



# Mucosal tolerance disruption favors disease progression in an extraorbital lacrimal gland excision model of murine dry eye



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## ABSTRACT

Dry eye is a highly prevalent immune disorder characterized by a dysfunctional tear film and a Th1/Th17 T cell response at the ocular surface. The specificity of these pathogenic effector T cells remains to be determined, but auto-reactivity is considered likely. However, we have previously shown that ocular mucosal tolerance to an exogenous antigen is disrupted in a scopolamine-induced murine dry eye model and that it is actually responsible for disease progression. Here we report comparable findings in an entirely different murine model of dry eye that involves resection of the extraorbital lacrimal glands but no systemic muscarinic receptor blockade. Upon ocular instillation of ovalbumin, a delayed breakdown in mucosal tolerance to this antigen was observed in excised but not in sham-operated mice, which was mediated by interferon  $\gamma$ - and interleukin 17-producing antigen-specific T cells. Consistently, antigen-specific regulatory T cells were detectable in sham-operated but not in excised mice. As for other models of ocular surface disorders, epithelial activation of the NF- $\kappa$ B pathway by desiccating stress was determinant in the mucosal immune outcome. Underscoring the role of mucosal tolerance disruption in dry eye pathogenesis, its prevention by a topical NF- $\kappa$ B inhibitor led to reduced corneal damage in excised mice. Altogether these results show that surgically originated desiccating stress also initiates an abnormal Th1/Th17 T cell response to harmless exogenous antigens that reach the ocular surface. This event might actually contribute to corneal damage and challenges the conception of dry eye as a strictly autoimmune disease.

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## 1. Main body

Ocular surface inflammation is one of the hallmarks of dry eye disease (DED), a highly prevalent disorder with an incompletely understood pathophysiology. The presence of a dysfunctional tear film is the other unifying feature of the different clinical patterns of DED (aqueous deficient, evaporative and mixed types), and several lines of evidence suggest that an abnormal tear film promotes a local inflammatory response (Hessen and Akpek, 2014). Intriguingly, a Th1/Th17 T-cell-driven adaptive immune response

eventually ensues and leads to ocular surface damage (Chauhan et al., 2009; Chen et al., 2014, 2011; Coursey et al., 2014; Zhang et al., 2011), which explains the efficacy of topical cyclosporine treatment in DED patients (Hessen and Akpek, 2014). Thus, an autoimmune etiology for DED has been proposed (Stern et al., 2013, 2012), but the actual specificity of the pathogenic T cells is yet to be determined. The ocular surface is continuously exposed to airborne particles and the commensal flora, but normally it drives the expansion of regulatory T cells (Tregs) specific for these innocuous antigens that suppress unwanted inflammation. This protective mechanism is generally termed mucosal tolerance, and we have previously shown that its disruption is involved in eye drop preservative toxicity (Galletti et al., 2013; Guzmán et al., 2014). An alternative, compelling hypothesis for DED pathophysiology is that the abnormal tear film disrupts homeostatic mucosal tolerance at the ocular surface, leading to a pro-inflammatory T-cell response to any available mucosal antigen. We have recently shown this to be true in a mixed-type murine model of DED (Guzmán et al., 2015),

*Abbreviations:* DED, dry eye disease; Treg, regulatory T cells; NF- $\kappa$ B, nuclear factor kappa B; SCO + AF, scopolamine + forced air flow; LGE, lacrimal gland excision; OVA, ovalbumin; DTH, delayed-type hypersensitivity; IFN, interferon; IL, interleukin; PDTC, pyrrolidine dithiocarbamate.

