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Regulation of T-Cell Chemotaxis by Programmed Death-Ligand 1 (PD-L1) in Dry Eye–Associated Corneal Inflammation

Jaafar El Annan,¹ Sunali Goyal,¹ Qiang Zhang,¹ Gordon J. Freeman,² Arlene H. Sharpe,³ and Reza Dana¹

PURPOSE. Given that dry eye disease (DED) is associated with T cell-mediated inflammation of the ocular surface and that PD-L1 is an important negative or inhibitory regulator of immune responses constitutively expressed at high levels by corneal epithelial cells, the authors studied the expression and function of PD-L1 in DED.

METHODS. Dry eye was induced in untreated wild-type mice, PD-L1^{-/-} mice, and wild-type mice treated with anti-PD-L1 antibody by exposing these mice to a desiccating environment in the controlled environment chamber modified with subcutaneous administration of scopolamine. Real-time PCR was used to quantify the expression of chemokine gene transcript levels of multiple CC and CXC chemokine ligands and receptors. Epifluorescence microscopy was used to evaluate corneal infiltration of CD3⁺ T cells after immunohistochemical staining.

RESULTS. The increased expression of specific chemokine ligands and receptors in PD-L1^{-/-} corneas of normal mice is associated with significant increases in T-cell homing into these corneas. Similar, and more enhanced, increases in T-cell infiltration were observed in PD-L1^{-/-} DED mice or DED mice treated with anti-PD-L1 antibody compared with controls. In addition, the authors found significantly decreased expression of PD-L1 by corneal epithelial cells in DED and significantly increased corneal fluorescein staining score with PD-L1 functional blockade using anti-PD-L1 antibody.

CONCLUSIONS. Downregulation of corneal epithelial PD-L1 amplifies dry eye-associated corneal inflammation and epitheliopathy by increasing the expression of chemokine ligands and receptors that promote T-cell homing to the ocular surface. (*Invest Ophthalmol Vis Sci.* 2010;51:3418–3423) DOI:10.1167/iovs.09-3684

Dry eye disease (DED) affects many millions of persons, in particular women, in the United States alone.¹ The current literature on the immunopathogenesis of DED focuses on the inflammatory milieu of the tear film or conjunctiva, whereas it is corneal inflammation that is the most clinically recognizable and important ocular manifestation of DED.^{2–8}

There is growing evidence that CD4⁺ T cell-mediated inflammation plays a critical role in amplifying the pathogenesis of DED.^{9–15} Still, the manner by which this inflammation can induce corneal pathology remains poorly understood.

PD-L1 is a member of the B7 family of receptors and has a role in regulating T cell-mediated immunity.¹⁶ In vivo studies using PD-L1^{-/-} mice and anti-PD-L1 blocking antibody have provided evidence for the inhibitory functions of PD-L1 in both autoimmunity and alloimmunity. For example, it has been shown that tissue-specific PD-L1 expression protects against autoimmune diabetes, ocular inflammation, and corneal allograft rejection by inhibiting autoreactive and alloreactive T cells.^{17–20} In DED, there is increased T-cell infiltration into the conjunctiva, but, remarkably, the cornea remains relatively resistant to this infiltration.^{9–13}

Herein we test the hypothesis that decreased PD-L1 expression is associated with increased chemokine expression, increased T-cell infiltration, and increased corneal fluorescein staining. The purpose of the present study was to determine the effect of PD-L1 on modulating the expression of chemokine gene transcripts in the cornea. Additionally, we investigated the potential role of PD-L1 in the pathogenesis of DED by inducing DED in PD-L1^{-/-} mice and in mice treated with anti-PD-L1 blocking antibody to determine how the blockade or elimination of PD-L1 affects the expression of the principal T-cell chemokines and the clinical aspects of DED.

MATERIALS AND METHODS

Mouse Model of Dry Eye

Eight- to 12-week-old female C57BL/6 mice were obtained from Taconic Farms (Germantown, NY), and Charles River Laboratory (Boston, MA). Similarly aged PD-L1^{-/-} C57BL/6 mice were generated as previously described.¹⁶ In all the experiments performed, the mice were age-matched among the different groups. The protocol was approved by the Institutional Animal Care and Use Committee, and all animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Dry eye was induced by placement of mice in a controlled-environment chamber modified with subcutaneous administration of scopolamine to maximize ocular dryness as previously described.^{13–15,21,22} Age-matched mice not placed in the controlled-environment chamber were used as controls.

