Role of IL-12 and IFN-γ in Pseudomonas aeruginosa Corneal Infection

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PURPOSE. In Pseudomonas aeruginosa ocular infection, T-helper cell 1-responsive mouse strains are susceptible (the cornea perforates), and neutralization of IFN-γ before infection has been shown to delay the onset of perforation. IFN-γ is the predominant cytokine induced by IL-12, and positive regulation of IL-12 by IFN-γ, if unchecked, leads to excessive cytokine production and toxicity. Despite its potential importance, the role of IL-12 in ocular infection with P. aeruginosa remains unexplored and was the purpose of this study.

METHODS. IL-12 knockout mice, histopathology, RT/PCR and ELISA analyses, immunocytochemistry, and quantitation of viable bacteria in cornea were used to examine the role of IL-12 in IFN-γ production and the susceptibility phenotype.

RESULTS. To directly test the effect of IL-12 on IFN-γ production, IL-12 knockout and wild-type C57BL/6 mice were used. Both groups of mice were susceptible to infection, with corneal perforation seen at 5 to 7 days after infection. RT-PCR and ELISA analyses confirmed that IL-12 message and protein levels were elevated after infection only in the wild-type mouse cornea. Other differences between the two groups were detected. Knockout versus wild-type mice showed a significant decrease in IFN-γ mRNA levels in the cornea and cervical lymph nodes and decreased TNF-α protein levels in cornea. Cornes of knockout mice also had a significant increase in bacterial load at 5 days after infection when compared with wild-type mice.

CONCLUSIONS. These data provide evidence that IL-12 is important in IFN-γ production and in the absence of the cytokine, both IFN-γ and TNF-α levels in cornea are significantly decreased, resulting in unchecked bacterial growth and perforation. (Invest Ophthalmol Vis Sci. 2002;43:419–424)

A Pseudomonas aeruginosa infection is a rapidly developing and destructive disease of the cornea. A higher incidence of disease occurs in extended-wear contact lens users with corneal transplantation potentially necessary. Previous studies from our laboratory demonstrated that a CD4+ T cell (Th1)-dominated response after P. aeruginosa corneal infection is associated with susceptibility and corneal perforation in C57BL/6 (B6) mice, and that IFN-γ is involved, at least partially, in this process. In addition, other mouse strains favoring a Th1 response, such as C57BL/10 and B10.D2/nSn are also susceptible to infection. This model is not unique in this regard; in other systems, development of distinct Th responses and cytokine profiles has been shown to critically influence the outcome of host defense against microbial and parasitic pathogens in mice.

Development of a Th1 response depends on both the presence of IL-12 and the ability of T cells to respond to this cytokine. As a multifunctional cytokine, IL-12 exhibits a number of bioactivities that may modulate infectious disease progression, including enhancing proliferation and cytotoxicity of NK and T cells, inducing Th1 T cell differentiation, and influencing the production of other immunoregulatory cytokines, particularly IFN-γ. In this study, we investigated whether IL-12 is associated with IFN-γ production and corneal perforation (susceptibility) in P. aeruginosa infection in mice. Because neutralization of IFN-γ, a predominant Th1-type cytokine, before infection in B6 mice prevented corneal perforation, we elected to test the expression of this cytokine in susceptible B6 mice after infection. In addition, IL-12 knockout (KO) mice on a B6 background were tested to examine disease progression in the absence of IL-12 expression.

MATERIALS AND METHODS

Infection of Mice

Eight-week-old female B6 and IL-12 KO mice (p35 and p40 KO) on the B6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in an isolator unit. Before corneal infection, mice were anesthetized with isoflurane (Aerane; Anaquest, Madison, WI) and placed beneath a stereoscopic microscope at 40 magnification. Using a sterile 25gauge needle, the central cornea of the left eye was scarified with three 1-mm incisions. A 5-μL bacterial suspension containing 1.0 × 108 colony forming units (CFU) of P. aeruginosa, American Type Culture Collection (ATCC) strain 19660, prepared as described before, was topically applied onto the scarified cornea. Eyes were examined macroscopically at 24 hours after infection (PI) and at times described in the following section to ensure that all mice were infected and to monitor the course of disease, respectively. All animals were treated humanely and in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reverse Transcription–Polymerase Chain Reaction

