

# Goblet cell loss abrogates ocular surface immune tolerance

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**Intestinal epithelial cells condition tolerogenic properties in DCs. Aqueous-deficient dry eye is associated with goblet cell (GC) loss and increased IFN- $\gamma$  expression in the conjunctiva. We hypothesized that loss of GCs reduces tolerance-inducing properties of antigen presenting cells (APCs) in the conjunctiva and draining nodes. Mice lacking the SAM pointed domain containing ETS transcription factor (*Spdef*) that is required for GC differentiation had an increased frequency of macrophages in the conjunctiva and CD11b<sup>+</sup>CD11c<sup>+</sup> DCs in the conjunctiva and draining nodes, and these cells had greater IL-12 expression than WT mice. Conditioned media from cultured WT conjunctival GCs suppressed LPS-induced IL-12 production by conjunctival APCs. OVA antigen-specific OTII CD4<sup>+</sup> T cells primed by *Spdef*-KO draining lymph node APCs showed greater proliferation, lower frequency of Foxp3<sup>+</sup>, increased frequency of IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup> cells, and greater IFN- $\gamma$  production than those primed by WT APCs. The immune tolerance to OVA antigen topically applied to the conjunctiva measured by cutaneous delayed type hypersensitivity (DTH) reaction, OVA-specific T cell proliferation, Foxp3 induction, and IFN- $\gamma$  production observed in WT mice was lost in the *Spdef*-KO mice. We concluded that conjunctival GCs condition tolerogenic properties in APCs that suppress IL-12 production and Th1 polarization.**

## Introduction

Dry eye is one of the most common medical problems, affecting tens of millions of individuals worldwide. There is a loss of mucus-producing conjunctival goblet cells (GCs) in aqueous-deficient dry eye due to increased IFN- $\gamma$  expression that causes GC secretory dysfunction and death (1–3). Dry eye is increasingly recognized as an immune-based inflammatory condition. Mouse dry eye models have shown that exposure of the ocular surface to a desiccating environment activates innate inflammatory pathways and promotes an adaptive immune response with infiltration of the conjunctiva and lacrimal gland by Th1 and Th17 cells (4–8). One theory for the generation of autoreactive CD4<sup>+</sup> T cells is that dry eye disrupts ocular surface immune tolerance. Loss of immune tolerance to antigens topically applied to the eye surface has been found in experimental models of dry eye induced by systemic cholinergic blockade and exposure to a desiccating environment, lacrimal gland excision, or topical administration of the detergent benzylkonium chloride (9–11). Similar to human dry eye, significant conjunctival GC loss has been documented in these mouse models (5, 12). GCs have been shown to serve as conduits for antigen passage from the surface to stroma of mucosal tissues, including the conjunctiva, and to produce immunomodulatory factors, such as TGF- $\beta$ 2 and MUC2, that suppress maturation and condition tolerogenic properties in DCs (13–15).

Mice with genetic deletion of the SAM pointed domain containing ETS transcription factor (*Spdef*) lack GCs (16). These mice have been found to have inflammatory cell infiltration of the conjunctiva and develop dry eye (17). We hypothesized that loss of conjunctival GCs in the *Spdef*-KO reduces tolerance-inducing properties of antigen presenting cells (APCs) in the conjunctiva and draining cervical nodes and abrogates immune tolerance to antigen applied topically to the conjunctiva.

The purpose of this study was to evaluate the effects of GC loss on the number and distribution of APCs in the conjunctiva, the number and T cell-polarizing properties of APCs in the draining lymph nodes, and the induction of tolerance to an antigen topically applied to the ocular surface.

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## Results

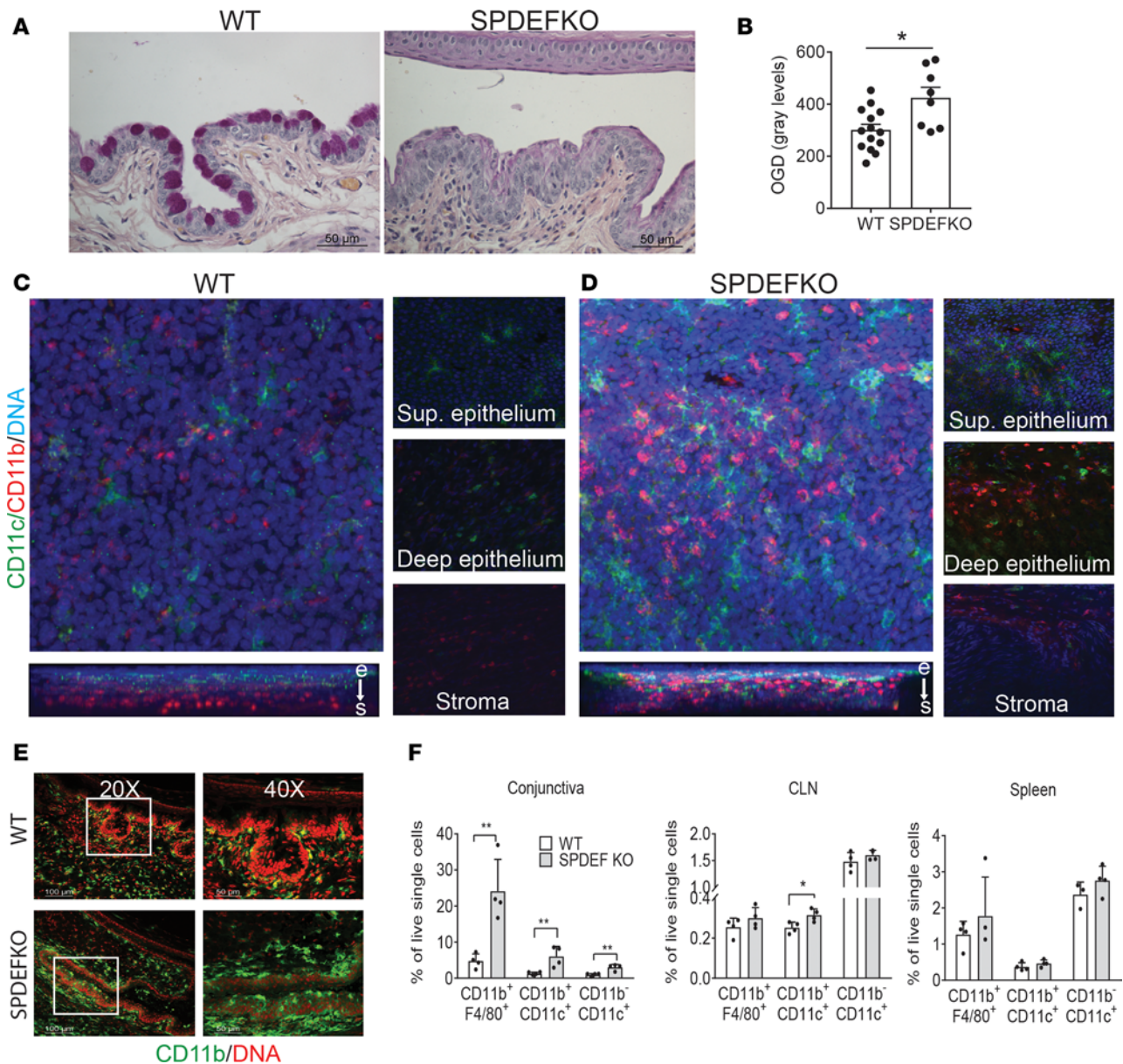
*Absence of GCs alters APC distribution in the conjunctiva.* The Spdef transcription factor is essential for GC differentiation (18), and as previously described, we found Spdef-KO mice have no formed conjunctival GCs (Figure 1A) (17). We also noted the Spdef-KO mice develop cornea epithelial disease with increased uptake of 70kDa Oregon green dextran (OGD) as an indicator of corneal epithelial barrier disruption, which is characteristic of dry eye (Figure 1B).

