• Clinical Research •

Corneal epithelial dendritic cells associated with ocular pain in dry eye disease

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Abstract

● **AIM:** To investigate the association between active corneal epithelial dendritic cells (CEDCs) and ocular pain in patients with dry eye disease (DED).

● **METHODS:** This cross-sectional study enrolled 67 DED patients, who were divided into two groups based on numerical rating scale (NRS) scores: the mild pain group (n=44) and the moderate-to-severe pain group (n=23). *In vivo* confocal microscopy (IVCM) was used to image the subbasal layer of the central cornea. Corneal nerve characteristics were analyzed using ACCMetrics software, while CEDCs were quantified manually with Image J software. Regression and correlation analyses were performed to assess the impact of active CEDCs on ocular pain. Additionally, the Luminex method was employed to compare the concentrations of inflammation-related cytokines in tears between patients with ≥2 CEDCs and those with <2 CEDCs. Differences in cytokine levels between the two groups were analyzed using Student's t-test.

● **RESULTS:** The study included 44 eyes of 44 patients with mild ocular pain (12 males and 32 females) and 23 eyes of 23 patients with moderate-to-severe ocular pain (3 males and 20 females). The mean age was 36.2±13.5y in the mild pain group and 39.7±12.4y in the moderate to severe pain group. There were no significant differences in age or sex between the two groups (P=0.30; P=0.19). Multivariable regression analysis showed that older age [odds ratio (OR) =1.05, 95% confidence interval (CI) 1.00–1.11] and a higher number of CEDCs (OR=1.80, 95%CI 1.17–2.76) were associated with ocular pain. Patients with ≥2 CEDCs had significantly higher tear concentrations of interleukin (IL)-6 (P<0.05), IL-8 (P<0.05), and tumor necrosis factor (TNF)-α (P<0.05) compared to those with <2 active CEDCs.

● **CONCLUSION:** The findings suggest that infiltrating CEDCs in the corneal subbasal layer are a potential risk factor for ocular pain in DED.

● **KEYWORDS:** dry eye disease; ocular pain; dendritic cells; inflammation

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INTRODUCTION

Dry eye disease (DED) is a multifactorial disease characterized by instability of the tear film and potential damage to the ocular surface, affecting nearly 50% of adults[1-2]. Patients with DED often exhibit visual impairment and eye irritation, resulting in a significant disease burden in terms of reduced quality of life and work[3]. Ocular pain is the most common symptom of ocular discomfort and irritation in these patients, with a reported prevalence of 89%[4]. Nonetheless, owing to the limited understanding of the pathophysiology of ocular pain[5-6], treatment of these patients with conventional dry eye medications is not effective.

Given the high density of nerve fibers, the cornea is one of the most innervated tissues in the human body[7-8]. Consequently, numerous studies have been undertaken to elucidate the

pathophysiological mechanisms underlying ocular pain in DED by investigating alterations in the structure and functionality of corneal sensory nerves. Shetty *et al*[9] found that the parameters of the corneal subbasal nerve plexus including corneal nerve fiber density (CNFD), were significantly reduced in DED patients with ocular pain. Numerous studies have shown that ocular surface inflammation plays a key role in the pathological progression of DED. However, the significance and role of inflammation, especially the neuro-immune interactions in this process, need to be further revealed, as this bidirectional communication is crucial for maintaining homeostasis in the ocular surface.

Dendritic cells (DCs) are the antigen-presenting cells, which make up the majority of immune cells in the corneal epithelium and have been considered the key to neuro-immune crosstalk in previous studies[10-11]. DCs serve as crucial mediators of adaptive immunity by initiating T cell responses to antigenic challenges. As specialized antigen-presenting cells, they first capture and process antigens in peripheral tissues before migrating to lymphoid organs, where they engage with antigen-specific T lymphocytes. Through this process, DCs not only present antigens but also provide the necessary co-stimulatory signals to activate naïve T cells, ultimately driving their proliferation and differentiation into effector cells[10-11]. Changes in the morphology of corneal epithelial dendritic cells (CEDCs) in both human and animal models serve as indicators of the immune response stage of ocular surface disorders[12-14]. Unactivated CEDCs contain linear cell bodies, samll sizes, no dendrites, or occasionally, a few short non-branched dendrites[15]. When the immune system is triggered, CEDCs broaden and generate longer dendrites to respond to the presence of antigens, which are also called active corneal epithelial dendritic cells (aCEDCs)[14,16].

