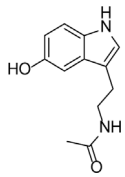
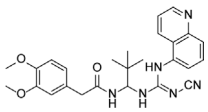
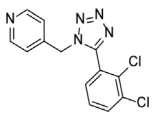
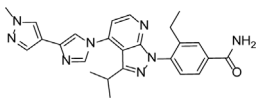
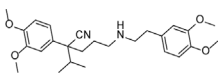
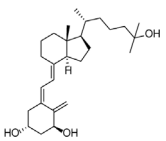
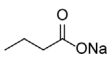
Inflammasomes involved in the processes of ocular pathogenesis play crucial roles in various eye diseases. Although the exact role of the inflammasome and its interacting proteins in human eye disease has not been fully elucidated, small-molecule inhibitors targeting the inflammasome and the pyroptosis process in human eye disease could help to design effective therapies assisting clinical practice. Excitingly, several pyroptosis inhibitors have been reported, including NLRP3 inhibitors, caspase-1 inhibitors, GSDMD inhibitors, and indirect inhibitors targeting inflammasome components or related signaling events.

TABLE 1 | Pyroptosis inhibitors in eye disease.

Inhibitor	Structure	Mechanism	Animal models of eye disease	References
NLRP3 Inhibitors				
MCC950		Specifically, it inhibits canonical and non-canonical NLRP3 inflammasome activation *in vitro*. Its central sulfonylurea group interacts with the Walker A motif of the NLRP3 nucleotide-binding domain	Ocular hypertension Oxygen-induced ischemic retinopathy Photo-oxidative damage-induced retinal degeneration	Zhang et al. (2019b), Sui et al. (2020), Wooff et al. (2020)
INF39		NACHT ATPase inhibitor. Reduces NLRP3, IL-1β, and caspase-1 expressions and attenuates the pyroptosis level	-	Huang et al. (2020)
Tranilase		Binds NACHT domain and inhibits the NLRP3–NLRP3 interaction and the expression of cytokines and chemokines	-	Liu et al. (2016b), Yumnamcha et al. (2019)
β-Carotene		Protects the eye from oxidative stress, apoptosis, mitochondrial dysfunction, and inflammation	-	Camelo et al. (2020), Johra et al. (2020)
Caspase-1 Inhibitor				
VX-765		Decreases caspase-1 and inhibits the mature IL-1β and IL-18 and caspase-1–mediated pyroptosis	-	Lin et al. (2018), Li et al. (2021)
GSDMD Inhibitors				
Necrosulfonamide		Inhibits the expression of NLRP3 and GSDMD and reverses the effects of high glucose on ARPE-19 cell proliferation	-	Xi et al. (2020)
Disulfiram		Blocks GSDMD pore formation, reduces the proportion of pyroptotic cells, and prevents cells against hyperosmotic stress–induced cytotoxicity	Diabetic retinopathy (OLETF rats) Ocular hypertension induced by rapid infusion of 5% glucose solution Endotoxin-induced uveitis	Ito et al. (2010), Kanai et al. (2010), Kanai et al. (2012), Deguchi et al. (2020), Zhang et al. (2021)
Other Indirect Inhibitors of the Inflammasome				
Glyburide		Inhibition of NLRP3 inflammasome activation and release of IL-1β by changing ion channel activity	Alu RNA–mediated geographic atrophy	Kerur et al. (2013), Gan et al. (2020a)
Ticagrelor		Induces degradation of Cl^- channel to block chloride outflow and inhibits the interaction of NLRP3 with ASC to inhibit activation of the NLRP3 inflammasome	ABCA4$^{-/-}$ mouse model of retinal degeneration	Lu et al. (2018), Lu et al. (2019), Huang et al. (2021)

(Continued on following page)

TABLE 1 | (*Continued*) Pyroptosis inhibitors in eye disease.

Inhibitor	Structure	Mechanism	Animal models of eye disease	References
TAK-242		TLR4 inhibitor, which decreases the expression levels of TLR4 downstream signaling molecules (MyD88, NF-κB, TRAF6, NLRP3) and inflammatory factors (IL-1β and IL-18)	-	Hu et al. (2017)
N-Acetylserotonin		TLR4 inhibitor, which alleviates the expression of IL-1β in retinal ischemia–reperfusion rats via the TLR4/NF-κB/NLRP3 pathway	-	Liu et al. (2021)
A740003		P2X7R inhibitor, which inhibits the activation of NLRP3 inflammasome and phosphorylation of IKBα	Oxidized low-density lipoprotein model (retinal inflammation and neovascularization)	Yang et al. (2021)
A438079		P2X7R inhibitor, which inhibits P2X7R–NLRP3 pathway reduced NLRP3 inflammasome expression	Ocular hypertension N-Methyl-D-aspartic acid–induced retinal injury	Sakamoto et al. (2015), Zhang et al. (2019b)
TAS-116		HSP90 inhibitor, which prevents the activation of caspase-1, subsequently reducing the release of mature IL-1β	-	Ranta-Aho et al. (2021)
Xanthone		Inhibits cross-link ASC oligomerization, endogenous NLRP3 oligomerization, the cleavage of GSDMD, and the release of IL-1β	LPS-induced keratitis	Cui et al. (2021)
Verapamil		Inhibition of thioredoxin-interacting protein and inflammasome assembly	STZ-induced diabetic retinopathy	Eissa et al. (2021)
Calcitriol		Alleviates hyperosmotic stress–induced corneal epithelial cell damage through inhibiting the NLRP3–ASC–caspase-1–GSDMD pyroptosis pathway	Corneal wound in STZ-induced diabetic mice	Wang et al. (2021b), Zhang et al. (2021)
Butyrate		Decreased NLRP3, caspase-1, and IL-1β mRNA transcripts and NLRP3 protein expression	Corneal alkali burn	Bian et al. (2017)
Epigallocatechin-3-gallate		Inhibits the ROS/TXNIP/NLRP3 inflammasome axis to reduce ROS accumulation, NLRP3 inflammasome activation, Müller cell proliferation, and production of the pro-angiogenic factors	STZ-induced diabetic retinopathy	Du et al. (2020)

OLETF rats, Otsuka Long Evans Tokushima Fatty rats; LPS, lipopolysaccharide; STZ, streptozotocin.

However, some pyroptosis inhibitors have yet to be tested in human or animal models of eye disease, and even some inhibitors tested in eye disease have a potential risk due to their incompletely elucidated precise target of inhibitory mechanisms. Furthermore, in the process of eye disease development, pyroptosis may have different weight values in different stages. For example, at the early stage of retinal neovascularization formation, the expression of NLRP3 and caspase-1 begins to increase and is significantly exacerbated later, while it shows a relatively low expression level at the stage of neovascular regression (Sui et al., 2020). Similarly, the expression of activated caspase-1 and activated IL-1β was also inconsistent at the time point. These factors may be associated with various factors, including the functional efficiency of activated caspase-1, pro-IL-1β expression, the degradation degree of activated IL-1β, and other complex regulatory

mechanisms in biological individuals. These factors might determine the time point of treatment to intervene in disease development. Inhibition of NLRP3, caspase-1, or GSDMD specifically to reverse the activation pattern of pro-IL-1β or pro-caspase-1 might have a protective effect on eye diseases. Here, we summarize the pyroptosis inhibitors that have been applied to animal models of eye disease (**Table 1**). MCC950, as the most potent and specific NLRP3 inhibitor, specifically inhibits canonical and non-canonical NLRP3 inflammasome activation in both human and mouse macrophages *in vitro* without impacting the other inflammasomes and strongly attenuates the release of inflammatory factors both *in vivo* and *in vitro* (Coll et al., 2019). INF39 inhibits NLRP3 inflammasome activation by inhibiting NLRP3 ATPase enzyme activity but with poor specificity (Cocco et al., 2017). The marketed drug "tranilast" directly binds to NLRP3 to inhibit NLRP3 polymerization and activation (Huang et al., 2018). β-Carotene was discovered to bind to the PYD of NLRP3 to block the direct interaction of NLRP3 and ASC to inhibit inflammasome complex formation (Yang G. et al., 2020). The caspase-1 inhibitor VX-765 could inhibit the maturation of the two cytokines IL-1β and IL-18 and reduce the secretion of inflammatory cytokines and chemokines (Wannamaker et al., 2007). The GSDMD inhibitors necrosulfonamide and disulfiram covalently bind to Cys^{191} on GSDMD to block pore formation and inflammatory factor release and inhibit pyroptosis progression (Rathkey et al., 2018). Other indirect inhibitors of the inflammasome, including related ion channel inhibitors, TLR4 inhibitors, P2X2R inhibitors, HSP90 inhibitors, and some small-molecule inhibitors impacting related signaling events, have been tested in eye disease models *in vivo* and *in vitro*. With thorough studies of the inflammasome and pyroptosis in various eye diseases and an increased number of individuals affected by inflammatory disease, direct and specific pyroptosis inhibitors will boost future clinical translation, epitomizing the use of precision medicine in inflammasome-related diseases.

CONCLUSION

In this review, we summarize the mechanisms and functions of pyroptosis and inflammasomes in various ocular disorders. Clearly, our current knowledge of pyroptosis occurring in eye disease is just the tip of the iceberg. Many questions remain to be answered. Inflammasomes play a crucial role in the pathogenesis of eye disease, and pyroptosis could aggravate inflammation and cellular lysis death. However, most of the evidence available on inflammasome assembly and pyroptosis occurrence in eye diseases derives from *in vitro* and experimental models in animals, and inhibitors targeting inflammasomes and pyroptosis are currently being tested at the level of preclinical trials. It is essential to understand the role of inflammasomes and pyroptosis in human eye diseases and whether the data from experimental models are translatable to suit humans. In sum, we highlighted the increasing evidence about the role of pyroptosis in various eye diseases. In the future, the application of pyroptosis inhibitors may help us design new classes of targeting therapeutic agents to combat eye diseases.

AUTHOR CONTRIBUTIONS

YZ and YJ drafted the manuscript. YZ, YJ, XL, and SG edited the manuscript. YZ drew the figures. YJ prepared the table. XL, MZ, and SG reviewed the manuscript. SG and NZ searched the databases. JD helped to draw the figures. MZ conceived the manuscript.

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Human Olfactory Mesenchymal Stem Cells Are a Novel Candidate for Neurological Autoimmune Disease

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Background: Human olfactory mesenchymal stem cells (OMSC) have become a novel therapeutic option for immune disorder or demyelinating disease due to their immunomodulatory and regenerative potentials. However, the immunomodulatory effects of OMSC still need to be elucidated, and comparisons of the effects of different MSCs are also required in order to select an optimal cell source for further applications.

Results: In animal experiments, we found neural functional recovery and delayed EAE attack in the OMSC treatment group. Compared with umbilical cord–derived mesenchymal stem cells (UMSC) treatment group and the control group, the OMSC treatment group had a better neurological improvement, lower serum levels of IFN-γ, and a lower proportion of CD4+IFN-γ+ T splenic lymphocyte. We also observed OMSC effectively suppressed CD4+IFN-γ+ T cell proportion *in vitro* when co-cultured with human peripheral blood–derived lymphocytes. The OMSC-mediated immunosuppressive effect on human CD4+IFN-γ+ T cells was attenuated by blocking cyclooxygenase activity.

Conclusion: Our results suggest that OMSC treatment delayed the onset and promoted the neural functional recovery in the EAE mouse model possibly by suppressing CD4+IFN-γ+ T cells. OMSC transplantation might become an alternative therapeutic option for neurological autoimmune disease.

Keywords: experimental autoimmune encephalomyelitis, olfactory mucosa–derived mesenchymal stem cells, immunomodulation, mesenchymal stem cell therapy, neuroinflammation

Abbreviations: CBA, cytometric bead array; CD29/34/44/45/73/90/105/166, cluster of differentiation 29/34/44/45/73/90/105/166; CNS, central nervous system; COX, cyclooxygenase; DMT, disease-modifying therapy; EAE, experimental autoimmune encephalomyelitis; IFN-γ, interferon γ; IL-6/10, interleukin-6/10; LFB, luxol fast blue; MOG, myelin oligodendrocyte glycoprotein; MSC, mesenchymal stem cells; OMSC, human olfactory mucosa–derived mesenchymal stem cells; PG, prostaglandin; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor α; UMSC, human umbilical cord–derived mesenchymal stem cells.

INTRODUCTION

In recent years, mesenchymal stem cell (MSC)–based therapy has become a new clinical approach for treating various diseases, but the specific mechanisms of action are still not fully elucidated. Previous studies have demonstrated that different sources of MSCs can improve the neurological function of various animal models through immunomodulation (Bai et al., 2009; Peron et al., 2012; Payne et al., 2013; Donders et al., 2015; Bravo et al., 2016; Shu et al., 2018; Ahmadvand Koohsari et al., 2021). However, the effects of MSCs differ in terms of the tissue source. Payne et al. compared the therapeutic effects of human bone marrow–derived MSC (BMSC), adipose-derived MSC, and umbilical cord–derived MSC (UMSC) and found that the immunosuppressive effects among these MSCs were different (Payne et al., 2013). Thus, these findings suggested that seeking the most appropriate MSC was of great significance.

The human olfactory mucosa is a tissue that promotes neurogenesis throughout life (Johnstone et al., 2015), and its cell components play an important role in repairing damaged olfactory nerves (Schwob, 2002). Olfactory mucosa–derived mesenchymal stem cells (OMSC) lay in the lamina propria of the olfactory mucosa (Lindsay et al., 2020). They present immunoregulatory and regenerative effects in animal experiments, which indicates that they are potential candidates for treating related diseases (Lindsay et al., 2013; Khankan et al., 2016; Rui et al., 2016). Recent research showed that OMSC not only shared common characteristics with other MSC (Lindsay et al., 2013; Rui et al., 2016), such as self-renewal, multi-differentiation, and immunoregulatory potential, but also had extra advantages in promoting neuronal affinity (Veron et al., 2018) and myelin regeneration (Lindsay et al., 2017). Lindsay et al. showed that human OMSC promoted the neural functional recovery *via* enhancing immune regulation and myelin repair in mice with spinal cord injury (Lindsay et al., 2016; Lindsay et al., 2017). For an *in vitro* study, Di Trapani et al. had demonstrated that OMSC had immunomodulatory effects on T cells, but its molecular mechanisms remain unknown (Di Trapani et al., 2013). Thus, the specific mechanisms of OMSC still need to be further elucidated.

In order to study the effect and immunoregulatory mechanism of OMSC, we established an experimental autoimmune encephalomyelitis (EAE) mice model and a co-culture system of human lymphocytes and OMSC. The EAE model is an autoimmune disease model mediated by CD4+ T helper (Th) cells, characterized by the local infiltration of T lymphocytes in the CNS, and recognized as a classical animal model to study the autoimmune disease (Gerdoni et al., 2007; Robinson et al., 2014). A previous study had demonstrated that UMSC ameliorated EAE by regulating the inflammatory response (Liu et al., 2013). Thus, in this study, we aimed to compare the therapeutic effects between UMSC and OMSC to select the potential seed cells and demonstrate the immunomodulatory effects of OMSC.

RESULTS

Characteristics of OMSC and UMSC

OMSC and UMSC isolated from the human olfactory mucosa and human umbilical cord appeared as spindle-shaped cells and presented adipogenic and osteogenic differentiation potential as determined by Oil Red O and Alizarin Red S staining, respectively (**Figure 1A**). Phenotype analysis by flow cytometry showed that OMSC expressed CD29, CD44, CD73, CD90, CD105, and CD166 but did not express CD34 and CD45 (**Figure 1B**).

OMSC Promotes Neural Functional Recovery *in vivo*

To check the therapeutic effect of OMSC and UMSC on EAE mice, their neurological function was evaluated daily, as shown in **Figure 2A**. During 31 days of EAE process, the neurological function of the mice treated with OMSC was significantly improved ($p_{\text{OMSC-UMSC}} = 0.001$,$p_{\text{OMSC-PBS}}<0.001$, **Figure 2B**). On days 16 to 20, daily clinical score differences among the OMSC, UMSC, and PBS treatment groups were statistically significant, showing the extraordinary therapeutic effects of OMSC. The day of EAE onset in each group was statistically significant (**Figure 2C**, $F = 5.942$, $p = 0.015$). The onset of the OMSC treatment group was later than that of UMSC treatment and control groups (**Figure 2C**; **Table 1**, $p_{\text{OMSC-PBS}} = 0.005$, $p_{\text{OMSC-PBS}} = 0.024$). The incidence within 30 days in OMSC treatment, UMSC treatment, and control groups were 66.7, 100, and 100%, respectively (**Table 1**), and the mortality were 16.67, 16.67, and 42.86% (**Table 1**), respectively. In conclusion, EAE mice had improved neurological function, delayed onset time, and reduced incidence and mortality rate after OMSC intervention.

Spinal cord sections of mice in OMSC, UMSC, and PBS treatment groups were stained with HE and LFB (**Figure 2D**), respectively. It was found that compared with PBS and UMSC groups, the inflammatory and demyelinating areas of the OMSC group showed a downward trend. However, there was no statistical significance in the histological inflammation score and demyelination score among OMSC, UMSC treatment groups, and control group (data not shown).

OMSC and UMSC Reduced the Serum Level of IFN-γ *in vivo*

In order to study the effect of OMSC and UMSC on serum inflammatory factors in EAE mice, serum IFN-γ, TNF-α, IL-6, and IL-10 levels were detected by CBA. Differences of serum IFN-γ levels between OMSC treatment, UMSC treatment, and control groups were statistically significant (**Figure 3**, $p_{\text{IFN-}\gamma} = 0.004$). The serum levels of IFN-γ in UMSC treatment and OMSC treatment groups were lower than those in the control group (**Figure 3**, $p_{\text{UMSC-PBS}} = 0.002$, $p_{\text{OMSC-PBS}} = 0.003$). Serum levels of TNF-α, IL-10, and IL-6 in the three groups were not statistically significant (**Figure 3**, $p_{\text{IL-10}} = 0.120$, $p_{\text{TNF-}\alpha} = 0.085$, $p_{\text{IL6}} = 0.646$).