PD-L1 Blockade

To block PD-L1-mediated signaling, five mice were treated 1 day before dry eye induction and every other day thereafter for 10 days with antimurine PD-L1 (10F.9G2, rat IgG2b; 150 µg/mouse intraperitoneally) or control rat IgG (MP Biomedicals, Santa Ana, CA).^{17,23,24}

Measurement of Corneal Fluorescein Staining

Corneal fluorescein staining was performed at baseline (day 0) and then at days 2, 5, 7, and 9. One microliter of 2.5% fluorescein was

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applied to the lateral conjunctival sac of the mice, as previously described.^{13-15,21,22} Eyes were examined for fluorescein staining after 5 minutes with a slit lamp biomicroscope under cobalt blue light. Punctate staining was recorded in a masked fashion using the standard National Eye Institute grading system. Each of the five areas of the cornea was given a score from 0 to 3, with 0 denoting no epitheliopathy and 3 denoting diffuse severe dry eye.²⁵

Immunohistochemical Staining

The following antibodies were used for immunohistochemical staining: purified hamster anti-mouse CD3 ϵ monoclonal antibody (T-cell marker, catalog no. 553057; isotype control purified hamster IgG1, catalog no. 11121D) with a secondary antibody Cy-3 goat anti-Armenian hamster antibody (catalog no. 127135-160). The primary antibody and isotype-matched control were purchased from BD PharMingen, (San Diego, CA). The secondary antibody was purchased from Jackson Laboratories (Bar Harbor, ME).

For cross-sectional staining of the cornea, whole eyeballs were excised, frozen in OCT, cut into 7- μ m frozen sections, and fixed in acetone for 15 minutes at room temperature. Immunostaining was performed as described previously.¹³ Four to six eyeballs from four to six mice per group were used for T-cell enumeration in the cornea. Serial cross-sections per slide were studied under the epifluorescence microscope (model E800; Nikon, Melville, NY) with a 40 \times objective lens. Positively stained cells were counted in the stroma of the cornea because the epithelium showed nonspecific background signal in all the cross-sections investigated, including the isotype-matched controls, over a length of 4 mm and to a depth of 100 μ m (the thickness of the cornea). Results were expressed as the number of positive cells per square millimeter.

RNA Isolation and RT-PCR

Corneas were harvested, and corneal epithelial layers were dissected as described previously.²⁶ Four to six corneas from four to six mice were involved in each group. Each experiment was repeated three times. A combined method for total RNA isolation was used, with reagent (Trizol; Invitrogen Corp., Carlsbad, CA) for tissue homogenization on ice, precipitation of RNA in the aqueous phase using 70% ethanol, subsequent extraction, purification (RNeasy Micro Kit; Qiagen, Valencia, CA), and storage at -80°C until future use. Fixed amounts of RNA were reverse transcribed into cDNA with random hexamers (SuperScript III Reverse Transcriptase; Invitrogen) according to the manufacturer's protocol.

Real-time PCR

Quantitative PCR was performed (TaqMan Universal PCR Master Mix; Applied Biosystems, Carlsbad, CA) with dye labeled predesigned primers (FAM-MGB; Applied Biosystems) for glyceraldehyde-3-phosphate dehydrogenase GAPDH (Mm99999915_g1), programmed death-ligand 1 (PD-L1; Mm01208505_m1), CC chemokine receptor 5 (CCR5; Mm01216171_ml), CXC chemokine receptor 3 (CXCR3; Mm00438269_ml), macrophage inflammatory protein 1 α (MIP-1 α)/CCL3 (Mm00441258_ml), macrophage inflammatory protein 1 β (MIP-1 β)/CCL4 (Mm00443111_ml), regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5 (Mm01302428_ml), monokine induced by IFN- γ (MIG)/CXCL9 (Mm00434946_ml), and IFN- γ -inducible protein 10 (IP-10)/CXCL10 (Mm00444235_ml), according to the manufacturer's recommendation. PCR conditions were 2 minutes at 50°C and 10 minutes at 95°C , followed by 35 cycles of 15 seconds at 95°C and 60 $^{\circ}\text{C}$ for 1 minute using a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems). One microliter of cDNA was loaded in each well, and assays were performed in duplicate. A nontemplate control was included in all experiments to evaluate DNA contamination. Results of quantitative real-time PCR were analyzed by the comparative threshold cycle (C_{t}) method and normalized to GAPDH as an internal control.