Marko previously reported that Spdef-KO mice have an increased number of inflammatory cells in the conjunctival stroma (17), and we hypothesized that some of these cells could be DCs and macrophages because conjunctival GCs have been found to produce immunomodulatory factors (13, 15). By confocal microscopy, an increased number of CD11c<sup>+</sup> cells in the superficial conjunctiva and increased CD11b<sup>+</sup> cells in the superficial and deep epithelium and stroma were observed in the Spdef-KO compared with WT mice (Figure 1, C and D). In tissue sections, CD11b<sup>+</sup> cells were primarily located below the conjunctival basement membrane in WT mice but were observed in the epithelium and stroma of the Spdef-KO mice (Figure 1E). The frequency of macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>) and DCs (CD11c<sup>+</sup>CD11b<sup>+</sup>, CD11c<sup>+</sup>CD11b<sup>-</sup>) was evaluated by flow cytometry in whole conjunctiva and cervical lymph node (CLN) suspensions, and a significant increase in all 3 populations was observed in the conjunctiva of the Spdef-KO compared with WT mice (Figure 1F). CD11b<sup>+</sup>CD11c<sup>+</sup> cells were also increased in draining CLN, but all populations had a similar frequency in the spleen. These findings demonstrate that the number and location of APCs in the conjunctiva is altered in the Spdef-KO mice.

*APCs from Spdef-KO mice prime Th1 cells.* Because GCs produce immunomodulatory factors and conjunctival GC loss in dry eye disease is associated with increased IFN- $\gamma$  expression (1), we investigated if Spdef-KO APCs prime different responses in antigen-specific CD4<sup>+</sup> T cells than those from WT mice. OVA presentation assays were performed using OVA peptide pulsed CLN APCs combined with CD4<sup>+</sup> T cells from OT II mice. Cells primed by APCs from the Spdef-KO CLN had greater proliferation (Figure 2A), a lower frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> cells, and increased frequency of CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD4<sup>+</sup>IL-17<sup>+</sup> cells (Figure 2B). A significant increase in IFN- $\gamma$  concentration was measured in culture supernatants from the Spdef-KO-primed group (Figure 2C), while the IL-17A concentration was below the detection threshold.

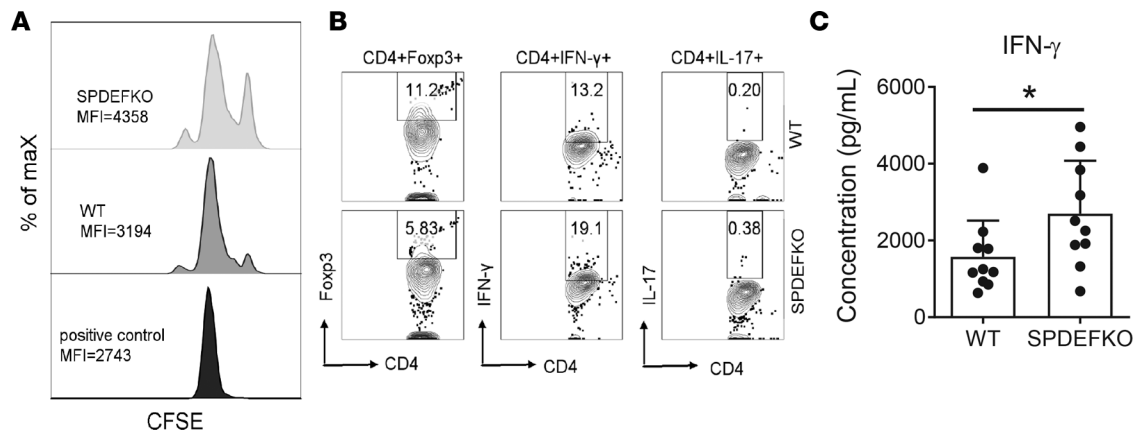
*Spdef-KO mice have higher IL-12 expression in conjunctiva.* Because IL-12 is critical in Th-1 induction, we asked if there is evidence of increased IL-12 expression by APC populations in the conjunctiva of Spdef-KO mice. IL-12<sup>+</sup> cells were evident in the stroma of immunostained conjunctival sections from the Spdef-KO mice, while they were absent in the WT mice (Figure 3A). Expression of IL-12 mRNA was 8.5-  $\pm$  0.4-fold higher ( $P < 0.001$ ) in whole conjunctival lysates of Spdef-KO compared with WT mice. These findings were confirmed by flow cytometry, where a significantly increased percentage of IL-12<sup>+</sup> cells and increased IL-12 mean fluorescent intensity (MFI) were detected in CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages and CD11b<sup>+</sup>CD11c<sup>+</sup> DCs (Figure 3B).

