
Linda D. Hazlett, Sharon McClellan, Ronald Barrett and Xiaowen Rudner

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B7/CD28 Costimulation Is Critical in Susceptibility to Pseudomonas aeruginosa Corneal Infection: A Comparative Study Using Monoclonal Antibody Blockade and CD28-Deficient Mice

Linda D. Hazlett, Sharon McClellan, Ronald Barrett, and Xiaowen Rudner

Evidence suggests that Pseudomonas aeruginosa stromal keratitis and corneal perforation (susceptibility) is a CD4+ T cell-regulated inflammatory response following experimental P. aeruginosa infection. This study examined the role of Langerhans cells (LC) and the B7/CD28 costimulatory pathway in P. aeruginosa-infected cornea and the contribution of costimulatory signaling by this pathway to disease pathology. After bacterial challenge, the number of LC infiltrating the central cornea was compared in susceptible C57BL/6 (B6) vs resistant (cornea heals) BALB/c mice. LC were more numerous at 1 and 6 days postinfection (p.i.), but were similar at 4 days p.i., in susceptible vs resistant mice. Mature, B7 positive-stained LC in the cornea and pseudomonas Ag-associated LC in draining cervical lymph nodes also were increased significantly p.i. in susceptible mice. To test the relevance of these data, B6 mice were treated systemically and subconjunctivally with neutralizing B7 (B7-1/B7-2) mAbs. Treatment decreased corneal disease severity and reduced significantly the number of B7-positive cells as well as the recruitment and activation of CD4+ T cells in the cornea. IFN-γ mRNA levels also were decreased significantly in the cornea and in draining cervical lymph nodes of mAb-treated mice. When CD28−/− animals were tested, they exhibited a less severe disease response (no corneal perforation) than wild-type B6 mice and had a significantly lower delayed-type hypersensitivity response to heat-killed pseudomonas Ag. These results support a critical role for B7/CD28 costimulation in susceptibility to P. aeruginosa ocular infection. The Journal of Immunology, 2001, 166: 1292–1299.

Keratitis caused by Pseudomonas aeruginosa is one of the most rapidly developing and destructive diseases of the cornea and is characterized by severe corneal ulceration and extensive stromal destruction. It is a serious problem for users of extended wear contact lenses (1, 2), who have an ~37 times higher risk of developing bacterial keratitis than individuals who use daily wear, rigid, gas-permeable contact lenses (3). Because of its rapid progression and the notorious resistance of P. aeruginosa to antibiotics, the infection is difficult to treat and leads to blindness in the infected eye, with the need for corneal transplantation.

Many clinical features of the disease are reproducible in rodent models, including the rat (4) and mouse (5, 6). The mouse scarring model of Pseudomonas keratitis has been well characterized, and the genetic susceptibility of C57BL/6 (B6) (7) and related inbred strains (8) to eye infection resulting in perforation of the cornea and blindness correlated with a CD4+ T cell (Th1)-regulated inflammatory response. That the tissue-destructive stromal inflammation is mediated by T lymphocytes was evidenced by the decreased stromal inflammation in mice that were mAb depleted of CD4+ T lymphocytes or in which IFN-γ was neutralized before corneal infection (7). In contrast, no T effector cells were detected in the infected cornea of resistant, Th2 responder mice, such as BALB/c; that also exhibited a significantly lower delayed-type hypersensitivity (DTH) than Th1 responder strains (8).

The normal, uninflamed cornea, unlike most other tissue sites, lacks professional APC that are capable of presenting foreign Ag to CD4+ T cells (9). However, Langerhans cells (LC) are present in the adjacent conjunctival epithelium. Following experimental extended wear contact lens usage (10) or P. aeruginosa infection (11), LC migrate from the conjunctiva into the central cornea. LC resident in the conjunctival tissue are immature cells with limited APC function, but cytokines that are produced by infected tissue or the products of the pathogen itself can stimulate the migration and maturation of these cells (12). In this regard, cytokine mRNA transcripts are up-regulated early after P. aeruginosa corneal infection (13), but the products may be labile without T cell stimulation.

