CD8⁺ T cells control corneal disease following ocular infection with herpes simplex virus type 1

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The role that T cell subsets play in herpetic stromal keratitis (HSK) has been the subject of intense research efforts. While most studies implicate CD4⁺ T cells as the principal cell type mediating primary corneal disease, recent reports using knockout mice have suggested that both CD4⁺ and CD8⁺ T cell subsets may play integral roles in modulating the disease. Furthermore, recent studies suggest that CD8⁺ T cells are directly involved in maintaining virus latency in infected trigeminal ganglia. This work has addressed these discrepancies by infecting the corneas of mice lacking CD4⁺ and CD8⁺ T cells with herpes simplex virus type 1 (HSV-1) and monitoring both corneal disease and latent infection of trigeminal ganglia. Results indicated that mice lacking CD8⁺ T cells had more severe corneal disease than either BALB/c or B6 parental strains. In contrast, mice lacking CD4⁺ T cells had a milder disease than parental strains. When mice were evaluated for persistence of infectious virus, only transient differences were observed in periocular tissue and corneas. No significant differences were found in persistence of virus in trigeminal ganglia or virus reactivation from explanted ganglia. These data support the following conclusions. CD4⁺ T cells are not required for resistance to infection with HSV-1 and probably mediate HSK. Mice lacking CD8⁺ T cells do not display differences in viral loads or reactivation and thus CD8⁺ T cells are not absolutely required to maintain latency. Finally, CD8⁺ T cells probably play a protective role by regulating the immunopathological response that mediates HSK.

INTRODUCTION

Herpetic stromal keratitis (HSK) is a potentially blinding corneal inflammation that accompanies herpes simplex virus (HSV) infection of the eye. The disease course in HSK begins with a primary infection by HSV followed by a period during which the virus enters latency in sensory and autonomic ganglia. Many studies have shown that clinical disease is the result of a cocktail of inflammatory cells consisting of polymorphonuclear leukocytes, macrophages and T cells (both CD4⁺ and CD8⁺) that are recruited to the corneas of patients with HSK (Maertzdorf et al., 2003; Pepose et al., 1996; Thomas & Rouse, 1997; Youinou et al., 1985, 1986).

Corneas removed from patients requiring corneal transplants due to HSK contain both CD4⁺ and CD8⁺ T cells that are specific for HSV-encoded antigens (Koelle et al., 2000). In most models of HSK, T cells are critical to the development of corneal lesions, as athymic mice do not display signs of HSK (Metcalf et al., 1979) unless adoptive transfer of T cells is performed (Russell et al., 1984). While most investigators believe that the CD4⁺ subset of T cells mediates this disease, some reports implicate CD8⁺ T cells as having a major role in primary HSV keratitis (Akova et al., 1993; Doymaz & Rouse, 1992; Hendricks & Tumpey, 1990; Niemialtowski & Rouse, 1992). Furthermore, when most corneas are immunohistochemically stained for T cell subsets, the predominant subset found appears to be dependent on the model system employed (Doymaz & Rouse, 1992; Hendricks & Tumpey, 1990). Further complicating the issue are reports by Ghiasi et al. (1999, 2000) showing that both CD4 and CD8 knockout (KO) mice made on the C57BL/6 background demonstrate increased disease when compared with normal C57BL/6 mice. They interpreted their results as indicating that both of these T cell subsets play a role in herpetic corneal disease and that their function can be both destructive and protective. Additional studies assessing the function of T cell subsets in HSV latency found that CD8⁺ T cells in mice with HSV-infected trigeminal ganglia...
are responsible for maintaining latency (Khanna et al., 2003; Liu et al., 2000, 2001).

An understanding of the cellular interactions between virus-specific immune cells and cells of the cornea and nervous system are crucial in determining the underlying mechanisms of HSK. To examine more fully the role of CD4⁺ and CD8⁺ cells during primary HSK, we utilized mice deficient in CD4⁺ or CD8⁺ T cells. To determine whether host genetic background influences the role of T cell subsets in recurrent corneal disease, we performed our experiments in HSV-susceptible (BALB/c) and HSV-resistant (C57BL/6) strains of mice. Our findings indicated that disease was associated with the presence of CD4⁺ T cells and that, when these cells were absent, little disease was evident. Furthermore, transfer of CD8⁺ T cells from infected mice provided significant protection against the development of primary HSK.