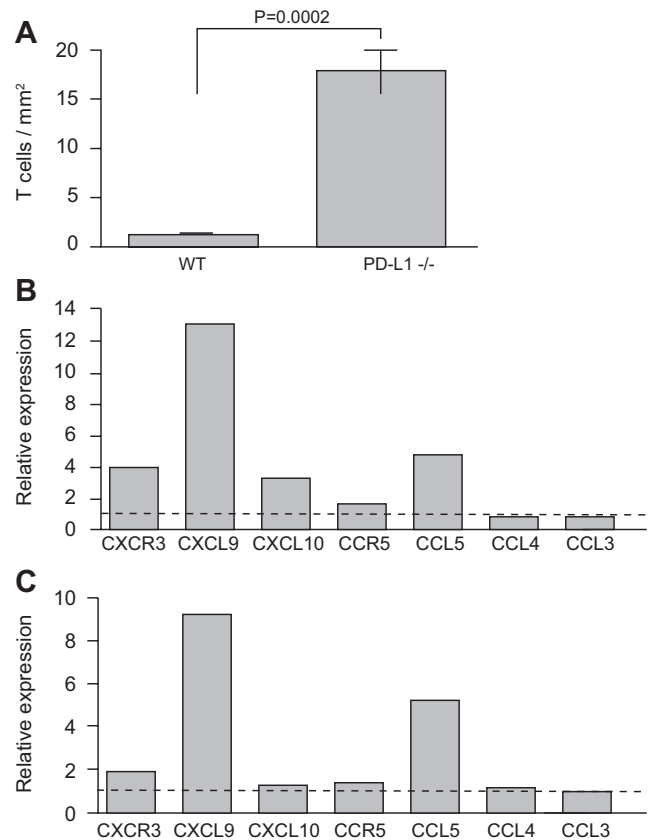


FIGURE 1. Increased T-cell infiltration and expression of chemokines in normal corneas of PD-L1^{-/-} mice. (A) T-cell counting per square millimeter of normal cornea in PD-L1^{-/-} compared with WT mice. Each group consisted of four to six mice, and data represented the mean \pm SEM. Real-time PCR analysis of chemokines (CXCR3, CXCL9, CXCL10, CCR5, CCL5, CCL4, CCL3) in whole cornea (B) and corneal epithelium (C) of normal PD-L1^{-/-} compared with normal WT mice (defined as 1, dotted line). Each group consisted of four to six mice, and data are representative of three independent experiments.

Statistical Analysis

A two-tailed Student's *t*-test was performed (Excel; Microsoft, Redmond, WA). $P < 0.05$ was taken as indicative of statistical significance.

RESULTS

Increased T-Cell Homing and Expression of Chemokines in PD-L1^{-/-} Corneas

The cornea is relatively unique in that it is largely resistant to T-cell homing under normal conditions compared with many other tissues (e.g., skin).^{27,28} To investigate the role of corneal PD-L1 in inhibiting T-cell homing to the cornea under normal conditions, we harvested globes from normal (non-dry eye) PD-L1^{-/-} and wild-type (WT) mice and immunohistochemically stained cryosections for CD3⁺ cells. We observed a significant (15.7-fold) increase in CD3⁺ T-cell homing to the cornea ($P = 0.0002$) in PD-L1^{-/-} compared with WT age-matched mice (Fig. 1A).

T cells migrate and home to peripheral tissues along a chemokine gradient.²⁹ The increased homing of T cells into the normal PD-L1^{-/-} cornea suggests that PD-L1 downregulates the expression of chemokines/receptors in the cornea. To evaluate this potential role of PD-L1, we compared the transcript level expression of chemokines/receptors in the cornea and corneal epithelium of PD-L1^{-/-} with that of WT age-