Because topical administration of LPS on the mouse ocular surface was found to be a potent inducer of IL-12 expression in the mouse conjunctiva (19), we hypothesized that topical application of conditioned media from cultured WT conjunctival GCs would suppress LPS-stimulated IL-12 expression in the Spdef-KO mice. Using our published culture protocol (3), WT conjunctival GCs were cultured for 14 days and conditioned media (CjCM) was collected. Spdef-KO mice were topically dosed with culture media alone (IMDM) or WT CjCM 4 times daily starting the day before and 1 hour prior (for a total of 5 topical administrations) to topical challenge with a single dose of LPS, and the suppressive effect was compared with a separate group of mice that received only a single topical application of either media or CjCM 1 hour prior to LPS challenge (1 $\times$ ). Conjunctivae and CLNs were collected 4 hours after LPS and digested with collagenase, and IL-12 was measured in CD11b<sup>+</sup>CD11c<sup>+</sup> cells by flow cytometry and intracellular staining. Compared with the water control, we observed a single administration of LPS increased the percentage and MFI of conjunctival IL-12<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells. IL-12 remained high after a single administration of CjCM, but topical administration of CjCM 5 $\times$  prior to LPS challenge significantly blunted the increase in IL-12 MFI compared with the media-alone control group (Figure 3, C and D). This effect was not observed in conjunctival CD11b<sup>+</sup>F4/80<sup>+</sup> cells (Figure 3C), and no change in IL-12 frequency or MFI was observed in the CLN (data not shown). These results indicate that IL-12 production in the conjunctiva increases with loss of GCs and that GC-produced factors inhibit LPS-stimulated IL-12 production by APCs.



**Figure 1. Lack of goblet cells alters DC distribution in Spdef-KO conjunctiva.** (A) Representative PAS staining of paraffin-embedded conjunctival sections. Magenta-colored PAS<sup>+</sup> goblet cells are present in WT but absent in Spdef-KO ( $n = 3/\text{group}$ ; scale bars: 50  $\mu\text{m}$ ). (B) Greater uptake of 70 kDa Oregon green dextran (OGD) by cornea epithelium, indicating altered corneal barrier function, was noted in the Spdef-KO ( $n = 8\text{--}14/\text{group}$ ). Mean  $\pm$  SEM,  $*P < 0.05$ . (C and D) Laser confocal microscopy of whole mount conjunctivas taken from fornices of WT (C) and Spdef-KO mice (D). Top left panels show surface view (200 $\times$  magnification); lower left panels show distribution of DCs in the conjunctiva using Z-stack option from epithelium (e) to stroma (s). Smaller right panels are of superficial (Sup) and deep epithelium and of stroma (400 $\times$  magnification). Nuclei are stained with Hoechst 33343 dye (blue) ( $n = 3/\text{group}$ ). (E) Representative conjunctival sections from WT and Spdef-KO stained for CD11b (green) and DNA with propidium iodide (red, 20 $\times$ ). White boxes in left images are seen in higher magnification in right images (40 $\times$ ,  $n = 3/\text{group}$ ). (F) Bar graphs of mean  $\pm$  SEM of macrophage (CD11b<sup>+</sup>F4/80<sup>+</sup>) and DC (CD11c<sup>-</sup>CD11b<sup>+</sup>, CD11c<sup>+</sup>CD11b<sup>-</sup>) percentages in conjunctiva, cervical lymph nodes (CLN), and spleen from WT and Spdef-KO measured by flow cytometry. Lack of GCs increases all 3 populations in the conjunctiva and CD11c<sup>-</sup>CD11b<sup>+</sup> DCs in the CLN of Spdef-KO ( $n = 4/\text{group}$ ). Between-group comparisons for each cell type in each tissue were performed with the Student *t* test,  $*P < 0.05$ ;  $**P < 0.01$

Conjunctival GCs have been previously found to produce TGF- $\beta$ 2 that suppressed DC maturation (15). We found that conjunctival GCs also express aldehyde dehydrogenase ALDH1A3, have aldehyde dehydrogenase activity, and produce biologically active retinoic acid (RA; Y. Xiao unpublished data). To determine the effects of GC-produced RA on IL-12 expression by unstimulated and LPS-stimulated BM-derived DCs (BMDCs), day 9 cultured BMDCs were treated with CjCM or RA, with or without the RAR- $\alpha$  antagonist Ro41-5253 (Ro). Both CjCM and RA significantly inhibited IL-12 production in LPS-treated BMDCs,



