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The Function of Donor versus Recipient Programmed Death-Ligand 1 in Corneal Allograft Survival

Linling Shen,* Yiping Jin,* Gordon J. Freeman, † Arlene H. Sharpe, ‡ and M. Reza Dana2*

Programmed death-ligand (PD-L)1 and PD-L2, newer B7 superfamily members, are implicated in the negative regulation of immune responses and peripheral tolerance. To examine their function in alloimmunity, we used the murine model of orthotopic corneal transplantation. We demonstrate that PD-L1, but not PD-L2, is constitutively expressed at high levels by the corneal epithelial cells, and at low levels by corneal CD45+ cells in the stroma, whereas it is undetectable on stromal fibroblasts and corneal endothelial cells. Inflammation induces PD-L1 up-regulation by corneal epithelial cells, and infiltration of significant numbers of PD-L1+ CD45+ CD11b+ cells. Blockade with anti-PD-L1 mAb dramatically enhances rejection of C57BL/6 corneal allografts by BALB/c recipients. To examine the selective contribution of donor vs host PD-L1 in modulating allorejection, we used PD-L1+/− mice as hosts or donors of combined MHC and minor H-mismatched corneal grafts. BALB/c grafts placed in PD-L1+/− C57BL/6 hosts resulted in pronounced T cell priming in the draining lymph nodes, and universally underwent rapid rejection. Allografts from PD-L1−/− C57BL/6 donors were also significantly more susceptible to rejection than wild-type C57BL/6 grafts placed into BALB/c hosts, primarily as a result of increased T cell infiltration rather than enhanced priming. Taken together, our results identify differential roles for recipient vs donor PD-L1 in regulating induction vs effector of alloimmunity in corneal grafts, the most common form of tissue transplantation, and highlight the importance of peripheral tissue-derived PD-L1 in down-regulating local immune responses. The Journal of Immunology, 2007, 179: 3672–3679.

There has been considerable recent interest in the roles of programmed death-ligand (PD-L)1 (B7-H1) and PD-L2 (B7-DC), members of the expanding B7 family, in regulating T cell-mediated immunity (1–4). Although PD-L1 is not expressed on the surface of most resting cells at high levels, its surface expression is inducible on both hematopoietic and parenchymal cells (2, 5–7). PD-L2 expression, however, is restricted to macrophages and dendritic cells (3, 4). A large body of in vitro evidence has demonstrated that ligation of PD-Ls with their receptor PD-1 on activated T cells down-regulates TCR-mediated T cell proliferation and cytokine production (2, 4, 6–9). However, other studies have shown that PD-Ls can stimulate the T cell response, suggesting the presence of another receptor, different from PD-1, that may also regulate T cell immunity (1, 3, 10). Studies with blocking Abs and knockout mice have provided important evidence for the inhibitory functions of PD-L1 in vivo in autoimmunity (11–13). As an example, Keir et al. (14) recently have provided in vivo evidence that tissue-specific PD-L1 expression protects against autoimmune diabetes by inhibiting autoreactive T cells.

The PD-L1/PD-1 pathway has also been linked to allograft survival. It has been shown that PD-L1.Ig, in combination with cyclosporin A, significantly prolongs cardiac allograft survival (15). Similarly, concurrent use of PD-L1.Ig and anti-CD154 is able to prevent rejection of allogeneic islet cells (16). Moreover, a more recent study by Watson et al. (17) demonstrated that treatment with PD-L1.Ig alone substantially prolongs corneal allograft survival. However, all of these manipulations have involved systemic immunomodulatory strategies, leaving open the specific role of tissue-expressed PD-Ls in regulating the alloimmune response.

