

Emerging Insights into Macrophage Roles in Dry Eye Disease Onset and Progression

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ABSTRACT

Dry eye disease (DED), also known as keratoconjunctivitis sicca, is a common chronic inflammatory disease of the eyes that causes discomfort, burning, and various other symptoms. Its prevalence is increasing, and the underlying mechanisms remain unclear. It is well established that inflammation on the ocular surface (OS) triggers DED. This is indicated by elevated levels of inflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF)- α , found in the tear fluid and the epithelial layers of the cornea and conjunctiva, as well as by the infiltration of numerous inflammatory immune cells, particularly macrophages. Any disruption in the planned functional harmony between OS structural cells and resident or trafficking macrophages can impair OS health. Over the past decade, researchers have studied the diversity and role of OS macrophages in DED. The OS, like other mucosal tissues, contains a diverse population of macrophages, some of which are altered in DED. In this review, we outline the origin of macrophages in DED and describe their specific roles in relation to the main stages of this disease process. We discuss how hyperosmolarity induces pathological changes in DED and how it is related to macrophage functions. We then explain how a high-fat diet affects macrophage-related signaling pathways and thus contributes to the development of DED. Finally, we discuss how exosomes can be used in the treatment of dry eye disease.

Keywords: Cytokines; Dry eye syndromes; Extracellular vesicles; Immune response; Inflammation; Macrophage; Ocular surface; Therapeutics

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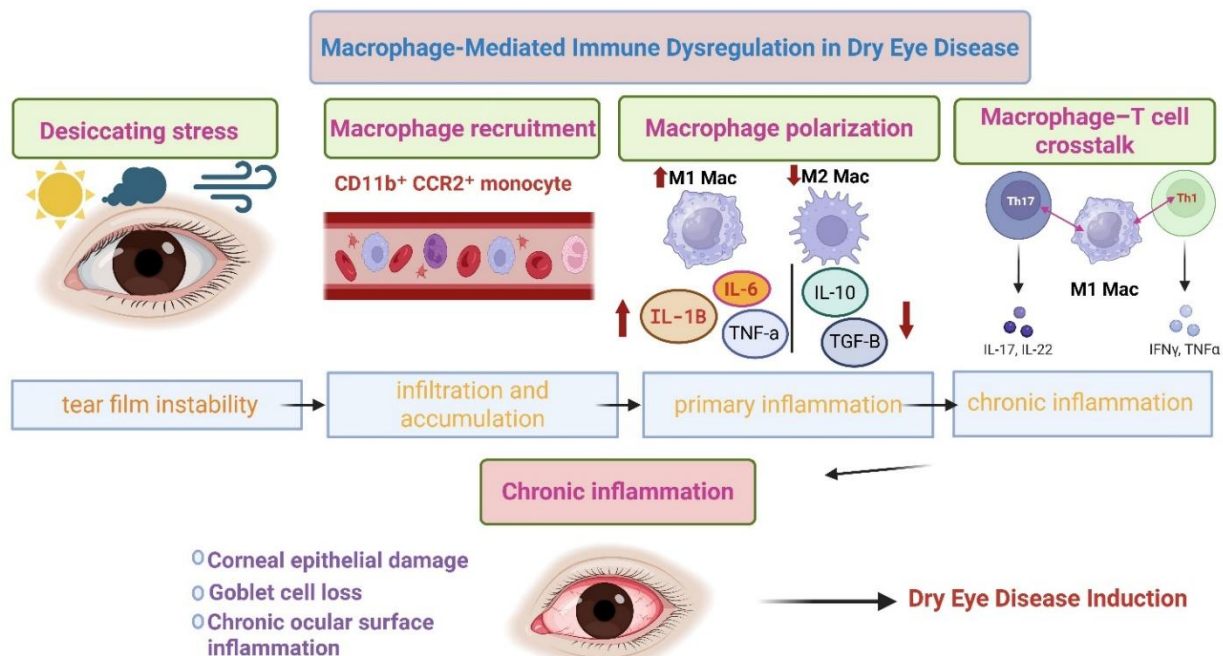
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INTRODUCTION

Dry eye disease (DED), which typically affects about 5%–50% of the population worldwide, depending on the population studied and the diagnostic criteria used,^{1,2} is a multifactorial disorder that involves the tears and the surface of the eye.³ It is an abnormality of the tear film involving the lacrimal glands, corneal surface, and eyelids, resulting in an inadequate tear film.⁴ It can result from diseases such as graft-versus-host disease (GVHD) and various autoimmune diseases, causing significant clinical problems by leading to symptoms and signs including discomfort, visual disturbances, tear film instability, increased tear film osmolarity, and ocular surface (OS) inflammation.⁵⁻⁷ The efficacy of currently available treatments, including artificial tears, corticosteroids, and non-specific immunosuppressive agents, remains limited, and a substantial proportion of patients fail to respond.⁸ This emphasizes the need to develop more effective immunotherapeutic drugs and biologics, as they can potentially improve clinical outcomes for patients with DED.

The immune response plays a pivotal role in the initiation and progression of DED.^{9,10} Different

populations of immune cells are involved in the development of DED and are immediately attracted to the site of damage.¹¹ Macrophages, as important immune cells, have a significant impact on the entire process from inflammation to healing in DED through the expression of specific phenotypes. Normally, macrophages are recruited in large numbers to the affected area in response to injury via the C-C chemokine receptor type 2 (CCR2) and become the predominant immune cells. These macrophages not only contribute significantly to the inflammatory response by engulfing tissue debris and releasing numerous pro-inflammatory cytokines and proteinases, but also produce various mediators that promote extracellular matrix (ECM) production, cell growth, and angiogenesis.¹² Advances in this field have shown that therapeutic approaches used or investigated for DED exert anti-inflammatory effects that may involve modulation of macrophage-associated inflammatory pathways, supporting the idea that macrophages may be a potential target in the treatment of inflammation-related DED.¹³ The evidence that inflammatory macrophages may act as independent triggers of the DED process and its clinical consequences makes it necessary to investigate this type of immune cell.



Graphical abstract.

In this review, we discuss the multiple roles and functions of macrophages in DED, focusing on their respective immunological profile, metabolism, and phenotype. In addition, we highlight key inflammatory pathways and strategies, both current and potential, to target them to treat DED.

Origins, Heterogeneity, and Polarization of OS Macrophages in Health and DED

Macrophages play a crucial role in the innate immune system and are known for their ability to present antigens, which helps to promote cell-mediated immunity to control infections, fight off pathogens such as microbes, and maintain the body's homeostasis.^{14,15} In addition, they are involved in regulating the healing process of wounds after tissue damage, from dampening inflammation to clearing cell debris and coordinating tissue repair.¹⁶ OS macrophages display three functionally intricate subtypes. M0 macrophages have the ability to polarize and produce classical M1 and M2-like macrophages in reaction to Th1/Th2 cytokines; however, M1 and M2-like macrophages do not completely represent the complex phenotype of macrophages. Research indicated that M ϕ C1 is similar to resident-like macrophages,¹⁷ M ϕ C2 is inclined to be M0 macrophages, and the M ϕ C3 subtype demonstrated characteristics of a pro-inflammatory nature along with robust features of a chronic inflammatory response.¹⁸ Macrophage populations at the ocular surface include both monocyte-derived macrophages that are recruited during inflammatory conditions such as DED and tissue-resident macrophages that support immune surveillance and homeostasis.^{19,20} Ocular macrophages are tissue-resident and contribute to epithelial maintenance, clearance of cellular debris, immunological tolerance, and tissue repair. In contrast, circulating monocytes are attracted to the ocular surface during inflammation via chemokine pathways, particularly CCL2/CCR2 signaling, and then differentiate into inflammatory macrophage populations. Although recruited macrophages can aid host defense and repair, prolonged activation may exacerbate chronic inflammation by producing excessive cytokines, chemokines, and matrix-remodeling enzymes.²¹

Recent advances in scRNA-seq techniques have provided significant insights into macrophage heterogeneity, particularly by identifying distinct transcriptional types of macrophages in the eye.

Specifically, subtypes of macrophages have been found with resident, inflammatory, and remodeling properties, indicating that macrophage phenotypes are dynamic functional states rather than discrete M1 and M2 types. In other words, although it is still useful in experimental settings to refer to M1/M2, understanding macrophage involvement in DED requires consideration of their specific features.^{22,23} Accordingly, the terms “M1-like” and “M2-like” in this review refer to macrophages with predominantly inflammatory and reparative characteristics, respectively, without specifying particular cell subsets.

Macrophages in response to a variety of factors can alter their phenotype into M1-like or M2-like macrophages in response to different stimuli, thereby influencing their functionality.²⁴ Lipopolysaccharide (LPS), which is found in the outer membrane of Gram-negative bacteria, can trigger the transformation of macrophages to the Pro-inflammatory macrophage state, whereas cytokines like interleukin 4 (IL-4) and IL-13 can enhance the differentiation of macrophages into the anti-inflammatory macrophage state.²⁴ The roles played by M1 and M2-like macrophages are closely linked to inflammatory responses, with M1-like macrophages primarily involved in pro-inflammatory responses and tissue damage by producing factors such as IL-6, IL-12 and tumor necrosis factor (TNF), while M2-like macrophages primarily trigger anti-inflammatory responses by producing cytokines such as IL-10 (an anti-inflammatory cytokine), and thereby lead to the tissue remodeling and healing.^{25,26} Inflammatory macrophage-associated mediators may contribute to disruption of corneal epithelium, cause apoptosis of epithelial cells, and leads to instability in the tear film system. Therefore, Pro-inflammatory macrophage activation is associated with reduced TBUT, increased corneal fluorescein staining, and ocular surface damage. Increased levels of pro-inflammatory cytokines in the tear film, including IL-1 β , IL-6, TNF- α , CXCL8 (IL-8), and CCL2, have been observed in moderate to severe DED and correlate with disease severity.^{27,28} Furthermore, persistent Pro-inflammatory macrophage activation may contribute to amplification of the inflammatory environment through interactions with dendritic cells and Th17 lymphocytes, resulting in epithelial damage, tear film instability, and symptom development.²⁹ The use of cytokine expression patterns is common when assessing the phenotype and functional status of macrophages.³⁰ However, it is

