Interleukin 17 Regulates Mer Tyrosine Kinase–Positive Cells in Pseudomonas aeruginosa Keratitis

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Purpose. To determine if IL-17 regulates Mer tyrosine kinase–positive (MerTK+) cells in Pseudomonas aeruginosa keratitis.

Methods. Interleukin 17 was tested in normal and infected cornea of susceptible C57BL/6 and resistant BALB/c mice. The latter were treated with recombinant mouse (rm) IL-17; both groups were treated with IL-17 neutralizing antibody. Mice were infected, and clinical score, PCR, ELISA, and myeloperoxidase (MPO) assays tested expression of proinflammatory and anti-inflammatory mediators and polymorphonuclear neutrophilic leukocyte (PMN) infiltrate. Fas and Fas ligand (FasL) protein levels were assessed in both mouse strains, while MerTK+ cells were examined by immunostaining and cell sorting before and after IL-17 neutralization.

Results. The IL-17 mRNA and protein were higher in C57BL/6 versus BALB/c cornea after infection. The rmIL-17 treatment of BALB/c mice modified proinflammatory and anti-inflammatory mediators, but clinical score and MPO assay revealed no differences. However, only BALB/c mice treated with IL-17 neutralizing antibody showed increased disease, macrophage inflammatory protein (MIP) 2, and MPO levels. Fas and FasL protein levels, elevated earlier in BALB/c versus C57BL/6 mice, correlated with significantly more MerTK+ cells in BALB/c cornea at 3 days after infection. Neutralization of IL-17 in C57BL/6 mice elevated MerTK+ cells, while similar treatment of BALB/c mice significantly decreased them.

Conclusions. These data provide evidence that IL-17 expression is higher in C57BL/6 versus BALB/c cornea after infection and that the latter group has more MerTK+ cells. Exogenous rmIL-17 failed to shift the disease response in resistant mice, but its neutralization resulted in worsened disease and reduced MerTK+ cells. Neutralization of IL-17 in C57BL/6 mice increased MerTK+ cells but did not dramatically shift the disease response.

Keywords: bacteria, keratitis, IL-17, MerTK+ cells, mice

Pseudomonas aeruginosa, an opportunistic, gram-negative bacteria, is one of the most common pathogens associated with microbial keratitis1 and whose occurrence is closely related to contact lens usage.2 Pseudomonas aeruginosa keratitis progresses rapidly and elicits an acute inflammatory response in cornea that contributes to eradication of the bacterium. Unless precisely regulated, this inflammatory response also leads to significant corneal damage such as stromal destruction and loss of vision. Interventions are needed to promote bacterial clearance, while limiting tissue damage due to a rapid and extensive influx of inflammatory cells, the majority of which are polymorphonuclear neutrophilic leukocytes (PMNs).

Experimental murine models of the disease have been established. T helper type 1 (Th1) responder mouse strains such as C57BL/6 are susceptible (cornea perforates), whereas Th2 responder strains such as BALB/c are resistant (cornea heals).3 Host innate responses to bacterial infection are primarily mediated by PMNs and macrophages. Studies4–5 have provided evidence that a key regulatory molecule associated with PMN infiltration and inflammation-associated tissue damage in infectious diseases is IL-17.

Interleukin 17 has been mainly regarded as a proinflammatory cytokine that contributes to the local inflammatory response through increased production of various chemokines and cytokines, including TNF-α, macrophage inflammatory protein (MIP) 2, IL-1β, IL-6, and intercellular adhesion molecule 1 (ICAM-1), which are essential for migration and activation of PMNs and tissue damage at the site of inflammation.6–8 Interleukin 17 is now emerging as critical for host defense against bacteria, virus, and fungi. Previous investigations have shown that topical IL-17 neutralization reduces corneal pathology, PMN influx, and intracellular bacterial levels and improves early outcome for P aeruginosa keratitis in C57BL/6 mice.9 Neutralization of IL-17 also reduces the corneal lesion severity in recurrent herpetic keratitis in BALB/c mice.10 Furthermore, Candida keratitis development was blocked after neutralization of IL-17 activity in BALB/c mice.11 Interestingly, there is now an accumulation of evidence for IL-17 being able to exert anti-inflammatory action as well, depending upon the tissue environment, nature of the host, and kinetics of the response. Evidence shows that IL-17 is a negative regulator of established allergic asthma.12 Neutralization of IL-17 augments the allergic response, while exogenous IL-17 reduces pulmonary eosinophil recruitment and bronchial hyperreactivity. Others also have reported that neutralization of IL-17 markedly enhances the severity of colitis in BALB/c mice13 and increases periapical inflammatory bone destruction.14
Earlier apoptosis of infiltrating PMNs and efficient clearance of apoptotic cells lead to a rapid resolution of inflammation and protect against tissue damage.\textsuperscript{15,17} Efficient clearance of apoptotic cells requires M2c polarization and Mer tyrosine kinase (MerTK) induction.\textsuperscript{17} Mer tyrosine kinase is a major macrophage apoptotic cell receptor and enables M2c macrophages to clear apoptotic cells more efficiently. One study\textsuperscript{18} has shown that IL-17 critically stimulates proinflammatory M1 macrophage programming during removal of \textit{Versinia pestis} infection in C57BL/6 mice; while another study\textsuperscript{19} reported that IL-17 can stimulate differentiation of anti-inflammatory MerTK+ macrophages in response to IL-10. The relationship between IL-17 and MerTK+ cells, including macrophages during \textit{P. aeruginosa} keratitis, remains untested to date.

