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Contact lens-induced corneal parainflammation involving Ly6G+ cell infiltration requires IL-17A and γδ T cells

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Abstract

Purpose—Previously, using a murine model, we reported that contact lens (CL) wear induced corneal parainflammation involving CD11c+ cells after 24 h and Ly6G+ cells (neutrophils) after 5–6 days. Here, we investigated the role of IL-17 and γδ T cells in the CL-induced neutrophil response.

Methods—CL-wearing C57BL/6 wild-type (WT) mice were compared to lens-wearing IL-17A/F single or double gene knock-out mice, or mice treated with UC7–13D5 monoclonal antibody to functionally deplete γδ T cells. Contralateral eyes served as no lens wear controls. Corneal Ly6G+ and γδ T cell responses were quantified as was expression of genes encoding pro-inflammatory cytokines IL-17A/F, IL-β, IL-18 and expression of IL-17A/F protein.

Results—After 6 days lens wear, WT corneas showed Ly6G+ cell infiltration while remaining free of visible pathology. In contrast, lens-wearing corneas of IL-17AF (−/−), IL-17A (−/−) mice and γδ T cell-depleted mice showed little or no Ly6G+ cell infiltration. No Ly6G+ cell infiltration was detected in contralateral eye controls. Lens-wearing WT corneas also showed a significant increase in γδ T cells after 24 h that was maintained after 6 days of wear, and significantly increased cytokine gene expression after 6 days versus contralateral controls: IL-18 & IL-17A (~3.9 fold) and IL-23 (~6.5-fold). Increased IL-17A protein (~4-fold) was detected after 6 days lens wear. γδ T cell-depletion abrogated these lens-induced changes in cytokine gene and protein expression.

Conclusion—Together, these data show that IL-17A and γδ T cells are required for Ly6G+ cell (neutrophil) infiltration of the cornea during contact lens-induced parainflammation.

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Keywords

Contact lens wear; Parainflammation; Ly6G+ cells (neutrophils); γδ T cells; IL-17 signaling; IL-18; Murine cornea; UC7–13D5

1. Introduction

Contact lens wear is a common form of vision correction with more than 140 million wearers worldwide. Contact lens-associated complications affecting the cornea and conjunctiva include inflammatory events, ocular discomfort, dry eye disease and infection (microbial keratitis) [1–7]. While significant progress has been made in understanding the mechanisms of contact lens-associated adverse events, there is still much to learn about their underlying etiology.

In recent years, it has become evident that contact lens wear can be associated with subclinical changes in corneal immune cells, e.g. increased numbers of Langerhans cells, as early as 2 h of wear [8], that are also evident after 7 days [4]. It was proposed that human contact lens wear is “intrinsically inflammatory” and that lens-induced parainflammation may serve to protect the cornea against noxious external stimuli [9]. More recently, we reported development of a contact lens wearing murine model of microbial keratitis, in which lens-induced parainflammatory events in the cornea were observed that resembled changes in Langerhans cells observed during lens wear in humans [10]. These contact lens-induced changes were: a significant increase in corneal CD11c+ (dendritic) cells after 24 h and recruitment of Ly6G+ cells (neutrophils) to the cornea after 5 to 6 days. Since both of these cellular responses occurred without obvious clinical signs, they were termed parainflammatory [10].

Parainflammation was described previously as a response to tissue stress that resided between the basal homeostatic state of a tissue and classical “symptomatic” inflammation [11]. Our long-term goal is to understand the mechanisms and clinical significance of contact lens-induced corneal parainflammation. Do these clinically silent changes in corneal CD11c+ cells and Ly6G+ cells during lens wear represent protective adaptations or the first step in predisposing the cornea to clinically evident adverse events?

In the present study, we focused on the mechanisms by which Ly6G+ cells are recruited to the cornea after 6 days of lens wear. Recruitment of Ly6G+ cells (neutrophils) to a site of injury or infection is typically associated with many different types of signaling *via* complement proteins (C5a), leukotrienes (LTB_4) cytokines (IL-6, IL-8, IL-17) and chemokines (CXCL1, CXCL2) [12–15]. The murine model of contact lens wear was used to test the hypothesis that IL-17 mediated signaling was involved. This hypothesis was based on two prior observations: 1) IL-17 produced by ocular surface γδ T cells in response to conjunctival colonization by a commensal *Corynebacterium* sp. was important for neutrophil homeostasis in the conjunctiva and recruitment of these cells to defend the cornea against *Pseudomonas aeruginosa* and *Candida albicans* infection [16], and 2) our recent observation that CD11c+ cell corneal parainflammatory responses to 24 h of contact lens

wear were prevented by antibiotic treatment correlating with changes in viability/activity of conjunctival commensal bacteria [17].

Using IL-17A- and F-deficient mice and antibody depletion of γδ T cells, we found evidence that an early recruitment of IL-17 producing γδ T cells to the cornea of contact lens-wearing mice precipitates IL-17A (but not F)-dependent recruitment of neutrophils to the cornea, serving as a trigger of contact lens-induced parainflammation.

2. Methods

2.1 Murine model of contact lens wear

Six-week old C57BL/6 wild-type (WT) female mice were used along with age- and sex-matched gene knockouts in IL-17A (−/−), IL-17F (−/−) or both IL-17AF (−/−). The IL-17 gene-knockout mice were provided *via* RRC under a MTA by Dr. Yoichiro Iwakura, Tokyo University of Science. The right eye of each mouse was fitted with a custom-designed silicone-hydrogel contact lens, developed by a contact lens manufacturer, as previously described [10]. Contralateral eyes were used as controls unless otherwise specified since we have previously established that these eyes were indistinguishable from naïve mice in this model [10]. Before fitting, contact lenses were removed from packaging solution and soaked for 1 h in filtered (0.45 μm) phosphate buffered saline (PBS). For lens fitting, mice were anesthetized with 2.5 – 3 % isoflurane delivered via a precision vaporizer (VetEquip Inc., Pleasanton, CA), an Elizabethan collar (Kent Scientific) was placed on each mouse, followed by lens placement using a Handi-Vac suction pen (Edmund Optics, Barrington, NJ) with a 3/32″ probe. After lens insertion mice were single-housed without enrichments to prevent lens removal using Pure-o'Cel paper bedding (The Andersons Inc., Maumee OH) to reduce dust levels. Mice wore the contact lenses for 24 h or up to 6 days depending on experimental design. Mice were monitored daily for retention of lenses and for any evidence of pathology, e.g. discharge or corneal opacity, using a stereomicroscope (Zeiss Stemi 2000-C) under short-term (~20 min) isoflurane anesthesia. For overnight lens wear mice were returned to the animal housing facility. After assigned times of lens wear, mice were euthanized using 5 % isoflurane for 10 min followed by cervical dislocation. Any mouse that had lost its contact lens prior to the desired time point was excluded from further experimentation. All procedures were carried out in accordance with the standards established by the Association for the Research in Vision and Ophthalmology, under a protocol AUP-2019-06-12322 approved by the Animal Care and Use Committee, University of California Berkeley. This protocol adheres to PHS policy on the humane care and use of laboratory animals, and the guide for the care and use of laboratory animals. This study is reported in accordance with the ARRIVE guidelines (Animals in Research: Reporting *In-Vivo* Experiments).

2.2 γδ T cell functional depletion

C57BL/6 mice were subjected to an intraperitoneal injection of UC7–13D5 (Bio X Cell; #BE0070), a monoclonal antibody which reacts with an epitope on the murine γδ TCR (γδ T Cell Receptor) to functionally deplete γδ T cells *in vivo* [16,18,19, 32]. UC7–13D5 was

administered in a dose of 500 μg per 200 μl (2.5 μg/μl) of PBS 24 h before contact lens fitting: PBS alone was injected similarly for control group mice.

