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CD8 T Cells Mediate Transient Herpes Stromal Keratitis in CD4-Deficient Mice

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Abstract

PURPOSE—To evaluate the role of CD4⁺ T cells in the development of murine herpes stromal keratitis (HSK).

METHODS—The corneas of wild-type (WT) BALB/c mice and three types of CD4-deficient BALB/c mice (CD4^{−/−}, CD4-depleted, CD4 and CD8 double-depleted) were infected with different doses of HSV-1 RE, and HSK incidence and severity were monitored. Corneal infiltrates were quantitatively and functionally assayed by flow cytometric analysis of individually digested diseased corneas and documented histologically.

RESULTS—At a relatively high infectious dose (1×10^5 pfu/cornea): (1) CD4-deficient and WT BALB/c mice had severe HSK with a similar incidence (80%–100%), whereas HSK did not develop in mice deficient in both CD4⁺ and CD8⁺ T cells; (2) neutrophils were the predominate leukocyte in the corneas of CD4-deficient and WT mice; (3) the corneas of WT mice had activated, HSV-1-specific CD4⁺ T cells, but few if any CD8⁺ T cells; (4) the corneas of CD4-deficient mice had activated, HSV-1-specific CD8⁺ T cells; and (5) HSK in CD4-deficient mice was transient, showing loss of CD8⁺ T cells at 2 to 3 weeks after infection (pi) followed by a loss of neutrophils. At a relatively low infectious dose of HSV-1 (10^3 pfu/cornea) severe HSK developed in 80% to 90% of WT mice, but in only 30% to 40% of CD4-deficient mice.

CONCLUSIONS—CD4⁺ T cells preferentially mediate HSK, but, in their absence, a high infectious dose of HSV-1 can induce histologically similar but transient HSK that is mediated by CD8⁺ T cells.

Herpes stromal keratitis (HSK) is a vision-threatening, recurrent disease of the cornea that is the leading infectious cause of corneal blindness in the United States. Most of our current

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knowledge of the immunologic processes that underlie HSK has arisen from studies in mice. Seminal studies by Metcalf et al.¹ demonstrated that HSK did not develop in congenitally athymic (and thus T-cell-deficient) nude mice. Subsequent studies confirmed the involvement of T cells by demonstrating that nude mice can be rendered susceptible to HSK by adoptive transfer of T cells before HSV-1 corneal infection.² It was then established independently in two laboratories that HSK induced by the RE strain of HSV-1 in either A/J or BALB/c mice is regulated primarily by CD4⁺ T cells^{3–5} and the Th1 cytokines IFN- γ and IL-2.^{6–9}

Although the capacity of CD4⁺ T cells to mediate HSK is well documented, the involvement of CD8⁺ T cells is less clear. One study showed that after corneal infection of A/J mice with the KOS strain of HSV-1, CD8⁺ T cells were the predominant infiltrating T cell population, and their depletion significantly reduced the incidence and severity of HSK.⁴ In contrast, studies using the RE and McKrae strains of HSV-1 to infect the corneas of BALB/c, and C57BL/6 mice, respectively, suggested a regulatory role in which CD8⁺ T cells reduce the incidence and severity of HSK.^{5,10} Of note, a recent study indicated that this regulatory role of CD8⁺ T cells may be expressed outside the cornea, perhaps involving control of HSV-1 infection of the trigeminal ganglion.¹¹

Several recent studies have added to the complexities surrounding the involvement of T-cell subpopulations in HSK. In one set of studies, a congenic BALB/c mouse strain developed HSK due to an autoimmune T-cell attack on the cornea induced by a molecular mimicry mechanism involving a homologous epitope on viral and corneal proteins.^{12–14} In a second study,¹⁵ the RE strain of HSV-1 induced HSK in T-cell- and B-cell-deficient SCID mice by a mechanism apparently involving only innate immunity. In a third study,¹⁶ HSK was induced in DO11.10 SCID mice, which are incapable of producing HSV-1-specific CD4⁺ T cells, through apparent cytokine-mediated bystander activation of Ova-specific CD4⁺ T cells in the infected cornea. The conclusion of these studies is that, depending on the strain of virus and the strain and immune competence of the mice used, HSK can result from an autoaggressive response of CD4⁺ T cells, an uncharacterized innate immune response, or bystander activation of CD4⁺ T cells of irrelevant specificity.