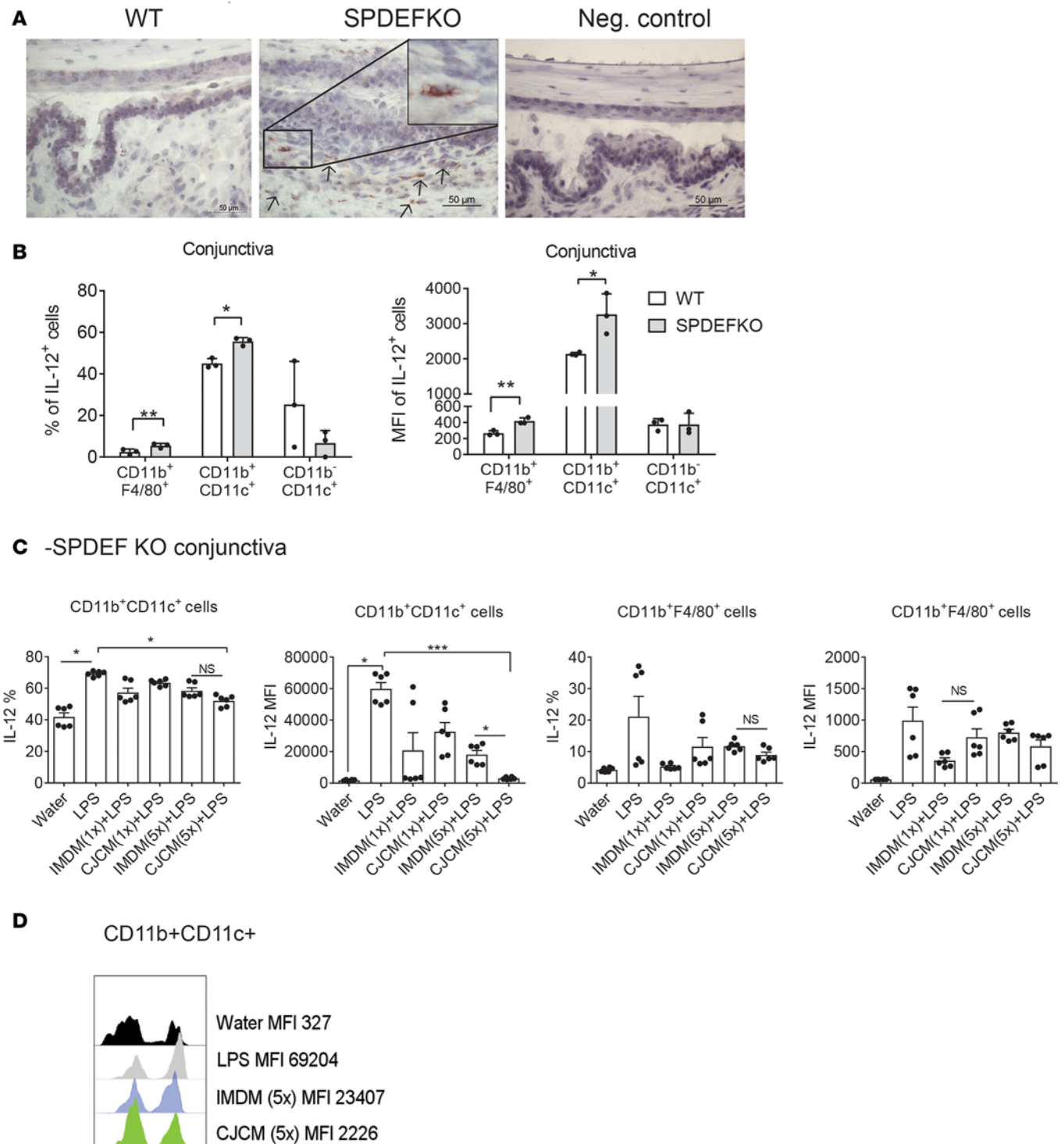
**Figure 2. Spdef-KO DCs prime Th1 and Th17 responses.** CLN suspensions were pulsed with OVA peptide for 1 hour prior to addition of OTII CFSE-labeled CD4<sup>+</sup> T cells. After 4–5 days, cells were collected, intracellular staining was investigated by flow cytometry, and IFN- $\gamma$  immunobead assay was performed in culture supernatants. **(A)** Representative histogram of CFSE median fluorescence intensity (MFI) in WT and Spdef-KO groups. **(B)** Flow cytometry dot plots of intracellular staining for Foxp3, IFN- $\gamma$ , or IL-17 in CFSE<sup>low</sup>CD4<sup>+</sup> OTII T cells after 4 days in culture ( $n = 3$ /group). **(C)** Production of IFN- $\gamma$  was measured by Luminex assay of coculture supernatants. Bar graph presents mean  $\pm$  SEM ( $n = 10$ ). \* $P = 0.03$ . Between-group comparisons were performed with the Mann-Whitney test. Results from a repeat experiment showed similar between-group difference.

and the suppressive effects of both CjCM and RA on LPS-stimulated IL-12 expression were significantly reversed by Ro (Table 1). This indicates that RA produced by conjunctival GCs can suppress stimulated IL-12 production by APCs.

*Altered induction of conjunctival immune tolerance in Spdef-KO mice.* Spdef-KO mice that lack GCs have been previously reported to develop ocular surface inflammation and dry eye disease (17). Additionally, loss of ocular surface immune tolerance has been observed in experimentally induced mouse dry eye models where GC loss occurs (9, 10). Based on these findings and the altered distribution of APCs in the conjunctiva of the Spdef-KO mice, we compared the ability to induce tolerance to topically applied OVA antigen in WT and Spdef-KO mice using a previously reported protocol with cutaneous delayed type hypersensitivity (DTH) response as the readout (Figure 4A). Compared with unimmunized control mice, both WT and Spdef-KO strains develop a significant DTH response (ear swelling) following s.c. OVA immunization followed by an intradermal ear lobe injection on day 15 (Figure 4B). As previously reported, WT mice developed tolerance to OVA applied topically to the ocular surface; however, tolerance induction was not observed in the Spdef-KO mice (Figure 4B). We also evaluated antigen-specific immune response. Immunization of WT B6 mice increased proliferation of OVA-specific T cells in their spleens, and this was significantly reduced in animals that received OVA drops on the conjunctiva prior to immunization. In contrast, this decrease in proliferation of OVA-specific T cells was not observed in the Spdef-KO mice (Figure 4C). Similar results were seen in CLN T cells in the B6 mice, but compared with WT mice, the difference in T cell proliferation between the control and mice receiving topical OVA did not reach significance in Spdef-KO mice (Figure 4D). As further evidence in support of defective tolerance induction in the Spdef-KO mice, an increase in CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the spleen was observed in WT mice topically treated with OVA, but this was not observed in the Spdef-KO mice (Figure 4E). Furthermore, IFN- $\gamma$  production was significantly higher in splenocytes from the immunized and the topically treated and immunized Spdef-KO than from the corresponding WT groups (Figure 4F).

