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Persistence of Contact Lens-Induced Corneal Parainflammation Following Lens Removal

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PURPOSE. Contact lens wear induces corneal parainflammation involving increased immune cell numbers after 24 hours' (CD11c+, Lyz2+, $\gamma\delta$ -T cells) and six days' (Ly6G+ cells) wear. We investigated the time course of onset and resolution of these responses.

METHODS. LysMcre or C57BL/6J mice were fitted with a contact lens (four to 48 hours). Contralateral eyes did not wear lenses. After lens removal, Lyz2+, MHC-II+ or Ly6G+ cells were examined by quantitative imaging. RT-qPCR determined cytokine gene expression.

RESULTS. Lens wear for 24 hours increased corneal Lyz2+ cells versus contralateral eyes approximately two-fold. Corneas remained free of visible pathology. The Lyz2+ response was not observed after four or 12 hours' wear, nor after 12 hours' wear plus 12 hours' no wear. Lens removal after 24 hours' wear further increased Lyz2+ cells (~48% after one day), which persisted for four days, returning to baseline by seven days. Lyz2+ cells in contralateral eyes remained at baseline. MHC-II+ cells showed a similar response but without increasing after lens removal. Lens wear for 48 hours showed reduced Lyz2+ cells versus 24 hours' wear with one day discontinuation, correlating with reduced IL-1 β and IL-18 gene expression. Lens wear for 24 hours did not induce Ly6G+ responses six days after removal.

CONCLUSIONS. Lens-induced corneal parainflammation involving Lyz2+ cells requires 24 hours' wear but persists after lens discontinuation, requiring seven days for reversal. Lens wear for 48 hours may suppress initial Lyz2+ cell and cytokine responses. The significance of parainflammation during and after lens wear remains to be determined.

Keywords: contact lenses, corneal epithelium, parainflammation, Lyz2+ immune cells, cytokines

Contact lens wear is a successful and generally well-tolerated form of vision correction. However, contact lenses can cause ocular complications, the most severe being sight-threatening corneal infection.¹⁻³ Despite extensive investigation, the mechanism(s) by which lens wear predisposes to corneal infection and other adverse events remain unclear.

In recent years, it was proposed that contact lens wear is "intrinsically inflammatory"⁴ with subclinical "parainflammatory" events occurring during human lens wear that include increased corneal Langerhans cell density, which gradually decreases after six hours of lens discontinuation,^{5,6} and increased tear fluid levels of proinflammatory cytokines (e.g., IL-1 β , TNF α , IL-17).⁷

To further understand contact lens-induced effects on the cornea we developed a murine model of lens wear,⁸ which not only demonstrated lens-associated corneal infections from one to 13 days after *Pseudomonas aeruginosa* inoculation, but lens-induced corneal parainflamma-

tion involving a significant increase in multiple types of immune cells without clinical signs or symptoms. Those parainflammatory responses occurred after 24 hours wear (CD11c+ cells, Lyz2+ cells) and five or six days of wear (Ly6G+ cells). Subsequent studies using this model have shown that topical antibiotics suppress 24-hour lens-induced CD11c+ responses, suggesting involvement of commensal bacteria,⁹ and that six-day Ly6G+ cell responses require $\gamma\delta$ -T cells (responding after 24 hours, remain present after six days) and IL-17.¹⁰ Most recently, we demonstrated that lens-induced parainflammation of the cornea after 24 hours (e.g., MHC Class-II+ cell responses) and six days of wear (Ly6G+ cell responses) requires transient receptor potential (TRP) ion channels with both TRPA1 and TRPV1, expressed primarily on corneal sensory nerves, being involved.¹¹

Parainflammation represents a low-grade inflammatory response that is intended to preserve or restore normal tissue function and promote adaptation to adverse conditions.¹² It can be induced by tissue stress or malfunction

and can lead to the development and progression of multiple disease conditions that include atherosclerosis, cancer, macular degeneration, type 2 diabetes, and several neurodegenerative diseases.^{13–15} The short- and long-term effects of corneal parainflammation remain unknown with potential for beneficial or harmful effects. Thus, in the present study, we investigated the time course of the onset and reversal of lens-induced corneal parainflammation in the murine model.

MATERIAL AND METHODS

Murine Contact Lens Wear Model

All procedures involving animals 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).

Six- to eight-week-old male and female LysMcre transgenic mice (Lyz2+GFP cells, myeloid-derived)^{3,6} were used along with C57BL/6J mice. Custom-made silicone-hydrogel mouse contact lenses were fitted onto the right eye of each mouse. Contralateral eyes were not fitted with contact lenses. Previously, using this model, we showed that contralateral corneas were morphologically indistinguishable from naïve mice.⁸ Before fitting, lenses were soaked in PBS for one hour to eliminate potential effects of packaging solution. Mice were anesthetized with 1.5% to 2% isoflurane delivered via precision vaporizer (VetEquip Inc., Pleasanton, CA, USA) and fitted with an Elizabethan collar (Kent Scientific Corporation, Torrington, CT, USA). Contact lenses were fitted using a Handi-Vac suction pen (Edmund Optics, Barrington, NJ, USA) and mice single-housed without enrichments to prevent lens removal. Pure-o'Cel paper bedding (The Andersons Inc., Maumee, OH, USA) was used to reduce dust.

Contact lenses were worn for various times from four to 48 hours. After assigned times of lens wear, lenses were removed and at various times after discontinuing lens wear (12 hours, one, four, or seven days), mice were euthanized, eyes enucleated and corneas subject to quantitative imaging *ex vivo*. For overnight lens wear and further monitoring mice were returned to the animal care facility. Mice were monitored daily for lens retention. After lens removal, mice were observed daily for evidence of pathology (e.g., discharge or corneal opacity). Any mouse that lost its contact lens was excluded from further experimentation, as was any mouse presenting with excessive weight loss or distress. Lenses were gently removed from anesthetized mice under a stereomicroscope (Stemi 2000-C; Zeiss, Oberkochen, Germany) using sterile forceps. All euthanasia was performed by isoflurane inhalation (5%) for 10 minutes followed by cervical dislocation.