Although the latter two studies^{15,16} incorporated a rather elegant design, there is an important caveat to accepting the authors' conclusions. Both studies used BALB/c SCID mice, in which HSV-1 replication in the cornea never comes under control, and which typically succumb to a lethal encephalitis by 12 days after infection. One cannot assume that the involvement of innate immunity and bystander activation of CD4⁺ T cells in corneas of BALB/c SCID mice that never control HSV-1 replication will also contribute to HSK in WT mice that control HSV-1 replication in the cornea within 7 days and uniformly survive the infection. Indeed, it was subsequently demonstrated that when HSV-1 replication is controlled in the corneas of SCID mice, bystander activation alone is not sufficient to cause HSK.¹⁷

In the current study, viral dose dictated whether HSK development in BALB/c mice occurred in a CD4-dependent or-independent manner. Differences in the HSK is regulated by CD4⁺ and CD8⁺ T cells were determined.

MATERIALS AND METHODS

Mice and Virus Infection

Female wild-type (WT) BALB/c mice (Frederick Cancer Research Center, Frederick, MD) and CD4^{-/-} BALB/c mice¹⁸ were used at 6 to 8 weeks of age in all experiments. All mice were anesthetized by intramuscular injection of 2 mg of ketamine hydrochloride and 0.04 mg of xylazine (Phoenix Scientific, St. Joseph, MO) in 0.2 mL of HBSS (BioWhittaker, Walkersville, MD). Topical corneal infection was performed by scarification of the central

cornea 15 times with a sterile 30-gauge needle in a crisscross pattern, and applying 3 μ L of RPMI (BioWhittaker) containing various doses of HSV-1. The RE strain of HSV-1 used in these studies was grown in Vero cells, and intact virions were purified on gradients according to the manufacturer's instructions (OptiPrep; Accurate Chemical and Scientific Corp., Westbury, NY), and stored at -80°C . The concentration of plaque-forming units (pfus) of HSV-1 was determined in a standard virus plaque assay. All experimental procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In Vivo CD4 Depletion

The mice were given 0.15 mg of anti-CD4 antibody (GK1.5) intraperitoneally (IP) starting 2 days before infection and repeated on days +1, +5, +8, +15, +22, and +29 after infection (dpi).

Monitoring of HSV-1 Corneal and Skin Disease

Corneal disease was monitored in a masked fashion by slit lamp examination on alternate days after HSV-1 corneal infection. By 2 dpi, the BALB/c mice uniformly exhibited dendritic-shaped corneal epithelial lesions that healed by 4 dpi. HSK was characterized by corneal opacity and neovascularization, beginning approximately 7 days after HSV-1 corneal infection. Opacity and neovascularization developed concurrently and were monitored by slit lamp examination. HSK was scored on the basis of opacity as 1+, mild corneal haze; 2+, moderate opacity; 3+, severe opacity obscuring the iris; or 4+, corneal perforation.

Preparation of Corneal Single-Cell Suspension

At various times after HSV-1 corneal infection, the mice were scored for HSK, and the corneas were removed and incubated with PBS-EDTA to separate the epithelial layer. Individual corneal stromas were rinsed, cut into quarters, treated with collagenase type I (84 U/cornea; Sigma-Aldrich, St. Louis, MO) for 1.5 to 2 hours at 37°C , and triturated until no apparent tissue fragments remained. The single-cell suspension of each cornea was then filtered through a 40- μm cell strainer cap (BD Labware, Bedford, MA) and washed.

Quantification of Leukocyte Populations in Corneal Cell Suspensions

Single-cell suspensions of individual corneas (one cornea/tube) were prepared on the designated day after HSV-1 infection, incubated with anti-mouse CD16/CD32 (Fc γ III/II Receptor; 2.4G2; BD PharMingen, San Diego, CA) to prevent nonspecific binding of fluoresceinated mAbs, and then stained for various leukocyte surface markers for 30 minutes at room temperature. The following antibodies were purchased from BD PharMingen: PE-conjugated anti-CD8 α (clone 53-6.7), PE-Cy7-conjugated anti-CD4 (RM4-5), PerCP-conjugated anti-CD45 (30-F11), and FITC-conjugated anti-CD69 (H1.2F3). APC-conjugated anti-GR-1 (RB6-8C5) was purchased from Caltag (Burlingame, CA). All isotype control antibodies were obtained from BD PharMingen and included PE-rat IgG2a, PE-Cy7 rat IgG2a, PerCP-rat IgG2b, FITC-hamster IgG1, and APC-rat IgG2b. After they were stained, the cells were fixed in 1% paraformaldehyde (PFA; Electron Microscopy Sciences, Fort Washington, PA) and analyzed on a flow cytometer (FACS Aria with FACSDIVA data analysis software; BD Bioscience). Gates were set based on staining with the appropriate isotype control antibodies, and data are listed as total number of cells per cornea (obtained by analyzing the entire extract), or as the proportion of CD45 $^{+}$ (bone marrow-derived) cells that coexpress a particular phenotypic marker.