The cornea is known as an immune-privileged tissue whose transparency is a prerequisite for vision. Corneal transplants enjoy a higher acceptance rate compared with other solid tissue transplants (18). In this study, we test the hypothesis that PD-L1 may serve as an immunoinhibitory factor protecting the cornea graft against T cell-mediated alloimmune responses. In a murine model of orthotopic corneal transplantation involving no immunosuppression or pharmacological intervention, nearly one-half of C57BL/6 corneal grafts survive indefinitely in BALB/c recipients (19–23), whereas 15–20% of BALB/c corneal grafts survive in C57BL/6 hosts (24). The indefinite survival of a significant portion of corneal allografts allows us to readily investigate constitutive negative immunoregulatory factors in allograft rejection. Additionally, this model has a number of other distinguishing features, as follows: first, the direct pathway of sensitization has a minimal role in graft rejection due to the low numbers of MHC class II+ APC in the cornea; in contrast, the indirect pathway is the principal route of T cell sensitization mediated by host APC (18, 20, 21). Second, CD4+ Th1 cells are the primary effectors of rejection in this model, whereas B cells and CTLs play no essential function in acute corneal transplant rejection (19–23). Corneal transplantation is, therefore, a very useful model to evaluate the specific role of PD-L1 in modulating CD4+ T cell-mediated alloimmune responses in vivo.
In the present study, we demonstrate that PD-L1 is constitutively expressed on corneal epithelial cells, as well as on infiltrating bone marrow (BM)-derived cells. Abrogating PD-L1-mediated signaling via systemic PD-L1 blockade significantly enhances corneal allograft rejection. Furthermore, using PD-L1-deficient mice, we provide evidence that corneal allograft survival relies not only on the suppression of the induction of alloreactive T cells by recipient PD-L1, but also significantly on attenuated allospecific T effector responses in the target tissue by the graft PD-L1, delineating distinct functions for host-vs-graft-derived PD-L1. We thus conclude that PD-L1 expression in donor corneal tissue, as well as in the recipient hemopoietic compartment, serves critical functions in maintaining the relative immune-privileged status of corneal grafts, the most common form of transplantation.

**Materials and Methods**

**Mice**

Eight- to 12-wk-old C57BL/6 and BALB/c mice were obtained from Taconic Farms. PD-L1+/− C57BL/6 mice were generated, as previously described (12). They were housed in a specific pathogen-free environment at the Schepens Eye Research Institute animal facility. All procedures were approved by the Institutional Animal Care and Use Committee.

**Cauterization of corneal surface**

Application of electrocautery to the cornea is a standard experimental method of inducing corneal inflammation (25). Briefly, mice were anesthetized and placed under the operating microscope. Using the tip of a handheld thermal cauter (Aaron Medical Industries), five light burns were applied to the central 50% of the cornea. Immediately after the procedure, antibiotic ointment was applied. Corneas were excised 7 days after cautery application and used for cell preparation and RNA extraction.

**Immunofluorescence staining**

Eyes were frozen in OCT compound (Miles), and 6-μm-thick sections were made. The sections were blocked with 2% BSA and anti-FcR mAb (BD Pharmingen), followed by biotin-avidin block (Vector Laboratories). The sections were then stained with biotin-labeled primary Abs to PD-L1 (MIH5) or PD-L2 (TY25) (eBioscience) in combination with anti-CD45 allophycocyanin (eBioscience) and rat IgG2b PE mAb (BD Pharmingen) (13). They were incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 1 h at 37°C in a humidified atmosphere of 5% CO2. Thereafter, the fragments were incubated with PBS, then four times with PBS containing 0.025% Tween 20. Biotinylated detection mAbs were added at 2 μg/ml and after 1 h of incubation with avidin-HRP, the plates were washed again by three thorough washings in PBS. Finally, the sections were covered with mounting medium (Vector Laboratories) and examined by the eclipse E800 epifluorescent microscope (Nikon).

**Isolation of corneal cells**

Single-cell suspensions were prepared from the corneal samples by collagenase digestion, as previously described (26). Briefly, corneal buttons were aseptically removed. To separate the epithelial layer from underlying stroma, they were incubated with PBS-EDTA for 1 h. The full-thickness corneal buttons, the epithelial sheets, or the combined sheets of stroma and endothelium were cut into small fragments and incubated with 2 mg/ml collagenase type IV (Sigma-Aldrich) and 0.05 mg/ml DNase I (Roche) for 1 h at 37°C in a humidified atmosphere of 5% CO2. Thereafter, the fragments were triturated through 21-gauge needle, followed by passing through 70-μm cell strainer to create a single-cell suspension. Cells were counted with trypan blue exclusion with high viability.