Preparation of Eyes for Imaging

Enucleated eyes of LysMcre mice (Lyz2+GFP cells) were fixed in 2% paraformaldehyde (PFA) overnight, followed by PBS washing for two to three hours at room temper-

ature with rotation. Whole-mount confocal imaging was performed as previously described.⁸

For immunolabeling of corneas, enucleated eyes were fixed in 2% PFA for one hour, washed in PBS for 10 minutes with rotation, and corneal dissection performed over ice using a dissecting microscope. Dissected corneas were washed once in PBS for 10 minutes with rotation and then incubated in blocking buffer (3% BSA with 0.3% Triton X-100 in PBS) for one hour at room temperature with rotation. Corneas were then incubated for one hour in blocking buffer containing EDTA (20 mM) at 37°C with rotation. Primary antibodies used were as follows: Rat anti-mouse MHC Class-II (no. 556999; BD Bioscience, Franklin Lakes, NJ, USA) and Rabbit anti-mouse β -Tubulin III (no. T2200; Sigma-Aldrich Corp., St. Louis, MO, USA) each diluted 1:500 in blocking buffer. Dissected corneas were incubated in primary antibody solutions overnight at 4°C with rotation. Corneas were then washed in PBS for 10 minutes with rotation then incubated with secondary antibody for two hours at room temperature with rotation (covered with aluminum foil). Secondary antibodies used were: Goat anti-Rat or Goat anti-Rabbit (no. A21434; Life Technologies, Carlsbad, CA, USA) diluted 1:800 in blocking buffer. DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (12.5 μ g/mL) (no. D1306; ThermoFisher, St. Louis, MO, USA) was included in secondary antibody buffer solution to label cell nuclei. Corneas were transferred to fresh PBS, washed three times for 10 minutes each with rotation, and flat-mounted with Prolong Diamond (no. P36970; ThermoFisher) to enhance visibility for imaging.

Ly6G+ cells were imaged and quantified in corneal cryosections as previously described.⁸ Enucleated eyes were transferred to 2% PFA for overnight fixation at 4°C, followed by overnight washing in PBS at 4°C to remove excess PFA. Eyes were then cryoprotected by immersion in sucrose (15% for four hours, then 30% for an additional four hours) at room temperature, followed by embedding in Tissue Tek O.C.T. medium (Sakura Finetek USA, Inc., Torrance, CA, USA), flash frozen in liquid nitrogen, and stored at -80°C until sectioned. Embedded eyes were sectioned at 10 μ m thickness using a Leica CM 1900 cryostat (Leica, Wetzlar, Germany), placed on a glass slide, and stored at -80°C , until antibody labeling. To label Ly6G+ cells, corneal sections were washed with PBS for five minutes at room temperature, followed by blocking with 3% BSA for 30 minutes at room temperature in a moist Petri dish. After blocking, sliced corneas were incubated with primary antibody: Rat NIMP-R14 antibody (10 μ g/mL, no. 1676494; ThermoFisher) at room temperature for one hour, followed by three rinses with PBS, and then incubated for one hour in Alexa 647-conjugated Goat anti-Rat antibody (5 μ g/mL, Life Technologies) as secondary antibody. Sections were counterstained with DAPI (12.5 μ g/mL) to visualize cell nuclei and ActiGreen (Phalloidin, 1:10; ThermoFisher) for cytoskeleton (F-actin) labeling. Sections were rinsed three times with PBS and mounted on a coverslip with Prolong Diamond as described above. Sections were allowed to dry for at least 30 minutes in the dark at room temperature before imaging.

Imaging and Image Analysis

Confocal imaging was performed using a 20x/1.00 NA water-dipping objective and an upright Olympus Fluoview FV1000 microscope (Olympus, Tokyo, Japan). Whole-mounted eyes

TABLE. Primers Used in This Study

Gene	Source	Forward (5'-3')	Reverse (5'-3')
IL-18	NCBI	GACAGCCTGTGTTCCGAGGATATG	TGTTCTTACAGGAGAGGGTAGAC
TNF- α	PB	AGG GAT GAG AAG TTC CCA AAT G	CAC TTG GTG GTT TGC TAC GAC
IL-1 β	NCBI	CAA CCA ACA AGT GAT ATT CTC CAT G	AT CGCA CAC TCT CCA GCT GCA
IL-17A	NCBI	CAGACTACCTCAACCGTTCCAC	TCCAGCTTTCCTCCGCATTGA
IL-17F	NCBI	AACCAGGGCATTCTGTCCAC	GGCATTGATGCAGCCTGAGTGT
IL-23	NCBI	CATGCTAGCCTGGAACGCACAT	ACTGGCTGTGTCCCTTGAGTCC
GAPDH	NCBI	TGC GAC TTC AAC AGC AAC TC	GCC TCT CTT GCT CAG TGT CC

NCBI, NCBI Primer-BLAST; PB, PrimerBank.

were imaged using a 488 nm laser for detection of Lyz2+GFP cells and a 559 nm laser for detecting red fluorescent cell membranes. Z stacks (0.5 or 1 μ m steps) were collected from four or more random fields per sample. Flat-mounted corneas were imaged using the 559 nm (red, MHC Class II+ cells) and 488 nm (green, corneal nerves) lasers. For Z stacks at 0.54 μ m steps, images were collected from four or more random fields per sample. Corneal nerve labeling helped locate the central cornea and four peripheral corneal images that were collected for each sample. Three- and four dimensional D image reconstructions, cell quantification, cell morphology analysis, and movie generation were performed using Image-J (MorpholibJ tools collection) and Imaris (Bitplane AG, Zurich, Switzerland). Protocols were similar to those described previously⁸ and maximum intensity projections (i.e., reducing a three-dimensional image into two-dimensions by projecting the maximum intensity of each pixel in a specific channel to the z plane, where indicated to visualize MHC Class-II+ cells and cell morphology). Imaging of corneal sections for Ly6G+ cells was accomplished using a Nikon Ti-E (Nikon Inc., Melville, NY, USA) inverted wide-field fluorescence microscope equipped with a SpectraX illumination source (Lumencor, Beaverton, OR, USA), and CFI Plan APO VC 20x/0.75 NA objective. Quantification was performed using Image-J (MorpholibJ tools) based on a maximum intensity projection of the Ly6G+ cells for a minimum of five to six fields per sample and three samples per condition and confirmed by manual counts.

