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Galectin-1–Mediated Suppression of Pseudomonas aeruginosa–Induced Corneal Immunopathology

Amol Suryawanshi,*† Zhiyi Cao,*† Thananya Thitiprasert,*† Tanveer S. Zaidi,‡ and Noorjahan Panjwani*†§

Corneal infection with Pseudomonas aeruginosa leads to a severe immunoinflammatory lesion, often causing vision impairment and blindness. Although past studies have indicated a critical role for CD4+ T cells, particularly Th1 cells, in corneal immunopathology, the relative contribution of recently discovered Th17 and regulatory T cells is undefined. In this study, we demonstrate that after corneal P. aeruginosa infection, both Th1 and Th17 cells infiltrate the cornea with increased representation of Th17 cells. In addition to Th1 and Th17 cells, regulatory T cells also migrate into the cornea during early as well as late stages of corneal pathology. Moreover, using galectin-1 (Gal-1), an immunomodulatory carbohydrate-binding molecule, we investigated whether shifting the balance among various CD4+ T cell subsets can modulate P. aeruginosa–induced corneal immunopathology. We demonstrate in this study that local recombinant Gal-1 (rGal-1) treatment by subconjunctival injections significantly diminishes P. aeruginosa–mediated corneal inflammation through multiple mechanisms. Specifically, in our study, rGal-1 treatment significantly diminished corneal infiltration of total CD45+ T cells, neutrophils, and CD4+ T cells. Furthermore, rGal-1 treatment significantly reduced proinflammatory Th17 cell response in the cornea as well as local draining lymph nodes. Also, rGal-1 therapy promoted anti-inflammatory Th2 and IL-10 response in secondary lymphoid organs. Collectively, our results indicate that corneal P. aeruginosa infection induces a strong Th17-mediated corneal pathology, and treatment with endogenously derived protein such as Gal-1 may be of therapeutic value for the management of bacterial keratitis, a prevalent cause of vision loss and blindness in humans worldwide. The Journal of Immunology, 2013, 190: 000–000.

C orneal infection with Pseudomonas aeruginosa is a leading cause of infectious microbial keratitis and associated vision impairment in humans worldwide (1–3). This bacterial keratitis is characterized by rapid inflammatory response with suppurative coagulative necrosis, often irreversibly damaging corneal tissue architecture, leading to vision loss (4). Widespread use of contact lenses and the ability of P. aeruginosa to grow in nutrient-deprived conditions such as various ophtalmic solutions poses a serious threat of vision impairment due to P. aeruginosa keratitis (5–7). Currently, bacterial keratitis is mainly controlled by therapeutic topical administration of antibiotics (8). However, constant emergence of antibiotic resistant bacteria poses a serious challenge for effective management of microbial keratitis (9, 10). Therefore, understanding the pathogenesis of P. aeruginosa corneal infection, particularly the different factors and cells that either contribute to or inhibit the corneal pathology, would reveal significant information to design novel therapeutics.

Past studies using a mouse model of P. aeruginosa–induced bacterial keratitis have shown the complex interplay of numerous factors secreted from both host as well as pathogen (4, 11). The inflammatory response in the cornea is mainly initiated and maintained by sequential migration and activation of proinflammatory cells from both innate and adaptive immune response against invading corneal P. aeruginosa infection (4). The role of CD4+ effector T cells, particularly CD4+ IFN-γ+ T (Th1) cells, is well documented in the pathogenesis of P. aeruginosa infection of the cornea (12–14). In this respect, it is known that mice strains such as C57BL/6, which are biased toward a strong Th1 response, are susceptible to a severe form of pathology characterized by corneal perforation (13). In contrast, the BALB/C mouse strain that dominantly induces Th2 (CD4+ IL-4+ T cells) response is more resistant to corneal perforation (13). Furthermore, studies have shown that shifting the balance from a Th1 to Th2 response in C57BL/6 mice significantly diminishes pathology and protects mice from corneal perforation after P. aeruginosa infection (15, 16). Although these reports indicate an obvious involvement of Th1 cells as a principal orchestrator of corneal immunopathology after P. aeruginosa infection, the actual tissue damage to the corneal epithelial and stromal tissue layers is primarily mediated by uncontrolled migration and activation of neutrophils and their secretion of various proinflammatory molecules in the cornea (4, 11, 17, 18).

Little is known about the role of recently discovered Th17 cells (CD4+IL-17A+ T cells) in the immunopathology of P. aeruginosa keratitis. Th17 cells, a new subset of CD4+ T cells that produce IL-17A, have been shown to play an important role in various autoimmune and immunopathological disorders by enhancing granulopoiesis as well as neutrophil activity at the site of inflammation (19). IL-17A is a proinflammatory cytokine that contributes to the local inflammatory response through increased production of

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Abbreviations used in this article: DLR, draining lymph node; Gal-1, galectin-1; MMP, matrix metalloproteinase; pi, postinfection; rGal-1, recombinant galectin-1; Treg, regulatory T cell.

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various chemokines and cytokines essential for migration and activation of neutrophils at the site of inflammation (20–22). Because the neutrophil is the predominant cell type that contributes to corneal tissue damage post-\(P.\) aeruginosa infection, it is plausible that Th17 cells and resulting IL-17A are key factors responsible for the severe tissue destruction in \(P.\) aeruginosa keratitis. In support of this notion, Zaidi et al. (23) have shown that topical neutralization of IL-17A during \(P.\) aeruginosa corneal infection reduces neutrophil influx and pathology.