**Flow cytometry**

The single-cell suspensions obtained from corneal samples were blocked with anti-FcR mAb for 30 min at 4°C in 1% BSA/0.02% NaN3/PBS. Cells were then stained with the following Abs for 45 min at 4°C: Anti-CD45 FITC, anti-CD11b FITC, anti-CD11c PE, and their isotype-matched rat IgG2a FITC and hamster IgG FITC were purchased from BD Pharmingen. Anti-PD-L1-PE (MIH5), anti-PD-L2-PE (TY25), anti-PD-1-FITC (J43), and their isotype-matched rat IgG2b PE and hamster IgG FITC were purchased from eBioscience. Cells were then washed and analyzed on an EPICS XL flow cytometer (Beckman Coulter).

**Orthotopic corneal transplantation and PD-L1 blockade**

Orthotopic penetrating keratoplasty was performed, as described previously, in one (right) eye of each mouse (16). Briefly, donor center corneas (2 mm diameter) were excised from wild-type C57BL/6, PD-L1−/−, or wild-type BALB/c mice using Vannas scissors (Storz Instruments) and placed in chilled PBS. The recipient graft bed was prepared by excising a 1.5-mm spot in the central cornea of BALB/c, C57BL/6, or PD-L1−/− C57BL/6 mice. The donor button was then placed onto the recipient bed and secured with eight interrupted 11-0 nylon sutures, followed by application of antibiotic ointment. The corneal sutures were removed 7 days after surgery. To block PD-L1-mediated signaling, allografted mice were treated from the day of transplantation with anti-murine PD-L1 (10F/9G2, rat IgG2b, 150 μg/mouse i.p. (7, 9) or control rat IgG (MP Biomedicals) three times per week for 8 wk. All grafts were evaluated using slit-lamp microscopy weekly over 8 wk. Grafts were defined as rejected when they became opaque and the iris details could not be recognized clearly using a standardized opacity-grading (ranging, 0–5) scheme (19).

**RT-PCR**

Total RNA was isolated from full-thickness corneas, cornea epithelial sheets, and the combined sheets of stroma and endothelium using RNeasy Micro Kit (Qiagen). Fixed amounts of RNA were reverse transcribed into cDNA with SensiScript reverse transcriptase (Qiagen). For PCR detection of PD-L1 and PD-L2, the following primers were used: PD-L1 forward, 5'-CATCTTCAGGCGGCAAC3'–PD-L1 reverse, 5'-dGCTTG TATCTTCAGGCGGCAAC3'–PD-L2 forward, 5'-TGCGGATTGTCGGACGC AGAG-3'–PD-L2 reverse, 5'-CTAGGATGTTGACAAAGACC3'–GAPDH forward, 5'-GAAGGGCATCTTTGGCTACAC-3'–GAPDH reverse, 5'-GCAGCGACTCTTTGTGATTAT3'–GAPDH was amplified by general settings 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. PCR products were analyzed by gel electrophoresis.

**Real-time PCR**

Frozen corneal buttons were homogenized, and RNA was isolated with RNeasy miniprep columns (Qiagen) and treated on-column with DNase I, according to the manufacturer’s protocol. RNA (300 ng) was reverse transcribed using Superscript III and random hexamers (Invitrogen Life Technologies) for 50 min at 50°C. Quantitative PCR was performed with Taqman Universal PCR Mastermix and preformulated primers for IFN-γ (assay ID Mm00801778_ml), TNF-α (assay ID Mm99999008_ml), and GAPDH (assay ID Mm99999915) (Applied Biosystems), according to the manufacturer’s recommendation. PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 60°C for 1 min using an ABI PRISM 7900 HT (Applied Biosystems). The results of real-time PCR were analyzed by the comparative threshold cycle method and normalized by GAPDH as an internal control.