Thus, the present study investigated expression and function of IL-17 in innate immunity to \textit{P. aeruginosa} keratitis in mice. Our data provide evidence that IL-17 mRNA and protein levels are disparately upregulated in C57BL/6 (more) versus BALB/c (less) cornea after infection and that BALB/c mice have increased MerTK+ cells in cornea. In addition, BALB/c mice treated with recombinant mouse (rm) IL-17 or IL-17 neutralizing antibody provided evidence that exogenous rmIL-17 does not significantly shift the disease response, while neutralization of IL-17 results in worsened disease. Furthermore, neutralization of IL-17 in BALB/c mice decreased MerTK+ cells compared with controls, while similar treatment in C57BL/6 mice increased them, albeit not significantly.

**METHODS**

**Corneal Infection**

Eight-week-old female BALB/c (resistant) and C57BL/6 (susceptible) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were anesthetized with ether, placed beneath a stereoscopic microscope at ×40 magnification, and the cornea of the left eye wounded (three 1-mm incisions) using a sterile 25\(^{\text{g}}\)-gauge needle. A 5-µL aliquot containing 1 \times 10\(^6\) CFU/µL \textit{P. aeruginosa}, strain 19660 (American Type Culture Collection, Manassas, VA, USA) prepared as described before,\textsuperscript{3} was topically applied to the corneal surface. Eyes were examined at 1 day after infection and/or at times described below to ensure mice were infected and/or to monitor disease. Animals were treated humanely and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Corneal Response to Infection**

Disease was graded using the following scale\textsuperscript{20}: 0 (clear or opacity, partially or fully covering the pupil), +1 (slight opacity, covering the anterior segment), +2 (dense opacity, partially or fully covering the pupil), +3 (dense opacity, covering the anterior segment), and +4 (corneal perforation). Clinical score was recorded for each mouse after infection (1, 3, and 5 days) for statistical comparison of disease severity, and photography with a slitlamp was used to illustrate disease.

**rmIL-17 Treatment**

The left eyes of BALB/c mice (\(n = 5\)/group/time) were injected subconjunctivally with 1 µg/5 µL rmIL-17 protein (R&D Systems, Minneapolis, MN, USA) or 5 µL PBS at 1 day before infection. At 1 and 3 days after infection, each mouse was injected intraperitoneally with 1 µg rmIL-17 diluted in 100 µL PBS. Control mice similarly received an equal volume of PBS.

**IL-17 and IL-10 Neutralizing Antibody Treatment**

Rat anti-mouse IL-17 neutralizing antibody (R&D Systems) (10 µg/5 µL) or control rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (10 µg/5 µL) was given subconjunctivally into the left eyes of BALB/c and C57BL/6 mice (\(n = 5\)/group/time) the day before infection. On 1 and 3 days after infection, an additional 150 µg/100 µL was injected intraperitoneally; controls were similarly injected with IgG. Rat anti-mouse IL-10 neutralizing antibody (R&D Systems) (10 µg/5 µL) or control rat IgG (Jackson ImmunoResearch Laboratories) (10 µg/5 µL) was given subconjunctivally into the left eyes of BALB/c mice (\(n = 5\)/group/time) the day before infection. At 1 and 3 days after infection, an additional 150 µg/100 µL was injected intraperitoneally; controls were similarly injected with IgG.

**Real-Time RT-PCR**

After killing, normal (uninfected) and infected corneas were removed at 1 and 5 days after infection in BALB/c and C57BL/6 mice (\(n = 5\)/group/time) for detection of IL-17 mRNA levels. Corneas were harvested at 5 days after infection after rmIL-17 treatment or PBS treatment and after IL-17 neutralizing antibody treatment or IgG treatment. Total corneal RNA was extracted (RNA STAT-60; Tel-Test, Friendswood, TX, USA) according to the manufacturer’s instructions and was used to produce a cDNA template for PCR reaction. After spectrophotometric quantification (260 nM), 1 µg of each RNA sample was reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase. The 20-µL reaction mixture contained 10 U RNasin, 500 µM dNTP, 10 mM deoxyribonucleotide triphosphates, 100 mM dithiothreitol, and M-MLV reaction buffer (all from Invitrogen, Carlsbad, CA, USA). The cDNA products were diluted 1:25 with diethylpyrocarbonate-treated water, and a 2-µL cDNA aliquot was used for real-time RT-PCR (10-µL total reaction volume). The mRNA levels of IL-17, MIP-2, IL-1β, TNF-α, IL-6, IFN-γ, ICAM-1, and IL-10 were tested by real-time RT-PCR (CFX Connect Detection System; Bio-Rad, Hercules, CA, USA). Real-time SYBR Green/Fluorescein PCR Master Mix (Bio-Rad) was used for PCR reaction with primer concentrations of 5 µM. Optimal conditions for PCR amplification of cDNA were established using routine methods.\textsuperscript{21} Relative transcription levels were calculated using the relative standard curve method that compares the amount of target normalized to the endogenous reference gene β-actin. The primer pair sequences used for real-time RT-PCR are listed in the Table.

