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Topical interferon-gamma neutralization prevents conjunctival goblet cell loss in experimental murine dry eye

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ABSTRACT

Evidence suggests that the cytokine interferon (IFN)-γ released by natural killer and $CD4^+$ T cells contributes to the conjunctival goblet cell (GC) loss in dry eye. The purpose of this study was to investigate if topical neutralization of IFN-γ prevents or alleviates GC loss in an experimental desiccating stress (DS) model of dry eye. In this study, we found that topical IFN-γ neutralization significantly decreased DS-induced conjunctival GC loss. This was accompanied by decreased epithelial apoptosis, and increased IL-13 and decreased FoxA2 expression in the forniceal conjunctiva. To establish that IFN-γ produced by pathogenic $CD4^+$ T cells contributes to DS-induced GC loss, adoptive transfer of $CD4^+$ T cells isolated from DS exposed donors to naïve RAG-$1^{-/-}$ recipient mice was performed. Similar to the donor mice, topical IFN-γ neutralization decreased conjunctival GC loss, suppressed apoptosis and increased IL-13 expression in adoptive transfer recipients. In summary, this study demonstrated that topical neutralization of IFN-γ prevents GC loss via modulating apoptosis and maintaining IL-13 signaling.

1. Introduction

Dry eye is one of the most prevalent eye diseases, affecting tens of millions of people worldwide. The pathogenesis of keratoconjunctivitis (KCS), the ocular surface disease of dry eye is a multifactorial process that includes activation of stress pathways in the ocular surface epithelia by desiccation, the hyperosmolar tear film and inflammatory cytokines, such as interleukin (IL)-17 and interferon gamma (IFN-γ) that are produced by resident intraepithelial lymphocytes and infiltrating $CD4^+$ T cells (De Paiva et al., 2007, 2009, 2010; Lam et al., 2009; Niederkorn et al., 2006). Conjunctival goblet cells (GCs) are simple columnar epithelial cells that secrete the gel-forming mucin MUC5AC that stabilizes the tear film and protects the cornea. GC loss in dry eyes is often associated with a poorly protected and irregular cornea and may lead to sight-threatening corneal ulceration and perforation (Murube and Rivas, 2003; Pflugfelder et al., 1997; Stern et al., 1998a, 1998b). The mechanisms responsible for GC loss in KCS are not completely understood; however, there is increasing evidence indicating that an altered balance of T helper cell (Th) cytokines has a prominent role this process. We previously demonstrated that $CD4^+$ T cells activated by exposure to desiccating stress (DS), when adoptively transferred to naïve T-cell-deficient nude mice, were sufficient to elicit autoimmune KCS with pronounced conjunctival GC loss (Niederkorn et al., 2006; Zhang et al., 2011b).

Th1 and Th2 cytokines have been found to have opposing effects on conjunctival goblet cell development. The Th2 cytokine IL-13 has been found to induce GC hyperplasia in nonocular mucosa, such as the gut and respiratory tracts (Atherton et al., 2003; Kanoh et al., 2011; Marillier et al., 2008). We have observed that IL-13 produced by resident NKT cells has a homeostatic function in promoting conjunctival epithelial goblet cell differentiation and mucus production (De Paiva et al., 2011). IL-13 signals through STAT6 and has been found to stimulate production of GC mucin MUC5AC directly or indirectly by suppressing production of the forkhead transcription factor FoxA2, a MUC5AC repressor (Kim et al., 2008; Oh et al., 2010; Rogers, 2003). In contrast, experimental desiccating stress increased the number of cells staining positively for the Th1 cytokine IFN-γ^+ in the goblet cell zones of the conjunctiva and increased the concentration of IFN-γ in tears (De Paiva et al., 2007). This was accompanied by a decrease in IL-13/IFN-γ ratio and progressive GC loss. No change in conjunctival GC density was noted in IFN-γ-knockout mice subjected to desiccating

Abbreviations: KCS, keratoconjunctivitis sicca; GC, goblet cell; MUC5AC, gel forming mucin secreted by goblet cells; IFN-γ, interferon gamma, a cytokine produced by natural killer (NK) and $CD4^+$ T cells.

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stress; however, loss of GCs similar to wild type was observed following subconjunctival injection of IFN-γ in these mice (De Paiva et al., 2007). These findings suggest that strategies to neutralize IFN-γ may prevent dry eye induced GC loss.

One mechanism for IFN-γ induced GC loss in KCS is induction of conjunctival epithelial apoptosis. IFN-γ-knockout mice were found to be resistant to DS-induced conjunctival apoptosis; however, exogenous administration of IFN-γ to this strain significantly increased apoptosis after DS (Zhang et al., 2011a). Meaningfully, apoptosis was greatest in the goblet cell area, and MUC5AC expression was inversely associated with level of apoptosis in experimental murine dry eye, suggesting that IFN-γ may cause GC loss in DS by promoting apoptosis under DS (Zhang et al., 2011a).

The purpose of this study was to investigate if topical neutralization of IFN-γ would alleviate or prevent GC loss by maintaining IL-13 expression and modulating apoptosis using a murine DS model with features similar to human KCS.

2. Methods

2.1. Mouse model of dry eye

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Desiccating stress (DS) was used to induce experimental dry eye in C57/BL6(B6) mice, 6–8 weeks of age of both genders, by subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma–Aldrich, St. Louis, MO) into alternating hindquarters administered four times a day (8:30 AM, 11 AM, 1 PM and 4:30 PM) with exposure to an air draft and <40% ambient humidity. Mice were euthanized after 5 days of desiccating stress (DS) treatment. A group of age- and gender-matched mice that did not receive any treatment to induce dry eye served as non-stressed (NS) controls.