**ELISPOT assay**

The ELISPOT assay was performed to delineate the contribution of direct and indirect allosensitization to graft immunity, as described previously (27). Briefly, 96-well ELISPOT plates (Multifiltronics) were coated with 4 μg/ml primary anti-IFN-γ mAb (BD Pharmingen) in sterile PBS overnight. The plates were then washed three times with PBS and blocked for 1.5 h with PBS containing 1% BSA. Next, cells were harvested from draining lymph nodes ipsilateral to the grafted eyes (n = 6). Cells harvested from lymph nodes of ungrafted animals served as controls. Responder T cells were depleted using anti-CD90 magnetic beads and an autoMACS cell separator (Miltenyi Biotec) and added to wells previously loaded with irradiated donor spleen cells as APC (direct response), or irradiated syngeneic spleen cells together with donor conditioned media (indirect response) in a final volume of 200 μl of AIM-V medium, as previously described (27). Cells were incubated for 1 h. The plates were washed and re-measured three times with PBS, then four times with PBS containing 0.025% TWEEN 20. Biotinylated anti-IFN-γ mAb (BD PharmAbs) were added at 2 μg/ml (BD PharmAbs) and incubated for 2 h at room temperature. The washing steps were repeated, and after 1 h of incubation with avidin-HRP, the plates were washed again three times with PBS/0.025% TWEEN 20 and then three times with PBS alone. The spots were then developed by the addition of the aminophthaleinborazole staining solution (Sigma-Aldrich). The resulting spots were counted and analyzed on a computer-assisted ELISPOT image analyzer (C.T.L.).

**Statistics**

Kaplan-Meier analysis was adopted to construct survival curves, and the log-rank test was used to compare the rates of corneal graft survival in different settings. Student’s t test was used for comparison of statistical
significance between groups; \( p \) values of <0.05 were considered significant.

Results

The cornea constitutively expresses PD-L1

To investigate the protein expression of PD-L1 and PD-L2 in normal corneas, we performed immunofluorescence microscopy on corneal cross-sections and flow cytometry of collagenase-digested corneal cells. Strong PD-L1 staining was detected primarily in the corneal epithelium with lower intensity staining in the stroma; there was no expression in the corneal endothelium (Fig. 1). Consistent with the immunohistochemistry results, flow cytometry revealed that PD-L1 was almost exclusively expressed by corneal parenchymal (CD45\(^+\)) rather than CD45\(^-\) BM-derived cells (Fig. 1D). In marked contrast, PD-L2 staining was negligible in the cornea (Fig. 1F). To confirm PD-L1 expression on the corneal epithelium, RT-PCR was performed; PD-L1 transcripts were readily detected in the epithelial layer, but barely identifiable in the combined layer of stroma/endothelium; no PD-L2 transcripts were detected in the cornea (Fig. 2).

PD-L1 is expressed on infiltrating CD45\(^+\) cells of the inflamed cornea

To determine whether the expression of PD-L1 is altered in the inflamed cornea, we induced corneal inflammation by application of electrosurgery, a commonly used laboratory method for the study of corneal inflammation (25). Profound corneal inflammation was evident, with attendant influx of monocytes 1 wk post-cauterization. Mice were euthanized, and their corneas were harvested. To delineate PD-L1 expression in the epithelium, corneas were separated into epithelial layers and the subjacent layer of stroma and endothelium for flow cytometric analysis. Comparing the inflamed to the normal corneas, PD-L1 expression was only slightly elevated on CD45\(^+\) epithelial cells and the few BM-derived CD45\(^+\) cells (previously shown by us to be comprised of Langerhans cells) (Fig. 3, A and C) (25). In contrast, corneal inflammation led to a dramatic increase in the number of PD-L1\(^+\) cells (>10-fold) in the stroma/endothelium, with nearly all being CD45\(^+\), suggestive of infiltrating BM-derived cells (Fig. 3, B and D). Staining with anti-CD11b further identified the majority of CD45\(^+\) cell infiltrates as monocytic cells (Fig. 3, E and F), compatible with the ingress of host APC into the graft, as previously demonstrated (28).

![FIGURE 1. Expression of PD-L1 in the normal uninflamed cornea. Cross-sections of normal corneas were dual stained with anti-CD45 FITC and biotinylated anti-PD-L1 (B), or with isotype-matched control Abs (A), followed by streptavidin-rhodamine. Normal corneas were incubated with 2 mg/ml collagenase IV for 1 h at 37°C, and the cells were harvested and stained with either anti-PD-L1-PE (D) or anti-PD-L2-PE (F), in combination with anti-CD45 FITC, and analyzed by flow cytometry. The appropriate control Abs were also used (C and E). Data shown are representative of three experiments. Ep, Epithelial layer; St, stromal layer; En, endothelial layer.](http://www.jimmunol.org/)