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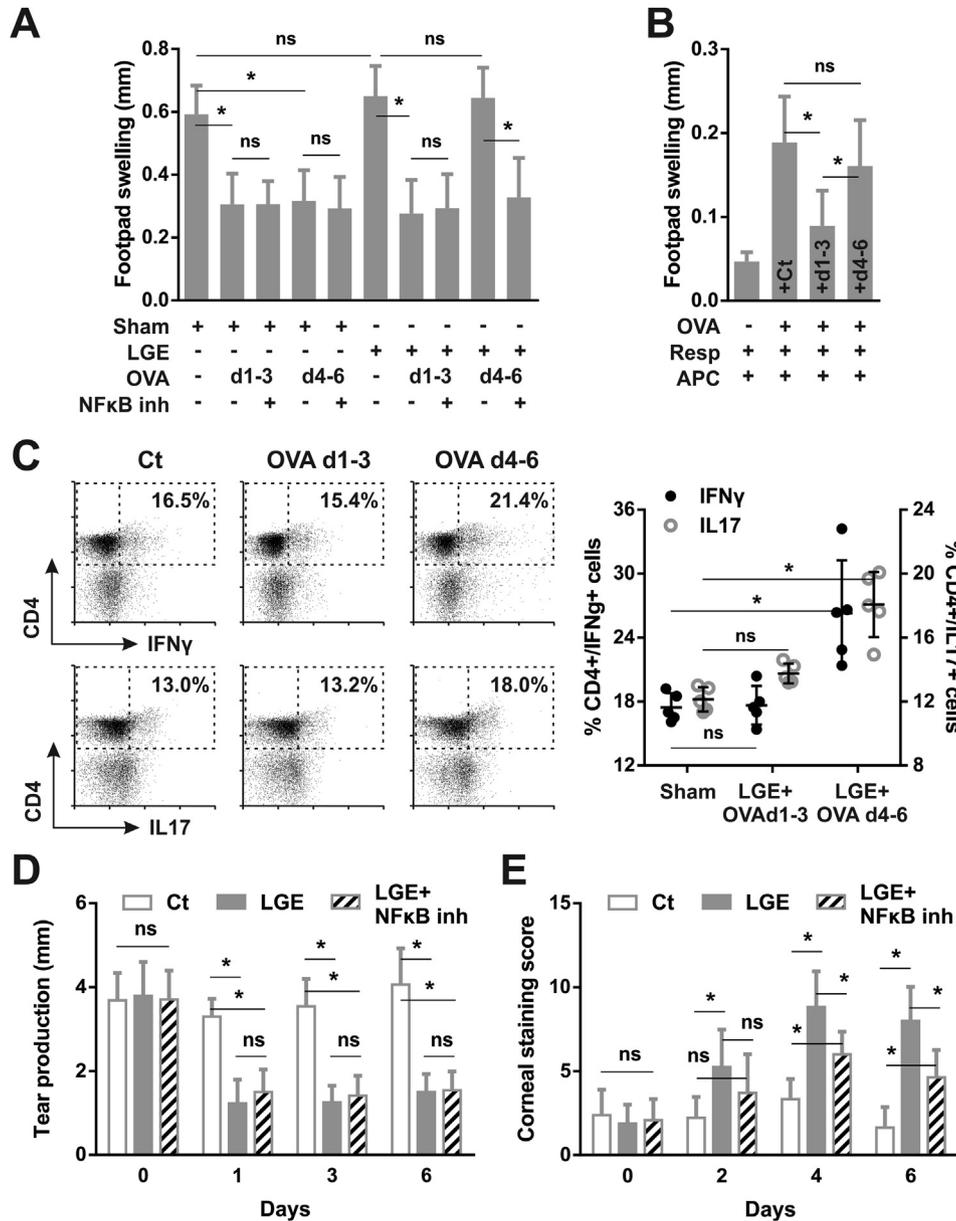
which involves suppression of tear production by systemic muscarinic blockade with scopolamine and increased tear evaporation by forced air flow (SCO + AF). This widely employed model, however, corresponds to a severe form of DED, with almost no tear secretion and lacrimal gland atrophy (Chen et al., 2013; Xiao et al., 2015). Moreover, there is the caveat that scopolamine could have direct effects on immune cells (Chen et al., 2013; Pitcher et al., 2011). Recently, a purely aqueous-deficient DED model was characterized that only involves extraorbital lacrimal gland excision (LGE), with many contrasting features with the SCO + AF model (Stevenson et al., 2014). We hypothesized that if ocular surface mucosal tolerance disruption is indeed responsible for the initiation of the pathogenic adaptive immune response in DED, this phenomenon should also be observed in a different DED model with an unrelated cause for the abnormal tear film. Therefore, in this work we set out to explore the ocular mucosal immune response to an exogenous model antigen in the LGE model of DED.