2.3 Immunolabeling of whole corneas

Enucleated eyes were fixed for 1 h using 2 % paraformaldehyde, then washed in PBS for 10 min with rotation. Corneal dissection was performed under a dissecting microscope over ice, the dissected corneas then washed once in PBS for 10 min with rotation, incubated in blocking solution (3 % bovine serum albumin, 0.3 % Triton X-100 in PBS) for 1 h at room temperature with rotation followed by incubation in EDTA (20 mM in PBS) for 1 h at 37 °C. The following primary antibodies were used to label corneal immune cells or neurons each diluted in blocking buffer; Hamster anti-Mouse TCR Gamma/Delta (1:500; BioRad; #MCA1366GA) and Rabbit anti-Mouse β-Tubulin III (1:500; Sigma-Aldrich; #T2200). Dissected corneas were incubated with primary antibody overnight at 4 °C with rotation, then with an appropriate secondary antibody diluted in blocking solution (1:800) for 2 h at room temperature with rotation and covered with foil. Secondary antibodies used were: Goat anti-Hamster (Abcam; #175716) and Goat anti-Rabbit (Life Technologies; #A21434). DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (12.5 μg/ml) (ThermoFisher; #D1306) was included in the secondary antibody buffer solution to label cell nuclei. After the secondary antibody incubation, corneas were transferred to fresh PBS and washed three times for 10 min with rotation. Corneas were flat-mounted with Prolong Diamond (ThermoFisher; #P36970) to enhance visibility during confocal imaging.

2.4 Immunolabeling of corneal cryosections

Ly6G+ cells (neutrophils) or cells producing IL-17A or IL-17F were labeled and imaged using corneal cryosections as previously described [10]. Enucleated eyes were fixed in 2 % paraformaldehyde overnight at 4 °C, followed by overnight washing in PBS at 4 °C to remove the excess paraformaldehyde. Eyes were cryo-protected by immersion in sucrose (15 % for 4 h, then 30 % for an additional 4 h) at room temperature, then embedded in OCT (Tissue Tek) and flash-frozen in liquid nitrogen and stored at −80 °C until sectioning. Embedded eyes were sectioned at 10 μm thickness using a Leica CM 1900 cryostat, placed on a glass slide and stored at −80 °C until labeling. For labeling of Ly6G+ cells or cells producing IL-17A or IL-17F, corneal cryosections were washed with PBS for 5 min at room temperature in a Petri dish containing a wet paper towel to provide moisture, followed by incubation in blocking solution (3 % BSA) for 30 min at room temperature while maintaining the moist environment. Corneal sections were then incubated with primary antibody diluted 1:100 in blocking buffer as follows; for Ly6G+ cells, Rat NIMP-R14 antibody (10 μg/ml; ThermoFisher; #1676494), for IL-17A Rabbit anti-mouse antibody (Abcam; #ab79056) or IL-17F Rabbit anti-mouse antibody (R&D Systems; #MAB2057-SP)at room temperature for 1 h, followed by three washes with PBS (10 min each) to remove excess unbound antibody. Sections were then incubated for 1 h with secondary antibody solution; either Alexa 647-conjugated Goat anti-Rat antibody or Alexa 555-conjugted Goat anti-Rabbit (5 μg/ml, Life Technologies; #A21247, #A21434 respectively) diluted 1:400 in blocking buffer. Corneal sections were counterstained with DAPI to visualize cell nuclei and Cell Navigator™ (AAT Bioquest; #22661) diluted 1:400 for corneal epithelial cell F-actin labeling. Sections were then washed three times with PBS

(10 min each) and mounted on a coverslip with Prolong Diamond. Sections were allowed to dry for at least 30 min in the dark at room temperature before imaging under Nikon bright field microscope.

2.5 Imaging and image analysis

Confocal imaging was performed using a 20x/1.00 NA water-dipping objective, and an upright Olympus FluoView FV1000 Confocal Microscope. Flat-mounted corneas were imaged using 559 nm (red, γδ T cells), and 488 nm (green, corneal nerves) lasers. For Z stacks at 0.54 μm steps, images were collected from 4 or more random fields per sample. Corneal nerve labeling was used to help locate the central cornea and four peripheral corneal images that were collected for each sample. The 3-D and 4-D image reconstructions, cell quantification, cell morphology analysis and movie generation were performed using Image-J (MorpholibJ tools collection) and Imaris (Bitplane). Protocols were similar to those described previously [10] and maximum intensity projections, i.e. reducing a 3-D image into 2-D by projecting the maximum intensity of each pixel in a specific channel to the z plane, where indicated to visualize γδ T cells and cell morphology.

Imaging of corneal sections for Ly6G+ cells or IL-17 producing cells was accomplished using a Nikon Ti-E inverted wide-field fluorescence microscope equipped with Lumencor SpectraX illumination source, and CFI Plan APO VC 20x/0.75 NA objective. Quantification was performed using Image-J (MorpholibJ tools collection) based on the maximum intensity projection of the Ly6G+ cells (or IL-17 producing cells) for at least 4 fields per sample, and at least 3 samples per condition and confirmed by manual counts. All samples were masked to minimize bias.

2.6 RT-qPCR

Following euthanasia, corneas were carefully dissected from enucleated eyes to remove all limbal tissue, and flash-frozen using liquid nitrogen and stored at −80 °C until sample preparation. To minimize enzymatic reactions, corneal dissection was performed using 2 % dithiothreitol. Two corneas from the same condition were pooled for RNA extraction. Corneas were disrupted using a hand-held tissue homogenizer (Kinematica Polytron, ThermoFisher) in Trizol. RNA was extracted from the homogenate in Trizol using liquid-liquid extraction and the aqueous phase collected for RNA isolation and purification. cDNA synthesis was performed using iScript (Bio-Rad) and RT-qPCR using Faststart Sybergreen (Roche) running on a Light Cycler 96 real-time PCR machine (Roche). Primers were designed to be separated by at least one intron to assure selective amplification of cDNA and tested for efficiency (greater than or equal to 1.90), and specificity under conditions used.

2.7 Statistical analysis

Data analysis was performed using Prism 9.0 for Mac, Microsoft Excel 2010, and the Statistical Package for Social Science for Mac version 27.0 (SPSS, Inc, Chicago, IL). The distribution of data was assessed by the normality test (Shapiro-Wilk test and Kolmogorov-Smirnov test), and since most of the data was not normally distributed, it was expressed as the median with inter-quartile range (IQR). The Mann-Whitney U test was used for two group comparisons or the Kruskal-Wallis test with Dunn’s multiple comparisons was used

for three or more groups. Gene expression data, however, were expressed as the mean with standard error of the mean (+/− SEM) and Student's t-Test used for two group comparisons. P values less than 0.05 were considered significant. Experiments were repeated twice unless otherwise stated.

3. Results

3.1 Contact lens-induced recruitment of Ly6G+ cells after 6 days of wear requires IL-17A

To explore the role of IL-17 in contact lens-induced recruitment of Ly6G+ cells to the murine cornea after 6 days of wear, wild-type (WT) mice were compared to IL-17 gene knockout mice; IL-17AF (−/−), IL-17A (−/−) and IL-17F (−/−). After 6 days continuous lens wear, immunolabeling for Ly6G+ cells showed recruitment of Ly6G+ cells to the corneal stroma as previously reported [10] with no change observed in the contralateral control eyes (Fig. 1). IL-17 AF (−/−) and IL-17A (−/−) mice, however, showed little or no Ly6G+ cell recruitment to the cornea, while Ly6G+ cells were recruited to the corneas of IL-17F (−/−) mice similar to WT eyes. Few or no Ly6G+ cells were observed in contralateral control corneas of any IL-17 gene knockout mice. Ly6G+ cells were detected in conjunctival and limbal regions of IL-17A (−/−) and IL-17F (−/−) mice (data not shown). These results show that IL-17A is required for contact lens-induced recruitment of Ly6G+ cells (neutrophils) to the murine cornea after 6-days of wear.