**ELISA Analysis**

All individual corneas taken for ELISA analysis were homogenized in 500 µL PBS with 0.1% Tween 20 (Sigma-Aldrich Corp., St. Louis, MO, USA) with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and centrifuged at 12,000g for 5 minutes. Normal (uninfected) and infected corneas were removed at 1, 3, and/or 5 days after infection from BALB/c and C57BL/6 mice (\(n = 5\)/group/time), and 50 µL of each sample was assayed in duplicate for IL-17 and 100 µL for Fas and Fas ligand (Fasl) (normal and 1 and 3 days after infection) according to the manufacturer’s instructions (R&D Systems). After rmIL-17 or PBS treatment in BALB/c mice or after IL-17 neutralizing antibody or IgG treatment of both mouse strains, normal and infected samples were harvested and prepared as described above at 3 and 5 days after infection. An aliquot (either 25 or 50 µL) of each supernatant was assayed in duplicate for TNF-α, MIP-2, and IL-1β protein (R&D Systems).
Quantitation of Corneal PMNs

A myeloperoxidase (MPO) assay was used to quantitate PMN number. Corneas (n = 5/group/time) were removed at 3 and 5 days after infection and homogenized in 1.0 mL 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich Corp.). Samples were freeze-thawed four times, and after centrifugation 100 μl supernatant was added to 2.9 mL 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 mL) and hydrogen peroxide (0.005%). The change in absorbency (460 nm) was monitored for 5 minutes at 30-second intervals. The slope of the line was determined for each sample and was used to calculate units of MPO per cornea. One unit of MPO activity is equivalent to approximately 2 × 10⁵ PMNs.²²

Immunofluorescent Staining

Eyes were enucleated (n = 3/group/time) at 3 days after infection from BALB/c and C57BL/6 mice, immersed in 0.1 M PBS, embedded in optimum cutting temperature compound (Tissue-Tek; Miles, Elkhart, IN, USA), and frozen in liquid nitrogen. Twelve-micrometer sections were cut, mounted to poly-L-lysine–coated glass slides, and stored at 37 °C overnight. After a 2-minute fixation in acetone, slides were blocked with 0.01 M PBS containing 2.5% BSA and goat IgG (1:100) for 30 minutes at room temperature. To identify IL-17-producing cells, sections were incubated for 1 hour with a 1:30 dilution of rat anti-mouse F4/80 antibody (Santa Cruz Biotechnology, San Jose, CA, USA) to label macrophages or rat anti-mouse NIMP-R14 (Abcam, Cambridge, MA, USA) to label PMNs. This was followed by the first secondary antibody, AlexaFluor 546–conjugated goat anti-rat antibody, at 1:1500 for 1 hour. Sections were then incubated for 2 minutes with SYTOX Green Nuclear Acid Stain (Lonza) as described above. Controls were similarly treated, but the primary antibodies were replaced with the same host IgG (Jackson ImmunoResearch Laboratories). This was followed by the corresponding secondary antibody, AlexaFluor 647–conjugated goat anti-rat antibody, at 1:1500 for 1 hour. Sections were incubated for 2 minutes with SYTOX Green Nuclear Acid Stain (Lonza) as described above. Controls were similarly treated, but the primary antibodies were replaced with the same host IgG as above. Finally, sections were visualized and digital images captured with a confocal laser scanning microscope (Leica TCS SP 8; Leica Microsystems, Buffalo Grove, IL, USA).

Histopathology

For histopathological analysis, whole eyes were enucleated from three mice from each experimental group (rmIL-17–treated and PBS-treated BALB/c mice) at 3 days after infection. Enucleated eyes were immersed in PBS, rinsed, and placed in a fixative containing 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorenson’s phosphate buffer, pH 7.4 (1:1:1), at 4 °C for a total of 3 hours. Eyes were transferred into fresh fixative after 1.5 hours, dehydrated in graded ethanols, and embedded in Epon-Araldite (Electron Microscopy Sciences, Hatfield, PA, USA). Sections (1.5 μm) were cut, stained with a modified Richardson’s stain, and observed. Representative sections were photographed with a Leica DM4000b bright-field microscope (Leica Microsystems).

Staining and Quantitation of F4/80+ and MerTK+ Cells

Individual corneas from C57BL/6 and BALB/c mice, corneas from both strains after IL-17 antibody or IgG control treatment, and BALB/c corneas after IL-10 antibody or IgG treatment were harvested at 3 days after infection and incubated in 1 mL (1 mg/mL) type I collagenase (Sigma-Aldrich Corp.) in Hanks’
balanced salt solution (Invitrogen) containing 5% fetal bovine serum (FBS) for 2 to 3 hours at 37°C. The samples were pipetted several times every 15 minutes to aid in dissociation of the cornea. Cell suspensions were centrifuged at 300g at 4°C for 5 minutes to pellet cells. Cells in suspension were fixed for 10 minutes in methanol at −20°C. Cells were centrifuged, and the cell pellet was washed with PBS containing 5% FBS. Cell suspensions were incubated in 100 μL PBS with 5% FBS containing goat anti-MerTK (R&D Systems) and rat anti-macrophage (F4/80; Santa Cruz Biotechnology) diluted 1:10 and 1:20, respectively, for 1 hour on ice. After washing in 500 μL PBS with 5% FBS, the cell suspension was incubated in 100 μL PBS with 5% FBS containing FITC-conjugated donkey anti-rat IgG (H + L) and R-phycocyanin-conjugated donkey anti-goat IgG (H + L) (Jackson ImmunoResearch Laboratories), each diluted 1:50 for 1 hour on ice. Cells were washed with 500 μL PBS with 5% FBS, and positive cells were counted on a Cellometer Vision (Nexcelom Biosciences, Lawrence, MA, USA). Data are expressed as the number of positively stained cells. Viability of cells for all experiments was greater than 85%.