Corneas and ipsilateral cervical lymph nodes (CLNs) were removed from B6 wild-type and p35 and p40 KO mice (before infection and at 6 and 12 hours and 1, 5, and 7 days after infection, for IL-12 and at 5 days after infection, for IFN-γ and IL-4, respectively) frozen in liquid nitrogen and stored at −70°C. Frozen tissue samples were homogenized in RNA STAT-60 (Tel-Test, Friendsville, TX) and total RNA was isolated according to the manufacturer’s instruction. Total RNA (50 ng) was reverse transcribed using random primers (Gibco BRL, Grand Island, NY) and reverse transcriptase (Sensicrypt; Qiagen, Valencia, CA) in the presence of 10 U of RNase inhibitor (Promega, Madison, WI). Amplification of cDNA was conducted with Taq polymerase (Gibco BRL) and specific primers for IL-12, IFN-γ, IL-4, and β-actin in a thermal cycler (GeneMate; ISC BioExpress, Kaysville, UT). The cycling
conditions used were 94°C for 45 seconds, 59°C for 30 seconds, 72°C for 1 minute 15 for 35 cycles, and a final extension at 72°C for 10 minutes.

The primers used were 5'-GGGTTCTCTCCGAGCATCAACATGCTG-3' (sense) and 5'-GCTGCGGTTGCGGCGGATTTCGGA-3' (antisense) for IL-12, 5'-TCCATGGCTCTGGTGAGCCTTCTCTG-3' (sense) and 5'-TGGAAGCTGTGATCTCTCATCTCCATGG-3' (antisense) for TNF-α.

After, an aliquot of each supernatant was cryopreserved as picograms of each cytokine per milligram of corneal tissue.

Integrated density values (IDVs) for IFN-γ and IL-4 PCR products were corrected for the amount of β-actin on each sample. Data are expressed as the mean IDV of samples from three separate mice.

Quantification of IL-12 p40 and TNF-α Levels

Corneal IL-12 p40 and TNF-α levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN), as described before.3,4 For these studies, individual corneas (n = 3/time point) were collected from mice at PI days 1 and 5. The total weight of each cornea was determined and the samples were immediately analyzed. Samples were homogenized with a glass pestle (Kontes; Fischer, Itasca, IL) and centrifuged. After, an aliquot of each supernatant was frozen at −20°C. Cytokine levels were measured using ELISA kit (Pierce, Rockford, IL) diluted 1:100 in stable peroxide buffer (Pierce) for 2 to 4 minutes, rinsed in distilled water, and mounted (Accu-mount; VWR Scientific Products, Chicago, IL). Control sections were incubated similarly with omission of the primary antibody. For morphological slides, were deparaffinized and rehydrated as for immunostaining, allowed to dry, stained with 1% methyl green for 1 minute at room temperature, rinsed, and mounted as for immunostained sections.

Histopathology

Histopathology. Eyes from three mice of each group were enucleated at PI days 5 and 7 for IL-12 p35 and p40 KO versus B6 wild-type mice. Eyes were immersed in PBS, rinsed, and placed in a fixative containing 1% osmium tetroxide, 2.5% glutaraldehyde and 0.2 M sodium phosphate buffer (pH 7.4; 1:1:1) at 4°C for 3 hours. Eyes were dehydrated in graded ethanol and embedded in Epon-Araldite as described.2 Thick sections (1.5 μm) were cut, stained with a modified Richardson’s stain, and observed. Representative sections for histopathology and from the immunostaining experiment above were photographed under a microscope (Axioskop; Carl Zeiss, Thornwood, NY).2

Quantification of Viable Bacteria in Cornea

At PI days 3 and 5, six corneas from each experimental group (B6 vs. p40 KO) were collected and the number of viable bacteria quantitated. Individual corneas were homogenized in sterile 0.9% NaCl containing 0.25% BSA.8,9 A portion (100 μl) of each sample was diluted serially 1:10 in the same solution, plated in triplicate on Pseudomonas isola
tion agar-coated plates (Difco, Detroit, MI), and incubated overnight at 37°C. The number of viable bacteria in an individual cornea was determined by counting individual colonies on plates from the various dilutions and multiplying the number of colonies by the appropriate dilution. Results are reported as log10 CFU per cornea ± SEM.