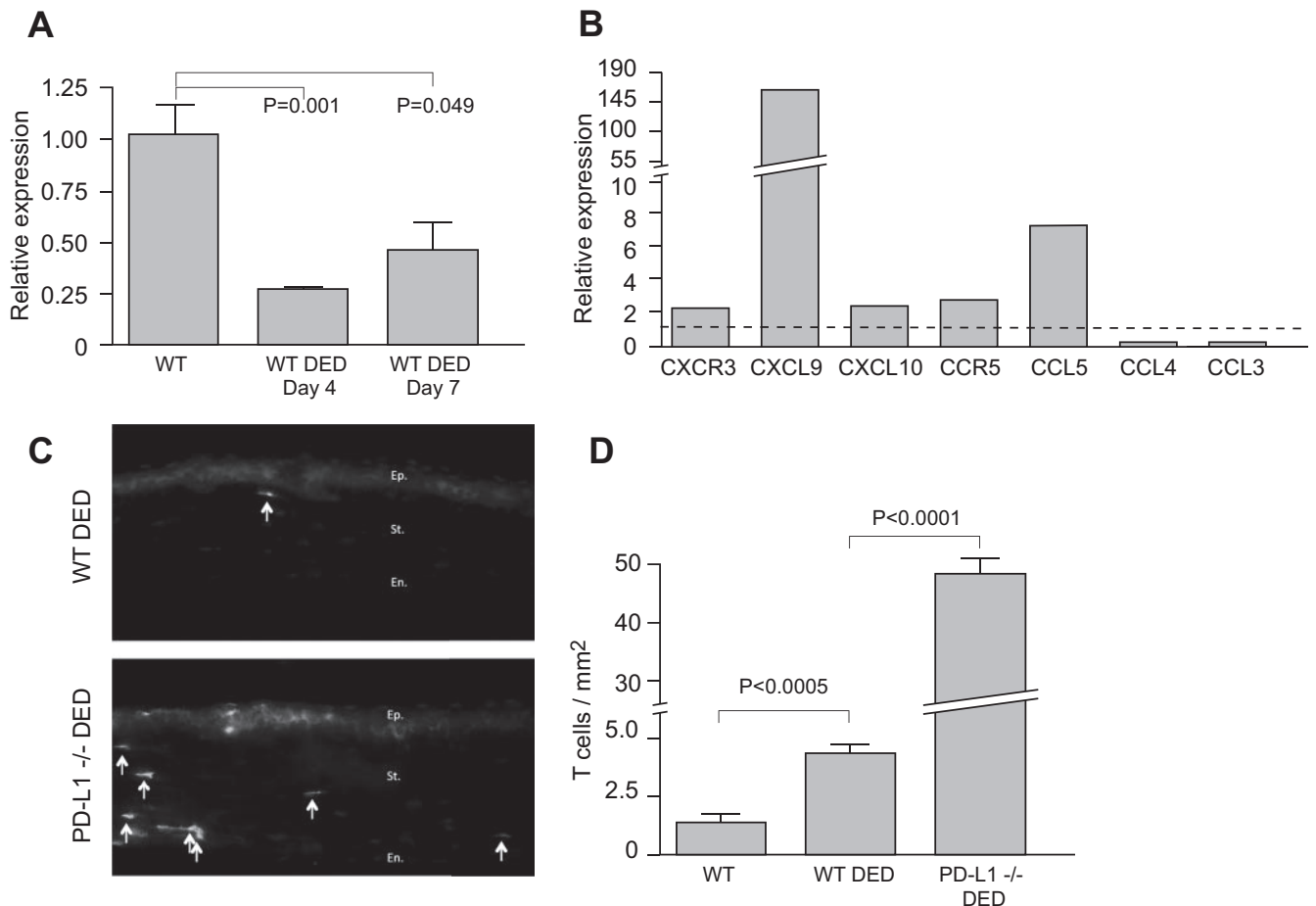


FIGURE 2. Decreased expression of PD-L1 and increased T-cell infiltration and expression of chemokines in DED. **(A)** Real-time PCR analysis of PD-L1 transcript in the corneal epithelium of DED compared with normal WT mice at day 4 and day 7 after DED induction. Data represent the mean \pm SEM and are representative of two independent experiments. **(B)** Real-time PCR analysis of chemokines (CXCR3, CXCL9, CXCL10, CCR5, CCL5, CCL4, CCL3) in whole cornea of PD-L1^{-/-} DED compared with WT DED mice (defined as 1, dotted line). Each group consisted of four to six mice, and data are representative of three independent experiments. **(C)** Representative micrographs of CD3 immunostaining of corneal cross-sections showing increased T-cell infiltration (arrows) in PD-L1^{-/-} DED compared with WT DED mice. The epithelium shows nonspecific background signal. Ep, epithelial layer; St, stromal layer; En, endothelial layer. **(D)** T-cell counting per square millimeter of cornea in PD-L1^{-/-} DED compared with WT DED compared with WT normal mice. Each group consisted of four to six mice, and data represent the mean \pm SEM.

matched mice by real-time PCR under normal conditions (Figs. 1B, 1C). We observed a marked increase in the expression level of most chemokines/receptors in the cornea and corneal epithelium of PD-L1^{-/-} compared with WT mice. This increase was especially notable for CXCL9 (13-fold, cornea; nearly ninefold, corneal epithelium) and CCL5 (nearly fourfold, cornea; nearly fivefold, corneal epithelium).

