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NKT Cells Are Critical to Initiate an Inflammatory Response after Pseudomonas aeruginosa Ocular Infection in Susceptible Mice

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CD4⁺ T cells produce IFN-γ contributing to corneal perforation in C57BL/6 (B6) mice after Pseudomonas aeruginosa infection. To determine the role of NK and NKT cells, infected corneas of B6 mice were dual immunolabeled. Initially, more NKT than NK cells were detected, but as disease progressed, NK cells increased, while NKT cells decreased. Therefore, B6 mice were depleted of NK/NKT cells with anti-asialo GM1 or anti-NK1.1 Ab. Either treatment accelerated time to perforation, increased bacterial cells were detected, but as disease progressed, NK cells increased, while NKT cells decreased. Therefore, B6 mice were depleted of NK/NKT cells with anti-asialo GM1 or anti-NK1.1 Ab. Either treatment accelerated time to perforation, increased bacterial

In humans, keratitis caused by Pseudomonas aeruginosa develops rapidly and may lead to corneal perforation, with a higher incidence of disease with extended contact lens wear (1, 2). Disease is initiated by bacterial pathogenic events, but often, sequential tissue destruction occurs mainly due to cytokines and chemokines released by both resident and inflammatory cells that have infiltrated the infected cornea (3). Activated polymorphonuclear neutrophils (PMN), macrophages (Mφ), NK, T lymphocytes, and other cells release cytokines including, but not limited to IL-1β, MIP-2, IL-12, IL-18, IFN-γ, and TNF-α, that fuel inflammatory events. If uncontrolled, such events contribute to tissue destruction, including corneal perforation. Experimental studies of the disease have shown that Th1 responder mouse strains (e.g., C57BL/6, B6) are susceptible (cornea perforates), whereas Th2 responder strains (e.g., BALB/c) are resistant (cornea heals) (3, 4). After bacterial infection, Th1 response development with IFN-γ production, in turn, often depends upon both IL-12 and the ability of T cells to respond to this cytokine (5–7). IL-12p40 has been detected in the infected cornea of susceptible B6 mice by real-time RT-PCR and protein analyses (8). We also have demonstrated that in B6 mice, either sustained IL-12 p40-driven IFN-γ production or endogenous absence of IL-12 p40 (or p35), resulting in reduced IFN-γ mRNA levels, leads to corneal perforation (8). In contrast, studies using similar procedures failed to detect IL-12p40 or T cells in the infected cornea of BALB/c mice that control infection and restore corneal clarity (9). Recently, in resistant BALB/c mice, NK cell-derived IFN-γ production has been shown as important in disease resolution and NK cells identified as a source of IFN-γ. NK cells were shown to express the NK-1R and participate in regulation of PMN infiltration. Evidence also was provided that in cornea, the neuropeptide substance P (SP), as well as IL-18, regulated NK cell production of IFN-γ, through interaction with the NK-1R (10).

NKT cells also are capable of IFN-γ production and represent a minor subset of T cells that share receptor structure with conventional T and NK cells (11). NKT cells regulate immune cells, such as T (12), NK (13), and dendritic cells (DCs) (14) because of their capacity to rapidly release large amounts of IL-4 and/or IFN-γ upon activation (15). NKT-derived IFN-γ plays an important role in both innate and acquired immunity (16–18) and the cells are important regulators of immune responses in many diseases, including antimicrobial immune responses (19–22) and Th1/Th2 differentiation (20). After corneal infection, whether NKT cells contribute to ocular inflammation and the Th1-like response of B6 mice through the early production of cytokines such as IFN-γ, remains untested. In this report, the role and interaction of NKT and NK cells were evaluated during P. aeruginosa keratitis in susceptible B6 mice.

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Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; Mφ, macrophage; SP, substance P; DC, dendritic cell; pAb, polyclonal Ab; p.i., postinfection; MPO, myeloperoxidase; LC, Langerhans cell; MMLV, Moloney murine leukemia virus; RT, reverse transcriptase; αGalCer, α-galactosylceramide.

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Materials and Methods

Experimental animal protocol

Eight-week-old female B6, RAG-1−/−, and IL-12p40−/− mice (purchased from The Jackson Laboratory) and Ino281−/− mice (a gift of Dr. J. Stein-Streilein, Schepens Eye Research Institute, Boston, MA) were housed in accordance with the National Institutes of Health guidelines. Mice were anesthetized with ethyl ether and the cornea of the left eye was wounded as previously described (23). A 5-μl aliquot containing 1 × 10⁶ CFU of P. aeruginosa ATCC strain 19660 (American Type Culture Collection) was topically delivered to the ocular surface.

Ocular response to bacterial infection

Corneal disease was graded using an established scale (24): 0, clear or transparent; 1, slight opacity, partially or fully covering the pupil; +2, dense opacity, partially or fully covering the anterior segment; +3, dense opacity, covering the entire anterior segment; and +4, corneal perforation or phthisis. Ab-treated mice (n = 5/group/time) were injected i.p. with 40 μl of naive rabbit serum 2 days before and on the day of infection. Depletion of NK and NKT cells (described below) was examined and a clinical score was calculated for each group to express disease severity. Slit-lamp photography was used to illustrate the disease response.