Recent discovery of CD4\(^+\) Foxp3\(^+\) regulatory T cells (Treg) and their critical role in the suppression of the effector T cell response represents a promising therapeutic strategy to control various autoimmune and chronic immunopathological disorders (24). Accordingly, recent studies have demonstrated that various strategies that promote increased expression of Treg over Th1 and/or Th17 can be beneficial to the host in various autoimmune and chronic pathological disorders (24–27). Moreover, the host produces anti-inflammatory molecules that curtail proinflammatory Th1 and Th17 response and promote anti-inflammatory Th2 response (28–30). In this regard, galectin-1 (Gal-1), a member of the galectin family of mammalian \(b\)-galactoside–binding proteins, has been shown to play an immunomodulatory role during various immunoregulatory processes (28, 31–33). Gal-1 exerts its anti-inflammatory effect through a variety of mechanisms including induction and differentiation of Treg, suppression of Th1 and Th17 differentiation, increased apoptosis of activated Th1 and Th17 cells, increased production of anti-inflammatory cytokines, and induction of CD4\(^+\)IL-10\(^+\) T cells (Tr1) (28, 34–41). Accordingly, Gal-1 has been shown to diminish Th1- and Th17-mediated immunopathology through increased representation of Treg/Tr1/Th2 cells over Th1 and Th17 responses (28, 36, 40, 42, 43). However, the role of Treg and modulation of different CD4\(^+\) T cell subsets during corneal \(P.\) aeruginosa–induced immunopathology is poorly understood.

In the present report, we demonstrate for the first time, to our knowledge, that both Th1 and Th17 cells infiltrate the cornea after \(P.\) aeruginosa infection. Moreover, we show that Th17 cells are far more numerous than Th1 cells during the late stage of corneal immunopathology. Additionally, Treg also migrate into the cornea during the early as well as the late stages of bacterial keratitis. Moreover, we show that application of recombinant Gal-1 (rGal-1) by subconjunctival injections after ocular \(P.\) aeruginosa infection significantly diminishes corneal lesion severity through modulation of Th17 and Treg responses. Interestingly, rGal-1 treatment shifted the proinflammatory Th17-mediated pathogenesis toward anti-inflammatory Th2- and Tr1-type immune responses.

Materials and Methods

Mice and bacterial strains

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care at Tufts University, and all experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. A \(P.\) aeruginosa cytotoxic strain 6077 was used for corneal infection. Different doses from 2–5 \(\times\) \(10^5\) CFU/eye were evaluated for severity of corneal pathology. For kinetics experiments, a dose of 3.5 \(\times\) \(10^5\) CFU/eye was used to infect mice, whereas for rGal-1 treatment experiments, a slightly lower dose of 2.5 \(\times\) \(10^5\) CFU/eye was used to infect mice. Bacterial strain was grown overnight on tryptic soy broth agar plates at 37˚C before each infection.

Corneal infection

Corneal infections of mice were conducted under deep anesthesia induced by i.p. injection of ketamine and xylazine. Central corneas of mice were scarified with three parallel 1-mm incisions using a 26-gauge needle, and a 5-µl drop containing the appropriate dose of bacteria was applied to the eye.

Clinical scoring

The eyes were examined on different days postinfection (pi) for the development of corneal opacity. The following scoring system, graded from 0–4, was used as previously described (44): 0, eye macroscopically identical to the uninoculated control eye; 1, partial corneal opacity covering the pupil; 2, dense corneal opacity covering the pupil; 3, dense opacity covering the entire anterior segment; and 4, perforation of the cornea, phthisis bulb (shrinkage of the globe post–inflammatory disease).

Reagents and Abs

CD4\(^+\)-allophycocyanin (RM4-5), IL-17–PE (TC11-18H10), IFN-\(\gamma\)-FITC (XMGl-1), CD45-PerCP (30-F11), CD45-allophycocyanin (30-F11), CD11b-PerCP (M170), Ly6G-PE (1A8), anti-CD3 (145.2C11), and anti-CD28 (37.51), and GolgiStop (brefeldin A) were purchased from BD Biosciences (San Jose, CA). The Fxsp-3-PE and ELISA kit for IL-4, IL-10, and IL-17A were purchased from eBioscience (San Diego, CA). The rGal-1 was purified by culturing transformed Escherichia coli with pQE-60/hGal-1 in Luria-Bertani containing ampicillin (50 \(\mu\)g/ml). The production of rGal-1 was induced by adding 1 mM isopropyl-\(D\)-thiogalactoside during last 4 h of bacterial culture. Gal-1 was purified from bacterial cell lysate by affinity chromatography using lactosyl-Sepharose beads. Briefly, bacterial cell lysates supernatants were incubated with 2 ml bead volume at 4˚C for 1 h, followed by loading of beads on column. The column was washed with 30 ml 20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.2 mM PMSF, and 4 mM 2-ME followed by a second washing with 10 ml wash buffer (PBS containing 0.2 mM PMSF and 4 mM 2-ME). Fractions containing rGal-1 were eluted with wash buffer containing 0.1 M lactose, dialyzed against PBS containing 2% glycerol and 4 mM 2-ME, and stored at –80˚C.