Decreased Expression of PD-L1 by Corneal Epithelium in DED

Corneal epithelial PD-L1 plays an important role in suppressing the corneal immune response.¹⁷ To investigate the expression of PD-L1 in DED, we determined the transcript level of PD-L1 by the corneal epithelium by real-time PCR (Fig. 2A) at several time points after DED induction. We observed a significant (nearly fourfold) decrease at day 4 ($P = 0.001$) and a greater than twofold decrease at day 7 ($P = 0.049$) in the transcript level of PD-L1 by the corneal epithelium of WT mice in which DED was induced compared with normal WT mice.

Enhanced Expression of Chemokines in DED PD-L1^{-/-} Corneas

To investigate the role of PD-L1 in the pathogenesis of DED in vivo, we induced DED for 10 days in PD-L1^{-/-} mice and

determined the expression levels of T-cell (both Th1 and Th2) chemokines and their receptors in the cornea by real-time PCR (Fig. 2B). We observed a marked increase in most of the chemokines/receptors in the corneas of PD-L1^{-/-} DED compared with WT DED age-matched mice. This increase was especially notable for (MIG)/CXCL9 and (RANTES)/CCL5, with 161-fold and sevenfold increases, respectively.

To detect whether the increase in expression of these chemokines/receptors was associated with increased T-cell infiltration, we enumerated T cells infiltrating the cornea 10 days after DED induction (Figs. 2C, 2D), as described. We observed a significant (more than 12-fold) increase in T cells infiltrating the corneas ($P < 0.0001$) of PD-L1^{-/-} DED compared with WT DED age-matched mice. Significantly more of these cells were CD4⁺ T cells (data not shown).

Increased Expression of Chemokines in DED Cornea after PD-L1 Blockade

To further investigate the in vivo function of PD-L1 in the immunopathogenesis of DED, we induced DED for 10 days in WT mice after systemic blockade of PD-L1 and determined the expression of the chemokines/receptors in the cornea by real-time PCR (Fig. 3A). We observed a marked increase in the expression of most chemokines/receptors in the corneas of