RT-qPCR

Corneas were carefully dissected from enucleated eyes to remove all limbal tissue, flash-frozen with liquid nitrogen, and stored at -80°C until sample preparation. To minimize enzymatic reactions, corneal dissection was performed using 2% dithiothreitol. Two to three corneas from the same condition were pooled for RNA extraction and 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 Life Science, Hercules, CA, USA) and qPCR using Faststart Sybergreen (Roche, Basel, Switzerland) running on a Light Cycler 96 real-time PCR machine (Roche). Primer pairs used are shown in the Table. Primers were sourced from PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>) or were custom-designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primers were designed to be separated by at least one

intron to ensure selective amplification of cDNA and tested for efficiency (≥ 1.90) and specificity under conditions used.

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, USA). The distribution of data was assessed by the normality test (Shapiro-Wilk test and Kolmogorov-Smirnov test), and because most data was normally distributed, it was expressed as the mean with standard deviation. One-way or two-way ANOVA was used with Tukey's multiple comparisons test for post-hoc analysis. *P* values < 0.05 were considered significant.

RESULTS

Lens-Induced Lyz2+ Cell Responses in the Murine Cornea Require 24 Hours of Continuous Wear

Previously we showed that contact lens wear increased Lyz2+ cell numbers in the murine cornea after 24 hours.⁸ The time course for onset of that response was explored. A contact lens was fitted on one eye of a LysMcre transgenic mouse for four, 12, or 24 hours with contralateral eyes not fitted with a lens. Quantification of corneal Lyz2+ cells (Fig. 1) showed that the contact lens-induced Lyz2+ response only occurred after 24 hours of lens wear (approximately twofold increase, *P* < 0.0001). The observation that corneas wearing lenses for 12 hours followed by 12 hours without wear showed no Lyz2+ cell response confirmed that 24 hours of continuous lens wear was required.

Lens Removal Further Increased Corneal Lyz2+ Cells Returning to Baseline After Seven Days

We next investigated the time course for reversal of corneal Lyz2+ cell responses in LysMcre mice after 24 hours of wear. Figure 2 shows that removal of the contact lens was associated with a further increase in corneal Lyz2+ cells at one day after lens discontinuation ($\sim 48\%$). This elevated level of Lyz2+ cells remained at four days after lens discontinuation (*P* < 0.0001 vs. no lens wear for one and four days after lens discontinuation), only returning to baseline after seven days of lens discontinuation. Lyz2+ cell numbers in non-lens-wearing contralateral corneas did not change from baseline levels shown after 24 hours in Figure 2 (see Supplemental Fig. S1A). Thus the corneal parainflammatory Lyz2+

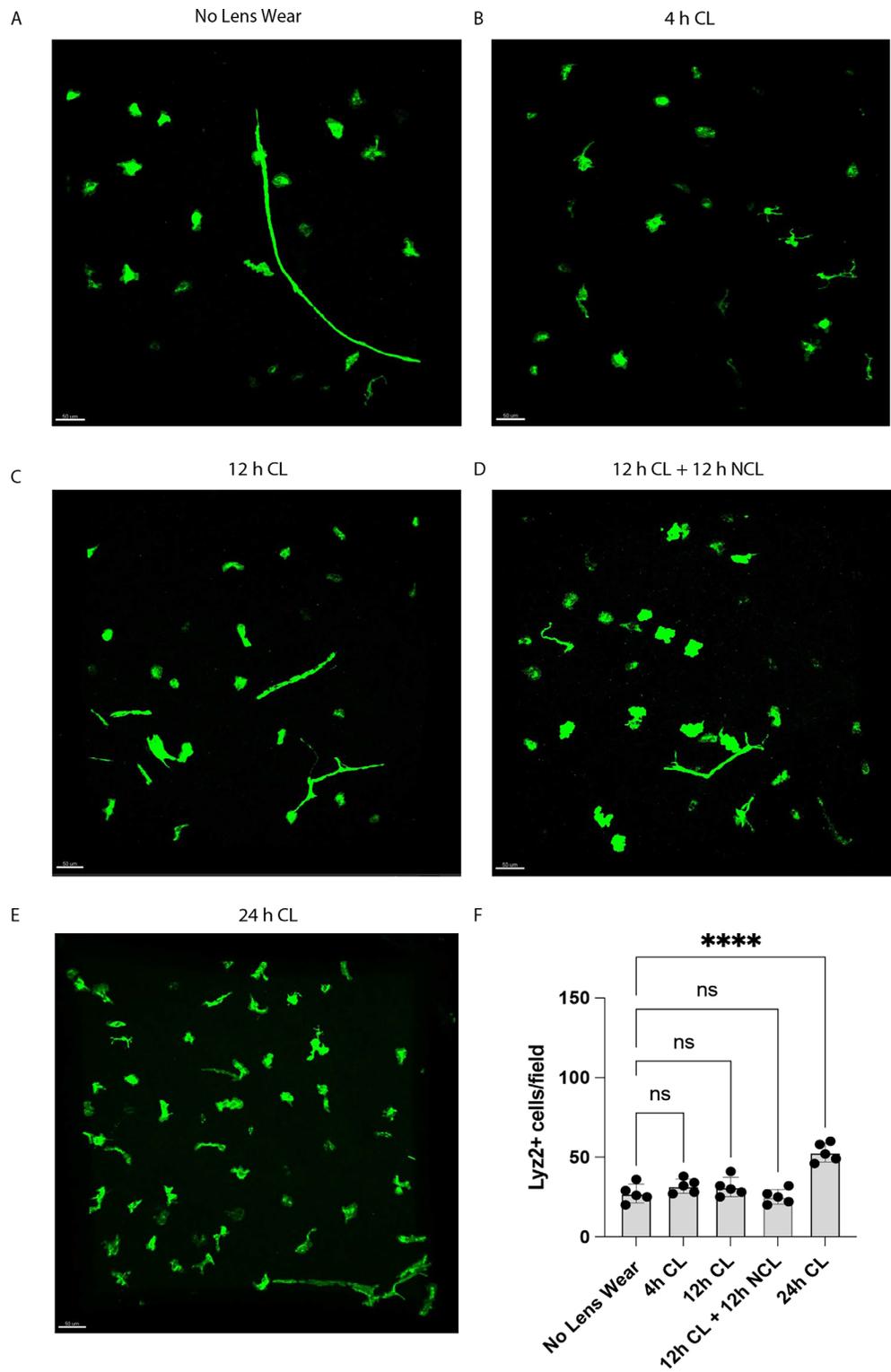


FIGURE 1. Onset of Lyz2+ cell responses in corneas of contact lens wearing LysMcre mice. (A–E) Maximum intensity z-projections of the GFP signal from Lyz2+ cells (*green*) in the central and peripheral cornea of healthy mouse eyes after lens wear for four, 12, or 24 hours compared to a no lens wear contralateral cornea (24-hour timepoint shown) or 12 hours' wear + 12 hours of discontinued wear. *Scale bar*: 50 μ m. (F) Quantification of Lyz2+ cells in contact lens wearing corneas under the above conditions. A significant increase in Lyz2+ cells was only observed after 24 hours of continuous wear. **** $P < 0.0001$; ns = not significant (one-way ANOVA with Tukey's multiple comparisons test).