Maturational events that enhance the capacity of LC to present Ag to CD4+ T cells include increased expression of the CD80 and CD86 (B7 family) costimulatory molecules (14, 15). In a mouse model of HSV keratitis, immunostaining for CD80 (B7-1) was first detected at 3 days postinfection (p.i.), whereas CD86 (B7-2) was constitutively expressed on conjunctival LC. Early LC migration and B7-1 expression were independent of T cells, but T cells were required for the massive accumulation of B7-1+ LC in the cornea at the onset of inflammation (16).

To discern the role of B7/CD28 costimulation in P. aeruginosa infection, we compared disease induced in mice receiving B7-1/B7-2 mAb therapy before and after infection with P. aeruginosa to disease induced in control mAb-treated animals and in mice deficient for expression of CD28 (CD28−/−). Disease severity was
significantly reduced in B7-1/B7-2 mAb-neutralized mice and correlated with decreased recruitment and activation of CD4+ T cells in cornea and reduced mRNA transcript levels of IFN-γ in both cornea and draining cervical lymph nodes (CLN) p.i. Disease also was reduced in CD28−/− vs wild-type B6 mice, and this correlated with a significantly reduced DTH response to pseudomonal Ag. Collectively, these data support a critical role for B7/CD28 signaling in the susceptibility response to *P. aeruginosa* ocular infection.

Materials and Methods

**Mice**

Female, B6, BALB/c, and CD28−/− mice on the B6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) at 8 wk of age. Mice were housed in accordance with the National Institutes of Health guidelines, and all procedures in this study conformed to the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Vision Research.

**Bacterial preparation**

*P. aeruginosa* strain 19660 was purchased from American Type Culture Collection (Manassas, VA) and were maintained and prepared as described previously (7, 8). The stock culture was maintained on tryptic soy broth (PSTB) slants (PSTB solidified with 1.7% agar: Difco, Detroit, MI) at 4°C, and fresh slants were prepared every 2 wk. Cultures were grown in PSTB at 37°C on a rotary shaker at 150 rpm for 18 h to an approximate OD of 1.6 at 540 nm, centrifuged at 6000 × g for 10 min at 15°C, and washed with, then resuspended in 5 ml of sterile saline to a concentration of 1.0 × 10^8 CFU/ml. For infection, two dilutions (1/10 each) were made in sterile saline for a final concentration of 1.0 × 10^7 CFU/ml.

**Infection and ocular response**

Mice were anesthetized, and the left cornea was scarified with a 25 5/8-gauge needle as described previously (7, 8). A 5-μl aliquot containing a 1.0 × 10^8 CFU/ml suspension of bacteria was applied to the wounded cornea, and ocular disease was evaluated daily up to 7 days p.i. Corneal disease was graded as previously described (17): 0 = clear or slight opacity, partially or fully covering the pupil; +1 = slight opacity, fully covering the anterior segment; +2 = dense opacity, partially or fully covering the pupil; +3 = dense opacity, covering the entire anterior segment; and +4 = corneal perforation or phthisis. Mean clinical scores were calculated by summation of the scores for each group (n = 5/group/time) of mice divided by the total number of mice scored at each time point.

**LC in epithelial flat mounts**

At 1, 4, and 6 days p.i., epithelia were harvested (n = 5/group/time) and stained with ADPase as previously described (18, 19). Briefly, enucleated eyes were placed in 0.02 M EDTA-PBS buffer, pH 7.2, at 37°C for 3 h. Sections were cut, mounted onto poly-L-lysine-coated slides (Poly-L, Immunotech, West Grove, PA). For B7-1 staining, the same epithe- 

**Dual labeling of CLN**

 Sections were incubated with DEC-205 mAb (1/200) for 1 h and washed, and goat anti-rat FITC-conjugated Ab (1/100; Jackson ImmunoResearch, West Grove, PA) was applied for 1 h. The secondary Ab, rabbit Pab, was obtained by Western immunoblot and found specific against *P. aeruginosa* strain 19660 heat-killed Ag (data not shown), was applied to the sections (1/25) and incubated for 1 h, followed by a biotinylated goat anti-rabbit IgG (1/500; Jackson ImmunoResearch) for 1 h. Control sections were similarly treated with naive rabbit serum (NRS) and photography was performed as described above.