### Statistical Analysis

The difference in clinical score between two groups at each time was tested by the Mann-Whitney U test. An unpaired, two-tailed Student's t-test was used to determine the statistical significance of real-time RT-PCR, MPO, ELISA, and cell-sorting data. Data were considered significant at \( P < 0.05 \). All experiments were repeated once to ensure reproducibility, and data from a representative experiment are shown as the mean ± SEM.

### Results

#### IL-17 Expression in Cornea of BALB/c and C57BL/6 Mice

To investigate the role of IL-17 in the mechanism by which corneas of BALB/c mice are able to more efficiently and effectively heal after \( P. aeruginosa \)-induced corneal infection, whereas C57BL/6 corneas typically perforate, we tested mRNA and protein levels of IL-17 in normal uninfected and infected BALB/c and C57BL/6 corneas by real-time RT-PCR and ELISA. Results indicated that relative IL-17 mRNA levels were significantly higher in C57BL/6 over BALB/c mouse corneas at 5 days after infection (Fig. 1A; \( P < 0.05 \)). Interleukin 17 was not detected in normal cornea of either group and was not different at 1 day after infection. To confirm these data, IL-17 protein was examined by ELISA (Fig. 1B). Interleukin 17 protein was elevated in C57BL/6 over BALB/c mouse corneas at 1, 3, and 5 days after infection (\( P < 0.01, P < 0.05, \) and \( P < 0.05 \), respectively). No IL-17 protein was detected in the normal cornea of either group. Dual immunostaining was used for both groups of mice to detect whether macrophages and PMNs expressed IL-17 in the infected cornea. Positive dual staining (magenta) for F4/80+ macrophages (red) that expressed IL-17 (blue) was seen in C57BL/6 (Fig. 1C) and BALB/c (Fig. 1D) corneal stroma at 3 days after infection. The F4/80+ macrophages that did not express IL-17 (F4/80 blue) were seen in C57BL/6 (Fig. 1F) and BALB/c (Fig. 1G) infected cornea, and controls were negative (Fig. 1H) for both groups but are only shown for C57BL/6 cornea.

#### rmIL-17 Treatment of BALB/c Mice

Given the divergent expression of IL-17 in resistant BALB/c versus susceptible C57BL/6 mice, as demonstrated at the mRNA (real-time RT-PCR) and protein (ELISA) levels in infected corneas, resistant BALB/c mice were treated with rmIL-17 to test whether increased levels of IL-17 resulted in worsened disease. Treatment with rmIL-17 did not significantly alter disease outcome at any time tested, as indicated by clinical score (Fig. 2A) and slitlamp photography comparing PBS-treated (Fig. 2B) and rmIL-17–treated (Fig. 2C) BALB/c mice at 5 days after infection. Next, we examined the effects of rmIL-17 treatment regarding corneal production of proinflammatory and anti-inflammatory cytokine expression after infection. After treatment with rmIL-17, corneal IL-17 mRNA levels were significantly decreased at 5 days after infection (Fig. 2D; \( P < 0.01 \)) compared with control-treated mice. Treatment with rmIL-17 compared with PBS also significantly decreased mRNA levels of proinflammatory cytokine IL-6 (Fig. 2E; \( P < 0.05 \)), while IFN-γ mRNA was elevated in the recombinant protein over control group at 5 days after infection; the difference was not significant (Fig. 2F). The ICAM-1 was decreased (Fig. 2G; \( P < 0.01 \)) at this time. The anti-inflammatory mediator IL-10 was downregulated in rmIL-17–treated mice compared with PBS-treated mice (Fig. 2H; \( P < 0.001 \)) at 5 days after infection. No differences between groups for normal cornea were detected (Figs. 2D–H). Moreover, corneal mRNA and protein levels for TNF-α, MIP-2, and IL-1β were tested. The PCR and ELISA data showed lower TNF-α mRNA (Fig. 3A; \( P < 0.05 \)) at 5 days after infection and protein (Fig. 3B), but the latter was not significantly decreased at any time tested. The MIP-2 mRNA (Fig. 3C; \( P < 0.001 \)) and MIP-2 protein (Fig. 3D; \( P < 0.05 \)) at 5 days after infection were significantly reduced. The IL-1β mRNA (Fig. 3E; \( P < 0.01 \)) was decreased after rmIL-17 treatment, but protein levels did not differ between the two groups (Fig. 3F). In addition, MPO levels, reflecting the PMN infiltrate, did not differ significantly between the two groups (Fig. 3G) at either 3 or 5 days after infection. The cell infiltrate was also examined histopathologically at 3 days after infection, and reflective of the MPO data, tissue sections showed a similarity of infiltrating cell type, mainly PMNs, in both PBS-treated (Fig. 4A) and rmIL-17–treated (Fig. 4B) BALB/c mouse cornea.