**METHODS**

**Virus and cells.** The viruses used in this study were the McKrae and KOS strains of HSV-1. A plaque-purified stock was grown and assayed on Vero cells in minimum essential medium with Earle’s balanced salts (MEM-EBS) containing 5% fetal bovine serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (Keadle et al., 2002b). Virus titres in eye swabs were determined by standard plaque assay (Keadle et al., 2002a).

**Mice and primary infection.** Investigations with mice conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 (B6) and BALB/c mice were purchased from the National Cancer Institute (Fredrick, MD, USA). The B6.129-cd4tm1Mak and B6.129-cd8tm1Mak mice were generously provided by Dr Tak Mak (University of Toronto) and maintained in our colony. For the purposes of this report, these mice are referred to as B6-CD4 KO and B6-CD8 KO mice, respectively. We also bred the B6-CD4 KO and B6-CD8 KO mice with BALB/c mice for a minimum of 10 generations. The resultant strains were designated C.129S(B6)-cd4tm1 and C.129S(B6)-cd8tm1, referred to here as C-CD4 KO and C-CD8 KO, respectively. Due to the occasional ‘leakiness’ of the KO phenotype, all mice underwent flow cytometric analysis of peripheral blood lymphocytes. Only those mice in which the targeted cell type could not be detected were used in these studies (Haskova et al., 2000). It should be noted that we have never observed a mouse that tested negative for the targeted cell type prior to infection display those cells at any time following infection (data not shown). Normal and KO mice (8–12 weeks old) were infected as described previously (Rader et al., 1993). Briefly, following corneal scarification, 2 × 10⁶ p.f.u. HSV-1 KOS strain or 5 × 10⁶ p.f.u. HSV-1 McKrae strain in 5 μl MEM-EBS was placed onto the surface of both corneas of BALB/c (HSV-sensitive) or C57BL/6 (HSV-resistant) mice, respectively.

**Clinical evaluation.** On the designated days after virus infection, a masked observer examined mouse eyes through a binocular dissecting microscope and scored clinical disease. Stromal opacification was rated on a scale of 0–4, where 0 indicated clear stroma, 1 indicated mild stromal opacification, 2 indicated moderate opacity with discernible iris features, 3 indicated dense opacity with loss of defined iris detail except pupil margins, and 4 indicated total opacity with no posterior view. Corneal neovascularization was evaluated as described previously (Stuart et al., 2003) using a scale of 0–8, where each of four quadrants of the eye is evaluated for the number of vessels that have grown into them. Pericellular disease, in the form of blepharitis, was measured in a masked fashion on a semi-quantitative scale as described previously (Smith et al., 2000).

**Titration of virus from tissues.** Eye swab material was collected and assayed for virus by standard plaque assay as described previously (Rader et al., 1993). Trigeminal ganglia and 6 mm biopsy punches of periorcular skin were removed and placed in pre-weighed tubes containing 1 mm glass beads and 1 ml medium. Trigeminal ganglia and periorcular skin homogenates were prepared by freezing and thawing the samples, mechanically disrupting in a Mini-Beadbeater-8 (Biospec Products) and sonicating. Homogenates were assayed for virus by standard plaque assay and the amount of virus was expressed as p.f.u. (ml tissue homogenate)⁻¹.

**Virus reactivation assay.** Trigeminal ganglia were removed from infected mice 30–40 days post-infection. To assess reactivation, individual trigeminal ganglia were dissociated (Kennedy et al., 1980) and plated on collagen-coated 12-well plates. Supernatants were assayed every 12 h for progeny virus from 1 to 5 days post-plating.

**Limiting dilution assay for latency.** Between 28 and 35 days post-infection, trigeminal ganglia were removed from infected mice. Trigeminal ganglia were pooled from three to four mice, washed once in Dulbecco’s minimal essential medium (DMEM) and 3 ml dissociation buffer was added per ganglion pool. The mixture was incubated in a shaking incubator at 37 °C for 1 h, then pelleted at 2500 r.p.m. and the supernatant discarded. The cells in the pellets were resuspended in DMEM and added to collagen-coated plates; twofold serial dilutions, starting with 5 × 10⁶ cells per well, were performed. Cells were incubated overnight at 37 °C. At this time, 5 × 10⁴ Vero cells were added in 1 ml serum-free DMEM containing penicillin, fungizone and streptomycin to each collagen well. Wells were monitored for cytopathic effect (CPE) and 100 μl supernatant was removed from each well on days 3, 5, 9, 12 and tested for the presence of virus by adding to 48-well plates containing 2 × 10⁴ Vero cells per well. These wells were then judged for CPE at day 6.

