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Role of IL-17 and Th17 Cells in Herpes Simplex Virus-Induced Corneal Immunopathology

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HSV-1 infection of the cornea leads to a blinding immunoinflammatory lesion of the eye termed stromal keratitis (SK). Recently, IL-17–producing CD4+ T cells (Th17 cells) were shown to play a prominent role in many autoimmune conditions, but the role of IL-17 and/or of Th17 cells in virus immunopathology is unclear. In this study, we show that, after HSV infection of the cornea, IL-17 is upregulated in a biphasic manner with an initial peak production around day 2 postinfection and a second wave starting from day 7 postinfection with a steady increase until day 21 postinfection, a time point when clinical lesions are fully evident. Further studies demonstrated that innate cells, particularly γδ T cells, were major producers of IL-17 early after HSV infection. However, during the clinical phase of SK, the predominant source of IL-17 was Th17 cells that infiltrated the cornea only after the entry of Th1 cells. By ex vivo stimulation, the half fraction of IFN-γ–producing CD4+ T cells (Th1 cells) were HSV specific, whereas very few Th17 cells responded to HSV stimulation. The delayed influx of Th17 cells in the cornea was attributed to the local chemokine milieu. Finally, HSV infection of IL-17R knockout mice as well as IL-17 neutralization in wild-type mice showed diminished SK severity. In conclusion, our results show that IL-17 and Th17 cells contribute to the pathogenesis of SK, the most common cause of infectious blindness in the Western world.

Ocular infection with HSV can cause a chronic inflammatory reaction in the corneal stroma that may culminate in blindness (1, 2). This stromal keratitis (SK) lesion in humans is suspected to represent an immunopathological reaction, a notion well supported by studies with animal models of SK (2–5). An inevitable consequence of ocular HSV infection is lifelong latency in neuronal cells of the trigeminal ganglion (6). In humans, periodic reactivation from some latently infected cells gives rise to replicating virus that acts as the most common stimulus for SK lesion development (2–5). A major objective of SK research is to identify the roles of cellular and molecular events involved in tissue damage and its resolution with a view to improving current means of management of this often distressing disease. Studies in mouse models of SK have firmly established an essential role for T cells as the principal orchestrators of SK lesions (7, 8). However, the actual tissue damage appears to be a consequence of inflammatory events that derive primarily from neutrophils that represent the major cellular component of lesions at all of the phases of SK pathogenesis (9, 10). The prominence of neutrophils in SK lesions could indicate that the recently identified proinflammatory cytokine IL-17 is a significant participant in the pathogenesis of lesion development. Accordingly, IL-17 functions indirectly to cause tissue infiltration by neutrophils, acts as a neutrophil survival factor, and also may drive the cells to produce and release tissue-damaging molecules such as matrix metalloproteinases and oxyradicals (11–15).

In human SK, the presence of IL-17 has been reported (16). Additionally, Molesworth-Kenyon et al. (17) showed that the severity of early SK lesions was diminished in mice unable to respond to IL-17 because they lacked IL-17R. However, the cellular source of IL-17 as well as the role that this cytokine plays compared with those of other inflammatory mediators remains to be further defined. This is the topic of the present article.

We show that HSV infection of the cornea leads to biphasic upregulation of IL-17. Initially, its source was innate cells that included γδ T cells, whereas later during the clinical phase Th17 cells were the predominant producer. The CD4+ T cell subset responsible for orchestrating SK appeared to be mainly Th1 cells at all of the stages of SK. However, very few Th17 cells infiltrated into the cornea during the early stages of SK (days 8 and 15 postinfection) but became more prominent during the very late stage of SK (day 21 postinfection) when SK lesions were fully evident. The late entry of Th17 cells was explained partly by the delayed upregulation of IL-6 and TGF-β, cytokines responsible for Th17 generation, as well as expression in the cornea of CCL20, a chemokine responsible for the migration of Th17 cells at the site of inflammation. On the basis of anti-cytokine suppression and comparison of lesion severity between wild-type (WT) and IL-17R knockout (IL-17RKO) mice, our results show that IL-17 contributes to inflammatory events during the pathogenesis of SK. Future therapies targeting the IL-17 response could be useful to alleviate SK lesion severity, an important cause of infectious blindness in humans.