This study aimed to investigate the potential role of aCEDCs as risk factors for ocular pain in patients with DED. Specifically, we examined the relationship between the number of aCEDCs and ocular pain and further explored their potential association with cytokines.

PARTICIPANTS AND METHODS

Ethical Approval The study was approved by the Peking University Third Hospital Medical Science Research Ethics Committee and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from each participant before participation in the study. This study was approved by the Ethics Committee of the Third Hospital of Peking University (Approval No.M2023073).

Participants This cross-sectional study enrolled 67 patients with DED-related ocular pain at the ophthalmic center of Peking University Third Hospital between January 2021 and July 2022. Participants who met the TFOS DEWS II diagnostic criteria for DED and had a numeric rating scale (NRS) score greater than 0 were included in the study[2]. Exclusion criteria included ocular inflammation (such as infection and allergy), history of ocular surgery, ocular or systemic pathologies causing pain (such as anxiety disorders, depression disorders, and chronic pain syndromes), contact lens wear, topical drug use other than artificial tears within 1mo, or systemic pain medications.

Clinical Parameters of DED Patients were requested to assess their ocular pain severity over the previous week using a widely utilized 10-point scale called the NRS[17]. Based on their NRS scores, ocular pain patients were categorized into two groups: a mild pain group (scores 1-4), and a moderate-to-severe pain group (scores 5-10)[18]. The subjective symptoms of DED were evaluated using the Ocular Surface Disease Index (OSDI) questionnaire. Fluorescein tear breakup time (TBUT) was determined by calculating the average of three consecutive breakup times, while corneal fluorescein staining (CFS) was assessed using the National Eye Institute grading system[19]. The Schirmer I test (SIt) was conducted using sterile Schirmer paper strips (5×35 mm) without anesthesia. The length of wetting after five minutes was recorded as the test score.

***In vivo* Confocal Microscopy** All patients underwent *in vivo* confocal microscopy (IVCM; Heidelberg Retina Tomograph 3/Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg, Germany) of the central cornea[20]. The acquired corneal images have a definition of 384×384 pixels over an area of 400×400 μm, with a lateral spatial resolution of 0.5 μm and a depth resolution of 1-2 μm. The operating process of the IVCM is described in our previous article[21]. Nearly 100 images of the subbasal layer were obtained per eye, typically at a depth of 50-70 μm.

Image Analysis For the selected eye, five images of the subbasal layer were randomly chosen for corneal nerve and DCs analyses. All images chosen for inclusion in the study adhered to the specified criteria, demonstrating excellent contrast and focus, the absence of artifacts, and accurately representation of the monolayer structure of the cornea. According to the study by Zhang *et al*[22], CNFD showed better sensitivity and specificity than other corneal nerve morphological parameters in discriminating between ocular pain and normal participants. For nerve analysis, we employed ACCMetrics software (M.A. Dabbah, Imaging Science and Biomedical Engineering, Manchester, UK), an automated platform that quantifies nerve fiber density and morphology through proprietary edge-detection algorithms. DC quantification was performed using Image J (National Institutes of Health, Bethesda, MD, USA) with customized macros that enabled semi-automated cell counting and morphological characterization based on intensity thresholding and particle analysis.

DCs were considered "active" if they had a thin body and at least three processes that extended from the cell trunk and were equal in size to or longer than the cell body itself[23]. Based on a previous study, individuals were grouped based on the presence or absence of ≥2 aCEDC in the central cornea (Figure 1)[14]. Two blinded researchers (Duan HY and Zhou YF) evaluated all images and the averaged values were used for the analysis.