Bone Marrow–Derived DC Preparation

Bone marrow was isolated from naïve BALB/c mice, depleted by antibody and complement treatment of T cells (anti-CD3, clone 145-2C11), neutrophils (anti Gr-1, clone RB6-8C5), macrophages (anti Mac-1, clone TIB-128), and B cells and mature antigen presenting cells (APCs; by anti-Ia, clone TIB-120). The enriched dendritic cell (DC) precursors were then cultured in GM-CSF (granulocyte macrophage–colony-stimulating factor, catalog 415-ML; R&D Systems, Minneapolis, MN) and stem cell factor (SCF, catalog 455-MC; R&D Systems) for 3 days. The cells were fed with fresh medium containing GM-CSF and SCF after 3 days, and nonadherent cells were harvested on day 6. These nonadherent cells were then cultured in medium containing GM-CSF, IL-4, and TGF- β , to generate Langerhans-like cells that were used for corneal CD4 T-cell stimulations.

Stimulation and Analysis of IFN- γ Production by Corneal T Cells

MKSA cells (a SV40-transformed BALB/c mouse kidney fibroblast cell line kindly provided by Robert Bonneau, Pennsylvania State University College of Medicine) were infected at a multiplicity of infection (MOI) of 5 for 5 hours with the HSV-1 RE strain and harvested, and 1×10^6 HSV-infected MKSA cells were used to stimulate the CD8⁺ T cells in each digested cornea. Alternatively, bone-marrow–derived (BMDCs) were pulsed overnight with an extract from Vero cells that were infected with HSV-1 for 10 hours, as previously described,¹⁹ and 0.25×10^5 BMDCs were used to stimulate CD4⁺ T cells in each cornea. The corneal cells were stimulated for 6 hours and assayed for intracellular IFN- γ production using BDPharmingen's intracellular cytokine staining protocol.

Histopathology

At various times after corneal HSV-1 infection, HSK severity was scored, and whole eyes were removed and frozen with OCT media (Sakura Finetek, Torrance, CA) in histoprep base molds (Fisher Scientific, Pittsburgh, PA). Sections (6–8 μ m) were cut on a cryostat (model CM2050S; Leica Microsystems, Bannockburn, IL), and stained with hematoxylin and eosin (H&E). Alternatively, the sections were stained with purified anti-CD8 α (53-6.7, 2 μ g/mL; BD PharMingen), or anti-CD4 (4 μ g/mL, GK1.5) overnight at 4°C, washed with PBS and fixed with 2% paraformaldehyde (Electron Microscopy Sciences), washed with PBS, and blocked with 2% normal goat serum, and incubated with goat anti-rat Alexa Fluor 488 (2 μ g/mL; Invitrogen, Eugene OR) in 2% normal goat serum. The sections were washed with PBS, mounted (Immu-mount; Thermo Shandon Corp., Pittsburgh, PA), and analyzed using an inverted microscope (model IX70; Olympus America, Inc., Melville, NY) equipped with a confocal system (Radiance Plus; Bio-Rad Laboratories, Richmond, VA). Confocal images were captured and recorded (LaserSharp software; Bio-Rad).

Detection of Infectious Virus on Corneas

The corneal surfaces of WT and CD4^{-/-} BALB/c mice were swabbed with sterile surgical spears (Weck-Cel; Medtronic Solan, Jacksonville, FL) on days +2, +4, +6, +8 +10, +12, and +14 after HSV-1 infection, spears were placed in 0.5 mL RPMI, and frozen at –80°C until assayed. The samples were added to confluent Vero cells, incubated for 1 hour at 37°C, and overlaid with 0.5% methylcellulose. The cultures were incubated for 72 hours, fixed with formalin, and stained with cresyl violet, and a viral cytopathic effect was detected with the aid of a dissecting microscope.

RESULTS

Role of CD4⁺ T Cells in HSK Development after High-Dose HSV-1 RE Infection

Because CD4⁺ T cells are thought to play a requisite role in HSK, we predicted that CD4-deficient mice would show reduced susceptibility to HSK. After corneal infection with a high dose (1×10^5 pfu) of HSV-1, untreated WT BALB/c mice exhibited progressive HSK that began at ~7 dpi (Fig. 1). HSK severity increased exponentially through 15 dpi and then was maintained or progressed slowly through 35 dpi. Maximum disease incidence (94%) was achieved in untreated WT BALB/c mice by 15 dpi and remained constant through 35 dpi (Table 1). During the first 15 days after high-dose corneal infection, CD4^{-/-} and CD4-depleted BALB/c mice exhibited HSK with kinetics (Fig. 1) and incidence (Table 1) that were surprisingly similar to those of untreated WT mice. However, unlike untreated WT BALB/c mice in which HSK was uniformly maintained, 33% and 39%, respectively, of CD4-depleted and CD4^{-/-} BALB/c mice demonstrated disease regression of at least 1 unit of severity between 15 and 35 dpi (Fig. 1, Table 1).