important to note that these functional phenotypes have the potential to reverse. The macrophage activation on the OS is increased in samples from humans with DED³¹ and in animals,^{32,33} contributing to the initiation and resolution of inflammatory processes. Experimental studies have shown an increased polarization of macrophages towards an inflammatory pro-inflammatory macrophage state with simultaneous suppression of the anti-inflammatory macrophage state on the OS in animals with DED.^{34,35} In one research study, an increase in TNF- α and *NOS2A* (nitric oxide synthase 2A) expression by corneal macrophages was noted, along with a reduction in *CSIF* (human cytokine synthesis inhibitory factor) and arginase 1 (*ARG1*) expression.³³ The transition to a greater proportion of the Pro-inflammatory macrophage state compared to the anti-inflammatory macrophage state could be due to the presence of pro-inflammatory cytokines and the activation of Th1 responses under acute desiccation stress, which induce macrophages to adopt an Pro-inflammatory macrophage state.³³ M1-like macrophages produce pro-inflammatory cytokines such as IL-6 and IL-23, which actively contribute to the amplification of Th17 cells and the onset of DED. In contrast, M2-like macrophages synthesize IL-10, playing a critical role in tissue remodeling and suppression of inflammatory responses.³⁵ Research in DED animals has shown a strong link between these macrophages and the spread of inflammation in DED, both in the lymph nodes (by supporting T cell activation) and on the OS.³⁶ Therefore, controlling macrophages' activation and movement patterns may be beneficial in treating DED. Recent studies have explored the indirect therapeutic use of M2-polarized macrophages in DED models. In a scopolamine-induced DED mouse model, M2 macrophage-conditioned media (M2-CM) increased the vitality, proliferation, and migration of primary mouse corneal ECs (CECs) in co-culture.³⁷ Topical M2-CM therapy improved OS integrity by increasing tear volume, reducing fluorescein staining scores, and delaying tear film break-up. Analyses showed decreased pro-inflammatory cytokines and cells (M1-like macrophages, Th1, Th17) in ocular tissues, as well as increased anti-inflammatory factors and M2-like macrophages, indicating a promising cell-free treatment for this chronic disease.³⁸

Crosstalk between Macrophages and Other Populations of Immune Cells in DED

Macrophages are key immune mediators within the

Ocular surface immune system and interact extensively with neutrophils, DCs, T lymphocytes, and ILCs. These interactions are bidirectional and involve cytokines, chemokines, lipid mediators, antigen presentation, and direct cell-to-cell contact. This immune crosstalk can either lead to resolution of inflammation or drive its progression into tissue damage at the ocular surface.

Macrophages and Neutrophils

Communication between macrophages and neutrophils is particularly important in acute and chronic inflammation of the ocular surface. Activated macrophages synthesize the chemokines CXCL1, CXCL2, CXCL8, and CCL2, as well as the cytokines TNF- α and IL-1 β , thereby promoting neutrophil recruitment and activation. In turn, neutrophils secrete ROS, proteases, neutrophil elastase, and release neutrophil extracellular traps (NETs), which stimulate macrophage activation and cause epithelial damage. This ongoing mutual activation of macrophages and neutrophils can result in an inflammatory cascade in DED.³⁹

Macrophages and DCs

Both macrophages and DCs play critical roles in innate immune surveillance and antigen presentation and could be located in different niches on the surface of the eye. Cytokines and other mediators released by macrophages, such as TNF- α , IL-1 β , IL-6, etc, can stimulate the differentiation of DCs into more mature antigen-presenting cells. Activated DCs, in their turn, stimulate T-cell responses and provide signals for macrophage activation through the secretion of cytokines and chemokines. This interaction between macrophages and DCs is especially crucial for autoimmune DED development, where ongoing antigen presentation leads to adaptive immune response initiation.⁴⁰

Macrophages and Th17 cells

An especially significant interplay exists between macrophages and Th17 cells. Cytokines IL-1 β , IL-6, and IL-23 released by macrophages have been shown to induce the differentiation and proliferation of Th17 cells, leading to an increase in IL-17A and other pro-inflammatory factors. IL-17 can then act on ocular surface epithelial and myeloid cells, causing an increase in the secretion of chemokines and matrix metalloproteinases and hence further inflammation and epithelial cell damage.⁴¹

Macrophages and T Regulatory Cells

The effect of Tregs on macrophages is primarily immunosuppressive. Through anti-inflammatory factors, including IL-10 and TGF- β , and direct cell contact, Tregs restrain macrophage overactivation and promote inflammation resolution. At the same time, depending on their activation state and cytokine expression pattern, macrophages can modulate Treg differentiation and function. Disruption of the balance between pro-inflammatory macrophages and Tregs may lead to immune dysregulation and chronic inflammation of the ocular surface.⁴²

Macrophages and Innate Lymphoid Cells

It is increasingly recognized that macrophages also interact with innate lymphoid cells (ILCs), particularly the ILC2 and ILC3 subsets, although this has been relatively less explored in DED compared with macrophage interactions with T cells and neutrophils. Cytokines and lipid mediators produced by macrophages can affect ILC activation, while cytokines produced by ILCs, such as IL-5, IL-13, IL-17, and IL-22, depending on the ILC subtype, may influence myeloid cell functions and epithelia. Because ILCs are associated with mucosal immunity and tissue repair, the interplay between macrophages and ILCs could represent an important yet understudied pathway of innate immune activation.⁴³

Taken together, these findings suggest that macrophages cannot be regarded as isolated effector cells in the context of DED. Instead, they are part of an intricate immune network in which interactions between macrophages and neutrophils drive acute inflammation, interactions between macrophages and DCs enable antigen presentation, interactions between macrophages and Th17 cells sustain inflammation, interactions between macrophages and Treg cells govern immune resolution, and interactions between macrophages and ILCs may contribute to immune homeostasis and repair at the ocular surface.

Macrophage-Associated Signaling Networks in Dry Eye Disease

Macrophage activation in DED involves several coordinated signaling pathways rather than a single molecular mechanism. Environmental stressors such as tear hyperosmolarity, oxidative stress, microbes, and endogenous damage-associated molecular patterns (DAMPs) are sensed by pattern recognition receptors,

including cell-surface, endosomal, and intracellular sensors on the surface of ocular macrophages.^{44,45} The recognition of DAMPs by TLRs activates downstream signaling through MyD88-dependent pathways that ultimately activate the NF- κ B and MAPK pathways.^{46,47} Inflammation and epithelial barrier dysfunction in DED are driven by increased expression of pro-inflammatory mediators, including TNF- α , IL-1 β , IL-6, IL-12, CCL2, CXCL8, and MMP-9 (Figure 1).^{48,49}

The transcription factor NF- κ B acts as the master switch of inflammatory responses in macrophages and is one of the major signaling nodes in DED. Continuous activation of NF- κ B drives ongoing cytokine synthesis and enhanced leukocyte recruitment, maintaining the chronic inflammatory process via positive feedback loops.^{45,46} Activation of MAPK family members, ie, ERK1/2, JNK, and p38 MAPK, regulates the transcription of inflammatory genes, macrophage survival and motility, and cytokine secretion.^{47,50} Moreover, inflammatory cytokines produced in association with DED also activate the JAK/STAT3 signaling pathway. Activation of STAT3 promotes macrophage activation, increases the production of inflammatory cytokines, and enhances interactions between macrophages and adaptive immune cells such as Th17 cells.^{51,52}

An important pathway activated in DED is the NLRP3 inflammasome, triggered by oxidative stress, mitochondrial dysfunction, potassium efflux, and ROS. NLRP3 inflammasome formation activates caspase-1 and induces the secretion and maturation of IL-1 β and IL-18, causing pyroptotic cell death under certain circumstances. There is increasing evidence that activation of the NLRP3 inflammasome plays an important role in exacerbating ocular surface inflammation and epithelial damage in DED (Figure 1).⁵³⁻⁵⁵ The study investigating the role of the NLRP3 inflammasome in humans included patients with Sjögren's syndrome dry eye disease (SSDE) and non-Sjögren's dry eye disease (NSSDE). Among 54 patients with SSDE, 50 patients with NSSDE, and 46 healthy controls, the expression levels of NLRP3 and caspase-1 were measured in conjunctival impression cytology samples, while IL-1 β and IL-18 were assessed in tear samples. Higher levels of NLRP3, caspase-1, IL-1 β , and IL-18 were found in the DED group, with particularly elevated NLRP3 levels observed in SSDE.⁵⁶

Recent evidence highlights a strong connection between macrophage phenotype and cellular

metabolism. Classically activated inflammatory macrophages depend heavily on aerobic glycolysis for their energy needs, while anti-inflammatory and reparative macrophages rely more on OXPHOS and FAO.^{57,58} Hyperosmolarity and oxidative stress drive metabolic switching toward glycolysis and increase inflammatory signaling via NF- κ B and HIF-1 α .^{59,60} These observations indicate that metabolic pathways are not merely consequences of macrophage activation but active regulators of macrophage function, and thus represent promising therapeutic targets in DED.

The rationale for using macrophage-targeted treatment is related to the underlying mechanisms of the innate immune response in DED at the molecular level. For instance, blocking the TLR pathway can limit

activation of the NF- κ B and MAPK pathways, thereby reducing the production of inflammatory mediators. Targeting the NF- κ B or MAPK pathways can further inhibit the production of cytokines and chemokines in activated macrophages, as well as in other ocular surface cells. Similarly, inhibiting the NLRP3 inflammasome can prevent activation of caspase-1 and the maturation of IL-1 β and IL-18.^{12,56,61,62} Collectively, TLR signaling, NF- κ B, MAPK, STAT3, NLRP3 inflammasome activation, and metabolic reprogramming form an integrated signaling network that governs macrophage plasticity, inflammatory mediator production, immune cell crosstalk, and progressive ocular surface damage throughout the course of DED (Table 1).