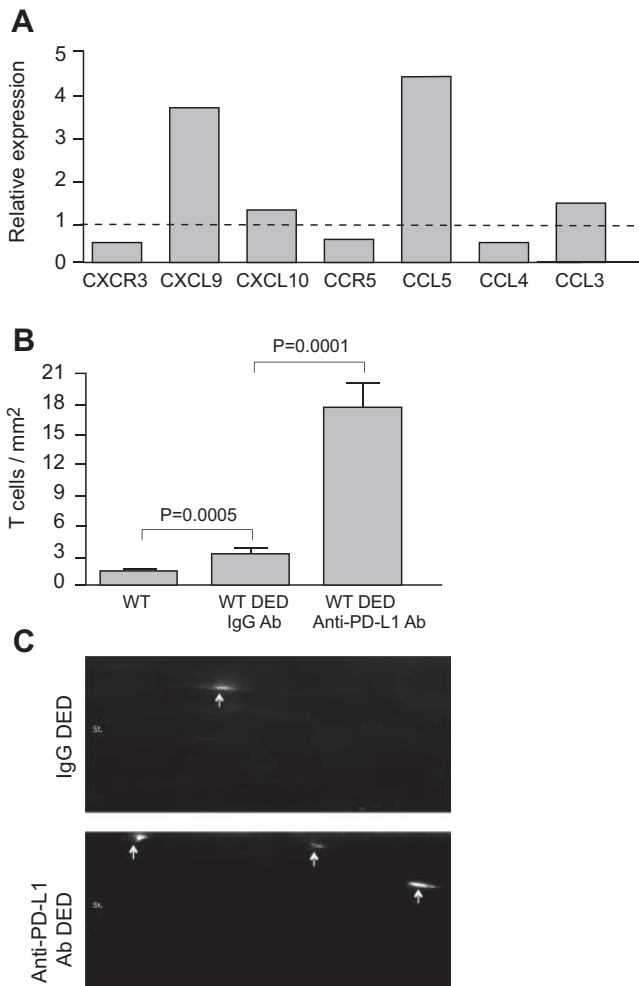


FIGURE 3. Increased T-cell infiltration and expression of chemokines after PD-L1 blockade in DED. (A) Real-time PCR analysis of chemokines (CXCR3, CXCL9, CXCL10, CCR5, CCL5, CCL4, CCL3) in whole cornea of anti-PD-L1 blocking antibody-treated DED compared with IgG (isotype)-treated DED mice (defined as 1, dotted line). (B) T-cell counting per square millimeter of cornea in anti-PD-L1 blocking antibody-treated DED compared with IgG (isotype)-treated DED compared with normal WT mice. Each group consisted of five mice, and data represent the mean \pm SEM. (C) Representative micrographs of CD3 immunostaining of corneal cross-sections with higher magnification on the stroma showing increased T-cell infiltration (arrows) in anti-PD-L1 blocking antibody-treated DED compared with IgG (isotype)-treated DED mice. St, stromal layer.

DED mice after systemic blockade of PD-L1 in DED mice compared with IgG (isotype)-treated DED age-matched mice. This increase (nearly fourfold) was especially notable for CXCL9 and CCL5.

To detect whether the increase in chemokines/receptors expression was associated with increased T-cell infiltration, we enumerated T cells infiltrating the cornea 10 days after DED induction (Figs. 3B, 3C), as described. We observed a significant (nearly sixfold) increase in T cells infiltrating the corneas ($P = 0.0001$) of anti-PD-L1 blocking antibody-treated DED compared with IgG (isotype)-treated DED age-matched mice.

Increased Severity of DED after PD-L1 Blockade

The data above suggest that corneal epithelial PD-L1 is tightly negatively correlated with the expression of chemokines and chemokine receptors. To determine the functional relevance

of corneal epithelial PD-L1 on the clinical signs of DED, we performed corneal fluorescein staining on DED mice treated with anti-PD-L1 blocking antibody compared with IgG-treated DED mice (Fig. 4). We observed a 40% increase in the CFS score at day 2 ($P = 0.005$), a 50% increase at day 5 ($P = 0.002$), a 70% increase at day 7 ($P = 0.0001$), and a 40% increase at day 9 ($P < 0.0001$) in the anti-PD-L1 blocking antibody-treated mice compared with the IgG (isotype)-treated DED age-matched mice, suggesting that inhibiting PD-L1 leads to significant exacerbation of DED.

DISCUSSION

We demonstrate here a novel function for PD-L1 in maintaining peripheral immune tolerance by downregulating the expression of chemokine gene transcripts. In addition, we demonstrate that in DED, one of the most common ophthalmic conditions, PD-L1 expression is suppressed. We also examine the functional significance of PD-L1 expression in DED by studying DED pathogenesis using a blocking anti-PD-L1 mAb and PD-L1^{-/-} mice showing that blockade or elimination of PD-L1 is associated with amplified dry eye-associated inflammation and overexpression of chemokines relevant for the recruitment of T cells (Fig. 5).

We have previously shown that corneal epithelial cells constitutively express high levels of PD-L1, which is capable of directly suppressing the local T-effector response, contributing to the immunoprivileged status of the cornea.¹⁷ Recently, it was reported that an extremely low number of T cells may be present in the cornea of the normal mouse.²⁶ Interestingly, in normal PD-L1^{-/-} mice, we found a significantly increased number of T cells (compared with normal WT mice), comparable to the numbers of T cells present in the dermis of the normal skin of WT mice, which, unlike the cornea, has a significant constitutive population of resident T cells and which, unlike the cornea, has no detectable PD-L1 expression in the uninfamed state.^{28,30-32}

The increased infiltration of T cells into the corneas of PD-L1^{-/-} mice suggested to us a potential role for PD-L1 in regulating the expression of chemokines in the cornea. Accordingly, we determined the transcript levels of chemokines and relevant receptors expressed by the cornea by real-time PCR in normal PD-L1^{-/-} mice and normal WT mice. Interestingly, we found a remarkable increase, primarily in the expression of CXCL9, CCL5, and their corresponding receptors in PD-L1^{-/-} corneas. In the cornea, chemokines can be expressed by epithelial cells, keratocytes, immune cells, and endothelial cells.³³⁻³⁵ In the PD-L1^{-/-} mice, most chemokines are expressed by the epithelial cells because chemokine expression in the stroma/endothelium (which contains keratocytes, immune cells, and endothelial cells) was below the detectable level detected by real-time PCR (data not shown).