Ab treatment

A nonactivating polyclonal Ab (pAb) against asialo GM1, expressed at high levels on the surface of NK cells (25), was used for NK (and NKT) cell depletion. B6 mice (n = 5/group/time) were injected i.p. with 40 μl of anti-asialo GM1 pAb (Wako) diluted in 100 μl of PBS, or an equivalent amount of naive rabbit serum 2 days before and on the day of infection. Depletion of NK cells with this dosage (0% cytotoxicity) was assessed and confirmed previously by a functional assay using YAC-1 cells (26) and in this laboratory using immunohistochemistry (10).

An NK cell-activating mAb from PK136 hybridoma (Serotec) against mouse NK 1.1 Ag also was used to deplete deplete (NK and NKT) cell depletion. B6 mice (n = 5/group/time) were injected i.p. with 200 μg of mAb 1 day before and 100 μg of mAb 1 day after infection or with an equivalent amount of mouse IgG. In the current study, depletion of NK and NKT cells was confirmed by immunostaining of splenic tissue from asialo GM1 and NK1.1 depletes and control groups (data not shown) using immunostaining techniques described below.

Bacterial quantification

Individual corneas (n = 5/group/time) were collected at 1 and 3 days postinfection (p.i.) from anti-asialo GM1, anti-NK1.1, naive serum, and IgG-treated control B6 mice and homogenized in sterile 0.25% BSA-saline. To quantitate viable bacteria, a 0.1-ml aliquot of each corneal homogenate was serially diluted 1/10 in sterile BSA-saline, plated in triplicate onto Pseudomonas isolation agar (Difco) plates and incubated overnight at 37°C. Individual colonies on plates from the various dilutions were counted and results reported as log₁₀ number of CFU/cornea ± SEM. Experiments were performed at least twice to confirm results.

Myeloperoxidase (MPO) assay

Corneas (n = 5/group/time) from anti-asialo GM1 and naive serum-treated mice were excised at 1 and 3 days p.i. and an MPO assay used to quantitate PMN. Tissue was homogenized in 1.0 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl-ammonium bromide. Samples were freeze-thawed and after centrifugation, 0.1 ml of the supernatant was added to 2.9 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml) and hydrogen peroxide (0.0005%). The change in absorbancy (460 nm) was monitored for 5 min at 30 s intervals. The slope of the line was determined for each sample and used to calculate units of MPO/sample. One unit of MPO activity is equivalent to ~2 × 10⁷ PMN (28).

Real-time RT-PCR

Table I. Primer sequences used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>5'-CAC AGC CAG ATT ACT TTC TTT GCC CTT GCT GCT GTA GAA G-3' sense</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>5'-ATT CTG CTT CCC CCG GTC TTC TAC CAA C-3' antisense</td>
</tr>
<tr>
<td>CD1d</td>
<td>5'-TGC CTA CTA AAG AAG ACT ACT CCA TTG-3' sense</td>
</tr>
<tr>
<td>CD80</td>
<td>5'-TAA AGA TTA TGA AGA TGC TCA GTP GCT-3' sense</td>
</tr>
<tr>
<td>CD86</td>
<td>5'-GAC GAC GAC GAC TCT TGG TAG AT-3' antisense</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5'-CAG GTA CTT GGC ATT CAC ACT ACT-3' antisense</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GCT TAC TCC TCT GGC TCC TAG C-3' sense</td>
</tr>
</tbody>
</table>

Infected corneas (n = 5/group/time) from anti-asialo GM1 and NK1.1 Ab-treated vs naive serum and IgG-treated control B6 mice were removed at 3 and 5 days p.i. Normal and infected corneas (n = 5/group/time) from RAG-1−/−, IL-12p40−/−, Ino281−/−, oGalCer mice (and their respective controls) were removed at 5 h, and at 1 and 5 days p.i, as specified below. Langerhans cells (LC) and peritoneal-elicited Mø (described below) also were processed for real-time RT-PCR. All specimens were frozen in RNA stabilization reagent and stored at −80°C until used. Total RNA was either isolated from individual corneas or collected from cells using RNA-Stat 60 (Tel-Test) in accordance with the manufacturer’s recommendations and quantitated by spectrophotometric determination (260 nm). One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The 20-μl reaction mixture contained: 200 U of MMLV-RT, 10 U of RNasin, 500 ng of oligo dT primers, 10 mM dNTPs, 100 mM DTT and MMLV reaction buffer. Next, cDNA was amplified using SYBR Green Master Mix per the manufacturer. Briefly, 20-μl reactions contained: 10 μl of 2× SYBR Green PCR Master Mix, 0.5 μM primers, 2 μl of cDNA (diluted 1/25) and diethyl pyrocarbonate water. All primers (sequences listed in Table I) for the PCR were designed using PrimerQuest (Integrated DNA Technologies). Quantitative real-time RT-PCR was performed using the MyiQ Single Color Real-Time RT-PCR Detection System (Bio-Rad).