**In vivo T cell depletion.** BALB/c mice were treated with either anti-CD4 (clone GK1.5) or anti-CD8 (clone H-35) to remove targeted T cell subsets, or with diluent as a control. Treatment consisted of three injections given on days 2, 3 and 5 post-infection. Mice were monitored for effectiveness of in vivo depletion between 2 and 3 weeks post-infection by flow cytometry of peripheral blood lymphocytes (Haskova et al., 2000). Treatment with anti-CD4 antibody resulted in 85–90% depletion of CD4⁺ T cells. Because this treatment did not remove more than 95% of the CD4⁺ T cells, representative HSV-infected mice were tested for the functional presence of CD4⁺ T cells by testing them for specific delayed-type hypersensitivity (DTH) responses to HSV antigenic preparations (Keadle et al., 2002b). For all mice tested, no mice displayed DTH responses above background levels (data not shown). Mice treated with anti-CD8 antibody resulted in more than 98% depletion of CD8⁺ T cells.

**T cell isolation and adoptive transfer.** C-CD4 KO mice were infected with 2 × 10⁶ p.f.u. HSV-1 KOS strain and killed 2–3 weeks following infection. Single-cell suspensions of spleen and lymph node cells were prepared. These cells were then fractionated as follows: a T cell-enriched population was isolated by passing cells over a nylon wool column followed by treatment with anti-B220 (clone J11d) and anti-MHC class II (clone 11.3.1) plus complement (Cedarlane Laboratories) to deplete B cells and monocytes further. A T cell-depleted population was isolated by treatment with anti-Thy-1 (clone HO 13-4.6) and anti-CD8 (clone 3.155) plus complement. A control population was treated with an irrelevant antibody (clone W6/32) plus complement. All of the antibodies used in these
depletions were derived from culture supernatants of the indicated hybridoma clones. Following depletion, each of the resultant groups was evaluated for the presence of the targeted cell population and less than 1% of the targeted cells were found. BALB/c and C-CD8 KO mice were then injected with these fractions using 2 x 10^7 cells for adoptive transfer. Mice were subsequently challenged with 2 x 10^7 p.f.u. HSV-1 KOS strain and the disease monitored for 5 weeks.

**Assays of antibody titres.** Serum was collected from mice at weekly intervals following infection and examined for HSV-specific antibody content as described previously (Geiss et al., 2000). Briefly, for ELISA, serial fourfold dilutions of mouse serum were incubated for 2 h in duplicate wells of a 96-well plate coated with purified HSV-1 glycoprotein. Biotinylated goat anti-mouse IgG was subsequently used in a colorimetric assay to determine specific IgG levels based on comparison with a standard curve generated as described previously (Geiss et al., 2000).

**Statistical analyses.** All statistical analyses were performed with the aid of Sigma Stat for Windows, version 2.0 (Jandel, Corte Madera, CA). Student’s unpaired t-test was used to compare corneal disease scores and virus and antibody titre data. Fisher’s exact χ² test was used to compare the limiting dilution assay data.

**RESULTS**

We initially infected BALB/c, C-CD4 KO and C-CD8 KO mice with HSV-1 KOS strain. Results indicated that mice without CD8⁺ T cells had significantly worse corneal disease than either normal or C-CD4 KO mice (Fig. 1a, b). Furthermore, these mice displayed more severe signs of blepharitis (Fig. 1c) and neurological disease as evidenced by hunched posture, changes in coat appearance, some paralysis and some death (data not shown). In contrast to the C-CD8 KO mice, C-CD4 KO mice did not show significant disease (Fig. 1a–c). To verify these results using a different method, we treated BALB/c mice with T cell subset-specific antibodies. Results for mice treated with anti-CD8 antibody paralleled those for C-CD8 KO mice (Fig. 1d–f). Surprisingly, the disease in BALB/c mice treated with anti-CD4 antiserum was indistinguishable from BALB/c mice treated with an irrelevant antibody and was not reduced, in contrast to the results in C-CD4 KO mice (compare Fig. 1a and d). Since anti-CD4 treatment of BALB/c mice was not as effective as anti-CD8 treatment in removing the targeted T cell subtype but did result in the functional depletion of CD4-mediated HSV-specific DTH responses (data not shown), we concluded that there may exist a population of T cells in antibody-treated mice that is not found in the gene-targeted mice and that this population contributes to disease.