![FIGURE 2. RT-PCR analysis of PD-L1 and PD-L2 transcripts in the normal cornea. Total RNA from full-thickness corneas (lane 1), corneal epithelial layers (lane 2), combined corneal stroma/endothelium (lane 3), and lymph nodes (lane 4) were used to generate cDNA, and PD-L1 and PD-L2 expression was analyzed by RT-PCR. Upper panel, PD-L1 transcripts; middle panel, PD-L2 transcripts; lower panel, GAPDH transcripts used as housekeeping internal control.](http://www.jimmunol.org/)

![FIGURE 3. Flow cytometric analysis of PD-L1 expression in the normal uninflamed (A and B) vs inflamed (C–F) corneas. Corneas received electrosurgery to induce inflammation and were excised after 7 days. After treatment with 20 mM EDTA, normal and inflamed corneas were separated into epithelial sheets (A and C) and combined sheets of stroma/endothelium (B and D). The cells were collected from collagenase digestion of cornea epithelial sheets and the combined sheets of stroma and endothelium, and dual stained with anti-PD-L1-PE and anti-CD45 FITC, or their control Abs. Cells derived from inflamed full-thickness corneas were stained with anti-PD-L1-PE, in combination with either anti-CD45 FITC or anti-CD11b FITC (E and F). Data shown are representative of three experiments.](http://www.jimmunol.org/)
PD-L1 blockade enhances T cell-mediated corneal allograft rejection

To examine the role of corneal PD-L1 in regulating the alloimmune response in vivo, we used PD-L1 blockade in an orthotopic model of murine corneal transplantation. Corneal grafts were prepared from eyes of C57BL/6 mice, and then transplanted orthotopically to BALB/c mice. To abolish the PD-L1/PD-1-mediated signaling, a blocking anti-murine PD-L1 mAb (10F.9G2) was administered systemically into BALB/c recipients three times per week from the time of engraftment for up to 8 wk, a time point at which tolerance to corneal grafts is well established (19–23). At weekly intervals thereafter, graft survival was assessed clinically by slit-lamp biomicroscopy. The results of this experiment, conducted over an 8-wk interval, are shown in Fig. 4. Administration of neutralizing anti-PD-L1 mAb to allograft recipients led to a cumulative survival of only 7% at 8 wk, a rate significantly lower than the 50% survival observed among the recipients treated with control rat IgG (p = 0.019). Corneal allografts placed in anti-PD-L1 mAb-treated recipients exhibited an accelerated rejection, beginning as early as 2 wk. Furthermore, the severity of the rejection, as measured by the mean opacity score of the grafts, was greater for the grafts in the anti-PD-L1 mAb-treated BALB/c recipients (mean opacity = 3.9 ± 0.24) as compared with the control Ab-treated group (mean opacity = 3 ± 0.52).

Corneal allograft survival relies not only on recipient PD-L1 expression, but also significantly on donor PD-L1 expression

The data above clearly demonstrate that PD-L1 blockade via systemic administration of anti-PD-L1 mAb accelerates the onset, and increases the severity, of corneal allograft rejection. However, because both recipient APC infiltrating the grafts (Fig. 3, D–F), as well as the donor tissue (Fig. 1) can express PD-L1, it remained unclear at which sites the blockade of PD-L1 affected enhanced alloantigen presentation. To investigate whether the protection of corneal allograft is directly achieved through PD-L1 on corneal tissue or through PD-L1 in the recipient hemopoietic compartment, we used PD-L1−/− C57BL/6 mice as either corneal allograft donors or recipients. When PD-L1-deficient C57BL/6 mice were used as recipients of allogeneic BALB/c corneal fragments, the rejection of the corneal grafts was enhanced compared with that seen in wild-type recipients (Fig. 5). Indeed, all corneal allografts were rejected in these recipients by 4 wk postengraftment, whereas 21% survived in wild-type recipients after 8 wk (p = 0.028). Interestingly, however, when wild-type BALB/c mice were used as recipients of allogeneic PD-L1−/− C57BL/6 corneal fragments, the rejection of PD-L1-deficient grafts was also enhanced compared with wild-type allografts by the end of 8 wk (20 vs 53%, p = 0.047; Fig. 6). These findings (Figs. 5 and 6), in conjunction with the fate of corneal grafts in anti-PD-L1-treated recipients...
PD-L1 expression in the recipient, but not the donor, enhances corneal allograft survival by suppressing the induction of alloreactive T cells

To gain further insight into the mechanisms by which recipient and donor PD-L1 contribute significantly to corneal allograft survival...