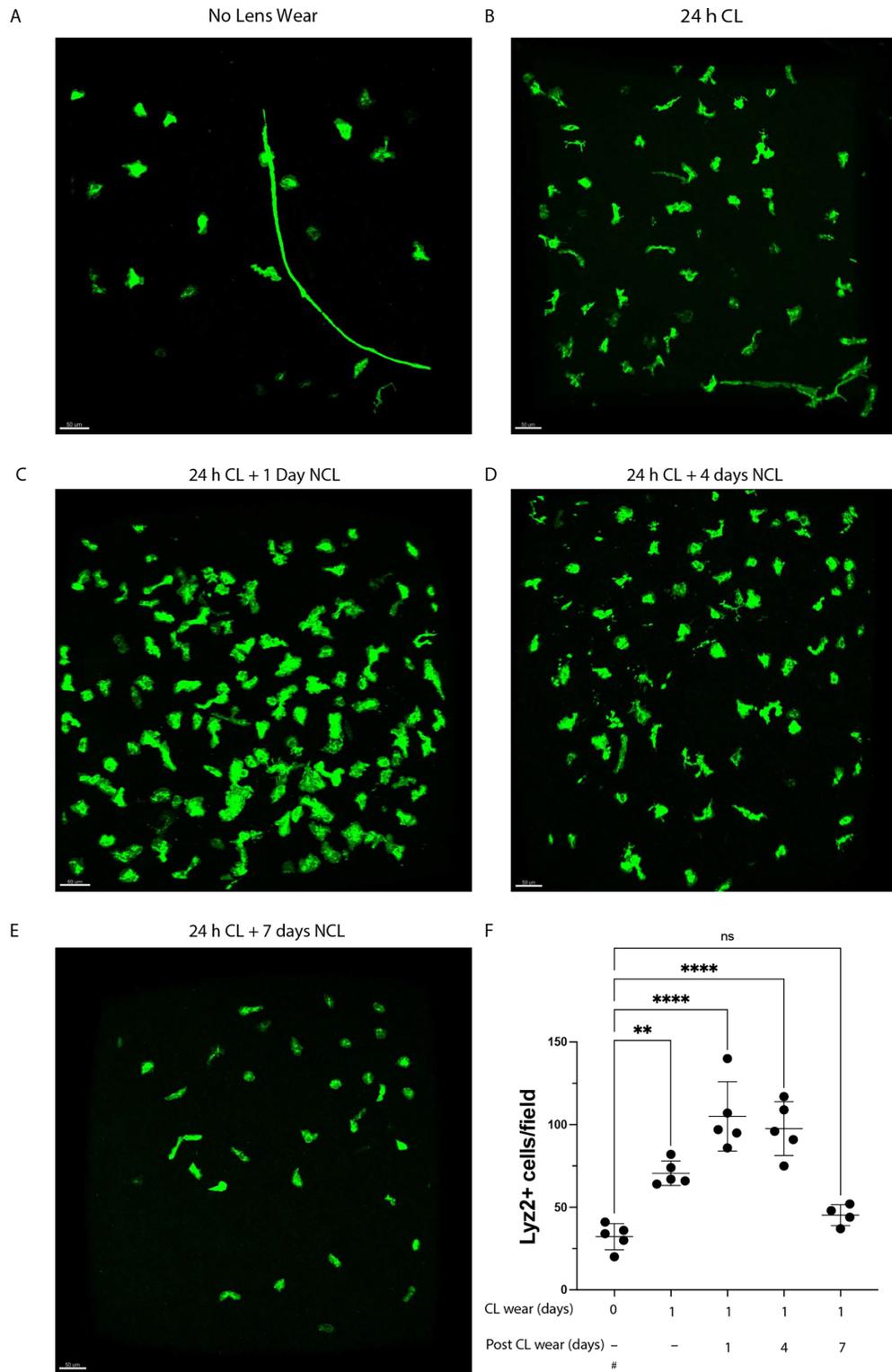


FIGURE 2. Persistence of lens-induced corneal Lyz2+ cell responses after lens removal. Maximum intensity z-projections of the GFP signal from Lyz2+ cells (green) in the central and peripheral cornea of healthy mouse eyes in (A) non-lens-wearing contralateral eye (24-hour timepoint shown), (B) after 24 hours lens wear, (C) 24 hours' lens wear plus one day of discontinued wear, (D) 24 hours' wear plus four days of discontinued wear, and (E) 24 hours' wear plus seven days of discontinued wear. Scale bar: 50 μ m. (F) Quantification of Lyz2+ cells in contact lens wearing corneas under the above conditions. Corneal Lyz2+ cell numbers remained significantly elevated at one and four days after lens discontinuation returning to baseline by seven days. ** $P < 0.01$; **** $P < 0.0001$; ns = not significant (one-way ANOVA with Tukey's multiple comparisons test). # This column shows Lyz2+ cell numbers in non-lens-wearing contralateral corneas (after 24 hours) versus 24 hours' lens wear (column 2). Contralateral cornea Lyz2+ cell numbers did not change at one, four, or seven days after lens discontinuation (see Supplemental Fig. S1A).