OMSC Suppressed CD4+IFN-γ+ T Cells *in vitro* and *in vivo*

We have found that OMSC better improved neurological function and reduced the serum IFN-γ level in EAE mice compared with UMSC. Since IFN-γ is a crucial cytokine secreted by Th1

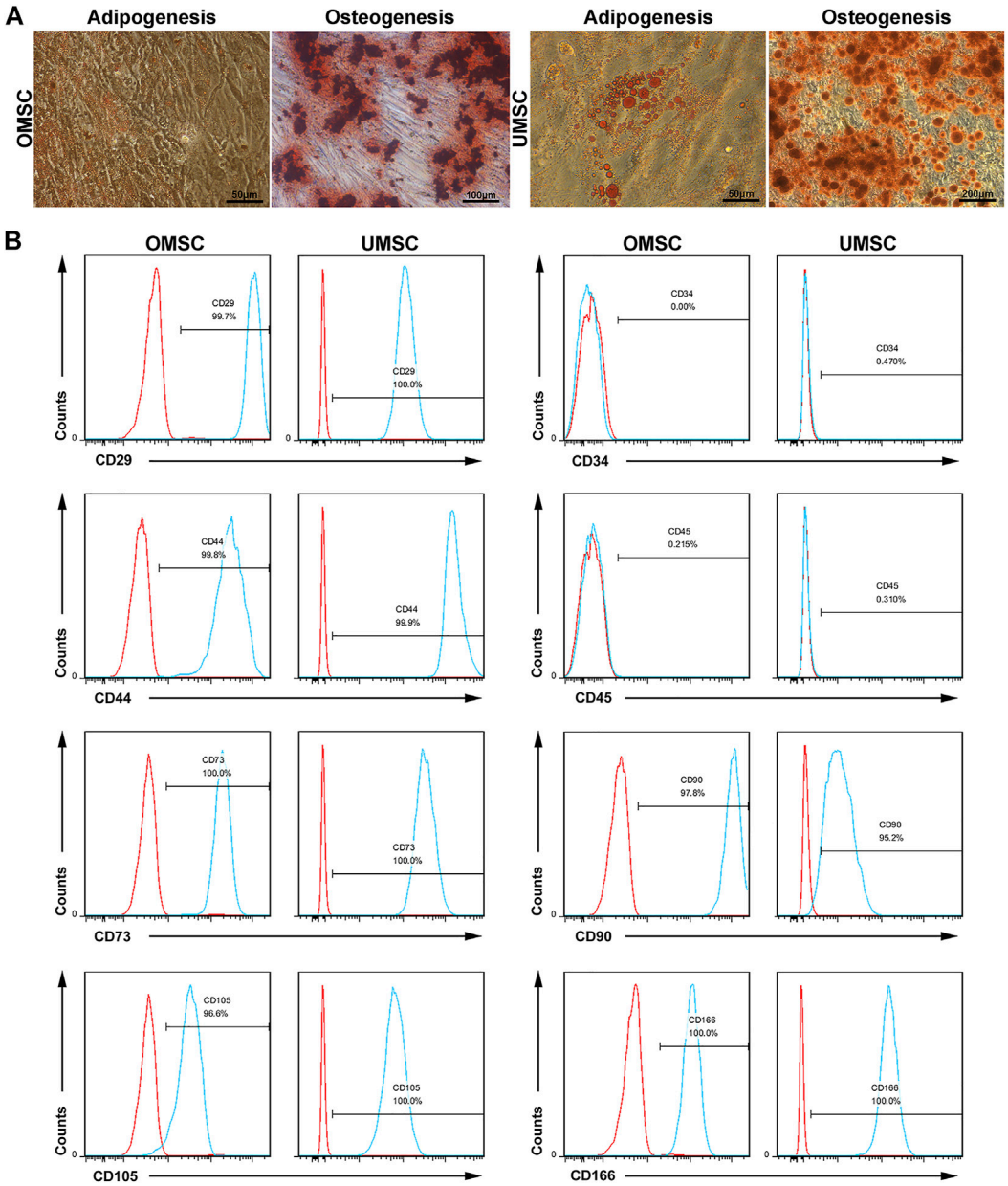

FIGURE 1 | Differentiation potential and phenotypes of the OMSC and UMSC: **(A)** MSCs captured by the microscope. Alizarin Red S chelates with calcium ions to form a red or purplish red complex, which represents the osteogenic formation of OMSC and UMSC. Oil Red O colorizes the lipid inside the MSCs and appears as red lipid droplets, which represents adipogenic formation of OMSC and UMSC. **(B)** Phenotypes of OMSC, including CD29, CD34, CD45, CD44, CD73, CD90, CD105, and CD166, were detected by flow cytometry.

lymphocytes, and Th1 lymphocytes, characterized as CD3+CD4+IFN-γ+ cells, play a pivotal role in the development of EAE; we assumed that early preventive treatment of OMSC could downregulate the proportion of CD4+IFN-γ+ T cells. We co-cultured UMSC, OMSC, and human peripheral blood mononuclear cells (PBMCs) and stimulated them with MOG_{35-55} for 48 h to observe the immunomodulatory effects of UMSC and OMSC under MOG_{35-55} stimulation. Since serum TNF-α showed a decreasing trend in EAE mice receiving OMSC treatment, and TNF-α is also an important marker for EAE, CD4+IFN-γ+ T cell proportion and CD4+TNF-α+ T cell proportion were all detected by flow cytometry (**Figure 4A**). The proportion of CD4+IFN-γ+ T cells (**Figure 4B**) and CD4+TNF-α+ T cells (**Figure 4C**) significantly decreased in MOG_{35-55}-stimulated PBMC co-cultured with UMSC and OMSC compared to MOG_{35-55}-stimulated PBMC cultured alone. Meanwhile, OMSC exhibited a stronger inhibitory effect on CD4+TNF-α+ T cells than UMSC (**Figure 4C**).

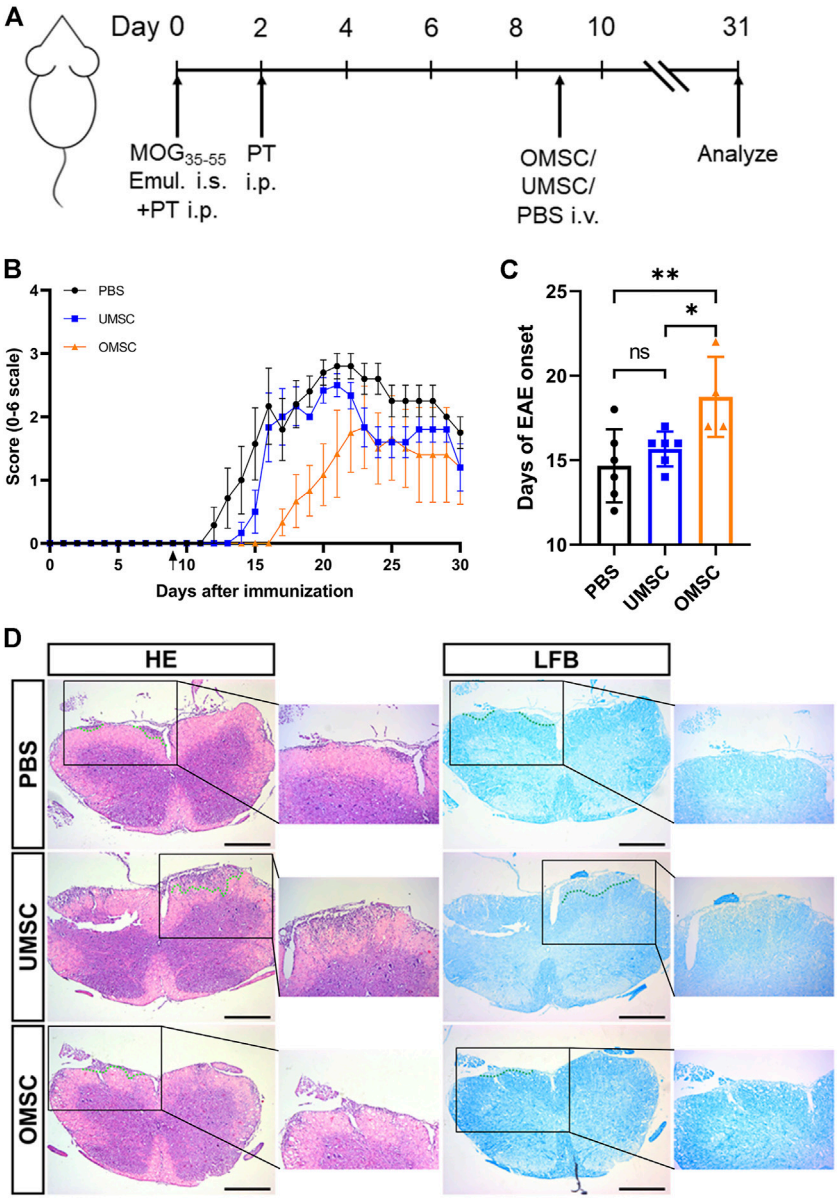

FIGURE 2 | Preventive effects of early intervention of OMSC and UMSC treatments on EAE mice: **(A)** Model diagram of the experimental process. **(B)** Effects of preventive treatment of OMSC and UMSC on the neurological function of EAE mice (n = 6–7). **(C)** Effects of preventive treatment of OMSC and UMSC on the onset of EAE in mice. **(D)** HE staining and LFB staining of the spinal cord of EAE mice. i.s.: subcutaneous injection; i.p.: intraperitoneal injection; i.v.: intravenous injection. Bar: 400 μm *p < 0.05; ns: the difference was not statistically significant.

For the *in vivo* study, the mice underwent OMSC preventive treatment, and the control group was sacrificed at the day of EAE onset, and splenic lymphocyte analysis was performed. The proportion of CD4+IFN-γ+ T cells (Th1 cells) in the PBS and OMSC treatment groups was analyzed (**Figure 4D**). The results showed that the percentages of CD4+IFN-γ+ T cells in the OMSC treatment and PBS groups were 7.61 ± 1.96 % and 28.97 ± 0.54 %, respectively, and the difference was statistically significant (**Figure 4E**, t = 10.51, p < 0.001). Interestingly, although EAE induced by MOG_{35-55} is mainly CD4+ T cell–dependent, the proportions of CD8+T lymphocytes were also lower in the OMSC treatment group, indicating a multi-immunomodulatory potential of the OMSC. There was no significant difference in the proportions of CD4+T lymphocytes and $CD8^+$ IFN-γ+ T lymphocytes between the two groups. The above mentioned studies highlighted the immunomodulatory effects of OMSCs.

OMSC Inhibited Human CD4+IFN-γ+ T Cells *in vitro* Partially *via* COX2 Activity

In order to study whether OMSC can regulate the T-cell subsets of human lymphocytes and their mechanism, we constructed a co-

TABLE 1 | Clinical characteristic of EAE mice treated with MSCs.

	OMSC	UMSC	PBS
Incidence (%)	4/6	6/6	7/7
	66.7%	100%	100%
Mortality (%)	1/6	1/6	3/7
	16.67%	16.67%	42.86%
Mean onset day (d)	18.75 ± 1.18	15.67 ± 0.42	14.67 ± 0.88
(Interval)	(14 ~ 21)	(14 ~ 17)	(12 ~ 18)
Average score	0.58 ± 0.12	0.94 ± 0.18	1.24 ± 0.20

culture system of OMSC and human PBMC and detected the proportion of CD4+IFN-γ+ T cells by flow cytometry (**Figure 5**). The results showed that after 48 h of co-culture, the proportion of CD4+IFN-γ+ T cells in the single culture group (**Figure 5A**) was 6.69 ± 0.39 % and that in the co-culture group (**Figure 5B**) was 1.14 ± 0.24 %. There was a significant difference in the proportion of CD4+IFN-γ+ T cells between the two groups (**Figure 5E**, $F = 119.60$, $p < 0.0001$), which confirmed the immunomodulatory effects of OMSC.

Besides, we used indomethacin and TGF-β1 inhibitors to elucidate the possible mechanism of OMSC that inhibited human CD4+IFN-γ+ T lymphocytes. Flow cytometry (**Figures 5A–E**) was used to detect the proportion of CD4+IFN-γ+ T cells in different groups. The results showed that the percentage of CD4+IFN-γ+ T cells in the COX inhibitor group (indomethacin) was 2.21 ± 0.12 % (**Figure 5C**) and that in the TGF-β1 inhibitor group was 1.25 ± 0.07 % (**Figure 5D**), respectively. The ratio of CD4+IFN-γ+ T cells in the single culture group was significantly higher than that in the OMSC co-culture group (**Figure 5E**, $p < 0.001$); the ratio of CD4+IFN-γ+ T cells in the COX inhibitor group was higher than that in the OMSC co-culture group (**Figure 5E**, $p = 0.014$). TGF-β1 inhibition has no obvious effect on OMSC-mediated inhibition of CD4+IFN-γ+ T cells. Taken together, these results suggest that OMSCs decreased the proportion of CD4+IFN-γ+ T cells *in vitro* partially *via* COX activity.

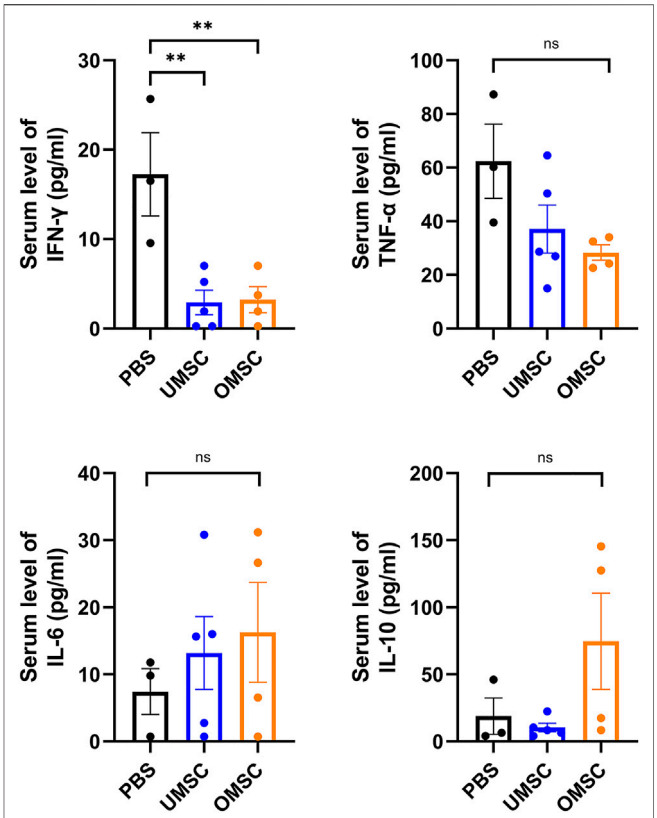

FIGURE 3 | Effects of early intervention of OMSC and UMSC treatments on serum inflammatory cytokines in EAE mice: serum IFN-γ, TNF-α, IL-10, and IL-6 levels in the OMSC treatment group ($n = 4$), UMSC treatment group ($n = 5$), and PBS group ($n = 3$) of EAE mice. *$p < 0.05$, **$p < 0.01$, ns: the difference was not statistically significant.

DISCUSSION

In our study, OMSC treatment before the EAE onset effectively increased the neurological improvement in EAE mice compared with PBS and UMSC, followed by a reduced serum level of IFN-γ. We also found that OMSC effectively suppressed CD4+IFN-γ+ T cell proportion *in vivo* and *in vitro*, and this effect is closely related to the COX pathway.

OMSC are a potential candidate for clinical application for their abilities in immunoregulation. At present, there are more than 10 clinical research reports on the olfactory mucosa and its cellular components, which preliminarily confirmed the safety of olfactory mucosa component application (Mackay-Sim et al., 2008) and the potential therapeutic effect (Wu et al., 2012; Chen et al., 2014). Andrews et al. (2016) evaluated the safety of the transnasal endoscopy olfactory mucosa sampling, suggesting that nasal endoscopic extraction of the olfactory mucosa does not affect nasal function and olfaction (IIa evidence). Therefore, autologous transplantation of OMSC is feasible, and it is necessary to further study the efficacy and mechanism of OMSC.

Various studies have shown that OMSC achieve therapeutic role by promoting immune regulation: Lindsay et al. found that the therapeutic effect of OMSC on demyelinating disease might be better than that of BMSC (Lindsay and Barnett, 2017). Meanwhile, Rui et al. (2016) reported that OMSC inhibited the proportion of Th1 cells and IFN-γ secretion in the spleen and suppressed autoimmune arthritis of mice. In addition, Lindsay et al. found that OMSC co-cultured *in vitro* promoted the polarization of SD rat microglia to an anti-inflammatory phenotype (Lindsay et al., 2016). As mentioned before, OMSC may have a better therapeutic effect on immune-related diseases.