## Discussion

Similar to the intestinal mucosa, the conjunctiva covering the ocular surface has a GC-rich epithelium. The conjunctival GCs have traditionally been considered to function as the source of gel-forming mucins that protect and lubricate the surface of the eye. GCs, including those in the conjunctiva, have also been found to serve as antigen passages to APCs and lymphatics in the underlying stroma (13, 20). Additionally, conjunctival GCs have been reported to secrete immunomodulatory factors (15). APCs are found in close proximity to the apex and the base of conjunctival GCs. Our study found that absence of GCs in the Spdef-KO mice is associated with an increased number of DCs and macrophages



**Figure 3. Increased IL-12 production in conjunctiva of Spdef-KO mice.** (A) Immunohistochemical staining demonstrates presence of IL-12<sup>+</sup> cells in the conjunctiva of Spdef-KO mice. Slides without primary antibody are shown as negative control (neg. control). Scale bars: 50  $\mu$ m. Arrows indicate IL-12<sup>+</sup> cells. Magnification of inset, 2 $\times$  (B) Flow cytometry analysis showing percentage and median fluorescence intensity (MFI) of IL-12<sup>+</sup> macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>) and DCs (CD11c<sup>+</sup>CD11b<sup>+</sup>, CD11c<sup>+</sup>CD11b<sup>-</sup>) in conjunctiva of WT and Spdef-KO ( $n = 3$ /group, means  $\pm$  SEM, between-group comparisons for each cell type performed with Mann-Whitney test). (C) Graphs showing percentage and MFI of IL-12<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>-</sup> (left) or CD11b<sup>+</sup>F4/80<sup>+</sup> cells in Spdef-KO mice with or without topical administration of media (IMDM) or CJCM prior to topical LPS challenge. Mice received a single dose of media as noted 1 hour (1 $\times$ ) or 1 day and 1 hour (for a total of 5 applications) prior to LPS stimulation. ( $n = 6$ /group, means  $\pm$  SEM, 1-way ANOVA followed by Holm-Sidak's multiple comparisons test corrected for repeated measures). (D) Histograms of median fluorescence intensity (MFI) of IL-12<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells in conjunctivae of Spdef-KO treated topically with water, LPS, and IMDM (media control) 5 $\times$  prior to LPS or WT conjunctival conditioned media (CJCM) 5 $\times$  prior to LPS.

**Table 1. Goblet cell–produced retinoic acid suppresses DC IL-12 production**

	UT	LPS	Ro	Ro LPS	CJCM	CJCM LPS	CJCM Ro LPS	RA	RA LPS	RA Ro LPS
IL-12 Relative Fold	1	13.2 <sup>A</sup>	1.9	8.9 <sup>A</sup>	0.1	0.3	8.7 <sup>A,B,C</sup>	1.6	3 <sup>A,B</sup>	11 <sup>A,B,C</sup>
SD	0.6	0.5	0.1	0.2	0.8	1.1	0.2	0.5	0.1	0.2

The retinoid receptor  $\alpha$  (RAR- $\alpha$ ) antagonist Ro was added 2 hours before CJCM or RA on day 6, and RNA was harvested for PCR following a 4-hour stimulation with LPS on day 8. Values are mean relative fold;  $n = 4$  per group. Comparisons were made with 1-way ANOVA followed by Holm-Sidak's multiple comparisons test corrected for repeated measures. <sup>A</sup> $P < 0.0001$  vs. UT; <sup>B</sup> $P < 0.0001$  vs. CJCM or RA within group; <sup>C</sup> $P < 0.0001$  vs. CJCM LPS or RA LPS within group. UT, untreated; LPS, lipopolysaccharide; CJCM, conjunctival (goblet cell) conditioned media; Ro, RAR- $\alpha$  inhibitor Ro41-5253; RA, retinoic acid.

in the conjunctiva, particularly an increased number of CD11b<sup>+</sup> DCs in the epithelium. The Spdef-KO mice also had a significantly higher percentage of IL-12<sup>+</sup>CD11b<sup>+</sup> DCs and macrophages in the conjunctiva, suggesting their potential role in Th1 induction. Consistent with their increased IL-12 production, we observed that APCs from the eye-draining CLNs of Spdef-KO mice caused greater Th1 and reduced Foxp3<sup>+</sup> priming of naive CD4<sup>+</sup> T cells than those from the WT mice. As evidence that GCs factors suppress IL-12 production, we found that topical reconstitution of the Spdef-KO mice with conditioned media from cultured WT GCs suppressed LPS-stimulated IL-12 production in APCs in the Spdef-KO conjunctiva. This suppressive activity may be attributed to one or more of the immunomodulatory factors produced by GCs. TGF- $\beta$ 2 produced by conjunctival GCs was reported to suppress DC maturation (15), and the GC mucin MUC2 was noted to inhibit production of inflammatory, but not tolerogenic, cytokines in LPS-treated human DCs (14). We found that RA produced by cultured conjunctival GCs contributed to the suppressive activity of GC conditioned media on LPS-stimulated IL-12 production by BMDCs because this suppressive activity was lost when the BMDCs were pretreated with a retinoid receptor antagonist. Epithelial cells in the intestine and stomach have also been noted to produce RA that functions to maintain immune tolerance in these tissues (21, 22). Our findings suggest that the conjunctival GCs have homeostatic immunomodulatory activity that promotes generation of Tregs, while suppressing generation of IFN- $\gamma$ -producing T cells that can amplify dry eye disease by promoting GC loss and secretory dysfunction (3, 23).