Although HSK severity was significantly lower in both groups of CD4-deficient mice after 15 dpi, disease reduction was more consistent and pronounced in the CD4^{-/-} mice than in the CD4-depleted mice, particularly beyond 25 dpi (Fig. 1). This finding may reflect reduced efficacy of CD4⁺ T cell depletion over time, although CD4⁺ T cells were never observed in the corneas of CD4-depleted mice.

Composition of the Inflammatory Infiltrate in Infected Corneas at 14 dpi

The inflammatory infiltrate in the infected corneas of WT mice 14 dpi consisted primarily of neutrophils (80%–90% of CD45⁺ cells) and CD4⁺ T cells (~10% of CD45⁺ cells), with few if any CD8⁺ T cells (<1% of CD45⁺ cells) present (Fig. 2). Despite similar HSK scores in WT and CD4-deficient mice at 14 dpi, the overall infiltrate (CD45⁺ cells) was somewhat higher in the CD4-depleted and CD4^{-/-} mice, although the difference was not statistically significant (Fig. 2). Neutrophils were the predominant leukocyte population in the infected corneas of CD4-deficient mice (Fig. 2). As expected, no CD4⁺ T cells were detected in the corneas of CD4^{-/-} or CD4-depleted mice. Instead, these corneas contained a prominent population of CD8⁺ T cells (~3 to 10% of CD45⁺ cells), demonstrating that, in the absence of CD4⁺ T cells, CD8⁺ T cells are capable of infiltrating HSV-1-infected corneas. The fact that CD8⁺ T cells mediated HSK in CD4-deficient mice was demonstrated by the complete resistance to HSK of mice simultaneously depleted of both CD4⁺ and CD8⁺ T cells (Table 1). Thus, CD8⁺ T cells mediate a milder and more transient form of HSK in CD4⁺ T cell-deficient mice.

Activation Phenotype and HSV-1 Specificity of T Cells in HSK Lesions at 14 dpi

At 14 dpi, the CD69 activation marker was expressed on 20% to 25% of CD8⁺ T cells in corneas of CD4-deficient mice and on 25% of CD4⁺ T cells in the corneas of WT mice (Fig. 3A). The extremely low number of CD8⁺ T cells in corneas of untreated WT mice precluded accurate analysis of their CD69 expression.

For the assessment of the antigen specificity and function of T cells in the infected corneas 14 dpi, dispersed corneal cells were stimulated with HSV-1 antigen-pulsed DCs (to stimulate HSV-1-specific CD4⁺ T cells in WT corneas, or with HSV-1 infected, major histocompatibility complex (MHC) class I-compatible MKSA cells, to stimulate HSV-1-specific CD8⁺ T cells in corneas of CD4-deficient mice (Fig. 3B). Approximately 16% of the CD4⁺ T cells in corneas of WT mice, and 5% to 10% of CD8⁺ T cells in corneas of CD4^{-/-} mice produced IFN- γ in response to viral antigens. Thus, a substantial portion of CD4⁺ T cells in infected corneas of WT mice, and CD8⁺ T cells in infected corneas of CD4^{-/-} mice were activated, HSV-1 specific, and capable of producing IFN- γ when stimulated with HSV-1 antigens.

Association between Chronic HSK and Persistence of Activated T Cells

The severe HSK (>3+) in WT mice persisted through 35 dpi, even though the total number of CD4⁺ T cells and neutrophils declined significantly (compare Fig. 4A and Fig. 2A). However, the percentage of the infiltrate that were CD4⁺ T cells ($13.3\% \pm 2.7\%$) and the percentage of CD4⁺ T cells that were activated as indicated by CD69 expression ($49.5\% \pm 3.1\%$) in infected corneas of untreated WT mice at 35 dpi were significantly higher than that at 14 dpi (compare Fig. 4B and Fig. 2B). Thus, chronic HSK appears to be more closely related to the persistent activation of CD4⁺ T cells than to the actual magnitude of the inflammatory infiltrate.