Our observation that CD4 KO mice on a BALB/c background had less severe corneal disease than wild-type BALB/c mice was not consistent with results reported for B6-CD4 KO mice infected with the McKrae strain of HSV-1 (Ghiasi et al., 1999, 2000). These investigators reported that both B6-CD4 KO and B6-CD8 KO strains of mice had more severe disease than the parental B6 mice. Consequently, we also infected B6, B6-CD4 KO and B6-CD8 KO mice with 5 x 10⁶ p.f.u. HSV-1 McKrae strain to determine whether our results were restricted to BALB/c mice infected with the KOS strain of HSV-1. Interestingly, the pattern of disease in B6 mice (Fig. 2) was more similar to that observed in BALB/c mice (Fig. 1) than the results reported previously (Ghiasi et al., 1999, 2000). Namely, B6-CD4 KO mice did not display significant disease at any time point, while both wild-type B6 and B6-CD8 KO showed corneal disease at early time points post-infection (Fig. 2). In addition, as was seen with C-CD8 KO mice, B6-CD8 KO mice had higher disease scores that persisted longer than had been noted previously in wild-type B6 mice.

The increased corneal disease seen in CD8 KO or anti-CD8-treated mice could be the result of several different, though not mutually exclusive, mechanisms. One possible mechanism could be that CD8⁺ T cells are responsible for much of the clearance of virus from infected corneas. Thus, in C-CD8 KO mice, HSV-1 would persist in the tissues longer than would ordinarily be the case, leading to the potential for stronger inflammatory responses due to persistence of viral antigen. To test this hypothesis, we titrated virus in eye swabs at days 3, 5, 7, 9, 14, 21 and 28 after infection of BALB/c, C-CD4 KO and C-CD8 KO mice and took periocular biopsies on days 5, 7, 9, 14, 21 and 28 following infection. Results indicated that while both CD4 and CD8 KO mice shed virus only slightly longer than was noted in wild-type BALB/c mice, none demonstrated active infection in corneal swabs or trigeminal ganglia by day 9 post-infection (Fig. 3a, b). These data strongly suggested that persistent active infection was not the likely mechanism for increased corneal disease observed in C-CD8 KO mice. A second possible mechanism is that CD8⁺ T cells might play a role in promoting and maintaining virus latency in infected neuronal cells (Khanna et al., 2003; Liu et al., 2000, 2001). Since the C-CD8 KO mice displayed increased incidence of neurological disease (data not shown), we thought it possible that there was increased virus replication in the trigeminal ganglia due to the absence of CD8⁺ T cells. When trigeminal ganglia were removed from BALB/c, C-CD4 KO and C-CD8 KO mice at days 3, 7, 9, 14, 21 and 28 post-infection, replicating virus was only detected in trigeminal ganglia up to day 7 post-infection (Fig. 4a).

Furthermore, no differences in titres of virus reactivating from infected trigeminal ganglia were detected (Fig. 4b). Since it remained a possibility that there could be differences in the numbers of trigeminal neurons that were latently infected, we also performed a limiting dilution analysis on infected neurons from BALB/c, C-CD4 KO and C-CD8 KO mice. Results of this assay also did not reveal significant differences between parental BALB/c mouse trigeminal ganglia and those from either CD4 or CD8 KO strains of mice (Fig. 4c). Therefore, in this model, we could find no evidence supporting a role for CD8⁺ T cells in promoting or maintaining latency.

Since the mechanism responsible for increased disease in C-CD8 KO mice appears to involve neither increased clearance of virus (Fig. 3) nor reduced latency (Fig. 4), we...
tested whether it involves differences in the inflammatory response initiated by viral infection. Previous work has suggested that CD4$^+$ T cells are the primary mediators of corneal disease in most models studied (Doymaz & Rouse, 1992; Hendricks & Tumpey, 1990; Niemialtowski & Rouse, 1992). Thus, one reason for increased disease could be an increased frequency of CD4$^+$ T cells that infiltrate the cornea to stimulate a more effective inflammatory response.