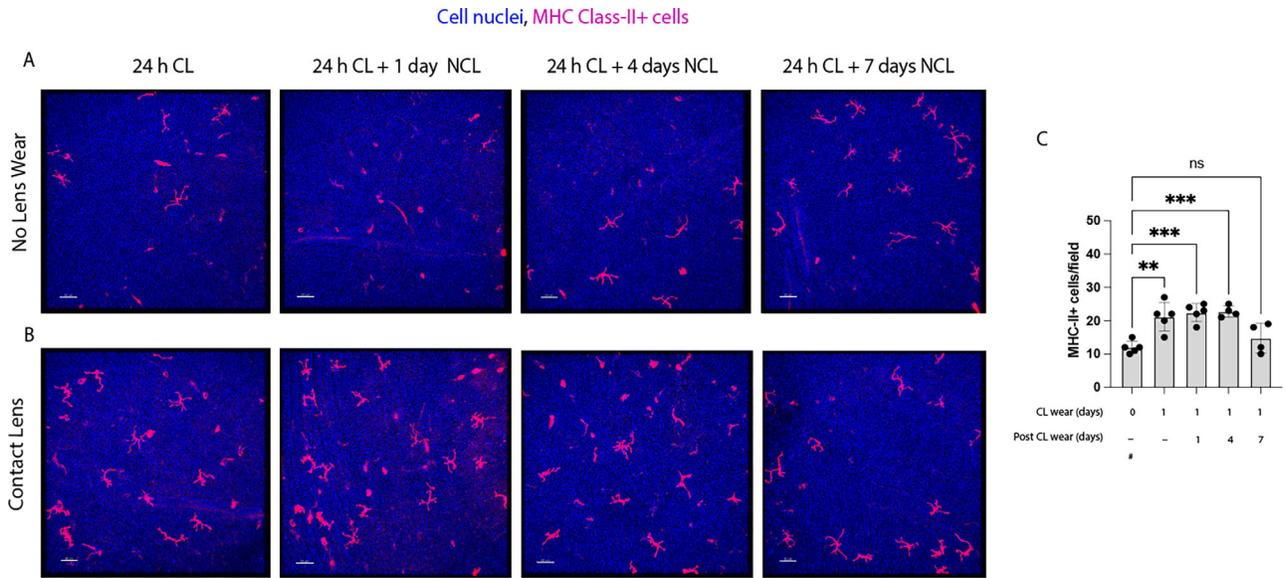


FIGURE 3. Immunohistochemistry of MHC-II+ cells in corneas of lens-wearing C57BL/6J mice. (A) No lens wear contralateral corneas (B) Contact lens wearing corneas. MHC-II+ cells (purple), cell nuclei (blue). Scale bar: 50 μ m. Increased numbers of MHC-II+ cells after 24 hours' wear persisted after lens removal at one day and four days after discontinuation returning to baseline by seven days. (C) Quantification of MHC-II+ cells under the above conditions. ** $P < 0.01$; *** $P < 0.001$; ns = not significant (one-way ANOVA with Tukey's multiple comparisons test). # This column shows MHC-II+ cell numbers in non-lens wearing contralateral corneas (after 24 hours) versus 24 hours' lens wear (column 2). Contralateral cornea MHC-II+ cell numbers did not change at one, four, or seven days after lens discontinuation (see Supplemental Fig. S1B).

cell response induced by 24 hours of lens wear can persist and amplify after lens discontinuation.

Corneal MHC Class-II+ Cells Induced by Lens Wear Also Persist After Lens Removal

Lens wear for 24 hours can also induce MHC Class-II+ cell responses in the murine cornea.¹¹ Thus we next explored whether these cells also persisted after discontinuation of lens wear. Figure 3 shows that 24 hours of lens wear increased MHC Class-II+ cell numbers in the corneas of wild-type mice as expected ($P < 0.01$). This response also persisted at one and four days ($P < 0.001$) after lens removal returning to baseline after seven days. Although the corneal MHC Class-II+ cell response to lens wear and discontinuation was similar to the Lyz2+ cell response in timing, numbers of corneal MHC Class-II+ cells were lower, and this response did not amplify at one day after lens removal. As observed for Lyz2+ cells, numbers of MHC Class-II+ cells in contralateral non-lens-wearing corneas did not change from the levels shown after 24 hours in Figure 3 (see Supplemental Fig. S1B). A similar pattern of results was found when this experiment (MHC Class-II+ cell analysis) was performed in LysMcre (Lyz2+cell-GFP) mice (data not shown).

Increased Circularity of Lyz2+ Cells After 24 Hours' Lens Wear Persists After Lens Removal

Shape analysis of corneal Lyz2+ cells was performed under the same experimental conditions shown in Figure 2. Increased Lyz2+ cell circularity was observed after 24 hours of lens wear consistent with our previous findings for Lyz2+ cells,⁸ and MHC Class-II+ or $\gamma\delta$ -T cells.¹¹ This increase in cell

Overall circularity Lyz2+ cells

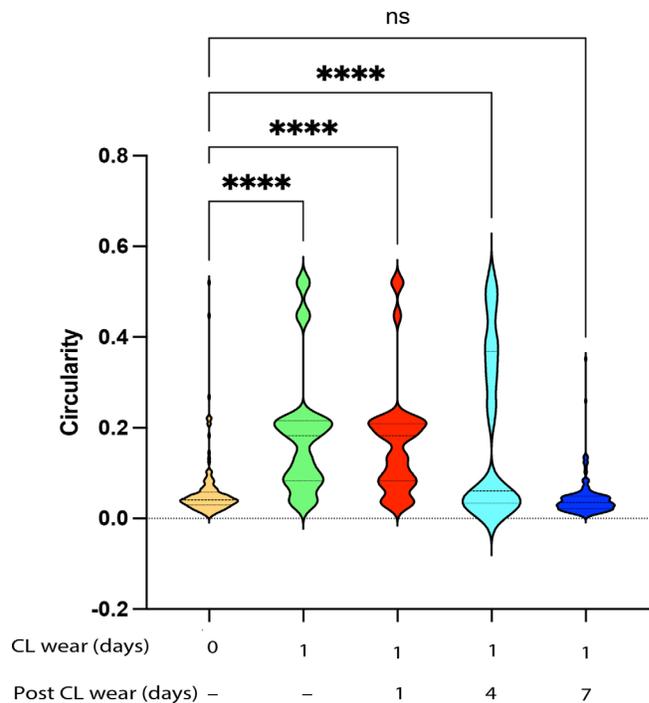


FIGURE 4. Quantification of overall Lyz2+ cell circularity per field of view in contact lens-wearing corneas after 24 hours wear versus contralateral eyes (24-hour timepoint shown) and at various timepoints after lens removal (one, four, or seven days). Increased circularity after 24 hours' wear persisted at 24 hours after lens removal, reverting toward baseline at day 4 and returning to baseline by day 7. **** $P < 0.0001$; ns = not significant (one-way ANOVA with Tukey's multiple comparisons test).