(Fig. 4), strongly suggest that both donor and recipient PD-L1 contribute significantly to corneal allograft survival.

So as to ensure that PD-L1 depletion does not lead to graft failure through mechanisms other than allorecognition, corneal transplantation was also performed from PD-L1−/− C57BL/6 to wild-type C57BL/6, and wild-type C57BL/6 to PD-L1−/− C57BL/6 mice, to exclude the possibility that PD-L1 expression modifies inflammation, angiogenesis, healing, or other relevant biological events that can affect graft survival without allospecificity. No graft failure occurred in any of these isograft groups (Figs. 5A and 6), suggesting that nonallospecific responses could not explain the enhanced rejection seen with PD-L1 KO donors or recipients. Upon sacrifice of the mice after 8 wk, these isografts showed negligible T cell infiltration or neovascularization (data not shown).

FIGURE 7. The indirect pathway of allore cognition in PD-L1−/− and wild-type C57BL/6 recipients grafted with BALB/c corneas. Responder T cells were isolated from lymph nodes of PD-L1−/− or wild-type C57BL/6 recipients (n = 6) 7 days after transplantation, or from ungrafted mice, and stimulated with irradiated syngeneic APC (wild-type or PD-L1−/− C57BL/6; ratio 1:3) and BALB/c donor sonicates. The frequency of indirectly activated T cells in grafted mice was measured by the IFN-γ-ELISPOT assay and compared with their respective background signals from ungrafted mice. Significantly stronger T cell priming was evident in PD-L1−/− recipients (*, p < 0.05; **, p < 0.01). The results are depicted as the mean number of spots per million responder T cells loaded ± SEM and represent one of three independent experiments.

PD-L1 expression in the recipient, but not the donor, enhances corneal allograft survival by suppressing the induction of alloreactive T cells

To gain further insight into the mechanisms by which recipient and donor PD-L1 exerts its protective effect on T cell-mediated graft rejection, we separated tested the effect of PD-L1 depletion in the hosts vs donors on the initial priming of T cells in lymph nodes using the IFN-γ-ELISPOT assay. Because the indirect pathway of sensitization represents the predominant mechanism of corneal graft rejection (18, 20, 21), indirect recognition of corneal allograft was measured by culturing responder T cells from corneotransplanted hosts with syngeneic APC and donor sonicates (as source of alloantigen). As shown in Fig. 7, a potent indirect response was triggered in the PD-L1−/− C57BL/6 recipients 1 wk after surgery (a time point just preceding the manifestation of clinical rejection), reflected by an 8- to 9-fold increase in the frequency of IFN-γ-producing T cells over ungrafted controls in response to either wild-type or PD-L1−/− syngeneic stimulators. In contrast, only a 2- to 3-fold increase in the frequency of IFN-γ-secreting T cells was observed in wild-type C57BL/6 allograft recipients as compared with ungrafted controls similarly stimulated, suggesting that corneal transplantation elicited a significantly more vigorous T cell sensitization in PD-L1-deficient as compared with wild-type recipients.

FIGURE 8. Indirect and direct sensitization in hosts grafted with either PD-L1−/− or wild-type corneas. The frequency of alloreactive T cells activated through the indirect (A) and the direct (B) pathways was evaluated by IFN-γ-ELISPOT analysis. A, Responder T cells, isolated from lymph nodes of BALB/c recipients grafted with either wild-type or PD-L1−/− C57BL/6 corneas (n = 6), were incubated with irradiated syngeneic BALB/c APC (ratio 1:3) and C57BL/6 donor or BALB/c (negative control) sonicates. B, Responder T cells isolated from lymph nodes of BALB/c recipients grafted with wild-type and PD-L1−/− C57BL/6 corneas were incubated with irradiated allogeneic C57BL/6 or syngeneic BALB/c stimulators (ratio 1:1). Ungrafted BALB/c mice served as controls. The frequencies of both the indirectly and directly primed IFN-γ-producing T cells were comparable in BALB/c mice transplanted with either PD-L1−/− or wild-type C57BL/6 corneas. The results are depicted as the mean number of spots per million responder T cells loaded ± SEM, and are representative of one of three independent experiments.
We then assessed the function of graft-derived PD-L1 in regulating host T cell priming by using PD-L1−/− or wild-type C57BL/6 mice as donors. Compared with ungrafted controls, a higher frequency of IFN-γ-producing T cells was observed in response to donor alloantigen stimulation in BALB/c hosts harboring either wild-type or PD-L1−/− C57BL/6 allografts (Fig. 8A). However, the number of these indirectly primed T cells was comparable in these two donor groups, suggesting that graft PD-L1 has minimal effect on indirect allorecognition. Finally, donor APC-dependent direct sensitization was measured to explore the possibility of enhanced T cell priming as a result of PD-L1 deficiency on graft-derived APC. Once again, no significant difference in the magnitude of the direct response was noted in BALB/c mice grafted with either wild-type or PD-L1−/− C57BL/6 corneas (Fig. 8B).