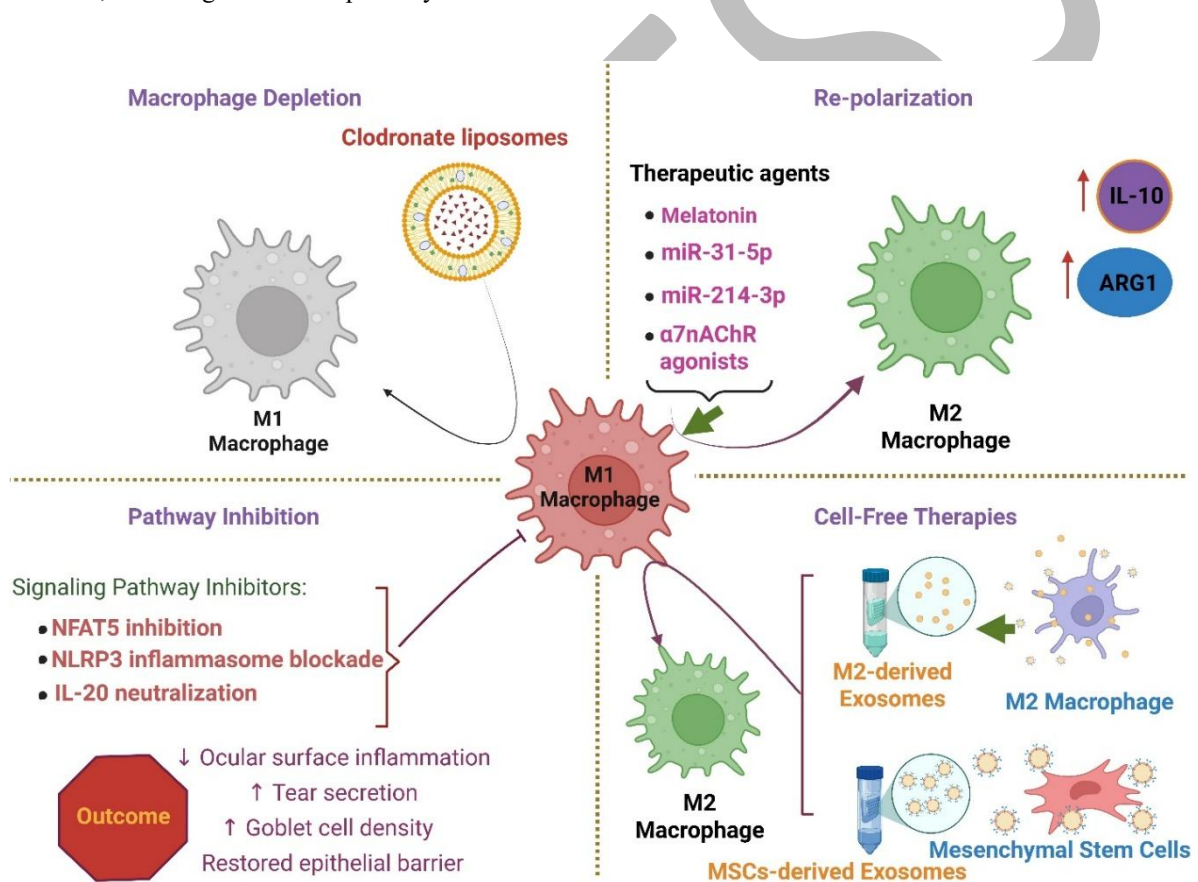


Figure 1. A schematic representation of innovative approaches to regulate macrophage-driven inflammation in dry eye disease (DED). Pro-inflammatory M1-like macrophages may contribute to damage on the ocular surface by releasing cytokines such as TNF- α , IL-1 β , and IL-6. The strategies involve the elimination of macrophages using clodronate liposomes, converting M1-like macrophages into an anti-inflammatory M2 subtype through agents like melatonin, miR-31-5p, miR-214-3p, and α 7 nicotinic acetylcholine receptor (α 7nAChR) agonists, as well as cell-free immunomodulation via exosomes released by mesenchymal stromal cells or M2-like macrophages. Concurrently, the inhibition of critical pro-inflammatory pathways, including NFAT5, the activation of the NLRP3 inflammasome, or IL-20 signaling, also plays a role in reducing macrophage-mediated inflammatory responses.

Macrophages in Dry Eye Disease

Table 1. Macrophage-associated molecular pathways and corresponding therapeutic strategies in DED.

Macrophage mechanism/pathway	Consequence in DED	Therapeutic strategy	Mechanistic rationale	Evidence
TLR/MyD88	NF- κ B/MAPK activation and inflammatory cytokine production	TLR inhibition	Suppresses upstream innate immune activation	Mainly preclinical
NF-κB	TNF- α , IL-1 β , IL-6 and chemokine production	NF- κ B inhibition	Reduces macrophage inflammatory transcription	Preclinical
MAPK (p38/JNK/ERK)	Cytokine production, migration and inflammatory activation	MAPK inhibition	Attenuates inflammatory signaling	Preclinical
JAK/STAT3	Persistent inflammatory macrophage activation	JAK/STAT inhibition	Limits cytokine-driven macrophage activation	Preclinical/experimental
NLRP3 inflammasome	IL-1 β /IL-18 maturation and pyroptosis	NLRP3/caspase-1 inhibition	Reduces inflammasome-mediated inflammation	Preclinical
ROS/oxidative stress	Macrophage activation and inflammasome signaling	Antioxidant therapy	Reduces oxidative activation signals	Preclinical/limited clinical
Glycolysis/HIF-1α	Pro-inflammatory macrophage state	Metabolic modulation	Shifts macrophage metabolism away from inflammatory glycolysis	Experimental
CCR2/CCL2	Recruitment of monocyte-derived macrophages	CCR2/CCL2 blockade	Reduces recruitment of inflammatory monocytes	Preclinical
Macrophage functional plasticity	Persistent inflammatory state	Macrophage repolarization	Promotes reparative/resolution-associated states	Preclinical
Extracellular vesicle signaling	Modulation of macrophage inflammatory phenotype	EV/exosome therapy	Transfers regulatory cargo and alters macrophage function	Predominantly preclinical; early clinical studies available
Pro-fibrotic macrophage signaling	Tissue remodeling/fibrosis, particularly GVHD	TGF- β /fibrosis pathway inhibition	Limits macrophage-associated fibrotic remodeling	Preclinical

Macrophage-Driven Mechanisms in DED Onset Hyperosmolarity as a Primary Trigger for Macrophage Activation

Tear hyperosmolarity is considered an early and critical event in the onset and progression of DED.⁶³ Elevated tear osmolarity induces hyperosmolar stress in epithelial cells (ECs), triggering the release of pro-inflammatory cytokines, including IL-1, IL-8, and TNF- α , as well as proteases such as gelatinase B.^{63,64} When tear osmolarity exceeds that of ECs, normal

osmoregulatory mechanisms that protect the OS and maintain visual function are disrupted. This hyperosmolar stress promotes apoptosis of corneal ECs, compromising the mucosal barrier and facilitating pathogen infiltration. Activated antigen-presenting dendritic cells (DCs) in the corneal epithelium capture antigens that penetrate the damaged barrier, undergo maturation, and migrate toward inflamed corneal and conjunctival tissues via upregulated epithelial and endothelial adhesion molecules. Moreover, epithelial

hyperosmolarity has been shown to induce metabolic reprogramming within ECs, further amplifying OS inflammation.⁶⁵

Besides, hyperosmolarity leads to the generation of oxidative stress in human corneal ECs (HCECs) by stimulating the formation of reactive oxygen species (ROS; it is determined through the use of a fluorogenic redox probe, which is a compound that can pass through membranes and be oxidized by oxidants within cells, resulting in the production of a highly fluorescent 2',7'-dichlorofluorescein (DCF)).^{66,67} Hyperosmolarity also leads to the increasing synthesis and release of two toxic products of lipid peroxidation such as 4-Hydroxynonenal (4-HNE) and malondialdehyde (MDA)⁶⁸-and suppressing levels of antioxidant enzymes such as superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPX1),⁶⁹ leading to a significant increase in biomarkers of cellular oxidative damage, including lipid peroxidation of cell membranes and mitochondrial DNA.⁶⁵ These effects could potentially be the main reason for the subsequent inflammation observed in macrophages after activation of relevant gene and protein expression.

Han et al demonstrated that hypertonic stress induces higher expression of glycolysis-related genes in OS ECs and promotes the expression of pyroptosis-related proteins and cytokines in macrophages via the accumulation of lactic acid in the microenvironment, ultimately leading to severe DED characterized by inflammatory damage to ocular tissues, underscoring the central role of glycolysis-dependent pyroptosis in DED.¹³ Pyroptosis is carried out by gasdermin D (GSDMD) and is characterized by a change in intracellular osmotic pressure and eventually the formation of holes in the cell membrane, which can lead to the release of IL-1 β /leukocytic pyrogen and IL-18, initiating cell death and a strong inflammatory response.⁷⁰ Pyroptosis is involved in many inflammatory responses in various organs, including the central nervous system and the kidney.⁷¹ Drugs capable of downregulating the glycolytic pathway are potential pharmacologic candidates for concomitant use with other conventional drugs, which may provide insights into tracking for novel therapeutic strategies and pharmacologic targets to enhance DED treatment.⁷² D-arabino-2-deoxyhexose is shown to interfere with the glycolysis of HCECs and subsequently repress the pyroptosis of macrophages. In vivo, D-arabino-2-deoxyhexose revealed effectiveness in easing DED and

could remarkably decrease the overexpression of genes and proteins related to glycolysis and pyroptosis.^{63,73} Similarly, silencing of PKM2 and HK1, important components of glycolytic metabolism, using small-interfering RNA in HCECs under hyperosmotic conditions reduced the extent of pyroptosis in macrophages. However, further investigation on the involvement of glycolysis in the corneal epithelium and the pathogenesis of DED is needed.

Hyperosmolarity in HCECs has been shown to play a role in the development of chronic inflammation by promoting the migration and recruitment of macrophage immune cells. Once activated, the macrophages can be mobilized through afferent lymphatic vessels (LVs) to the draining lymph nodes, where they stimulate Th17 cells to migrate through efferent LVs to the OS. Subsequently, Th17 cells exacerbate epithelial damage by producing pro-inflammatory factors and matrix metalloproteinases/matrixins. In addition, the ability of Th17 cells to counteract the suppressive function of regulatory T cells leads to the release of more Th17 and Th1 cells that amplify the inflammatory response and damage the epithelium.³³ Tear film (TF) instability and hyperosmolarity also trigger the release of various molecules such as damage-associated molecular patterns (DAMPs), cytokines, and metalloproteinases from HCECs.⁷⁴ These molecules are recognized by pattern recognition receptors (PRRs) such as RAGE (receptor for advanced glycation endproducts)/AGER. Examples of DAMPs are amphoterin protein, members of the S100 protein family, cell-free DNA and AGEs (advanced glycation endproducts). Henrioux et al showed that the presence of S100A4, S100A9, and HMGB1 led to an independent increase in macrophage migration. MCP-1 (monocyte chemoattractant protein-1), a chemokine that attracts monocytes/macrophages, also showed a comparable chemotaxis effect.⁽⁷⁵⁾ In the presence of the RAP inhibitor (RAGE inhibitor), they observed a significant inhibition of macrophage migration for each DAMP studied, revealing the influence of RAGE on both migratory effect and DED inflammation.⁷⁵ The researchers also examined the function of NFAT5 (nuclear factor of activated T Cells 5, also known as tonicity-responsive Enhancer-Binding Protein), a member of the Rel family of transcription factors, in the inflammation seen in DED.⁷⁵ NFAT5 serves as a crucial transcription factor that controls the transcription of genes responsive to osmotic stress in mammalian cells, resulting in an elevation of

intracellular osmolytes.^{76,77} The emergence of mouse models lacking NFAT5 and other recent developments have revealed additional functions of the *NFAT5* gene, including the production of inflammatory cytokines and the development of immune cells.⁷⁸ The results of their study showed an increase in the expression of NFAT5 (both mRNA and protein) and an escalation in the activation of NFAT5 in a hyperosmolar environment. NFAT5 could be categorized as a prompt-response gene in terms of transcription/translation, as activation of NFAT5 was observed in HCE and WKD cells within only 6 hours when exposed to hyperosmolar pressure. Suppression of *NFAT5* resulted in the elimination of excessive expression and synthesis of MCP-1 (CCL2) and S100A4 in both cell types. In HCE cells, NFAT5 inhibition led to a decrease of MCP-1 and TNF α mRNA, providing evidence on osmoprotective role of NFAT5 in mammals.⁷⁵