rGal-1 treatment

Subconjunctival injections of rGal-1 in \(P.\) aeruginosa–infected mice were performed as described previously (45). Briefly, a 2-cm needle attached to a 32-gauge syringe (Hamilton, Reno, NV) was used to penetrate the perivascular region of the conjunctiva, and rGal-1 (20 \(\mu\)g in 10 \(\mu\)l volume) was delivered into the subconjunctival space. \(P.\) aeruginosa–infected mice were scored for corneal opacity on day 1 pi, and animals with similar opacity were randomly divided into two groups. One group of mice was treated with rGal-1 by local subconjunctival injections every alternate day starting from day 1 until day 11 pi. Control mice were injected with 10 \(\mu\)l vehicle (PBS containing 2% glycerol and 4 mM 2-ME).

Detection of corneal-infiltrating cells and flow cytometry

\(P.\) aeruginosa–infected corneas were harvested from different groups of mice at indicated time points pi. Six to eight corneas per group were excised, pooled group wise, and digested with 60 U/ml Liberase (Liberase TL; Roche Diagnostics, Indianapolis, IN) for 35 min at 37˚C in a humidified atmosphere of 5% CO\(_2\). After incubation, the corneas were disrupted by grinding with a syringe plunger on a cell strainer, and single-cell suspensions were made in complete RPMI 1640 medium. The single-cell suspensions obtained from corneas, draining lymph nodes (DLN), and spleen were stained for different cell-surface molecules using conjugated Abs as described below. Briefly, cells were blocked with an unconjugated anti-CD3/CD16 mAb for 30 min in FACS buffer (2% PBS in PBS) and then incubated with different cocktails of Abs depending on cell type for 30 min on ice. For CD4\(^+\) T cells, CD4-allophycocyanin (RM4-5) and CD45-PerCP (30-F11) were used. For neutrophils, CD45-allophycocyanin (30-F11), CD11b-PerCP (M170), and Ly6G-PE (1A8) mAbs were used. The cell samples were washed three times using FACS buffer followed by fixation in 1% paraformaldehyde.

To analyze the number of CD4\(^+\), CD8\(^+\), IFN-\(\gamma\)–, IL-17–, and IL-4–producing CD4\(^+\) T cells, intracellular cytokine staining was performed as previously described with minor modifications (46). Briefly, 1 million cells from DLN and spleen or total corneal cells were left untreated or stimulated with anti-CD3 (1 \(\mu\)g/ml) and anti-CD28 (0.5 \(\mu\)g/ml) for 5 h in the presence of GolgiStop at 37˚C in 5% CO\(_2\). At the end of the stimulation period, cell-surface staining for CD4 was performed as described above. This was followed by cell permeabilization and intracellular cytokine staining using Cytotox/Cytoperm kit (BD Biosciences) in accordance with the manufacturer’s recommendations. For intracellular staining, PE-labeled IL-4, FITC-labeled IFN-\(\gamma\), and PE-labeled IL-17 Abs were used. To enumerate...
ate Foxp3^+CD4^+ T cells, cell-surface staining was performed for CD4 (allophycocyanin) followed by intranuclear staining using the fixation/ permeabilization kit and an anti–Foxp3-PE mAb (eBioscience) as per the manufacturer's recommendations. Samples were acquired with an FACSCalibur (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**ELISA**

The pooled corneal samples were homogenized using a tissue homogenizer, and supernatant was used for analysis. For DLN and spleen, single-cell suspensions were collected on day 12 pi. Aliquots of single-cell suspensions containing 1 million cells were stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) for 48 h at 37°C. The concentrations of IL-4, IL-10, and IL-17A were measured in supernatants by using sandwich ELISA kits (eBioscience) as per the manufacturer's instructions.

**Statistical analysis**

An unpaired two-tailed Student t test was performed to determine statistical significance for disease severity between different groups. The p values ≤0.05 were considered significant. All experiments were repeated at least two times, and results are expressed as means ± SEM.

**Results**

**Corneal P. aeruginosa infection induces a dominant Th17 cell response in the cornea**

Past studies have shown that after corneal P. aeruginosa infection, CD4^+ T cell infiltration in the cornea becomes readily apparent as early as day 3 pi with subsequent migration of activated CD4^+ T cells by day 5 pi (4, 12–14). Moreover, it has been noted that Th1 cells contribute to corneal pathology and perforation (4, 12–14). Although these studies implied the critical role of IFN-γ-producing Th1 cells in P. aeruginosa–induced corneal immunopathology, the relative contribution of recently discovered Th17 cells is not defined. To evaluate the role of different CD4^+ T cell subsets in P. aeruginosa–mediated corneal pathology, corneas of mice were infected with P. aeruginosa, and the severity of corneal opacity and CD4^+ T cell infiltration was measured at various days pi (Fig. 1). As shown in Fig. 1A and 1B, P. aeruginosa–infected mice showed corneal opacity on day 1 pi, with increasing severity as infection progressed. The peak severity of keratitis was noted on day 7 pi (Fig. 1A, 1B). To characterize the role and relative contribution of different CD4^+ T cell subsets, mice were sacrificed on days 5 and 8 pi, and single-cell suspensions were prepared from collagen-digested pooled corneal samples for cellular analysis by flow cytometry. As shown in Fig. 1C, CD4^+ T cells were evident by day 5 pi with continued infiltration in the cornea on day 8 pi as the disease progressed to a more severe form. Interestingly, intracellular cytokine staining revealed the infiltration of both Th1 and Th17 cells in the cornea on days 5 and 8 pi (Fig. 1D, 1E). However, as the disease progressed to a more severe form, the Th17 cells became far more prominent than Th1 cells (Fig. 1F). Taken together, these results demonstrate that both Th1 and Th17 cells infiltrate the cornea early after P. aeruginosa infection and that there is an increased representation of Th17 cells with the more severe P. aeruginosa keratitis during the late phase of corneal P. aeruginosa infection.