At 35 dpi, leukocytes were virtually undetectable in corneas of CD4^{-/-} or CD4-depleted mice in which HSK regressed below a score of 1+ (not shown). Corneas of CD4-deficient mice that still expressed more than a 2+ level of HSK had neutrophil counts similar to those observed in corneas of WT mice with 3+ HSK (Fig. 4A). However, in these corneas, blood vessels were observed to be highly attenuated (not shown), and the number of CD8⁺ T cells was markedly diminished (often undetectable) when compared with corneas of CD4-deficient mice at 14 dpi (compare Fig. 4 and Fig. 2). As already noted, HSK regression was more pronounced and consistent in CD4^{-/-} mice than in CD4-depleted mice, and this was associated with a more pronounced loss of CD8⁺ T cells in the former group (Fig. 4A). We conclude from these observations that the regression of inflammation in CD4-deficient mice is characterized by a loss of CD8⁺ T cells, followed by a gradual reduction in neutrophils and attenuation of blood vessels.

Histopathologic Confirmation of HSK Regression in CD4^{-/-} Mice

Histopathologic studies were performed to confirm the clinical and flow cytometry evidence of HSK regression in CD4^{-/-} mice. Corneas were excised from WT and CD4^{-/-} mice at 14 and 35 dpi, and inflammation was evaluated by both slit lamp examination and microscopic examination of H&E-stained frozen sections. Based on slit lamp examination, the corneas of WT and CD4^{-/-} mice showed similar kinetics of HSK development to that shown in Figure 1. Representative corneas with 2+ HSK at 14 dpi were examined microscopically (Fig. 5). Corneas of WT and CD4^{-/-} mice showed comparable thickness and cellularity, with most infiltrating cells expressing a neutrophilic morphology. At 35 dpi, all infected corneas of WT mice showed persistent inflammation with swelling and a heavy neutrophil infiltration. Corneas of CD4^{-/-} mice either maintained 2+ HSK or showed HSK regression to 1+ disease or below. As noted earlier, corneas from the latter group were not amenable to flow cytometric analysis, but microscopic examination revealed markedly reduced corneal swelling and cellularity (Fig. 5).

CD4⁺ T Cell Dependency of HSK

WT and CD4-deficient mice were infected with HSV-1 at doses of 1×10^5 , 5×10^4 , 1×10^4 , 5×10^3 , and 1×10^3 and were monitored for the development of HSK. HSK developed in untreated WT mice with 80% to 100% incidence and similar kinetics and severity over the full range of HSV-1 doses tested (only data for the lowest dose are shown in Fig. 6). In contrast, the incidence of disease began to decline in CD4-deficient mice at an infectious dose of 5×10^3 pfu (not shown), reaching a 20% to 40% incidence at the 1×10^3 tested (Fig. 6). In those few CD4-deficient mice in which HSK developed, the composition of the inflammatory infiltrate was identical (predominantly neutrophils and CD8⁺ T cells) to that seen in CD4-deficient mice infected with a high dose of HSV-1 (not shown). Immunohistochemistry showed CD8⁺ T cells scattered throughout the corneal stroma of CD4^{-/-} mice (Fig. 7). We conclude from these findings that CD4⁺ T cells are preferentially activated in HSV-1-infected corneas and can efficiently mediate HSK over a wide range of infectious doses. In the absence of CD4⁺ T cells, CD8⁺ T cells can also mediate a transient form of HSK, but the propensity of CD8⁺ T cells to mediate HSK is diminished at lower infectious doses.

Clearance of Infectious HSV-1 from CD4-Deficient Mice by 10 dpi

Swabs of infected corneas of CD4-deficient and WT BALB/c mice were obtained on 2, 4, 6, 8, 10, 12, and 14 dpi, and assayed for infectious HSV-1. As shown in Figure 8, HSV-1 clearance was delayed by approximately 2 days in both CD4^{-/-} and CD4-depleted mice; however, both WT and CD4-deficient mice had virus cleared from the cornea by 10 dpi.

DISCUSSION

Studies of HSK in mice have led to the general conclusion that T cells play a requisite role¹ and that CD4⁺ T cells are the primary mediators of disease.^{3–5} The involvement of CD8⁺ T cells in HSK is less clear. Several studies have demonstrated a large preponderance of CD4⁺ T cells over CD8⁺ T cells in HSK lesions. Consistent with those findings, we show that CD8⁺ T cells are either undetectable or outnumbered by CD4⁺ T cells by almost 10:1 in HSV-1 infected corneas of wild-type BALB/c mice. However, we demonstrate that in the absence of CD4⁺ T cells, CD8⁺ T cells can infiltrate HSV-1-infected mouse corneas, in which HSK subsequently develop. Two major conclusions that can be drawn from these findings are: (1) CD8⁺ T cell infiltration or retention in infected corneas is regulated, and (2) CD8⁺ T cells can mediate transient HSK when CD4⁺ T cells are deficient.