Measurement of Cytokines in Tears To further investigate the cytokines associated with the activation of CEDCs, 5 μL basal tears were collected by placing a microcapillary glass tube (Microcaps; Drummond Scientific Co, Broomall, PA, USA) over the temporal side of the lower lid. Tear samples were transferred to microcapillary glass tubes and immediately stored at -80°C. A Luminex 200™ System (Luminex, Austin, TX, USA) was used to measure the cytokine levels in tear samples, including nerve growth factor (NGF), interleukin (IL)-6, insulin, leptin, IL-8, macrophage inflammatory protein (MIP)-1, tumor necrosis factor (TNF)-α, IL-1β, α-melanocyte-stimulating hormone (α-MSH), β-endorphin, neurotensin, oxytocin, and substance P (SP). The concentration values were acquired from the mean fluorescence intensity (MFI) by using Luminex200 IS V2.1.

Statistical Analysis Statistical analyses were performed using the R 4.0.4 software. Univariate logistic regression was used to explore the risk factors for ocular pain in DED, including age, sex, aCEDCs, TBUT, SIt, and CNFD. Candidate variables for clinical data with $P<0.1$ in the former analysis were further included in the multivariable model. The correlation of the identified risk factors with the NRS score was also evaluated using Spearman's rank correlation test. Differences in cytokine levels between groups were compared using Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Demographic data and ocular surface parameters are shown in Table 1. The study included 44 eyes of 44 patients with mild ocular pain (12 males and 32 females) and 23 eyes of 23 patients with moderate-to-severe ocular pain (3 males and 20 females). The mean age was 36.2±13.5y in the mild pain group and 39.7±12.4y in the moderate to severe pain group. There were no significant differences in age or sex between the two groups ($P=0.30$; $P=0.19$). Compared with patients in the mild pain group, participants in the moderate to severe pain group showed higher NRS (6.9±0.9) and OSDI scores (48.0±19.3; both $P<0.05$). In addition, there were no significant differences in TBUT, CFS, and SIt between the two groups (all $P>0.05$).

Figure 2 shows the outcomes of the regression analysis investigating the impact of various clinical features on ocular pain. Univariate regression results indicated that the number of aCEDCs (OR=1.50, 95%CI 1.00-2.26) and the wetting length of SIt (OR=1.06, 95%CI 1.00-1.12) were risk factors for ocular pain in DED. Further analysis of the multivariable regression showed that age (OR=1.05, 95%CI 1.00-1.11) and the number of aCEDCs (OR=1.80, 95%CI 1.17-2.76) were associated with the occurrence of ocular pain in DED, while older age and higher aCEDCs counts were associated with greater odds of ocular pain.

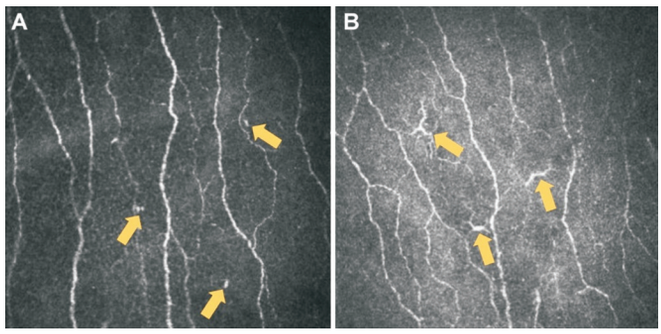

Figure 1 Corneal epithelial dendritic cells Yellow arrow indicates unactivated CEDCs without dendritic processes (A) and aCEDCs with long dendritic processes (B) in DED patients with ocular pain. The panels shown are representative IVCM images with a frame size of 400×400 μm.

Table 1 Demographics and ocular surface parameters of participants

Characteristics	Mild group	Mod-Sev group	*P*
Patients/eyes, *n*	44/44	23/23	-
Age, y	36.2±13.5	39.7±12.4	0.30
Sex, men	12 (27.3%)	3 (13.0%)	0.19
NRS score	2.7±1.8	6.9±0.9	<0.05
OSDI score	35.5±18.1	48.0±19.3	<0.05
TBUT, s	4.7±2.5	3.9±2.0	0.14
CFS	1.8±2.1	2.9±3.0	0.08
SIt, mm/5min	12.3±10.4	19.0±11.4	0.05

Mod-Sev: Moderate to severe; NRS: Numerical rating scale; OSDI: Ocular surface discomfort index; TBUT: Fluorescein tear breakup time; CFS: Corneal fluorescein staining; SIt: Schirmer I test.