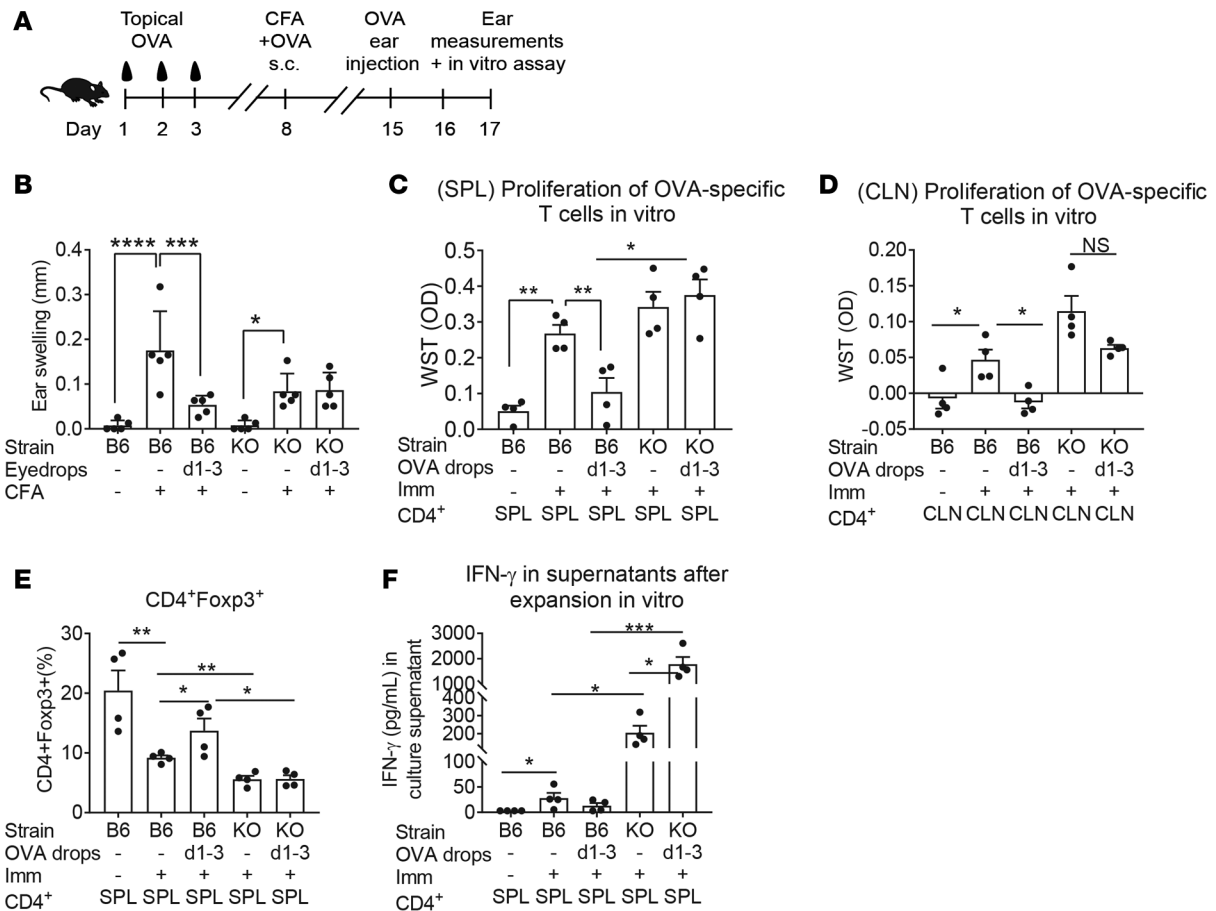
We also found that absence of GCs abrogated induction of conjunctival immune tolerance in the Spdef-KO group. Loss of immune tolerance has been implicated in the immune-based conjunctival inflammation that develops in dry eye (10, 11). Tolerance to antigens, such OVA, topically applied to the ocular surface has been reported to develop through anergy induction and generation of antigen-specific Tregs (10, 11, 24). Guzman and associates found that induction of tolerance to topically applied OVA antigen was lost after 3 days of experimental dry eye induced by pharmacological suppression of tear secretion and exposure to a desiccating environment, a model in which GC loss occurs (10). Loss of tolerance in the dry eye group was confirmed by increased DTH to OVA antigen, reduced T cell suppression of antigen-specific T cell proliferation, and increased generation of IFN- $\gamma$ - and IL-17–positive CD4<sup>+</sup> T cells (10). Both of these T helper cytokines have been implicated in the pathogenesis of the ocular surface epithelial disease that develops in dry eye (6, 23, 25).

As an exposed mucosa, the conjunctiva is subjected to a variety of foreign antigens, as well as the myriad of self-antigens that are secreted by the lacrimal glands into the tears. Under physiologic conditions, the GCs may serve as conduits for these antigens to tolerogenic APCs in the underlying stroma. The loss of tolerance in the Spdef-KO mice is evidence that GC secretions may condition tolerance in the conjunctival APCs.

In summary, these findings suggest that GCs have an important role in maintaining immune homeostasis and modulating antigen-specific immune reactions on the ocular surface.

## Methods

**Reagents.** Antibodies used for immunofluorescent staining and flow cytometry and topically applied antigens are listed in Supplemental Table 1 (supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.98222DS1>). WST-1 cell proliferation reagent was from Roche Diagnostics (catalog 11644807001).



**Figure 4. Lack of goblet cells abrogates conjunctival mucosal tolerance.** (A) Conjunctival immune tolerance was measured by delayed type hypersensitivity (DTH) to OVA using the following protocol: OVA eyedrops were administered topically for 3 days (d1–d3). Mice received s.c. immunization (imm) with OVA + complete Freund’s adjuvant on day 8 and challenge with antigen by i.d. ear injection (OVA right ear and PBS left eye) on day 15. Ear swelling was measured after 48 hours. (B) In vivo DTH assay (ear swelling) measured 48 hours after challenge. Results are the difference between the antigen-injected and PBS-injected ears of mice in each group. ( $n = 5/\text{group}$ , mean  $\pm$  SEM, Kruskal-Wallis followed by Dunn’s multiple comparisons test corrected for repeated measures). (C–F) Conjunctival immune tolerance suppresses expansion of OVA-specific T cells in vitro. CD4<sup>+</sup> T cells isolated from spleens (SPL) and cervical lymph nodes (CLN) of mice ( $n = 5$ ), as described in A, were pooled and cocultured for 7 days with naive CD3-depleted splenocytes as antigen presenting cells pulsed with 100  $\mu\text{g}$  of OVA. Proliferation was measured by WST-1 (C and D), frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> cells was investigated by flow cytometry (E), and IFN- $\gamma$  was measured in culture supernatants by Luminex immunobead assay at culture day 7 (F). Proliferation of CD4<sup>+</sup> T cells from control Spdef-KO (no immunization and no culture) was not significantly different than B6 and is not shown; ( $n = 4/\text{group}$ , means  $\pm$  SEM, Kruskal-Wallis followed by Dunn’s multiple comparisons test corrected for repeated measures). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  comparison as indicated. Imm, immunization; B6, C57BL/6 WT; KO, Spdef-KO

*Mice.* Breeder pairs of C57BL/6J and B6.Cg-Tg (Tcr $\alpha$ Tcr $\beta$ )425Cbn/J (OTII) mice 6–9 weeks old were purchased from The Jackson Laboratory for establishing breeder colonies. Spdef-KO mice on a B6 background were a gift of Hans Clevers (Hubrecht Institute, Utrecht, Netherlands) and obtained from Jeffery Whitsett (Cincinnati Children’s Hospital, Cincinnati, Ohio, USA). Spdef-KO mice were bred in a conventional vivarium and backcrossed to C57/B6J (WT) for at least 6 generations before these experiments were performed. Female C57BL/6 ( $n = 56$ ) and Spdef-KO ( $n = 77$ ) mice aged 6–9 weeks were used for experiments.