Relative mRNA levels were calculated using the relative standard curve method that compares the amount of target normalized to an endogenous reference, β-actin. Briefly, the mean and SEM values of replicate samples were calculated. Samples were then normalized to β-actin. Results are expressed as the relative amount of mRNA between experimental test samples and normal control samples. Before using this method, a validation experiment was performed comparing the standard curve of the reference and the target to demonstrate that efficiencies were approximately equal. The correct size of the amplified products was checked by electrophoresis using an agarose gel (data not shown).

Cell culture and isolation

LC (XS52) cell line (29) (a gift of G. Murakawa, Somerset Skin Center, Troy, MI) were routinely cultured in complete RPMI 1640 (with 10% FCS) supernatant. Peritoneal Mø were isolated from B6 mice that had been injected i.p. with 1.0 ml of 3% Brewer’s thioglycolate medium (Difco) 5–7 days previously (30). Cells were collected by peritoneal lavage with DMEM medium, stained with trypan blue (1:1), and viable cells (>95%) were enumerated using a hemacytometer. After a differential cell count, cells were seeded in 12-well plates at a density of 1 × 10⁶ cells/well. Nonadherent
cells were removed 4–6 h later and isolated Mφs were used for in vitro stimulation assays, described below.

In vitro stimulation assay

LC (XS52, 7.5 × 10^6 cells/well) and thioglycolate-elicited peritoneal Mφs (2 × 10^6 cells/well) from B6 mice were stimulated with LPS (0.5 or 25 μg/ml) for 18 h (30), with or without rIFN-γ (2.5 U/ml). Cells were collected and assayed by real-time RT-PCR (described above) for gene expression levels of CD80, CD86, IL-12p40, CD1d, and NF-κB. In a separate experiment, peritoneal Mφs were harvested, plated (50,000 cells/well) onto Lab-Tek slides, stimulated as above, and immunostained for CD1d localization, described below.

NKT, NK, and Mφ cell immunolabeling

Infected eyes of wild-type B6 mice (n = 3/time) were removed at 5 h and 1, 3, 5, and 7 days p.i., embedded in OCT, frozen in liquid nitrogen, stored at –20°C and sectioned (6–10 μm). Sections were dried overnight, fixed in acetone (2 min), washed in TBS and nonspecific binding blocked in TBS containing 5% normal goat serum, 1% BSA, and 0.1% Triton X-100 for 30 min. Sections were incubated with anti-asialo GM1 pAb (1/50) in TBS containing 1% BSA, rinsed with TBS, and then incubated with FITC-labeled goat anti-rabbit IgG (1/250; Jackson ImmunoResearch Laboratories) for 1 h. Sections were blocked with TBS containing 1% rat IgG and 1% BSA for 30 min and then incubated with Armenian hamster anti-mouse CD3 (1/50; from BD Pharmingen) Ab for 1 h and washed with TBS. Then sections were incubated with rhodamine-labeled goat anti-hamster IgG (1/200; Jackson ImmunoResearch Laboratories) for 1 h, rinsed with TBS, and mounted with Vectashield (Vector Laboratories). Control sections were similarly treated but without either primary Ab. Representative sections were photographed with a Zeiss Axioskop microscope. Identical FITC- and rhodamine-stained fields were digitized with a SPOT digital camera and overlaid using MetaMorph (Universal Imaging). For each cell population at each time point, six slides from each infected eye (n = 3) were immunolabeled and three fields from each slide were observed and evaluated. Results (Table II) are reported as qualitative grades (+1 = 1–3 cells/section; +2 = 4–10 cells/section; +3 = 11–20 cells/section; +4 = 21–30 cells/section; +5 = 31–50 cells/section; +6 = >50 cells/section).

Infected eyes from IL-12p40−/− and B6 wild-type mice were enucleated at 5 days p.i., embedded, and sectioned as above. Sections were incubated for 1 h with anti-CD4 mAb (rat IgG2a, clone H129.19, 1/10), and anti-CD25 (IL-2R, rat IgM, clone 7D4, 1/50; BD Pharmingen). Sections were incubated with anti-asialo GM1 pAb (1/50) in TBS containing 1% BSA, rinsed with TBS, and then incubated with FITC-labeled goat anti-rabbit IgG (1/250; Jackson ImmunoResearch Laboratories). Control sections were similarly treated using HLA-DR-5, a nonspecific mAb.

Stimulated peritoneal Mφs (described above) were fixed for 10 min with 3.7% formaldehyde. After rinsing in 0.01 M phosphate buffer, a biotin-conjugated rat anti-mouse CD1d (BD Biosciences) Ab diluted 1/100 in phosphate buffer containing 1.5% BSA was added to the wells and incubated for 1 h. Chambers were rinsed and a streptavidin-conjugated Alexa Fluor 594 secondary Ab (Invitrogen Life Technologies) diluted 1/200 in Tris-HCl was added to each well and incubated in reduced lighting for 1 h. Nuclear staining was done as above. Negative controls were similarly treated, but with omission of the primary Ab. Cells were visualized and digital images were captured as above.