Figure 3 demonstrates the linear correlation between the NRS score and the risk factors identified by regression analysis in all patients. With increasing age ($R=0.44$, $P<0.001$) and aCEDCs counts ($R=0.34$, $P<0.01$), the NRS score also increased accordingly. In addition, there was an upward trend in the association between aCEDCs counts ($R=0.42$, $P<0.001$) and the NRS score after adjusting for age and sex to reduce bias.

Figure 4 shows the levels of inflammation-related cytokines in the presence and absence of ≥2 aCEDCs. The results revealed that the concentrations of IL-6, IL-8, and TNF-α in the tears of patients in the presence of ≥2 aCEDCs group were significantly higher than those in the group with aCEDCs counts <2. No intergroup differences were found in the levels of other cytokines.

DISCUSSION

Ocular pain is a prevalent symptom in patients with DED,

Variables	Univariable		Multivariable	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age, year	2.38(0.46,12.30)	0.30	1.05(1.00,1.11)	<0.05
Sex, men	1.02(0.98,1.07)	0.19	3.11(0.54,17.91)	0.20
aCEDCs	1.50(1.00,2.26)	0.05	1.80(1.17,2.76)	<0.01
TBUT, s	0.86(0.65,1.13)	0.28	-	-
SIT, mm/5min	1.06(1.00,1.12)	0.05	1.05(0.99,1.12)	0.09
CNFD, fibres/mm^2	0.94(0.86,1.03)	0.20	-	-

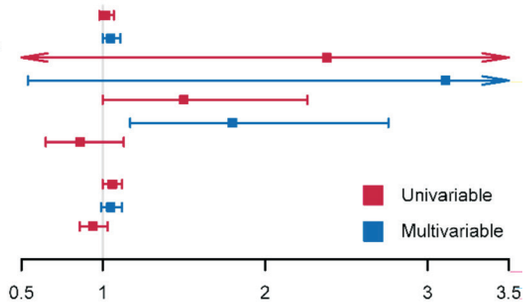

Figure 2 Univariable and multivariable regression analysis of factors associated with ocular pain in DED patients OR: Odds ratio; aCEDCs: Active corneal epithelial dendritic cells; TBUT: Tear breakup time; SIt: Schirmer's I test; CNFD: Corneal nerve fiber density.

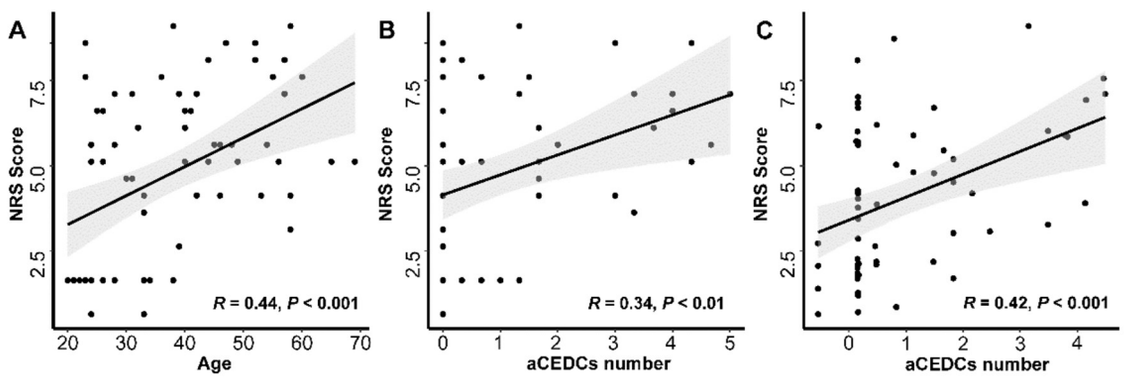

Figure 3 Spearman correlation between NRS score with age (A), aCEDCs counts (B) and aCEDCs counts after adjusting age and sex (C) NRS: Numerical rating scale; aCEDCs: Active corneal epithelial dendritic cells.