#### IL-17 Neutralizing Antibody Treatment of BALB/c and C57BL/6 Mice

Since treatment with rmIL-17 did not result in significantly changed disease outcome, BALB/c mice were treated with IL-17 neutralizing antibody. No significant difference in clinical scores was seen early (1 and 3 days after infection), but by 5 days after infection clinical score was significantly higher in antibody-treated compared with IgG-treated mice. Corneal perforation (+) occurred in 40% of mice after IL-17 neutralizing antibody treatment versus lower scores (+2) and no perforation in IgG control mice (Fig. 5A; \( P < 0.05 \)). Photographs taken using a slitlamp at 5 days after infection (Figs. 5B, 5C) illustrate the disease response in IgG control mice (+2) (Fig. 5B) versus IL-17 antibody-treated mice (+3) (Fig. 5C). Antibody treatment also significantly increased mRNA levels of IL-17 (Fig. 5D; \( P < 0.01 \)) at 5 days after infection, while levels of IL-6 (Fig. 5E) were decreased but not significantly. The IFN-γ (Fig. 5F; \( P < 0.01 \)) was increased 5 days after neutralization, while ICAM-1 (Fig. 5G; \( P < 0.05 \)) was slightly decreased at 5 days after infection. The IL-10 (Fig. 5H; \( P < 0.05 \)) mRNA levels were significantly upregulated in IL-17 antibody-treated compared with IgG-treated BALB/c mice at 5 days after infection. Moreover, PCR and ELISA data showed that...
TNF-α mRNA was decreased (Fig. 6A; P < 0.05), while protein levels were enhanced (Fig. 6B; P < 0.01) at 5 days after infection in antibody-neutralized over control-treated mice. Protein levels of IL-1β (Fig. 6C; P < 0.05) and IL-1b (Fig. 6E; P < 0.05) mRNA levels decreased at 5 days after infection, while MIP-2 protein (Fig. 6D) decreased at 3 days after infection (P < 0.05) but increased at 5 days after infection (P < 0.01) in antibody versus IgG controls. The IL-1β protein levels did not change significantly between the two groups (Fig. 6F) at 3 or 5 days after infection. The MPO levels, indicative of PMN infiltrate (Fig. 6G), decreased at 3 days after infection (P < 0.05) but increased at 5 days after infection (P < 0.001) after IL-17 neutralization.

The C57BL/6 mice were similarly treated with IL-17 neutralizing antibody (Figs. 7A–G). The mean clinical scores between the two groups did not differ at any time tested (Fig. 7A), nor were there visible differences between IgG-treated (Fig. 7B) and IL-17 antibody-treated (Fig. 7C) groups (visualized by slitlamp photography). No differences in protein levels were observed between groups for MIP-2 (Fig. 7D), IL-1β (Fig. 7E), or TNF-α (Fig. 7F). Consistent with these data, MPO levels also did not differ between groups at either 3 or 5 days after infection (Fig. 7G).

**Fas and FasL Expression and the Number of MerTK⁺ Cells in BALB/c Versus C57BL/6 Mice**

Since we have shown before that the pattern of apoptosis occurs earlier in BALB/c versus C57BL/6 mice and because among MerTK⁺ cells macrophages expressing the receptor are critical in apoptotic PMN removal and IL-10 secretion, we tested protein levels of Fas and FasL in the normal cornea and
after infection in both strains of mice. Results indicated that Fas (Fig. 8A) protein levels were elevated earlier in BALB/c versus C57BL/6 mice. At 1 day after infection, higher levels of Fas protein were detected in BALB/c versus C57BL/6 mice (P = 0.02), while at 3 days after infection more Fas was detected in C57BL/6 mice (P = 0.0001); a higher level of protein also was seen in the normal BALB/c mouse cornea (P = 0.0001). The FasL protein levels were greater in the normal cornea of BALB/c versus C57BL/6 mice and at 1 day after infection (Fig. 8B; P = 0.0002 and P = 0.008, respectively). At 3 days after infection, C57BL/6 mice showed higher levels of the protein over BALB/c mice (P = 0.007). Immunostaining and cell sorting were used to detect whether differences in MerTK+ cells correlated with the apoptotic pattern described above. Dual-label immunostaining at 3 days after infection revealed that MerTK+ macrophages are present in cornea of C57BL/6 (Fig. 8C) and BALB/c (Fig. 8E) mice; the respective controls (Figs. 8D, 8F) were both negative. In order to detect whether differences in the number of F4/80 and MerTK+ cells existed between the two groups, cell sorting was used, and at 3 days after infection more F4/80+ macrophages were detected in BALB/c over C57BL/6 mice (P = 0.007). The MerTK+ cells (macrophages among them) also were sorted, and at 3 days after infection BALB/c mice had significantly more positive cells (P = 0.003) than C57BL/6 mice.