3.2 Contact lens wear recruits γδ T cells to the murine cornea after 1 and 6 days of wear

Since IL-17A was required for Ly6G+ cell recruitment to the lens-wearing cornea after 6 days, we explored possible sources of IL-17. Previously, it was shown that a conjunctival commensal bacterium (*Corynebacterium mastitidis*) was required for γδ T cells in the ocular mucosa to produce IL-17 that recruited neutrophils to defend against *P. aeruginosa* corneal infection [16]. Therefore, γδ T cell recruitment to the murine cornea was explored after 1 and 6 days of wear. Fig. 2 shows that contact lens wear was associated with a significant increase in γδ T cells in the cornea (primarily at the peripheral cornea and limbus) after 1 day (Fig. 2a, b) as well as after 6 days (Fig. 2c, d) compared to contralateral controls. The magnitude of the γδ T cell response was not greater after 6 days versus 1 day. In each instance there were no obvious clinical signs or symptoms of corneal disease, i.e. this lens-induced γδ T cell response also appeared to be parainflammatory.

Contact lens-induced γδ T cell responses were also examined in corneas of IL-17A (−/−) mice which had previously failed to show Ly6G+ cell infiltration after 6 days of lens wear (Fig. 1). Supplemental Fig. S1 shows that IL-17A (−/−) mice also demonstrated lens-induced corneal γδ T cell responses after 1 and 6 days of lens wear showing that these responses do not require IL-17A.

3.3 Functional depletion of γδ T cells using UC7–13D5 monoclonal antibody abrogates contact lens induced γδ T cell responses after 1 and 6 days of wear

To further explore the role of γδ T cells in contact lens-induced corneal parainflammation, UC7–13D5 monoclonal antibody was administered to functionally deplete γδ T cells. Control group mice were injected with PBS. Fig. 3 images show that after UC7–13D5

treatment, there was reduction in the number of γδ T cells detected in the central (Fig. 3a) and peripheral (Fig. 3b) cornea after 1 day and 6 days of wear. This was confirmed by image quantification (Fig. 3c).

Control (PBS treated) and γδ T cell depleted (UC7–13D5 treated) mice were then fitted with a contact lens for 1 and 6 days. Lenses were fitted 1 day after UC7–13D5 administration and contralateral (non-lens wearing eyes) were also assessed to compare lens-induced responses from baseline in each group. Fig. 4 imaging (Fig. 4a, c) and quantification (Fig. 4b, d) show that UC7–13D5 treatment abrogated lens-induced γδ T cell responses after 1 day and 6 days of wear.

3.4 Functional γδ T cell depletion prevents contact lens-induced Ly6G+ cell recruitment into the cornea after 6 days of wear

It was next determined if UC7–13D5 administration to functionally deplete γδ T cell responses would affect contact lens-induced Ly6G+ cell recruitment to the cornea after 6 days of lens wear. Fig. 5 shows that mice treated with UC7–13D5 failed to show a significant Ly6G+ cell infiltrative response to contact lens wear after 6 days compared to the control group. Few Ly6G+ cells were observed in contralateral control corneas in either group. Thus, these data indicate that γδ T cells are required for contact lens-induced recruitment of Ly6G+ cells to the cornea after 6 days of wear.

3.5 Functional γδ T cell depletion attenuates contact lens-induced increases in corneal expression of IL-17A, IL-23 and IL-18 genes *in vivo* after 6 days of wear

With prior experiments showing a role for IL-17A and γδ T cells in contact lens-induced recruitment of Ly6G+ cells to the cornea after 6 days wear, the transcriptional profile of IL-17 signaling in the cornea was evaluated. Fig. 6 shows changes in gene expression in lens-wearing corneas expressed relative to contralateral controls (i.e. baseline normalized to zero) in control (treated with PBS) mice versus γδ T cell depleted mice (treated with UC7–13D5). Contact lens wear for 6 days was associated with a significant increase in the expression of genes encoding IL-17A (~3.9-fold), IL-23 (~6.5-fold), and IL-18 (~3.9-fold) versus respective contralateral controls (Fig. 6; Black bars with *, **). In each instance, this response was significantly reduced in γδ T cell depleted mice (Fig. 6; Compare black bars to white bars with *, **). IL-6 responses were also significantly reduced in γδ T cell depleted mice versus controls. IL-1β gene expression increased ~3-fold, and that of the transcription factor Ror-γ by ~4-fold, but these changes were not significant versus contralateral controls, nor were they affected by γδ T cell depletion. Interestingly, IL-17F gene expression decreased in lens-wearing corneas versus contralateral controls, a decrease that appeared to be attenuated by γδ T cell depletion. However, neither of those changes reached statistical significance. IL-21 gene expression was not significantly affected under any condition.

3.6 IL-17 cytokine responses to 6 days of lens wear are attenuated by γδ T cell depletion

Having observed an increase in IL-17A gene expression with 6 days of contact lens wear and decreased expression of the IL-17F gene, quantitative immunofluorescence imaging was used to assess corneal expression of these proteins under the same conditions. Contact lens

wearing corneas showed a significant increase in IL-17A expression versus contralateral controls (~4-fold) that was attenuated by γδ T cell depletion (Fig. 7a, b). In contrast to its gene expression (Fig. 6), a small, but statistically significant increase in IL-17F protein was observed after 6 days of lens wear, that was also attenuated by γδ T cell depletion (Fig. 7c, d). The reason(s) for these apparently contradictory findings for IL-17F gene and protein expression is/are unknown. Nevertheless, lens-induced changes in corneal IL-17A protein expression correlated with those in gene expression and are consistent with a role for IL-17A in mediating Ly6G+ cell recruitment to the cornea after 6 days of lens wear.

4. Discussion

In this study, we investigated the mechanisms underlying contact lens-induced parainflammation of the murine cornea involving Ly6G+ cell (neutrophil) recruitment after 6 days of continuous lens wear. Results showed that the proinflammatory cytokine IL-17A was required since lens-wearing IL-17A (−/−) gene knockout mice did not show a Ly6G+ cell response. Mice treated with UC7–13D5 to functionally deplete γδ T cells also failed to show a Ly6G+ cell response to 6 days of lens wear showing that γδ T cells were also required. These findings were supported by data showing a lens-induced increase in IL-17A (gene and protein) expression after 6 days of wear, along with increased IL-18 and IL-23 gene expression, and a corneal γδ T cell response after 1 and 6 days of lens wear. Each of these cytokine and cellular responses to lens wear were abrogated in mice treated with antibody to functionally deplete γδ T cells, correlating with the loss of Ly6G+ cell recruitment after 6 days of wear. Together, these results suggest that the contact lens-induced parainflammation involving Ly6G+ cell recruitment to the cornea after 6 days of wear requires a prior and likely ongoing concurrent γδ T cell corneal response to lens wear, with IL-17A secretion by these cells driving subsequent Ly6G+ cell recruitment.