The relative lack of CD8⁺ T cells in HSK lesions is particularly interesting in view of the fact that these cells are prominently present in the infected trigeminal ganglia of the same mice.^{20–24} Such findings illustrate regional influences on leukocytic infiltration of infected tissue. The nature of the regional influence of the HSV-1-infected cornea on CD8⁺ T cell infiltration is unclear, but it does not appear to reflect an intrinsic capacity of infected mouse corneas to exclude CD8⁺ T cells. In the absence of CD4⁺ T cells, CD8⁺ T cells infiltrate the infected cornea in large numbers, actually exceeding the number of CD4⁺ T cells in infected corneas of WT mice. These findings invite conjecture that the presence of CD4⁺ T cells within the infected cornea in some way inhibits the infiltration of CD8⁺ T cells. However, CD4⁺ T cells are present in the infected trigeminal ganglia of these mice and do not appear to regulate CD8⁺ T cell infiltration of that tissue. Thus, if CD4⁺ T cells directly or indirectly regulate CD8⁺ T-cell infiltration, this function appears to be restricted to the cornea.

Results of a recent study are consistent with this concept and further suggest that this putative capacity of CD4⁺ T cells to exclude CD8⁺ T cells from the cornea does not require the presence of cognate antigen within the cornea. HSV-1-specific CD8⁺ T cells were adoptively transferred into DO11.10 SCID mice after HSV-1 corneal infection. The HSK developed in the mice due to the apparent bystander activation of OVA-specific CD4⁺ T cells that were prominently present in the cornea. However, HSV-1-specific CD8⁺ T cells were completely excluded from the cornea, but infiltrated the trigeminal ganglia in large numbers. If indeed activated CD4⁺ T cells are able to exclude CD8⁺ T cells from the cornea, exertion of this function does not require T-cell receptor signaling.

An alternative though not mutually exclusive possibility is that activated CD8⁺ T cells are discouraged from entering the cornea by a CD4⁺ T cell-independent mechanism. Supporting this concept, HSV-1 corneal infection of OT-1 mice on a RAG^{-/-} background resulted in infiltration and apparent bystander activation of Ova-specific CD8⁺ T cells, leading to the development of HSK.¹¹ That the OT-1/RAG mice are deficient in CD4⁺ T cells again suggests that in the absence of CD4⁺ T cells, CD8⁺ T cells can infiltrate the infected cornea and be activated. Of interest, when HSV-1-specific CD8⁺ T cells were adoptively transferred into these mice, they were found in the trigeminal ganglion, but not in the infected corneas. One can reasonably conclude from these findings that HSV-1-specific CD8⁺ T cells were excluded from the infected corneas of these mice, whereas Ova-specific, presumably naïve CD8⁺ T cells could enter and be activated. In that regard, it is noteworthy that although CD8⁺ T cells are

prominently present in the corneas of CD4-deficient mice, the frequency of HSV-1-specific CD8⁺ T cells is quite low, relative to their frequency in the trigeminal ganglia of the same mice (Lepisto AJ, unpublished observation, 2002), and relative to the frequency of HSV-1-specific CD4⁺ T cells in the infected corneas of wild-type mice (Fig. 2, Fig. 6). Together these observations suggest that activated CD8⁺ T cells may be preferentially, though not absolutely, excluded from the cornea.

The concept that CD8⁺ T cells that infiltrate the corneas of CD4⁺ T cell-deficient mice mediate HSK is supported by our observation that HSK does not develop in BALB/c mice that are T-cell deficient, including nude,¹ SCID,⁵ or, as in our experiments, WT mice depleted of both CD4⁺ and CD8⁺ T cells. Although CD8⁺ T cells were able to mediate HSK, the disease was somewhat milder and decidedly more transient than that mediated by CD4⁺ T cells in wild-type mice. A possible explanation for the reduced duration of HSK in CD4-deficient mice is that the HSV-1-specific CD8⁺ T cells that infiltrate the corneas destroy the APCs that are necessary for antigen presentation and/or bystander activation that leads to prolonged inflammation. This would account for the normal initiation but short duration of HSK in CD4-deficient mice.

A second explanation for the short duration of HSK in CD4-deficient mice may relate to the impaired CD8⁺ T-cell memory response that is induced in the absence of CD4⁺ T cells.^{25, 26} In general, it was found that CD4⁺ T cells are not necessary for the initial development of effector CD8⁺ T cells, but a long-term memory CD8⁺ T cell response was not generated when CD4 T cells were absent during priming. We noted a virtually complete loss of CD8⁺ T cells in the corneas of the CD4^{-/-} mice late in infection when HSK was diminished. In contrast, CD4⁺ T cells were continuously present in the infected corneas of wild-type mice, in which HSK was chronic. In this scenario, the diminished HSK may result from a gradual exhaustion of the HSV-1-specific effector CD8⁺ T cells and failure to establish long-term memory. These possibilities are under investigation.