**Ocular P. aeruginosa infection induces a strong Treg response in the cornea**

Past studies have shown that Treg control both Th1- and Th17-mediated cellular response and suppress various autoimmune and immunopathological disorders associated with excessive effector T cell responses (24). In the cornea, primary HSV infection results in expansion of the Treg population, and increasing the representation of Treg over effector T cells diminishes corneal immunopathology (25–27, 47). To study the role of Treg during corneal P. aeruginosa infection, we analyzed corneal infiltration of Treg at various days pi. As shown in Fig. 2A and 2B, Treg infiltration in the cornea was far greater than effector T cells (both Th1 and Th17; see Fig. 1D, 1E) during both early and late stages of P. aeruginosa infection (day 5: Th1, 7.8%; Th17, 9.8%; Treg, 65%; day 8: Th1, 5.14%; Th17, 18.1%; Treg, 38.8%). When the ratio of total number of Treg to effector T cells was compared during the early and late stage of P. aeruginosa infection, there was no change in the total number of Treg per Th1 cell (Fig. 2C) or Treg per Th17 cell (Fig. 2D). Collectively, these results indicate that corneal P. aeruginosa infection induces a steady Treg response in the cornea. However, the total number of Th17 cells is significantly increased during the late stage of corneal P. aeruginosa infection, indicating that increased representation of Th17 cells could be responsible for a more severe form of corneal pathology.

**Treg and effector T response in lymphoid organs post–corneal P. aeruginosa infection**

Next, we examined the kinetics of effector T and Treg responses in local DLN and spleen at various days pi. The results in Fig. 3 show the frequency and total numbers of Th17 and Treg populations in DLN and spleen of P. aeruginosa–infected mice at different days pi (days 5 and 8 pi) as compared with uninfected control mice. As shown in Fig. 3A–D, there was a significant increase in the frequency as well as the total cell number of Th17 cells in DLN and spleen of P. aeruginosa–infected mice. Consistent with the observations made in the cornea, the frequency as well as total number of Th17 cells was significantly higher on day 8 as compared with day 5 pi in DLN. These data suggest that corneal P. aeruginosa infection induces a strong Th17-dominant immune response in the local DLN (Fig. 3A, 3B). In contrast, when the frequency and total cell number for Th1 cells were compared, we observed a significant increase only in spleen of P. aeruginosa–infected mice as compared with uninfected mice (data not shown).

As shown in Fig. 3E and 3F, there was no significant difference in the total number of Treg in DLN and spleen from uninfected and P. aeruginosa–infected mice at various days pi. However, when the ratio of total number of Treg to Th17 cells was compared between uninfected and P. aeruginosa–infected mice, a significant decrease in the proportion of Treg per Th17 cell was noted in both DLN and spleen at days 5 and 8 pi (Fig. 3G, 3H). In contrast to Th17 cells, there was no significant change in the number of Treg per Th1 cell in DLN as well as spleen of corneal P. aeruginosa–infected mice as compared with uninfected mice (data not shown). Taken together, these results indicate that local corneal P. aeruginosa infection results in the expansion of Th17 cell response in DLN and spleen. Our data also suggest that a significant reduction in the total number of Treg per Th17 cell in DLN and spleen during later stage of keratitis may induce a strong Th17 cell–mediated immunopathology in the cornea.