Materials and Methods

Mice, viruses, and cell lines

IL-17RA−/− mice on a C57Bl/6 background were obtained from Amgen (Thousand Oaks, CA). C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed in animal facilities approved by the Association for Assessment and Accreditation of Laboratory
Animal Care at the University of Tennessee, and all of the experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-1 RE Tumpey and HSV-KOS viruses were grown in Vero cell monolayers (CCL81; American Type Culture Collection). The virus was concentrated, titrated, and stored in aliquots at −80°C until use.

Corneal HSV infection and clinical scoring

Corneal infections of mice were conducted under deep anesthesia induced by i.p. injection of tribromomethanol (Avertin) as described previously (18). Mice were scarified on their corneas with a 27-gauge needle, and a 3-μl drop containing 1 × 10⁶ PFU of virus was applied to the eye. The eyes were examined on different days postinfection for the development and progression of clinical lesion by slit-lamp biomicroscope (Kowa Company, Nagoya, Japan). The progression of SK lesion severity and angiogenesis of individually scored mice was recorded. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing keratitis. The severity of angiogenesis was recorded as described previously (19). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The scores of the four quadrants of the eye then were summed to derive the neovessel index (range 0–16) for each eye at a given time point.

Reagents and Abs

CD4–allophycocyanin (RM4-5), IL-17–FITC (XMG1.2), γδ TCR–FITC (GL3), anti-CD3 (145.2C11), anti-CD28 (0.5 μg/ml) and anti-CD2 (37.51), purified anti–IFN-γ and GolgiStop (brefeldin A) were purchased from BD Biosciences. Foxp3–PE, IL-17, and IFN-γ ELISA kits were purchased from eBioscience. Anti-γδ TCR mAb (UC3-10A6) and hamster IgG isotype control Ab were purchased from Sigma-Aldrich.

γδ T cell depletion

C57BL/6 mice were injected on day 2 before infection with 500 μg per mouse of anti-mouse γδ TCR (clone UC3-10A6) followed by 250 μg per mouse on the day of infection.

Subconjunctival injection

Subconjunctival injections of anti–IL-17 were performed as described previously (20). Briefly, subconjunctival injections were done using a 2-cm, 32-gauge needle and syringe (Hamilton, Reno, NV) to penetrate the perivascular region of the conjunctiva, and the required dose of anti–IL-17 mAb was delivered into the subconjunctival space. Control mice received isotype mAb.

IL-17 and IFN-γ neutralization

C57BL/6 mice were infected ocularly, and 0.05 mg of anti–IL-17 or anti–IFN-γ was given i.p. starting from day −1 followed by days 2 and 5 postinfection or starting from day 7 followed by days 10 and 13 postinfection. For local depletion, 5 μg of anti–IL-17 mAb was administered by subconjunctival injection following the same regimes as described above.

Isolation of corneal-infiltrating cells and flow cytometry

HSV-infected corneas were harvested from different groups of mice at the indicated time points postinfection. Six to eight corneas per group were excised, pooled group wise, and digested with 60 U/ml Liberase for 35 min at 37°C in a humidified atmosphere of 5% CO2. 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 the corneas, draining lymph nodes (DLNs), and spleens were stained for different cell surface molecules for FACS. All of the steps were performed at 4°C. Briefly, cell suspensions first were blocked with an unconjugated anti-CD32/CD16 mAb for 30 min in FACS buffer. After being washed with FACS buffer, samples were incubated with CD4–allophycocyanin (RM4-5) for 30 min on ice. Finally, the cells were washed three times and resuspended in 1% paraformaldehyde.