Oxidative Stress and Mitochondrial Dysfunction in Early Macrophage Responses

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of ROS in cells and the ability of a cell to detoxify these reactive products. The release of ROS, a hallmark of DED, may serve as a potential signal for activation of the NLRP3 inflammasome. There are three possible mechanisms: (1) Recognition of ROS by a complex involving TXNIP (thioredoxin and thioredoxin-interacting protein), leading to its dissociation and subsequent binding of TXNIP to the leucine-rich repeat region of NLRP3, initiating NLRP3 activation.^{79,80} (2) Mitochondria are the primary targets of ROS, leading to the generation of free mtDNAs that negatively impact the electron transport chain. Free mtDNAs translocate into the cytoplasm, which then binds to NLRP3 and activates it.⁸¹ (3) NLRP3 activators induce mitochondrial damage by increasing ROS production and converting mtDNA into an oxidative form that can bind to NLRP3.⁸² Once activated, the NLRP3 protein enters into molecular interactions via the pyrin domain with ASC, the adaptor molecule known as apoptosis-associated speck-like protein containing a CARD. The CARD domain of ASC then recruits the CARD domain of pro-caspase-1, leading to the formation of the NLRP3 inflammasome. Following inflammasome formation, caspase-1 undergoes hydrolysis into two fragments that unite to form a dimer and ultimately mature into cleaved caspase-1. Caspase-1's cleavage of GSDMD results in

the generation of both the C-terminus and the N-terminus. The N-terminus is accountable for generating non-selective pores in the cell membrane (with a diameter of approximately 10–14 nm), triggering the onset of pyroptosis.⁸³ On the other hand, caspase-1 is additionally responsible for the proteolytic cleavage of the precursor forms of IL-1 β and IL-18, resulting in the generation of mature IL-1 β and IL-18.⁸⁴ These processed cytokines are subsequently liberated via the pores facilitated by GSDMD, subsequently initiating pyroptosis.⁸⁵ Remarkably, after inflammasome activation, cellular entities have the potential to undergo pyroptosis or release cytokines without cell death, although the exact mechanism is not yet known. Several studies have shown that the toll-like receptor adaptor protein SARM (sterile alpha and armadillo motif) plays a role in this regulatory process.⁸⁶ A novel self-assembled nanodrug has been created by conjugating a polyphenolic antioxidant, caffeic acid, with a small peptide, which facilitates the formation of nanoparticles for targeted delivery. In HCECs, this nanodrug predominantly enters through caveolin-mediated endocytosis, successfully evades lysosomal degradation, and achieves accurate localization of the antioxidant within the mitochondria. It exhibits a robust capacity for scavenging free radicals, thereby effectively reducing oxidative stress. In a model of inflammation induced by LPS utilizing RAW 264.7 macrophage cells, which are pertinent to ocular inflammation, the nanodrug markedly inhibits pro-inflammatory cytokines such as NO, IL-6, and TNF- α , while simultaneously enhancing the expression of anti-apoptotic proteins like Bcl-2. These results highlight the therapeutic promise of mitochondrial-targeted antioxidants in the modulation of cellular redox balance and lay the groundwork for the development of sophisticated drug delivery systems aimed at treating ocular inflammatory disorders driven by oxidative stress. The research is primarily conducted in vitro, emphasizing lysosomal escape and mitochondrial targeting as pivotal innovations for improved efficacy.⁸⁷ ROS overproduction can activate redox-sensitive signaling pathways, such as NF- κ B and MAPK, which in turn induce cytokine and chemokine production. Mitochondrial dysfunction can further amplify this stimulation through mtROS production, impaired oxidative phosphorylation, altered mitochondrial membrane potential, and the release of mtDAMPs. All of these changes may contribute to NLRP3

inflammasome activation, caspase-1 activation, and the production of mature IL-1 β and IL-18. Therefore, mitochondrial dysfunction can be not only a consequence of macrophage activation but also a factor that stimulates the inflammatory activity of macrophages.⁸⁸⁻⁹¹

Macrophage-epithelial Interactions and Barrier Disruption in Onset

The OS mucosa is directly and continuously exposed to external stimuli and plays a crucial role in regulating immune tolerance and defense responses, providing insights into the regulatory mechanisms associated with ocular mucosal immunity.^{37,92} ECs, especially the FL Epi subtype, make a significant contribution to the pro-inflammatory immune microenvironment; their interactions with macrophages and CD4⁺ T cells form the basis for the subsequent DED inflammation cascade.

DED induction directly affects OS ECs, causing damage, apoptosis, and the production of inflammatory cytokines due to exposure to hypertonic tears.⁹³ In response, basal ECs actively proliferate and differentiate, showing strong expression of aquaporin 3 (*AQP3*). Research indicates that *AQP3* is essential for facilitating the migration and proliferation of both skin and conjunctival ECs.⁹⁴ Basal ECs exhibit characteristics of stem/progenitor cells similar to conjunctival stem cells found in the basal layer of the conjunctival epithelium, suggesting that *AQP3* may serve as a marker for identifying activated stem cells within the regenerating epithelium. Recent studies have reported a significant increase in EC proliferation in the corneas of DED mice, suggesting that this EC activation following injury may be a common phenomenon across the OS.⁹⁵ Numerous studies have confirmed the essential role of conjunctival ECs in the inflammatory responses associated with DED, as they show upregulated MHC-II molecules.¹⁷ Cells in the conjunctival basal layer may acquire fibroblast-like properties (i.e., FL Epi, a relatively stable intermediate epithelial-mesenchymal transition (EMT) cell) and serve as the primary source of inflammatory mediators such as chemokines (CCL2) in DED patients. Topical application of CCR2 antagonists has been shown to significantly reduce infiltration of corneal CD11b⁺ cells and conjunctival T cells on the ocular surface of DED mice. The FL Epi subtype can interact with immune cells, potentially serving as a critical link between innate and adaptive immunity.¹⁷ Furthermore, the Ccl2-Ccr2

pathway can facilitate epithelial healing by attracting inflammatory monocytes that may contribute to tissue regeneration and wound repair through inflammatory clearance and the release of growth factors. However, excessive or prolonged activation can lead to persistent infiltration of monocyte-derived macrophages, which exacerbates inflammation and results in excessive extracellular matrix deposition and fibrosis.^{96,97} Therapeutic strategies targeting this pathway, such as CCR2 antagonists, have been shown to decrease fibrosis while maintaining adequate repair mechanisms in chronic conditions.⁹⁸ The conjunctival epithelium exhibits significant features of epithelial-mesenchymal transition (EMT) during DED, and the formation of FL Epi cells may be associated with this process. During EMT, differentiated epithelial cells undergo a marked transformation into a mesenchymal phenotype. They lose their polarized epithelial characteristics, including tight junctions involving E-cadherin and claudins, while acquiring migratory and invasive properties. This transformation is characterized by changes in morphology, function, and polarity, with increased expression of mesenchymal markers such as vimentin and fibronectin, enabling cells to transition into a fibroblast-like state and may contribute to tissue fibrosis, which is initiated by TGF- β signaling.^{99,100} Previous research has indicated the involvement of EMT in the pathogenesis of DED in patients with GVHD, where ruptures in the basal epithelial layer and abnormal downward extension of collagen fibers were observed in the conjunctival epithelium. This suggests that EMT may contribute to conjunctival fibrosis in chronic GVHD.¹⁰¹ Macrophages are significantly increased in DED, indicating a heightened need for both pro-inflammatory and anti-inflammatory regulation. Periostin (POSTN), a non-structural matricellular protein, showed particularly amplified signaling from FL Epi to the M ϕ C3 subtype in DED. Periostin signaling can activate various pathways by interacting with integrins, thereby stimulating or inhibiting downstream molecules and performing its biological functions. It plays a crucial role in chronic inflammatory diseases such as asthma and allergic conditions.^{18,102} This may represent one mechanism by which conjunctival ECs manage chronic inflammation through interactions with macrophages. This regulation is reciprocal, as research has shown that macrophages help regulate EC differentiation and promote EC expression of *Igfbp2* (insulin like growth factor binding protein 2)

by expressing *Tgfb1* (transforming growth factor beta 1) and *ApoE* (apolipoprotein E). *Igfbp2* is involved in macrophage differentiation and T-cell activation.¹⁰³ Li et al. discovered that inhibiting *Igfbp2* in lupus nephritis significantly increased Treg ratios and suppressed CD4⁺ T-cell proliferation and activation.¹⁰⁴ An elevated expression of *Igfbp2* has also been observed in Fib of pterygium, which is linked to conjunctival fibrovascular proliferation.¹⁰⁵ Consequently, signals from macrophages may promote the reprogramming of conjunctival epithelial cells, modifying expression patterns and amplifying pro-inflammatory effects.

