**CCR6-Positive γδ T Cells Provide Protection Against Intracorneal HSV-1 Infection**

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**PURPOSE.** γδ T cells offer an important early immune defense against many different pathogens, both bacterial and viral. Herein, we examined the capacity of γδ T cell subsets to provide protection in the cornea against herpes simplex virus-1 (HSV-1).

**METHODS.** C57BL/6 (wild-type [WT]), γδ T-cell deficient (TCRδ−/−) and CCR6-deficient (CCR6−/−) mice were infected intracorneally with HSV-1. At multiple time points following infection, corneas were excised, and cells were immunostained for surface markers, intracellular cytokines, and analyzed using flow cytometry. WT and CCR6−/− γδ T cells were adoptively transferred into TCRδ−/− mice and corneal scores and survival were measured.

**RESULTS.** Intracorneal infection of mice lacking γδ T cells exhibited increased corneal opacity scores, elevated viral titers, and higher mortality compared with WT mice. Both CCR6−/− and CCR6−/− γδ T cell subsets were observed in corneas after virus infection. CCR6−/− γδ T cells produced IL-17A and were predominantly CD44+/CD62L−, consistent with natural IL-17+ γδ T cells. In contrast IL-17A production by CCR6+/− γδ T cells was infrequent, and this subset was largely single positive for CD62L or CD44. The CCR6+ subset appeared to provide protection against HSV-1 as follows: (1) CCR6+/− mice had more severe corneal opacity compared with WT mice; and (2) adoptive transfer of γδ T cells from WT mice restored protection in TCRδ−/− mice whereas transfer of γδ T cells from CCR6−/− mice did not.

**CONCLUSIONS.** γδ T cells in the cornea can be divided into CCR6+ and CCR6−/− subsets with the former conferring protection early after intracorneal HSV-1 infection.

Keywords: gamma delta T cells, herpes simplex keratitis, CCR6, IL-17A
South Alabama. All protocols involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Alabama, and all procedures were in compliance with IACUC guidelines. In addition, all procedures performed in the current studies were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

HSV-1 Infection

Corneal infections were performed using an intrastromal route to inject the desired dose of HSV-1 strain RE, as previously described. Briefly, a pilot hole was produced through the corneal epithelium into the stroma using a 30-G disposable needle. A 32-G/30-cm needle attached to a repeating dispenser (Hamilton, Reno, NV) was then inserted and 1 µL of HSV-1 was injected (1 × 10^5 or 1 × 10^6 PFU HSV-1 were used for all infections).

Corneal Opacity Score

Eyes were monitored in a coded fashion for corneal opacity under a dissecting microscope and graded as follows: 0 = clear, 1 = slight haze, 2 = moderate opacity, 3 = severe opacity, 4 = severe opacity with iris obscured, 5 = necrotizing stromal keratitis. Scores were determined by blinded analysis.

Flow Cytometry Analysis of Immune Cells

Immune cells were isolated from corneas by digesting in Dulbecco’s modified Eagle’s medium (DMEM) with Liberase (Sigma-Aldrich Corp., St. Louis, MO, USA). Single-cell suspensions from lymph nodes were produced by disruption of the tissues with a glass slide and washing with Hanks’ balanced salt solution (HBSS). Single cells were resuspended in HBSS and stained using antibodies specific for purified Fc receptor (2.4G2), TCRγδ (GL3), CD3ε (145-2C11), CCR6 (29-2L17), IL-17A (eBio17B7), IFN-γ (XMG1.2), NK1.1 (PK156), CD4 (GK1.5), CD8 (Ly-2), Ly6G (RB6-8C5), CD11b (M1/70), CD44 (IM7), CD62L (MEL-14), IL-1R1 (35F5), IL-1R2 (4E2), and IL-23R (O78-1208). Various conjugates of antibodies were acquired from Invitrogen (San Diego, CA, USA), BD Biosciences (San Jose, CA, USA), and Biolegend (San Diego, CA, USA). Intracellular staining for cytokines was performed using a FoxP3 staining kit according to the manufacturer’s instructions (Invitrogen). Isotype and minus one controls were used to validate immunostaining specificity. Cell numbers were enumerated using whole-tissue cell counts and population frequencies obtained by flow cytometric analysis.