We used human OMSC and UMSC for preventive treatment on EAE mice at day 9 and found that the neurological function of mice in the OMSC preventive treatment group was better than that in the UMSC preventive treatment and control groups. The neurological function of the UMSC preventive treatment group showed a certain improvement trend compared with that of the control group, which was consistent with the previous research. In addition, compared with the control group, the mortality of

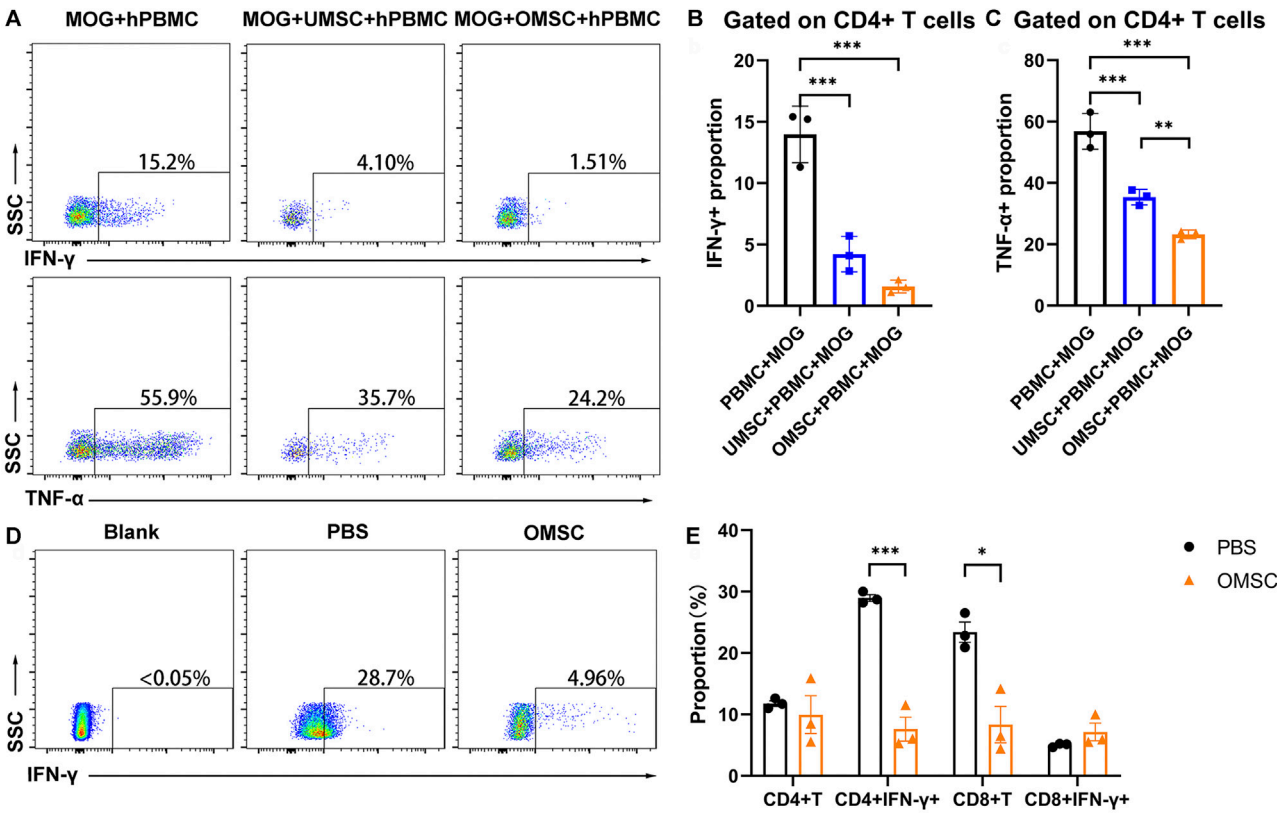

FIGURE 4 | OMSC treatment reduced CD4+IFN-γ+ T lymphocytes: **(A)** Proportion of CD4+IFN-γ+ T cells (Th1) and CD4+TNF-α+ T cells in human lymphocyte single culture, UMSC, and OMSC co-culture groups. **(B, C)** CD4+IFN-γ+ T cells and CD4+TNF-α+ T cells of the co-culture system. $^{**}p < 0.01$ and $^{***}p < 0.001$. **(D)** Proportion of CD4+IFN-γ+ T cells in mice splenic lymphocytes in the OMSC treatment group and PBS group. **(E)** CD4+T cells and CD8+T cells and their IFN-γ expression by splenic lymphocytes in the mice of the OMSC treatment group and PBS group ($n = 3$). $^{*}p < 0.05$ and $^{***}p < 0.001$.

the two MSC preventive treatment groups was lower, and the OMSC prevention group had the lowest incidence (66.7%) among the three groups within 30 days. We also found that the preventive treatment of OMSC and UMSC effectively delayed the onset of EAE in mice. These results also confirmed that the preventive treatment of OMSC improved the neurological function of mice and prevented the occurrence of EAE in mice.

Bai et al. found that stem cells played a therapeutic role mainly through a paracrine secretion–related immune regulation, rather than self-regeneration (Bai et al., 2012). Th1 lymphocytes, which are known as CD4+IFN-γ+ T cells and characterized by the secretion of IFN-γ, have been proven to be closely linked to the pathogenesis of EAE (Cummings et al., 2018). Li et al. found that MSCs could promote the recovery of neural function in EAE mice by increasing the proportion and function of CD5+IL-10 + B cells (Li et al., 2019). Other studies have shown that Th17 lymphocytes were also one of the main inflammatory cells involved in the pathogenesis of MS and EAE mice (Waisman et al., 2015; Liu et al., 2019), and the secretion of IL-17 could promote astrocytes to produce IL-6 and aggravate the inflammatory infiltration of EAE mice (Shan et al., 2017). Some researchers have proposed that MSCs might inhibit the proliferation of peripheral pathogenic T lymphocytes or Th1 lymphocytes (Gerdoni et al., 2007; Bai et al., 2009). These results suggest that inflammatory factors such as IFN-γ, TNF-α, IL-6, and IL-10 may participate in the recovery progress of the neural function in EAE mice mediated by MSCs. In our study, serum levels of IFN-γ, TNF-α, IL-17, IL-6, and IL-10 in OMSC preventive treatment, UMSC preventive treatment group, and control groups were analyzed. However, the serum level of IL-17 in all groups could not be detected accurately using CBA due to its low concentration. It was found that the serum IFN-γ level of EAE mice was affected by OMSC or UMSC preventive treatment. The levels of serum IFN-γ in the OMSC and UMSC preventive treatment groups were lower than those in the control group. There were no significant differences in the serum levels of TNF-α, IL-6, and IL-10 among these three groups, but the serum TNF-α level of OMSC and UMSC showed a certain downward trend ($p_{\text{TNF-α}} = 0.089$). Combined with previous studies and our results,

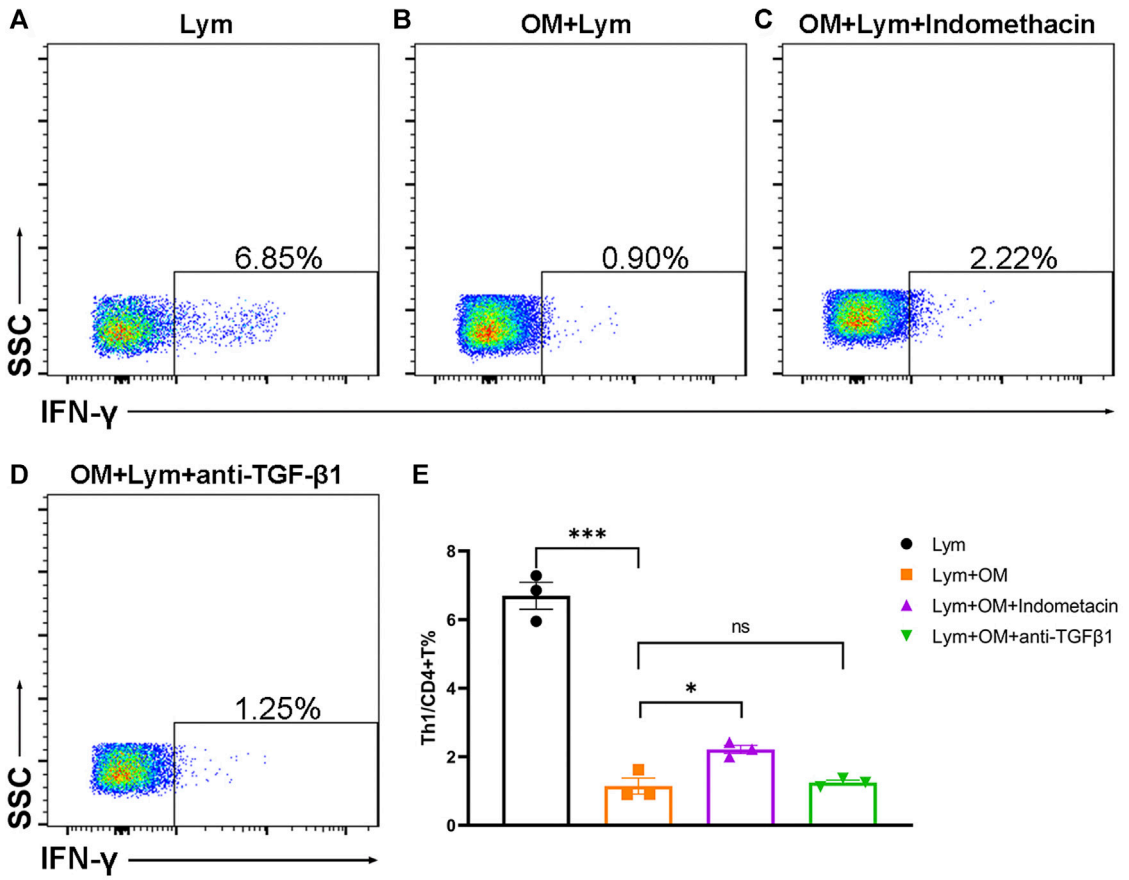

FIGURE 5 | OMSC inhibits the CD4+IFN-γ+ T lymphocyte *in vitro*: **(A–D)** CD4+IFN-γ+ T cell ratio in the human lymphocyte single culture group **(A)**, OMSC co-culture group **(B)**, COX inhibitor group **(C)**, and TGF-β1 inhibitor group **(D)**. **(E)** Difference of CD4+IFN-γ+ T cell ratio among the lymphocyte single culture group, OMSC co-culture group, COX inhibitor group, and TGF-β1 inhibitor group ($n = 3$). Lym: lymphocytes; OM: OMSC. $^{*}p < 0.05$; $^{***}p < 0.001$; ns: the difference was not statistically significant.

OMSC preventive therapy may improve the neurological function of EAE mice by suppressing CD4+IFN-γ+ T cells and its IFN-γ secretion.

Recent studies have shown that transforming growth factor-β1 (TGF-β1) is an important factor acting on the immunosuppressive function of MSCs (Wu et al., 2020). The transforming growth factor-β (TGF-β) family has extensive and diverse effects on metazoan cells and plays a crucial role in regulating immune responses (Liénart et al., 2018). Among family members such as TGF-β1, TGF-β2, and TGF-β3 isoforms, TGF-β1 is the main subtype secreted by immune cells (Travis and Sheppard, 2014).

Di Trapani et al. demonstrated that the immunosuppressive effect of other stem cells (leptomeningeal-derived stem cells) on T cells was also related to the cyclooxygenase (COX) pathway (Di Trapani et al., 2013). The arachidonic acid pathway is a core pathway in the process of human immune regulation. Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin (PG), standing as the key enzyme in the reaction (Mahboubi Rabbani and Zarghi, 2019). It was believed that PG was mainly involved in the regulation of acute inflammation, but some studies have shown that PG aggravates the progress of arthritis and inflammatory bowel disease and also participates in the occurrence and development of chronic inflammation (Wang and DuBois, 2018; Yao and Narumiya, 2019). Besides, Tonby et al. demonstrated that COX inhibitors effectively inhibited the activation of Th1 cells and reduced inflammatory response (Tonby et al., 2016). At present, the mechanism of OMSC regulating human lymphocytes remains unclear. We assumed that the suppressive effect of OMSC on human CD4+IFN-γ+ T cells (Th1 cells) might be related to TGF-β1 or COX.

In order to elucidate the mechanism of the immunosuppressive effect of OMSCs on CD4+IFN-γ+ T cells, we used indomethacin, a COX inhibitor, or TGF-β1 inhibitor to intervene the OMSC and lymphocyte co-culture system, respectively. After COX inhibition, the proportion of CD4+IFN-γ+ T cells in the COX inhibitor group and co-culture group was statistically different, which indicated that the COX pathway was involved in the inhibitory effect of OMSC on CD4+IFN-γ+ T lymphocytes, but the TGF-β1 inhibitor had no similar effect. This study found that the regulation effects of OMSC improved neurological function and reduced IFN-γ

secretion in EAE mice. The downregulation of IFN-γ by OMSC may be related to the inhibition of CD4+IFN-γ+ T cells, and the COX pathway may be involved in this progress.

In this study, although the role of the COX pathway in the regulation of human CD4+IFN-γ+ T cells by OMSC was explored, the effects of COX-1 and COX-2 inhibitors and their downstream pathway were not further studied. In addition, the immunomodulatory effect of OMSC on CD4+IFN-γ+ T lymphocytes in the spinal lesions and spleen of EAE mice, as well as the immunomodulatory effect of OMSC on human lymphocytes and the related mechanisms, need to be further studied, which is also an essential way to achieve clinical application of OMSC.

CONCLUSION

In conclusion, we have demonstrated that OMSC transplantation delayed the onset and promoted neural recovery in the EAE model. OMSC modulate CD4+IFN-γ+ T cells, and the COX pathway is involved in the immunomodulatory progress. Thus, OMSC are a potential candidate for the treatment of neurological autoimmune disease.

METHODS

Acquisition of Human OMSC

The olfactory mucosa of healthy donors was obtained through a nasal endoscope under local infiltration anesthesia with 2% lidocaine. The olfactory mucosa was cut with sterile instruments, digested with collagenase IV at 37°C for 1 h, and then passed through a 70-μm cell sieve (BD, CA, United States). After centrifugation at 4°C, 300 g, the cell suspension was resuspended and transferred into a culture bottle. The low-glucose DMEM (Gibco, Grand Island, NY, United States) was prepared with 10% FBS (Gibco, Grand Island, NY, United States), 100 U/ml penicillin (Gibco, Grand Island, NY, United States), and 0.1 g/L streptomycin (Gibco, Grand Island, NY, United States) (Ge et al., 2016). At the fifth passage, surface molecular identification and multi-differentiation staining and cell suspension preparation were performed.

Acquisition of Human UMSC

The umbilical cord tissue was taken from puerpera and washed with normal saline three times, with blood vessels removed. The umbilical cord tissue was cut and soaked in a solution containing 100 U/ml penicillin (Gibco, Grand Island, NY, United States) and 0.1 g/L streptomycin (Gibco, Grand Island, NY, United States). After digesting with collagenase I and hyaluronidase at 37°C for 5 h, centrifuging at 4°C, 300 g, and passing through a 70-μm cell sieve, the cells were resuspended and transferred into a culture bottle (Corning, NY, United States). The low-glucose DMEM (Gibco, Grand Island, NY, United States) was prepared with 10% FBS (Gibco, Grand Island, NY, United States), 100 U/ml penicillin (Gibco, Grand Island, NY, United States), and 0.1 g/L streptomycin (Gibco, Grand Island, NY, United States). At the fifth passage, surface molecular identification and multi-differentiation staining and cell suspension preparation were performed.

Acquisition of Peripheral Blood Lymphocytes From Healthy Donors

After peripheral blood was acquired, polysucrose solution (Serumwerk Bernburg AG, Alere Technologies, Oslo, Norway) and density gradient centrifugation were used to separate peripheral blood lymphocytes. After purification using red blood cell lysis buffer (Solarbio, Beijing, China), the peripheral blood lymphocytes were resuspended and counted, and cultured in a CO_2 incubator at 37°C.

Construction of the Co-Culture System

OMSC (or UMSC) were transferred into a 24-well plate (Thermo Fisher Scientific, Waltham, MA, United States) at a density of 1×10^5 cells/well; CD3+ lymphocytes were purified using CD3 MicroBeads (Catalog # 130-050-101, Miltenyi Biotec, Bergisch Gladbach, Germany) and transferred into a 24-well plate at a density of 5×10^5 cells/well. The total culture volume was 500 μl. For the *in vitro* co-culture study, each well was stimulated with 12.5 μg MOG_{35-55}. For the *in vitro* mechanism study, each well was stimulated with 500 ng anti-CD28 (BD, CA, United States) and 100 ng anti-CD3 (BD, CA, United States). For the COX inhibitor group, indomethacin (1 uM, Sigma-Aldrich, St. Louis, MO, United States) was added to the culture system, and for the TGF-β inhibitor group, anti-TGF-β (1 ug/ml) antibody was added to the culture system. At the last 6 h, the cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml). Brefeldin A (BFA, 10 μg/ml) was used to inhibit the secretion of cytokines. Each experiment was repeated three times (n = 3).

Detection of Surface Markers and Intracellular Factors of Human Lymphocytes

The cells were collected and washed with PBS (Gibco, Grand Island, NY, United States) for staining. The cells were resuspended with 100 μl staining buffer, stained with APC-CD8 (BD, CA, United States) at 4°C for 30 min, fixed with 4% paraformaldehyde (Boster Biological Technology, Wuhan, China), stained with PE-Cy7-IFN-γ (BD, CA, United States), permeabilized (Invitrogen, CA, United States) at 4°C for 30 min, and washed and resuspended with PBS.

Surface Molecular Identification

The fifth passage of UMSC were digested with 0.25% trypsin (Gibco, Grand Island, NY, United States), and OMSC were digested with 0.125% trypsin at 37°C. The concentration of the cell suspension was 1×10^6/ ml; it was filtered by a 70-μm sieve (BD, CA, United States) and transferred to flow tubes. UMSC and OMSC were labeled with PE-CD29 (BD, CA, United States), PE-CD90 (BD, CA, United States), PE-Cy7-CD34 (BD, CA, United States), PE-Cy7-CD45 (BD, CA, United States), APC-CD44 (BD, CA, United States), APC-CD166 (BD, CA, United States), FITC-CD73 (BD, CA, United States), and

FITC-CD105 (BD, CA, United States) at room temperature for 30 min. The surface molecules were detected by flow cytometry.

Alizarin Red S Staining

The fifth passage of UMSC (or OMSC) were transferred into 6-well plates (Thermo Fisher Scientific, Waltham, MA, United States) at the density of 3×10^5/well. After 80% density, the old culture medium was removed, with a culture medium for the induction of osteoblasts by a previous study (Lei et al., 2013). The induction culture was carried out in the incubator with 5% CO_2 under 37°C, and half of the medium was changed every 2 days. At the 21st day of culture, the medium was sucked out, and the cells were washed with PBS buffer three times, fixed with 4% paraformaldehyde (Boster Biological Technology, Wuhan, China) for 15 min, stained with 0.1% Alizarin Red S (Rolex-Bio, Guangzhou, China) for 10 min, and washed with PBS three times.

Oil Red O Staining

The fifth passage of UMSC (or OMSC) were transferred into 6-well plates at the density of 3×10^5/well. After 80% density, the culture medium was removed, with a culture medium for the induction of lipoblasts by a previous study (Lei et al., 2013). After induction with medium A for 3 days, fat induction medium B was used for 1 day. After circulation for 21 days, the medium was sucked out, and the cells were washed gently with PBS once and fixed with 4% paraformaldehyde for 15 min. After staining the cells using 0.5% Oil Red O (Sigma-Aldrich, St. Louis, MO, United States) for 10 min, they were washed carefully with isopropanol (Guangzhou chemical reagent factory, Guangzhou, China), gently rinsed with PBS three times, and then observed and photographed under a microscope.

Animals

C57BL/6 female mice were provided by Charles River, Ltd. All animal experiments, breeding, and care were performed according to Animal Experiment Center Guidelines and approved by the Animal Ethics Committee of Sun-Yat sen University.