Macrophage Roles in DED Progression and Chronicity

The Macrophage-T Cell Axis in Amplifying Chronic Inflammation

Recent research has highlighted the importance of the macrophage-T cell axis in the progression of chronic OS inflammation seen in DED. Desiccating stress recruits CD11b⁺ CCR2⁺ macrophages, which infiltrate the conjunctiva and cornea and then differentiate into antigen-presenting cells (APCs). These APCs, including DCs and macrophage-lineage cells, can contribute to antigen trafficking in draining lymph nodes. There, they activate naïve CD4⁺ T cells, inducing their differentiation into pathogenic Th1 (producing IFN- γ) and Th17 (producing IL-17) effector subsets, and also stimulate CD8⁺ cytotoxic T cells.¹⁰⁶⁻¹⁰⁸ IFN- γ derived from Th1 cells significantly polarizes macrophages toward a pro-inflammatory Pro-inflammatory macrophage state and facilitates their recruitment.¹⁰⁹ In contrast, IL-17 produced by Th17 cells directly compromises corneal barrier integrity,¹¹⁰ exposes additional autoantigens, stimulates macrophages to produce matrix metalloproteinases,¹¹¹ increases VEGF-C/D expression to promote lymphangiogenesis,¹¹² and further enhances macrophage infiltration and antigen presentation. This complex interplay between macrophages and T cells is demonstrated by strong positive correlations between macrophage density and the numbers of CD4⁺ and CD8⁺ T cells observed in human in vivo confocal microscopy and murine models. Inhibition of macrophage migration (via anti-CCR7), neutralization of IL-17, or depletion of T cells leads to a significant reduction in infiltration and activity of the corresponding population.^{112,113} Consequently, this reciprocal amplification loop transforms an initially reversible desiccation injury into persistent immune-

mediated inflammation, loss of goblet cells, and tear film instability, highlighting the macrophage-T cell axis as a crucial therapeutic target for disrupting the vicious cycle in DED. Macrophages closely interact with CD4⁺ T cells, functioning both as phagocytes that target these cells and as antigen-presenting cells (APCs) that activate them.¹¹⁴ They present antigenic peptides via MHC class II molecules to antigen-specific CD4⁺ T cells and, in turn, are activated by these T cells.¹¹⁴ In addition, macrophage-derived IL-6 and BAFF (B-cell activating factor) promote B-cell activation and autoantibody production, as well as the formation of ectopic lymphoid follicles in the lacrimal glands. Persistent macrophage activation and the resulting gland inflammation led to progressive destruction of glandular secretory acinar structures, reduced tear production, and the development of severe aqueous-deficient dry eye. In individuals with Sjögren's syndrome (SS), an autoimmune condition associated with DED, macrophages are among the most abundant innate immune cells infiltrating the salivary glands and play a dual role in disease pathogenesis. In the early stages, M1-like macrophages induce glandular inflammation by secreting TNF- α , IL-6, IL-12, and CXCL13, which promote Th1 responses, CD4⁺ T-cell activation, and ectopic germinal center formation.¹¹⁵ As the disease advances, M2-like macrophages produce IL-10 and TGF- β 1, which suppress acute inflammation but significantly contribute to irreversible glandular fibrosis through SMAD2/3/4-dependent and non-canonical TGF- β 1 signaling that transforms fibroblasts into myofibroblasts.¹¹⁶ The M1/M2 imbalance is positively correlated with the severity of localized sialadenitis and fibrotic remodeling, highlighting macrophage polarization as a key therapeutic target for controlling inflammation and preventing progressive salivary gland damage in Sjögren's disease.¹¹⁷ Notably, Toll-like receptor 8 (TLR8)-expressing monocytes and tissue macrophages are significantly increased in both peripheral blood and minor salivary glands of Sjögren patients, with infiltration severity correlating with focus score and EULAR (European League Against Rheumatism) Sjögren's syndrome disease activity index (ESSDAI), underscoring a previously underappreciated TLR8-driven inflammatory axis in Sjögren's-associated DED that may also operate in non-Sjögren DED states with prominent myeloid activation.¹¹⁸ In non-autoimmune DED, macrophage activation is driven primarily by environmental stressors such as tear

hyperosmolality, oxidative stress, bacteria, viruses, endotoxins, and pollutants.⁴⁵ In contrast, autoimmune DED, especially Sjögren, is characterized by persistent systemic immune dysregulation, in which macrophages mediate interactions between innate and adaptive immunity.^{12,119} In a study, the reduction of M2b macrophages, which are also known as regulatory macrophages, led to an increase in the population of IL-17- and IFN- γ -producing T cells, heightened expression of inflammatory mediators linked to M1-like macrophages (such as IL-1B, TLR4, and Nos2), a decrease in IL-10 levels, and exacerbated loss of goblet cells.¹²⁰ This indicates that M2b macrophages play a role in moderating T cell-mediated inflammation in DED. It implies that macrophages affect T cell responses not only through antigen presentation but also by influencing the inflammatory milieu—where the absence of M2b macrophage activity fosters the production of pathogenic T cell cytokines and leads to tissue damage in DED. Importantly, these regulatory M2b macrophages are functionally different from classical wound-healing M2-like macrophages, as their primary role lies in immune suppression and T cell regulation rather than tissue remodeling alone. From a therapeutic perspective, methods designed to restore the function of regulatory macrophages or to selectively interfere with the communication between macrophages and T cells could provide better disease management than strategies that focus solely on T cells or cytokines.

Environmental Triggers as Accelerators of Macrophage-Mediated Progression

High-fat Diet and Meibomian Gland Dysfunction

Overeating and significant changes in people's lifestyles have led to a widespread health problem in which obesity is often correlated with the occurrence of various diseases, such as type 2 diabetes, heart disease, and neurodegenerative disorders.¹²¹ Several epidemiologic studies have also shown that excessive consumption of dietary fats, also known as a high-fat diet (HFD), is a contributor to the increased prevalence of DED.¹²² Meibomian glands (MGs) are large sebaceous glands within the eyelids that can secrete lipids to maintain clear optical surfaces, protect the eye from microorganisms, and sustain homeostasis. MG dysfunction (MGD) is a contributor factor of DED, and most common cause of lipid deficient DED. It is usually characterized by the obstruction of the terminal ducts and/or qualitative/quantitative changes in the glandular

secretory content. Zhao et al. investigated the role of TNFAIP8L2 (TNF- α -induced protein 8-like 2) in the polarization of macrophages in MGD.¹²³ They found that the expression levels of TNFAIP8L2 were positively associated with the existence of M2-like macrophages in both MGD patients and MG mouse models. TNFAIP8L2 mRNA levels dropped in LPS-stimulated M1-like macrophages. In contrast, TNFAIP8L2 mRNA levels rose in IL-4-stimulated M2-like macrophages, suggesting that TNFAIP8L2 may have a probable anti-inflammatory function in controlling the progression of MGD by facilitating the polarization of M2-like macrophages.¹²³ Studies have shown that HFD-induced lipid accumulation in MGs exacerbates MGD by potentially inducing apoptosis in MG cells and disrupting their normal physiological functions.¹²⁴ In addition, HFD may lead to persistent inflammation in MG mediated by activation of signaling pathways such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B), as well as a reduction in peroxisome proliferator-activated receptor gamma (PPAR- γ) expression.¹²⁵ Rosiglitazone, commercially known as Avandia, is an antidiabetic drug of the thiazolidinedione class that acts as a selective agonist of PPAR- γ .¹²⁶ When it restores downregulated PPAR- γ levels to normal, it significantly reduces HFD-induced macrophage infiltration and the expression of proinflammatory cytokines and chemokines in the MG.¹²⁷ This observation suggests that HFD-induced downregulation of PPAR- γ may be a contributing factor to the increased infiltration of inflammatory cells and upregulation of proinflammatory cytokine and protein expression. Furthermore, the pathophysiological consequences of HFD on MG were confirmed by the finding that these negative effects improved when transitioning from HFD to a standard diet (SD).^{127,128}

Fat and muscle tissue show an accumulation of macrophages, which leads to an increase in pro-inflammatory cytokines and is another feature of HFD-associated inflammation.¹²⁹ According to studies, the MGs of mice with DED are mainly composed of macrophages with decreased M2 polarization and increased M1 polarization, and the imbalance between the two correlates with disease progression.¹³⁰ The development of ophthalmopathy in mice could be significantly slowed down by the administration of M2-like macrophages.¹³¹ HFD has also been shown to alter the immune response and influence the inflammatory processes associated with it. In regard to obesity, in

addition to converting macrophages to the Pro-inflammatory macrophage state, studies have shown that HFD can also lead to increased infiltration of Th17 cells, CD103⁺ dendritic cells, increased levels of proinflammatory cytokines, and a concomitant reduction in regulatory T cells (Tregs), all of which are correlated with inflammation in adipose tissue.¹³² The pathways by which HFD exposure affects the Th17/Tregs ratio in adipose tissue and the effects on macrophage recruitment and polarization in the context of obesity remain poorly understood. In cases of severe malnutrition in humans and corresponding mouse models, M2-like macrophages are in the majority, releasing anti-inflammatory cytokines and utilizing oxidative metabolic pathways to sustain adipose tissue homeostasis.

Emerging Triggers: Air Pollution and Particulate Matter

Air pollution, particularly from particulate matter with a diameter of ≤ 10 micrometers are a well-established environmental risk factor for DED.^{133,134} Recent research has shown that fine particulate matter, specifically PM2.5, which is rich in metals (such as Ca, Na, K, Zn, and Mg) and polycyclic aromatic hydrocarbons (for example, BbF and BbF), triggers dose-dependent activation, phagocytosis, and morphological changes, including pseudopodia extension and cytoskeletal expansion.¹³⁴ One study indicated that exposure to PM2.5 increases levels of pro-inflammatory cytokines (MCP-1, TNF- α , IL-6) and mRNA levels of M1 markers (iNOS, TNF- α), thereby promoting M1 polarization and the risk of DED. Furthermore, PM2.5 increases the expression of TLR4, NF- κ B, and COX-2 over time, suggesting that PM2.5 promotes macrophage inflammation through the TLR4/COX-2/NF- κ B pathway and highlighting the potential of these inhibitors as therapeutic options for inflammatory diseases associated with PM2.5.¹³⁵ The findings also revealed seasonal fluctuations in PM2.5 composition, with summer samples showing the highest metal concentrations due to increased photochemical reactions and higher temperatures, while winter samples had higher levels of PAHs due to reduced volatility. This resulted in varying toxicity, with summer PM2.5 causing the most significant M1 macrophage polarization, increased ROS, cytokine production, cell cycle arrest, and apoptosis. Human activities, such as traffic and industrial emissions, contribute to metal

enrichment, and restrictions imposed during the COVID-19 pandemic have further increased metal concentrations in PM2.5, worsening macrophage toxicity. The conclusions from this research emphasize that meteorological seasonal factors and human activities play a crucial role in modulating the immunotoxic effects of PM2.5 on macrophages, thereby increasing the risks of oxidative stress and inflammation.¹³⁶ In cultured human CECs exposed to PM2.5, increases in ROS are observed that are both dose- and time-dependent, along with heightened mitochondrial ROS production, decreased mitochondrial membrane potential, and cellular damage. This is accompanied by increased corneal fluorescein staining, superficial punctate keratopathy, and elevated IL-1 β levels.¹³⁷ The excessive ROS generated by PM2.5 inhibits the anti-inflammatory transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), which in turn activates the NF- κ B pathway and its associated inflammatory molecules, leading to persistent corneal inflammation and symptoms resembling DED.¹³⁷ In a DED mouse model, Rosarin treatment alleviated DED by inhibiting NF- κ B-mediated inflammation and M1 polarization in macrophages.¹³⁸ In the draining cervical lymph nodes, exposure to PM2.5 facilitated the maturation of DCs, as evidenced by an increase in MHC-II high CD11c⁺ DCs, and led to a Th2-dominant allergic immune response. This response is marked by elevated production of Th2 cytokines, which contrasts with the usual Th1-dominant profile observed in traditional DED models.¹³⁹ These findings highlight the urgent need for targeted pollution control measures to protect immune health.