ELISA

IL-17A and IFN-γ levels were measured by ELISA reagents obtained from R&D Systems (Minneapolis, MN, USA). Corneal lysates were added to 96-well plates coated with anti–IL-17A, or anti–IFN-γ antibody. Biotinylated antibody specific for either cytokine was subsequently added followed by streptavidin-horseradish peroxidase (HRP; R&D Systems). HRP substrate was added for detection and the reaction was terminated with stop solution (Thermo Scientific, Waltham, MA, USA). The OD measurements were acquired at 450 nm using a Synergy 2 plate reader (BioTek, Winooski, VT, USA). A standard curve using recombinant mouse IL-17A or IFN-γ was generated to determine the concentration of cytokine in experimental samples.

Plaque Assay

Trigeminal ganglia were collected and frozen at −80°C in 500 µL of DMEM containing 5% fetal bovine serum. Tissue lysates were then prepared by thawing the tissue, sonicating on ice, and then centrifuging at 47,300 g for 10 minutes at 4°C. Fifty microliters of undiluted supernatant was then added to vero cells (ATCC, Manassas, VA, USA) that were grown to confluency in 6-well plates. The lysates incubated on the cells for 1 hour at room temperature with gentle shaking, then a methylcellulose overlay was added for 48 hours. After 48 hours plaques were developed with crystal violet and counted.

Adoptive Transfer

Single cells were isolated from cervical lymph nodes and spleens of WT or CCR6−/− mice. γδ T cells were enriched for using magnetic column separation (Miltenyi Biotec, Bergisch Gladbach, Germany); 1 × 10^6 γδ T cells were transferred intravenously into TCRγδ−/− recipient mice. Mice were infected 1 day after cell transfer.

Natural Killer Cell Depletion

Natural killer (NK) cells were depleted in C57Bl/6 mice by intraperitoneal administration of 0.1-mg anti-NK1.1 antibody (BD Bioscience) per mouse. Mice were infected with HSV-1 ic 24 hours following administration of antibody.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism (V6.0; La Jolla, CA, USA) and individual statistical tests are indicated in the figure legends.

RESULTS

γδ T Cells in the Cornea Expand Markedly During the First 72 Hours Post HSV-1 Infection

We used flow cytometric analysis to identify innate and adaptive immune cell populations in the cornea prior to and following HSV-1 infection (Fig. 1A). As shown in Figure 1B, γδ T cells are detectable at low frequency in uninfected mouse corneas. Though the frequency remains low, the number of these cells increases significantly following HSV-1 infection (Fig. 1C), 4 ± 3 cells in uninfected cornea to 760 ± 285 cells by 48 hours after infection. Similarly, the cell numbers of other populations, such as NK cells and CD8+ T cells, also increased in the first 48 hours of infection. Additionally, the frequency and number of macrophages and neutrophils increased after infection compared with uninfected corneas.

Mice Lacking γδ T Cells Have Reduced Survival Following HSV-1 Corneal Infection

To determine the importance of γδ T cells in ic responses to HSV-1, we compared survival of WT mice with that of mice lacking γδ T cells (TCRγδ−/−). While approximately 72% of WT mice survived out to 21 days postinfection (PI), all mice lacking γδ T cells succumbed to infection by day 14 PI (Fig. 2A). In addition, TCRγδ−/− mice had significantly higher corneal opacity scores and increased virus load in the trigeminal ganglia (TG) at day 7 PI compared with WT mice (Figs. 2B, 2C). However, viral load in the cornea at day 7 PI were comparable between TCRγδ−/− and WT mice (data not shown). Collectively, these
data suggest that γδ T cells have an important role in limiting virus infection by preventing spread to and/or suppressing replication within the TG.

γδ T Cells Produce IL-17 in the Cornea Following HSV-1 Infection

γδ T cells are known to produce IL-17A and/or IFN-γ. We examined the expression of these cytokines within γδ T cells in the cornea of infected mice. Representative flow cytometric analysis at 24 hours PI is shown in Figure 3A, demonstrating that γδ T cells produce IL-17A and do not produce IFN-γ in the cornea during early HSV-1 infection. To address whether γδ T cells and NK cells are major producers of IL-17A and IFN-γ, respectively, we examined cytokine production in mice lacking these cell populations. WT mice treated with antibody to NK1.1 (to deplete NK cells) demonstrated significant reduction in IFN-γ production following HSV-1 ic. Notably IL-17A was comparable to untreated mice (Fig. 3B). To determine the relative contribution of γδ T cells for IL-17A production following HSV-1 infection, we compared IL-17A protein levels in corneas of WT and TCRδ−/−/− mice. At 24 hours PI, IL-17A protein levels were significantly reduced in corneas of TCRδ−/−/− mice compared with corneas from WT mice, while IFN-γ levels were comparable between the two strains (Fig. 3C). Though immunostaining from draining lymph nodes showed no change in the frequency or number of IL-17A+ γδ T cells (Fig. 3D), the number of IL-17A producing γδ T cells was significantly increased in corneas after HSV-1 infection (Fig. 3E). These data indicate that γδ T cells significantly contribute to IL-17A production in the cornea early following HSV-1 infection.