EAE Model Induction

EAE was induced in 6 to 8-week-old female C57BL/6 mice. The mice were anesthetized using 10 g/L pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, United States) at a dose of 50 mg/kg. The pedal reflex and tail pinching reflex were observed to ensure that the mice were anesthetized. The skin was prepared on the back of the mice, and the mice were labeled with ear markers. An emulsion containing 1 mg/ml myelin oligodendrocyte glycoprotein (MOG) $_{35–55}$ (Sigma-Aldrich, St. Louis, MO, United States) together with complete Freund adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, United States) with 4 mg/ml of *M. tuberculosis* H37R (BD, CA, United States) was injected subcutaneously on both sides of the back of the mice. In addition, 500 ng of pertussis toxin (List Biological Laboratories, New Delhi, INDIA) was injected intraperitoneally on days 0 and 2. All mice were randomly allocated to the OMSC treatment (n = 6), UMSC treatment (n = 6), and PBS groups (n = 7).

The mice were scored daily for neurological function evaluation according to the 6-point EAE scale as mentioned in a previous study (Ben-Zwi et al., 2019): 0, asymptomatic; 1, partial loss of tail tonicity; 2, tail paralysis; 3, hind limb weakness; 4, hind limb paralysis; 5, 4-limb paralysis; and 6, death.

Cell Therapy

On the 9th day after immunization, the third to fifth passage of OMSC and UMSC were digested, filtered, and counted. According to the counting results, an appropriate number of cells were resuspended to equal concentration (1×10^6/200 μl) of the cell suspension. The mice were fixed on the tail vein injection instrument, and the injection points were treated with 75% alcohol (Guangzhou Chemical Reagent Factory, Guangzhou, China). A 1-ml insulin needle (BD, CA, United States) was used to inject OMSC, UMSC cell suspension (1×10^6/mouse), or PBS. The mice in OMSC treatment, UMSC treatment, and control groups were injected with cells or PBS *via* the tail vein.

Histopathology

After the mice were sacrificed, PBS and 4% paraformaldehyde were used in turns to perfuse, and spinal cords were carefully removed and fixed with 4% paraformaldehyde for 24 h, and then dehydrated using 75–95% ethanol. And the tissue was cleared with xylene, and a paraffin-embedded tissue block was made and was cut into paraffin sections.

Hematoxylin and eosin (HE) staining: The paraffin sections were put into ethanol and xylene for gradient dehydration. The hematoxylin staining solution was added to dye for 5 min and then washed with running tap water, followed by eosin dye staining for 5 min. After dehydrating, the sections were sealed with a sealing agent. Inflammation was scored as follows: 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organization of inflammatory infiltrates around blood vessels; and 3, extensive perivascular cuffing with extension into the adjacent parenchyma, or parenchymal infiltration without obvious cuffing.

Luxol fast blue (LFB) staining: The paraffin sections were put into ethanol and xylene for gradient dehydration. Myelin staining solutions A and B were preheated, and the slices were put into dye A, dyed for 3 h, and then taken out and washed with water. After being immersed in staining solution B, the differentiation was terminated. After dehydrating, the sections were sealed with a sealing agent. Demyelination was scored as follows: 0, none; 1, rare foci; 2, a few areas of demyelination; and 3, large (confluent) areas of demyelination (Calida et al., 2001).

Cytometric Bead Array

After anesthetization, the mice were sacrificed and peripheral blood was collected through the inner canthus vein with a capillary collecting vessel. After centrifugation at 4°C for 10 min, the upper serum was transferred to a new EP tube. The standard sample was diluted, and the standard curve was made according to the CBA kit's (BD, CA, United States) operation instructions. The concentration of IFN-γ, TNF-α, IL-17, IL-6, and IL-10 was detected by flow cytometry.

Analysis of the Spleen Lymphocyte of EAE Mice

On the day of EAE onset, the mice were anesthetized and sacrificed. The spleen of mice of OMSC treatment and control groups was observed and ground (n = 3). Red blood cell lysis buffer was used to remove red blood cells. After washing and centrifugation, the lymphocytes were resuspended in 1640 medium (Gibco, Grand Island, NY, United States) and counted by using the cell counting plate. Purified lymphocytes were transferred into 24-well plates at a density of 1×10^6 cells/well. The volume of the culture system was 500 μl. Five microliters of leucocyte activation cocktail (BD, CA, United States) was added to each well to stimulate the cells for 6 h. After washing with PBS twice, the cells were resuspended in 100 μl PBS. After staining with FITC-CD3 (BioLegend, San Diego, CA, United States) and APC-CD4 (BioLegend, San Diego, CA, United States) at 4°C for 30 min, the lymphocytes were fixed with 4% PFA at room temperature for 15 min, then permeabilized, and stained with PE-Cy7-TNF-α (BioLegend, San Diego, CA, United States) and PE-IFN-γ (BioLegend, San Diego, CA, United States) at 4°C for 30 min. After washing with PBS, the lymphocytes were resuspended with PBS. The proportions of T lymphocyte subsets were detected by flow cytometry. Each experiment was repeated three times (n = 3).

Statistical Analysis

GraphPad Prism 8 and SPSS 20.0 were used for graphing and statistical analysis. All data were expressed as mean ± SEM. The Kruskal–Wallis H test was used to evaluate the neurological function score of mice among three groups; one-way ANOVA was used for detecting differences in the mean values of the three groups, and the *LSD-t* test was used for pairwise comparison. A p value < 0.05 was considered statistically significant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Sun-Yat sen University.

AUTHOR CONTRIBUTIONS

HZ and QL designed the study. CX and DL performed the animal study. JC collected the data. MH, SC, and YW prepared the MSC. QL performed the in vitro study. XC and HL performed the staining procedures of the MSC. HZ, QL, and CX drafted the manuscript or substantively revised it. All authors read and approved the final manuscript version.

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REVIEW
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Cationic Nanomaterials for Autoimmune Diseases Therapy

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Cationic nanomaterials are defined as nanoscale structures smaller than 100 nm bearing positive charges. They have been investigated to apply to many aspects including clinical diagnosis, gene delivery, drug delivery, and tissue engineering for years. Recently, a novel concept has been made to use cationic nanomaterials as cell-free nucleic acid scavengers and inhibits the inflammatory responses in autoimmune diseases. Here, we highlighted different types of cationic materials which have the potential for autoimmune disease treatment and reviewed the strategy for autoimmune diseases therapy based on cationic nanoparticles. This review will also demonstrate the challenges and possible solutions that are encountered during the development of cationic materials-based therapeutics for autoimmune diseases.

Keywords: nanomaterials, cationic polymer, autoimmune diseases, nanoparticles, cell-free DNA

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INTRODUCTION

Nanomaterials with particles size smaller than 100 nm and bearing positive charges or synthesized in the presence of novel cationic entities, incorporated on their backbone and/or as side chains, are considered as cationic nanomaterials (Rezaei et al., 2019). Cationic nanomaterials are generally divided into two categories: natural or synthetic (Samal et al., 2012). Poly (amidoamine) (PAMAM), polyphosphoramidate (PPA), poly [2-(N,N-dimethylamino) ethyl methacrylate] (PDMAEMA), hexadimethrine bromide (HBMBr) and β-cyclodextrin-containing polycation (CDP) are widely studied among them. For their inherent bioactive properties such as antimicrobial, stimuli responsiveness, antioxidant, antitumor, and anti-inflammatory, cationic polymers are expected to possess further enhanced therapeutic potential (Samal et al., 2012). Furthermore, the unique features of cationic nanomaterials such as desirable size, greater solubility, easier to pass through cellular barriers, and more reactivity make them become attractive options for therapeutic applications (Yonezawa et al., 2020).

Autoimmune diseases are defined as a clinical syndrome caused by the activation of T cells or a loss of B-cell tolerance to particular antigens without infection or other discernible causes (Davidson and Diamond, 2001). Autoimmune diseases vary greatly and are complicated in clinical manifestations, with some appear to be systemic such as systemic lupus erythematosus, some are limited to organ-specific like type 1 diabetes mellitus (Rosenblum et al., 2015). Autoimmunity is initiated by a combination of genetic predisposition and environmental triggers and followed by epitopes spread and inflammatory loop give rise to a vicious cycle (Davidson and Diamond 2001; Rosenblum et al., 2015). Nowadays, disease-modifying anti-rheumatic drugs, glucocorticoids, analgetics, non-steroidal anti-inflammatory drugs, and biological agents are the primary therapeutic method in autoimmune diseases, but the drugs used to suppress the immune

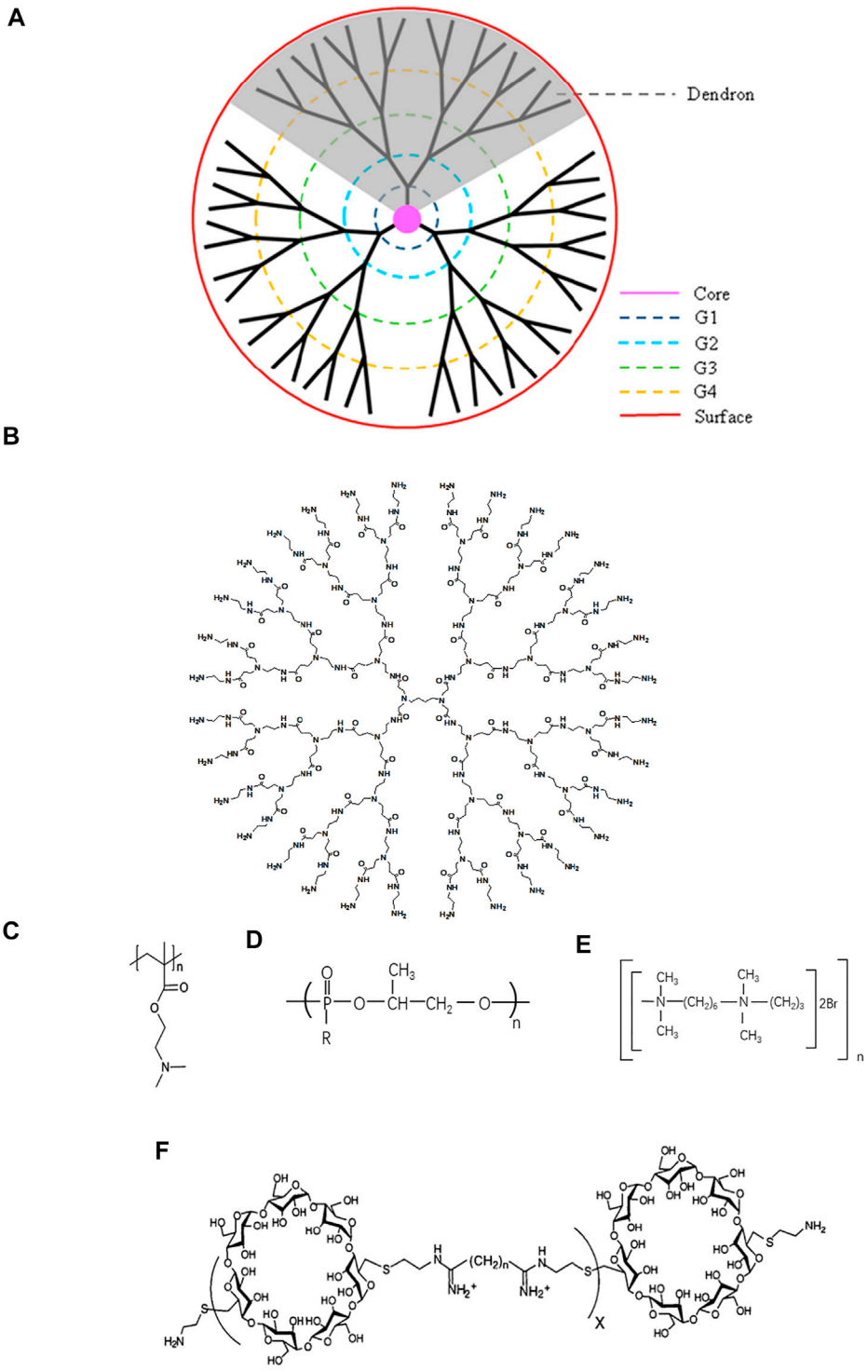

FIGURE 1 | Schematic structure of typical cationic polymers. **(A)** Dendrimer (Reprint from Reference (Wu et al., 2015)). **(B)** Genearlation 3 poly (amidoamine) (PAMAM). **(C)** Poly (2-(diethylamino) ethyl methacrylate) (PDMA) (Reprint from Reference (Samal et al., 2012). **(D)** Polyphosphoramidate (PPA) (Reprint from Reference (Zhang PC. et al, 2005)). **(E)** Hexadimethrine bromide (HBMBr) (Reprint from Ref(Aubin et al., 1997)). **(F)** β-cyclodextrin-containing polycation (CDP) (Reprint from Ref (Hwang et al., 2001)).

response have numerous side effects with large doses and continuous therapy is not conducive to long-term host survival (Miller et al., 2007). Hence, searching for novel therapeutic methods is crucial.

Recently, with the advancement of our understanding of nanotechnology, nanomaterials have become a promising approach for the treatment of autoimmune diseases. Cationic nanomaterials have become one of the important pillars of nanomaterials. The therapeutic applications of cationic nanomaterials mainly focus on three aspects: gene delivery, drug delivery and tissue engineering (Samal et al., 2012). Recently, successful attempts have been reported that cationic nanomaterials possessed the therapeutic potential severed as drugs. In this review, we highlight progress on the therapeutic potential of cationic nanomaterials in autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), autoimmune skin inflammation, and discuss the dilemma of cationic nanomaterials in the therapy of autoimmune diseases.

CATIONIC MATERIALS

Poly (amidoamine) (PAMAM)

The dendrimers were first synthesized by Tomalia et al. taking advantage of the architecture including monodispersity, extraordinary symmetry, hyper branch with tree-like structures (Tomalia et al., 1985). They consist of a central core and branches emanating from the core terminated with functional surface groups (**Figure 1B**). With the increase of generation, dendrimers form 3D spheres, thus creating supramolecular void spaces that can bind and transfer other molecules (Quadir and Haag 2012; Dzmitruk et al., 2018). In addition, it has multiple surface functional groups that can be modified, which is different from linear structures (Menjoge et al., 2010). A study had shown that linear structure had stronger DNA binding and cellular uptake, but dendritic structure mediated gene expression is higher than linear structure, which may be related to the escape of sufficient DNA amount of effective gene expression into the cytoplasm (Yamagata et al., 2007).

PAMAM is one of the most widely studied dendrimers. Because of the ability to combine with nucleic acid, numerous effort has been developed in applying PAMAM to the treatment of diseases including gene delivery and nucleic acid scavenge (Abedi-Gaballu et al., 2018). However, the binding efficiency is associated with the positive charge density. Higher generation PAMAM with more primary amines possess higher positive charge density on the surface (Jensen et al., 2011). G3-G10 PAMAM dendrimers are the optimal choices resulted from their remarkable stability to combine with nucleic acid and higher transfect efficiency (Palmerston Mendes et al., 2017). Successful attempts have been made to use PAMAM-G3 as antithrombotic agents and anti-metastatic agents. Due to the property of combination with nucleic acid, PAMAM-G3 attenuated the activation of blood coagulation and inflammation induced with nucleic acid through the Toll-like receptor (TLR) pathway, which was related to the thrombosis, lung metastasis in breast cancer, and liver metastasis in pancreatic cancer (Jain et al., 2012; Naqvi et al., 2018; Holl et al., 2020).

Furthermore, surface modification and tri-block modification have been applied to PAMAM. Compared with polyamidoamine (PAMAM), PEGylation modification of PAMAM could increase the transfection efficiency and stabilization with lower cytotoxicity (Reyes-Reveles et al., 2013; Sun et al., 2014). A novel tri-block nanocarriers consisting of PAMAM, poly(ethylene glycol) (PEG), and poly-l-lysine (PLL) were developed to deliver siRNA. PLL replaced the role of PAMAM to form polyplexes with siRNA and PAMAM severed as a proton sponge. PEG was used to stabilize nanocarriers in plasmas (Patil et al., 2011). Biswas et al. also developed another triblock nanocarrier PAMAM-G4-D-PEG-DOPE to deliver siRNA. Different from the former, PAMAM-G4 worked for efficient siRNA condensation (Biswas et al., 2013). Similarly, PAMAM, PEG, and lactobionic acid (Gal) were used to construct a delivery system to carry AEG-1 siRNA, and it was proved that PAMAM-AEG-1si nanoplexes restrain tumor growth (Rajasekaran et al., 2015).

Poly(2-(diethylamino)ethyl Methacrylate) (PDMA)

As an important pH bioresponsive functional cationic nanomaterials, as well as the character of excellent stability and safety profile (Huang et al., 2018), PDMA (**Figure 1C**) has been widely applied as a delivery system. Possessing a high affinity for nucleic acid, PDMA is increasingly studied in the gene delivery and neutralization of nucleic acid. A biodegradable cationic micelles PDMAEMA-PCL-PDMAEMA was established to deliver siRNA and paclitaxel into cancer cells, which diminished the expression of VEGF (Zhu et al., 2010). Rungsardthong et al. demonstrated that the DNA affinity of PDMA could be fine-tuned by varying the PH and the polymer/DNA ratios (Rungsardthong et al., 2003).

Researchers anticipated that the properties of PDMA could be optimized by modifying different groups. Deshpande et al. utilized 2-(dimethylamino) ethyl methacrylate (DMAEMA) to construct three different polymers: DMAEMA-PEG (a diblock copolymer), DMAEMA-OEGMA 7 (a brush-type copolymer), and DMAEMA-stat-PEGMA (a comb-type copolymer). Compared with PDMAEMA, all of them exhibited more excellent binding ability with oligonucleotide while DMAEMA-stat-PEGMA showed the best. But DMAEMA-PEG and DMAEMA-OEGMA 7 own better long-term colloidal stability (Deshpande et al., 2002). PEO-PPO-PEO-pDMAEMA (L92-pDMAEMA) and PEO-pDMAEMA copolymers basing on PDMA, poly (propylene oxide) (PPO), and poly (ethylene oxide) (PEO) were also developed to deliver genes. It has been reported that modification with PEO could reduce the unfavorable interactions with complement factors or cellular components (Bromberg et al., 2005). Furthermore, PEO-b-PDMAEMA could form soluble complexes with DNA of a much smaller size resulted from the amphiphilic nature of the polymer (Tan et al., 2006).