All procedures were approved by the Institute of Experimental Medicine Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Six- to eight-week-old C3H mice of either sex housed under standard conditions were used for all experiments. Each experiment was carried out at least once on either sex to account for gender-specific differences. On day 0, mice underwent anesthesia, bilateral extraorbital LGE or sham surgery, and post-operative analgesia as described by Stevenson et al. (Stevenson et al., 2014). Ovalbumin (OVA), an inert model antigen, was delivered to the ocular surface by daily instillation (5  $\mu$ l/eye of 2 mg/ml OVA in saline) either on days 1–3 (d1–3 group) or on days 4–6 (d4–6 group), because we have previously reported that at least 3 days of desiccating stress are required for mucosal tolerance disruption in the SCO + AF model (Guzmán et al., 2015). The introduction of OVA to the ocular surface milieu is required to track the specific immune response to a known antigen because the actual pathogenic antigens that drive DED remain unidentified (Stern et al., 2013). As we have previously observed that conjunctival tolerance fully develops after 48 h, mice were s.c. immunized on day 8 with 0.1 ml of 100  $\mu$ g OVA in a 1:1 saline/complete Freund's adjuvant emulsion. Then, their antigen-specific responses were quantified on day 15 by a delayed-type hypersensitivity (DTH) assay after injection of heat-aggregated OVA (500  $\mu$ g in 50  $\mu$ l of saline) in the foodpad, as previously described (Guzmán et al., 2015). For other experiments, mice were euthanized on day 7, their eye-draining (submandibular) lymph nodes were excised and T cells isolated for assaying antigen-specific cytokine production (Supplemental Table 1) or Treg activity. Corneal staining (as a surrogate for corneal epithelial damage) was assessed by fluorescein instillation and grading under cobalt-blue illumination by a masked observer according to the National Eye Institute scoring system (Chun and Park, 2014; Dursun et al., 2002). Tear production was also recorded with the aid of phenol red-impregnated filter paper strips. The details for all these techniques have been thoroughly described elsewhere (Guzmán et al., 2015).

Regarding ocular mucosal tolerance, we tested the immune response to a known antigen by instilling OVA at different time points, and then we performed systemic immunization by inoculating the same antigen in the presence of adjuvant. The lack of a strong immune response after such treatment when the antigen is delivered first through a mucosal surface is commonly referred to as mucosal tolerance. It should be highlighted that OVA was instilled in these experiments to be handled by the immune system as any other antigen indigenous to the ocular surface. As expected after immunization (Fig. 1A), LGE and sham-surgery mice developed full-blown DTH responses, whereas sham-surgery mice whose ocular surfaces had been exposed to OVA developed reduced

antigen-specific swelling (i.e., exhibited mucosal tolerance to the antigen). LGE mice exposed to OVA during the first 3 days also showed mucosal tolerance, but when the antigen was delivered on days 4–6, LGE mice failed to suppress the DTH response. Since we have previously observed that disruption of ocular surface immune tolerance was dependent on epithelial activation of the NF- $\kappa$ B pathway in the SCO + AF dry eye model (Guzmán et al., 2015), we also assessed the effect of topical inhibition of this pathway in the LGE model. As expected, the delayed change in the immune response to OVA was not observed when an NF- $\kappa$ B inhibitor (5  $\mu$ l/eye of 0.1 mM pyrrolidine dithiocarbamate [PDTCT] in saline) was instilled daily from day 1 onwards, whereas the NF- $\kappa$ B inhibitor by itself did not affect tolerant responses (Fig. 1A). These findings altogether are in line with our report on the SCO + AF model, which also exhibits a late NF- $\kappa$ B –dependent breakdown in ocular surface immune tolerance (Guzmán et al., 2015).