**Monoclonal Ab neutralization**

B6 mice (n = 5/group/time) were injected subconjunctivally and i.p. with neutralizing hamster and rat anti-mouse mAbs against B7-1/B7-2 (PharMingen). Intraportal injection (50 μg of each mAb) were given every other day beginning on day 14 before *P. aeruginosa* challenge and ending 5 days p.i. Before challenge, anti-B7-2 (3 days, 10 μg/10 μl) and anti-B7-1 mAbs (1 day, 10 μg/10 μl) were injected subconjunctivally. Control B6 mice (n = 5/group/time) were injected with a nonspecific anti-HLA-DR5 mAb. Eyes were examined, and mean clinical scores were calculated.

**Immunostaining**

Infected eyes from B7 (B7-1/B7-2) or HLA-DR5 mAb-treated (n = 2/group/time) B6 mice were enucleated at 5 days p.i. Eyes were embedded in Tissue-Tek, as described above and previously (7, 8). Ten-micrometer sections were cut, mounted onto poly-l-lysine-coated slides (Poly-sciences), and incubated for 1 h with primary mAbs specific for CD4 (rat IgG2a, clone H129.19, 1/100) and CD25 (IL-2R, rat IgM, clone 7D4, 1/50; all from PharMingen). Sections were incubated with 0.3% hydrogen peroxide for 30 min, blocking endogenous peroxidase activity, and for 1 h with a biotinylated secondary Ab, anti-rat IgG2a (1:25; CD4) or anti-rat IgM (IL-2R, 1:100; all from Pharmingen). Sections were counterstained with hematoxylin. Sections were incubated for 30 min before applying 3,3′-diaminobenzidine tetrahydrochloride (Pierce, Rockford, IL) for 10–15 min. Control sections were incubated similarly with HLA-DR5, as described previously (7, 8).

**RT-PCR**

Total RNA was isolated from corneas and CLN of B6 mice treated with anti-B7-1/B7-2 vs anti-HLA-DR5 mAb (n = 5 mice/group) at 5 days p.i. and from corneas and CLN of B6 and wild-type B6 mice (n = 5/group/time) at 3, 5, and 7 days p.i. Total RNA (50 ng) was reversed transcribed using Random primers (Life Technologies, Grand Island, NY) and Sensiscript reverse transcriptase (Qiagen, Valencia, CA) in the presence of 10 U of RNase inhibitor (Promega, Madison, WI). Amplification of cDNA was described above at 3 and 6 days p.i. from B6 and BALB/c (n = 5/group/time) mice and at 5 days p.i. from B7-1/B7-2 and HLA-DR5 mAb-treated (described below) mice (n = 5/group) and were fixed in 2% paraformaldehyde/0.1 M cacodylate buffer for 4°C for 15 min. Sheets were washed four times at 4°C in 0.1 M cacodylate buffer for 10 min each time and were blocked with 0.1 M PBS containing 1% BSA (Sigma). Sheets were incubated in DEC-205 mAb (1/2) at 4°C overnight, washed in PBS/BSA, and incubated for 1 h at 37°C in FITC-conjugated goat anti-rat IgG (1/100; Jackson ImmunoResearch, West Grove, PA). For B7-1 staining, the same epithelial sheets for washed 15 min in PBS/BSA, blocked with rat IgG (1/10; Jackson ImmunoResearch) for 1 h, and incubated for 2 h at 37°C with biotin-conjugated B7-1 mAb (1/25). Three PBS/BSA washes (10 min each) were performed before incubation in streptavidin-conjugated rhodamine (1/40; Jackson ImmunoResearch) at 37°C for 1 h. B7-2 staining was performed as described for B7-1. Sheets were placed on glass slides in a mountant containing 10 mg p-phenylenediamine, 0.01 M PBS, and glyc erol. Representative areas were photographed with a Zeiss Axiopt photomicroscope. Identical FITC- and rhodamine-stained fields were digitized with a SPOT digital camera (Sterling Heights, MI) and overlaid using Meta-Morph (Universal Imaging, West Chester, PA). Dual-labeled cells were counted on at least eight fields (each at ×200) for each group of mice as described above, and the number of positive cells was expressed as the total number of cells counted.