Polyphosphoramidate (PPA)

Polyphosphoramidate (PPA) (**Figure 1D**), a biodegradable cationic material, has been investigated as drug delivery and gene delivery for years. Their structures, different side chains, molecular weight, and positive charge on the surface can influence the complexation with nucleic acids. PPA owned higher DNA binding efficiency when molecular weight and positive charge density increased (Ren et al., 2010). Zhang et al. developed a series of cationic polymers which had an identical backbone and different side chains including primary, secondary, tertiary, and quaternary amino groups, and demonstrated that PPA with primary amino group possessed uppermost ability to complex with nucleic acids (Wang et al., 2004). Furthermore, the same team synthesized ternary complexes, consisting of PPA backbone, primary and tertiary amino group, and quaternary complexes, containing PPA, primary, secondary, and tertiary amino groups. And the results showed that the coexistence of primary and other amino groups could elevate the combination with the nucleic acid (Zhang PC. et al, 2005).

PEGylation modification is also applied in PAA. PEG-b-PPA/DNA micelles with lower surface charge and smaller particle size ranging from 80 to 100 nm maintained similar transfection efficiency while showing lower cytokines and better biocompatibility compared with PPA/DNA (Jiang et al., 2007). Moreover, galactosylated PPA was prepared to enhance the targeted capacity as a delivery system. However, the transfection efficiency of gal-PPA reduced with the increase of galactose substitution degree, presumably resulting from the decreased DNA binding capacity and particle stability (Zhang XQ. et al, 2005). Hence, modification of PPA needs to be further explored.

Hexadimethrine Bromide (HBMBr)

Hexadimethrine bromide (HDMBR) (**Figure 1E**) has been used as an antiheparin agent for many years (Pai and Crowther 2012) and has been rediscovered in recent years to neutralize nucleic acids and deliver genes due to its positive charge. A combination of HDMBr and dimethyl sulfoxide facilitated DNA transfection into chicken embryo fibroblast cells and human fibroblast (Kawai and Nishizawa 1984; Aubin et al., 1997). After intraperitoneally administered, HDMBR neutralized extracellular nucleic acids and thereby reduced lung injury, restrained disruption of alveolar-capillary barrier, and increased blood oxygenation in acute respiratory distress syndrome (ARDS) model rats exposed to CEES, a toxic chemical (Mariappan et al., 2020). Similarly, HDMBr could scavenge mitochondrial DNA (mtDNA) in an *in vivo* model of trauma hemorrhage, and the ability to inhibit inflammation and apoptotic cell death emerged (Aswani et al., 2018). But a crucial concern was the toxicity of HDMBr, including the nephrotoxicity and neurotoxicity, which may be a critical challenge for its biomedicine application (Pai and Crowther 2012; Bao et al., 2018).

β-Cyclodextrin-Containing Polycation(CDP)

β-cyclodextrin-containing polycation (CDP) (**Figure 1F**), a classical cationic compound, is widely studied in gene delivery. The introduction of β-cyclodextrin, which is itself a large carbohydrate, reduced the toxicity of the polymer (Reineke and Davis 2003). A delivery system, consisting of a CDP, a polyethylene glycol (PEG) steric stabilization agent, and human transferrin (Tf) encapsulated ribonucleotide reductase subunit M2 siRNA, was administrated in non-human primates and the result showed that the nanoparticles could be safely used in non-human primates (Heidel et al., 2007). The same team conducted a phase I clinical trial and the nanoparticle indeed diminished the expression of mRNA (Davis et al., 2010).

It's has been reported that the DNA binding efficiency of CDP was related to the structure. Hwang et al. synthesized five compounds composed of dicysteamine-β-cyclodextrin and other difunctionalized comonomers. And DNA affinity, DNA protective ability, and the toxicity of polymers altered with the number of methylene groups within the difunctionalized comonomers. When the number of methylene groups was six, the spacing between the cationic amidine groups is optimal for DNA binding (Hwang et al., 2001). Besides, maintaining stability *in vivo* is also a concern for CDP. Researchers have found that the introduction of PEG or Adamantane (AD) could enhance the stability of CDP (Heidel 2011).

THERAPEUTIC POTENTIAL IN AUTOIMMUNE DISEASES

The plasmas cell-free DNA (cf DNA) was first described in 1948 (Mandel and Metais 1948) and the elevated level of cfDNA was observed in patients with rheumatic disease (Tug et al., 2014). Endogenous sources of cfDNA include apoptotic bodies, exosomes, microvesicles, neutrophil extracellular traps (NETs), necrosis (Kubiritova et al., 2019) (**Figure 2A**). The imbalance of generation and clearance of the cell-free DNA is closely associated with the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) (Munoz et al., 2010; Dong et al., 2020). If cfDNA is not properly cleared, they can trigger activation of endosomal TLRs such as TLR7, 8, and 9, and thereby induce inflammatory responses (Barrat et al., 2005; Dong et al., 2020; Fillatreau et al., 2021) (**Figure 2B**).

Due to the capacity of interacting with nucleic acids and forming electrostatic complexes, numerous cationic nanoparticles have been widely used for the non-viral transfection of cells with plasmid DNA, miRNA, and siRNA (Yonezawa et al., 2020). Recently, the interest in exploiting cationic nanomaterials as the nucleic acid-binding polymer to inhibit inflammatory immune diseases emerged. It's been reported some cationic nanomaterials possess the ability to attenuate nucleic acid-mediated activation of TLRs on macrophages if binding nucleic acids (**Figure 3**). Sullenger et al. evaluated six of them and found that PAMAM-G3 and HDMBr inhibited the nucleic acid-mediated TLR activation through neutralizing extracellular inflammatory nucleic acids and altering the uptake and intracellular distribution of immune stimulatory nucleic acids (Lee et al., 2011) (**Figure 4**). Consistently, another research demonstrated that CDP, PAMAM-G3, and HDMBr can inhibit the binding of Lupus

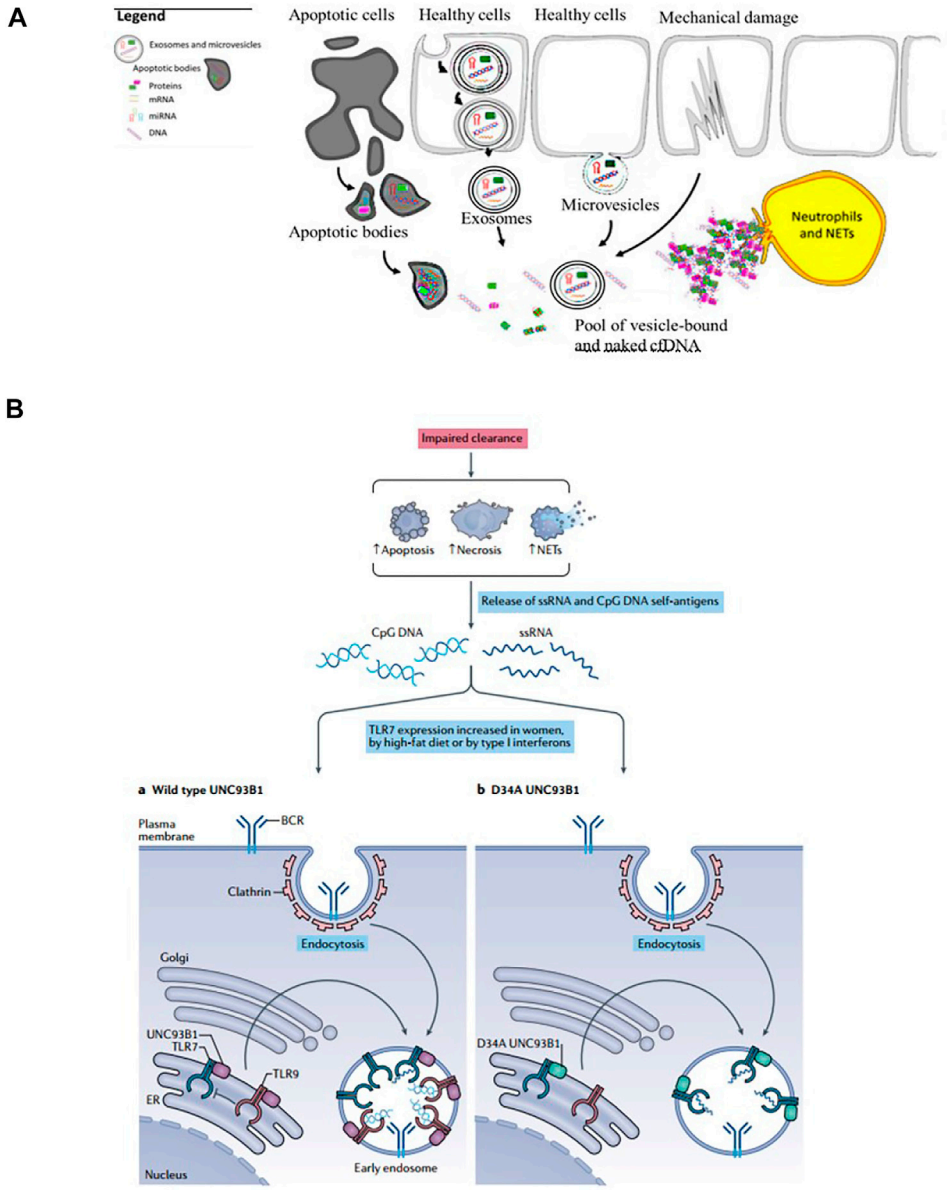

FIGURE 2 | The sources and mechanism of cfDNA. **(A)** Endogenous sources of cfDNA including apoptotic bodies, exosomes, microvesicles, neutrophil extracellular traps (NETs), necrosis (Reprint from Ref (Kubiritova et al., 2019)). **(B)** cfDNA induces inflammatory responses and the occurrence and development of inflammatory diseases (Reprint from Reference (Fillatreau et al., 2021)).

anti-DNA antibody and DNA by displacing antibodies from preformed complexes (Stearns et al., 2012). They also observed that nucleic acid scavenging polymers only limited the activation of the immune system by accessible extra-cellular nucleic acid and do not engender non-specific immune suppression (Holl et al., 2013). In some inflammatory diseases such as sepsis, studies had also found that cationic nanomaterials as nucleic acid scavengers could effectively reduce the severity of the disease (Dawulieti et al., 2020; Liu et al., 2021).

For the ability to scavenge nucleic acids, cationic nanomaterials were attempted for the treatment of autoimmune diseases.

Systemic Lupus Erythematosus (SLE)

SLE is characterized by increased apoptosis and impaired clearance of apoptotic cells. Many factors can influence the clearance of the cell-free DNA in SLE patients, including the abnormalities of DNase activity to clear cf DNA, the combination of the cfDNA with the antibodies, proteins, and nucleosomes, and thus potentially activate inflammatory pathways (Courtney et al., 1999; Duvvuri and Lood 2019). PAMAM-G3 was proved that local administration of it facilitated wound healing in a cutaneous lupus erythematosus (CLE) prone animal by diminishing extracellular nucleic acids and inhibiting TLR7 and TLR9 activation (Holl et al., 2016).

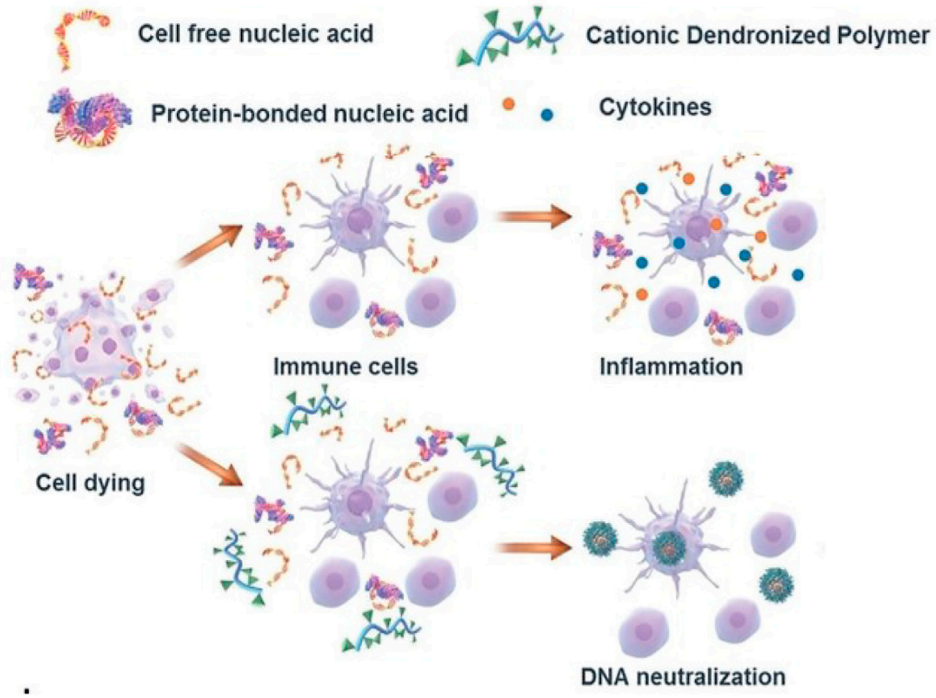

FIGURE 3 | Mechanism of cationic nanomaterials bind nucleic acids to attenuate nucleic acid-mediated activation of immune cells (Reprint from Reference (Peng et al., 2019)).

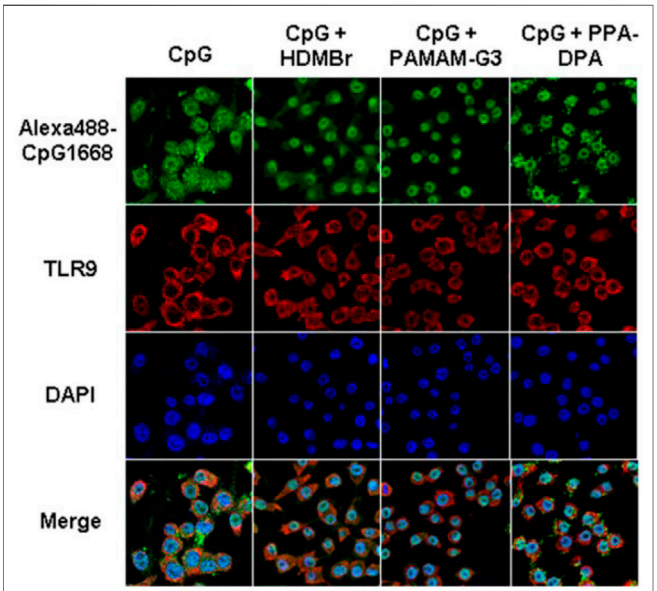

FIGURE 4 | PAMAM-G3, HDMBr, and PPA-DPA altered the uptake and intracellular distribution of immune stimulatory nucleic acids (Reprint from Reference (Lee et al., 2011)).

Furthermore, researchers also evaluated the ability of PAMAM-G3 to reduce glomerulonephritis and circulating autoantibody levels in MRLlpr mice (Holl et al., 2016).

Rheumatoid Arthritis

The elevated level of cf DNA was discovered in Rheumatoid arthritis patients and whole-genome shotgun sequencing showed SFcfDNAs in RA are enriched with specific CMR sequences, which are hypomethylated (Dong et al., 2020). Therefore, neutralizing cfDNA may be a potential treatment for Rheumatoid arthritis. In a recent study, the researchers prepared a self-assembly PLGA-block-PDMA block copolymer, PLGA-b-PDMA463, and they discovered that it could neutralize cfDNA derived from RA patients and inhibit nucleic acid-mediated activation of primary synovial fluid monocytes and fibroblast-like synoviocytes by restraining the activation of TLR9 (**Figure 5**). After intravenous injecting PLGA-b-PDMA463 into a CpG-induced mouse model or collagen-induced arthritis rat model (CIA model), successful prevention of RA symptoms, which was evaluated by inflammation, swelling, and deformities of the paws, was achieved and it might be attributed to capacity to scavenge cfDNA and a more favorable biodistribution (Liang H. et al, 2018). With the intent to boost the binding affinity and avoid potential systemic toxicities of PDMA-based cationic nanoparticles (cNPs), the same team tuned the proportion of PLGA and PDMA and introduced poly (ethylene glycol) (PEG) segments to the cNPs' PDMA shell. The introduction of PEG segments translated into a lower DNA binding efficacy while preserving the ability to hamper joint inflammation. Moreover, due to a greater accumulation and longer retention at the inflamed joints, new NPs were allowed for a lower frequency of administration (Wu JJ. et al, 2020). And another cationic nanoparticle, PCL-g-PAMAM, was also developed to inhibit synovial inflammation and relieve joint inflammation and

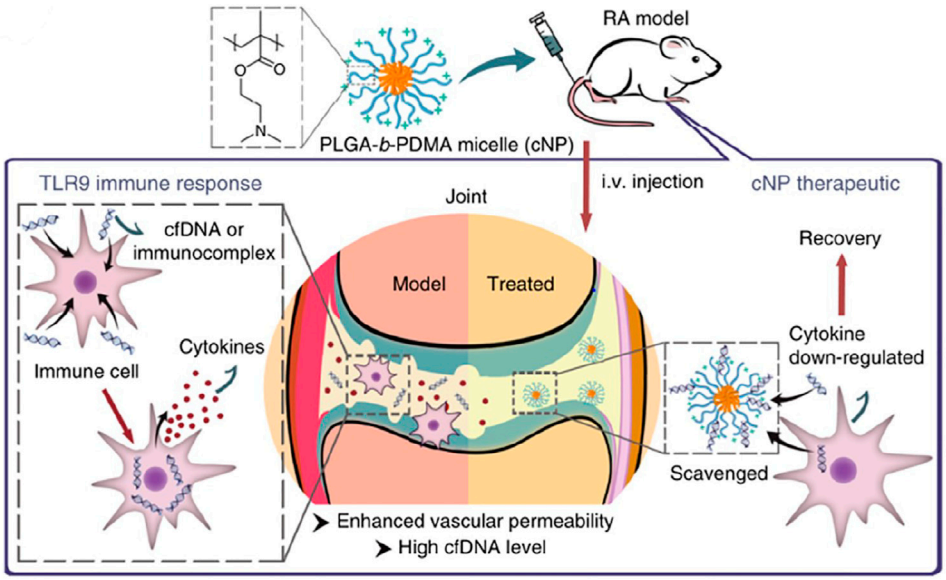

FIGURE 5 | Mechanism of applied cationic nanoparticles to scavenge cfDNA or immunocomplex and thereby prevent the activation of immune cells, down-regulation the expression of cytokines, and alleviate the symptoms of RA (Reprint from Reference (Liang H. et al, 2018)).