Therapeutic Targeting of Macrophages in DED Pharmacological Approaches to Modulate Macrophage Polarization and Depletion

The role of macrophages in the pathogenesis of DED triggered by autoimmune diseases such as psoriasis and experimental uveitis is gaining growing research interest. Clodronate liposomes (CL) were effectively used to deplete macrophages in vivo, leading to a reduction in the ocular manifestations of the psoriasis disease as well as an improvement in the lacrimal gland in psoriasis.¹⁴⁰ In a DED mouse model induced by iron overload, mice received dextran iron injections (total: 100 mg), which resulted in corneal epithelial defects, increased inflammation index, reduced tear volume, decreased conjunctival goblet cells (CGCs), and

elevated M1-like macrophages and cytokines such as IL-1 β , IL-6, and TNF- α .¹⁴¹ CL treatment effectively ameliorated DED symptoms by reducing CD11b/F4/80-positive macrophages by 50–70%, preferentially targeting M1 subtypes while promoting M2 polarization. The study lowered fluorescein and inflammation scores (to 4–6 and 2–4, respectively), restored tear production (up to 2–3 mm), preserved epithelial integrity and goblet cells, and decreased iron deposits by reducing oxidative stress and cytokine levels.¹⁴¹ The findings emphasize that M1-like macrophages are central mediators in iron overload-induced DED through inflammation and ROS generation, indicating that CL-mediated M1 macrophage depletion is a promising strategy to restore ocular homeostasis and balance macrophage phenotypes. Similarly, a study addressing oxidative stress and inflammation-related disorders associated with DED investigated the potential of tetrahedral framework nucleic acids (tFNA), a self-assembled nucleic acid compound.¹⁴² Mechanistically, tFNA has been shown to effectively alleviate DED by enhancing corneal epithelial healing, restoring goblet cell function, and promoting tear secretion recovery. tFNA treatment reduces excessive ROS production and modulates the inflammatory microenvironment, particularly through the cGAS-STING pathway, resulting in decreased inflammatory cytokines such as MMP9 and IL-6. Additionally, tFNA demonstrated excellent safety, with no overt ocular toxicity in the experimental model.¹⁴² A recent investigation identified a significant decrease in miR-31-5p levels in the lacrimal glands of rabbits with autoimmune dacryoadenitis and in the peripheral blood mononuclear cells (PBMCs) of patients with SS-related DED. Overexpression of miR-31-5p showed both prophylactic and therapeutic effects in rabbit autoimmune dacryoadenitis. Further analyses demonstrated that miR-31-5p overexpression substantially restored the M1/M2 macrophage balance both in vivo and in vitro (Figure 1). Mechanistically, miR-31-5p directly interacts with the P2x7 receptor (P2RX7)/purinergic receptor P2X 7, leading to attenuation of p38 mitogen-activated protein kinase (MAPK) signaling and reduced expression of M1 markers. Additionally, methylated RNA immunoprecipitation and luciferase reporter assays showed that *FTO* (the fat mass and obesity-associated gene, also known as alpha-ketoglutarate-dependent dioxygenase)-mediated m6A demethylation, which

preserves the stability of pri-miR-31, accounts for the observed reduction in miR-31-5p levels in autoimmune DED. PBMC samples from patients with SS-related DED further support the association between reduced miR-31-5p levels and Pro-inflammatory macrophage activation observed in the rabbit model, highlighting the critical role of the FTO/miR-31-5p/P2RX7/p38 MAPK signaling pathway in autoimmune DED and indicating their potential as therapeutic targets for autoimmune DED.¹⁴³ Besides of miR-31-5p, miR-214-3p also serves a protective function in DED by inhibiting macrophage inflammation through the modulation of ferroptosis (Figure 1).¹⁴⁴ Ferroptosis is a form of regulated cell death that is dependent on iron and is distinct from other cell death modalities such as apoptosis, necrosis, and autophagy. This process is marked by the buildup of harmful lipid peroxides within cellular membranes, which is facilitated by iron-driven Fenton reactions and lipid peroxidation.¹⁴⁵ The overexpression of miR-214-3p notably reduced the severity of DED by directly targeting and downregulating *TFRC* (transferrin receptor)/*CD71*, which in turn diminished iron accumulation, inhibited ferroptosis, reduced M1 polarization, and decreased the release of inflammatory cytokines; in contrast, the knockdown of miR-214-3p worsened the inflammatory response. These results underscore a novel mechanism that connects miRNA-mediated regulation of ferroptosis to macrophage-induced inflammation in DED, suggesting that the upregulation of miR-214-3p could be a promising therapeutic approach for mitigating the progression of DED (Figure 1).

According to Cai et al, one month of melatonin (N-acetyl-5-methoxytryptamine) eye drops can control macrophage polarization and reduce the symptoms of DED (Figure 1). In RAW 264.7 cells, a macrophage cell line, melatonin reduced lipopolysaccharide-induced pro-inflammatory responses and increased the release of anti-inflammatory molecules.¹⁴⁶ By influencing macrophage development¹⁴⁷ and altering macrophage polarization, MLT affects macrophage function. Specifically, MLT inhibits M1 macrophage function while increasing M2 macrophage function, which affects macrophage phenotype.¹⁴⁸ The mechanism of action of MLT in HFD-induced DED was investigated, and RNA sequencing revealed that MAPK signaling pathways were suppressed and *RABL4/IFT27* (Intraflagellar Transport 27) were upregulated after melatonin therapy. The MGs of mice with DED treated

with melatonin showed decreased ERK (extracellular signal-regulated kinase)/JNK (c-Jun amino-terminal kinases) phosphorylation, while RAW 264.7 cells in which RAB4 was knocked down showed increased phosphorylation, suggesting that melatonin may have a protective effect in MGD. Melatonin increases tear production and improves tear film stability while reversing dilatation of MG ducts in hyperlipidemic mice. In addition, melatonin has shown a strong antioxidant effect. Excessive production of ROS through oxidation can lead to inflammatory tissue damage and the activation of inflammatory cytokines.⁸⁹ In the study by Cai et al., hyperlipidemic mice had higher ROS levels in the MG and lacrimal gland (LG) tissue, which could be related to lipid deposition in the alveoli. In contrast, oxidative stress causes inflammation, leading to alveolar cell death and disruption of the OS microenvironment. However, melatonin induced antioxidant stress and increased the level of anti-apoptotic proteins.¹⁴⁹ Melatonin is a potent endogenous ROS scavenger and can minimize the excessive formation of ROS and maintain mitochondrial function.^{149,150} Intraperitoneal injection of melatonin protects corneal and retinal pigment epithelial cells, suggesting that melatonin may attenuate inflammation in the OS microenvironment triggered by elevated lipid levels by altering macrophage polarization and scavenging excess ROS.

Studies have shown that hyperosmolarity plays a critical role in the production of IL-20 in corneal ECs and macrophages during DED. IL-20 serves as a key factor that triggers the production of various other proinflammatory factors, such as IL-1 β , TNF- α , and MMP-9, suggesting that corneal cells and macrophages could be targeted by using anti-IL-20.⁽¹⁵¹⁾ Moreover, IL-20 is shown to promote the polarization and activation of macrophages into proinflammatory M1-type. NFAT5 is known for its involvement in upregulating the release of IL-20 under conditions of hyperosmolarity by binding directly to the promoter of the *IL-20* gene.¹⁵¹ A study demonstrated that treatment with 7E, a monoclonal antibody against IL-20, effectively inhibited the upregulation of proinflammatory factors induced by hyperosmotic stress (Figure 1).¹⁵¹ This treatment also proved successful in protecting corneal ECs from hyperosmotic stress- and benzalkonium chloride (BAC)-induced cell death by modulating the expression of Bcl-2-associated X protein (BAX) and B-cell lymphoma 2 (BCL-2).¹⁵¹ In addition to its protective

properties on corneal ECs, targeting 7E to IL-20 resulted in a decrease in polarization and activation of M1-like macrophages under hyperosmotic stress conditions in vitro.^{151,152} Remarkably, IL-20 was identified in the population of infiltrated F4/80+ macrophages, indicating its potential role as an autocrine or paracrine factor in macrophage activation, contributing to the maintenance of inflammatory responses in DED.¹¹⁴ Treatment with 7E in a mouse model of BAC-induced DED resulted in a reduction of F4/80+ macrophage infiltration in corneal tissue.¹¹⁴ In addition, treatment with 7E and lifitegrast in a DED model resulted in a decrease in the population of Th17 cells in both the conjunctiva and draining lymph nodes. Notably, 7E showed a significant decrease in the expression of *CD4* and *Il17a* in DED models, suggesting that IL-20 may play a role in the activation of Th17 cells and that 7E effectively inhibits the Th17 cell response in DED (Figure 2).¹⁰⁸ The process through which BAC may contribute to DED involves the substantial release of alarmins from damaged CECs. These alarmins trigger the activation of the nucleotide-binding domain, leucine-rich-containing family, and pyrin domain-containing-3 (NLRP3) inflammasome within neutrophils (NEUs) and macrophages that have infiltrated the eye, which in turn results in an elevated production of inflammatory cytokines such as IL-1 β , IL-6, IL-1 α , and TNF- α . This cascade ultimately leads to an increased recruitment of circulating leukocytes to the inflamed eyes of animals treated with BAC.¹⁵³ A study used a mouse model induced by lacrimal gland excision (LGE) to investigate the therapeutic potential of the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) in treating DED through macrophages (Figure 1).³⁶ The experiment employed the selective agonist PNU-282987, the antagonist methyllycaconitine (MLA), a diterpenoid alkaloid, and CLs for macrophage depletion as controls. Activation of $\alpha 7$ nAChR reduced pro-inflammatory M1-like macrophages and their cytokines, including IL-1 β , IL-6, TNF- α , and MMP9. It also suppressed TRPV1 (transient receptor potential cation channel subfamily V member 1, also known as the capsaicin receptor) expression, restored corneal nerve density, improved barrier function by ZO-1 (zonula occludens protein-1) expression, enhanced tear production, alleviated ocular inflammation, and reduced anxiety-like behaviors. These effects were reversed by MLA and partially mimicked by macrophage depletion, highlighting $\alpha 7$ nAChR's potential to restore neuroimmune