CCR6 Identifies IL-17A Producing γδ T Cells in the Cornea

CCR6 is a chemokine receptor associated with immune cell migration to sites of injury and infection. CCR6 is also a marker that can associate with γδ T cells capable of producing IL-17A. To determine whether CCR6+ γδ T cells participate in responses to HSV-1, we examined corneas from uninfected and infected mice by flow cytometry. We observed CCR6+ and CCR6− γδ T cell subsets in corneas before and after infection (Fig. 4A). The total number of cells for both subsets increases in the cornea during the first 2 days of infection. At 48 hours PI, the number of CCR6+ γδ T cells was significantly elevated compared with the number of CCR6− γδ T cells (Fig. 4C). To address whether one or both subsets were producing IL-17A we performed intracellular cytokine staining. Though few IL-17A+ producing CCR6− γδ T cells were observed in corneas from uninfected mice, the number increased markedly at 24 and 48 hours PI. Comparatively, a much lower number of CCR6− γδ T cells stained positive for IL-17A before and after infection.
infection (Fig. 4D). These data show that CCR6⁺ cd T cells increase in corneas after virus infection, and this subset of cd T cells is producing IL-17A.

**TCRδ⁻/⁻ Mice Can Be Rescued From Lethal HSV-1 Infection by WT, But Not CCR6⁻/⁻ T Cells**

To determine whether the CCR6⁺ γδ T cell subset contributes to protection against corneal HSV-1 infection, we initially compared corneal opacity scores in mice lacking CCR6 (CCR6⁻/⁻) with those of WT mice. At both days 3 and 7 PI, CCR6⁻/⁻ mice had modest, but significantly increased corneal opacity scores (Fig. 5A).

To directly test whether CCR6⁺ γδ T cells protect against HSV-1 intracorneal infection, we adoptively transferred γδ T cells from either WT or CCR6⁻/⁻ mice into TCRδ⁻/⁻ mice. TCRδ⁻/⁻ mice not receiving cell transfer had 80% mortality by day 7 PI. Similarly, TCRδ⁻/⁻ mice receiving CCR6⁻/⁻ γδ T cells had 60% mortality at day 7 PI. Comparatively, none of the TCRδ⁻/⁻ mice receiving WT γδ T cells died by day 7 PI (P < 0.05), and only 40% succumbed by day 15 PI (Fig. 5B). Corneal opacity scores were also significantly reduced in mice receiving WT γδ T cells compared with no transfer controls at day 10 PI (Fig. 5C). These data suggest that CCR6⁺ γδ T cells contribute to protection against HSV-1 corneal infection.

**CCR6⁺ γδ T Cells in the Cornea Have a Natural γδT17 Phenotype**

To further characterize the activation phenotype of γδ T cells in the cornea, we examined CD44 and CD62L levels on CCR6⁺ and CCR6⁻/⁻ γδ T cells. The small number of CCR6⁺CD44⁺CD62L⁻ TCRδ⁻/⁻ mice not receiving cell transfer had 80% mortality by day 7 PI. Similarly, TCRδ⁻/⁻ mice receiving CCR6⁻/⁻ γδ T cells had 60% mortality at day 7 PI. Comparatively, none of the TCRδ⁻/⁻ mice receiving WT γδ T cells died by day 7 PI (P < 0.05), and only 40% succumbed by day 15 PI (Fig. 5B). Corneal opacity scores were also significantly reduced in mice receiving WT γδ T cells compared with no transfer controls at day 10 PI (Fig. 5C). These data suggest that CCR6⁺ γδ T cells contribute to protection against HSV-1 corneal infection.
**Figure 3.** γδ T cells produce IL-17A in the cornea following HSV-1 infection. (A) At 24 hours PI, NK cells (blue) and γδ T cells (red) were identified in the cornea using NK1.1 and GL3 and intracellular production of IFN-γ and IL-17A was evaluated. IFN-γ and IL-17A were measured by ELISA at 24 hours PI in (B) C57Bl/6 mice treated with γNK1.1 and in (C) TCRδ−/− mice. (D) Flow cytometric analysis comparing the frequency of IL-17A producing cells in draining lymph nodes of WT and TCRδ−/− mice at 24 and 48 hours PI. (E) Enumeration of IL-17A producing γδ T cells in corneas of HSV-1–infected WT mice. (B–D) Data from one of two independent experiments with similar results are shown; n = 4 mice per strain for (B, C); n = 2 samples per strain for (D). (E) Data combined from 3 independent experiments (n = 8 samples per time point). Corneas from two mice were pooled for each sample (D, E). *P < 0.05, **P < 0.01, t-test (B, C), 1-way ANOVA with Dunn’s posttest (E).
T cells observed in uninfected corneas were markedly expanded by 48 hours PI (Fig. 6; Table 1). In contrast CCR6 neg T cells initially were CD44 negCD62L+ at 24 hours PI. By 48 hours PI, these cells tended to remain CD44 negCD62L+ or switch to CD44+CD62Lneg. Therefore, while CCR6 neg T cells conform to more conventional naive and effector phenotypes, CCR6+ T cells exhibit a natural CD17 phenotype.