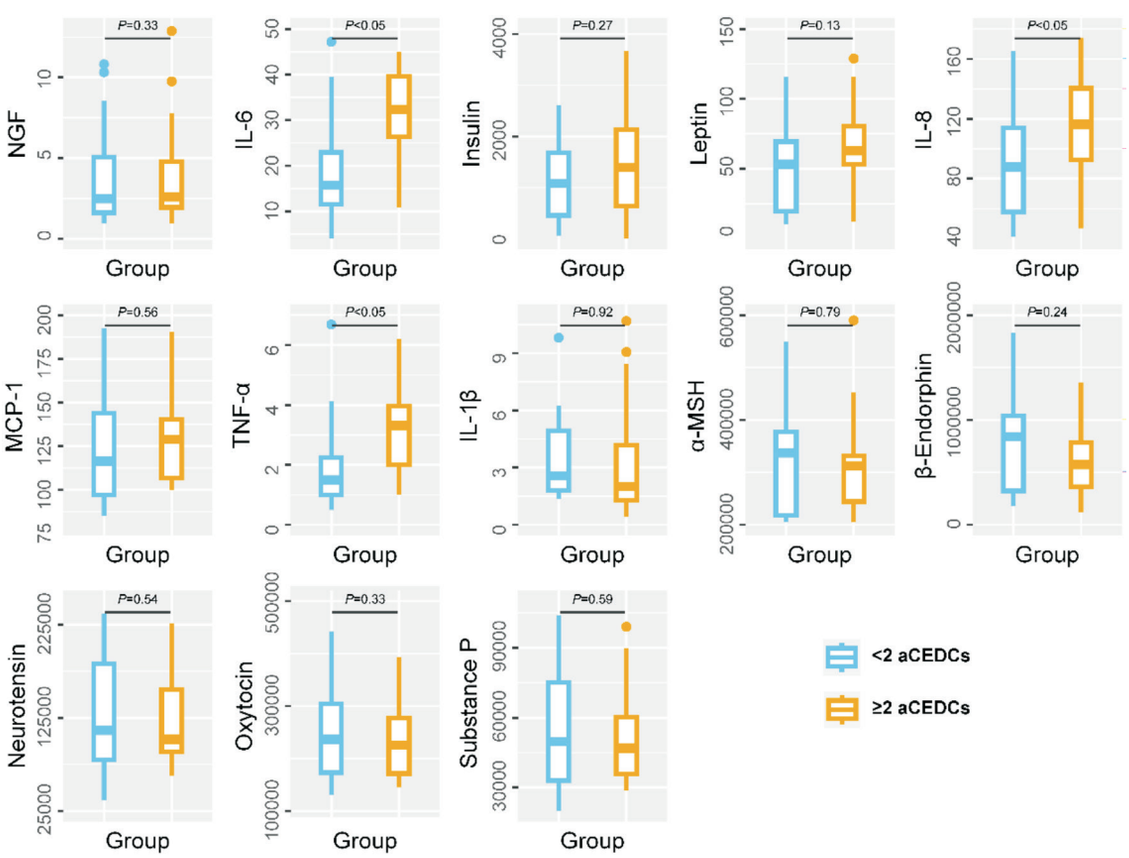

Figure 4 Differences in cytokines content between <2 aCEDCs and ≥2 aCEDCs groups NGF: Nerve growth factor; IL: Interleukin; MCP-1: Monocyte chemoattractant protein-1; TNF: Tumor necrosis factor; α-MSH: α-melanocyte-stimulating hormone; aCEDCs: Active corneal epithelial dendritic cells.

however, its underlying mechanism remains elusive. Previous research has shown significant changes in the sub-basal nerves of individuals experiencing DED-related ocular pain[24]. In this study, we revealed the contribution of infiltrated aCEDCs to ocular pain. These infiltrated DCs were found to be associated with ocular pain in DED, as supported by the higher expression of inflammatory factors such as IL-6, IL-8, and TNF-α. Notably, these cytokines have been previously linked to DCs activation[25], providing further insight into the pathophysiology of ocular pain in DED. We speculate that aCEDCs' production of NGF promotes nerve sprouting and hyperexcitability; and they form functional synapses with corneal sensory nerves, facilitating bidirectional signaling that amplifies pain perception. This neuroinflammatory cascade leads to the characteristic symptoms of ocular dysesthesia and pain in DED patients. These findings suggest that, apart from the attributes of the corneal sub-basal nerve plexus, the immune microenvironment, encompassing DCs and inflammatory factors, may also play a role in the development of ocular pain in individuals with DED.