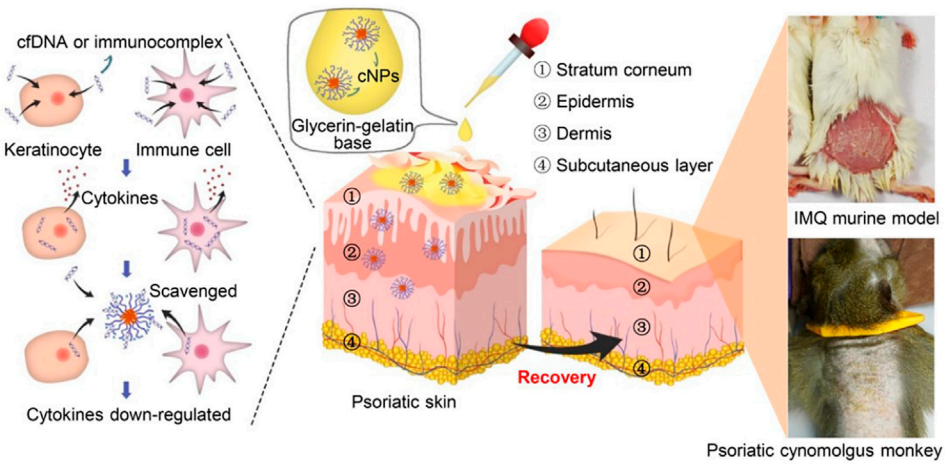

FIGURE 6 | Mechanism of topical administration of PLGA-b-PDMA on psoriasiform skin in both mouse model and cynomolgus monkey model by competing for DNA from the DNA-LL37 immunocomplex and suppressing DNA-LL37-induced cell activation (Reprint from Reference (Liang et al., 2020)).

damage in the CIA mouse model (Peng et al., 2019). Moreover, differences in surface groups of cationic nanomaterials could change their DNA scavenging ability and anti-inflammatory effect in RA, which may be related to different adsorption of opsonin protein. Hydroxylated nanoparticles could prolong the retention in joints and enhance anti-inflammatory effects (Liu. et al., 2021).

Autoimmune Skin Diseases

It's has been reported that a significantly elevated level of cfDNA in psoriasis patients (Beranek et al., 2017). Topical administration of PLGA-b-PDMA on psoriasiform skin of an IMQ-induced mouse model could alleviate psoriatic symptoms by efficiently competing for DNA from the DNA-LL37 immunocomplex and suppressing DNA-LL37-induced cell activation. Consistent with this result, the application of PLGA-b-PDMA in a cynomolgus monkey model relieved the symptoms of psoriasis (Liang et al., 2020) (**Figure 6**). A series of cationic materials, poly (2-(dimethylamino) ethyl methacrylate) grafted hairy silica particles (cSPs), with different PDMA lengths and different particle sizes had been studied. These cationic materials also had the ability to scavenge cfDNA and effectively inhibited

psoriatic skin inflammation and inflammatory cytokines secretion. In addition, they showed that different particle sizes and the ratio of PDMA affect DNA binding affinity, which was related to anti-inflammatory effects and the ability to enter the dermis (Yan et al., 2021).

Challenge of Cationic Nanomaterials

The appearance of cationic nanomaterials gives a novel direction in the treatment of disease, which is closely linked to the broad range of properties they offer. However, the safety profiles of cationic nanomaterials have been a critical concern in therapeutic researches (Wu LP. et al, 2020).

Toxicity

Cytotoxicity, immune-related toxicity, and systemic toxicity are the main barriers to the application of cationic nanomaterials. The application of cationic nanomaterials was restricted due to toxicity such as cell necrosis, inflammatory toxicity, pulmonary toxicity, leukopenia, and thrombocytopenia (Liang X. et al, 2018).

The cytotoxicity of cationic nanomaterials is mainly attributed to the positive charges. Compared with the anion group, cationic nanomaterials exhibit higher cytotoxicity and lethal effects (Calienni et al., 2017; Pereira et al., 2019). Cationic nanomaterials destroy plasma membrane integrity, mitochondrial and lysosomal damage, and more autophagosomes (Mecke et al., 2005; Frohlich 2012). It has been reported that cationic surfactants incorporated into nanoparticles induced cell necrosis and the release of mediators, which resulted from accelerating cell membrane lysis and Ca^{2+} influx *via* the interaction with the cell membrane (Hwang et al., 2015). Another study demonstrated that cytotoxicity decreased in the presence of increases in serum which was based on serum masking of the PEI surface and decrease of the interaction with cell (McConnell et al., 2016). Both of them illustrated that high positive charge density could increase cytotoxicity. A study found that cationic nanomaterials induced cell necrosis rapidly through inhibition of Na^+/K^+-ATPase and subsequent leakage of mitochondrial DNA from necrotic cells. Mitochondrial DNA triggered severe inflammation *in vivo* by a pathway involving TLR9 and MyD88 signaling (Wei et al., 2015). But the same team also discovered that the inflammatory response induced by cationic nanocarriers was gradually and spontaneously regressed within 1 week. They hypothesized that cationic nanoparticles negatively regulated inflammation and the result demonstrated that leaked mtDNA altered the phenotype of monocyte via a STING- or TLR9 pathway and PEG2 secreted from $Ly6C^+$mancytes inhibited neutrophil activation (Liu et al., 2018).

Similarly, Immunotoxicity limits the application of cationic nanomaterials. Cationic nanomaterials could alter the immune state *via* suppressing innate immunity such as inhibition of natural killer (NK) cell activity, reduction of CD4+/CD8+ ratio, and inflammation cytokines (Kim et al., 2014). Macrophages were also been proved that they could be activated by cationic nanomaterials depending on TLR4 (Toll-like receptor 4) and ROS (reactive oxygen species) signaling (Zhang et al., 2014; Mulens-Arias et al., 2015).

In animal models, the pulmonary toxicity of cationic nanomaterials was also described. Acute lung injury induced by the intratracheal instillation of cationic polyamidoamine dendrimer (PAMAM) nanoparticles had been reported and the model demonstrated cationic nanoparticles suppressed the activity of ACE2 via binding with ACE2, resulting in an imbalance of the renin-angiotensin system (Sun et al., 2015).

Although cationic nanomaterials have not yet entered the stage of clinical research in the treatment of autoimmune diseases, certain systemic toxicity has been found in clinical studies of cationic nanomaterials in tumors. Fatigue, chills, fever, and nausea were mostly described in clinical trials (Zuckerman et al., 2014; Autio et al., 2018). Cardiovascular symptoms such as sinus bradycardia, tachycardia, and hypotension have been reported in a phase Ia/Ib clinical data with polymer-based nanoparticle containing siRNA and a Phase I study of systemically delivering p53 nanoparticle in advanced solid tumors, respectively (Senzer et al., 2013; Zuckerman et al., 2014). In a phase I study for advanced solid tumors, patients experienced infusion-related hypersensitivity which could have been controlled by the frequency after pretreatment with drugs (Rudin et al., 2004). Besides, hematologic disorders including thrombocytopenia and lymphocytopenia occurred in the clinical trials of cationic nanomaterials (Rudin et al., 2004; Zuckerman et al., 2014).

Strategies to Minimize Toxicity

To minimize the toxicity, two strategies have been presented: designing and synthesizing biodegradable nanomaterials or masking of peripheral charge of nanomaterials by surface engineering (Jain et al., 2010).

It is generally accepted that poly (d,l-lactide-co-glycolide) (PLGA), taking advantage of remarkable biocompatibility, biodegradability, solubility, and stability, plays a pivotal role as delivery systems for drug and gene, scaffold in tissue engineer and drug in treatment. Aragao-Santiago et al. compared the toxicity of biodegradable and non-biodegradable nanoparticles *via* nebulization and discovered that biodegradable PLGA mostly accumulated in lung and eliminated to half in 17.5 to 19.9 h without an elevated level of IL-6 and TNF-α in bronchoalveolar lavage (BAL) supernatant while non-biodegradable nanoparticle induced overexpression of pro-inflammation cytokines and the recruitment of polymorphonuclear to BAL (Aragao-Santiago et al., 2016). Sun et al. constructed a biodegradable micellar nanoparticle consisting of monomethoxy poly (ethylene glycol), poly (epsilon-caprolactone) (PCL), and poly (2-aminoethyl ethylene phosphate) to deliver siRNA and the nanoparticles showed non-toxicity even at high concentrations (Sun et al., 2008). Biocompatible and biodegradable polymers provide non-toxic building blocks for the treatment of diseases, such as PLGA, PLA, PCL we have mentioned above (Hu et al., 2014). The toxicity of cationic materials can be effectively alleviated by introducing these groups.

Zhang et al. synthesized a series of terpolymer with low charge density and high molecular weight, which possessed low toxicity

and high conversion efficiency (Zhou et al., 2011). Consistent with this finding, another study illustrated that the toxicity decreased with the increase of particle size (Yan et al., 2021). Using high molecular weight and increased hydrophobicity to compensate for low charge density may be a good strategy to balance performance and toxicity (Mastrobattista and Hennink 2011).

Another strategy for the reduction of toxicity is the modification of the nanoparticle surface. In addition to this, modification of nanomaterial surface possesses extra properties such as prolongation of the retention time, improvement of biodistribution and efficiency, and so on (Jain et al., 2010). Polyethylene glycol (PEG) is the most widely used to coat cationic nanomaterials. Karabasz et al. confirmed that five-layer positively charged poly-l-lysine-terminated nanocapsules (NC5) with rapid hematotoxicity did not show cytotoxicity after being incorporated with PEG (Karabasz et al., 2018). PEG could invest in cationic nanomaterials stealthiness without inducing blood, kidney, spleen, and liver acute and extended acute toxicity (Perret et al., 2018). However, it has been reported that modification of nanomaterials with PEG could trigger activation of both the complement and coagulation systems (Pham et al., 2011). But Pannuzzo et al. put forward the solution. They modified nanomaterials with appropriate combinations and proportions of carboxyPEG2000 and methoxyPEG550 can and indeed inhibited activation of complement (Pannuzzo et al., 2020). Besides, other polymers have gradually been developed as modifications to cationic materials such as poly [N-(2-hydroxypropyl)methacrylamide], poly (carboxybetaine), poly (hydroxyethyl-L-asparagine), or poly-L-glutamic acid (Hu et al., 2014). Toy et al. modified primary amines with imidazole-acetic-acid (IAA) to secondary and tertiary amines and demonstrated that introduction of IAA could abate toxicity and immunotoxicity from branched polyethylenimine (bPEI) and chiton through the TLR4 pathway (Toy et al., 2019). A biodegradable, polyelectrolyte m ultilayer shell consisting of poly-L-lysine (PLL) and poly-L-glutamic (PGA) acid was coated with PGA(NC-PGA) and PEG (NC-PEG), respectively. The biochemical and histopathological evaluation suggested that neither of them showed acute or chronic hematotoxicity, hepatotoxicity, or nephrotoxicity. Compared with NC-PEG, NA-PGA didn't provoke activation of immune system (Karabasz et al., 2019).

CONCLUSION

In conclusion, extracellular nucleic acid is an important trigger mechanism in the development and progression of autoimmune diseases, and scavenging extracellular nucleic acid may be one of the candidates attempt to suppress the occurrence and severity of autoimmune diseases. However, the relevant research is still deficient. The use of cationic compounds, scavengers of extracellular nucleic acids, is only in its infancy as a novel treatment for autoimmune diseases. The applied cationic materials are concentrated on the several materials mentioned in the article, but more potential materials were not be studied. More research in the future can focus on other materials, including structural improvements and proportion optimization. At present, cationic materials have been thoroughly studied in various fields, and clinical studies have been carried out on some drugs and gene delivery. Despite the current challenges in cationic nanomaterials, continued improvements will likely yield achieving the new balance between low toxicity and high therapeutic efficacy *in vivo*, which enormous effort need to be devoted to. In addition, the structure construction, proportion distribution, dosage, usage, and pharmacokinetics of cationic compounds also need further exploration. The overall development prospect is considerable.

AUTHOR CONTRIBUTIONS

LW and ZL conceived the idea. BX and KD drafted the original article with contributions from all authors. FH offered significant suggestions for revisions. All authors revised and approved the final article.

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REVIEW
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TNF-α in Uveitis: From Bench to Clinic

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Uveitis is an inflammation of the iris, ciliary body, vitreous, retina, or choroid, which has been shown to be the first manifestation of numerous systemic diseases. Studies about the immunopathogenesis and treatment of uveitis are helpful to comprehend systemic autoimmune diseases, and delay the progression of systemic autoimmune diseases, respectively. Tumor necrosis factor-alpha (TNF-α), a pleiotropic cytokine, plays a pivotal role in intraocular inflammation based on experimental and clinical data. Evidence of the feasibility of using anti-TNF-α agents for uveitis management has increased. Although there are numerous studies on TNF-α in various autoimmune diseases, the pathological mechanism and research progress of TNF-α in uveitis have not been reviewed. Therefore, the objective of this review is to provide a background on the role of TNF-α in the immunopathogenesis of uveitis, as well as from bench to clinical research progress, to better guide TNF-α-based therapeutics for uveitis.

Keywords: uveitis, TNF-α, anti-TNF-α agents, infliximab, adalimumab, golimumab, certolizumab pegol, experimental autoimmune uveitis (EAU)

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INTRODUCTION

Uveitis is a heterogeneous nosological entity. Although the uvea is defined as the middle membrane (Jabs et al., 2005) of the ocular wall comprising the iris, ciliary body and choroid, the term uveitis is broad and encompasses inflammatory damage to the uvea, retina, retinal vessels, vitreous body, and optic papilla (**Figure 1**). The incidence of uveitis in the United States is 52.4/100,000 population, with a prevalence of 115/100,000 population (Gritz and Wong, 2004). One of the primary causes of blindness in developing countries is inefficacious control of or untreated uveitis, mainly owing to complications such as macular edema, glaucoma, and retinal ischemia (Dick et al., 2016). Uveitis is often the first manifestation of many systemic autoimmune diseases. According to recent studies, although 23–63% of uveitis cases are idiopathic (Bodaghi et al., 2001; Jakob et al., 2009; Keino et al., 2009; Barisani-Asenbauer et al., 2012; Bajwa et al., 2015; Llorenç et al., 2015; Luca et al., 2018; Hermann et al., 2021; Sonoda et al., 2021), up to 40% of uveitis patients also have systemic autoimmune diseases. The transparency of the eye allows the vascular lesions to be observed directly with the help of certain devices. Direct visualization of the vessels allows ophthalmologists to assess the inflammatory process in depth before serious tissue damage occurs. Therefore, investigating the immunopathological mechanism of uveitis based on a better understanding of autoimmune diseases is important and enables the development of better treatment methods to decrease the blindness rate and control the progression of autoimmune diseases.

Tumor necrosis factor-alpha (TNF-α) is an acidic protein that is mainly produced by macrophages in response to infection and inflammatory irritation. It is related to chronic inflammation and tissue damage in uveitis and is critical for initiating immunity to pathogens (Vassalli, 1992). TNF receptor I (TNFR1) and TNFR2 are expressed by the intraocular pigment epithelial cells. Further, these cells can produce TNF-α and the matrix metalloprotease, which can

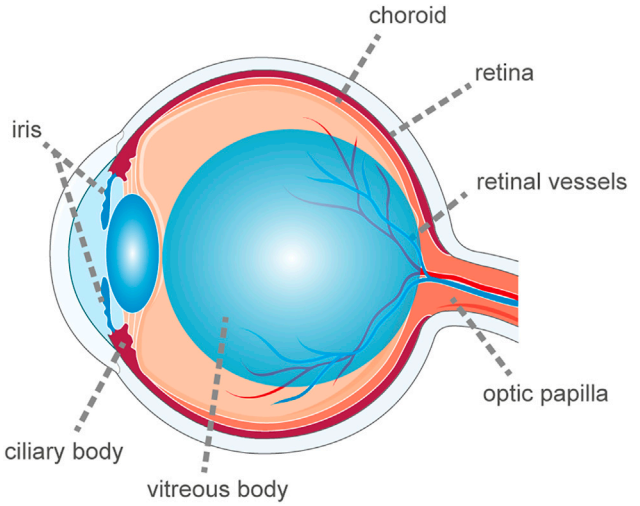

FIGURE 1 | The schematic diagram of ocular anatomy. The term uveitis is broad and encompasses inflammatory damage to the uvea, retina, retinal vessels, vitreous body and optic papilla.

cleave TNF-α from the transmembrane form into a soluble form circulating within the eye. These factors constitute the basis through which TNF-α can cause intraocular inflammation. TNF-α is of vital importance for the intraocular immune reaction, which is referred to as "anterior chamber associated immune deviation" and for the autoregulation of intraocular cell apoptosis (MacEwan, 2002).

Although uveitis represents a group of intraocular inflammatory conditions, each with its own phenotypic heterogeneity, the commonality is the increased expression of TNF-α in both the serum and aqueous humor. Over the past decade, studies have increasingly emphasized the effectiveness of anti-TNF-α agents for patients with uveitis. Although there are numerous studies on TNF-α in various autoimmune diseases, the pathological mechanism and research progress with respect to the role of TNF-α in uveitis have not been reviewed. Thus, in this review, we aimed to provide a background of the role of TNF-α in the immunopathogenesis of uveitis and an account of the progress from bench to clinical research progress to better guide TNF-α-based therapeutics for uveitis.

THE ORIGIN AND BIOLOGY OF TUMOR NECROSIS FACTOR-ALPHA

TNF-α is a cytokine with diverse functions, including inflammation, immunity, cellular communication, cell differentiation, cell death, and survival, and a variety of signaling pathways. Although TNF was identified as early as 1975, the true identity of TNF was unclear until 1984 when Aggarwal et al. reported the isolation of cytotoxic factors, one of which was derived from macrophages, named TNF (Gray et al., 1984; Pennica et al., 1984; Kelker et al., 1985; O'Malley et al., 1988; Aggarwal et al., 2012). Using the same assays, Aggarwal et al. reported the isolation of a cytotoxic factor and named human TNF-α (Aggarwal et al., 1985). In 1990, two immunological TNF-binding proteins, namely 55 kDa (TNFR1) and 75 kDa (TNFR2), were identified, and subsequently, the cDNAs for both human proteins have been cloned (Hohmann et al., 1989; Schall et al., 1990).