**Gal–1–mediated suppression of P. aeruginosa keratitis**

Past reports have shown that the severity of CD4^+ T cell–mediated immunopathological disorders could be influenced by shifting the balance between effector T cells and Treg (25, 27, 42). More recently, various in vivo studies using mouse models have demonstrated that Gal–1, a member of the β-galactoside–binding lectin family, suppresses CD4^+ effector T cell–mediated different autoimmune and immunopathological disorders such as HSV-induced corneal stromal keratitis (36), experimental autoimmune encephalomyelitis (48), autoimmune diabetes (49), collagen-induced arthritis (50), and experimental colitis (51). To evaluate whether the administration of rGal–1 after corneal P. aeruginosa infection could influence the balance between Th17 cells and Treg and limit the severity of corneal immunopathology, corneas of C57BL/6 mice were infected with P. aeruginosa, and rGal–1 (20 μg in 10 μl/eye) was administered by subconjunctival injections every other day.
**FIGURE 1.** Ocular *P. aeruginosa* infection induces a strong Th17 cell response in the cornea. C57BL/6 mice corneas were scarified and infected with 3.5 × 10^5 CFU of *P. aeruginosa*. The progression of bacterial keratitis was monitored by measuring corneal opacity scores on day 1, 3, 5, and 7 pi followed by flow cytometric analysis of CD4+ T cells infiltrating the cornea on days 5 and 8 pi. (A) Corneal opacity scores on day 0 (uninfected) and days 1, 3, 5, and 7 pi. (B) Representative eye images showing the disease severity at various days pi. (C) Representative FACS plots (left panel) and cell numbers (right panel) for CD4+ T cells gated on CD45+ cells obtained from pooled corneal samples on days 5 and 8 pi. Representative FACS plots (left panel) and cell numbers (right panel) for IFN-γ–secreting (D) or IL-17A–secreting (E) CD4+ T cells from pooled corneas stimulated with anti-CD3/anti-CD28 for 5 h from days 5 and 8 pi. (F) Total number of Th1 and Th17 cells on day 8 pi. Data are representative of two independent experiments and show mean values ± SEM (combined results of two separate experiments are shown; five to six corneas per group were used in each experiment). Statistical levels of significance were analyzed by the Student t test (unpaired). *p ≤ 0.05.
beginning day 1 pi (Fig. 4A). The severity of corneal pathology was recorded every alternate day and compared with the vehicle-treated control mice (Fig. 4B). All infected mice on day 1 pi developed corneal opacity to a similar extent. However, as infection progressed, the animals in the rGal-1–treated group showed significantly diminished corneal pathology as compared with control mice (Fig. 4B–D). Interestingly, continued rGal-1 treatment during the course of P. aeruginosa infection not only inhibited the further progression of the corneal lesion but also reversed the increased corneal opacity and corneal pathology to a normal clear cornea (Fig. 4D). Taken together, our results demonstrate that administration of rGal-1 treatment after corneal P. aeruginosa infection significantly reduces the corneal pathology.

rGal-1 treatment diminishes infiltration of various immune cell types involved in pathology

To further characterize the mechanisms of the inhibitory effect of rGal-1 treatment on P. aeruginosa–induced bacterial keratitis, the corneal immune cell composition of both control and rGal-1–treated groups was analyzed at day 12 pi by flow cytometry. As shown in Fig. 5A–E, the frequencies and total numbers of CD45+ cells, CD4+ T cells, and neutrophils (CD45+CD11b+Ly6G+) were significantly reduced in the corneas of the rGal-1–treated group when compared with the vehicle-treated control animals. These results show that rGal-1 treatment after corneal P. aeruginosa infection significantly reduces the corneal infiltration of total leukocytes, CD4+ T cells, and neutrophils.

rGal-1 treatment diminishes the proinflammatory Th17 cell response in the cornea and lymphoid organs

To assess whether Gal-1 suppresses P. aeruginosa–induced keratitis through a shift in the balance between effector T cells and Treg, CD4+ T cell subset composition of infected corneas of rGal-1–treated and control animals was analyzed by FACS at day 12 pi. As shown in Fig. 6A and 6B, the frequency and total number of Th17 cells (Fig. 6B) were reduced in the corneas of animals treated with rGal-1 compared with the control animals. In contrast, there was no difference in the frequency of Treg population in the rGal-1–treated group as compared with control animals (Fig. 6C). Also, when the ratio of total number of Treg per Th17 cells was compared between rGal-1–treated and control groups, there was no change in the total number of Treg per Th17 cell in rGal-1–treated group (Fig. 6D). In another set of experiments of the same design, the corneas were pooled groupwise and processed to analyze the levels of IL-17A by ELISA. As shown in Fig. 6E, the levels of proinflammatory cytokine IL-17A were significantly diminished in the rGal-1–treated group as opposed to vehicle-treated control group. In contrast, there was no difference in the levels of the anti-inflammatory cytokine IL-10 between the rGal-1–treated and control group (Fig. 6F). Taken together, these data indicate that rGal-1 treatment modulates the severity of P. aeruginosa–induced corneal pathology by reducing the magnitude of proinflammatory Th17 response.

Additionally, studies were performed to quantify the relative composition of Th17 and Treg in DLN and spleens from the same mice as those used for corneal studies. As shown in Fig. 7A–D, rGal-1 treatment reduced the percentages of Th17 cells in both DLN and spleen as compared with the control group but did not significantly influence the percentage of Treg populations. Also, there was a significant reduction in total CD4+ T cells and Th17 cell numbers in DLN of rGal-1–treated mice as compared with control group animals (Fig. 7E). Similarly, the total cell number of CD4+ T cells and Th17 cells was reduced in spleen of rGal-1–treated animals (Fig. 7F). Although there was significant reduction in total Treg cell numbers in the rGal-1–treated group (Fig. 7E, 7F), there was no change in the total number of Treg per Th17 cell
Figure 3. Corneal *P. aeruginosa* infection induces Th17 cell and Treg response in lymphoid organs. C57BL/6 mice corneas were scarified and infected with $3.5 \times 10^5$ CFU of *P. aeruginosa*. Flow cytometry was used to detect IL-17A production by CD4$^+$ T cells (anti-CD3/anti-CD28 stimulated for 5 h) obtained from DLN and spleen of uninfected (day 0) and animals from days 5 and 8 pi. The frequencies (A) and total cell numbers (B) of IL-17A–producing CD4$^+$ T cells in DLN of uninfected (day 0) and *P. aeruginosa*–infected (days 5 and 8 pi) mice are shown. The frequencies (C) and total cell numbers (D) of IL-17A–producing CD4$^+$ T cells in spleen of uninfected (day 0) and *P. aeruginosa*–infected (days 5 and 8 pi) mice are shown. Flow cytometry was used to detect Foxp3-expressing CD4$^+$ T cells obtained from DLN and spleen of uninfected (day 0), day 5, and day 8 pi. The total cell numbers of Foxp3$^+$ CD4$^+$ T cells in DLN (E) and spleen (F) of uninfected (day 0) and *P. aeruginosa*–infected (days 5 and 8 pi) mice are shown. Cell ratios for total numbers of Treg per Th17 cell in DLN (G) and spleen (H) of uninfected (day 0) and *P. aeruginosa*–infected (days 5 and 8 pi) mice are shown. Data are representative of two independent experiments and show mean values ± SEM ($n = 8$ mice at each indicated time point). Statistical levels of significance were analyzed by one-way ANOVA test with Tukey’s multiple comparison test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. 