To measure the number of IFN-γ–, IL-17–, and IL-2–producing CD4+ T cells, intracellular cytokine staining (ICC) was performed. Briefly, 10⁶ freshly isolated cells from the corneas, DLNs, or spleens were left untreated or stimulated with PMA plus ionomycin along with GolgiStop for 4 h at 37°C in 5% CO2. To quantify IL-2–producing CD4+ T cells, cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) for 5 h in the presence of GolgiStop at 37°C in 5% CO2. To enumerate the HSV-specific Th1 and Th17 cells, cells were stimulated with 2× UV-inactivated HSV and incubated overnight at 37°C in 5% CO2. GolgiStop (10 mg/ml) was added for the last 5 h of the stimulation. At the end of the stimulation period, cell surface staining was performed as described above, followed by intracellular cytokine staining using the BD Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer’s recommendations. FITC-labeled IFN-γ and PE-labeled IL-17 Abs were used. After the final wash, cells were resuspended in 1% paraformaldehyde. The stained samples were acquired with a FACScalibur (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Quantitative real-time PCR

Corneal cells were lysed, and total mRNA was extracted using TRIzol LS reagent (Invitrogen). Total cDNA was made with 500 ng of RNA using oligo (dT) primer. Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the iQ5 real-time PCR detection system (Bio Rad, Hercules, CA). The expression levels of different molecules were normalized to that of β-actin using Δ cycle threshold (Ct) calculation. The relative expression between experimental groups was calculated using the 2−ΔΔCt formula. The PCR primers used were as follows: β-actin, forward 5′-TCCCTAAGAACATCTCC-3′; reverse 5′-GATATTTCCTCCATGGAAGG-3′; IL-17, forward 5′-GGATGATGGCATGAGATCTG-3′; reverse 5′-GCTTT GCCCTC-3′; IFN-γ, forward 5′-GATTTCCACACTGACTTTG-3′; reverse 5′-GCAATGACGCTTACG-3′; IL-2, forward 5′-GGGATGGAGTTTCTGG-3′; reverse 5′-TCTGTGCCGGTGTG-3′; ELISA, forward 5′-CTGTTGTAAGCGGCAACGG-3′; reverse 5′-CCAGTCTGTTGGAGCC-3′.

ELISA

The pooled corneal samples were homogenized using a tissue homogenizer, and supernatant was used for analysis. The concentrations of IFN-γ were measured using sandwich ELISA kits (eBioscience) as per the manufacturer’s instructions.

Statistical analysis

Student t test was performed to determine statistical significance, and data are expressed as mean ± SEM.

Results

IL-17 is upregulated in the cornea of mice ocularly infected with HSV-1

Total mRNA isolated from the corneas of HSV-infected mice was analyzed for the relative fold changes in IL-17 mRNA expression by real-time PCR. As is evident in Fig. 1A, the expression profile revealed that IL-17 was upregulated in a biphasic manner. During the early phase, IL-17 expression was elevated at day 2 postinfection but returned to near basal levels around day 5 postinfection. This was followed by subsequent upregulation of IL-17 starting from day 7 postinfection with a steady increase until day 21 postinfection (last observed time point), which corresponds to the chronic phase of the disease. In addition, quantification of IL-17 by ELISA revealed a similar pattern of expression as observed with real-time PCR (Fig. 1B). Early after HSV-1 infection, innate cells infiltrate the cornea, and CD4+ T cells start infiltrating the infected cornea around day 7 postinfection (1). Recent studies have implicated innate cells, particularly γδ T cells, as one of the major sources of IL-17 (21–25). Therefore, to investigate this possibility in our system, IL-17–producing γδ T cells were enumerated by ICCS after stimulation with PMA/ionomycin. Approximately 16% of total γδ T cells produced IL-17 (Fig. 1C), and depletion of γδ T cells using a specific mAb diminished the levels of IL-17 as measured by ELISA (Fig. 1D). Collectively, these data show that IL-17 expression is upregulated in a biphasic manner after HSV infection and that innate γδ T cells contribute to the early source of IL-17.
Th1 cells predominate during early stages of SK pathogenesis followed by Th17 cells in later stages