**Fig. 1.** Corneal disease in BALB/c mouse strains lacking CD4$^+$ or CD8$^+$ T cells (a–c) or treated *in vivo* with either anti-CD4 or anti-CD8 antibody (d–f) following HSV-1 infection. Following infection, mice were monitored for corneal opacity (a, d), corneal neovascularization (b, e) and blepharitis (c, f) for 28 days. The numbers of mice used for these studies were as follows: BALB/c, $n = 15$ (a–c) or $n = 10–20$† (d–f); C-CD4 KO, $n = 15$ (a–c); C-CD8 KO, $n = 15$ (a–c); anti-CD4-treated, $n = 12–30$† (d–f); and anti-CD8-treated, $n = 12–30$† (d–f). Results indicate mean ± SEM. *, $P = 0.001–0.05$ compared with normal BALB/c mice (a–c) or normal BALB/c mice treated with control antibody. †Each group of mice started with the higher number and mice were randomly killed throughout the experiment for tissue and virus titre measurements. (a–c) ●, BALB/c mice; ▲, C-CD4 KO mice; ■, C-CD8 KO mice. (d–f) ●, Control Ab; ▲, anti-CD4-treated; ■, anti-CD8-treated.
Fig. 2. Corneal disease following HSV-1 infection in B6, B6-CD4 KO and B6-CD8 KO mice. B6 (n=10–25), B6-CD4 KO (n=10–25) and B6-CD8 KO (n=10–25) mice were infected with HSV-1 McKrae strain and monitored for corneal disease for 28 days. Results indicate mean±SEM. *, P=0.01–0.05 compared with normal B6 mice. ●, C57BL/6 (HSV-resistant) mice; ■, B6-CD8 KO mice; ▲, B6-CD4 KO mice.

Fig. 3. In vivo growth of HSV-1 KOS strain following infection of BALB/c (solid bars), C-CD4 KO (open bars) and C-CD8 KO (cross-hatched bars) mice. Results indicate means±SEM of virus titre in eye swabs (a) and periocular skin (b) for five mice per time point. *, P=0.01–0.05 compared with normal BALB/c mice.

Fig. 4. In vivo growth and reactivation in trigeminal ganglia of BALB/c, C-CD4 KO and C-CD8 KO mice infected with HSV-1 KOS strain. (a, b) Results indicate mean±SEM of virus titre in trigeminal ganglia (a; n=10 per time point) or culture supernatants of dissociated ganglia (b; n=20 per time point). (a) Solid bars, BALB/c mice; open bars C-CD4 KO mice; cross-hatched bars, C-CD8 KO mice. (b) ●, BALB/c mice (10/10); ▲, C-CD4 KO mice (9/10); ■, C-CD8 KO mice (8/10). (c) Reactivation frequency from pools of dissociated ganglia from six normal BALB/c, 24 C-CD4 KO and 16 C-CD8 KO mice. The horizontal line indicates the 63.2% Poisson distribution line at which the frequency of reactivation was calculated (the point at which one reactivation event is likely to occur per well). ●, C-CD4 KO mice; ▼, C-CD8 KO mice; ■, BALB/c mice. No significant differences were detected when comparing groups in any of these assays.
in C-CD8 KO mice. Alternatively, CD8+ T cells could act as regulatory cells to reduce the capacity of CD4+ T cells to stimulate inflammation (Filaci & Suciu-Foca, 2002; Gilliet & Liu, 2002a, b; Jiang & Chess, 2000; Nicholson et al., 1996). To address these possibilities, we performed adoptive transfer experiments from infected C-CD4 KO mice to C-CD8 KO recipients prior to corneal infection. C-CD4 KO mice were used as donors for three reasons. Firstly, these mice displayed the least corneal disease (Fig. 1a, b) and neurological dysfunction (data not shown). Secondly, transferred cells would not include any CD4+ T cells, which might lead to increased pathology in the recipients. Thirdly, these mice displayed significantly lower anti-HSV antibody responses (Fig. 5) and thus contaminating B cells producing anti-HSV antibodies would be unlikely to confound the interpretation of results.

Spleen and lymph node cells were removed from HSV-1-infected C-CD4 KO mice 17 days post-infection and fractionated into separate groups by treatment with subset-specific antisera and complement. Cells were transferred the same day that recipients were infected with HSV-1. Results indicated that C-CD8 KO mice receiving either control cells (treated with complement only) or B cell/macrophage-depleted cells from C-CD4 KO mice showed little corneal disease (Fig. 6). Conversely, C-CD8 KO mice receiving either no cells or T-depleted cells showed severe disease pathology as expected for C-CD8 KO mice (compare Fig. 1a with Fig. 6). Results from these studies indicated that a T cell population prevents abnormally severe HSK in recipient mice. While these results do not preclude the possibility that C-CD8 KO mice might have higher precursor frequencies of CD4+ T cells mediating corneal disease, they did indicate that these cells must be rendered ineffective when CD8+ T cells are transferred from infected C-CD4 KO mice. Furthermore, the lack of difference in viral swab titres from any of the groups of mice described in this experiment failed to support the argument that mice receiving CD8+ T cells clear virus more efficiently from the cornea (data not shown).