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**IMMUNOREGULATORY ROLE OF GALECTIN-1 IN PSEUDOMONAS KERATITIS**

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in both DLN and spleen of rGal-1–treated and control mice (Fig. 7G, 7H). Furthermore, when the cytokine levels of IL-17A and IL-10 from cell supernatants of DLN and spleen cells stimulated with anti-CD3 and anti-CD28 were compared, there was a significant decrease in IL-17A production by DLN cells from the rGal-1–treated group as opposed to control mice (Fig. 7I). In contrast, we observed significant increase in IL-10 production by DLN as well as spleen cells from the rGal-1–treated group as compared with the control-treated group (Fig. 7J). Taken together, these data suggest that rGal-1 treatment post-P. aeruginosa corneal infection significantly decreases the total number of Th17 cells and IL-17A production at the site of infection as well as in lymphoid organs. Interestingly, rGal-1 treatment also increases production of the anti-inflammatory cytokine IL-10 by DLN and spleen cells.

rGal-1 treatment shifts the balance from proinflammatory Th17 to anti-inflammatory Th2 response in secondary lymphoid organs

Past studies have indicated that C57BL/6 mice are more susceptible to P. aeruginosa–induced corneal keratitis as compared with BALB/C mice (13). This increased susceptibility of B6 mice was attributed to a dominant Th1 cell–mediated immune response, whereas the resistant phenotype in BALB/c mice was due to a strong Th2 cell–mediated immune response (13, 14, 16). Furthermore, shifting the balance from Th1 to Th2 immune response in B6 mice diminishes the susceptibility to P. aeruginosa–induced corneal pathology, indicating that a Th2-biased response protects mice from severe keratitis (15, 16). Interestingly, Gal-1 has been shown to mediate its anti-inflammatory effect by suppressing Th1 and Th17, while promoting the Th2 response (35, 42, 50). To assess whether rGal-1 treatment during P. aeruginosa–induced bacterial keratitis affects the severity of corneal pathology by shifting the proinflammatory Th17 response toward an anti-inflammatory Th2 response, we evaluated the relative composition of Th2 cells in DLN and spleen of rGal-1–treated mice as compared with control-treated mice on day 12 pi. The results shown in Fig. 8A and 8B clearly indicate that the rGal-1 treatment resulted in increased percentage of Th2 cell population in both DLN and spleen as compared with control-treated mice. Moreover, the ratio of total number of Th2 cells per Th17 cell was significantly increased in DLN and spleen of rGal-1–treated mice as compared with control mice (Fig. 8C, 8D). Also, the cell supernatants of DLN and spleen cells stimulated with anti-CD3 and anti-CD28 showed a significant increase in IL-4 production by DLN cells from the rGal-1–treated group as opposed to control mice (Fig. 8E). However, there was no significant change in IL-4 production by spleen cells (Fig. 8F). Collectively, these results suggest that rGal-1 treatment after corneal P. aeruginosa infection modulates corneal immunopathology by suppressing proinflammatory Th17 immune response and promoting protective Th2 cell response in secondary lymphoid organs.

Discussion

In this report, we have shown for the first time, to our knowledge, that Th17 cells predominate over Th1 cells and can orchestrate various critical events involved in the pathogenesis of corneal P. aeruginosa infection. Our data also indicate that Treg migrate into the cornea postinfection during acute and chronic stages of corneal pathology. Interestingly, treatment of P. aeruginosa–infected mice with Gal-1, an endogenously derived immunoregulatory molecule, significantly diminishes the number of pathogenic Th17 cells and neutrophils with a concomitant reduction in corneal...
pathology. Furthermore, Gal-1 shifts the balance from proinflammatory Th17 cells toward Th2 cells, indicating the potential therapeutic value of Gal-1 in vision-impairing P. aeruginosa–induced bacterial keratitis.

Corneal transparency is an essential prerequisite for optimal vision. However, corneal infection with various pathogens such as P. aeruginosa induces a strong immunoinflammatory reaction characterized by severe alteration in corneal tissue architecture and vision loss (4). Past studies have shown the critical role of IL-17A, the source of IL-17A as well the role of Th17 and Treg populations were not defined. Our data demonstrate that Th17 cells migrate into the cornea as early as day 5 pi, with increased numbers as the disease progresses to a more severe form. These findings, in conjunction with published studies showing that IL-17A plays a central role in the migration and activation of neutrophils (19–22), lead us to propose that an increase in the number of Th17 cells in P. aeruginosa–infected corneas most likely contributes to corneal pathology through increased migration of neutrophils at the site of inflammation.