Past studies showed that CD4+ T cell infiltration of the corneal stroma becomes readily apparent by 7 d postinfection (1, 7, 8), but the relative composition of the CD4+ effector T cells was not recorded. Through the use of the ICCS approach with cells isolated from collagen-digested pooled corneas, the relative frequencies of Th1 and Th17 cells were measured in the HSV-infected corneas at days 8, 15, and 21 postinfection. As shown in Fig. 2A, stimulation of corneal cells with PMA and ionomycin revealed a large proportion of CD4+ T cells producing either IFN-γ or IL-17 within the corneal infiltrate. However, the ratios of frequencies of Th1 to Th17 cells were higher at all of the tested time points postinfection. Additionally, Th1 cells showed an initial increase at day 15 over day 8 postinfection followed by a gradual increase in their frequency with the progression of SK (Fig. 2B, middle panels). The total cell numbers for each population revealed similar kinetics (Fig. 2B). Furthermore, intracellular staining of corneal cells stimulated with a UV-inactivated viral Ag preparation revealed a high percentage (≥24.4%) of HSV-specific Th1 cells as compared with a lower percentage (≤0.35%) of HSV-specific Th17 cells (Fig. 2A, right panels). In addition, analysis of local DLNs for Th1 and Th17 cells revealed a pattern similar to that observed for the cornea (Fig. 2C, 2D). These results indicate that Th1 cells initially infiltrate the site of infection followed by increased infiltration of Th17 cells during late stages of SK.

Local cytokines/chemokines direct delayed entry and/or differentiation of Th17 cells in the cornea

The delayed migration of Th17 cells in the cornea could be the result of the early conditions suppressing Th17 cells or conditions such as the local cytokine and chemokine milieu favoring Th17 cell generation and/or migration during later stages of SK. To investigate this issue further, studies were carried out for the corneal quantification of different cytokines as well as chemokines, particularly those that are involved in the generation, migration, and suppression of Th17 cell responses. Because Th17 cells differentiate from naive CD4+ T cells in the presence of IL-6 and TGF-β with antigenic stimulation (26–28), we quantified the relative fold change of IL-6 and TGF-β at various days postinfection as compared with mock-infected cornea (Fig. 3A, 3B). The results showed relatively low levels of IL-6 at day 8 postinfection followed by a peak at day 15 postinfection (Fig. 3A). TGF-β levels were upregulated from day 8 postinfection and peaked at day 15 postinfection. As compared with day 15 postinfection, both IL-6 and TGF-β levels were reduced by day 21 postinfection. Furthermore, quantification of CCL20, a known ligand for CCR6 that is expressed preferentially by homeostatically proliferating Th17 cells as well as regulatory T cells (29, 30), was expressed significantly only after day 15 postinfection (Fig. 3C). Furthermore, because a recent study has shown the suppressive role of IL-2 in the generation of Th17 cells (31), we quantified CD4 T cells producing IL-2 in the local DLNs and spleens by ICCS assay. As shown in Fig. 3D and 3E, IL-2–producing CD4+ T cells were significantly higher in frequency as well as numbers at day 8 postinfection followed by a reduction at days 15 and 21 postinfection. This early and robust IL-2 production by CD4+ T cells could be responsible for the
promotion of Th1 responses as well as the suppression of Th17 responses in lymphoid organs early after HSV infection. However, during the progression of SK, increased IL-6, TGF-β, and chemokine CCL20 expression in the cornea could be responsible for either generation or migration of Th17 cells during the later stages of SK.

The dual role of IFN-γ in HSV-induced immunopathology

Because IFN-γ suppresses the induction and expansion of Th17 cells (17, 32–34), we investigated the role of IFN-γ in relation to IL-17 as well as Th17 cells. Similar to IL-17, IFN-γ showed biphasic upregulation. However, the second peak expression was observed around day 7 postinfection followed by a steady decline with the progression of SK (Fig. 4A). Measurement of cytokine IFN-γ by ELISA revealed similar kinetics of expression to those observed with quantitative PCR analysis (Fig. 4B). To demonstrate the role of IFN-γ in SK, IFN-γ depletion was carried out from day 21 followed by depletion on days 2 and 5. The severity of SK lesions was scored on days 8 and 11. As shown in Fig. 4C, early
depletion of IFN-$
\gamma$
significantly increased the severity of SK on day 8. However, on day 11, it reached that of the isotype Ab-treated control mice group. Furthermore, all of the mice from the IFN-$
\gamma$
depleted group showed signs of severe encephalitis, which necessitated termination of experiments. In contrast, depletion of the second peak of IFN-$
\gamma$ during later stages of SK (on days 7, 10, and 13) showed significantly diminished SK lesion severity on days 11 and 15 postinfection (Fig. 4D). The analysis of various cell types from the corneas showed reduced frequencies of total CD4+ T cells, the Th1 subset, but an increased percentage of the Th17 subset (Fig. 4E, 4F). However, the total numbers of total CD4+ T cells, Th1 cells, and Th17 cells were reduced in IFN-$
\gamma$–depleted mice as compared with those in isotype Ab-treated mice. These results demonstrate an early protective role of IFN-$
\gamma$, likely acting by controlling virus replication in the cornea. However, IFN-$
\gamma$ exerts a pathological role once virus is cleared from the cornea during the later stages of SK. Taken together, our data indicate that Th1 cells are the initial main orchestrators of SK lesions followed later on in the chronic phase by the contribution of both Th1 and Th17 cells. Early infiltration of Th1 cells initiates the inflammatory condition, particularly a cytokine and chemokine environment that promotes generation and/or migration of highly pathogenic Th17 cells. This late migration of Th17 cells maintains the SK pathology via their secretion of IL-17, which acts as a proinflammatory cytokine through various mechanisms.