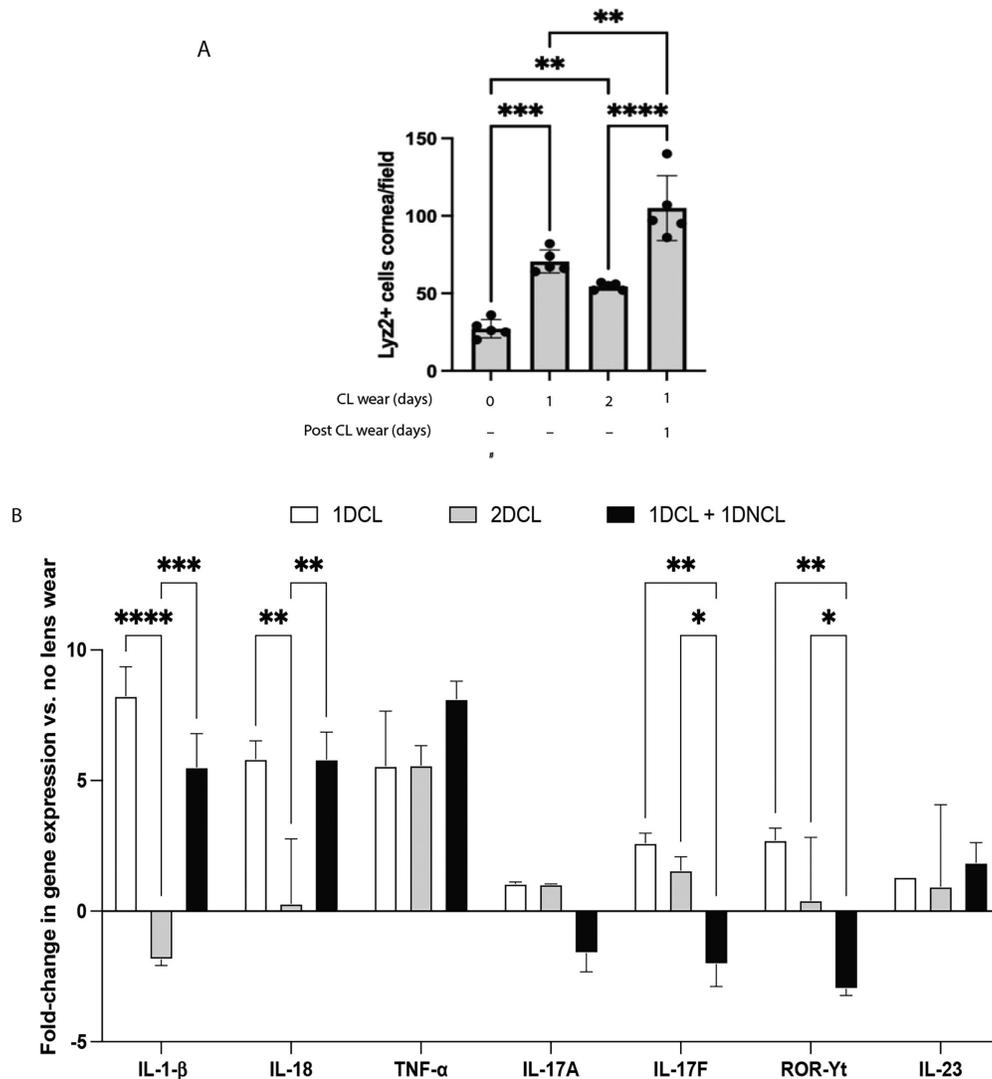


FIGURE 5. (A) Quantification of corneal Lyz2+ cell responses to 48 h of continuous lens wear vs. 24 hours of wear plus 24 hours discontinued wear. Lens wear for 48 h did not generate the same Lyz2+ cell response observed after 24 hours wear plus 24 hours discontinued wear. $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$ (one-way ANOVA with Tukey's multiple comparison test). # Lyz2+ cell numbers in contralateral corneas remained similar to baseline (24-hour timepoint shown) under each condition. (B) qPCR analysis of corneal cytokine gene expression under the same conditions as panel A. Data expressed as fold-change in lens-wearing corneas relative to their respective contralateral eyes. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$ (two-way ANOVA with Tukey's multiple comparison test).

circularity persisted after one and four days of lens wear discontinuation ($P < 0.0001$), returning to baseline after seven days (Fig. 4). Supplemental Figure S2 shows further analysis of changes in Lyz2+ cell numbers within three arbitrarily defined morphological subgroups during the course of the same experiment. The results demonstrate increased numbers of more circular Lyz2+ cells over the course of the experiment along with the appearance of more dendriform cells at four and seven days after lens removal.

Continuous Lens Wear for 48 Hours Does Not Reproduce the Lyz2+ Cell Response That Follows 24 Hours of Wear with 24 Hours of Lens Wear Discontinuation

We next explored whether continuous lens wear for 48 hours would reproduce the same level of Lyz2+ cell response

observed in the cornea by 24 hours of lens wear followed by 24 hours of discontinued wear (see Fig. 2). A similar response under both conditions would eliminate potential physical effects of lens removal as being involved in amplification and persistence of Lyz2+ cells in the cornea after 24 hours of wear. Figure 5A shows, however, that lens wear for 48 hours did not reproduce the same level of Lyz2+ cell response in the cornea that followed 24 hours of wear followed by lens removal for 24 hours. A comparison of corneal cytokine gene expression under the same experimental conditions revealed that 48 hours of continuous lens wear was associated with loss of IL-1β and IL-18 expression relative to 24 hours of lens wear or 24 hours of wear followed by 24 hours of lens wear discontinuation (Fig. 5B). Interestingly, the latter condition was associated with significant reduction in expression of IL-17F and the transcription factor ROR-γt versus 24 or 48 hours of lens wear. No significant changes in