Induction of NKT cell anergy

To induce NKT cell anergy, 8-wk-old B6 mice received an i.p. injection of PBS containing 5 μg of α-galactosylceramide (αGalCer; Toronto Research Chemicals) and 0.025% Tween 20 in a total of 200 μl (32). Control mice received 200 μl of PBS with 0.025% Tween 20. Per this protocol, 8 days later, mice were infected and five corneas from each group were harvested at 5 h and 1 day p.i. for IFN-γ detection using real-time RT-PCR.

Table II. Qualitative evaluation of NK and NKT cell infiltrate after P. aeruginosa infection

<table>
<thead>
<tr>
<th>Time</th>
<th>Conjunctiva</th>
<th>Corneal stroma</th>
<th>NK+CD3−/CD3+ (NK)</th>
<th>NK−CD3−/CD3+ (NKT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 h</td>
<td>0</td>
<td>0</td>
<td>+2</td>
<td>0</td>
</tr>
<tr>
<td>1 day</td>
<td>0</td>
<td>0</td>
<td>+2</td>
<td>0</td>
</tr>
<tr>
<td>3 day</td>
<td>+1</td>
<td>+1</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>5 day</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
</tr>
<tr>
<td>7 day</td>
<td>+6</td>
<td>+6</td>
<td>+6</td>
<td>+6</td>
</tr>
</tbody>
</table>

*+1 = 1–3 cells/section; +2 = 4–10 cells/section; +3 = 11–20 cells/section; +4 = 21–30 cells/section; +5 = 31–50 cells/section; +6 = >50 cells/section.

FIGURE 1. Dual immunostaining of NK+CD3− and NK+CD3+ cells at 5 h (A, C, and E) and 7 days (B, D, and F) p.i. NK+ cells (A and B) (positively stained green by FITC label); CD3+ cells (C and D) (positively stained red by Rhodamine-label); NK+CD3+ cells (E and F) (overlay, yellow stained cells are merged images from A and C, and B and D, respectively). Primary Ab was omitted in control sections (5 h and 7 days p.i.) that showed slight background staining with either filter (data not shown) or both filters (G and H). Magnification, ×260.
A significant difference was seen between the two groups (n = 5/group/time, performed twice) at 3 days p.i. (Fig. 1) in anti-asialo GM1- or NK1.1-treated vs control-treated mice (p = 0.008 for both). Results are reported as individual clinical scores. Slit lamp photomicrographs (B, C, E, and F) were taken at 3 days p.i. from representative eyes from each group. The corneas of Ab-treated mice (C and F) had perforated while controls (B and E), although opaque, had not.

**Statistics**

The difference in clinical score at each experimental time point between two groups was tested by the Mann-Whitney U test. An unpaired, two-tailed Student’s t test was used to determine the significance for all other data. Differences were considered significant at p < 0.05. Each experiment was performed at least twice for reproducibility and representative data from a single experiment are shown.

**Results**

**NK/NKT cell depletion**

Immunostaining for NKT and NK cells was not detected in the spleens of anti-asialo GM1 or anti-NK1.1 Ab-treated mice. In contrast, positively stained cells were detected in the spleens of control-treated animals (data not shown).

**Immunostaining and qualitative evaluation**

Dual immunolabeling was used to detect and qualitatively evaluate NK+/CD3− (NK) and NK+/CD3+ (NKT) cells in the cornea and conjunctiva from 5 h-7 days p.i. (Table II and Fig. 1). Positively labeled cells were observed after corneal infection and were localized within the conjunctiva and corneal stroma. NK+CD3+ (NKT) cells (Fig. 1, E and F) were qualitatively more frequently observed at 5 h p.i. (Fig. 1E, overlay of Fig. 1, A and C) in the peripheral cornea. This staining pattern remained relatively constant throughout 5 days of observation, with positively labeled NK+/CD3+ cells primarily seen in the stroma; by 7 days p.i. (Table II and Fig. 1F, overlay of Fig. 1, B and D) fewer of these cells were observed. NK+/CD3− (NK) cells also were seen at 5 h p.i., but very few cells were detected (Fig. 1E and Table II) in conjunctiva or corneal stroma. By 1–7 days p.i., these cells continued to increase and appeared most numerous in the 7 day p.i. cornea, filling the infected stroma (Fig. 1, B and F). Control sections at 5 h p.i. (Fig. 1G) and 7 days p.i. (Fig. 1H) in which the primary Abs were omitted showed slight nonspecific background staining with both filters.