homeostasis in DED by shifting macrophage polarization.³⁶

Biological and Cell-Free Therapies: Exosomes and Conditioned Media

Exosomes play a crucial role in the paracrine signaling pathway and can produce either inflammatory or non-inflammatory responses.¹⁵⁴ Numerous experimental investigations have demonstrated significant diagnostic and therapeutic effectiveness of exosomes concerning DED (Figure 1).¹⁵⁵ A study examined the therapeutic effectiveness of extracellular vesicles derived from M2-polarized macrophages (M2-EVs) in a mouse model of DED induced by BAC.¹⁵⁶ M2-EVs significantly improved DED symptoms by restoring OS immunological homeostasis, as evidenced by reduced corneal fluorescein staining scores, increased tear production, preserved goblet cell density, and decreased inflammatory cell infiltration. The results showed that pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) and M1 macrophage markers (iNOS, CD86) were downregulated, while anti-inflammatory factors (IL-10, Arg-1) and M2 markers (CD206) were upregulated, indicating a shift from M1 to M2 polarization. M2-EVs reduced LPS-induced inflammation in RAW264.7 cells and increased HCEC viability under hyperosmotic stress in vitro, highlighting their potential as a non-steroidal, immunomodulatory treatment for BAC-induced DED by reducing inflammation and promoting epithelial repair.¹⁵⁶ The suppression of harmful immune responses in inflamed eyes is also facilitated by MSC-Exos. These MSC-Exos inhibit the expression of NLRP3, IL-1 β , and IL-18, thereby reducing the inflammatory phenotype in NEUs and monocytes that infiltrate the eye.¹⁵⁷ The MSC-Exo-mediated reduction of NLRP3-driven inflammation leads to the suppression of cysteinyl aspartate specific proteinase-3 (caspase-3), which in turn prevents the loss of CECs, likely due to the prevention of pyroptosis caused by caspase-3-mediated cleavage of GSDME/gasdermin E. By inhibiting the activation of the NLRP3 inflammasome, MSC-Exos promote the generation of M2-like macrophages that interact with other anti-inflammatory cells infiltrating the eye, such as tolerogenic DCs and CD4⁺ CD25⁺ regulatory T cells (Tregs).¹⁵⁷ This interaction fosters an immunosuppressive microenvironment in the inflamed eyes, which is essential for mitigating eye inflammation and alleviating symptoms associated with DED. The

immunosuppressive TGF- β derived from M2-like macrophages, along with IL-10 sourced from MSC-Exosomes, plays a crucial role in inhibiting the maturation of DCs.¹⁵⁸ This process encourages the differentiation of DCs into tolerogenic variants, which are characterized by a reduced expression of MHC Class II and costimulatory molecules. Rather than producing pro-Th1 and pro-Th17 cytokines, these tolerogenic DCs secrete IL-10 and IDO (indoleamine 2,3-dioxygenase), thereby facilitating the generation and proliferation of immunosuppressive Tregs within inflamed tissues.¹⁵⁹ Furthermore, IDO produced by DCs reduces the concentration of tryptophan (TRP) by metabolizing it to generate kynurenine in the inflamed microenvironment, leading to the activation of general control non-repressible 2 (GCN2) kinase. This activation inhibits the signaling pathway involving protein kinase B (PKB/Akt) and mammalian target of rapamycin (mTOR)2 in Tregs, thereby preventing their transdifferentiation into inflammatory Th17 cells. In addition, IDO leads to increased expression of the FoxP3 transcription factor in naïve CD4⁺ T lymphocytes, enabling the increase of immunosuppressive Tregs in the inflamed eyes, which results in the weakening of enduring inflammation.¹⁵⁸ Additionally, TGF- β secreted in MSC-Exos inhibits activation of the Janus kinase/ signal transducer and activator of transcription (JAK/STAT) signaling pathway in IL-17-producing Th17 cells, resulting in G0/G1 cell cycle arrest.^{160,161} MSC-Exos also upregulate the expression of immunoregulatory factors such as *ARG1* and *NO* (Nitric Oxide) in myeloid-derived suppressor cells (MDSCs), which in turn inhibit the proliferation of activated Th1 (T helper 1), and Th17 cells. IFN- γ produced by Th1 cells induces apoptosis of salivary gland epithelial cells (SGECs) and may contribute to the development of SS-related symptoms.¹⁶² MSC-Exos upregulate the expression of aquaporin 5 (AQP5), a member of the AQPs family of transmembrane water channels, on SGECs, activate the protein kinase A/cyclic AMP response element binding (PKA/CREB) pathway, restore salivary gland function, and alleviate DED-related symptoms in experimental mice.¹⁶² NO produced by MDSCs also inhibits serine/threonine protein kinases that play a crucial role in regulating the cell cycle, thereby impeding cell cycle progression and triggering apoptosis in inflammatory T cells through activation of Caspase-3.¹⁶³ In addition, NO diminishes the expression of IL-2, an essential cytokine

that promotes the proliferation of activated T cells.¹⁶⁴ Furthermore, ARG1 derived from MDSCs alters arginine metabolism toward the synthesis of polyamines and proline, which aids in tissue repair and helps restore compromised OS barriers.¹⁶⁴ In a study, exosomes (CD9/CD63 positive) derived from olfactory ecto-MSC (OE-MSC-Exos) significantly enhanced the suppressive capacity of MDSCs by upregulating levels of arginase, ROS, and NO, while simultaneously reducing maturation markers such as CD40 and MHCII. This led to reduced disease progression in a murine model of SS. OE-MSC-Exos have been shown to improve salivary flow rates, decrease autoantibodies (including anti-SG, anti-SS related antigen A, and anti-muscarinic type 3 receptor), reduce glandular lymphocytic infiltration, and suppress Th1/Th17 responses. OE-MSC-Exos secrete IL-6, which activates the JAK2/STAT3 pathway in MDSCs. Additionally, exosomal calcium-binding protein A4 (S100A4) binds to Toll-like receptor (TLR 4) on MDSCs, triggering autocrine IL-6 production and thereby amplifying immunosuppression. The therapeutic effects were confirmed through the adoptive transfer of MDSCs treated with OE-MSC-Exos, suggesting that OE-MSC-Exos represent a promising cell-free strategy for the treatment of SS and other autoimmune diseases.¹⁶⁵ A recent investigation of DED has revealed that topical eye drops containing MSC-exos can effectively reduce symptoms associated with GVHD related DED. In experimental mouse models of DED, MSC-exo suppressed inflammation, facilitated the recovery of the corneal epithelium, and improved clinical indicators of the condition. A prospective clinical trial that included 28 eyes from patients with refractory GVHD-associated DED reported significant improvements, including decreased corneal fluorescein staining, prolonged tear-film breakup time, increased tear production, and lower OS disease index (OSDI) scores. The underlying mechanisms of these therapeutic effects are primarily attributed to miR-204 present in the exosomes, which reprograms proinflammatory M1-like macrophages into anti-inflammatory macrophage states by targeting the IL-6/IL-6 receptor/STAT3 signaling pathway; inhibiting miR-204 negated these benefits, whereas engineering control exosomes to overexpress miR-204 replicated the beneficial effects. This underscores the potential of MSC-exos, particularly through the action of miR-204, as a promising cell-free therapeutic approach for dry eye associated with GVHD.¹⁶⁶ In addition, a cohort of 14 patients (28 eyes)

with DED associated with GVHD was recruited to a clinical trial (NCT04213248) designed to evaluate the therapeutic efficacy of MSC-derived exosomes in the treatment of DED in humans. Exosomes were extracted from human umbilical cord MSCs (UC-MSC-exos) and subsequently prepared as eye drops after optimizing osmotic pressure and pH. Study participants were advised to administer the eye drops 4 times daily in each eye for a duration of 2 weeks. Remarkably, none of the 28 eyes of patients with DED due to GVHD had responded to topical corticosteroids, artificial tears, or even treatment with autologous serum. The results showed a statistically significant decrease in corneal epithelial damage coupled with marked restoration of the epithelial layer.^{166,167} Symptoms of discomfort associated with DED, such as burning or redness, disappeared after treatment. No treatment-related adverse events were reported during the study period; however, the small sample size and limited follow-up preclude conclusions regarding long-term safety. Similarly, in a randomized clinical trial, investigators showed that eye drops containing MSC-derived exosomes are both safe and highly effective for managing severe DED in patients with SS.¹⁶⁸ In this triple-blinded study, 8 women diagnosed with primary SS received exosome drops (10 µg) in one eye and a placebo (PBS) in the other, administered twice daily for 2 weeks. Eyes treated with exosomes showed notable improvements in symptoms, tear production, tear film stability (TBUT), and corneal staining compared with control eyes, with benefits lasting up to 3 months. Levels of pro-inflammatory cytokines (IL-6, MMP-9) in tears significantly decreased, while healing factors (EGF, thrombospondin-1) increased. No adverse events were observed, highlighting the potential of MSC-derived exosomes as an innovative cell-free treatment for SS-related DED, warranting broader and longer-term studies.¹⁶⁸ Similarly, a study investigated the protective role of exosomes derived from periodontal ligament MSCs (PDLSC-Exos) in DED by modulating macrophage polarization and preserving CGC function. The researchers found that PDLSC-Exos were efficiently absorbed by pro-inflammatory M1-like macrophages, facilitating their transition toward an anti-inflammatory macrophage state, as demonstrated by elevated levels of IL-10 and Arg1, coupled with a decrease in TNF-α. When CGCs were subjected to supernatants from M1-like macrophages treated with PDLSC-Exos, there was a notable increase in the