Age was found to be the greatest risk factor for DED[1]. The prevalence of DED in women and men increases every five years after the age of 50[26-27]. In our study, we noted that patients with moderate-to-severe pain were older than those with mild pain, suggesting that age is also associated with ocular pain in DED. Previous studies have also reported that the prevalence of pain, including lower back pains, joint pain, and lower extremity pain, tends to increase with age[28]. In addition, many biological changes associated with aging, such as systemic inflammation, oxidative stress, and alterations in neuronal structure and function, may be responsible for the increased clinical pain and altered pain modulatory balance[29].

The cornea is one of the most highly innervated tissues in the body. The ocular pain associated with DED is likely to be neuropathic, which is closely related to the dysfunction of the ocular somatosensory nerves, particularly the subbasal nerve plexus[30]. Nerves and DCs are likely to constantly communicate and interact with each other in the cornea. The absence of DCs in the corneas results in a notable impairment in corneal nerve regeneration after epithelial debridement[31]. Moreover, in animals subjected to desiccating stress, those with DC-depleted corneas exhibited decreased paracentral corneal nerve density and reduced levels of neurotrophic factors compared with mice with intact DC populations[32]. These findings provide further evidence to support an association between corneal DCs and nerve structures. To determine the factors affecting ocular pain associated with DED, regression analyses were performed. Interestingly, no correlation was observed between ocular pain and the CNFD. This difference may be due to racial differences in the measurement strategies. Notably, the number of aCEDCs was significantly associated with the severity of ocular pain in patients with DED. This is consistent with previous studies, which indicated that infiltrated DCs in the tumor microenvironment promote neuropathic pain[33]. These groundbreaking findings shed light on novel mechanisms through which DCs may contribute to the development of ocular pain in DED.

DCs, as professional antigen-presenting cells, play a crucial role in surveillance within the immune system, maintaining immune quiescence of the ocular surface and contributing to the immune privilege of the cornea[34]. Previous studies have reported that DCs density is associated with increased ocular inflammation and is significantly higher in patients with DED[35]. In addition, compared to healthy controls, higher numbers of DCs have also been found in the central cornea of patients with other autoimmune diseases, such as Behcet's disease[36], systemic lupus erythematosus[37], and rheumatoid arthritis[38]. In DED, the elevation of inflammatory cytokines on the ocular surface induces the transformation of resident antigen-presenting cells into an activated and mature phenotype known as aCEDCs. These aCEDCs possess an enhanced ability to present antigens through major histocompatibility complex class II molecules displayed on their surface[39]. Subsequently, these aCEDCs migrate to the cervical lymph nodes where they facilitate the differentiation of naïve T cells, which subsequently migrate to the cornea. The aCEDCs express co-stimulatory molecules such as CD40, CD80, and CD86, thereby priming themselves to efficiently presentation of antigenic peptides to T cells and initiate adaptive immune responses[40]. DC dendrites are recognized as morphological indicators of "activation." In the presence of antigens, DCs undergo changes such as increased size and elongation of dendrites, giving rise to "active" DCs[23,41-42]. Based on our study findings, the increased number of aCEDCs in the moderate-to-severe ocular pain group indicated a higher number of DCs in this group. Given the involvement of DCs in inflammatory responses and T-cell activation, these results suggest that reducing the number of aCEDCs in the cornea could be a potentially effective approach for managing ocular pain in DED and may have long-term beneficial effects on ocular surface health.

In relation to the cytokines linked to ocular pain in DED, our study established a noteworthy correlation between elevated levels of IL-8, TNF-α, and IL-6 and an increase in aCEDCs. This finding is consistent with previous research suggesting an association between these cytokines and activated DCs in the context of DED. These cytokines, secreted by the ocular surface under desiccation stress or hyperosmolar conditions, have been implicated in the activation of DCs[25]. Li *et al*[43] reported that IL-8 expression positively correlated with the

DC activation markers CD80 and CD86. Furthermore, several studies have reported that IL-8 released after inflammation or chronic desiccation stress is strongly associated with DED severity[44]. Notably, IL-8 is also crucial for the development of neuropathic pain after disc herniation or nerve damage[45].