**Anti-asialo GM1 pAb and NK1.1 mAb treatment**

Next, to test the role of these cells in bacterial keratitis, Ab-depletion studies were initiated. Disease progression was observed and graded at 1, 3, and 5 days p.i. in anti-asialo GM1 pAb and anti-NK1.1 mAb-treated vs control (naive rabbit serum or mouse IgG-treated) mice. These data, and slit lamp photomicrographs to document clinical score disease grades, are shown in Fig. 2. Similar disease progression was observed in anti-asialo GM1 pAb (Fig. 2A) and NK1.1 mAb-treated (Fig. 2D) vs control-injected mice at 1 day p.i. By day 3 p.i., both anti-asialo GM1- and NK1.1-treated mice similarly showed significantly more severe disease compared with controls (p = 0.008 for both). At this time, all corneas of the anti-asialo GM1-treated and 90% of the anti-NK1.1-treated mice were perforated, compared with only 10% perforation in mouse IgG-treated controls. By 5 days p.i., no significant differences in disease response (perforation) were observed between the experimental and control-treated groups.

**FIGURE 2.** Ocular disease response in anti-asialo GM1 or NK1.1 Ab vs control-treated B6 mice after P. aeruginosa infection. A significant difference was seen between the two groups (n = 5/group/time, performed twice) at 3 days p.i. (A and D) in anti-asialo GM1-or NK1.1-treated vs control-treated cornea (p = 0.008 for both). Results are reported as individual clinical scores. Slit lamp photomicrographs (B, C, E, and F) were taken at 3 days p.i. from representative eyes from each group. The corneas of Ab-treated mice (C and F) had perforated while controls (B and E), although opaque, had not.

**FIGURE 3.** Bacterial plate counts (A and B). There was a significantly greater number of viable bacteria in the cornea of anti-asialo GM1 and NK1.1 vs control-treated mice only at 3 days p.i. (p < 0.001 and p < 0.001, respectively). Results are reported as mean of log10 CFU/cornea ± SEM. Corneal MPO activity (C) in individual corneas of anti-asialo GM1 vs control-treated mice after P. aeruginosa challenge. After asialo GM1 pAb treatment, there was no significant difference in MPO activity at day 1 p.i. (p = 0.166); however, significantly increased MPO activity was detected at 3 days p.i. (p = 0.001).
whether NK/NKT cell depletion before infection affected IFN-βacterial killing/stasis (33) in the cornea, we next determined mRNA expression was detected in anti-asialo GM1 (A) and NK1.1 (B) vs control-treated mice after challenge (n = 5/group/time). IFN-γ mRNA levels were significantly lower in anti-asialo GM1 vs control-treated cornea at days 3 and 5 p.i. (p = 0.007 and p = 0.0001, respectively) (A). However, in the NK1.1-treated group (B), IFN-γ mRNA levels were significantly reduced only at day 5 p.i. (p = 0.002). At day 3 p.i., IL-12 p40 mRNA levels in cornea were significantly decreased in anti-asialo GM1 (C) and NK1.1 (D) vs control-treated mice (p = 0.007 and p = 0.02, respectively). At day 5 p.i., reduced IL-12p40 mRNA expression was detected in anti-asialo GM1 (C) and NK1.1 (D) vs control-treated mice (p = 0.05 and p = 0.006, respectively).

Quantitation of bacteria
Because of the earlier perforation time in both the anti-asialo GM1 and NK1.1 Ab-treated groups, we next tested whether depletion of NK/NKT cells led to increased bacterial growth in the infected cornea. Data, expressed as the mean log10 number of viable bacteria per cornea (±SEM), are shown in Fig. 3. (A) Similar bacterial growth was observed at 1 day p.i. in anti-asialo GM1 and NK1.1 Ab vs control-treated mice. However, a significant increase in bacterial load was evident in the corneas of experimental vs control-treated mice at 3 days p.i. (p < 0.001 for both).

MPO activity assay
Because more severe disease and a greater bacterial burden was observed in the cornea after anti-asialo GM1 or NK1.1 Ab vs control treatments, we next quantitated the number of PMN in the cornea of experimental vs control treatments, we next quantitated the number of PMN in the cornea of the two mouse groups using an MPO assay. Data from a representative experiment using anti-asialo GM1 Ab treatment are shown in Fig. 3C and are reported as units of MPO/cornea ± SEM. At day 3 p.i., MPO activity was significantly increased in the cornea in the anti-asialo GM1 Ab vs control-treated mice (p = 0.001). No difference in MPO activity was detected at 1 day p.i.

IFN-γ mRNA expression
Because we have shown before that IFN-γ indirectly regulates bacterial killing/stasis (33) in the cornea, we next determined whether NK/NKT cell depletion before infection affected IFN-γ mRNA expression levels. Real-time RT-PCR was used and data from a representative experiment are shown in Fig. 4, A and B. No IFN-γ mRNA was detected in the normal cornea of either group of mice (data not shown). At day 3 p.i., decreased IFN-γ mRNA levels were detected in both the Ab-treated groups. However, the decrease was significant only after anti-asialo GM1 (p = 0.007), but not NK1.1 Ab treatment (p = 0.12). At 5 days p.i., IFN-γ mRNA levels were significantly reduced in corneas from both anti-asialo GM1 and NK1.1 Ab vs control-treated mice (p = 0.0001 and p = 0.002, respectively).