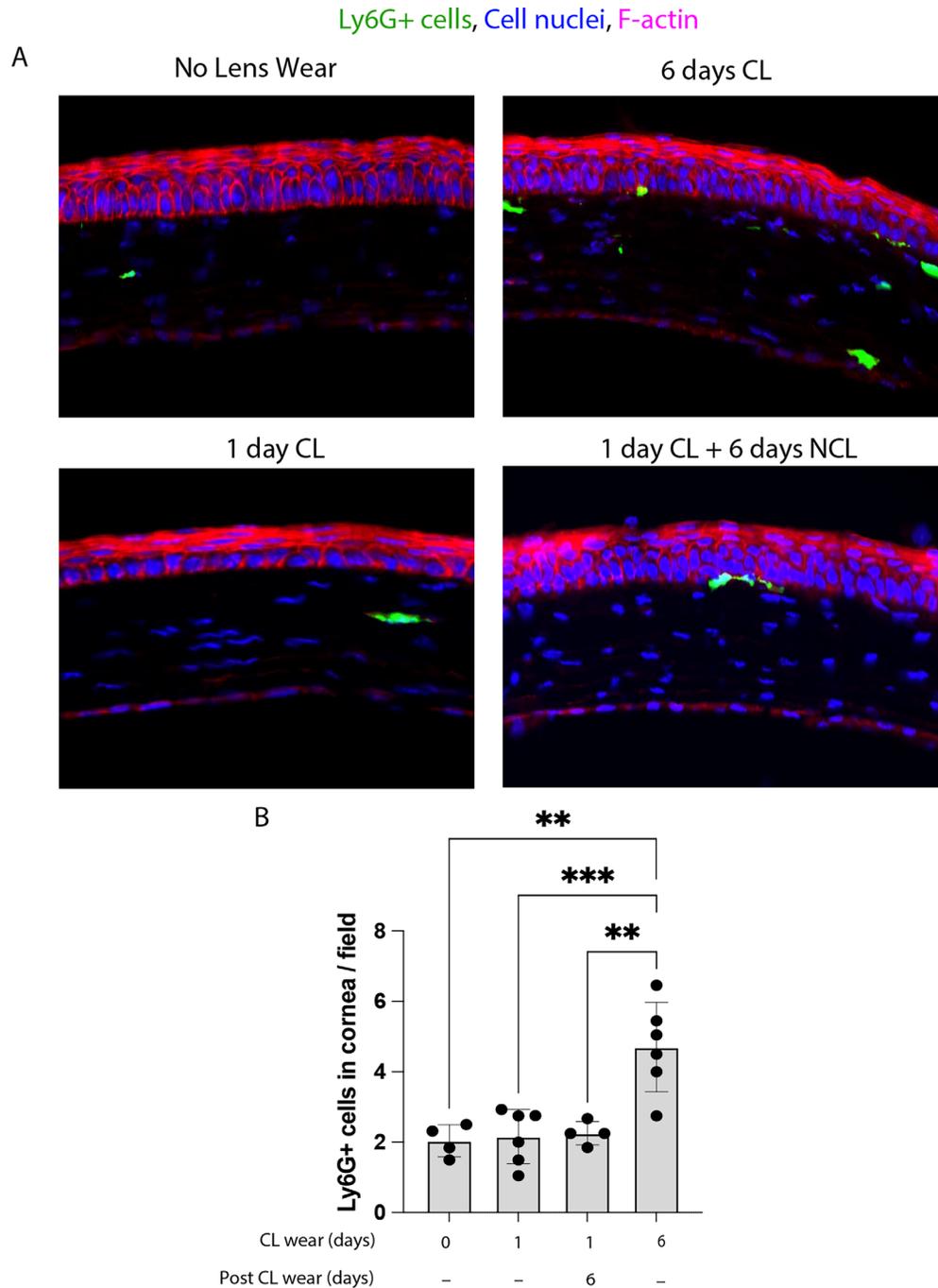


FIGURE 6. (A) Immunofluorescence imaging of Ly6G+ cell infiltration of lens-wearing mouse corneas after 24 hours and six days of wear versus 24 hours of wear plus six days of discontinued wear. No lens wear contralateral eye shown at six days. Ly6G+ cells (green), cell nuclei (blue), and cell F-actin (red). Scale bar: 30 μ m. (B) Quantification of Ly6G+ cells per field of view in corneas under the same experimental conditions as panel A. ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA with Tukey's multiple comparisons test).

expression of TNF- α , IL-17A, or IL-23 were observed in this experiment.

Lens Wear for 24 Hours Is Not Sufficient To Induce Six-Day Ly6G+ Cell Responses

Previously we have shown that continuous lens wear for five or six days induced a Ly6G+ (neutrophil) response in

the cornea⁸ that requires IL-17A and $\gamma\delta$ -T cells.¹⁰ Given the persistence of Lyz2+ and MHC Class-II+ cell responses after 24 hours of lens wear (Figs. 2, 3), we tested whether lens wear for 24 hours would be sufficient to induce a Ly6G+ cell response at six days after lens removal. Figure 6 shows that corneal Ly6G+ responses were only induced by six days of continuous lens wear as previously reported ($P < 0.01$). Lens wear for 24 hours was not sufficient to induce a six-day Ly6G+ cell response.

DISCUSSION

The purpose of this study was to determine the time course of contact lens-induced corneal parainflammation in our previously published murine model⁸: both onset after lens insertion and reversal after lens removal. Results showed that parainflammation involving a Lyz2+ immune cell response was not induced by either four or 12 hours of continuous lens wear requiring 24 hours for onset. Moreover, 12 hours' lens wear followed by 12 hours of no lens wear remained insufficient. Results also showed that lens removal after 24 hours wear was not associated with an immediate reduction in Lyz2+ or MHC Class-II+ cell parainflammatory responses: both continuing for four days after lens removal and only returning to baseline after seven days. Indeed, Lyz2+ responses appeared to amplify over one day after lens removal, an effect not observed when compared to 48 hours of continuous lens wear. The latter condition (48 hours' continuous wear) was associated with the loss of IL-1 β and IL-18 gene expression compared to 24 hours' wear plus 24 hours' discontinued wear, which in turn was associated with loss of IL-17F and ROR- γ t expression. Finally, lens-induced Lyz2+ and MHC Class-II+ cell responses after 24 hours of wear, and their continued persistence at four days after lens removal, were not sufficient to induce the Ly6G+ cell response observed after six days of continuous wear.