Statistical Analysis

An unpaired, two-tailed Student’s t test was used to determine statistical significance for data from RT-PCR, ELISA, mean clinical score, and bacterial count analyses. Differences (mean ± SEM) were considered significant at the confidence level of P ≤ 0.05. All experiments were repeated at least twice to ensure reproducibility. Representative data from a single experiment are shown.

Results

IL-12 mRNA and Protein Expression in Cornea after P. aeruginosa Infection

In wild-type B6 mice, IL-12 mRNA transcripts were undetectable in the uninfected cornea (time = 0) or in corneas at 6 hours after infection. IL-12 was first detected in the infected cornea of wild-type B6 mice at 12 hours after infection and remained detectable at all later times tested (Fig. 1). No message was detected in either infected or uninfected corneas from either group of IL-12 KO mice (p40 data shown), as expected (Fig. 1). To substantiate the mRNA data, IL-12 p40 protein levels in B6 mouse cornea were tested by ELISA (Fig. 2), and low levels of protein were detected at PI day 1. At PI day 5, levels were significantly elevated (P = 0.0163) when compared with PI day 1 data. Immunostaining coupled with methyl green staining of alternate sections, confirmed polymorphonuclear neutrophils (PMNs) as the predominant cellular source of IL-12 p40 in the B6 mouse corneal stroma at PI day 3 (Fig. 3). The experiments were performed similarly twice, with consistent results.
IL-12 KO Mice

IL-12 p35 and p40 KO and wild-type B6 mice also were tested for ocular disease response differences after *P. aeruginosa* challenge. A significant difference was observed in mean clinical scores between B6 wild-type versus p40 (Fig. 4) or p35 (data not shown) KO mice at PI day 1 only (*P* = 0.0004). At this time, the corneas of the two groups of KO mice exhibited slightly reduced opacity covering the anterior segment of the eye when compared with wild-type mice. However, at PI day 3, all groups of mice exhibited similar mean clinical scores (+3) indicating worsening disease. By PI days 5 to 7, all the corneas of the three groups of mice had perforated (+4). Because the p35 KO response was the same as the p40 KO, only the latter data are illustrated (Fig. 4).

Corneas from KO and B6 mice were also examined histopathologically at PI days 5 and 7—times when corneas of wild-type B6 mice began to perforate or had all perforated, respectively. The data from the p40 KO and wild-type B6 mice are presented in Figure 5. At PI day 5, the corneas of B6 (Fig. 5A) and p40 KO (Fig. 5C) B6 mice exhibited central thinning and edema of the corneal stroma. In both groups, the epithelium was denuded, and numerous inflammatory cells occupied the cornea and were present in the anterior chamber. By PI day 7, corneas of B6 (Fig. 5B) and p40 KO (Fig. 5D) mice had perforated, with inflammatory cells persisting in the cornea.

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**Figure 1.** IL-12 mRNA expression in *P. aeruginosa*-infected cornea. Total corneal RNA from B6 wild-type and p40 KO mice was analyzed for IL-12 expression by RT-PCR before and at 6 hours to 7 days after infection. *Lane 1:* B6, 0 hours; *lane 2:* B6, 6 hours; *lane 3:* B6, 12 hours; *lane 4:* B6, 7 days; *lane 5:* p40 KO, 7 days.

**Figure 2.** Corneal IL-12 p40 protein levels in B6 wild-type mouse cornea after *P. aeruginosa* infection. Three individual corneas from B6 wild-type mice were analyzed for IL-12 p40 protein levels by ELISA at PI days 1 and 5. Results are reported as picograms IL-12 p40 per milligram cornea ± SEM (*P* = 0.0165).

**Figure 3.** IL-12 p40 immunostaining. (A) Positively stained section from a B6 wild-type mouse cornea at PI day 3. Positive cells were scattered throughout the stroma. *Inset:* a methyl green-stained alternate section providing evidence that the immunostained cells are predominantly PMNs, due to their multilobated nuclei. (B) Control section in which the primary antibody was omitted with negative staining. Magnification, ×125.