IL-12p40 mRNA expression
Because we have shown that IL-12 contributes to sustained production of IFN-γ and leads to corneal perforation in B6 mice (8), we next determined whether NK/NKT cell depletion modulated IL-12p40 mRNA levels. At days 3 and 5 p.i., significantly reduced IL-12p40 mRNA expression was observed in the anti-asialo GM1 and the NK1.1 Ab-treated groups, (p = 0.007 and 0.02; p = 0.05 and 0.006, respectively) (Fig. 4, C and D) when compared with controls.

IFN-γ in NKT cell-deficient mice
We next determined whether NKT cells regulated NK cell IFN-γ production. To do this, we used several knockout mouse models. RAG-1−− (NK cells present, no T or NK/T cells) mice were tested for mRNA levels of IFN-γ at 5 h and 1 day p.i. (Fig. 5A). Compared with wild-type B6 mice, significantly decreased levels of IFN-γ were seen in the knockout mice at both time points (p = 0.004 and p = 0.01, respectively). NKT cell-deficient mice also were tested (Fig. 5B) and had significantly decreased IFN-γ mRNA levels when compared with B6 wild-type control mice at 5 days p.i. (p = 0.001), a time of optimum IFN-γ production in B6 mice. We also anergized NKT cells using a single injection of
αGalCer, a synthetic glycolipid, and found no IFN-γ at 5 h (p = 0.0002) and reduced mRNA levels at 1 (p = 0.003) and 5 days (p = 0.002) p.i. compared with PBS injected B6 wild-type mice (Fig. 3C).

Because activated CD4+ T cells, capable of producing IFN-γ, also are present in the B6 cornea at 5 days p.i. and because IL-12 drives a Th1-type response in these mice (8), we next tested IFN-γ mRNA levels at 5 days p.i. and immunostained corneal sections in B6 wild-type vs IL-12 p40−/− mice. In the absence of IL-12p40, mRNA levels of IFN-γ were reduced significantly (p = 0.016, Fig. 6A) and more activated CD4+ T cells were detected in the B6 wild-type (Fig. 6B, a and c) vs knockout mouse cornea (Fig. 6B, b and d), confirming the importance of IL-12p40 for IFN-γ production leading to a Th1 T cell response in these mice (8). Control sections (Fig. 6B, e and f) in which the primary Abs were omitted were negatively stained.

Dual Ab staining also was used to test whether IL-12p40 deficiency resulted in a reduction of NK cells in the cornea, potentially contributing to the observed decreased levels of IFN-γ. Figure 7 shows that NK CD4− cells are numerous in the peripheral cornea of both B6 wild-type (Fig. 7, A, C, and E) and IL-12p40−/− mice (Fig. 7, B, D, and F), at 5 days p.i., suggesting that the decreased IFN-γ mRNA levels observed were not due to fewer infiltrating NK cells. Controls (Fig. 7, G and H) which were not incubated with the primary Abs, were negative. Nuclear label with SYTOX green also is shown (Fig. 7, E–H).

LC and Mφ

To elucidate the cell type and source(s) of IL-12 and other molecules involved in NKT cell interactions and activation, we next tested for CD80, CD86 (costimulatory molecules), IL-12p40, and NF-κB mRNA levels using LC (Fig. 8) and B6 peritoneal elicited Mφ (Fig. 9). LC were stimulated with LPS and mRNA levels were significantly increased (p < 0.0001) for each of the above molecules. Mφ isolated from B6 mice, similarly treated with LPS and

FIGURE 6. IFN-γ mRNA levels and T cell immunostaining. Cytokine levels were reduced significantly at 5 days p.i. in B6 IL-12p40−/− vs B6 wild-type mice (p = 0.001) (A). B. In wild-type B6 mice, infiltrated CD4+ (a), CD25+ (c) T cell immunostaining was heavier than in IL-12p40−/− mice (b and d). e and f. Controls in which the primary Abs were omitted were negative. Magnification, ×53.

FIGURE 7. Immunofluorescence staining for corneal expression of CD4+ and asialo GM1+ (NK) cells in B6 wild-type (A, C, E, and G) vs IL-12 p40−/− (B, D, F, and H) mice at 5 days p.i. Both B6 wild-type (A) and IL-12 p40−/− (B) mice showed positive staining for asialo GM1 in the peripheral corneal epithelium. In contrast, little positive staining was visible for the CD4 marker in B6 wild-type (C) or IL-12p40−/− (D) mouse cornea. Composite images of B6 wild-type (E) and IL-12 p40−/− (F) mouse cornea show asialo GM1 labeling localized to the cell membranes and SYTOX green labeling the nuclei. Control eyes incubated in the absence of either primary Ab were negative with only the nuclear label visible (G and H). Magnification, ×265.