IL-17 signaling contributes to the recruitment and activation of neutrophils (and other immune cells) during autoimmune diseases, or in response to infection, in multiple tissues including the eye [16,20–25]. The present study adds to our understanding of the inflammatory role of IL-17 signaling by showing its involvement in corneal parainflammation, i.e. a Ly6G+ cellular response to contact lenses after 6 days of wear without overt clinical signs or symptoms. Further work will be needed to identify specific signaling pathways involved and help explain why IL-17A, but not IL-17F, was required for this parainflammatory response since both cytokines are proinflammatory and share many similar functions including neutrophil attraction [20,26]. However, distinct roles for IL-17A and IL-17F have been previously demonstrated. One study showed that IL-17F was not involved in the pathogenesis of several autoimmune diseases or allergic responses that were IL-17A-dependent, yet both cytokines contributed to epithelial defense against bacterial infection [27]. In contrast, studies have shown that IL-17F plays a distinct role in promoting inflammatory disease in the airways and intestinal tract, that is not shared with IL-17A [28,29]. In our study, data were consistent with a role for IL-17A, but not IL-17F, in mediating contact lens-induced corneal parainflammation. Only a small increase in IL-17F protein expression was observed in the cornea after 6 days of lens wear that did not align with gene expression data. Moreover, IL-17F (−/−) mice still showed Ly6G+ cell infiltration. As our understanding of IL-17 signaling events involved in contact lens-induced corneal

parainflammation improves, an explanation for these IL-17F findings may become more apparent. Other work is also needed to delineate the role(s) of cytokines such as IL-18 and IL-23 each of which showed significant upregulation of corneal gene expression in response to 6 days of lens wear, and to establish the relationship between these findings and our previous data showing that IL-1R and MyD88 are also required for Ly6G+ cell infiltration after 5 to 6 days of wear [10]. All of the above factors are part of the classical innate immune response to bacteria which could be derived from the contact lens or ocular surface as discussed below.

Gamma-delta (γδ) T cells are well known for IL-17 production in response to a variety of stimuli [16,20,30,31]. In the present study, the role of γδ T cells in corneal parainflammation was shown using UC7–13D5, a monoclonal antibody that binds to the γδ T cell receptor. A *caveat* to using this antibody, is that it remains unclear whether UC7–13D5 physically depletes γδ T cells or masks their presence while inhibiting their function (i.e. a “functional depletion”) [32], and this antibody can even induce markers of γδ T cell activation [32]. That said, several subsequent studies have utilized this monoclonal antibody *in vivo* to functionally deplete γδ T cells and demonstrate their contribution to immune-mediated responses during allergy or infection [16,18,19]. Thus, while it is possible that UC7–13D5 interfered with our ability to detect γδ T cells, our data showing that UC7–13D5 administration abrogated 6 day lens-induced corneal IL-17A responses (gene and protein expression), along with the Ly6G+ cell infiltrative response, support the conclusion that γδ T cells represent the primary source of IL-17A that drives contact lens-induced Ly6G+ cell corneal parainflammation. This conclusion was also corroborated by our data showing a significant corneal γδ T cell response to lens wear after 1 and 6 days, a response that was also present in lens-wearing IL-17A (−/−) mice (Supplemental Fig. S1), confirming that IL-17A was not required for γδ T cell infiltration. However, other potential cellular sources of IL-17A may also contribute. Previously, we reported that lens-induced corneal parainflammation in this murine model also involves a CD11c+ (dendritic) cell response after 1 day of wear. It is well established that dendritic cells, and other antigen presenting cells, in the cornea and at other sites, function to drive CD4+ T lymphocyte responses through IL-12 (Th1 responses) or IL-23 (Th17 responses) the latter resulting in IL-17 production [31,33–35]. Therefore, it remains possible that the 1 day lens-induced CD11c+ response is also involved in the subsequent 6 day Ly6G+ cell infiltration *via* Th1 or Th17 responses. However, these types of adaptive immune response would be more likely to contribute to chronic parainflammation beyond 6 days of wear.

Our results showed that γδ T cell depletion also abrogated 6 day lens-induced upregulation of corneal IL-18 and IL-23 gene expression suggesting that these parainflammatory responses are also dependent on γδ T cells. It is known that both IL-18 and IL-23 can help activate γδ T cells [21,23,31,36]. Activated γδ T cells can in turn secrete direct antimicrobial factors or activate inflammatory and tissue repair responses via cytokine secretion [37,38]. Future studies that help define the signaling and cellular pathways involved in contact lens-induced Ly6G+ cell corneal parainflammation will also likely shed light on the contribution of γδ T cells to lens-induced IL-18 and IL-23 responses.

Could commensal bacteria play a role in the Ly6G+ cell corneal parainflammation observed in this study *via* activation of γδ T cells and IL-17A production? It was shown previously that a conjunctival *Corynebacterium* sp. helped maintain homeostatic Ly6G+ (neutrophil) cell populations in the conjunctiva that depended on γδ T cell-derived IL-17 [16]. The commensal-driven, γδ T cell-derived IL-17 response also drove neutrophil and other antimicrobial responses against corneal infection [16]. Others have also shown a role for commensal bacteria in supporting effective tumor surveillance in the lung *via* γδ T cells producing IL-17, with antibiotics compromising that immune defense against cancer [39]. Recently, we showed that topical ocular antibiotic treatment reduced baseline levels of corneal CD11c+ cells and blocked the CD11c+ cell parainflammatory response to contact lens wear after 24 h [17]. Those changes correlated in part with changes in viability of conjunctival bacteria suggesting that conjunctival commensals can modulate lens-induced parainflammation. Given the above findings and the ability of γδ T cells respond to microbes and their ligands, it is possible that commensal bacteria (conjunctival, lens associated, or elsewhere) are also involved in initiating and/or maintaining lens-induced Ly6G+ cell corneal parainflammation involving γδ T cells and IL-17A reported here. However, other factors associated with contact lens wear may also contribute including mechanical stimulation, hypoxia or changes to the tear fluid. For example, hypoxia is known to favor the induction of Th17 cells over regulatory T cells [40]. While the mouse lenses used in our model are made of silicone hydrogel material with high oxygen transmissibility, actual oxygen levels at the corneal epithelium in our model are unknown, leaving the potential for hypoxia to contribute to contact lens-induced corneal parainflammation. There may also be a relationship between contact lens-induced parainflammation and fluctuations in tear fluid composition associated with lens wear (tear stagnation) [41,42] and/or the closed eye environment, the latter well known to induce subclinical infiltration of neutrophils and proinflammatory components [43–45].

In our original study of lens-induced corneal parainflammation in this model, lens colonization by conjunctival and skin commensal bacteria was consistently observed over 1–11 days of wear (mostly *Corynebacterium* spp.) [10]. Indeed, in a sub-group of 5 mice each showing Ly6G+ cell corneal infiltration after 5 days or more of wear, *Corynebacterium* spp. were detected on 4 of 5 lenses. A possible ligand for activation of γδ T cells and IL-17 production by *Corynebacterium* spp. is the cell wall glycolipid trehalose dicorynomycolate (TDCM). TDCM can activate murine macrophages [46], and is an analog of Mycobacterial trehalose-6,6-dimcolate (TDM), the latter a potent adjuvant for induction of Th1 and Th17 responses dependent on IL-1R and MyD88 [47]. Contact lens wear associated reductions in tear exchange mentioned above [41,42] could allow TDCM, or other potential microbial triggers, to accumulate between the lens and cornea even if the microbe itself is not adherent to the lens. While these possibilities are intriguing, the relationship between commensal bacteria and corneal parainflammation remains to be determined.

5. Conclusion

This study furthers our understanding of contact lens-induced parainflammation in the murine cornea involving Ly6G+ cell (neutrophil) infiltration after 5–6 days of lens wear by demonstrating the requirement for both IL-17A and γδ T cells. Data suggest that

γδ T cells are the foundation of this corneal para-inflammatory response and associated IL-17 signaling events since they respond to lens wear after 1 and 6 days and their functional depletion abrogates Ly6G+ cell infiltration and IL-17 gene expression and protein expression. The relationship between these events and the presence of commensal bacteria on the conjunctiva or elsewhere remains to be determined. From a broader perspective, it will also be important to determine the extent to which the mechanisms of contact lens-induced corneal parainflammation in mice relate to those occurring during lens-wear in humans. The latter include an increased number of Langerhans cells in the cornea and conjunctiva [4,8,9], and an elevation of multiple proinflammatory cytokines in the tear fluid including IL-17A [48–50]. Increased tear levels of IL-17A have also been associated with contact lens discomfort (CLD) in patients without clinical signs of dry eye [51], and attenuation of CLD associated with a reduction of tear IL-17A (and IL-6) levels via topical corticosteroid application or via topical/oral administration of omega-3 supplements [52]. Lens-induced adverse events are also associated with polymorphisms in genes encoding pro- and anti-inflammatory cytokines, e.g. IL-1β, IL-6, IL-10 [53,54]. Murine immunological models have been of considerable valuable in facilitating our understanding of human immune-mediated diseases in the eye and elsewhere. As such, it is likely that lens-wearing murine models will similarly facilitate our understanding of the significance of corneal parainflammation in the context of adverse events occurring during lens wear in humans: does corneal parainflammation offer protection against an external threat, or provide a foundation for adverse events, or both depending on circumstances?