As mucosal tolerance at the ocular surface (and elsewhere) is mediated by peripherally induced Tregs, we tested for the presence of OVA-specific Tregs in LGE mice. We initially could not detect by flow cytometry a statistically significant difference in the number of CD4<sup>+</sup> Foxp3<sup>+</sup> CD25<sup>+</sup> T cells in the eye-draining lymph nodes of LGE mice unexposed to OVA and those instilled with OVA mice on days 1–3 and 4 to 6 (data not shown). In our experimental setting, however, OVA-specific Tregs would be expected to represent a minute proportion of the total Treg population in the lymph nodes, so we performed a local adoptive transfer of DTH assay, which is very sensitive for detecting low-frequency antigen-specific Tregs (Jankowska-Gan et al., 2013). To this aim, we isolated T cells from the submandibular lymph nodes of mice one week after LGE surgery, mixed them at 1:1:1 ratio with T cells from OVA + CFA immunized mice and OVA-pulsed T-cell-depleted splenocytes (as a source of antigen presenting cells) and injected the resulting suspensions into the footpads of naïve mice (Fig. 1B). Compared to T cells from LGE mice unexposed to OVA (Ct), T cells from LGE mice that were instilled OVA on days 1–3 markedly suppressed the inflammatory reaction elicited by the effector T cells from the immunized donor in the presence of OVA. By contrast, T cells from LGE mice that were instilled OVA on days 4–6 had no such suppressive effect. As OVA-specific regulatory T cells were readily detectable in mice exposed early to the antigen but not when done later, we then tested whether antigen-specific effector T cells were being expanded in the latter group. Given the skewing of the mucosal immune response towards a Th1/Th17 profile under desiccating stress (Chauhan et al., 2009; Chen et al., 2014, 2011; Coursey et al., 2014; Zhang et al., 2011), it would be expected that naïve OVA-specific T cells were expanded and converted to interferon (IFN)  $\gamma$ - and interleukin (IL) 17A-producing effector cells in LGE mice exposed late to the antigen. Because of the low frequency of antigen-specific T cells in wild-type mice, we resorted to in vitro amplification of the in vivo induced effector T cells prior to cytokine profiling (Supplemental Table 1). To this aim, submandibular lymph node cells were isolated as for the previous experiment and cultured for 6 days in the presence of 100  $\mu$ g/ml OVA. Then, cells were stimulated with phorbol-myristate-acetate and ionomycin in the presence of brefeldin A for 5 h, fixed and permeabilized as detailed elsewhere (Guzmán et al., 2015) and stained for IFN $\gamma$  and IL17A for flow cytometry. As depicted in Fig. 1C, there was no expansion of cytokine-producing CD4<sup>+</sup> cells in the cultures from LGE + OVA d1–3 mice compared to unexposed LGE mice (Ct), whereas those from LGE + OVA d4–6 mice exhibited ~50% increase in IFN $\gamma$ - and IL17A-producing CD4<sup>+</sup> T cells. This increase is comparable to that observed in cultures from mice immunized with OVA in complete Freund's adjuvant (data not shown), underscoring the magnitude of the response. Moreover, these results are consistent with the detectable OVA-specific Treg activity in



**Fig. 1. Mucosal tolerance at the ocular surface is disrupted in an NFκB-dependent manner.** **A**) Extraorbital lacrimal gland excision (LGE) or sham surgery was performed on day 0, and then ovalbumin (OVA) was instilled daily on days 1–3 (d1–3) or on days 4–6 (d4–6). An NF-κB inhibitor (inh) or saline as a control were instilled daily from day 1. Ocular mucosal tolerance to OVA was measured by s.c. immunization with OVA + adjuvant on day 8 and challenge with antigen one week later in one footpad. Swelling was measured after 48 h and expressed as the difference with the saline-injected contralateral footpad. **B**) Local adoptive transfer assay in which different combinations of T cells isolated on day 7 from submandibular lymph nodes of LGE mice, OVA-specific T cells from an immunized mouse and OVA-pulsed antigen presenting cells were injected into the footpads of naïve mice. Surgery was performed on day 0 and then OVA was instilled on days 1–3 (d1–3) or on days 4–6 (d4–6). Mice that underwent LGE at the same time but received no OVA served as source of control cells (Ct). Swelling was measured 24 h and expressed as the difference with the preinjection measurement. **C**) Intracellular flow cytometry data for groups Ct, OVA d1–3 and OVA d4–6 operated and instilled as described for panel B. Representative dot plots (left) and individual data (right) of submandibular lymph node cells expanded in vitro with OVA for 6 days, activated for 5 h with phorbol-myristate-acetate and ionomycin in the presence of brefeldin A, and then stained for interferon γ (IFNγ), interleukin 17 (IL17) and CD4. **D**) Tear production as measured by phenol red test and **E**) corneal fluorescein staining as graded by the National Eye Institute scoring system on days 0, 2, 4 and 6 in sham-surgery (empty bars), LGE + saline instilled mice (filled bars) and LGE + NF-κB inhibitor mice (hatched bars). For all panels, results are expressed as mean ± SD of at least 3 independent experiments with 3–6 mice per group (ANOVA with Dunnett's post hoc test). \* and ns indicate statistically significant (p < 0.05) and non-significant difference in means, respectively.