**Donor PD-L1 promotes corneal allograft survival via attenuating allospecific effector T cell responses**

The increased susceptibility of PD-L1−/− corneal grafts to rejection and the minimal involvement of graft PD-L1 in T cell priming raised the question as to whether corneal graft-expressed PD-L1 is immunoprotective by directly suppressing the effector phase of alloimmunity, which in corneal transplantation is orchestrated almost exclusively by Th1 effectors (17, 18, 20, 21, 28). We therefore examined the Th1 cytokine IFN-γ levels in rapidly rejected wild-type and PD-L1−/− corneal allografts in addition to the frequency of allograft-infiltrating T cells. Of note, during the time of acute rejection, significantly higher IFN-γ mRNA levels were detected in PD-L1−/− allografts as compared with rejecting wild-type allografts. By contrast, the expression of IFN-γ was not detected in accepted wild-type allografts (Fig. 9A). Correspondingly, we observed an increase in T cell infiltration in PD-L1−/− allografts compared with wild-type allografts 4 wk posttransplantation (Fig. 9B). Because the normal cornea is naturally devoid of T cells (25), the T cells identified in these grafts were comprised of infiltrating alloreactive cells.

**Discussion**

There is now mounting evidence that PD-L1 plays a crucial role in down-regulating immune responses and maintaining or promoting peripheral tolerance. A noteworthy feature of PD-L1 is its broad expression on both lymphoid and nonlymphoid tissues. The peripheral tissue-specific PD-L1 expression indicates that it may have a key role in regulating or terminating immune responses in inflamed tissues. Although a majority of in vitro studies to date show that PD-L1 up-regulation in nonlymphoid tissues can inhibit T cell activity as assessed by proliferation and cytokine secretion (6, 7), the exact function of nonlymphoid tissue PD-L1 expression in vivo is still unfolding. The preponderance of in vivo evidence supports the role of parenchymal PD-L1 as a negative regulator of T cell responses in tolerance and autoimmunity, whereas other studies indicate its role as a positive regulator, suggesting the presence of potentially another receptor, different from PD-1, that may also regulate T cell-mediated immunity (10, 14).

The use of PD-L1−/− mice as both donors and recipients in the orthotopic model of corneal transplantation provides a highly useful means to dissect the in vivo functional significance of PD-L1 expression on peripheral tissues vs the lymphoid compartment. We show in this study that in the cornea, PD-L1 is constitutively expressed on epithelial cells at high levels, and is further up-regulated during inflammation (Fig. 3). Differing significantly from other tissues, MHC class II+ APC are absent in the central portions of the cornea (18, 25, 28); hence, donor APC play no appreciable role in priming T cells unless the host graft bed is significantly inflamed (29), in which case the highly immature APC in the corneal graft can express adequate MHC class II and accessory molecule expression for T cell priming. For this reason, the protective role of corneal tissue PD-L1 against potentially alloreactive effector T cells can be specifically tested using PD-L1-deficient mice as donors. As shown in Fig. 6, PD-L1−/− corneal allografts underwent significantly more rapid rejection, concurrent with increased IFN-γ levels and enhanced recruitment of effector T cells in the grafts (Fig. 9). The priming of T cells, however, was not affected by the absence of PD-L1 expression in the grafted tissue. Both PD-L1−/− and wild-type corneal allografts produced a comparable increase in the frequency of indirectly primed T cells in the regional lymph nodes (Fig. 8A). Furthermore, the lack of PD-L1 on graft-borne APC did not render them any more capable of mounting a direct allosequence than wild-type controls (Fig. 8B). This finding offers additional evidence that donor passenger leukocytes do not play a significant role in corneal alloantigen presentation. Finally, PD-L1−/− isografts remained clear indefinitely, further validating that the rapid graft rejection seen with PD-L1 depletion in the host or donor tissue was allospecific and not due to nonspecific modulation of tissue inflammation by PD-L1. Taken together, these data strongly indicate that the increased vulnerability of PD-L1−/− grafts to alloregression is not due to the priming phase, and provide strong in vivo evidence that PD-L1 expression on corneal parenchymal cells is an important immunoinhibitory factor in limiting the allospecific T effector response in the target tissue.