*Laser scanning confocal scanning microscopy.* Freshly harvested whole-mount conjunctivas were fixed with cold acetone for 10 minutes, washed, blocked with 20% goat serum (GS) for 45 minutes, and incubated with primary anti-CD11c or anti-CD11b antibodies (Supplemental Table 1) for 45 minutes at room temperature (RT). Tissues were washed with 1% PBS and then incubated with appropriate secondary antibodies diluted 1:100–1:300 in PBS for 60 minutes at RT in the dark and then counterstained with DNA binding Hoechst 33342 dye diluted 1:500 for 5 minutes. After washing with PBS, conjunctivas were flattened on slides and coverslips mounted with Gel/Mount (Thermo Fisher Scientific). Digital confocal images were captured with a laser scanning confocal microscope (NIKON A1 RMP, Nikon) wavelength 400–750 nm and 1  $\mu\text{m}$  z-step. The images were processed using NIS Elements 4.20 version (Nikon).

*Histology, PAS staining, and IHC.* Eyes from each group (WT and Spdef-KO) were surgically excised and fixed in 10% formalin or flash-frozen in liquid nitrogen as previously described (19, 26). Paraffin-embedded sections were stained with PAS reagent (Thermo Fisher Scientific) to identify filled GCs.

IHC was performed to detect IL-12<sup>+</sup> cells in the conjunctiva. Cryosections were stained with the primary antibody (IL-12 $\alpha$ , ab203031; Abcam) and appropriate biotinylated secondary antibody (1:100 biotin goat  $\alpha$ -rabbit; BD Pharmingen) using a Vectastain Elite ABC kit and Nova Red reagents (Vector). Sections from each experimental group were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DSQi1Mc; Nikon).

*Flow cytometry analysis.* Single-cell suspensions of conjunctiva or CLNs were prepared as previously described (27). Conjunctiva were excised and incubated with 0.1% collagenase and 0.05 mg/ml of DNase I for 1 hour, followed by 2 sequential washes with complete RPMI. Cell suspensions were stained with anti-CD16/CD32 at 4°C for 10 minutes, followed by staining with anti-CD45, anti-CD11c, anti-CD11b, or anti-F4/80 antibodies (Supplemental Table 1). Negative controls consisted of fluorescence minus one (FMO) splenocytes. Cells were resuspended in PBS/1% FBS containing 1  $\mu$ l of live/dead violet reconstituted fluorescent reactive dye (Invitrogen-Molecular probes, Life Technologies). The cells were kept on ice until flow cytometry analysis was performed. The gating strategy used in this study was as follows: dead cells were excluded by gating violet dye versus CD45<sup>+</sup> cells, subsequently gated on the basis of forward scatter height versus forward scatter area (singlets 1), and then gated on side scatter height versus side scatter area (singlets 2). CD11b and CD11c were then plotted.

Single-cell suspensions of conjunctiva or CLNs were prepared as previously described (27). Conjunctiva were excised and incubated with 0.1% collagenase and 0.05 mg/ml of DNase I for 1 hour, followed by 2 sequential washes with complete RPMI. Cell suspensions were stained with anti-CD16/CD32 at 4°C for 10 minutes, followed by staining with anti-CD45, anti-CD11c, anti-CD11b, or anti-F4/80 antibodies (Supplemental Table 1). Negative controls consisted of FMO splenocytes. Cells were resuspended in PBS/1% FBS containing 1  $\mu$ l of live/dead violet reconstituted fluorescent reactive dye (Invitrogen-Molecular probes, Life Technologies). The cells were kept on ice until flow cytometry analysis was performed. The gating strategy used in this study was as follows: dead cells were excluded by gating violet dye versus CD45<sup>+</sup> cells, subsequently gated on the basis of forward scatter height versus forward scatter area (singlets 1), and then gated on side scatter height versus side scatter area (singlets 2). CD11b and CD11c were then plotted. CD11c<sup>+</sup>CD11b<sup>+</sup> cells were gated against F4/80, and CD11b<sup>+</sup>F4/80<sup>+</sup> cells were identified.

For IL-12 intracellular staining, single cell suspensions were obtained, and  $2 \times 10^6$  cells were incubated for 5 hours with 1  $\mu$ l Golgi Stop (BD Bioscience) and 1  $\mu$ l Golgi Plug (BD Bioscience) in 1 ml complete RPMI. Cells were stained with violet fluorescent reactive dye (Invitrogen-Molecular probes, Life Technologies) for 30 minutes, prior to fixation. Cells were then stained with CD16/CD32 followed by incubation with anti-IL-12 and anti-CD45, anti-CD11b, anti-CD11c, or anti-F4/80 antibodies. The gating strategy used in this study was as follows: dead cells were excluded by gating live dye versus CD45<sup>+</sup> cells, subsequently gated on the basis of forward scatter height versus forward scatter area (singlets 1), and then gated on side scatter height versus side scatter area (singlets 2). CD11b and CD11c were then plotted. CD11c<sup>+</sup>CD11b<sup>+</sup> cells were gated against F4/80, and CD11b<sup>+</sup>F4/80<sup>+</sup> cells were identified. IL-12 percentage and MFI were then calculated in each cell subset.

A BD FACS CANTO II cytometer (Becton Dickinson) was used, and data were analyzed using FlowJo software (TreeStar Inc.). Biological replicates were averaged.

*Cell proliferation assay.* CLN cell suspensions from each WT and Spdef-KO mice, prepared as previously reported, were used as APCs (28). CLN cells ( $0.5 \times 10^6$ ) were pulsed with 10 ng/ml of OVA<sub>323-339</sub> peptide for 1 hour. The MHCII<sup>+</sup> population in the CLN suspension was found to include CD11c<sup>+</sup>CD11b<sup>-</sup>, CD11c<sup>+</sup>CD11b<sup>+</sup>, and CD11b<sup>+</sup>F4/80<sup>+</sup> cells. CD4<sup>+</sup> T cells from 6- to 8-week-old B6.Cg-Tg(TcraTcrb)425Cb-n/J (OTII) mice were isolated according to the manufacturer's instructions (untouched CD4<sup>+</sup> T cell isolation kit, Stemcell Technologies) and labeled with CFSE, and  $0.5 \times 10^6/100$   $\mu$ l per well were added to the CLN APCs in 96-well plates and incubated for 4–5 days. Supernatants were collected and stored at  $-80^\circ\text{C}$  until use. Cell proliferation was measured by CFSE dilution assay as previously described (13).