**Figure 4.** Ocular disease response in B6 wild-type and p40 KO mice. Ocular disease grades were averaged at individual times after infection. Results are reported as mean clinical score ± SEM. A significant difference was observed only at PI day 1. *P* = 0.0004, *P* = 1.000, *P* = 5.504, and *P* = 1.000 at PI days 1, 3, 5, and 7, respectively.
and the anterior chamber. Histopathology of the p35 KO mouse cornea was similar to the data shown for the p40 KO mouse, and thus the data are not shown.

**IFN-γ Expression in p40 KO and B6 Mice**

We next tested for the level of IFN-γ mRNA production in p40 KO and wild-type B6 mice. To do this, IFN-γ expression in the cornea and CLNs was analyzed by RT-PCR. Figure 6A shows that IFN-γ transcripts were significantly reduced in the cornea (P = 0.0495) and CLNs (P = 0.0396) of p40 KO mice when compared with wild-type B6 mice at PI day 5. IL-4 levels were similarly tested to determine whether in the absence of IL-12, KO mice had elevated levels of a prototypic Th2-type cytokine (Fig. 6B). However, no differences in IL-4 mRNA transcript levels were detected in corneas (P = 0.6726) or CLNs (P = 0.3665) between the two groups of mice at PI day 5. To resolve the question of why, in past studies, reduction in levels of IFN-γ by neutralization delayed the time to perforation, whereas in this study we saw no similar effect, we also tested protein levels in cornea for TNF-α, another Th1-type cytokine that may be regulated by IL-12. KO mice had significantly reduced levels of TNF-α protein at both PI days 1 (P = 0.0509) and 5 (P = 0.0072), when compared with B6 wild-type mice (Fig. 7).

**Quantitation of Viable Bacteria in P. aeruginosa–Infected Corneas of B6 Versus p40 KO Mice**

We next tested whether the absence of IL-12 expression resulting in reduced IFN-γ and TNF-α levels in p40 KO versus wild-type B6 mice contributes to increased bacterial growth in the cornea. Direct plate count was used to quantitate bacterial load in the cornea of these two groups of mice at PI days 3 and 5. The mean log2 CFU of viable bacteria per cornea (± SEM) is shown in Figure 8. A significant increase in bacterial load (1–2 log increase) was found in the cornea of p40 KO versus B6 wild-type mice at PI days 3 (P = 0.0276) and 5 (P = 0.0089), respectively.

**DISCUSSION**

Previous studies from this laboratory have indicated that in B6 mice, a Th1-dominated response is associated with susceptibility to corneal perforation after P. aeruginosa infection and that treatment with anti-IFN-γ monoclonal antibody (mAb) delays this response. Further studies have shown that several other mouse strains favoring development of a Th1-type response were susceptible to corneal infection, whereas strains favoring Th2 responsiveness were resistant. Current work, reported herein, was focused on the regulatory role of IL-12 in induction of IFN-γ and the susceptible phenotype. During inflammation, IL-12 participates in setting the stage and influences the characteristics of the ensuing adaptive immune response. Its activities have been clearly documented in mice in vivo. After lipopolysaccharide (LPS) activation of phagocytic cells, accumulation of IL-12 p40 mRNA is seen within 2 to 4 hours, subsiding after several hours or in the first 2 days after infection, when sufficient levels of microorganisms are present in vivo. IFN-γ is the predominant cytokine induced, and positive regulation of IL-12 by IFN-γ, if unchecked or unbalanced, represents a dangerous loop that leads to excess proinflammatory cytokine production and toxicity. To unravel the role of IL-12 in our model system, RT-PCR was used to detect IL-12 levels in infected mice. Our data showed that IL-12 mRNA was undetectable in both groups of IL-12 KO mice, as expected, and that it was elevated in the cornea in susceptible wild-type B6 mice, beginning at 12 hours to 7 days after infection—the latter time coincident with corneal perforation. ELISA confirmed the presence of IL-12 p40 protein in the B6 wild-type mouse cornea. Furthermore, immunostaining with an IL-12 p40-specific antibody, together with methyl green staining of alternate sections for morphology, showed that PMNs, in the main, were the source of the cytokine in B6 wild-type mice at PI day 3. Nonetheless, many other cell types, such as macrophages, dendritic cells, and B cells, can also produce IL-12.