**Figure 2.** (A–H) The rmIL-17 treatment of BALB/c mice. Similar clinical scores (A) were seen at 1, 3, and 5 days after infection in rmIL-17–treated compared with PBS-treated mice. Photographs taken with a slitlamp at 5 days after infection showed similar disease after PBS (B) compared with rmIL-17 (C) treatment. After rmIL-17 treatment, relative mRNA levels of IL-17 (D), IL-6 (E), and ICAM-1 (G) were significantly decreased in the infected cornea of BALB/c mice at 5 days after infection. There was no difference in mRNA expression of IFN-γ (F) between treatment groups at 5 days after infection. The mRNA levels of IL-10 (H) were significantly decreased in rmIL-17 compared with PBS controls at 5 days after infection. The IL-17, IL-6, IFN-γ, and IL-10 mRNA was not detected in the normal cornea of either group; no differences between groups were seen for ICAM-1 in the normal cornea. Magnification: ×5 (B, C).
FIGURE 3. (A–G) Effects of rmIL-17 treatment on TNF-α, MIP-2, IL-1β, and MPO levels. The TNF-α mRNA (significantly) and protein (not significant) levels were decreased (A, B) at 5 days after infection in rmIL-17–treated over PBS control mice. In addition, MIP-2 mRNA (C) and protein (D) levels were both significantly decreased at 5 days after infection. No differences in protein levels were detected at 3 days after infection. The IL-1β mRNA (E) was significantly decreased after rmIL-17 treatment at 5 days after infection, while protein levels (F) did not differ between groups at either 3 or 5 days after infection. The MPO (G) levels did not differ between the two groups at either 3 or 5 days after infection. No differences were detected for normal cornea between the two groups.
Human patients with hypercells are macrophages, nor are all macrophages keratitis showing that IL-27 Interleukin 17 (and IL-17F) mediates immunologic... effects of IL-17 on the number of MerTK+ and F4/80+ cells. IL-17 neutralizing antibody was used in C57BL/6 and BALB/c mice. Immunostaining and cell sorting to determine the number of F4/80 and MerTK+ cells at 3 days after infection showed that neutralization of IL-17 in C57BL/6 mice increased F4/80+ and MerTK+ cells (Fig. 9A) but not significantly, while similar treatment of BALB/c mice decreased both F4/80 (P = 0.003) and MerTK+ (P = 0.02) cells significantly. However, as shown in Figures 9A and 9B, not all MerTK+ cells are macrophages, nor are all macrophages MerTK+. To determine in BALB/c mice whether IL-10 was of importance in the latter response, IL-10 was neutralized, and data showed (Fig. 9C) that IL-10 neutralization compared with IgG treatment did not affect either F4/80 or MerTK+ cell number.

**DISCUSSION**

The IL-17 family members are emerging as key players in immune responses. The first member to be identified, IL-17A (also commonly called IL-17), is the prototype of the IL-17 family. Interleukin 17 is a proinflammatory cytokine that contributes to the local inflammatory response through increased production of various chemokines and cytokines that are essential for migration and activation of PMNs and tissue damage at the site of inflammation. It has been reported that IL-17 is produced in cornea during *P. aeruginosa* keratitis. Results presented in this study revealed that IL-17 was not detected in normal cornea and that after *P. aeruginosa* infection its expression is discretely upregulated in C57BL/6 (more) versus BALB/c (less) corneas. These data, suggesting a potential role for IL-17 in the development of susceptible versus resistant phenotype, are consistent with results in a previous study from another laboratory showing that *P. aeruginosa* keratitis induced a strong Th17 cell response in C57BL/6 mice that results in IL-17 production in cornea. Our findings also are consistent with those in previous investigations in a murine model of *Candida* keratitis showing that IL-17 was induced after infection and correlated with the development of disease. In line with data from animal investigations, IL-17 expression levels also were elevated in corneas of herpetic stromal keratitis patients, while no IL-17 was detected in normal corneas.

In our study, despite the disparate levels of IL-17 in cornea between the two groups, we found that PMNs and macrophages in both strains of mice produced IL-17 in corneal stroma after infection. While PMNs are thought to be the predominant effector of Th17 cell responses, IL-17 is also known to induce myelotropic chemokines and growth factors. In fact, IL-17 signaling may elicit unique macrophage functions based upon the anatomical compartment in which they reside. The greatest expression of IL-17 receptors was observed on mucosal macrophages, supporting that macrophages from different anatomical locations direct IL-17-mediated responses. We did not examine other cell types, but classically gamma delta T cells and another innate subtype of T cells that expresses IL-17 during intestinal bacterial infection have recently been identified. In fact, IL-17 can be produced by several other innate immune cell types such as lymphoid tissue-inducer cells, natural killer and natural killer T (NKT) cells, macrophages, and Paneth cells. The functional importance of the IL-17 produced by these cell types during inflammation is not well characterized.

In this regard, IL-17 (and IL-17F) has protective roles in host defense against several pathogens at epithelial and mucosal barriers. A defect in IL-17 or IL-17RA results in increased bacterial dissemination, correlating with reduced inflammatory mediators and PMN recruitment. Human patients with hyper-IgE syndrome, who have defective IL-17A/F production due to a genetic mutation in the STAT3 gene, have high susceptibility to *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Candida albicans* infection, further supporting the protective roles of IL-17 (and IL-17F) in immune responses against these pathogens. Interleukin 17 (and IL-17F) mediates immunological function by inducing proinflammatory cytokine, antipathogenic peptide, and chemokine secretion by responder cells. The release of these molecules signals recruitment of innate immune cells to the site of infection and elimination of the pathogen.