**IL-17 neutralization diminishes SK lesion severity**

To investigate the role of IL-17 in SK pathogenesis further, the effects of neutralizing IL-17 with mAb was studied over a 15-d observation period. Treatment i.p. was began on the day before infection followed by injections on days 2 and 5 postinfection. The early neutralization of IL-17 reduced SK lesion severity on day 11 postinfection (Fig. 5A). To confirm further the role of IL-17 during later stages of SK, IL-17 neutralization was started from day 7 postinfection when SK lesions become evident, followed by depletion on days 10 and 13 postinfection. This therapeutic neutralization of IL-17 also reduced the severity of SK lesions, further confirming the pathogenic role of IL-17 during the later stages of SK (Fig. 5B). In addition, therapeutic local depletion of IL-17 by subconjunctival administration of anti–IL-17 Ab also resulted in significantly diminished SK lesion scores (Fig. 5C). An examination of corneal sections by histopathological analysis revealed a reduced inflammatory response in the IL-17–depleted corneas, with less fibrosis of the corneal stroma, no or minimal hypertrophy of epithelial layers, and diminished infiltration of leukocytes, particularly neutrophils (Fig. 5D). Moreover, the frequencies as well as total numbers of CD4+ T cells, Th1 cells, and Th17 cells also were reduced in the anti–IL-17–treated group (Fig. 5E, 5F). The reduced numbers of Th1 cells could be the result of reduced infiltration of neutrophils, which may act as a source of CXC chemokines essential for Th1 infiltration (35). Taken together,
these data indicated the pathogenic role of IL-17 during early as well as late phases of SK.