mRNA expression and protein secretion of Mucin-5AC (MUC-5AC), the predominant gel-forming mucin in the ocular system,¹⁶⁹ both in the presence and absence of cholinergic stimulation. This indicates a boost in mucin production and enhanced protection of goblet cells against inflammation. Conversely, the direct addition of PDLSC-Exos to the supernatants from control macrophages yielded minimal effects unless the exosomes were diluted. Collectively, these findings imply that PDLSC-Exos alleviate M1 macrophage-induced inflammation on the OS, partially through the indirect reprogramming of macrophages, thereby providing valuable therapeutic insights for DED.⁽¹⁶⁹⁾ In a recent study, the anti-inflammatory properties of a multifunctional eye drop consisting of gold nanoparticles (AuNPs) reduced by ascorbic acid (AA) and bound to the phospholipid membrane of MSCs-derived exosomes (mExo@AA) were investigated.¹⁷⁰ The results showed that LPS-stimulated peritoneal mouse macrophages treated with mExo@AA exhibited a marked increase in the expression of CD206, a marker associated with M2 inflammation-resolving macrophage immunophenotypes.¹⁷¹ In addition, treatment with mExo@AA resulted in a marked decrease in pro-inflammatory cytokines IL-6 and IL-1 β in the supernatant, suggesting that mExo@AA promotes M2 macrophage polarization and consequently attenuates inflammation. In vivo, the researchers investigated the ROS scavenging ability of mExo@AA and found that mExo@AA had superior ROS scavenging ability compared with the saline-treated group. Consistent with the LPS-induced macrophage immunophenotype observed in vitro, histological analysis revealed a significantly increased number of CD206 in the corneas of mice treated with mExo@AA. This likely contributed to the suppression of inflammatory responses and the promotion of subsequent repair processes, thus slowing the progression of DED.¹⁷⁰ In another study, milk-derived exosomes were prepared by ultracentrifugation and filtration to encapsulate lutein for the treatment of DED.¹⁷² The results showed that Lutein@Exosomes exhibited excellent biocompatibility, easy cellular uptake, and restoration of mitochondrial membrane potential, which effectively reduced ROS and inflammation levels in HCECs and macrophages. In addition, Lutein@exosomes were found to be highly effective in repairing corneal epithelial damage, restoring tear film balance, and enlarging tear glands in

a chronic dry eye model compared with a commercial product.¹⁷² Similarly, Wang et al. examined the efficacy of mouse adipose-derived mesenchymal stem cell (MSC)-derived exosomes (mADSC-Exos) in the treatment of DED.¹⁷³ The outcomes showed that mADSC exosomes highly expressed genes such as Tetraspanin-30, MIC3, and Tetraspanin-28, leading to repair of damaged OSs while preventing cell apoptosis. Following treatment, there was an outstanding reduction in the levels of interleukins 6, IL-1 α , IFN- γ , and TNF- α and an increase in the anti-inflammatory cytokine IL-10/ a class-2 cytokine in the OS. Furthermore, the activation of NLRP3 and the upregulation of caspase-1, IL-1 β , and IL-18 were decreased by mADSC-Exos.¹⁵⁷ GUO et al investigated the therapeutic effects of MSCs-derived vesicles (MSC-EVs) and their inhibitory effect on the functionality of DCs in DED.¹⁷⁴ In vitro experiments showed that MSC-EVs have a protective effect on HCECs exposed to hyperosmotic stress while reducing the level of pro-inflammatory cytokines. In vivo, mouse models given MSC-EVs topically showed a reduction in the severity of DED compared with PBS-treated animals. Application of MSC-EVs resulted in decreased expression of inflammatory cytokines, particularly TNF- α , IL-6, and IL-1 β , as well as a decrease in the prevalence of Th17 cells in the inflammatory environment. Further studies revealed that MSC-EVs inhibit the maturation, activation, and proliferation of DCs associated with DED, highlighting the great therapeutic potential of MSC-EVs in the treatment of DED and other OS diseases.¹⁷⁴ The therapeutic capabilities of human adipose-derived stem cell vesicles (hADSC-Evs) were also investigated in a murine model of DED. Topical hADSC-Evs eye drops were administered to mice subjected to stress induced by dehydration. Compared with the control group, mice treated with hADSC-Evs showed less damage to the corneal epithelium, improved tear production, preserved goblet cell counts, and reduced production of inflammatory cytokines. In vitro, hADSC-Evs protected HCECs from hyperosmotic stress-induced cell death. Mechanistically, administration of hADSC-Evs inhibited the activation of Caspase-1 and the maturation of IL-1 β , indicating the potential of hADSC-Evs eye drops to attenuate the NLRP3 inflammatory response and to alleviate the OS damage associated with DED.¹⁷⁵ The administration of Labial gland-derived MSCs (LGMSCs) or LGMSC-Exosomes to SS mice model via tail vein injections resulted in a significant enhancement

of saliva flow rates, a reduction in lymphocytic infiltration foci, and a decrease in apoptosis within the salivary glands, comparable to the effects observed in hydroxychloroquine controls. In addition, there was a decrease in the proportions of Th17 cells (CD4+IL-17A+) alongside an increase in regulatory T cells (Treg) (CD4+CD25+FoxP3+) in the spleen, as well as a shift in cytokine profiles characterized by a reduction in serum levels of IL-17, IFN- γ , IL-6, and TNF- α , while TGF- β levels were elevated (with IL-10 showing variable increases). In vitro, co-culturing of PBMCs from SS patients with LGMSCs or LGMSC-Exos similarly inhibited the differentiation of Th17 cells and promoted the induction of Treg cells in PBMCs from SS patients, leading to the suppression of pro-inflammatory cytokines and the enhancement of anti-inflammatory cytokines; these effects were partially reversed by GW4869, an exosome release inhibitor, suggesting a paracrine mechanism of exosomal mediation. In summary, these findings underscore the potential of LGMSCs and LGMSC-Exosomes as immunomodulatory agents that mitigate the pathology of SS by restoring the balance between Treg and Th17 cells, with LGMSC-Exosomes presenting a promising and safer cell-free therapeutic alternative.¹⁷⁶ Despite the positive therapeutic outcomes of exosomes or EV administration in DED models, several problems impede translation of these findings into routine practice. One major issue is the lack of standardized methods for EV preparation and characterization. EVs are highly heterogeneous, and their content depends on several factors, including the parental cells used, donor properties, culture conditions, number of passages, and purification and storage protocols.^{177,178} Consequently, depending on the EV isolation and purification protocol employed, preparations can have significantly different compositions and functions. EVs also differ from traditional drugs in their ability to quantify dosage using various metrics such as particle number, total protein amount, lipid content, or cargo content. In other words, even when the same nominal dose is used in different trials, it does not necessarily mean that the biological dose is the same. In addition, the effects of EV dose, cargo content, and tissue exposure on treatment response are not yet understood. Therefore, potency assays need to be developed.¹⁷⁹

Challenges, Limitations, and Future Directions

Despite the accumulating evidence of the role of

macrophages in the onset and development of DED, there remain many challenges and limitations to overcome before the role of macrophages is clearly understood and the findings can be effectively utilized to develop efficient therapeutic strategies. The first is the existence of various macrophage phenotypes that constantly polarize and turn from one type to another. In animal models of DED, macrophages can easily polarize to the pro-inflammatory M1 type during the onset of the disease, thereby causing perpetual inflammation of the ocular surface due to the secretion of cytokines and the infiltration of inflammatory cells, while the M2 type are commonly reduced.^{34,37} Hyperosmolarity, typical of DED, can also interfere with the polarization of the M2 type, thus making it difficult to restore equilibrium. Most animal studies employ the desiccating-stress model, which partly mimics the human disease but is far from mimicking the complex variety of human disease, such as autoimmune diseases, aging, or other environment-related conditions that cause the onset and progression of human disease.¹⁸⁰ Another challenge is the ability to specifically target pro-inflammatory macrophages while sparing tissue-resident protective cells, as currently available therapeutic methods lack adequate cellular specificity. Moreover, several other macrophage-modulating treatment modalities, such as cytokine blockers, extracellular vesicles, nanoparticles, and macrophage programming, are still in the preclinical stage, where their safety profiles, pharmacokinetics, dosing regimens, manufacturing processes, and immunological adverse effects outside the target tissues have not been sufficiently investigated.^{181,182} Excessive immunomodulation can render the eye more susceptible to ocular infections, impair epithelial cell recovery, and disrupt normal immunological processes. Additionally, considerable biological disparities between preclinical models of dry eye disease and humans limit the clinical relevance of preclinical studies.^{19,183}

Technological limitations also limit the pace of advancements. Single-cell RNA sequencing studies have identified a remarkable shift in gene expression patterns during the monocyte-to-macrophage transition in DED, including the induction of pro-inflammatory markers.¹⁸⁴ However, the lack of ease in sample processing and the small number of cells that can be derived from the ocular surface tissues might create biases or artifact results. Furthermore, while the depletion or modulation of macrophages in mice holds

promise in preclinical studies to reduce iron overload-induced DED symptoms, the lack of specificity and the need for targeted ocular surface delivery limit the application of these observations to humans.¹⁸⁵

Moving forward, the future research should aim to explore how to modulate macrophage polarization—such as tipping the balance towards M2 by utilizing conditioned media, exosomes derived from mesenchymal stem cells, or novel immunomodulatory compounds. Also insightful would be the decoding of how macrophages activate during the progression of DED, including how they communicate with the adaptive immune component (such as Th17 cells) and neuroimmune signaling, which would provide insights into optimal timing. Advanced technologies such as spatial transcriptomics and the fusion analysis of multiple omics platforms would allow us to identify the topography of macrophage heterogeneity in humans. Ultimately, the strategy would be macrophage-targeting therapies—a dampening strategy aimed at inhibiting M1 excessive activation or an M2-promoting strategy aimed at facilitating resolution, coupled with nanotechnology that would target the eye.^{180,185}

Although significant advances have been made in understanding the involvement of macrophages in DED, most available information on molecular mechanisms comes from experiments using animal models and in vitro systems. These approaches have enabled the identification of signaling cascades, including NF- κ B, MAPK, JAK/STAT3, activation of the NLRP3 inflammasome, and metabolic remodeling in macrophages. However, the extent to which these results reflect human disease remains unknown. Studies in humans have shown higher concentrations of inflammatory cytokines, such as IL-1 β , IL-6, TNF- α , CCL2, and MMP-9, in tears and ocular surface tissue samples, indicating inflammation associated with macrophages. Nonetheless, direct characterization of macrophage subtypes, their transcriptional profiles, and phenotypic plasticity in patients with DED is still lacking. Future research involving single-cell and spatial transcriptomics and longitudinal clinical cohorts will help validate findings from experimental models and translate them into treatments for humans.

CONCLUSION

As a core member that has a role in the pathogenesis of dry eye disease, macrophages play a crucial role by

releasing pro-inflammatory cytokines (TNF- α , IL-8/CXCL8, IL-12, and IL-6), chemoattractants (CCL2 and IL-8), and tissue remodeling enzymes (MMP-3 and MMP-1), attracting significant attention from researchers and leading to extensive exploration of this area. Emerging therapeutic strategies aim to modulate macrophage inflammatory activity, enhance pro-resolution responses, or interfere with macrophage-associated signaling pathways, which has yielded promising outcomes. The findings from these investigations not only offer new targets for the prevention and management of dry eye disease but also pave the way for novel avenues in future research endeavors.

STATEMENT OF ETHICS

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

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