Previous investigators have reported that neutralization of IL-17 during corneal *P. aeruginosa* infection reduced PMN influx and pathology at 48 hours after infection in C57BL/6 mice. In order to fully determine the role of IL-17 in corneal *P. aeruginosa* infection, rmIL-17 protein was given to resistant BALB/c mice that had lower IL-17 expression than C57BL/6 mice to determine whether rmIL-17 increased the disease response. Clinical score and slitlamp data showed that injection of BALB/c mice with rmIL-17 protein did not significantly alter disease outcome. Nonetheless, treatment with rmIL-17 compared with PBS significantly decreased mRNA levels of proinflammatory molecules such as IL-17, MIP-2, IL-1β, TNF-α, IL-6, and the anti-inflammatory mediator IL-10, while IFN-γ mRNA levels did not differ between the two groups. We also found lower MIP-2 protein levels after rmIL-17
treatment at 5 days after infection, but protein levels for IL-1β, TNF-α, and MPO (indicative of PMN infiltrate) did not differ between the two groups. These findings are consistent with a previous study showing that administration of rmIL-17 did not significantly alter MPO activity and mRNA levels of IFN-γ on Helicobacter pylori–induced gastritis. Our in vivo data suggest that exogenous IL-17 in a resistant mouse disrupts the balance of proinflammatory and anti-inflammatory mediators but is not sufficient to shift the disease response to susceptibility or perforation. Our results provide evidence that exogenous IL-17 may have multiple roles in the pathogenesis of corneal P. aeruginosa infection in BALB/c mice, which is consistent with the study by Ke et al. showing that IL-17 had both proinflammatory and anti-inflammatory effects in experimental autoimmune uveitis.

Since treatment with rmIL-17 did not significantly change disease outcome, IL-17 neutralizing antibody was used to test if decreasing IL-17 in BALB/c mice had an effect. Data provided evidence that IL-17 neutralizing antibody did not significantly change early disease (1 and 3 days after infection) clinical scores, but at 5 days after infection clinical scores were significantly higher in IL-17 antibody–treated compared with IgG-treated mice. Our findings are consistent with studies showing that neutralization of IL-17 markedly increased the severity of colitis in BALB/c mice and that IL-17 neutralization induced a significant increase in periapical inflammatory bone destruction. In contrast, Zaidi et al. reported that neutralization of IL-17 in C57BL/6 mice during corneal P. aeruginosa infection reduced PMN influx and pathology at 48 hours after infection. Thus, we also neutralized IL-17 in C57BL/6 mice, but

**Figure 5.** (A–H) Interleukin 17 neutralizing antibody treatment of BALB/c mice. Clinical scores (A) were similar at 1 and 3 days after infection but were significantly higher in IL-17 antibody–treated compared with IgG-treated mice at 5 days after infection. Photographs taken by slitlamp at 5 days after infection illustrate the disease response in IgG control mice (±2) (B) versus IL-17 antibody–treated mice (±3) (C). After IL-17 neutralization, relative mRNA levels of IL-17 (D), IFN-γ (F), and IL-10 (H) were significantly increased in the infected cornea of BALB/c mice at 5 days after infection, while ICAM-1 (G) mRNA levels decreased. There was no difference in mRNA expression of IL-6 (E) between the two groups at 5 days after infection. There were no differences between groups for normal cornea. Magnification: ×5 (B, C).
there was no change in clinical score, in levels of proteins (including MIP-2, IL-1β, and TNF-α), or in MPO assay following infection. The reason for these inconsistencies may be the use of different bacterial strains, routes of delivery, and concentrations of the neutralizing antibody, as well as the extended time points used for our versus their experiments. Similar inconsistency between neutralization of IL-17 and gene deletion of IL-17 was also found. Flierl et al. reported that neutralization of
IL-17 by antibody was protective by reducing bacteremia and systemic levels of pro-inflammatory cytokines and chemokines and by improving survival during polymicrobial sepsis in C57BL/6 mice. In contrast, Ogiku et al. reported that the survival rate of IL-17 knockout mice was significantly reduced after sepsis and that a more severe degree of acute lung injury was detected in the IL-17 knockout mice than in the wild-type mice after sepsis. In addition, we found that IFN-γ mRNA levels were significantly upregulated in IL-17 antibody-treated compared with IgG-treated BALB/c mice at 5 days after infection. The MPO levels also did not differ between the two groups for either time tested. Magnification: ×5 (B, C).

**Figure 7.** (A–G) Interleukin 17 neutralizing antibody treatment of C57BL/6 mice. Similar clinical scores (A) were seen at 1, 3, and 5 days after infection in IL-17 antibody-treated compared with IgG-treated mice. Photographs taken with a slitlamp at 5 days after infection showed similar disease after IgG (B) compared with IL-17 antibody (C) treatment. After IL-17 neutralization, protein levels of MIP-2 (D), IL-1β (E), and TNF-α (F) did not differ between the two groups at 3 or 5 days after infection. The MPO levels (G) also did not differ between groups for either time tested.

IL-17 increased MPO activity and expression of mRNA for IFN-γ on H. pylori-induced gastritis. The IL-17RA knockout mice expressed significantly higher levels of MIP-2 and elevated accumulation of PMNs in infection-stimulated periapical bone destruction. Our neutralization data showed that IL-17 is needed for the resistance response of BALB/c mice and that IFN-γ mRNA levels were significantly upregulated in IL-17 antibody-treated compared with IgG-treated BALB/c mice at 5 days after infection. The MIP-2 protein and MPO decreased at 3 days after infection but increased at 5 days after infection in IL-17 neutralizing antibody versus IgG controls. These findings are consistent with a study showing that neutralization of IL-17 increased MPO activity and expression of mRNA for IFN-γ on H. pylori-induced gastritis. The IL-17RA knockout mice expressed significantly higher levels of MIP-2 and elevated accumulation of PMNs in infection-stimulated periapical bone destruction. Our neutralization data showed that IL-17 is needed for the resistance response of BALB/c mice and consistent with studies showing that IL-17 had anti-inflammatory effects on H. pylori-induced colitis and gastritis.