CXCL9 is an essential chemokine for corneal T-cell infiltration, as demonstrated in a murine model of HSV keratitis in which CXCL9^{-/-} corneas are extremely resistant to T-cell infiltration.³⁶

Our data suggest that corneal PD-L1 expression is tightly inversely correlated with the expression of major T-cell chemokine gene transcripts such as CXCL9. The mechanism by which PD-L1 exerts its inhibitory function on chemokine expression requires further investigation. It is known that there are two ligands for PD-L1, CD80 and PD-1. PD-1 is not expressed by the cornea, but CD80 is expressed at low levels under normal conditions.^{17,37,38} CD80 expression is increased in corneal antigen-presenting cells (Langerhans cells in the corneal epithelium and dendritic cells in the corneal stroma) under inflammatory conditions.^{37,38} Therefore, it is possible

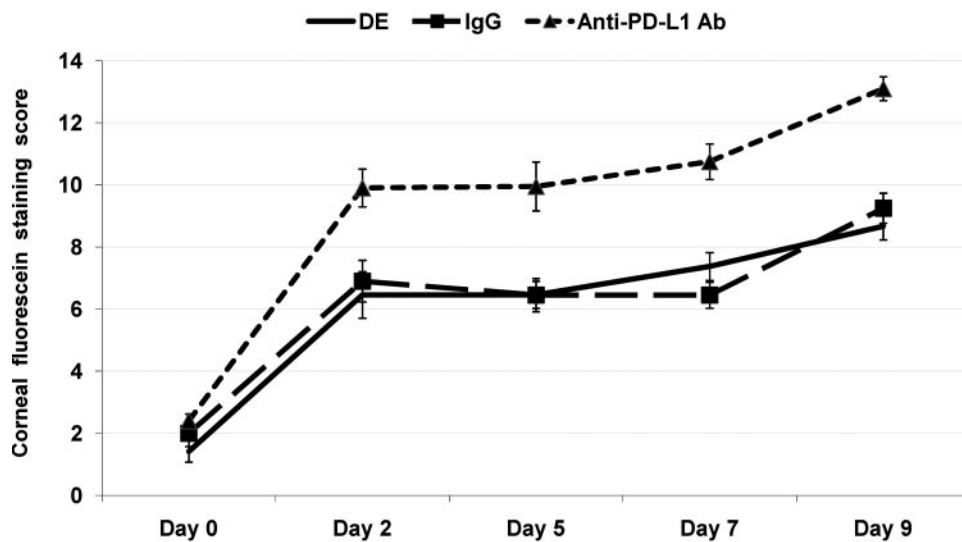


FIGURE 4. Increased severity of DED after PD-L1 blockade. Corneal fluorescein scores were significantly higher in anti-PD-L1 antibody-treated mice compared with isotype (IgG)-treated and untreated (DE) mice during induction (day 2) and during the progression phase of disease. $n = 5$ mice in each group. P -values between anti-PD-L1 antibody-treated compared with isotype (IgG)-treated group at different time points: day 2, $P = 0.005$; day 5, $P = 0.002$; day 7, $P = 0.0001$; day 9, $P < 0.0001$. Data represent the mean \pm SEM.

that PD-L1 may exert its function by binding to CD80 in the cornea or to an unidentified ligand on the corneal epithelium. On the other hand, there are myriad questions to be determined, not the least of which is the precise receptor-ligand interactions in the PD-L1 response system. Additionally, future studies are clearly needed to determine the downstream PD-L1-mediated signaling mechanisms that regulate chemokine gene expression. In many chronic inflammatory and autoimmune diseases, IFN- γ leads to increased expression of PD-L1 by parenchymal cells, which is believed to be a protective mechanism against T cell-mediated inflammation.³⁹ Interestingly, however, in DED, corneal expression of PD-L1 is suppressed,

thus disinhibiting chemokine expression normally afforded by high constitutive expression of PD-L1. PD-L1/PD-1 or PD-L1/CD80 interaction decreases the production of IFN- γ by T cells.⁴⁰ Hence, we propose that the decreased expression of PD-L1 in DED corneas facilitates T-cell infiltration and IFN- γ production in the cornea. In addition, in DED, the ocular surface epithelial expression of molecules that inhibit T-cell function is also suppressed.¹² This suggests that the decreased expression of the corneal immune privilege properties, such as PD-L1, is a precedent step that promotes corneal T-cell infiltration and IFN- γ secretion in DED.