Corneal γδ T Cells are IL-1R+ and IL-23R+ Following HSV-1 Infection

IL-1 and IL-23 are required for IL-17A production by γδ T cells. Therefore, we examined the CCR6+ and CCR6neg γδ T cell subsets for the presence of receptors for IL-1 and IL-23. We found that at 48 hours PI there were significantly more CCR6+ γδ T cells expressing IL-1R1, -1R2, and -23R than in uninfected corneas (Fig. 7). Additionally, though some CCR6neg γδ T cells also expressed IL-1R1, -1R2, and -23R there were significantly fewer compared with CCR6+ γδ T cells at 48 hours PI.

FIGURE 4. CCR6 is an identifying marker for IL-17A producing γδ T cells following corneal HSV-1 infection at 24 and 48 hours. (A) Representative flow cytometric analysis to identify CCR6+ and CCR6neg γδ T cells, and IL-17A+ γδ T cells at 48 hours PI. (B) Representative staining for IL-17A on γδ T cells in draining lymph nodes (dark gray histograms); isotype controls are indicated (light gray histograms). (C) Enumeration of CCR6+ (gray open circles) and CCR6neg (black squares) γδ T cells in the cornea following HSV-1 infection. (D) Number of IL-17A producing γδ T cells within CCR6+ or CCR6neg subsets in the cornea following HSV-1 infection. Data are combined from four to six independent experiments. Each symbol represents data from an individual sample; corneas from two mice were pooled for each sample. *P < 0.05, **P < 0.01, ***P < 0.001, 2-way ANOVA with Sidak’s posttest.

DISCUSSION

Previous studies have shown that γδ T cells can be either protective or pathogenic depending upon the pathogen-infection model examined. These cells mediate their biological effect via the production and release of various effector molecules including IL-17A, IFN-γ, and granzyme B. A hallmark of γδ T cells is that they are able to respond quickly to infectious agents due to local tissue residency and surveying the body through the circulation waiting for a migratory signal. Our survey of immune cells showed that this cell type was present in the uninfected cornea, albeit at very low levels. HSV-1 corneal infection stimulated a γδ T cell increase of nearly 100-fold within the first 48 hours (Fig. 1). Even though these cells were still only a minor population of the immune cells infiltrating and expanding in the cornea they constituted an important combatant in the early antiviral immune response. This is documented by the observations that mice which lacked functional γδ T cells displayed an elevated viral load in the trigeminal ganglion, more severe corneal opacity, and a
significantly increased rate of mortality (Fig. 2). In contrast, viral loads in the cornea were comparable between TCR d−/− and WT mice (data not shown), suggesting that corneal γδ T cells limit spread of HSV-1 from the cornea to the TG and/or suppress virus replication in the TG.

IL-17A is an inflammatory cytokine that has protective or pathogenic effects depending on the stimuli, the site of inflammation, and the T-cell source.41–45 The importance of IL-17A for ocular infections demonstrates context-dependent activity. In naïve mice, basal levels of IL-17A are maintained in the conjunctiva by γδ T cells stimulated by commensal bacteria.44 IL-17A production in the conjunctiva stimulates production of antimicrobial peptides, thereby imparting protection against challenge with either Candida albicans or Pseudomonas aeruginosa.44 During, intracorneal HSV-1 infection, IL-17A production by CD4+ T cells is pathogenic.45–47 Interestingly, the study by Suryawanshi et al.47 reported that at 48 hours PI, γδ T cells were the principle producers of IL-17A, but CD4+ Th17 cells were the cellular source at later timepoints. While their study concluded that IL-17A produced...
by CD4+ Th17 cells contributes to a pathogenic response the importance of early IL-17A production by γδ T cells was not addressed. We find that diminished early IL-17A production in TCRδ−/− mice coincided with enhanced susceptibility to HSV-1 corneal infection (Figs. 2, 3). This suggests that IL-17A may contribute to the protective effect in mice with functional γδ T cells, perhaps via its known capacity to stimulate production of other cytokines and chemokines.42,43