Our previous studies have shown that PMN apoptosis occurs earlier in BALB/c versus C57BL/6 mice. Early apoptosis of PMNs leads to rapid resolution of inflammation. In addition, we reported herein that Fas and FasL protein levels were elevated earlier in BALB/c versus C57BL/6 mice, consistent with earlier apoptosis in BALB/c mice. In this regard, work...
from another laboratory has shown that, among the cells that express MerTK, macrophages (a subset of M2 macrophages) expressing this receptor are critical in apoptotic PMN removal and IL-10 secretion. The MerTK receptor mediates phagocytosis of apoptotic cells and modulates cytokine production; it is also required for prevention of systemic autoimmune disease. Using a Mer-specific antibody, Behrens et al. confirmed the presence of Mer on macrophages but also detected its expression on other innate immune cells, including NK, NKT, and dendritic cells (DCs). They found that DCs do not
require Mer for ingestion of apoptotic cells because DCs from Mer-deficient mice phagocytose apoptotic cells normally. Mer apparently participates in NKT cell antigen–induced signaling because NKT cells from Mer-deficient mice demonstrated much lower cytokine production after in vivo α-galactosylceramide stimulation; this defect was intrinsic to the Mer-deficient NKT cells. Taken together, these studies show Mer expression on many cells of the innate immune system. Mer, through its binding of lipid antigens, may not only mediate ingestion of apoptotic cells but also signal events in NK cells, NKT cells, and DCs. Little is known regarding MerTK cells and their role in P. aeruginosa keratitis, but previous studies from this laboratory have shown the importance of both NK cells and NKT cells in bacterial keratitis. In BALB/c mice, NK cells were shown to express the NK-1R and were required for disease resolution through production of IFN-γ. In C57BL/6 mice, NKT cells were critical to initiate an inflammatory response, and their depletion (NKT or NK cells) resulted in earlier corneal perforation. To date, nothing is known regarding MerTK-expressing cells in the infected cornea.

Using dual immunostaining, we found both MerTK{sup+} and F4/80{sup+}, as well as MerTK{sup+}–F4/80{sup+} macrophages, in both the C57BL/6 and BALB/c infected corneal stroma. These data are consistent with other reports that not all human macrophage subsets (e.g., M1 cells secreting IL-12 and promoting Th1 differentiation) were positive for MerTK.

Using cell sorting, significantly more MerTK{sup+} cells were seen in BALB/c over C57BL/6 infected cornea at 3 days after infection. Then, to determine the effect of IL-17 on this population of MerTK{sup+} cells further, antibody neutralization and cell sorting were used. The IL-17 neutralization in C57BL/6 mice raised the level of both F4/80 and MerTK{sup+} cells but did not reach significance. In contrast, similarly treated BALB/c mice showed a significant decrease in both F4/80 and MerTK{sup+} cell number and again confirmed that not all macrophages were positive for MerTK. These data complement another study showing that administration of IL-17 neutralizing antibodies led to decreased M1 macrophages and increased M2 (anti-inflammatory) macrophages (a decreased ratio of M1 to M2) in local necrotic lesions of the jawbone in C57BL/6 mice. These data suggest that lower levels of IL-17 in BALB/c mice promote MerTK{sup+} cells (including F4/80 macrophages) to enhance the resistance response. Our findings are also complementary to other investigations showing that IL-17 is involved in stimulating macrophages from BALB/c mice to phagocytize aged PMNs, but further work in the keratitis model will be required to test that more specifically.

In addition, since investigators have reported that IL-17 can stimulate differentiation of anti-inflammatory MerTK{sup+} cells in response to IL-10, we neutralized IL-10 in BALB/c mice and found no difference in the MerTK cell population at 3 days after infection. These data were surprising because we have shown that IL-10 recombinant protein together with rapamycin treatment can rescue BALB/c mice from the deleterious effects of inhibition of mammalian target of rapamycin by administration of rapamycin, whereby IL-10 is reduced and IL-12 is increased.

In summary, the data presented herein indicate that IL-17 is more highly expressed in C57BL/6 versus BALB/c mouse cornea after infection. Exogenous rmIL-17 in a resistant (BALB/c) mouse disrupts the balance of proinflammatory and anti-inflammatory mediators but does not shift the disease response to susceptibility or perforation. However, its neutralization resulted in worsened disease in these mice. In addition, we provide evidence that neutralization of IL-17 in C57BL/6 mice increases MerTK{sup+} cells, albeit not significantly, while similar treatment of BALB/c mice decreases them, with significant consequences and enhanced disease. Continued studies of IL-17–expressing and MerTK-expressing cells appear critical and may significantly enhance our understanding of protective inflammation during pathogen infection. Further dissection of the source and regulation of IL-17 expression and signaling mechanisms may provide novel ways to modulate the immune system in infectious and inflammatory diseases.

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