LGE + OVA d1-3 mice, and altogether show that a marked change in the ocular surface's immune response to an exogenous antigen takes place in the LGE model of DED after 3 days.

As disruption of mucosal tolerance at the ocular surface after LGE could be prevented by topical treatment with an NF-κB inhibitor (PDTIC), we then assessed the effect of such treatment on DED severity. To this aim, corneal damage was measured by surface staining with fluorescein and graded as reported elsewhere

(Guzmán et al., 2015). As shown in Fig. 1D, tear production (as measured by wetting of phenol red-impregnated filter paper) significantly decreased to about 30% of sham-surgery mice by day 1 and remained so throughout the observation period. Moreover, topical daily instillation of saline or NF-κB inhibitor had no significant effect on tear production after 6 days. However, as depicted in Fig. 1 E, a significant increase in corneal staining was observed by day 2 in LGE mice instilled with saline but not in those treated with

NF- $\kappa$ B inhibitor. Furthermore, the latter exhibited less corneal staining than their saline-treated littermates on days 4 and 6. Such reduction in corneal staining could not be ascribed to differences in tear production or in desiccating stress, as both groups measured comparably and were housed under the same conditions. It should be noted that this LGE model exhibits a similar reduction in induced tear production (as measured by the wetting test) to the SCO + AF model of DED (Guzmán et al., 2015), but the latter includes forced air flow or a low-humidity environment to increase tear evaporation. This distinction probably accounts for the lower corneal staining scores observed in the LGE model, which could be interpreted as a milder form of DED.

Despite the obvious differences in the cause of DED in both models, here we show that the LGE model exhibits the same pattern of late-onset disruption in mucosal immune tolerance at the ocular surface. The surgical DED model also seems to be driven by a change in the immune stance of the ocular surface that is triggered by desiccating stress. Our findings on the tempo of this phenomenon in DED are in agreement with the increase in Th1-associated chemokines in corneal and conjunctival cells (Coursey et al., 2014) and the early IFN $\gamma$  release by conjunctival resident NK cells (Chen et al., 2011), both of which peak during the first three days. Desiccating stress has also been associated with a stress response in the ocular surface epithelium, marked by activation of mitogen-activated protein kinases (Luo et al., 2004) and pro-inflammatory cytokine release (Li et al., 2006). NF- $\kappa$ B activation is a common downstream event in epithelial stress responses (Swamy et al., 2010), and increased epithelial NF- $\kappa$ B signaling favors pro-inflammatory conditioning of mucosal dendritic cells and effector T cells (Wullaert et al., 2011; Zaph, 2007). It is therefore conceivable that the aforementioned events induced by desiccating stress lead to sufficient epithelial NF- $\kappa$ B signaling at the ocular surface to drive the mucosal adaptive immune response towards a Th1/Th17 profile. In line with this reasoning, we here show that in the LGE model, ocular surface tolerance disruption can be prevented by topical NF- $\kappa$ B inhibition and that such blockade reduces corneal damage. At any rate, the fact that desiccating stress sways the mucosal immune response to an exogenous antigen towards a Th1/Th17 profile in both models challenges the notion of DED as a strictly autoimmune disorder (Stern et al., 2013, 2012). By contrast, it suggests that the disease could also be caused by a generalized disruption of mucosal tolerance to the many exogenous antigens (both airborne and from the ocular microbiota) that reach the ocular surface.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.exer.2016.07.004>.

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