To further substantiate the role of PD-L1 in the pathogenesis of DED *in vivo*, we induced DED in PD-L1^{-/-} mice and WT mice treated with anti-PD-L1 blocking mAb and found a significant increase in the expression levels of CXCL9 and CCL5 and relevant receptors. This was associated with a further significant increase in T-cell infiltration into the corneas of these mice and a significant increase in corneal fluorescein staining score as a result of dry eye. The systemic administration of anti-PD-L1 antibody does not allow precise localization of where this antagonism is effecting the immune response. However, the data derived from both (normal and challenged) PD-L1^{-/-} mice strongly suggest that PD-L1 does in fact regulate corneal immune response locally. In the aggregate, these data suggest that the downregulation of corneal epithelial PD-L1 amplifies dry eye-associated corneal inflammation and epitheliopathy by modulating the expression of chemokine gene transcripts, thus facilitating T-cell infiltration into the cornea in DED (Fig. 5).

Data from several laboratories have strongly suggested that tissue-specific expression of PD-L1 protects against autoimmune diseases, improves the success of tissue grafts, and promotes immune quiescence.¹⁶⁻²⁰ In addition, it has been shown that the gene transfer or treatment with molecules that stimulates PD-L1 signaling prolongs allogeneic and xenogeneic organ transplant survival.^{41,42} However, the potential therapeutic usefulness of overexpressing PD-L1, or PD-L1-mediated signaling, in ocular disease remains unknown, although topical blockade of corneal chemokine expression has been shown to suppress inflammation in microbial keratitis and to promote the survival of corneal transplants.⁴³⁻⁴⁵ Hence, our data not only shed light on important immunoregulatory mechanisms in dry eye disease, they suggest potentially novel therapeutic strategies based on enhancing PD-L1 signaling (e.g., through PD-L1 agonistic fusion protein).

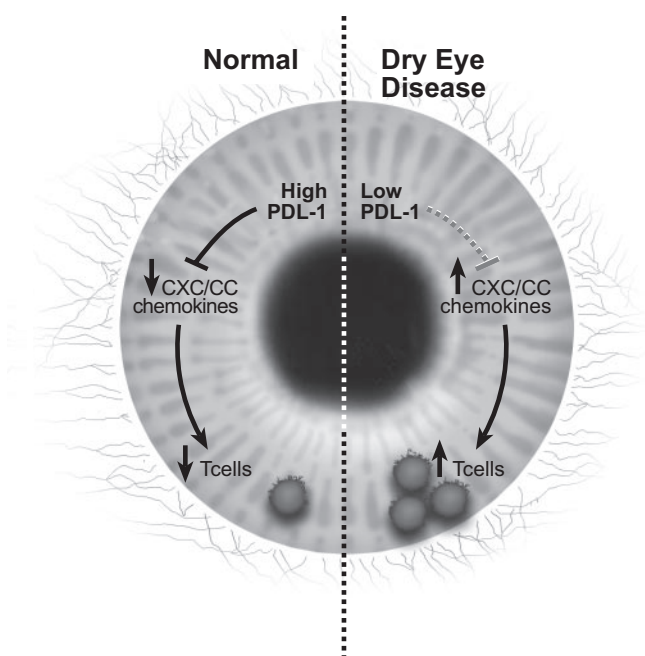


FIGURE 5. Conceptual figure summarizing proposed role of PD-L1 in the pathogenesis of DED. Under normal conditions, the high constitutive expression of PD-L1 by the corneal epithelium downregulates the expression of chemokine gene transcripts and limits T-cell infiltration into the cornea. In DED, the decreased expression of PD-L1 by the corneal epithelium suppresses the regulatory effect of PD-L1 on CXC/CC chemokine expression, which facilitates T-cell infiltration into the cornea and further amplifies DED-associated inflammation.

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