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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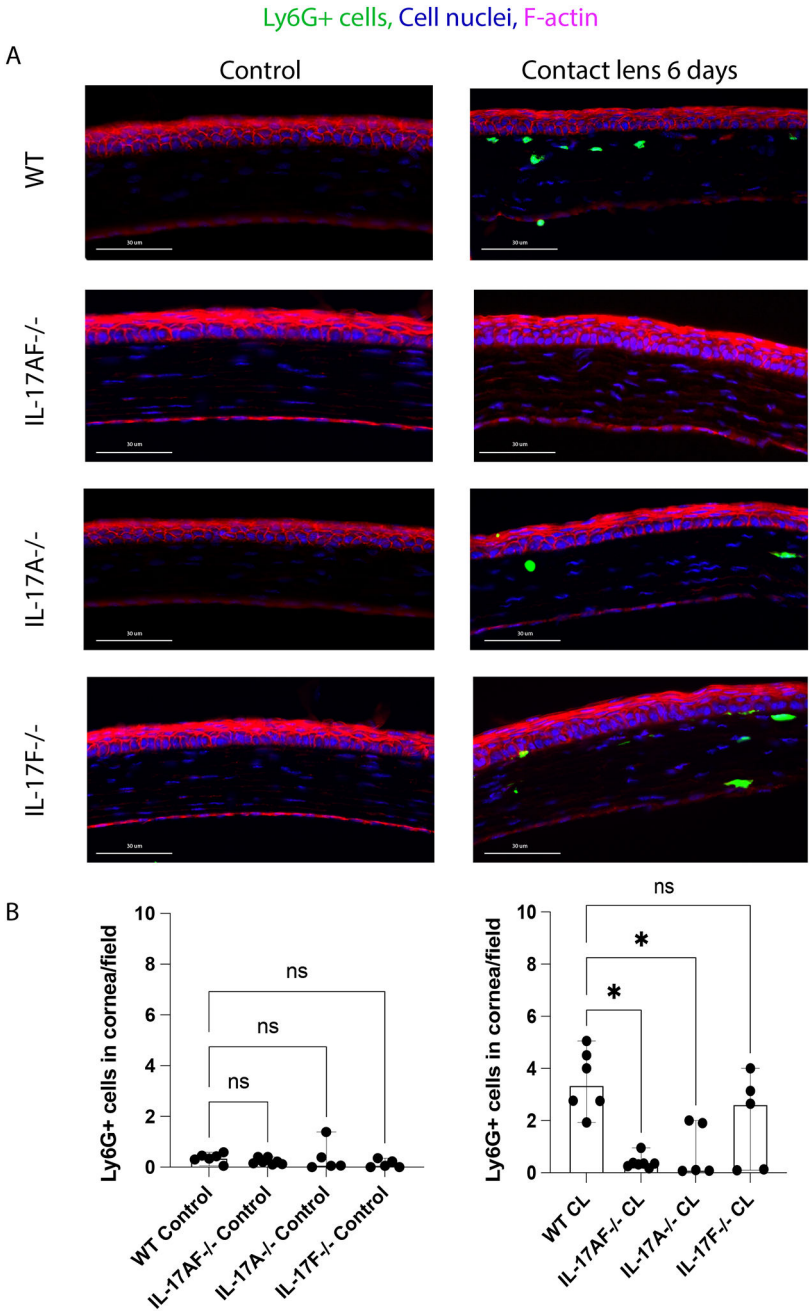

Fig. 1.

(A) Immunofluorescence imaging showing Ly6G+ cell (neutrophil) infiltration of contact lens wearing mouse corneas after 6 days of wear versus contralateral controls. Cryosections of wild-type (WT) corneas were compared with IL-17 gene-knockout mice; IL-17AF (−/−), IL-17A (−/−) and IL-17F (−/−). Ly6G+ cells (green), cell nuclei (blue), cell F-actin (red). Scale bar = 30 μm (B) Quantification of Ly6G+ cells (per field of view) in WT versus IL-17AF (−/−), IL-17A (−/−) and IL-17F (−/−) corneas after 6 days of contact lens-wear (right panel) versus contralateral controls (left panel). * $P < 0.05$, ns = Not Significant (Kruskal-Wallis Test with Dunn's multiple comparisons).

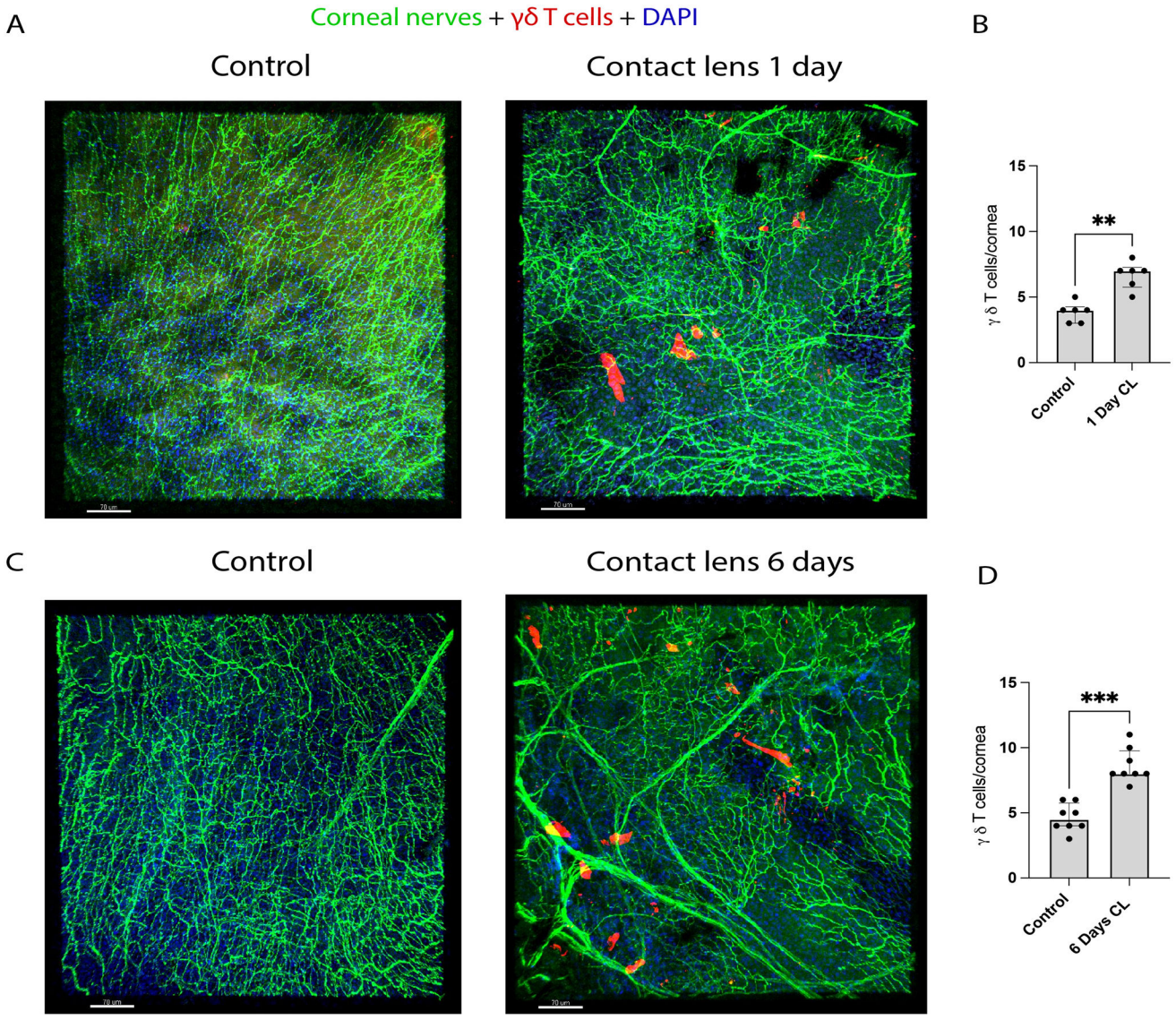

Fig. 2.