2.2. In vivo neutralization of IFN-γ in B6 mice subjected to DS

In vivo neutralization of IFN-γ was performed using topical application of rat anti-mouse IFN-γ IgG1 (1 mg/mL, hybridoma R4-6A2; catalog no. HB-170; American Type Culture Collection, Rockville, MD). This antibody has been used in previously reported studies to neutralize IFN-γ by inhibiting MHC class II (Ia) antigen expression in IFN-γ treated Ch3/HeJ mouse macrophages (Vogel et al., 1986) and it significantly reduced serum TNF-α concentration and brain inflammation in an experimental murine cerebral malaria model (Grau et al., 1989). Antibody neutralization of IFN-γ on the ocular surface in our model was confirmed by comparing expression of MHC Class II Ia gene in the conjunctiva of adoptive transfer recipients of $CD4^+$ T cells from donors exposed to DS for 5 days.

To evaluate the role of IFN-γ in GC loss in dry eye induced by DS, in vivo neutralization of IFN-γ was performed in B6 mice subjected to DS by topical application of 10 μL of anti-IFN-γ (DS+αIFNγ) or isotype control (rat IgG, 1 mg/mL; Vector Laboratories) (DS + IC) to the ocular surface four times daily from −3–5 days of DS.

2.3. $CD4^+$ T cell isolation and adoptive transfer

To evaluate the role of pathogenic $CD4^+$ T cells in conjunctival GC loss induced by DS, adoptive transfer experiments were performed after $CD4^+$ T cell isolation.

Spleens and cervical lymph nodes were collected from NS and DS B6 donor mice, and their cell suspensions were enriched for $CD4^+$ T cells by negative selection using a cocktail of antibodies conjugated to magnetic microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described (Zhang et al., 2012). Untouched $CD4^+$ T cells (5×10^6) were washed with PBS and adoptively transferred (i.p.) to RAG-1–deficient mice ($RAG\text{-}1^{-/-}$; B6.129S7/J; Jackson Laboratories) that lack mature B and T lymphocytes. $RAG\text{-}1^{-/-}$ recipient mice were divided into two treatment groups: DS that received $CD4^+$ T cells from B6 DS exposed donors ($n = 80$) and NS ($n = 80$) that received $CD4^+$ T cells from NS B6 donors. All $RAG\text{-}1^{-/-}$ mice were euthanized 72 h after adoptive transfer.

2.4. In vivo neutralization of IFN-γ in the recipients

To evaluate the role of IFN-γ produced by pathogenic $CD4^+$ T cells in GC loss in $RAG\text{-}1^{-/-}$ mice after adoptive transfer of $CD4^+$ T cells, in vivo neutralization of IFN-γ was performed in DS $RAG\text{-}1^{-/-}$ mice by topical application of 10 μL of anti-IFN-γ (1 mg/mL) or vehicle isotype control (rat IgG, 1 mg/mL) four times daily from −3 to 3 days of $CD4^+$ T cell adoptive transfer.

2.5. Histology

The eyes and adnexa of mice were excised and embedded in OCT compound (VWR, Suwanee, GA) or paraffin. OCT-embedded samples were cut into sagittal sections (8 μm thick) and placed onto glass slides that were stored at −80 °C. Periodic acid Schiff (PAS) staining was performed on paraffin sections and immunostaining was performed on frozen sections (2 sections per slide, 3 slides per animal, 5 animals per experimental group).

2.6. Measurement of goblet cell density

The goblet cell density was measured in PAS-stained sections from the fornix to the mid-palpebral conjunctiva overlying the distal meibomian glands superiorly and inferiorly using NIS Elements software and expressed as the number of positive cells per millimeter.

2.7. Immunohistochemistry

Cryosections were immunostained for mouse CD4 (rat anti-mouse CD4 antigen; 10 μg/mL; rat IgG2a, k; clone H129.19; BD Pharmingen). Sections in three slides (at least 100 μm apart) from each experimental group were evaluated. Positively stained cells in digital images were counted in the area where the GCs are found (fornix to mid palpebral conjunctiva) over a length of at least 500 μm in the epithelium and in the stroma and to a depth of 75 μm below the epithelial basement membrane using NIS Elements (version 4.1, Nikon, Melville, NY) and were expressed as the number of positive cells per millimeter.

2.8. Immunofluorescent staining

Cryosections were incubated with polyclonal rabbit anti-AC caspase-3 (1:100; BD Pharmingen, San Diego, CA), rabbit anti-AC caspase-8 (1:100; Novus Biologicals, Littleton, CO) or rabbit anti-FOXA2 (1:100, Abcam, Cambridge, MA) primary antibodies at 4 °C overnight. The next day, samples were incubated with goat anti-rabbit FITC-conjugated antibody for 45 min in the dark at room temperature, washed and nuclei were counterstained using propidium iodide (PI) (0.7 μg/mL). Negative controls were performed at the same time and consisted of sections incubated with secondary antibody alone. Digital images (512 × 512 pixels) of representative areas of the conjunctiva were captured with a laser-scanning confocal microscope with krypton-argon and He–Ne laser (LSM 510; Carl Zeiss Meditec, Ltd., Thornwood, NY). They were acquired with a 40/1.3× oil immersion objective. The intensity of the staining was graded in sections on three slides that were at least 100 μm apart. Briefly, in each digital image, seven elliptical regions