Our study shows that complete viral clearance from the cornea is delayed by about 2 days in CD4-deficient mice. This finding is consistent with those of another group.²⁷ How CD4⁺ T cells contribute to the clearance of virus is not established. It is known that CD4⁺ T cell-deficient mice do not generate an IgG anti-HSV-1 antibody response.¹⁶ Moreover, we have seen activated CD4⁺ T cells (defined by OX40 expression) in HSV-1-infected mouse corneas as early as 3 days after infection (Lepisto AJ, unpublished observation, 2002). Thus, CD4⁺ T cells could contribute to viral clearance through a local reaction in the cornea or by promoting an anti-HSV-1 antibody response.

An important observation in this study is that the requisite involvement of CD4⁺ T cells in HSK was dependent on the dose of HSV-1 used to infect the cornea. Relatively low (perhaps more physiologic) doses of HSV-1 that uniformly induced HSK in wild-type mice were inefficient at inducing HSK in CD4-deficient mice. How virus dose influences the capacity of CD8⁺ T cells to mediate HSK is unclear. It is possible that a higher dose of virus is necessary to induce a potent effector CD8⁺ T cell response in the absence of CD4⁺ T cell help. Alternatively, less viral protein production in the cornea may be needed to activate infiltrating CD4⁺ T cells. Regardless of the explanation, it is important to note that most studies of HSK probably incorporate doses of HSV-1 that are more than 2 logs higher than that necessary to induce disease and that the HSK induced at high doses of virus is not necessarily CD4⁺ T cell dependent.

It should be noted that in some studies CD8⁺ T cells appeared incapable of mediating HSK.^{11,17} It is not clear why this discrepancy exists, but it may in part reflect the effective dose of HSV-1 corneal infection used in different models. The effective dose would include the amount

of virus applied to the cornea and the degree of virus replication within the corneal epithelium. The latter could be influenced by the degree of scratching of the cornea, the ability of the virus strain to replicate in mouse corneal epithelial cells, and the ability of the host immune system to control replication of the virus used. A relatively low effective dose might favor CD4⁺ T-cell involvement. We suggest that greater consistency in infectious dose, virus strain, and mouse strain used in studies of HSK may resolve some of the confusion that has hampered progress in understanding this blinding disease.

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FIGURE 1.

Kinetics of HSK. Wild-type (WT), CD4 knockout (CD4^{-/-}), and CD4-depleted mice were infected with a high dose of HSV-1 RE. (1×10^5 pfu), and HSK was monitored by a masked observer with a slit lamp biomicroscope between 7 and 35 dpi. The mean disease severity $\pm$ SEM is shown for each group. The significance of differences in disease severity between WT mice and CD4^{-/-} or CD4-depleted mice is indicated as * $P < 0.05$ or ** $P < 0.01$, as assessed by ANOVA and Tukey's post test.

FIGURE 2.

Composition of the corneal infiltrate 14 days after infection. WT, CD4^{-/-}, and CD4-depleted mice were infected with a high dose of HSV-1 RE. (1×10^5 pfu), individual corneas with HSK were removed on 14 dpi, and single-cell suspensions were stained with antibodies to CD45, CD4, CD8, and Gr-1, and analyzed by flow cytometry. Data are represented as the number of each cell type/cornea (**A**), and as the percentage of CD45⁺ (bone marrow-derived) cells represented by each cell type (**B**). Infiltrate data are pooled from two experiments with four individual mice per group. *Significant ($P < 0.05$) difference between WT and CD4-deficient mice, as assessed by ANOVA and the Tukey post test.

FIGURE 3.

Activation phenotype and specificity of T cells in the corneal infiltrate 14 days after infection. WT, CD4^{-/-}, and CD4-depleted mice were infected with 1×10^5 pfu of HSV-1 RE. Individual corneas with HSK were removed at 14 dpi, and single-cell suspensions were either (A) stained directly for CD45, CD4, CD8, and CD69 or (B) stimulated for 6 hours in the presence of a protein transport inhibitor (Golgiplug; BD Biosciences, Piscataway, NJ) with either viral antigen-pulsed bone marrow-derived DCs for CD4 T cell stimulation, or with HSV-infected MKSA cells for CD8 stimulation. The corneal cells were then stained for the surface markers CD45, CD4, and CD8 and for intracellular interferon γ and were analyzed by flow cytometry. Gating was based on the isotype control, as shown. Controls for intracellular cytokine staining include unpulsed bone marrow derived DCs for CD4⁺ T cells or uninfected MKSA target cells for CD8⁺ T cells. Graphed data representing two experiments with two individual mice per group as well as representative dot plots are shown. **Significant ($P < 0.01$) difference between WT and CD4-deficient mice, as assessed by an unpaired Student's *t*-test.