Immunofluorescence imaging showing γδ T cell infiltration of the mouse cornea at 1 day (A) and 6 days (C) of contact lens wear (right panels) versus contralateral controls (left panels). γδ T cells (red), cell nuclei (blue), corneal nerves (green), Scale bar = 70 μm. Quantification of γδ T cells in WT corneas at 1 day (B) and 6 days (D) of contact lens wear versus contralateral controls. ** $P < 0.01$, *** $P < 0.001$ (Mann-Whitney U Test).

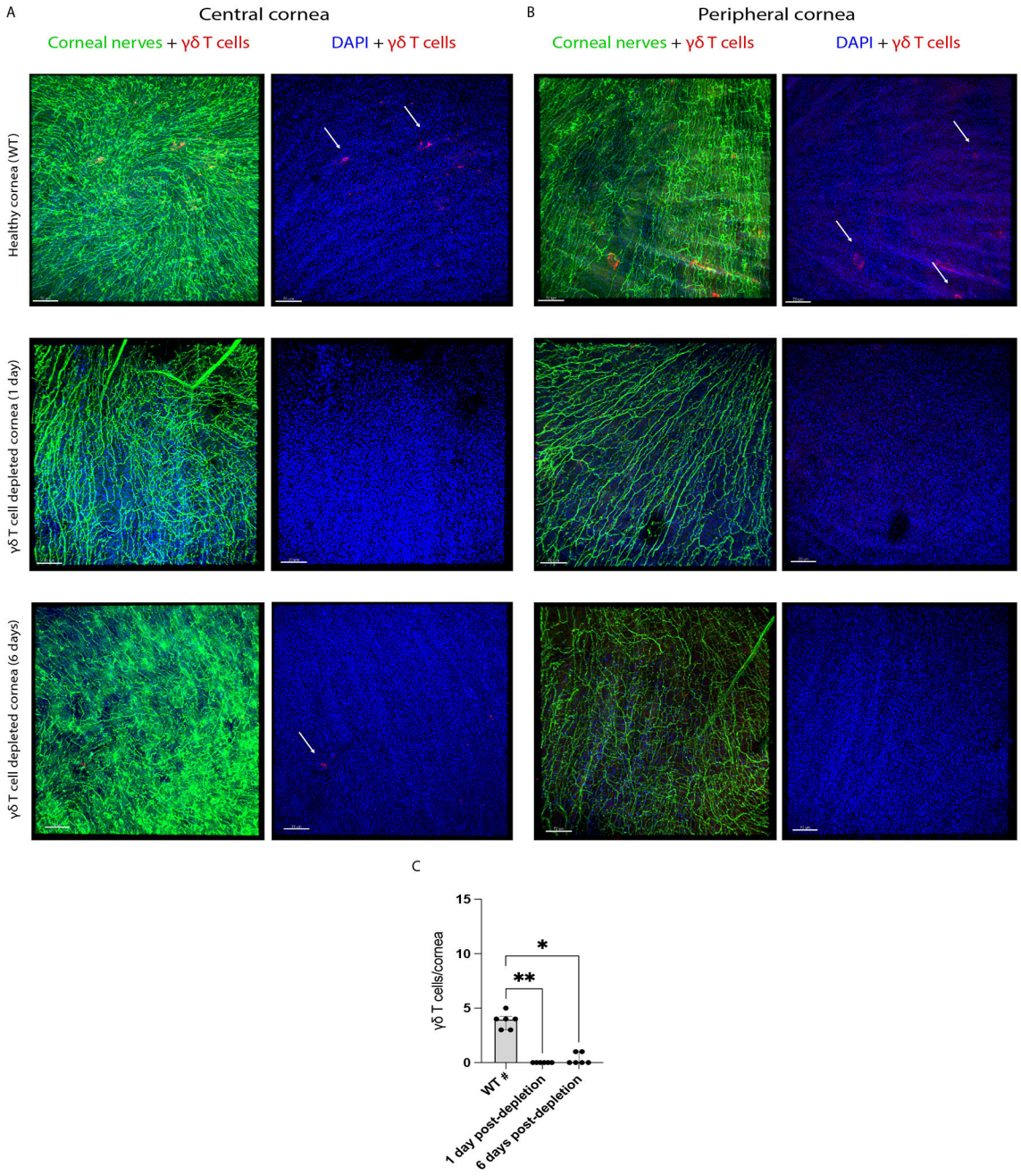

Fig. 3.

Immunofluorescence imaging of γδ T-cells in the central cornea (A) and peripheral cornea (B) at 1 day (middle panels) and 6 days (lower panels) after treatment with UC7–13D5 monoclonal antibody to functionally deplete γδ T cells versus PBS-treated controls (upper panels). γδ T cells (red), cell nuclei (blue), corneal nerves (green), Scale bar = 70 μm. (C) Quantification of total γδ T cells in the corneas under the above conditions. # Data shown from 1 and 6 day PBS-treated controls. * $P < 0.05$, ** $P < 0.01$ (Kruskal-Wallis Test with Dunn's multiple comparisons).