In our study, we also observed a higher expression of TNF-α in individuals with increased numbers of aCEDCs. Both TNF-α and IL-8 contribute to the persistence of mechanical nociceptor hypersensitivity[46]. Following peripheral nerve injury, Schwann cells release endogenous TNF, which participates in a rapid immune response. In nerve-injured animals, activation of the tumor necrosis factor receptor (TNFR) leads to nociceptive hypersensitivity and ectopic firing[47]. Moreover, local application of TNF to the dorsal root ganglion (DRGs) and sciatic nerve results in ectopic firing of Aδ-, Aβ-, and C-fibers[48]. Based on these findings, it is hypothesized that targeting the TNF-α or IL-8 downstream pathways may have therapeutic potential for alleviating ocular pain in DED. Further investigations are warranted to thoroughly evaluate the intricate relationship between TNF-α and IL-8 levels and ocular pain in patients with DED.

The levels of IL-6 were elevated in patients with more aCEDCs. Emerging evidence supports the dual role of IL-6 as a modulator and mediator of DCs in the immune and inflammatory processes. IL-6 within the inflammatory microenvironment can modulate the activation status of DCs, whereas aCEDC-secreted IL-6 predominantly mediates the immune activities of aCEDCs, particularly their interactions with T cells[49]. In DED, IL-6 drives the differentiation of naive T cells into Th17 cells[50], and its increased expression has been observed in DED lymph nodes[51]. Therefore, targeting IL-6 may be a potential therapeutic approach for alleviating DED-related ocular pain by reducing the induction of Th17 cells by aCEDCs. Following spinal nerve injury, IL-6 knockout mice have reduced pain hypersensitivity, whereas their nociceptive responses to thermal and mechanical stimuli are normal, suggesting that IL-6 plays a role in the development of neuropathic chronic pain rather than an acute response[52]. Because of the diverse roles of IL-6 in neuropathic pain, further studies are needed in the future to elucidate the mechanism of IL-6 in dry eye pain. Above all, within the corneal immune microenvironment of dry eye pain, an overwhelming prevalence of pro-inflammatory responses is evident, characterized by heightened DCs activity and elevated pro-inflammatory factors. Consequently, there is a pressing need to effectively inhibit these pro-inflammatory processes or discover novel approaches to activate inherent anti-inflammatory mechanisms.

Our study has several limitations that should be acknowledged. First, we utilized a basic 10-point scale to quantify ocular pain, which provided a measure of pain severity[17], but may not have captured all dimensions of pain experience. The use of more comprehensive pain questionnaires, such as the Ocular Pain Assessment Survey, could provide a more nuanced assessment of ocular pain in future studies[53]. Second, the cross-sectional and observational design of our study limited our ability to establish a causal relationship between aCEDCs and ocular pain. While we observed an association between aCEDCs and ocular pain, further longitudinal research is required to validate our findings in patients with DED with ocular pain. Finally, our findings carry important clinical implications for DED management. The demonstrated association between aCEDCs and ocular pain suggests that: 1) aCEDC density could serve as an objective biomarker for stratifying patients with neuropathic ocular pain components, particularly in cases with discordant symptoms and clinical signs; 2) targeted anti-inflammatory therapies (*e.g.*, topical cyclosporine 0.05%, lifitegrast 5%) should be prioritized for patients showing elevated aCEDC counts, as these agents may directly modulate DC activity; 3) combined neuro-immune evaluation (aCEDCs+corneal nerve analysis) could optimize treatment selection in refractory DED cases. However, further research is needed to confirm the effectiveness of these treatments. In conclusion, our study sheds light on the relationship between aCEDCs and ocular pain in DED patients. However, it is crucial to address these limitations in future research to further elucidate the underlying mechanisms and enhance treatment strategies for ocular pain in patients with DED.

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Authors' Contributions: Zhou YF: Conceptualization, Investigation, Writing-original draft; Yu HZ: Formal analysis, Data curation, Methodology; Duan HY: Formal analysis, Data curation, Writing-original draft; Yang TT: Methodology; Validation; Zhao L: Resources; Ma BK: Writing-review and editing; Chen JW: Resources; Qi H: Supervision, Funding acquisition.

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