FIGURE 4.

Composition of the corneal infiltrate at 35 dpi. Wild-type (WT), CD4 knockout (CD4^{-/-}), and CD4-depleted mice were infected with 1×10^5 pfu of HSV-1 RE, and individual corneas with HSK were removed at 35 dpi. Single-cell suspensions were simultaneously stained with antibodies to CD45, CD4, CD8, Gr-1, and CD69 and analyzed by flow cytometry. Infiltrate data are from five individual mice per group. **(A)** Data are presented as the total number of cells of each type per cornea. **(B)** Data are presented as the percentage of CD45⁺ (bone marrow-derived) cells represented by each cell type.

FIGURE 5.

Representative histopathology of HSK in WT and CD4^{-/-} mice. At 14 and 35 dpi, whole eyes of WT and CD4^{-/-} mice infected with 1×10^5 pfu of HSV-1 RE were removed and frozen sections prepared. The sections were stained with H&E, to observe the inflammatory infiltrate. At 14 dpi, corneal stromas of both WT and CD4^{-/-} mice exhibited heavy neutrophil infiltration and swelling. At 35 dpi, the WT corneas maintained swelling and heavy leukocyte infiltration, whereas both swelling and leukocyte infiltration were markedly reduced in corneas of CD4^{-/-} mice, in which HSK regressed to 1+ or below.

FIGURE 6.

HSK after low-dose HSV-1 RE corneal infection. WT, CD4^{-/-}, and CD4-depleted mice were infected with 1×10^3 pfu of HSV-1 RE and HSK was monitored by a masked observer with a slit lamp biomicroscope. The percentage of HSK incidence is based on pooled data (WT, $n = 15$; CD4^{-/-}, $n = 10$; and CD4-depleted, $n = 10$)

FIGURE 7.

Representative images of anti-CD8 immunohistochemistry of ocular frozen sections. Whole eyes of WT and CD4^{-/-} mice infected with 1×10^3 pfu HSV-1 RE were removed at 21 dpi and frozen sections prepared. The sections were immunostained with anti-CD8 antibody and a fluorescently labeled secondary antibody. Sections were examined with epifluorescence (*left*) and differential interference contrast (DIC; *right*) optics. CD8⁺ T cells are scattered throughout the stroma of CD4^{-/-} mice, but are very sparsely represented in corneas of WT mice. No staining of corneas of CD4^{-/-} mice was observed with an isotype control antibody.

FIGURE 8.

Viral clearance in the corneas of WT and CD4-deficient mice. WT, CD4^{-/-}, and CD4-depleted mice were infected with 1×10^5 pfu of HSV-1 RE. The corneas were swabbed with a sterile surgical spear on 2, 4, 6, 8, 10, 12, and 14 dpi and assayed for live virus by standard plaque assay. Data represent the percentage of mice positive for live virus in eye swabs and are from 10 individual animals in each group at each time point. Data are from one of two experiments with identical results.

TABLE 1 Effect of CD8⁺ T Cells on the More Transient HSK

Group [*]	Animals Examined	HSK Incidence 15 dpi [†]	Percent Regression [‡]
WT	43	94%	0
CD4 ^{-/-}	43	83% <i>P</i> = 0.1344	39% <i>P</i> < 0.0001
CD4-depleted	19	77% <i>P</i> = 0.0462	33% <i>P</i> = 0.0018
CD4/CD8-depleted	10	0% <i>P</i> = 0.0002	N/A

^{*} WT, CD4 knockout (CD4^{-/-}), CD4-depleted, and CD4 and CD8-depleted mice were infected with a high dose of HSV-1 RE. (1×10^5 PFU), and HSK was monitored by a masked observer with a slit lamp biomicroscope between 7 and 35 dpi.

[†] The maximum incidence of HSK (observed at 15 dpi for all groups) is shown. Probabilities were obtained by Fisher exact test and indicate the significance of differences in disease incidence comparing each experimental group with untreated WT mice.

[‡] The percentage of mice in each group showing HSK regression of at least 1 point in clinical score between 7 and 35 dpi is presented along with the probability as compared with untreated WT mice.