FIGURE 8. LC challenged with LPS. mRNA levels of CD80, CD86, IL-12p40, and NF-κB were measured in LC after LPS exposure (18 h). Significantly elevated mRNA levels for CD80 (A), CD86 (B), IL-12p40 (C), and NF-κB (D) over controls was seen (p < 0.0001 for each).
constitutive levels (35). Based upon past studies (4, 8), it is likely that all of these, including a decreased expression of CD1d, that levels increased significantly \((p = 0.03)\) after LPS stimulation, and that rIFN-\(\gamma\) together with LPS provided an additive (2-fold; \(p = 0.006)\) stimulatory effect on these cells to up-regulate CD1d mRNA levels.

Immunolabeling for CD1d following similar stimulation supported these results (Fig. 10, B–E). CD1d was constitutively expressed in Mφ (Fig. 10B) increased after LPS (Fig. 10C) and qualitatively appeared further increased after LPS and rIFN-\(\gamma\) combined treatment (Fig. 1D). Cells incubated similarly but in the absence of the primary Ab, appeared similar to cells stained only with SYTOX green (Fig. 10E).

**Discussion**

The innate immune system is characterized by rapid responses to pathogens and these are mediated mainly by PMN, Mφ, DC, and NK cells (22). Regarding innate immunity, past work from this laboratory has shown that in resistant BALB/c mice, corneal infection with \(P.\ aeruginosa\) results in direct activation of NK cells by the neuropeptide, SP, through its binding to the NK-1R and indirectly by SP regulation of IL-18, in turn regulating production of IFN-\(\gamma\) that is required for the resistance response (10). Neither T cells nor IL-12p40 are detectable in the infected cornea of these resistant mice.

In contrast, in susceptible B6 mice, activated CD4\(^+\) T cells are detected in the infected cornea by 5–7 days p.i (8, 34), correlating with peak corneal IFN-\(\gamma\) levels and perforation. Because CD1d NKT-restricted cells have been proposed to link the innate with the acquired immune system (35, 36), we examined the cornea of susceptible B6 mice early after infection and found by dual immunostaining that the predominant cell detected at 5 h p.i. was the NK cell, shifting with time to perforation (5–7 days p.i.) to a predominantly NK cell population. Ab depletion using either asialo GM1 or NK1.1 Ab, which deplete both cell populations, resulted in an accelerated time to perforation, a significantly greater bacterial load, and increased number of PMN in the cornea. In addition, both IFN-\(\gamma\) and IL-12p40 corneal mRNA levels were reduced significantly in mice treated with either Ab. In this regard, IL-12 has been shown as essential for the activation of NK cells and their subsequent production of IFN-\(\gamma\) during an infection (21). Furthermore, NKT cells, but not naive T cells or NK cells, express a substantial amount of IL-12R components (35). Based upon past studies (4, 8), it is likely that all of these, including a decreased host inflammatory response (less IFN-\(\gamma\)) coupled with a greater bacterial load and more PMN in cornea, contributed to the earlier time to perforation in these Th1 responder mice depleted of NK and NKT cells. Alternately, although we have not tested our model for the potential role of IL-23, composed of an IL-12 p40 and a p19 subunit, others have shown that IL-23 plays a critical role in generating airway inflammation observed in mucoid \(P.\ aeruginosa\) lung infections in mice (37). Thus, the role of this cytokine in bacterial ocular infection remains unresolved.

To determine the specific role of NKT and NK cells in this model, RAG-1−/− mice with NK, but lacking NKT and T cells, were tested. Early after infection, the knockout mice produced significantly less IFN-\(\gamma\) compared with B6 wild-type controls. These data are consistent with other studies (38) which found that NK cell activation and IFN-\(\gamma\) production were not observed in RAG-deficient mice, suggesting that despite rapid induction and the ability of the NK cell to produce IFN-\(\gamma\), it was a secondary event that depends on IFN-\(\gamma\) release by NKT cells. In this regard, work by

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**FIGURE 9.** Mφ challenged with LPS. mRNA levels of CD80, CD86, IL-12p40, and NF-κB were measured in Mφ from B6 mice after LPS exposure (18 h). Significantly elevated mRNA levels for CD80, CD86 (B), IL-12p40 (C), and NF-κB (D) over controls was seen \((p \leq 0.01)\).

**FIGURE 10.** Mφ challenged with LPS with or without rIFN-\(\gamma\). A. mRNA levels of CD1d were constitutively expressed, enhanced significantly with LPS \((p = 0.03)\) and addition of rIFN-\(\gamma\) together with LPS enhanced levels 2-fold \((p = 0.006)\) over LPS alone and almost 3-fold over constitutive levels \((p = 0.00002)\). B–E. Immunofluorescence staining for Mφ expression of CD1d; nuclei labeled with SYTOX green. B. Unstimulated cells; C. LPS stimulated; D. LPS and rIFN-\(\gamma\) stimulated. E. Control cells incubated in the absence of primary Ab appeared similar to SYTOX green nuclear staining alone. Magnification, \(\times 730\).
Eberl et al. (13) has shown that activated NKT cells selectively induce NK cell proliferation and cytotoxicity in an IFN-γ- and IL-12-dependent pathway. When the IL-12R is engaged, NK cells can produce IFN-γ which in turn activates a variety of antimicrobial responses designed to limit pathogen growth, but without the NKT cell, they saw weak production (similar to our studies) of the cytokine by NK cells and by mice which have no NKT cells but have both NK and T cells (39).