Previously we have shown the presence of multiple corneal immune cell responses after 24 hours of lens wear (CD11c+, Lyz2+ and MHC Class-II+ cells).^{8,9,11} Here, we show that 12 hours of lens wear was insufficient to induce a Lyz2+ cell response. Moreover, waiting a further 12 hours after lens wear discontinuation to match the 24-hour time-point did not induce a Lyz2+ response, suggesting that the trigger(s) for the 24-hour response were absent after 12 hours of lens wear or of insufficient magnitude after 12 hours to perpetuate a Lyz2+ response by 24 hours. This finding suggests that physical placement of the lens on the eye (i.e., mechanical effects) or disruption of the murine tear film by lens insertion did not activate immediate danger/alarm signals to the cornea. Events occurring during the 12- to 24-hour period of continued lens wear that could provide a necessary trigger(s) include (1) the gradual destabilization or compartmentalization of the post-lens tear fluid resulting in an accumulation of proinflammatory mediators (e.g., IL-1 β) or the loss of critical protective factors that modulate corneal inflammation (e.g., IL-10) or (2) changes in conjunctival microflora associated with lens placement/tear fluid changes that allow the accumulation of microbial triggers that activate a Lyz2+ response. In support of the latter hypothesis, we have previously shown that corneal CD11c+ cell responses to 24 hours' lens wear can be suppressed by topical antibiotic treatment correlating with a reduction in viable conjunctival bacteria suggesting involvement of microbial triggers.⁹ Nevertheless, further studies will be needed to decipher epithelial and tear fluid changes associated with lens wear during the first 24 hours that define the initial events leading to the onset of lens-induced immune cell parainflammatory responses (CD11c+, Lyz2+, MHC Class-II+, etc.).

Our results showed that once induced, the corneal Lyz2+ cell parainflammatory response persisted for at least four days after lens removal, only returning to baseline by seven days. The same was true for MHC Class-II+ cell responses. These findings suggest that once the "alarm" had been trig-

gered by 24 hours of lens wear, proinflammatory signals (whether chemoattractant or activation) remained in place. That assertion was supported by cytokine gene expression results showing that corneal IL-1 β and IL-18 responses present after 24 hours of wear persisted for one day after lens wear discontinuation. It is not clear, however, why Lyz2+ cell responses amplified after lens removal whereas elevated MHC Class-II+ cell levels remained steady. That difference was not reflected in the cytokine gene expression results, although a limited number were analyzed in this study. Because Lyz2+ cells (e.g., monocytes, macrophages, neutrophils, some dendritic cells)^{16,17} represent a different but partially overlapping group of immune cells compared to MHC Class-II+ cells (e.g., macrophages, dendritic cells), these data suggest that a subset of Lyz2+ cells was further influenced by lens removal.

To explore whether lens removal itself triggered amplification of the Lyz2+ cell response after 24 hours of wear, lens wear was continued for 48 hours in some experimental groups and compared to 24 hours of wear followed by one day of no lens wear. Similar responses in each group would have indicated that lens removal was not involved. However, amplification of the Lyz2+ cell response was not evident after 48 hours of continuous wear suggesting that the amplified response after 24 hours' wear could involve either mechanical forces of lens removal or further changes (albeit restorative) to tear fluid composition. Alternatively, it is possible that lens wear beyond 24 hours modulates parainflammatory events. In support of that hypothesis, lens wear for 48 hours was associated with a significant decline in IL-1 β and IL-18 gene expression. Further studies are needed to determine cytokine-, chemokine- and cellular-mediated events driving persistence of lens-induced corneal parainflammation after lens removal, the nature of immune cell types involved and if extended lens wear (beyond 24 hours) exerts immune-modulatory effects.

As we have previously reported,^{8,11} contact lens wear was associated with changes in immune cell morphology with increased circularity of Lyz2+ immune cells observed after 24 hours of wear. These changes remained even after four days of discontinued lens wear returning to baseline after seven days. However, quantitative analysis of arbitrarily divided subgroups of Lyz2+ cells revealed an increase in the number of dendriform cells at four and seven days after discontinuation of lens wear (see Supplemental Fig. S2) that suggest recruitment of new cells into the cornea. Although the significance of the increase in overall circularity of corneal Lyz2+ cells with lens wear is yet to be determined, rounding of corneal CD11c+ (dendritic) cells was associated with acquisition of CD86, a marker of maturation or activation.¹⁸

Previously we showed that lens-induced Ly6G+ cell parainflammatory responses require five to six days of continuous wear,⁸ that $\gamma\delta$ -T cells and IL-17A were required,¹⁰ along with TRPV1 and TRPA1 ion channels expressed on sensory nerves.¹¹ Corneal $\gamma\delta$ -T cell responses were present after 24 hours of lens wear and six days of continuous wear, with the latter resulting in increased IL-17A gene and protein expression.¹⁰ In the present study, 24 hours of lens wear followed by six days' discontinued wear was insufficient to induce a Ly6G+ response despite persistence of Lyz2+ and MHC Class-II+ cell responses for four days after lens wear discontinuation suggesting that those responses are not required or their involvement requires additional signals generated during six days of continuous wear. Further stud-

ies of factors driving persistence of lens-induced corneal parainflammation after lens removal mentioned above could also help determine the inter-relationship between 24 hours' and six days' parainflammatory responses. Results of this study provide some insights into the potential complexities involved. For example, in addition to modulation of Lyz2+ cell numbers and IL-1 β and IL-18 gene expression by 48 hours of lens wear, 24 hours of lens wear followed by one day's discontinued wear decreased the expression of genes encoding IL-17F and ROR γ t, the latter a key transcription factor regulating Th17 cell development, immune homeostasis, and mucosal immune defense.^{19,20}

Contact lens wear in humans also induces corneal parainflammation involving an increased density of Langerhans cells.^{5,6} In one study, this response occurred as early as two hours of wear and continued for the time course of the study (up to eight hours of wear).⁵ In the other study, increased corneal Langerhans cell density was also present after one week of daily disposable lens wear⁶ but not evident at later assessment times (four weeks and 24 weeks). It is difficult to make direct comparisons between those studies and results of this study because, in addition to comparing humans with mice, immune cell types observed will likely differ (Langerhans cells vs. Lyz2+ cells) and sample sizes are small in each instance. It remains evident, however, that both human and murine lens wear involve corneal parainflammatory responses involving resident or infiltrating immune cells and that studying each will provide further insights into the pathogenesis of lens-induced adverse events. Advances in live in vivo imaging of the cornea (e.g., Functional In Vivo Confocal Microscopy²¹) will likely help bridge the gap between in vivo models by helping to better define immune cell types and their relative roles.

In conclusion, this study demonstrates that contact lens-induced corneal parainflammation in mice involving Lyz2+ and MHC Class-II+ cells after 24 hours of wear can persist for at least four days after lens wear is discontinued and only returns to baseline by seven days. However, these responses are not sufficient to induce later (six-day) Ly6G+ (neutrophil) responses that require continuous wear. Further study is warranted to define differences in parainflammatory signaling and cellular events in the cornea between 24 hours of wear and continuous wear beyond that time.

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