**IL-17RKO mice show reduced SK lesion severity**

To confirm further the role of IL-17 in SK pathogenesis, mice lacking IL-17R signaling were infected ocularly with HSV, and SK lesion severity was monitored and compared with that of WT mice. The disease severity differed significantly at all of the tested time points postinfection between WT and IL-17RKO mice (Fig. 6A). The onset of SK was delayed, and SK lesions were less severe in IL-17RKO mice as compared with those in their WT counterparts. The severity of SK lesions (scores of ≥3) was reduced in mice in the IL-17RKO group (three of eight eyes at day 21) compared with those in their WT counterparts (seven of eight eyes at day 21) (Fig. 6A, right panel). Furthermore, histopathological analysis of WT corneas at day 15 postinfection showed loss of the corneal...
epithelial layer, extensive stromal fibrosis, and infiltration of a large number of leukocytes in the stromal layers with the resultant thickening of the corneal stroma (Fig. 6B, upper panels). In contrast to WT corneas, a section from IL-17RKO corneas showed no damage to the epithelial layer with minimal fibrosis as well as infiltration by inflammatory leukocytes at day 15 postinfection (Fig. 6B, lower panels). Moreover, immunofluorescence staining of corneal sections for CD4+ T cells at day 15 postinfection...
FIGURE 6. IL-17RKO mice are resistant to SK. C57BL/6 and IL-17RKO mice were infected with $1 \times 10^4$ PFU of HSV. A, The disease progression kinetics and SK severity were assessed on days 8, 11, 15, and 21 postinfection (left panel), and the scores of individual eyes on day 15 postinfection also are shown (right panel). B, Representative H&E-stained corneal sections from WT (upper panels) and IL-17RKO (lower panels) mice collected on day 15 postinfection. The figure shows pictures of the sections taken at ×10 (left panels), ×20 (middle panels), and ×100 (right panels) of the original magnification. C, Representative immunofluorescence micrographs of corneas for CD4$^+$ T cells (green) from WT and IL-17RKO mice on day 15 postinfection. The sections were counterstained with propidium iodide (red). Scale bar, 75 μm. Mice were sacrificed on day 15 postinfection, and corneas were harvested and pooled group wise for the analysis of various cell types ($n = 6–8$ per group). D, Representative FACS plots for corneal infiltrating total CD4$^+$ T cells. E, Intracellular staining was conducted to quantify Th1 and Th17 cells by stimulating them with PMA/ionomycin (upper panels) and HSV-KOS (lower panels). F, The bar diagram shows the average frequencies of Th1 and Th17 cells after stimulation with PMA/ionomycin or UV-inactivated HSV-KOS from two independent experiments. G, The bar diagram is a summary for total numbers of corneal infiltrating CD4$^+$ T cells, IFN-γ$^{+}$CD4$^+$ T cells, HSV-stimulated IFN-γ$^{+}$CD4$^+$ T cells, IL-17$^{+}$CD4$^+$ T cells, and HSV-stimulated IL-17$^{+}$CD4$^+$ T cells in the corneas of WT and IL-17RKO mice. Data are shown by guest on April 17, 2017 http://www.jimmunol.org/ Downloaded from
showed diminished infiltration of CD4+ T cells in IL-17 KO mice as compared with that in WT mice (Fig. 6C). To quantify further the different cell types infiltrating the cornea, experiments were terminated on day 15 to study cellular parameters. As shown, the frequencies (Fig. 6D, 6F) and absolute numbers (Fig. 6G) of total CD4+ T cells, Th1 cells, and Th17 cells were decreased in the IL-17RKO mice compared with those in the WT mice. Additionally, the expression of proinflammatory cytokines such as IL-6, IL-17, and IL-1β was diminished in IL-17RKO mice corneas as compared with that of the WT samples (data not shown). Analysis of DLNs and spleens of WT and IL-17RKO mice showed reduced frequencies but not total cell numbers of each population (Fig. 7). As shown in Fig. 7A–D, the frequencies of Th1 and Th17 cells showed 2-fold reduction in DLNs and spleens from IL-17RKO mice as compared with those from WT mice. However, the total numbers of each subpopulation were equal in both IL-17RKO and WT mice DLNs and spleens (Fig. 7E, 7F). These observations revealed that although the total cell numbers were equal in DLNs and spleens from IL-17RKO and WT mice, the frequencies and total cell numbers were reduced at the inflammatory site (cornea) in IL-17RKO mice as compared with those in their WT counterparts. Taken together, these results indicated the proinflammatory role of IL-17 in SK pathogenesis.

Discussion
In this study, we show that HSV infection of the cornea leads to a biphasic increase in corneal expression of IL-17. Moreover, the subsequent HSV-induced SK immunopathology is the outcome of the effector functions mediated by both Th1 and Th17 cells. The proinflammatory cytokine IL-17 drives initial as well as late events in SK pathogenesis and also contributes significantly to the pathology of SK. However, the sources of IL-17 during early and late stages of SK were different. Initially, innate cells such as γδ T cells were the main producers, and later on in the chronic phase Th17 cells were the producers. Additionally, IFN-γ was shown to be important for early protection from HSV infection, but cells producing IFN-γ also contribute to the pathology once the virus is absent. Furthermore, HSV-induced immunopathological lesions involve both Th1 and Th17 cells with their respective importance changing with time. Accordingly, in line with previous published reports, our data demonstrate that Th1 cells mainly initiate and orchestrate SK lesions at least during the early stages followed by the entry of Th17 cells when the disease is at its peak. Interestingly, a significant proportion of the Th1 cells secreted IFN-γ upon stimulation with UV-inactivated HSV virus, but most Th17 cells failed to produce IL-17 under similar conditions, indicating that Th17 cells could be involved in a bystander fashion in SK pathogenesis. Furthermore, depletion of IL-17 during both the early and the chronic phases of SK pathogenesis and infection of IL-17RKO mice showed reduced SK lesion severity. The findings indicate an important role for IL-17 at all of the stages of SK pathogenesis. Accordingly, targeting IL-17 production represents a logical target to control SK, an important cause of human blindness.