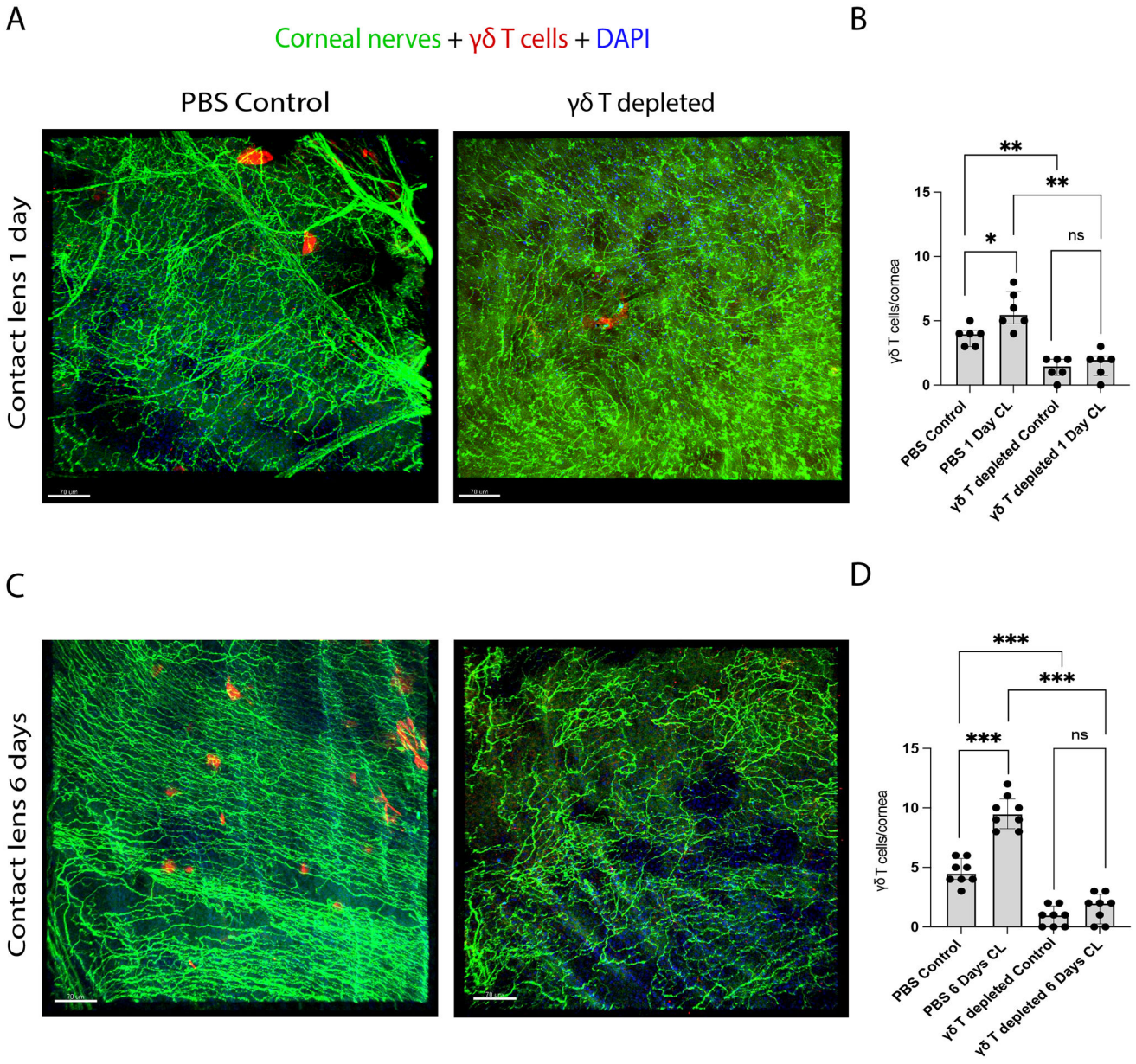

Fig. 4.

Immunofluorescence imaging showing γδ T cells in the contact lens wearing corneas of γδ T cell-depleted mice versus PBS-treated controls after (A) 1 day and (C) 6 days of lens wear. γδ T cell depletion was performed 1 day prior to lens wear. Lens-wearing corneas of γδ T cell depleted mice (right panels) showed few or no γδ T cells at both time points versus PBS-treated controls. (B, D) Quantification of γδ T cells after 1 and 6 days of lens wear respectively under the above conditions. Lens-induced γδ T cell responses observed after 1 and 6 days of wear in the PBS-treated control group were absent in the γδ T cell depleted group. γδ T cells (red), cell nuclei (blue), corneal nerves (green), Scale bar = 70 μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = Not Significant (Mann-Whitney U Test).

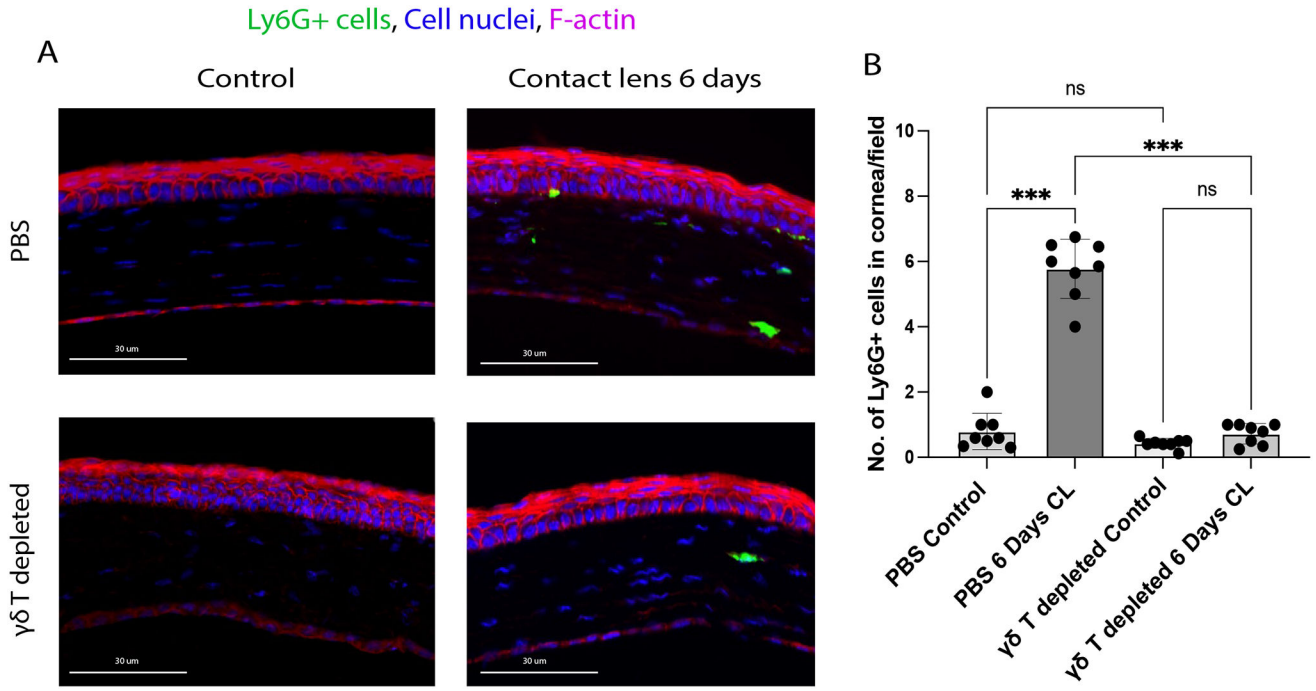

Fig. 5.

Immunofluorescence imaging (A) and quantification (B) of Ly6G+ cell infiltration of the corneas of contact lens wearing mice after 6 days of wear versus contralateral controls (without lens wear) for γδ T cell depleted mice versus PBS-treated controls. Lens-wearing corneas of γδ T cell depleted mice showed little or no Ly6G+ infiltration, a significant reduction from the 6 day response to lens wear observed in mice treated with PBS only. Ly6G+ cells (green), cell nuclei (blue), cell F-actin (red). Scale bar = 30 μm. *** $P < 0.001$, ns = Not Significant (Mann-Whitney U Test).