Based on previous mAb neutralization data, we next hypothesized that an absence of IL-12 might lead to a sufficient reduction in IFN-γ production, resulting in decreased pathogenesis. To ensure the complete absence of endogenous cytokine, IL-12 KO (p55 and p40 KO) mice on a B6 background were tested. As shown in Figure 5, except at PI day 1, when KO mice had slightly less inflammatory infiltrate, similar corneal disease was detected in the p55 (data not shown), p40 KO, and B6 wild-type mice after bacterial challenge. Further-
more, RT-PCR analysis revealed that IFN-γ transcripts remained detectable, albeit at a significantly lower level, in the cornea and CLN of p40 KO versus wild-type mice (Fig. 6A), suggesting that IL-12, although not required for IFN-γ induction, must play a dominant role in augmentation of its production. Furthermore, testing for IL-4 mRNA levels in corneas and CLNs of B6 wild-type versus IL-12 p40 KO mice, confirmed that in the absence of IL-12, there was no detectable upregulation of this predominantly Th2-type cytokine in KO versus wild-type mice (Fig. 6B).

Previous studies in this laboratory have shown that neutralization of IFN-γ in infected B6 mice delays the onset of corneal perforation.2 In contrast, we now show that p40 KO mice, despite expressing a significantly lower level of IFN-γ, have no better disease outcome than wild-type B6 mice. To resolve what may appear as disparate data, we propose the following scenario: It is well known that IL-12 is necessary for innate immune responsiveness,16 and as reported in other infectious disease models,17–22 the absence of IL-12 often results in production of an insufficient level of IFN-γ, critical for bacterial clearance. In contrast, BALB/c mice (Th2-responsive strain)3 fail to express mRNA for IL-12 after infection with P. aeruginosa, yet bacteria are efficiently cleared and corneal integrity restored.23 Knowledge of the precise mechanism(s) by which disease resolution occurs in this bacterial infection model, however, remains incomplete.
We next predicted that in the B6 mouse bacterial infection model, a sufficient level of IFN-γ, not available when IL-12 is knocked out, may be required for elimination of the bacteria from the cornea. When tested, this prediction was correct: p40 KO mice had a significantly higher (1–2 log increase) number of bacterial CFU in the cornea when compared with wild-type B6 mice. These data suggest that in the absence of IL-12, there is insufficient production of IFN-γ to control bacterial growth, and corneal perforation resulted due to bacterial versus host-driven factors. Nonetheless, we still had not resolved why in past studies, decreased IFN-γ ameliorated disease, and now, despite low mRNA levels of the cytokine in the KO mouse cornea, no difference in disease progression to perforation was detected between the two experimental groups. Therefore, we next tested for protein levels of another Th1-type cytokine, TNF-α. Unexpectedly, we found that in the KO versus wild-type mouse cornea, TNF-α protein levels were significantly reduced at both PI days 1 and 5 (Fig. 7).

TNF-α can be induced for release in human leukocytes by porins of *P. aeruginosa* and is produced by epithelial cells of the mouse cornea in response to endotoxin. TNF-α has been implicated in contributing to the pathogenesis of acute *Pseudomonas pneumonia*, but in a lung model, others have found that TNF-α not only significantly improves PMN recruitment, but that efficient activation of TNF-α is critical for prompt clearance of the bacteria. TNF-α shares many biological properties with IL-1, already shown by this laboratory to be of critical importance in the B6 ocular infection model for prompt clearance of the bacteria. Both TNF-α and IFN-γ are required for generation of sufficient IFN-γ and TNF-α and occurs via a nitric oxide-dependent mechanism. In summary, these data provide evidence that in B6 mice, IL-12 plays a disparate role in the host response to *Pseudomonas aeruginosa* corneal infection in mice infected with *Histoplasma capsulatum* through induction of IFN-γ and TNF-α and is produced by epithelial cells of the mouse cornea in response to endotoxin. On the one hand, data from the KO experiments suggest strongly that IL-12 is required for generation of sufficient IFN-γ and TNF-α to promote bacterial clearance. On the other, when IL-12 is present during the course of infection, as in the wild-type B6 mouse, its own augmentation and regulation of unchecked IFN-γ and increased TNF-α production results in corneal perforation and the susceptibility phenotype.

### References