NKT cells belong to a specialized population of lymphocytes that coexpress the TCRαβ chain and NK markers (40, 41). A major subpopulation of murine NKT cells also expresses a unique variant Vα14Jα281 Ag receptor not expressed by conventional T cells (40, 42–44). Taking advantage of this, and to see whether the absence of NKT cells influenced Th1 CD4⁺ T cell IFN-γ production, we next tested NKT cell-deficient Jα281⁻/⁻ mice similarly for IFN-γ levels. The cornea was examined at 5 days p.i. when activated CD4⁺ T cells are present in the B6 wild-type cornea (8, 34). Cytokine levels were significantly decreased in the NKT cell-deficient compared with B6 control mice, confirming the importance of NKT cells in development of the Th1 T cell response in B6 mice. Collectively, these studies indicate the importance of the NKT cell in regulation of both NK and T (Th1 type response) cell IFN-γ production in the cornea.

Others have shown the critical role of CD1d-restricted NKT cells in microbial immunity (45) and somewhat similar to our study, Nieuwenhuis et al. (46) showed that P. aeruginosa-induced pneumonia in mice activated CD1d-restricted NKT cells. Twenty-four hours after infection by the respiratory route, the bacterial burden was 100-fold higher in the lungs of either CD1d⁻/⁻ mice or anti-CD1d Ab-treated than in untreated control mice. In contrast with our study, the lungs of infected CD1d⁻/⁻ mice had decreased rather than increased PMN, consistent with their detecting less of the chemotactic factor, MIP-2, for which we did not test. Their data suggested that early during an immune response, the Mφ functions in a CD1d-restricted manner to regulate immune protection against P. aeruginosa at the lung mucosal surface through recruitment of PMN. We also have shown the importance of the Mφ (by depletion with clodronate) in regulation of PMN number in the BALB/c cornea and its ability to produce IL-10, critical to influence the IL-12 induced development of Th1 cells producing high levels of IFN-γ in the BALB/c mice (47).

To further test NKT cell deficiency and its effects on outcome to corneal infection, we used a single administration of the synthetic glycolipid αGalCer which has recently been shown by Parekh et al. (32) to induce long-term NKT cell unresponsiveness (anergy) in mice. It has been shown before by this group using their injection protocol that NKT cells failed to proliferate, produce cytokines such as IFN-γ, and transactivate other cell types upon rechallenge with αGalCer (32). When NKT cells were anergized in the infected B6 cornea, we saw reduced IFN-γ at 5 h and 1 and 5 days p.i., agreeing with our data using the Jα281-deficient mice and attesting to the requirement of NKT cells to induce high IFN-γ levels in the cornea. Using IL-12p40⁻/⁻ mice, we also showed that this cytokine is critical to production of IFN-γ by the NKT cell, and showed that in knockout mice, fewer activated (CD25⁺) CD4⁺ T cells and lower IFN-γ mRNA levels were seen. Admittedly, regulatory T cells also are characterized by expression of CD4 and high levels of the high-affinity IL-2R α-chain (CD25(high)) (48). However, the scope of this study did not include examination of these cells in the keratitis model. Use of dual immunostaining provided evidence that the lower levels of IFN-γ were not due to a dearth of NK cells in the cornea.

Despite the importance of NKT cells, the first cells in the innate immune system to be activated during an infection are DC and other APC, and this is often mediated by Toll receptors that sense bacterial products, leading to activation of the transcription factor NF-κB and the production of proinflammatory cytokines such as IL-12 and up-regulation of costimulatory molecules CD80 and CD86. Our work in vitro using LPS stimulation showed that LC and Mφ are sources of both costimulatory molecules, as well as IL-12p40, all contributing potentially to activate NKT cells. In addition, similar to the work of Skold et al. (49), we found that LPS alone up-regulated CD1d expression on Mφ in vitro. We also found that together with rIFN-γ, expression was enhanced 2-fold over LPS alone. These data suggest that modulation of cell surface CD1d levels on Mφ and other APC could be a mechanism that contributes (along with IL-12) to NKT cell activation.

Collectively, we have shown that: NKT cell production of IFN-γ is required for optimum early (NK) as well as later (NK and CD4⁺ T cell) IFN-γ production in the cornea; that without both NKT and NK cells, the cornea perforates earlier due to bacterial growth and increased PMN in the cornea; that in the absence of NKT and NK cells, IFN-γ and IL-12p40 levels are decreased; that the Mφ and LC are likely sources of IL-12p40 and other costimulatory molecules in cornea for NKT cell activation; and that IFN-γ potentiates Mφ CD1d levels synergistically with LPS.

Disclosures
The authors have no financial conflict of interest.

References