**Infection and ocular response**

Mice were challenged with *P. aeruginosa* strain 19660 heat-killed Ag (data not shown), was applied to the sections (1/25) and incubated for 1 h, followed by a biotinylated goat anti-rabbit IgG (1/500; Jackson ImmunoRese
conducted with Taq polymerase (Life Technologies) in a GeneMate Thermal Cycler (ISC BioExpress, Kaysville, UT). All primers were synthesized by Life Technologies. The primer sequences were 5’-TGCACTGGCTTGTGACAGCTCTCCTC-3’ for IFN-γ (36 bp) and 5’-GTGGGCC GCTCTAGGCAACCAA-3’ and 5’-CTCTTTGGAAGTACGAGGATTTC-3’ for β-actin (539 bp). The cycling conditions used were 94°C for 45 s, 59°C for 30 s, and 72°C for 1 min for 35 cycles, with a final extension at 72°C for 10 min. The same amount of RNA (without RT reaction) was subjected to PCR amplification as a negative control to verify the absence of DNA contamination in the RNA samples. Twenty microliters of final PCR products were analyzed by electrophoresis (1.2% agarose gel with ethidium bromide). Bands were visualized under UV transillumination and quantitated using an AlphaImager 2000 Documentation and Analysis system (a Innotech, San Leandro, CA). Integrated density values (IDV) for the IFN-γ PCR product were corrected for the amount of β-actin on each sample. Data are expressed as the mean IDV of three to five samples from separate mice.

DTH assay
For Ag challenge, a similarly prepared culture (as described above, 1.0 × 10^8 CFU/µl) was heat-killed (60 min at 56°C) and diluted 1/10 in sterile saline to a final concentration of 1.0 × 10^7 CFU/µl. At 5 days p.i., 1.0 × 10^8 CFU heat-killed P. aeruginosa cells (10 µl in 0.01 M PBS) were injected into the ear of CD282 mice and wild-type B6 mice ipsilateral to the infected eye. PBS was injected similarly into the contralateral ear as a control. Ear thickness was measured with a dial pocket gauge (L.S. Starrett, Small Parts, Miami Lakes, FL) before and at 24 and 48 h after Ag challenge. DTH was calculated as previously described (20).

Statistical analysis
Unpaired Student’s t test was used to determine the significance of the mean clinical scores, ADPase, B7, and dual staining of LC, RT/PCR, and DTH data. A p = 0.05 confidence interval was used to determine the level of significance. All experiments were repeated at least once, and representative data typical of a single experiment are shown.

Results
Infection in B6 and BALB/c mice
At 3–7 days p.i. significant differences were observed in the mean clinical scores between the two groups of mice, with a milder course of disease (no grade above +2 throughout 7 days p.i.) in BALB/c mice. Initially (1 day p.i.), BALB/c mice had faint opacity covering the anterior segment (+1), while B6 mice exhibited faint to dense central opacity covering the pupil (+2; p = 0.0948). By 3–7 days p.i. BALB/c mice had slightly increased (+2) opacity. Disease in B6 mice was more severe (+3) during this time (p = 0.0001 at 3 and 5 days p.i., respectively), and, as expected, only the corneas of B6 mice perforated (+4) by 7 days p.i. (p = 0.0001) (7, 8).

ADPase staining
Because of the important role of APC in Ag presentation to T cells, we next tested whether the number of LC infiltrating the cornea p.i. differed between the two groups. The data are shown in Fig. 1. A significant (p = 0.0001) 2-fold greater number of LC was seen in the cornea of B6 vs BALB/c mice at 1 day p.i. By 4 days p.i. LC in cornea had increased in both groups, but was not significantly different (p = 0.176). By 6 days p.i. LC were increased slightly in BALB/c mice, but had doubled in number in corneal flat mounts of B6 mice (p = 0.0005).