Innate cells such as γδ T cells act as one of the major early sources of IL-17, as shown in some other systems (21–25). Thus our data are in line with previously published reports where γδ T cells were shown to be the main source of innate IL-17 (21–25). IL-17 orchestrates the local inflammatory response by stimulating epithelial, endothelial, and fibroblastic cells to produce and release various inflammatory cytokines (12, 36–39). Furthermore, IL-17 drives increased influx as well as survival of neutrophils at the site of inflammation by the induction of neutrophil chemoattractants as well as GM-CSF-mediated granulopoiesis (12, 39–41). In ocular HSV infection, an initial influx of neutrophils plays an important role in virus clearance (42). The diminished expression of neutrophil chemoattractants for neutrophils and subsequent reduced infiltration of neutrophils occur in IL-17RKO mice after HSV infection (17). Similarly, we found reduced infiltration of neutrophils in HSV-infected IL-17RKO mice as well as after IL-17 neutralization during early and late stages of SK pathogenesis (data not shown). Taken together, the data show that during HSV infection of the cornea IL-17 contributes to the early innate cell response by promoting the migration of neutrophils.

Recent work has discovered a critical role for Th17 cells in various chronic and autoimmune inflammatory conditions (33, 34, 43–45), and our data show that Th17 cells do participate in SK pathogenesis. However, in contrast to autoimmune as well as some chronic inflammatory conditions, where Th17 cells may play a dominant role, HSV-induced immunopathology results from the participation of both Th1 and Th17 cells, with the relative importance changing as the disease progresses. Accordingly, our data from IFN-γ and IL-17 neutralization as well as IL-17RKO mice show that both Th1- and Th17-mediated immune responses contribute to SK pathogenesis. Th1 cells were the first to infiltrate the cornea, and significant proportions of them were HSV specific. They set the stage for the development of complex SK lesions that additionally involve Th17 cells. However, the Th17 cells could migrate only after the entry of Th1 cells, indicating their role in the later stages of SK. Our data accord with a recent study showing that only Th1 cells could gain entry in the noninflamed tissue and initiate inflammatory responses followed by entry of the Th17 cells into the inflamed CNS (46). Furthermore, almost none of the Th17 cells responded to HSV-specific Ag when stimulated ex vivo, which may indicate their bystander role in SK pathogenesis. However, diminished SK severity in the absence of IL-17R signaling as well as after IL-17 neutralization during early and late stages of SK indicated that IL-17 does play a proinflammatory role in SK pathogenesis.

Although presently we cannot detect the Ag specificity of these Th17 cells, they could be reactive against yet unidentified unmasked self-corneal Ags or functioning in a bystander fashion. A recent study on an autoimmune condition of the retina using an experimental autoimmune uveitis model showed that both retinal Ag-specific Th1 and Th17 cells could drive autoimmunity depending upon the local conditions at the time of Ag exposure as well as the type of effector T cell present (47). However, our data show that during viral immunopathology higher levels of IFN-γ occur early after HSV infection that could promote Th1 immune responses and suppress Th17 immune responses. During autoimmune conditions, exposure of self-unmasked Ags occurs that provides constant stimulation to the effector T cells at the site of inflammation (48). The body might sense this chronic persistence of Ag as the need arises for stronger and more pathogenic immune responses for the removal of such persistent Ags. The highly pathogenic nature of IL-17 and/or Th17 cells meets this criterion and might be the outcome of such persistent antigenic stimulation as observed in many autoimmune conditions. However, during