TNF-α primarily exists as a trimeric transmembrane protein, transmembrane TNF-α (tmTNF-α), which is subsequently cleaved by TNF-α converting enzyme (TACE; also known as ADAM17) into a soluble form (sTNF-α) (Black et al., 1997). TNF-α has multifunctional bioactivity achieved by binding and activating two different receptors (TNFR1 and TNFR2). TNFR1, which is activated by sTNF-α and tmTNF-α, is ubiquitously expressed. TNFR1 bears the death domain that allows TNFR1 to organize the molecule TNF receptor-associated death domain (TRADD), which is a vital component of the TNFR1 signaling complex. In contrast, TNFR2 expression is limited to certain cell types (e.g., immune cells and endothelial cells). TNFR2 lacks a death domain resulting in its inability to recruit TRADD, and instead, it enlists TNFR-associated factor 1 (TRAF1) and TRAF2. TNFR2 is speculated to be activated primarily by tmTNF-α (Grell et al., 1995; Krippner-Heidenreich et al., 2002; Bystrom et al., 2018). However, there is evidence that sTNF-α might induce biological effects by transferring onto TNFR1 when binding to TNFR2. TNFRs can also be cleaved by TACE to produce soluble forms (sTNFRs), which bind to sTNF-α to exert effects. Studies have shown that sTNFRs are significantly increased in the ocular fluids of patients with active uveitis (Sugita et al., 2007). For TNF-α, the disparate distributions and binding characteristics to receptors are the pathological foundations for the occurrence and development of intraocular inflammation, which could indicate why systematic autoimmune diseases and uveitis have different responses to anti-TNF-α agents.

SIGNALING PATHWAYS ACTIVATED BY TUMOR NECROSIS FACTOR-ALPHA

When TNF-α binds to TNFR1, it assembles different signaling complexes consisting of the complexes I, IIa, IIb (ripoptosome), and IIc (necrosome), resulting in different functional outcomes (Pasparakis and Vandenabeele, 2015). TNF-α complex I signaling primarily mediates homeostatic bioactivities, which comprise tissue regeneration, cell proliferation and survival, inflammation, and immune defense. Similar effects can be caused by the combination of TNF-α and TNFR2, which may be related to the overlapping downstream pathways of TNFR1 signaling pathways. However, the formation of the complex IIa and IIb leads to the activation of a caspase cascade and results in apoptosis, whereas the necrosome induces necroptosis and inflammation. The signaling transduction pathways of the complexes are described briefly below and summarized in **Figure 2**.

Tumor Necrosis Factor-Alpha Signaling in Complex I

With the binding of TNF-α to TNFR1, TRADD (Hsu et al., 1995), receptor-interacting protein kinase 1 (RIPK1), TRAF2, cellular

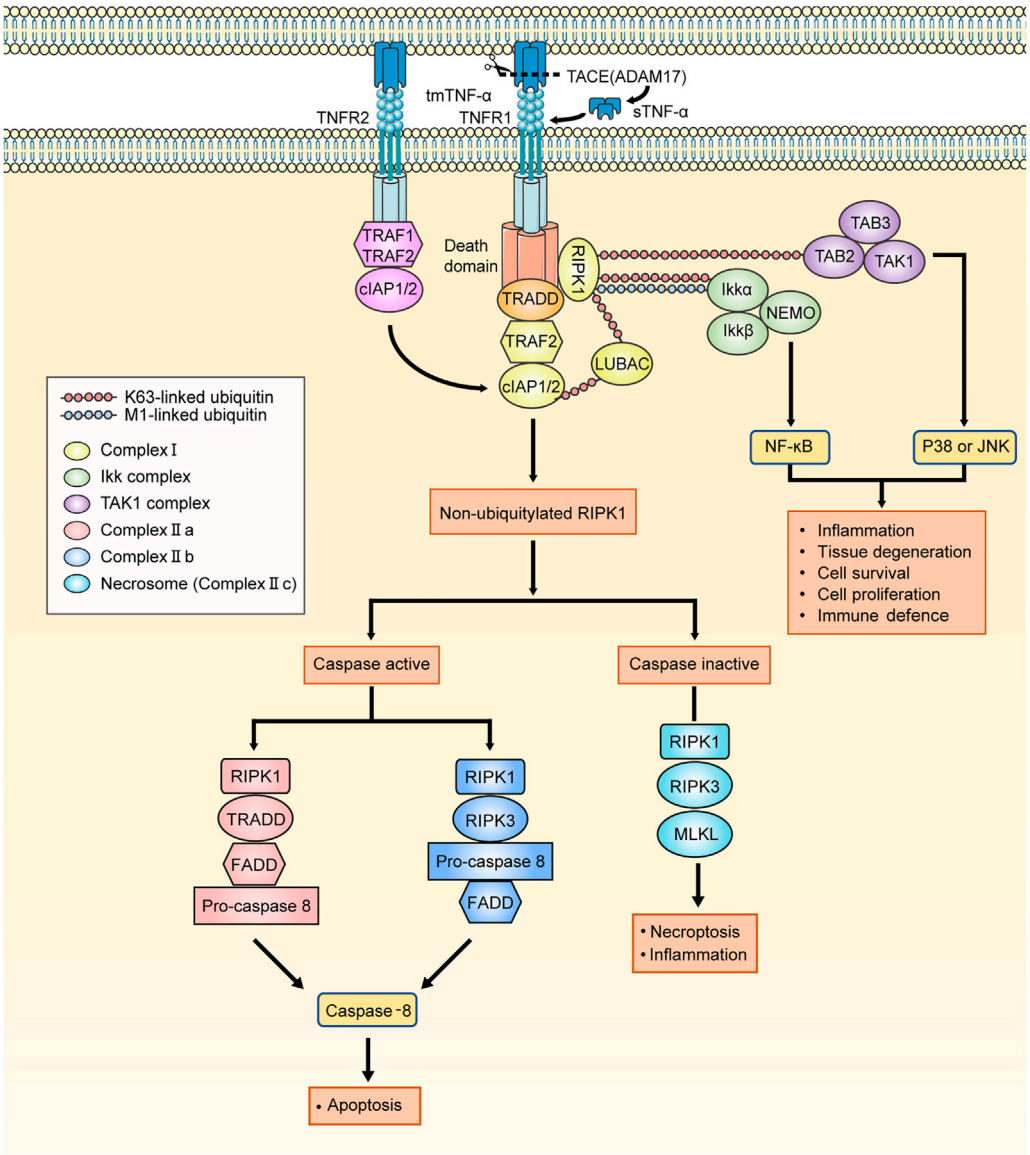

FIGURE 2 | The signaling pathways activated by TNF-α. The tmTNF-α is cleaved by TACE into sTNF-α. The TNFR1 signaling is activated by both tmTNF-α and sTNF-α. When the binding of TNF-α to TNFR1, TNFR1 ligation leads to the recruitment of TRADD, RIPK1, LUBAC, TRAF2, cIAP1/2 and initiate the assembly of TNFR1 complex I. The TNFR2 signaling is almost activated by tmTNF-α. On account of the lack of TRADD, TNFR2 binds to TRAF1/2 directly to recruit cIAP1/2 and affiliate the TNFR1 signaling. The K63 ubiquitin ligase activities which is owned by cIAPs are required for LUBAC recruitment, and cIAPs add M1-linked linear polyubiquitin chains to RIPK1 which makes TAK1 complex and IKK complex assemble to respectively mediate JNK/p38 and NFκB pathways. And RIK1 deubiquitylates under conditions in which the K63-linked and M1-linked polyubiquitin chains are removed by the deubiquitylating enzyme CYLD from RIPK1. The residuum recruits TRADD, FADD and pro-caspase 8, thereby forming the complex IIa. When the cIAPs are depleted, there is no RIPK1 is deubiquitylated and leaves residuum to recruit FADD, pro-caspase 8 and RIPK3, assembling complex IIb. Following the assembly of complex II, pro-caspase 8 conducts autocatalytic cleavage, releasing active caspase 8 to trigger the implementation of the apoptotic program. When deubiquitylated RIPK1 exists but caspase is devitalized, RIPK1/3 cannot be inactivated. Instead, RIPK1 and RIPK3 cluster together to form the complex IIc (necrosome) and necroptosis program is initiated. TNFR1: TNF-α receptor 1; tmTNF-α: transmembrane TNF-α; sTNF-α: soluble TNF-α; TACE: the matrix metalloprotease TNF-α converting enzyme; TNF-α: tumour necrosis factor-alpha; TRADD: TNFR1-associated death domain protein; RIPK1: receptor-interacting serine/threonine-protein kinase 1; LUBAC: linear ubiquitin chain assembly complex; TRAF1/2: TNFR-associated factor 1/2; cIAP1/2: cellular inhibitor of apoptosis protein 1/2; TAK1 complex: TGF-β activated kinase 1 complex, consisting of TAK1, TAK1-binding protein 2 (TAB2) and TAB3; IKK complex: the complex comprising kinases IKKα and IKKβ, nuclear factor-κB (NF-κB) essential modulator (NEMO); JNK: Jun N-terminal kinase; CYLD: cylindromatosis; FADD: FAS-associated death domain protein.

inhibitor of apoptosis proteins 1 (cIAP1), cIAP2, and linear ubiquitin chain assembly complex (LUBAC) sequentially integrate into TNFR1 to form complex I (Silke and Brink, 2010; Brenner et al., 2015). The cIAPs have ubiquitin ligase activity, which is required for LUBAC recruitment, adding M1-linked linear polyubiquitin chains to RIPK1 (Komander

and Rape, 2012). K63-polyubiquitylated RIPK1 associates with the TAK1 complex to activate Jun N-terminal kinase (JNK) and p38-mediated signaling. Furthermore, recruitment of the K63-polyubiquitylated RIPK1 and the IKK complex, which comprises kinases IKKα and IKKβ and nuclear factor-κB (NF-κB) essential modulator (NEMO), activates NF-κB-mediated anti-apoptotic signaling (Micheau and Tschopp, 2003). TNFR2 lacks the death domain sequence, rendering it incapable of recruiting TRADD, and instead, it recruits TRAF1/2 and cIAP1/2 directly. The TNFR2 signaling pathway will overlap with the subsequent TNFR1 signaling pathways here.

A recent study showed that MST1 negatively regulates TNF-α-induced NF-κB signaling by modulating LUBAC activity (Lee et al., 2019). Another report showed that TBK1 and IKKε (NEMO, as mentioned previously herein) prevent TNF-induced cell death via RIPK1 phosphorylation (Lafont et al., 2018). A similar study has shown that H-RN inhibits ocular inflammation in experimental autoimmune uveitis (EAU) by contributing to the attenuation of IKK complex activation and IκB degradation and significantly restraining the phosphorylation of NF-κB (Wang et al., 2014). More studies have been conducted to improve experimental uveitis by inhibiting the NF-κB signaling pathway, such as with lutein (Izumi-Nagai et al., 2007; Kijlstra et al., 2012), growth hormone (Liang et al., 2020), aminooxy-acetic acid (Meka et al., 2015; Mei et al., 2020), dehydroxymethylepoxyquinomicin (Ando et al., 2020), astaxanthin (Suzuki et al., 2006), silibinin (Chen et al., 2017), and aryl hydrocarbon receptor (Huang et al., 2018). Interestingly, a recent study showed that interleukin (IL)-17A inhibits the pathogenicity of Th17 cells by inducing the activation of IL-24 and the transcription factor NF-κB in EAU (Chong et al., 2020). However, clinical trials targeting IL-17A in uveitis have not been successful, which might be because the IL-17A-targeted drug improved EAU by inducing IL-24 *in vivo*, but silencing IL-24 in Th17 cells enhanced the disease. Some studies on inhibiting EAU by blocking the p38 signaling pathway, cannabidiol (El-Remessy et al., 2008), and IL-27 (Meka et al., 2015) have reported related results. These experiments have verified that the TNF-α signaling pathway is related to the pathogenesis of experimental uveitis, especially the NF-κB pathways (Li S. et al., 2010). The regulation of various signaling components in the TNF-α signaling pathways also seems to be promising for controlling the progression of uveitis when there is a poor response to TNF-α-agents. However, these ideas need to be verified with additional *in vivo* and *in vitro* experiments.

Pathways Leading to Apoptosis and Necroptosis

RIPK1, as a pivotal molecular switch, determines whether TNF-α signaling pathways result in cell apoptosis or necroptosis (Ea et al., 2006). RIPK1 is not ubiquitinated under the action of the deubiquitination enzyme cylindromatosis (CYLD) (Kovalenko et al., 2003; Komander et al., 2009) or the depletion of cIAPs (Bertrand et al., 2008), and it recruits different signaling molecules to form complex IIa and IIb, respectively. Following the assembly of complex II, pro-caspase 8 conducts autocatalytic cleavage, releasing active caspase 8 to trigger the implementation of the apoptotic program (Wang et al., 2008). When deubiquitylated RIPK1 exists but caspase is deactivated, RIPK1 and RIPK3 cannot be inactivated. Instead, they cluster together to form complex IIc (necrosome) and the necroptosis program is initiated (He et al., 2009; Li et al., 2012). The level of RIPK3 in cells is responsible for cell necroptosis rather than apoptosis (Vandenabeele et al., 2010). TNF-α-induced cell necroptosis at various barrier surfaces impairs barrier function and leads to inflammation, such as retinal pigment epithelial (RPE) cells (Yumnamcha et al., 2019). However, it has been suggested that apoptosis and subsequent phagocytosis are of vital significance for the clearance of infiltrating cells from the eyes and the dissipation of EAU (Jha et al., 2007).

KEY ROLE OF TUMOR NECROSIS FACTOR-ALPHA IN UNDERSTANDING UVEITIS

In patients with active uveitis or uveitis animal models, TNF-α levels in serum and aqueous humor are elevated, which is correlated with disease status (Fleisher et al., 1991; Kaufmann et al., 2012). TNF-α results in uveitis after intravitreal injection into the rabbit eye (El-Asrar et al., 2011). Evidence suggests a marked association between TNF-α and uveitis. TNF-α induces the release of secondary cytokines, such as IL-6 (Sironi et al., 1989; Sugita et al., 2007) and IL-8 (Sancéau et al., 1990), as well as a monocyte chemotactic and activating factor (Larsen et al., 1989a), to initiate a cascade of events integral to the inflammatory process. TNF-α also induces the release of bioactive lipids, such as eicosanoids (Larsen et al., 1989b; Zavoico et al., 1989), and platelet-activating factor (Bussolino et al., 1986; Camussi et al., 1987; Meyer et al., 1990) and increases the expression of adhesion molecules on vascular endothelial cells (e.g., vascular cell adhesion molecule-1,VCAM-1) (Pober et al., 1986; Bevilacqua et al., 1987; Valone and Epstein, 1988; Carlos et al., 1990). Some investigators reported that TNF-α plays an important role in the upregulation of matrix metalloproteinases (MMPs) in RPE cells and accounts for a directional shift in the balance between MMPs and tissue inhibitors of MMPs (Iademarco et al., 1995; Eichler et al., 2002). Moreover, MMPs, as a type of enzyme that degrades the extracellular matrix, are closely related to the integrity of the blood-retinal barrier (BRB) in uveitis patients (Li H. et al., 2010). T cells are important producers of TNF-α, and TNF-α regulates T cell responses (Nussenblatt, 1991; Cope et al., 1997). Studies have shown that anti-TNF-α therapy suppresses the differentiation of T-helper type 17 cells (Th17) and prevents severe eye inflammation (Masters et al., 2009). In brief, TNF-α, as a key link to intraocular inflammation, recruits leukocytes by mediating the production of intraocular chemokines, increases the adhesion of leukocytes to the vascular endothelium, enhances the antigen extraction ability of dendritic cells, activates macrophages and T cells, and eventually leads to the destruction of the BRB. The following part will summarize the previous research progress on the role of TNF-α in EAU in

chronological order based on basic experiments, with emphasis on the aforementioned points.

Progress on Tumor Necrosis Factor-Alpha in EAU

EAU was first described in 1965 (Wacker and Lipton, 1965; Sugita et al., 2012). It can be induced by many autoantigens of intraocular cells. Animal models have identified retinal S-antigen/arrestin (S-Ag) (Caspi, 2011), interphotoreceptor retinoid-binding protein (IRBP) (Bieganowska et al., 1997), rhodopsin (de Smet et al., 1990), opsin (Yamamoto et al., 1993), phosducin (Gery et al., 1994), recoverin (Dua et al., 1992), Rpe65 (Nityanand et al., 1993), melanin (Nakamura et al., 2005), and lens proteins and cellular retinaldehyde-binding protein (Broekhuyse et al., 1993) as "uveitogenic". Now, EAU is generally used as experimental models of uveitis to study the immunopathologic mechanisms of human intraocular inflammatory diseases. Many studies have observed a constant increase in TNF-α expression in inflammatory cell infiltrates, not only in various models of experimental uveitis, but also in RPE and Müller cells, which causes these cells to possess uveitogenic properties and might decisively influence the course of EAU (de Kozak et al., 1997; de Smet and Chan, 2001; Holtkamp et al., 2001).

Tumor Necrosis Factor-Alpha and TNF-α Blockade in Different Animal Experimental Models

In 1993, a team observed that TNF-α could protect against the inflammatory processes of endotoxin-induced uveitis (EIU) (Huang et al., 2018). By contrast, Nakamura et al. reported that the injection of recombinant huTNF in mouse models increases susceptibility to EAU (Kasner et al., 1993). One experiment demonstrated that mice deficient in TNFR retain their susceptibility to EIU (Nakamura et al., 1994). However, another study indicated that mice with TNF receptor deficiency show decreased inflammation in an immune complex model of uveitis (Smith et al., 1998). In 1997, a study confirmed that TNF-α is not essential for inducing experimental autoimmune diseases (Brito et al., 1999), and a chronic low level of TNF-α might exert protective effects.