IL-17A is a proinflammatory cytokine secreted by various cells such as Th17 cells, γδ T cells, NK cells, NKT cells, neutrophils, and mast cells (19–22). IL-17A activates epithelial, endothelial, and stromal cells to secrete various chemokines (CXCL1, CXCL2, IL-6), matrix metalloproteinases (MMP-1, MMP-3, MMP-9, MMP-13), and growth factors (G-CSF, GM-CSF, vascular endothelial growth factor-A) essential for migration and activation of various immune cell types at the site of inflammation (20, 53–58). Although our data indicate the involvement of IL-17A after corneal infection by P. aeruginosa in activation of neutrophils (19–22), it is possible that one or multiple cell types could contribute to corneal pathology after P. aeruginosa infection in addition to Th17 cells, other cell types such as γδ T cells, macrophages, and neutrophils can act as a potential source of IL-17A after corneal P. aeruginosa infection. In this respect, it has been shown that innate γδ T cells infiltrate the cornea and produce IL-17A after corneal HSV infection (46). In pulmonary infection by P. aeruginosa, γδ T cells have been shown to promote IL-17A production during the innate immune response (59). Thus, it is possible that one or multiple cell types could contribute to the early source of IL-17A after corneal P. aeruginosa infection.

Another important issue that needs to be addressed is why corneal P. aeruginosa infection induces a strong Th17 cell response compared with Th1 cells. Th17 cells differentiate from naive
CD4+ T cells after cognate Ag stimulation in the presence of IL-6, TGF-β, IL-21, and IL-23 and are characterized by a lineage-specific transcription factor, retinoic acid–related orphan receptor γt (19–22). Previous studies have shown that corneal epithelial cells express various TLR, and ligation of TLR5 with flagellin on corneal epithelial cells induces expression of IL-6, IL-23, IL-1β, and TGF-β (60–62). Thus, corneal P. aeruginosa infection through flagellin-mediated activation of TLR5 on corneal epithelial cells may generate a local cytokine milieu conducive for Th17 induction and differentiation. Although we have not determined Ag specificity of the Th17 cells, it is possible that these cells recognize one or more Ags from the bacterial outer cell membrane or secreted proteins. In support, one study using live attenuated P. aeruginosa vaccine identified three bacterial proteins, OprL, PopB, and FpvA, that induced a strong Th17 response (63). However, further studies are needed to confirm the Ag specificity of Th17 cells in corneal immunopathology.

Past studies using the mouse model of lung P. aeruginosa infection have shown that the Th17 response is induced after bacterial infection, and this contributes to either protection or pathology depending on the severity of infection. Accordingly, acute pulmonary P. aeruginosa infection induces an IL-17A response, and Th17 cells act as a source of IL-17A (64). This study showed that IL-17A protects mice from acute pulmonary infection through increased migration of neutrophils and subsequent control of bacterial load (64). Furthermore, IL-17A has been shown to play an important role in vaccine-induced protection against acute lung infection by LPS-heterologous strains of P. aeruginosa (65).

In contrast, cystic fibrosis, a chronic lung infection with P. aeruginosa, induces a strong IL-17A and IL-23 response and plays an

**FIGURE 6.** rGal-1 treatment diminishes Th17 response in P. aeruginosa–infected corneas. C57BL/6 mice infected with 3.5 × 10^7 CFU of P. aeruginosa were treated with rGal-1 or control vehicle by local subconjunctival injections from days 1–11 pi. Representative FACS plots (A) and total cell numbers (B) for IL-17A–secreting CD4+ T cells from day 12 pi pooled corneas stimulated with anti-CD3/anti-CD28 for 5 h of control (left panel) and rGal-1–treated (right panel) animal groups. Representative FACS plots (C) for Foxp3+ CD4+ T cells from day 12 pi pooled corneas of control (left panel) and rGal-1–treated (right panel) mice group. (D) Cell ratios for total numbers of Treg per Th17 cell. IL-17A (E) and IL-10 (F) protein levels per cornea from control and rGal-1–treated group. Data are representative of three independent experiments and show mean values ± SEM (n = 3, each sample is representative of 5–10 corneas). Statistical levels of significance were analyzed by the Student t test (unpaired). *p < 0.05.
FIGURE 7. Local rGal-1 treatment after corneal *P. aeruginosa* infection modulates Th17 and Treg differentially in lymphoid organs. C57BL/6 mice infected with $3.5 \times 10^5$ CFU of *P. aeruginosa* were therapeutically treated with rGal-1 or control vehicle by local subconjunctival injection from days 1–11 pi. Representative FACS plots for IL-17A–secreting CD4$^+$ T cells on day 12 pi DLN (A) and spleen (B) cells stimulated with anti-CD3/anti-CD28 for 5 h from control (left panel) and rGal-1–treated (right panel) groups are shown. Representative FACS plots for Foxp3$^+$CD4$^+$ T cells on day 12 pi DLN (C) and spleen (D) from control (left panel) and rGal-1–treated (right panel) animal groups are shown. Total cell numbers for CD4$^+$ T cells, IL-17A$^+$CD4$^+$ T cells, and Foxp3$^+$ T cells on day 12 pi in DLN (E) and spleen (F) from control (clear bar) and rGal-1–treated (gray bar) groups. Cell ratios for total numbers of Treg per Th17 cell in DLN (G) and spleen (H) from control (clear bar) and rGal-1–treated (gray bar) groups. IL-17A (I) and IL-10 (J) protein levels analyzed by sandwich ELISA from *P. aeruginosa*–infected rGal-1–treated and control DLN and spleen cells harvested at day 12 pi and stimulated with anti-CD3/anti-CD28 for 48 h. Data are representative of three independent experiments and show mean values $\pm$ SEM (n = 12–14). Statistical levels of significance were analyzed by the Student t test (unpaired).
important role in airway damage and lung inflammation through increased migration of neutrophils (66, 67). However, data from our study as well as others indicate that IL-17A and the Th17 response in the cornea appears to be pathogenic and may contribute to the corneal tissue damage and vision loss (23, 46, 57, 68, 69). Accordingly, HSV infection of the cornea induces a biphasic IL-17A expression and contributes to the corneal pathology and corneal neovascularization through increased migration of neutrophils, increased expression of proinflammatory cytokines, as well as proangiogenic molecules (46, 57, 70). Moreover, dry eye disease induces a strong IL-17A/Th17 response through increased production of IL-1β, IL-6, and TNF-α by corneal epithelial cells and contributes to the corneal pathology by increasing epithelial cell expression of MMP-3 and MMP-9 and subsequent increased corneal permeability (71). Collectively, data from these studies indicate that the corneal surface, particularly epithelial cells, is endowed with capabilities such as expression of TLRs as well as production of various proinflammatory cytokines essential for generation of strong Th17-biased immune response after inflammatory stimulus.