Dual Ab staining
B7-2 staining (data not shown) confirmed the results of an earlier report (16) that found B7-2 constitutively expressed in uninfected mouse conjunctiva. We next tested whether B7-1 expression differed between the two groups. B7-1 immunostaining was not detected in the cornea or conjunctiva until 1 day p.i. (data not shown) in either group. Dual labeling, representative of staining in BALB/c (data not shown) and B6 mouse cornea, is shown in Fig. 2 at 3 days p.i., a time when T cells begins to infiltrate the cornea of B6 mice (7). Fig. 2 illustrates FITC label for the DEC-205-specific LC marker (Fig. 2A), rhodamine staining for B7-1 (Fig. 2B; identical field as Fig. 2A), yellow-stained cells (Fig. 2C) that are dual labeled, and an HLA-DR5-reacted negative control (Fig. 2D). All cells that express the DEC-205 Ag do not stain for B7-1. Table I depicts the quantitative data from this experiment. No significant difference in the mean number of dual-labeled LC was seen in B6 vs BALB/c mice at 3 days p.i. (30% for both), but significantly (p = 0.0001) more of these cells were present in B6 (24%) vs BALB/c (18%) corneal epithelial flat mounts at 6 days p.i.

Ag in draining CLN
LC migrate from cornea to draining CLN where they present viral Ag to naive T cells (16). Whether bacterial Ag from the infected cornea...
could be detected associated with DEC-205-positive cells in draining CLN had not been examined. Therefore, CLN ipsilateral to the infected eye in both groups of mice were removed at 1 and 6 days p.i. Tissue sections were dual labeled with a DEC-205 mAb and anti-
Pseudomonas pAb. These data are shown in Fig. 3. Immunostaining for DEC-205 and that for Pseudomonas Ag were observed in the outer and paracortical regions of the CLN of B6 (Fig. 3, A and B, respectively) and BALB/c (data not shown) mice at all times examined. All DEC-205-positive cells did not dual label (Fig. 3 C), suggesting that some may not have been associated with Ag. Immunostaining was similar in distribution in CLN of B6 and BALB/c mice, but was patchy and sparse in the latter group. Control sections incubated with NRS were negative (Fig. 3 D). Table II depicts quantitative data from this experiment at 1 and 6 days p.i. Approximately 2-fold more dual-labeled Ag-associated LC were present in the CLN of B6 vs BALB/c mice at both 1 day (84 vs 33%) and 6 days (78 vs 37%) p.i. (p = 0.0175 and p = 0.0001, respectively).

**Table I. Labeling with DEC-205 and B7-1 at 3 and 6 days p.i.**

<table>
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<th>No. Labeled Cells</th>
<th>Strain</th>
<th>Significance</th>
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<tbody>
<tr>
<td></td>
<td>BALB/c</td>
<td>C57BL/6</td>
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<td>393&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Dual</td>
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<td>117</td>
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<tr>
<td>6 days p.i.</td>
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<td></td>
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<td>DEC-205</td>
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<td>289&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dual</td>
<td>52</td>
<td>69</td>
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<sup>a</sup> BALB/c, n = 9 fields, B6, n = 8 fields.
<sup>b</sup> The number of dual labeled cells in BALB/c vs B6 was not significantly different at 3 but was at 6 days p.i.
<sup>c</sup> BALB/c n = 6 fields, B6, n = 10 fields.

**FIGURE 3.** CLN stained with DEC-205 mAb and anti-
pseudomonas pAb. DEC-205-positive LC (A) and P. aeruginosa Ag-positive cells (B) were detected in draining CLN, and some, but not all, the cells exhibited dual staining (C) in B6 and BALB/c (data not shown) mice at all times tested p.i. (6 days p.i. illustrated). Control tissue, similarly incubated, but with NRS instead of the specific primary Ab was negative (D). Magnification, ×234.

**FIGURE 4.** Mean clinical scores of ocular disease in B7-1/B7-2 vs HLA-DR5 mAb-treated B6 mice. Significant differences were observed at 3, 4, and 5 days p.i. (p = 0.0007, p = 0.004, and p = 0.002, respectively).