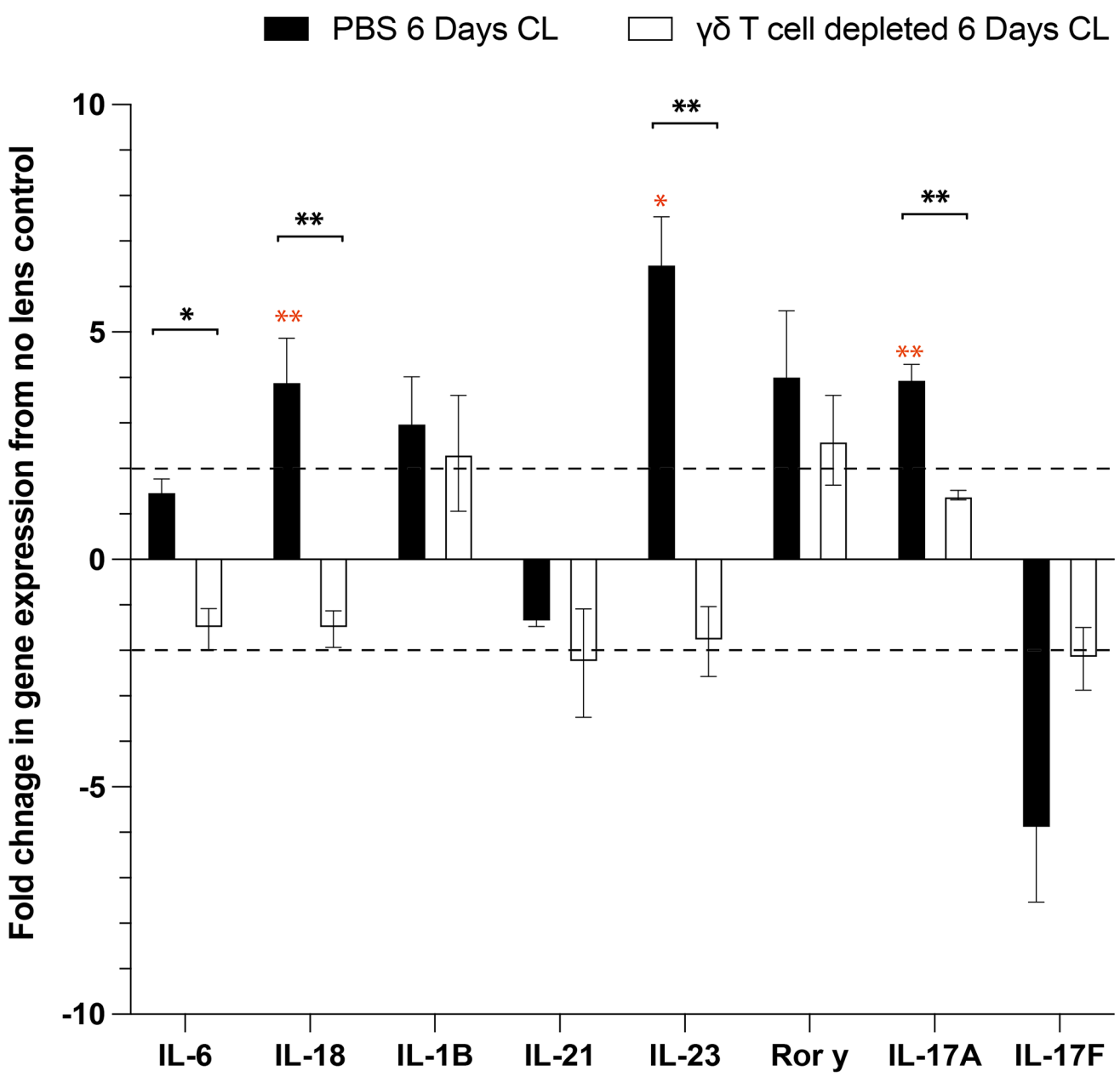

Fig. 6.

qPCR analysis of cytokine gene expression in the mouse cornea after 6 days of contact lens wear in γδ T cell depleted mice versus PBS-treated controls. Data expressed as the fold-change in the lens wearing cornea relative to the contralateral control (i.e. normalized to zero) with the dashed lines indicating a 2-fold change. * $P < 0.05$, ** $P < 0.01$ (Students t-Test). Lens-wearing PBS-treated control mice (black bars) were also compared to lens wearing γδ T cell depleted mice (white bars). * $P < 0.05$, ** $P < 0.01$ (Student's t-Test). ns = Not Significant.

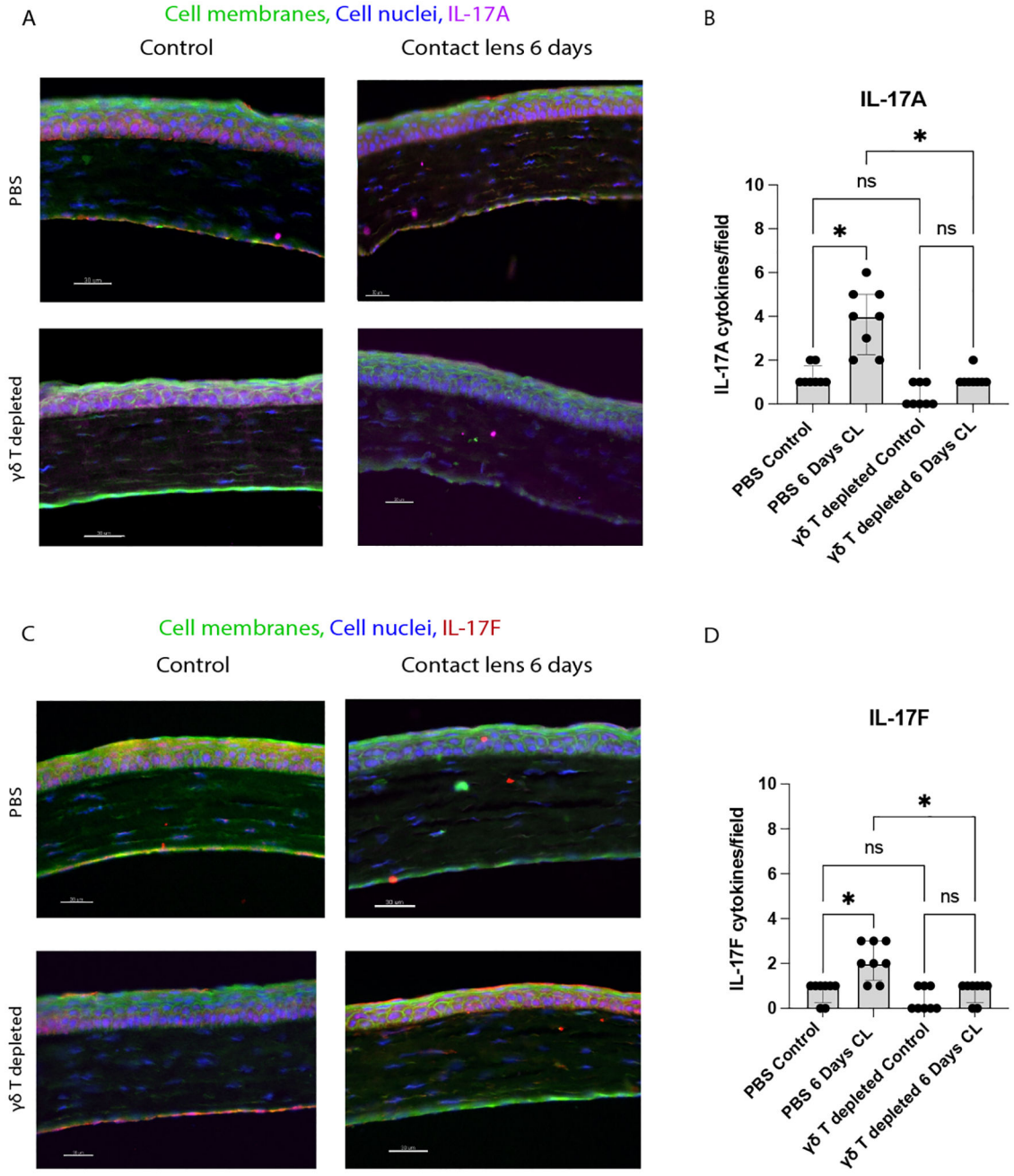

Fig. 7.

Immunofluorescence imaging of corneal cryosections with fluorescence quantification for the expression of IL-17A protein (A, B) and IL-17F protein (C, D) in contact lens wearing versus contralateral control corneas of γδ T cell depleted mice versus those treated with PBS only after 6 days of wear. γδ T cell depletion was performed 1 day prior to lens wear. Lens wear for 6 days caused a significant increase in both IL-17A and IL-17 F in the corneas of PBS-treated mice, but these responses were lost in γδ T cell depleted mice. IL-17A or

IL-17 (red), cell nuclei (blue), F-actin (green). Scale bar = 30 μm. * $P < 0.05$, ns = Not Significant (Mann-Whitney U Test).