The recent discovery of suppressor Treg and their critical role in suppressing Th1 and Th17 effector T cells represent a promising therapeutic target in various autoimmune and inflammatory disorders (24, 25, 30, 47). Accordingly, shifting the balance from an effector T cell response toward a Treg response has shown promising therapeutic outcomes in murine models of various au-

**FIGURE 8.** rGal-1 treatment after corneal *P. aeruginosa* promotes Th2 response in lymphoid organs. C57BL/6 mice infected with 3.5 × 10^5 CFU of *P. aeruginosa* were treated with rGal-1 or control vehicle by local subconjunctival injections from days 1–11 pi. Representative FACS plots for IL-4–secreting CD4^+ T cells on day 12 pi DLN (A) and spleen (B) cells stimulated with anti-CD3/anti-CD28 for 5 h from control (left panel) and rGal-1–treated (right panel) animal groups are shown. Cell ratios for total numbers of Th2 cells per Th17 cell in DLN (C) and spleen (D) on day 12 pi from control (left panel) and rGal-1–treated (right panel) group. Data are representative summary of two independent experiments and show mean values ± SEM (n = 8). IL-4 protein levels analyzed by ELISA from *P. aeruginosa*–infected rGal-1–treated and control DLN (E) and spleen (F) cells harvested at day 12 pi and stimulated with anti-CD3/anti-CD28 for 48 h. Data are representative summary of three independent experiments and show mean values ± SEM (n = 12). Statistical levels of significance were analyzed by the Student *t* test (unpaired).
to immune and chronic inflammatory disorders (24, 25, 27, 30). In addition, different host-derived endogenous molecules have been identified that control the excessive effector T cell response and promote Treg response (28, 30). One such family of carbohydrate-binding proteins, galectins, has been identified and characterized, which plays an important role in the regulation of both innate and adaptive immune response (28, 31–33). Gal-1 was the first identified member of this family. It plays an important immunomodulatory role in various autoimmune and pathological disorders (28, 33). Gal-1 is prominently expressed at the site of T cell activation as well as immune-privileged sites and is secreted by a range of different innate and effector cells (28, 33). Furthermore, Gal-1 controls immune response and host tissue damage by suppressing effector T cell differentiation and proliferation and promoting increased apoptosis of effector T cells, increased generation of Treg, and increased synthesis of anti-inflammatory molecules (28, 33). Additionally, Gal-1 induces tolerogenic dendritic cells through increased synthesis of IL-27, which, in turn, promotes induction of Tr1 cells (28, 33, 72). Moreover, it has been shown that Gal-1 can promote IL-10 synthesis by CD4+ T cells during differentiation or after lineage commitment to Th1 and Th17 cells through induction of transcription factors c-Maf and aryl hydrocarbon receptor (39). Recently, Toscano et al. (42) showed that Gal-1 treatment shifts the balance of IL-12 production of proinflammatory cytokines, as well as a reduced expression of Th17 and increased expression of Th2 cytokines. In addition, different host-derived endogenous molecules have been identified that control the excessive effector T cell response to immune and chronic inflammatory disorders (24, 25, 27, 30). Gal-1 has been shown to diminish HSV-induced Th1-mediated corneal immunopathology by shifting the balance from uveitogenic Th1 response toward a nonpathogenic anti-inflammatory Th2 response (35), and promote Treg response toward a protective anti-inflammatory Th2 response toward a nonpathogenic anti-inflammatory Th2 response (35).

References

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