**Monoclonal Ab neutralization**

To determine the relevance of these data, B6 mice were treated with B7-1/B7-2 or HLA-DR5 mAb before and after infection with P. aeruginosa. Ocular disease was graded, and mean clinical scores were calculated (1–5 days p.i.). These data are presented in Fig. 4. At 1 day p.i. both groups had similar disease grades (+1), but beginning on day 2, control mice began to consistently show slightly increased opacity (+2). At 3, 4, and 5 days p.i., B7-1/B7-2 mAb-treated mice had a significantly milder course of disease compared with control mice (p = 0.007, 0.004, and 0.002 at 3, 4, and 5 days p.i., respectively). Representative eyes from both groups were photographed at 5 days p.i. using a slit lamp, and the data are shown in Fig. 5. B7-1/B7-2 mAb-treated mice had slight opacity covering the anterior segment (Fig. 5A), while HLA-DR5 mAb-treated animals had dense opacity covering the anterior segment (+3 score) or corneal perforation (Fig. 5B). To confirm the neutralizing effects of mAb treatment, we examined B7-1 and B7-2 immunostaining in the cornea of the two groups of treated mice at 5 days p.i. These data are shown in Table III. B7-1 and B7-2 expression in cornea was reduced significantly (71% (p = 0.0009) and 82% (p = 0.0001), respectively) in the experimental vs control mAb-treated group, confirming the effectiveness of the blockade.

**FIGURE 4.** Mean clinical scores of ocular disease in B7-1/B7-2 vs HLA-DR5 mAb-treated B6 mice. Significant differences were observed at 3, 4, and 5 days p.i. (p = 0.0007, p = 0.004, and p = 0.002, respectively).
Immunostaining

Previously we showed that depletion of CD4+ T cells in B6 mice induced disease outcome after *P. aeruginosa* infection (7). Therefore, we tested whether T cells were affected by treatment with B7-1/B7-2 vs HLA-DR5 mAb, contributing to the improved outcome in the B7-treated animals. Representative examples of immunostaining for CD4 and IL-2R T cell surface markers in B6 mice at 5 days p.i. are shown in Fig. 6. Qualitatively fewer T cells were detected in the cornea of B7-1/B7-2 mAb-treated (Fig. 6B) vs control Ab-treated B6 mice (Fig. 6A). A few of these cells in anti-HLA-DR5-treated (Fig. 6C) mice were IL-2R+ but no activated T cells were detected in the cornea of B7-1/B7-2 mAb-treated mice (Fig. 6D). No immunostaining was detected in the cornea of B6 mice when anti-HLA-DR5 was substituted for either specific primary mAb (data not shown).

**RT-PCR**

As we had previously correlated a Th1 response and increased levels of IFN-γ with susceptibility in B6 mice (7), we next used RT-PCR to test B7-1/B7-2 vs HLA-DR5 mAb-treated B6 mice for mRNA transcript levels of IFN-γ in cornea and draining CLN at 5 days p.i. The results are shown in Fig. 7 as a representative agarose gel and a graph plotting the band IDV. Neutralization of B7-1/B7-2 significantly reduced IFN-γ mRNA expression in the cornea (51%; *p* = 0.04556) and CLN (31%; *p* = 0.01778) compared with HLA-DR5 mAb treatment.

**CD28-deficient mice**

Because of the potential for anti-B7-1/B7-2 mAb itself to provide signaling in interaction with CD28, we tested the response to infection in CD28−/− mice. These data are presented in Fig. 8. At 1 day p.i., the responses of both groups were similar and in the +1 to +2 range. By 3–7 days p.i., CD28−/− mice had lower disease grades, which differed significantly compared with that in wild-type mice (*p* = 0.0054, *p* = 0.0014, and *p* = 0.0001 at 3, 5, and 7 days p.i., respectively). CD28−/− mouse corneas did not perforate, while all the corneas of wild-type mice perforated by 5–7 days p.i. We also tested the responses of the two groups to heat-killed bacterial Ag challenge at 5 days p.i. by measuring DTH. This assay was an additional measure of host T cell responsiveness to *P. aeruginosa* infection. These data are shown in Fig. 9. DTH measured at 24 and 48 h after Ag challenge was significantly (*p* = 0.0002 and *p* = 0.0106, respectively) elevated in wild-type B6 vs CD28−/− mice.