*Cytokine immunobead assay.* Culture supernatants from each experimental group were collected, and IFN- $\gamma$  concentration was measured by an immunobead assay. Samples were added to wells containing cytokine beads coated with anti-IFN- $\gamma$  or anti-IL-17 (MilliporeSigma). Serial dilutions of IFN- $\gamma$  were added to wells in the same plate as the mouse samples to generate a standard curve. The plate was incubated overnight



at 4°C. After 3 washes with assay buffer, 25 µl of biotinylated secondary cytokine antibody mixture was applied for 1.5 hours in the dark at RT. The reactions were detected with streptavidin–phycoerythrin using a Luminex 100 IS 2.3 system. The limit of detection of this assay was 3.2 pg/ml for IFN-γ and IL-17.

**Conjunctival GC culture.** Conjunctival GCs were cultured as previously described (3) with modifications. Briefly, explants were excised from the fornical conjunctiva of 6- to 8-week-old female C57BL/6 mice and incubated for 15–20 minutes at 37°C in keratinocyte serum-free media (Thermo Fisher Scientific; catalog 10724-011) supplemented with 3% defined FBS, 1.25 mg/ml amphotericin B (Thermo Fisher Scientific; catalog 15290-018), 0.5 ml/ml gentamicin (Thermo Fisher Scientific; catalog 15750-060), and 5 mg/ml dispase II (Roche Diagnostics; 04942078001). Conjunctival explants were plated 1 explant per well in 48-well plates and 200 µl of keratinocyte serum-free media supplemented with 80 ng/ml mouse epidermal growth factor (BD Biosciences; catalog 354001). Four explants (upper and lower conjunctival fornix from each eye) were obtained per animal. Conjunctival conditioned media was collected after 14 days in culture and kept at –80°C until use.

**Topical reconstitution.** One hour (1 application) or 1 day (4 times) and 1 hour (for a total of 5 applications) before LPS challenge, Spdef-KO mice were topically administered Iscove's Modified Dulbecco's medium (IMDM) or CjCM (2 µl/eye). Mice were held in place for 1 minute to allow eye drops to distribute.

One hour after the last application, Spdef-KO mice were topically administered 5 µl of ultrapure LPS (1 µg/µl, Invitrogen) from Salmonella minnesota LPS dissolved in endotoxin-free water (MilliporeSigma) and compared with mice receiving 5 µl/eye of endotoxin-free water as vehicle control or naive mice. After 4 hours, mice were euthanized for IL-12 cytokine staining and flow cytometry.

**DC culture.** BMDCs were prepared by flushing cells from femurs of female C57BL/6J mice with ice-cold PBS. After RBC lysis, BM cells were cultured for 9 days at  $3 \times 10^6$  cells/well in 10-cm diameter plates in complete RPMI 1640 medium (10% FBS, 50 µg/ml gentamicin, 1.25 µg/ml amphotericin B) supplemented with mouse GM-CSF (20 ng/ml) and IL-4 (5 ng/ml; all from PeproTech). On day 3, fresh media containing GM-CSF and IL-4 was added. CjCM or RA (10 nM), with or without 2-hour pretreatment with RAR-α antagonist Ro (1 µM), was added to the cultures on day 6. On day 8, BMDCs were stimulated with LPS (1 µg/ml) for 4 hours before cells were lysed and RNA extracted for PCR.

**RNA isolation and PCR.** Conjunctiva was surgically excised and total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's protocol. Concentration of isolated RNA was measured using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). After RNA isolation, cDNA was synthesized using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) as previously reported (6).

After cDNA synthesis, PCR was run on a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene expression was analyzed by the comparative CT ( $\Delta\Delta CT$ ) method. CT values for each gene were normalized by the CT value of the housekeeping gene for each sample. The housekeeping gene used for these experiments was hypoxanthine guanine phosphoribosyl transferase (Hprt). Taqman Probes (Invitrogen) used in this study include IL-12α (Mm00434165\_m1) and Hprt (Mm00446968\_m1).

**Conjunctival immune tolerance and DTH assay.** Conjunctival immune tolerance was measured by cutaneous DTH to OVA (Invivogen) using the following protocol: OVA eyedrops (5 µl/eye of 2 mg/ml solution) were administered topically for 3 days in both WT and KO groups ( $n = 5$ /group). On day 8, mice received immunization with an emulsion of OVA + complete Freund's adjuvant (Thermo Fisher Scientific) prepared 1:1, administered s.c. in the neck under general anesthesia using isoflurane gas dispensed through a nose cone using the SomnoSuite Vaporizer (Kent Scientific). On day 15, mice were challenged with antigen by intradermal ear injection (10 µg of OVA right ear and PBS left eye). Ear swelling was measured after 24 and 48 hours using a gauge micrometer (Mitutoyo). Results show ear swelling at 48 hours as measured by the difference between the antigen-injected and PBS-injected ears for each mouse. A group of naive unimmunized mice and a group of mice immunized s.c. without OVA drops served as controls.

**Statistics.** Statistical testing was performed with Prism 7.0 (GraphPad). Normality was calculated using the D'Agostino and Pearson normality test, and the appropriate statistical test was used based on the result.  $P \leq 0.05$  was considered statistically significant. Multiple comparison tests were corrected for repeated measures.

**Study approval.** The IACUC at Baylor College of Medicine approved all animal experiments. All studies adhered to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Protocol AN 2032, "Role of dry eye in ocular surface inflammation," was approved 9/14/2017 by the Baylor College of Medicine IACUC.

## Author contributions

BYK, CSDP, and SCP performed mouse studies; FLB and BYK performed confocal microscopy; YX and BYK performed goblet cell and DC culture experiments; and BYK, CSDP, and SCP drafted the manuscript that was reviewed and approved by YX and FLB.

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