CCR6 associates with IL-17A producing γδ T cells.19 We hypothesized that CCR6+ γδ T cells in the cornea would be immunoprotective against HSV-1. Consistent with studies in other tissues, we observed that CCR6+ γδ T cells in the cornea are CCR6+18. In support of our hypothesis, CCR6−/− mice had more severe corneal opacity scores compared with WT mice at days 3 and 7 following HSV-1 infection (Fig. 5A). Importantly, TCRδ−/− mice receiving WT γδ T cells by adoptive transfer survived longer (Fig. 5B) and had lower corneal opacity scores compared with TCRδ−/− receiving CCR6−/− γδ T cells (Fig. 5C). The above findings support our hypothesis that CCR6+ γδ T17 cells protect against corneal HSV-1 infection.

Further characterization of CCR6+ γδ T17 cells in the cornea documented that they are primarily CD44+ CD62L+. In conventional γδ T-cell biology, CD44 is upregulated upon activation and CD62L is downregulated. Memory γδ T cells are an exception to this rule in that they retain both CD44 and CD62L.48 Interestingly, some γδ T cells leave the thymus as CCR6+ CD44+ CD62L+, while other CCR6+ γδ T cells are traditionally naive (CD44− CD62L+).19,49,50 It has been pro-

**TABLE.** Frequency and Enumeration of CCR6+ and CCR6neg γδ T Cell Populations in the Cornea

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<td>CD44+CD62L+</td>
<td>41.0 ± 9.8</td>
<td>38.1 ± 15.0</td>
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<td>20.2 ± 6.3</td>
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CCR6+ CD44+ CD62L+ increase at 48 hours compared with uninfected (P < 0.05). CCR6neg CD44+ CD62L− increase over uninfected at 48 hours PI (n = 6 samples per time point, corneas from two mice were pooled for each sample). P < 0.05 2-way ANOVA Dunnett’s posttest.
posed that γδ T17 cells can be divided into natural and inducible IL-17A producers, where natural γδ T17 cells are CCR6⁺CD44⁺CD62L⁺, and inducible γδ T17 cells are CCR6⁺CD44negCD62L⁻. Natural γδ T17 cells can be activated by cytokine alone, or in addition to a TCR signal, whereas inducible γδ T17 cells need both TCR and cytokine signal for IL-17A production. Our data suggest that there is a natural γδ T17 population present before HSV-1 infection, and that this population expands following infection. Additionally, there is a CCR6⁺CD44⁺CD62Lneg population that is present in the cornea at 24 and 48 hours following HSV-1 infection that is consistent with an inducible γδ T17 phenotype (Fig. 6). These data suggest that in the cornea following HSV-1 infection both natural and inducible γδ T17 cells are participating in protection.

A defining phenotype of γδ T17 cells is the presence of IL-1R and -23R. Natural γδ T17 cells can be activated to produce IL-17A by IL-1α or IL-1β and IL-23 signaling with or without TCR signaling. We examined CCR6⁺ γδ T cells from the cornea by flow cytometry and found that the number of IL-1R⁺, -R2⁺, and -23R⁺ cells increased in the cornea at 48 hours following HSV-1 infection, while CCR6neg γδ T cells presenting these receptors did not significantly increase (Fig. 7). Consistent with previous reports, IL-1α protein levels in
cornea lyses following HSV-1 infection increased by 24 hours PI (data not shown).52–54 IL-23 is also observed in corneae early after HSV-1 infection.55 Thus, the corneal microenvironment is altered during HSV-1 infection to potentiate IL-17A production by natural γδ T17 cells.

A central question in the field of γδ T cell biology is whether protective γδ T cell responses are antigen-specific or cytokine-driven. Sciammas et al.4,56 identified a γδ T cell clone that recognized an HSV-1 glycoprotein backbone in a conformationally dependent manner on the surface of dendritic cells. Using an HSV-1 footpad infection model, the same group of investigators showed that total T-cell depletion rendered mice susceptible to lethal encephalitis while depletion of only γδ T cells did not, suggesting γδ T cells are protective against HSV-1.28 However, they did not determine whether glycoprotein recognition by γδ T cells was important for protection against HSV-1 footpad infection. Our studies with corneal infection are consistent with their early work and build upon it by identifying a CCR6+ γδ T17 protective subset. Our data suggest that this subset can be further divided into natural and inducible populations. Future work is required to determine whether activation of either of these populations is TCR-mediated.

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