**Discussion**

 Recently, increased attention has been drawn to dendritic cells (DC) as immunostimulatory cells. Although a wide variety of MHC class II-positive cells induce proliferation of primed T cells, only DC are capable of clustering with and stimulating the proliferation of unprimed or resting (naive) T cells (22). Both in vitro and in vivo studies have confirmed that epidermal LC play a central role in the induction of skin-related immunological events (23, 24). However, the role of LC as APC in the bacterial susceptible vs resistant genetic model (7) in which T cells are detected in the cornea of susceptible, but not resistant, mice p.i. had not been tested. In the study reported herein significantly more LC were detected by ADPase staining in the cornea of B6 mice at 1 and 6 days p.i. compared with that in BALB/c mice. At 4 days p.i. LC number was similar in the two groups, indicating that the BALB/c mouse is capable of mobilizing LC in the cornea. However, unlike in the B6 mouse, LC only modestly increased in BALB/c mice from 4–6 days p.i., while in B6 mice the number of LC doubled. In another

**Table II. Labeling with DEC-205 and anti- *P. aeruginosa* pAb at 1 and 6 days p.i.**

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<th>Strain</th>
<th>No. Labeled Cells</th>
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<td>Dual</td>
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<tr>
<td>6 days p.i.</td>
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<tr>
<td>Dual</td>
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 a *n* = 7 fields for each strain.
 b *n* = 7 fields for each strain.

**Table III. Labeling with DEC-205 and B7-1 or B7-2 at 5 days p.i.**

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<tr>
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 a *n* = 7 fields, B7-1/B7-2, *n* = 8 fields.
 b *n* = 7 fields, B7-1/B7-2, *n* = 8 fields.

FIGURE 6. Immunostaining of T cells in corneas at 5 days p.i. Immunostaining for CD4+ T cells is shown in anti-HLA-DR5 (A) and B7-1/B7-2 mAb-treated (B) mice. IL-2R+ cells were detected in the cornea of control (C), but not the B7-treated (D), B6 mice. The latter (D) appeared similar to corneal tissue stained with HLA-DR5 mAb. Magnification, ×275.

FIGURE 5. Slit lamp photomicrographs representative of ocular disease observed at 5 days p.i. in B7-1/B7-2 mAb-treated (A) vs HLA-DR5-treated (B) mice. Corneal perforation is obvious in the control (B) vs B7 mAb-treated eye. Magnification, ×7.
model, data showed that the density of LC in the central cornea during the time of T cell infiltration may determine the relative contributions of CD4 and CD8 T lymphocytes to the immunologic reactions to HSV that occur in that tissue (25). Additionally, in a model of corneal transplant (26) data suggested that a high density of LC in the graft bed (recipient cornea) are in part responsible for the frequency of graft failures in inflammatory corneal diseases.

We were also interested in whether P. aeruginosa Ag associated LC in draining CLN could be detected in our bacterial model. Depending on the type of tissue in which they reside and the character of the pathogen-related migration-inducing signals, DC have been shown to reach lymph nodes within 4–48 h (23, 27–30). The cells can act in the inductive phase of an immune response as well as APC for peripheral tissue-homing memory T cells (24, 31). In both B6 and BALB/c mice, dual staining for bacterial Ag in association with DEC-205 cells was confirmed in draining CLN at 1 and 6 days p.i., with a greater number of dual-labeled cells at both times in susceptible vs resistant mice (Table II). These data suggest that LC may carry bacterial Ag from the cornea to the CLN. They do not rule out the possibility that Ag drained to CLN from the eye and associated with DEC-205-expressing cells in the node. Nonetheless, these data do show that both groups of mice are capable of carrying out an inductive response to pseudomonal Ag, but are disparate regarding the number of cells engaged in this process.

Among the maturational events that increase the capacity of LC to present Ag to CD4+ T cells is an increase in expression of the B7 family of costimulatory molecules (14–15, 32). The capacity of B7 to deliver a costimulatory signal to T cells by binding to CD28 is well documented both in vitro and in vivo (33–35). Systemic treatment with mAb to B7 or with the ligand CTLA4-Ig can reduce the severity of T cell-mediated inflammatory processes such as experimental lupus (35) and autoimmune encephalomyelitis (36, 37). However, such studies addressing the differential functional role of B7-1 and B7-2 in these autoimmune models have resulted in conflicting data (38). These models led us to ask whether there were differences in the maturation of corneal LC in susceptible vs resistant mice, as evidenced by expression of B7 costimulatory molecules. In our studies, as in HSV keratitis, B7-1 was not detected in the mouse ocular epithelium until after infection. However, in our model, positively stained cells were detected 2 days earlier (1 vs 3 days) p.i. than reported in the HSV model (16).