In 1996, a study showed that the neutralization of systemic TNF-α ameliorates the pathology of EAU, and interference with afferent processes, especially antigen priming, is important to protect against EAU through anti-TNF-α treatment (Frei et al., 1997). A similar result was observed in a 2001 study, in which IRBP-induced EAU in mice with a TNFR1-Ig fusion protein reduces damage to the retina (Sartani et al., 1996). However, TNF-α neutralization is ultimately not curative in experimental models of relapsing disease (Hankey et al., 2001). In 2003, Baker et al. (1994) identified that etanercept (an anti-TNF-α agent) decreases leukocyte rolling, leukocyte adhesion, and vascular leakage in a rat model of EIU. This outcome suggested that TNF-α is involved in the pathogenesis of uveitis and its potential use as a therapeutic drug to reduce ocular inflammation. In 2019, a study showed that intravitreal infliximab injection exacerbates inflammation in EIU models, whereas systemic infliximab treatment suppresses inflammation effectively and rapidly (Koizumi et al., 2003). It can be seen that the results of both TNF-α and TNF-α blocking experiments are inconsistent in different animal models. These opposite conclusions might be dependent on the experimental model, EAU or EIU. Moreover, these contradictory findings could suggest the different responses of patients with uveitis to certain therapies because of the diversity of uveitis pathogenesis.

Adhesion Molecule Regulation and BRB Rupture

In 1990, some investigators showed that TNF-α antagonists prevent adhesion molecule upregulation on the vascular endothelial cells in rheumatoid arthritis (RA) and experimental allergic encephalomyelitis (Ruddle et al., 1990; Liversidge et al., 2000). In 2011, investigators found that TNF-α expression decreases in aldehyde reductase-deficient mice, downregulating VCAM-1 expression (Elliott et al., 1994). In 2014, a study demonstrated that H-RN, a novel antiangiogenic peptide derived from hepatocyte growth factor which is an important angiogenic factor in vascular retinopathies, suppresses TNF-α-induced adhesion molecule expression (such as VCAM-1) in EAU (Wang et al., 2014). Further, silibinin was shown to prevent EIU and the subsequent production of ICAM-1 by blocking the NF-κB-dependent signaling pathway in 2017 (Chen et al., 2017).

In 1997, a study showed that TNF-α causes BRB rupture by opening tight junctions between retinal vascular endothelial cells and possibly by increasing transdermal vesicle transport in EAU (Yadav et al., 2011). In 2010, a team reported that TNF-α downregulates AQP1 protein expression in the retina, resulting in BRB breakdown (Luna et al., 1997). In 2017, chrysin (5,7-dihydroxyflavone) was reported to maintain the integrity of the BRB via suppression of the expression of inducible nitric oxide synthase (NOS) and macrophage infiltration in the retina, significantly decreasing the percentage of Th17 cells and $CD4^+$ cells, increasing the percentage of Treg cells, and suppressing ocular inflammation during EAU (Motulsky et al., 2010). In 2018, a report indicated that aryl hydrocarbon receptor-knockout mice show a decrease in pro-inflammatory cytokines, such as TNF-α, thereby inhibiting retinal cell apoptosis and reducing BRB decomposition during EAU (Meng et al., 2017).

Effects of Tumor Necrosis Factor-Alpha on Macrophage and Th17 Activity

In 1998, Dick et al. observed that the inhibition of TNF-α activity protects against organ destruction without suppressing retinal T cell infiltration during EAU in Lewis rats. To demonstrate whether neutralizing TNF activity leads to a change in macrophage activation, some trials have used TNFR1, resulting in reduced nitrite in macrophages infiltrating the retina of the treated animal, thereby reducing target tissue damage and destruction (Dick et al., 1998). In these experiments, NOS2 inhibition induced by a nonspecific inhibitor of NOS resulted in a reduction in EAU (Robertson et al., 2003). The role of TNF-α in macrophages was also demonstrated in a 2009 study, which reported that high

mobility group box 1 protein can stimulate TNF-α production in macrophages to promote and amplify ocular inflammation in EAU (Liversidge et al., 2002).

In 2007, Amadi et al. first described Th17 cells in EAU. They confirmed that IL-17 is increased in EAU, regulating TNF-α in retinal cells, suggesting a mechanism in which Th17 might contribute to ocular immunopathology (Watanabe et al., 2009). In 2019, a team reported that although TIPE2-deficient (TIPE2, one member of TNF-α-induced protein) T cells produce more IL-17, they do not migrate to the skin as efficiently. Instead, they migrate to the inflamed eye in a similar manner to TIPE2-deficient T cells and thus exacerbate the development of EAU in TIPE2-deficient mice but reduce the severity of psoriasis in these animals (Amadi-Obi et al., 2007).

TUMOR NECROSIS FACTOR-ALPHA AS A THERAPEUTIC TARGET FOR UVEITIS

Systemic immunomodulatory therapy (IMT) has been used to treat specific patients with uveitis over the last decades. Corticosteroids are an important component of IMT and are also the first-line treatment for uveitis. However, patients with uveitis are at risk of long-term complications caused by long-term uncontrolled inflammation and corticosteroid therapy, which can reduce the treatment success rate for the disease itself. Therapeutic strategies have evolved over the last few years, and anti-TNF-α agents have become well accepted for the treatment of refractory uveitis. Anti-TNF-α agents have fewer adverse effects than corticosteroids. Studies have shown that when used properly, dependence on corticosteroids can be significantly reduced to prevent uveitis recurrence (Liu et al., 2019).

Development of anti-TNF-α Agents in Uveitis

The first use of anti-TNF-α agents was reported in the 1980s in experimental models of sepsis (Beutler et al., 1985; Tracey et al., 1987; Calandra et al., 1991; Hu et al., 2020). In 1985, Feldmann et al. identified TNF-α as a therapeutic target for RA and reported the first proof of concept trials (Feldmann and Maini, 2003). In 1991, Keffer et al. reported the effectiveness of anti-TNF-α therapy for arthritis (Keffer et al., 1991). The success of phase I/II trials of anti-TNF-α antibodies announced in 1992 contributed to the performance of clinical trials for other chronic diseases. Since the first reported use of infliximab in 2001 for uveitis treatment, several new anti-TNF-α agents have been developed for the treatment of refractory uveitis (Muñoz-Fernández et al., 2001; Sfikakis et al., 2001). Four monoclonal anti-TNF-α antibodies, namely, infliximab (IFX; Remicade®), adalimumab (ADA; Humira®), golimumab (GOL; Simponi®), and certolizumab pegol (CZP; Cimzia®), are available. Etanercept (Enbrel®) is the only commercially available receptor fusion protein (Sandborn et al., 2001). In 2011, Cordero-Coma et al. first reported two cases of treatment with GOL, which both achieved satisfactory results (Tracey et al., 2008; Cordero-Coma et al., 2011). In 2016, the United States Food and Drug Administration (FDA) approved ADA as the first anti-TNF-α agent for the treatment of non-infectious intermediate, posterior, and panuveitis (Hasegawa et al., 2019). In the same year, clinical trials were performed on the effectiveness of CZP for refractory spondyloarthritis-related uveitis, but no significant advantages were found over other anti-TNF-α agents (Rudwaleit et al., 2016). Different inhibitors have different functional profiles. IFX, ADA, and GOL are humanized monoclonal antibodies, whereas CZP is a monovalent fragment linked to polyethylene glycol. ADA and GOL are fully human monoclonal antibodies; however, IFX is a chimeric protein with both human and murine components. The lack of the fragment crystallizable (Fc) portion suppresses the high immunogenicity of CZP and makes it less likely to cross the placenta in pregnant patients. Etanercept is a recombinant fusion protein composed of the extracellular portions of TNFR2 combined with the Fc portion of human immunoglobulin G-1. The most frequent side effect was determined to be infusion reaction, with infectious diseases including tuberculosis being second most common; the occurrence of demyelinating or autoimmune diseases was seldom reported. The associated risk of cancer has been debated. To date, anti-TNF-α agents have made more progress for uveitis treatment. The different characteristics of anti-TNF-α agents derived from clinical trials are summarized as below (summarized in **Supplementary Table S1**).

ADA (Humira®)

The advantages of ADA are listed as follows:

1) Compared with IFX, ADA is a fully human monoclonal antibody that causes almost no allergic reactions, and subcutaneous injection is safer and more convenient than intravenous injection (Ming et al., 2018).
2) During steroid tapering, ADA significantly reduces the relapse rate, visual deterioration, and anterior chamber flare, and has relatively good tolerance (Díaz-Llopis et al., 2012; Jaffe et al., 2016).
3) The use of ADA in the treatment of uveitis associated with Behçet's disease (BD) is not affected by the concomitant application of antirheumatic agents (Nguyen et al., 2016; Fabiani et al., 2018).
4) Numerous studies have shown that ADA is superior to immunosuppressive agents in decreasing the relapse rate and occurrence of retinal vasculitis and improving visual acuity (Sota et al., 2021).
5) ADA is safe and efficacious for the treatment of non-infectious uveitis in elderly patients (Moll-Udina et al., 2020).
6) ADA seems to be associated with better outcomes after follow-up, although both IFX and ADA are efficacious for refractory BD-related uveitis (Atienza-Mateo et al., 2019).
7) ADA plus conventional therapy outperforms conventional therapy alone in patients with retinal vasculitis due to refractory BD-related uveitis (Yang et al., 2021a; Yang et al., 2021b).
8) In children and adolescents with active juvenile idiopathic arthritis (JIA)-related uveitis, the treatment failure rate of

ADA is lower than that of the placebo (Ramanan et al., 2017; Angeles-Han et al., 2019).

The disadvantages of ADA are as follows:

1) Adverse events were reported in patients who received ADA (Díaz-Llopis et al., 2012). The most frequently reported treatment-emergent adverse event is infection (Al-Janabi et al., 2020).
2) The use of ADA for undifferentiated uveitis might result in premature discontinuation on account of side effects (Al-Janabi et al., 2020; Llorenç et al., 2020).

The indications are as follows:

1) Non-infectious uveitis, intermediate uveitis, posterior uveitis, and panuveitis in adult patients with underreaction and contraindications to steroids, as well as steroid dependence in Europe (Leclercq et al., 2020).
2) Non-infectious uveitis, intermediate uveitis, posterior uveitis, and panuveitis in adult patients in the United States (Leclercq et al., 2020).
3) As a first-line immunomodulator for the treatment of ophthalmic manifestations of BD (Touhami et al., 2019).
4) As a second-line immunomodulator for the treatment of uveitis associated with JIA (Angeles-Han et al., 2019; Llorenç et al., 2020).
5) ADA is approved for RA, ulcerative colitis, psoriatic arthritis, ankylosing spondylitis (AS), Crohn's disease, and plaque psoriasis in adults (Llorenç et al., 2020).

IFX (Remicade®)

The advantages of IFX are listed as follows:

1) IFX showed commendable efficacy for refractory non-infectious uveitis and severe uveitis cases associated with BD whether it was used as monotherapy or with other immunosuppressive agents (Vallet et al., 2015; Vallet et al., 2016).
2) IFX showed a significantly higher capacity to resolve macular edema in treating sight-threatening retinal vasculitis when compared with the effects of ADA (Levy-Clarke et al., 2014).
3) A report indicated that IFX is effective as a treatment for visually threatening refractory posterior uveitis (Joseph et al., 2003).
4) Multiple studies have shown that IFX is superior to immunosuppressive agents in reducing recurrence rates and ameliorating visual acuity (Vallet et al., 2015).
5) Arida et al. reported that 40% of BD cases remained in remission 3 years after the discontinuation of IFX (Vallet et al., 2015). In the event of relapse, good response rates were obtained after the resumption of IFX therapy (Markomichelakis et al., 2011).

The disadvantages of the IFX are listed as follows:

1) Tolerance is low owing to the relatively frequent infusion reactions (Lichtenstein et al., 2015; Leclercq et al., 2020).
2) Tuberculosis as an adverse effect was reported in patients treated with IFX (Tugal-Tutkun et al., 2005).
3) One study reported a higher rate of IFX toxicity in patients with uveitis (Markomichelakis et al., 2011).

The indications are as follows:

1) Numerous experts have recommended IFX as first-line therapy for visually threatening BD (macular ischemia, cystoid macular edema, serious vasculitis, monophthalmic patients) (Arida et al., 2011; Hatemi et al., 2018).
2) As a second-line immunomodulator for the treatment of uveitis related to JIA (Angeles-Han et al., 2019).
3) For the treatment of severe ocular inflammatory conditions including posterior uveitis, panuveitis, severe uveitis associated with seronegative spondyloarthropathy, and scleritis in patients requiring immunomodulation (Levy-Clarke et al., 2014).
4) Infliximab is authorized by the FDA for the treatment of RA, AS, Crohn's disease, psoriatic arthritis, plaque psoriasis in adults, and ulcerative colitis (Sobrin et al., 2007; Arida et al., 2011).

GOL (Simponi®)

The advantages of the GOL are listed as follows:

1) Compared with IFX, GOL is a fully human monoclonal antibody that causes almost no allergic reactions (Ming et al., 2018).
2) GOL is effective in improving visual acuity and controlling ocular inflammation (Cordero-Coma et al., 2014).
3) GOL has been proven to be conducive to AS-related anterior uveitis, ameliorating macular edema and inflammation, and decreasing the relapse rate (Calvo-Río et al., 2016; Fabiani et al., 2016).
4) The control of intraocular inflammation with multi-refractory uveitis associated with BD (Hatemi et al., 2018).
5) GOL represents an efficacious and secure therapy choice for uveitis with a significant reduction in the frequency of ocular flares while preserving visual function with a satisfactory long-term retention rate (Fabiani et al., 2019).
6) The effective treatment of JIA and idiopathic retinal vasculitis by GOL has been reported, whereas other anti-TNF-α agents are ineffective (Tosi et al., 2019).

CZP (Cimzia®)

The advantages of the CZP are listed as follows:

1) CZP can be an effective alternative to long-lasting chronic relapsing uveitis (Llorenç et al., 2016).
2) Some studies have shown a significant decrease in ocular flares with a satisfactory long-term retention rate with CZP compared to that with placebo (Tosi et al., 2019).
3) A national multicenter observational study supported the efficacy of CZP for the management of uveitis during pregnancy (Prieto-Peña et al., 2021). In terms of pregnancy safety, CZP displayed advantageous properties over other

anti-TNF-α agents because of its limited transport across the placenta (Mariette et al., 2018).
4) One study showed that the relative *in vitro* neutralizing potency is higher for CZP than for ADA (Berkhout et al., 2020).
5) One study observed positive outcomes using CZP as therapy for patients with refractory, non-infectious uveitis when other anti-TNF-α agents proved inadequate or when tolerance issues were present (Sharon and Chu, 2020).

Etanercept (Enbrel®)

The disadvantages of etanercept are listed as follows:

1) Owing to its poor intraocular permeability and limited effectiveness, it is not recommended for uveitis (Dick et al., 2018).
2) Granulomatosis, as a side effect, has been reported in the treatment of uveitis with etanercept (Leal et al., 2019).
3) Meta-analyses have shown that etanercept is inferior to other anti-TNF-α agents for uveitis treatment (Leal et al., 2019).
4) Paradoxical occurrences of uveitis have also been reported after etanercept administration in patients with AS-related acute anterior uveitis (Fabiani et al., 2016).
5) Etanercept might be less efficient than other anti-TNF-α agents in decreasing the risk of HLA-B27-related acute anterior uveitis in patients with spondyloarthritis (Mitulescu et al., 2018).

The indications are as follows:

1) Etanercept received FDA approval for RA, polyarticular JIA, AS, psoriatic arthritis, and plaque psoriasis (in patients aged 17 years and older) (Fabiani et al., 2016).
2) International guidelines concluded that the use of etanercept for the treatment of uveitis is not supported (Dick et al., 2018).

CONCLUSION

Inefficiently controlled or untreated uveitis is one of the primary causes of blindness in developed countries. Corticosteroids remain the first-line treatment; however, their chronic use can result in side effects. These complications have led investigators to seek corticosteroid-sparing treatments. Although uveitis represents a group of intraocular inflammatory conditions with distinct phenotypic heterogeneity, its common feature is increased expression of TNF-α in both the serum and aqueous humor. Over the past decade, studies have increasingly emphasized the effectiveness of anti-TNF-α agents for patients with uveitis. However, the lack of clinical trials and the rarity and heterogeneity of uveitis make their utilization in ophthalmology more challenging, particularly for first-line therapy.

Most international studies have focused on ADA and IFX, which are the most commonly recently employed biological agents for patients with uveitis. Authoritative experts recommended the use of ADA in cases of nullity or intolerance to immunosuppressive agents for non-infectious non-anterior uveitis. IFX was proposed as a first-line treatment for sight-threatening uveitis associated with BD. Nevertheless, knowing which of the two has a better effect in combating uveitis is an unmet demand. ADA is well tolerated with acceptable side effect profiles, and its costs have also decreased to acceptable levels. These properties make it an excellent option as second-line and reserved steroid therapy for uveitis. However, whether the earlier introduction of ADA would confer additional benefits in the management of uveitis and the preservation of visual function is unclear. GOL seems to have more evident advantages as a therapy for spondylitis-related uveitis. In terms of pregnancy safety, CZP has favorable characteristics over other anti-TNF-α agents owing to its limited transport across the placenta.

Furthermore, there are still many questions regarding the use of anti-TNF-α agents as a therapy for uveitis, including the following: treatment duration, when to stop using, the necessity to monitor drug levels regularly, alternative biological agents if anti-TNF-α failure occurs, how to reduce the immunogenicity against anti-TNF-α molecules, and how to ameliorate efficacy. Moreover, treatment failure when using one anti-TNF-α agent does not indicate that other agents in the same group will also be ineffective. Some studies have reported that agents in a group can be replaced with each other by changing novel routes of drug administration to less intense places such as subcutaneous injections. The development of monoclonal antibodies that simultaneously recognize multiple targets allows for more effective treatment of uveitis at a lower dose than that with any single biological drug. Alternatively, a secure and efficient sustained-release device can be developed that will enable the topical treatment of idiopathic immune-mediated uveitis with immunomodulators, including biological response modifiers. The pathogenic effect of TNF-α is caused by complex signaling pathways composed of cascades of signaling molecules. When the efficacy of anti-TNF-α agents is not good, changing therapeutic targets to different signaling molecules in the pathway is also a good alternative. However, these ideas need to be verified with additional *in vivo* and *in vitro* experiments.

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SUPPLEMENTARY MATERIAL

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