as a summary of two independent experiments with six to eight mice per group. Statistical levels of significance were analyzed by Student t test. *p < 0.05, **p < 0.005, ***p < 0.001. Error bars are SEM. ns, not significant.
acute infections, effective Th1 and Th2 responses could clear the Ag from the site of infection, avoiding the Th17-mediated immunopathology. In contrast, chronic persistence of Ag could be sensed by the body, with a resultant robust effector T cell response such as a Th17 cell-mediated immune response, which can cause damage and subsequent immunopathology. SK, being a chronic disorder, could mimic this condition, where initial acute viral infection is controlled by the Th1 cell response that initiates the inflammatory milieu in the cornea. This initial damage to the corneal tissue could uncover self-antigens that help to drive the subsequent Th17-mediated highly inflammatory reaction. Alternatively, one recent study showed that Th17 cells can be activated in the context of the local microenvironment and could cause tissue-specific damage in an Ag nonspecific way that depended upon the presence of IL-6 (49). Thus, it might be possible that these Th17 cells during late stages of SK could be nonspecific to the corneal Ag and acting in a bystander fashion stimulated in the presence of the complex inflammatory cytokine milieu. However, this issue needs to be addressed further using adoptive transfer of nonspecific Th17 cells in the context of HSV-induced SK immunopathology. Such studies are ongoing currently in our laboratory.

Our data also demonstrate a dual role for IFN-γ during HSV-mediated immunopathology. Innate cells contribute to the early source of IFN-γ after HSV infection and Th1 cells from day 7 postinfection (1, 50). Thus, IFN-γ neutralization during the preclinical phase when replication virus is still present in the cornea

**FIGURE 7.** IL-17RKO mice exhibit reduced frequencies but not numbers of Th1 and Th17 cells in DLNs and spleens after HSV infection. C57BL/6 and IL-17RKO mice were infected with $1 \times 10^4$ PFU of HSV. Mice were sacrificed on day 15 postinfection, and single-cell suspensions of the individual spleens and cervical DLNs were prepared ($n = 4$). A. Representative FACS plots for Th1 and Th17 cells stimulated with PMA/ionomycin (upper panels) or HSV-KOS virus (lower panels). B and C, Summary for average frequencies of different CD4+ T cell types as depicted in A from DLNs (B) and spleens (C). D and E. The total numbers of IFN-γ+CD4+ T cells, HSV-stimulated IFN-γ+CD4+ T cells, IL-17+CD4+ T cells, HSV-stimulated IL-17+CD4+ T cells, and Foxp3+CD4+ T cells from DLNs (D) and spleens (E) of WT and IL-17RKO mice. Statistical levels of significance were analyzed by Student t test. *p < 0.05, **p < 0.005, ***p < 0.001. Error bars are SEM. ns, not significant.
(1) leads to the transient increased tissue damage presumably driven by the virus. Furthermore, this early depletion caused severe encephalitis, possibly an effect of higher viral titers, indicating the early protective role of IFN-γ as documented previously (1, 49, 51). Interestingly, neutralization of IFN-γ after day 7 postinfection resulted in significantly reduced SK severity, indicating a crucial role for IFN-γ and/or Th1 cells as the initiators and main orchestrators of early immunopathological lesions of SK. Because IL-2 and IFN-γ suppress Th17 responses (17, 31–34), conceivably during HSV-induced immunopathology higher levels of IFN-γ in the cornea might be suppressing the early development of Th17 responses. Furthermore the chronic inflammatory situation in the cornea might be creating appropriate conditions for the generation and or migration of Th17 cells. Naive CD4+ T cells differentiate into Th17 cells under appropriate antigenic stimulation in the presence of IL-6 and TGF-β (26–28). Furthermore, Th17 cells express the chemokine receptor CCR6 and migrate to the site of inflammation through the specific chemokine CCL20 (29, 30). Indeed, our data for the expression levels of IL-6, TGF-β, and CCL20 indicated that conditions favoring either migration and/or generation of Th17 cells occur only during the very late stage of SK pathogenesis. This might explain the delayed appearance of Th17 cells in the cornea.

Taken together, our results demonstrate the relative contributions of IL-17/Th17 cells and IFN-γ/Th1 cells in the pathogenesis of SK. Both cell types participate in the pathogenesis of SK depending on the stage of SK. The demonstration of the pathogenic role of IL-17/Th17 cells in later stages of SK is a novel finding and could have implications for future therapeutical interventions for HSV-induced immunopathology, an important cause of infectious human blindness.

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References