Although the total number of dual-labeled cells was decreased at 6 vs 3 day p.i., significantly more such cells were present at the latter

FIGURE 7. IFN-γ expression in cornea and CLN from B7-1/B7-2 vs HLA-DR5 mAb-treated B6 mice at 5 days p.i. Data are expressed as the mean IDV of three PCR of samples from five separate mice in each group. Values are the mean ± SEM; p = 0.01778 and 0.04556 in CLN and cornea, respectively.

FIGURE 8. Mean clinical scores in CD28−/− and B6 wild-type mice. Significant differences were observed at 3, 5, and 7 days p.i. p = 0.0054, p = 0.0014, and p = 0.0001, respectively.

FIGURE 9. P. aeruginosa-specific DTH in wild-type B6 vs CD28−/− mice. When challenged with heat-killed pseudomonal Ag at 5 days p.i., wild-type mice had a significantly greater DTH (p = 0.0002 and p = 0.0106 at 24 and 48 h, respectively) than similarly challenged CD28−/− mice.
time in B6 vs BALB/c cornea. This decrease in labeled cells may reflect corneal healing in BALB/c vs perforation in B6 mice, but this remains untested. Further, some, but not all, migrating LC in cornea expressed B7-1, suggesting that LC migration and B7-1 expression are independently regulated in bacterial as well as in ocular viral infection (16).

The requirement for costimulation of T effector cells at an inflammatory site remains controversial. It has been suggested that during acute infections, cytokines/chemokines that are produced by cells (resident and inflammatory) within the lesion may supplant the need for costimulation of effector T cells (39). In the HSV model, replicating virus and viral Ag are detectable in the cornea at 5 days p.i., whereas T cell-mediated inflammation is initiated at around 10 days p.i. (16). This is in contrast to the Pseudomonas model used herein, in which viable bacteria have been detected in the cornea throughout 9 days of infection (8) with a significantly greater bacterial load in susceptible mice (8). Thus, in susceptible B6 mice, activation of infiltrating effector T cells could occur in the inflamed tissue site via other mechanisms besides B7 costimulation. To determine the role of B7/CD28 pathway costimulation in our bacterial model, systemic and intraocular mAb neutralization of B7-1/B7-2 was performed. mAb-treated mice had a milder course of disease compared with control Ab-treated animals and dual Ab staining confirmed that both B7-1- and B7-2-positive cells were significantly reduced in cornea and CLN by the Ab blockade. We also tested whether T cell recruitment to cornea was affected and found that in B7-1/B7-2 mAb vs control treated mice, T cell immunostaining for CD4- and IL-2R (activated)-positive cells was significantly reduced qualitatively or absent, respectively, compared with control Ab-treated mice. RT-PCR data also supported the importance of costimulation through this pathway in the disease response, as mRNA transcripts for IFN-γ were reduced in both cornea and CLN in B7-1/B7-2 vs control mAb-treated mice. These data also are consistent with an earlier report from this laboratory showing that neutralization of IFN-γ correlated with reduction of corneal disease in B6 mice (7). Reduction of other cytokines/chemokines also has been reported using anti-B7 mAbs. Production of macrophage inflammatory protein-1α induced following TCR-mediated T cell activation was inhibited by coculture with anti-B7 mAbs, indicating that full production of this C-C chemokine depended on interaction with B7 ligand through CD28 (21).

Ligation of CD28 together with TCR stimulation can prevent cell death and stimulate cytokine production and proliferation as well as recruitment of other cells to inflammatory sites by producing chemokines, important mediators of chemotaxis in vivo (40–44). The role of CD28 is unique, because IL-2 does not provide costimulatory signals needed for chemokine production despite its ability to enhance expression of chemokine receptors (21). Due to the potential of the B7-1/B7-2 mAbs to initiate costimulatory signaling through CD28, we next tested CD28 −/− mice. Ocular disease and DTH were reduced in these mice, and none of the corneas of deficient vs wild-type mice perforated p.i., confirming the mAb neutralization studies.

In summary, our findings strongly suggest that in the susceptibility response to P. aeruginosa, B7/CD28 costimulation appears critical in the induction of an inflammatory response resulting in corneal perforation and blindness.

References