Given the primacy of the indirect route of allosensitization in corneal transplantation (22, 23), it is likely that PD-L1 expression
by recipient APC serves to inhibit recipient T cell priming, providing an explanation for enhanced rejection of grafts in PD-L1−/− hosts. Our data clearly suggest that systemic PD-L1 blockade with anti-PD-L1 produces a significantly more rapid and higher rate of graft rejection than can be explained by loss of PD-L1 expression in the graft alone. Therefore, to examine any protection exerted by recipient PD-L1, we performed corneal transplantation in recipient mice lacking PD-L1 expression, and noted a significant augmentation in allograft rejection as compared with wild-type recipients (Fig. 5). ELISPOT studies revealed a greater expansion of indirectly primed allogeneic Th1 cells in the draining lymph nodes in PD-L1-deficient recipients as compared with wild-type recipient controls (Fig. 7). Hence, our results indicate that PD-L1 signaling within the recipient also plays a critical role in allograft survival by regulating the induction of host allogeneic cells. Other possibilities certainly exist because of the broad expression of PD-L1 on a variety of nonhemopoietic cells. For example, it is possible that vascular endothelial cell expression of PD-L1 could engage PD-1 on T cells and regulate their effector function or peripheralization; these and other possibilities require further investigation.

The results in this study are largely consistent with a recent study by Horii and coworkers (30) showing that blockade of PD-L1 accelerated conal allograft rejection using a different mAb specific for PD-L1. However, in their study, the exact mechanisms by which systemically administered anti-PD-L1 exerted its effect on graft survival remained unclear because of widespread PD-L1 expression in both the immune compartment and corneal tissues, and by both hemopoietic and parenchymal cells of the cornea. Although these investigators detected some degree of apoptosis in infiltrating T cells in control IgG-treated, but not anti-PD-L1-treated grafts, they failed to explore the suppressive action of recipient PD-L1 on T cell priming in the host lymphoid compartment. Accordingly, because the possibility remained that graft PD-L1 alone may be insufficient to prolong corneal survival unless synergized with the function of recipient PD-L1 in vivo, the prolongation of graft survival using systemic anti-PD-L1 treatment could not be wholly explained by graft-derived PD-L1 expression. Our study using PD-L1−/− mice as donors and recipients in corneal transplantation conclusively demonstrates that each mechanism alone is sufficient to provide considerable protection to corneal allografts from immune rejection through distinct mechanisms.

In the aggregate, our data highlight the importance of peripheral tissue PD-L1 in down-regulating immune response. Interestingly, the skin, another surface ectoderm-derived tissue, has no detectable PD-L1 expression in the uninflamed state, unlike the cornea, where we report profound and constitutive expression of PD-L1. In the skin, inflammation can provoke PD-L1 expression by keratinocytes, potentially providing a means to control excessive inflammatory damage in the skin (2, 6, 31). Although the cornea and skin are both constantly exposed to environmental pathogens, constitutive overexpression of PD-L1 is unique to the cornea, perhaps as the consequence of immunogenic damage, which, in the cornea, is an unavoidable cause of blindness. Our discovery of constitutive and abundant PD-L1 expression by corneal epithelial cells and its ability to directly suppress local T cell-mediated immune destruction suggests that PD-L1 is an important factor contributing to the immune-privileged status of the cornea. This study also suggests the therapeutic potential of manipulating PD-L1 expression in peripheral tissues, possibly through tissue-specific overexpression, to improve the success of tissue grafts and to promote immune quiescence.

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