**DISCUSSION**

The relative role that T cell subsets play in herpetic keratitis and the development of latency remains an active topic of investigation. In humans (Koelle et al., 2000; Maertzdorf et al., 2003; Verjans et al., 1998) and animal models (Miller et al., 1996), both CD4+ and CD8+ T cells infiltrate corneas with HSK. Most studies implicate CD4+ T cells as the primary mediators of the disease process (Doymaz & Rouse, 1992; Hendricks & Tumpey, 1990; Niemialtowski & Rouse, 1992; Russell et al., 1984). This conclusion was drawn from studies wherein removal of CD4+ T cells resulted in reduced disease (Newell et al., 1989). It is known that during the early stages of the adaptive immune response that occurs following HSV-1 infection of the eye, CD4+ T cells bearing the Th1 phenotype are generated (Niemialtowski & Rouse, 1992). These cells appear to be the principal mediators of the lesions seen in primary HSK that occur from 7 to 21 days post-infection (Niemialtowski & Rouse, 1992). In addition, animals depleted of CD8+ T cells prior to corneal infection with HSV-1 have a more severe disease (Doymaz & Rouse, 1992; Hendricks & Tumpey, 1989; Newell et al., 1989). These studies suggest that CD4+ T cells can mediate HSK, but are
normally held in check by CD8^+ T cells. Thus, CD8^+ T cells would play a protective role. In contrast to these results, when Ghiasi et al. (2000) infected B6-CD4 KO or B6-CD8 KO mice with over twofold less HSV-1 McKrae strain than used in our experiments, both KO strains demonstrated significant corneal scarring, while parental B6 mice had healthy corneas. These authors hypothesized that both CD4^+ and CD8^+ T cells in B6 mice regulate corneal disease and only when both are present is corneal disease prevented. Our data do not support the existence of a CD4^+ population of T cells that protects the cornea from HSV-1-induced corneal disease. Mice that lacked CD4^+ T cells did not experience more severe disease than the parental mice, regardless of the strain of mouse or strain of virus used. In fact, their disease tended to be less severe than wild-type mice. Thus, our data support the notion that CD4^+ T cells are the likely mediators of HSK and that they play only a minor role, if any, in regulating disease.

The role of CD8^+ T cells remains a complicated and controversial question. Mice that have CD8^+ but not CD4^+ T cells exhibit similar or reduced corneal disease when compared with normal mice and they also display no differences when compared with wild-type mice in signs of neurological disease or increased mortality following HSV-1 infection. In contrast, mice lacking CD8^+ T cells have a higher incidence of gross neurological pathology and have a slightly, although not significantly, greater incidence of mortality. Since the disease phenotype for mice lacking CD8^+ T cells does not appear to involve compromised virus clearance or increased viral loads in the trigeminal ganglia, we propose that CD8^+ T cells might be acting as regulators of disease by controlling the activity of those cells that mediate HSK. Data shown in Fig. 6 are consistent with this hypothesis. When we transferred T cells from infected C-CD4 KO mice, recipient C-CD8 KO mice were significantly protected from disease. Thus, the addition of CD8^+ T cells to CD8 KO mice prevented the development of HSK following infection.

It is unlikely that protection from HSK in mice receiving T cells by adoptive transfer was due to contaminating B cells producing protective levels of anti-HSV-1 antibodies (Keadle et al., 2002b; Shimeld et al., 1990). Firstly, there were no differences in protection when C-CD8 KO mice were given unfraccionated cells or B cell-depleted cells from infected CD4 KO mice. Secondly, when C-CD4 KO mice were evaluated for anti-HSV antibodies (Fig. 5), there were significantly less than seen in either C-CD8 KO or BALB/c mice, which is consistent with previous reports that mice lacking CD4^+ T cells are significantly compromised in their ability to generate anti-HSV-1 protective antibody responses (Chan et al., 1985; Ghiasi et al., 1997; Irie et al., 2002; Morrison & Knipe, 1997).

Since several reports (Khanna et al., 2003; Liu et al., 2000, 2001) have implicated a direct role for CD8^+ T cells in maintaining the latent phenotype of HSV-1 in trigeminal ganglia, we thought it an attractive hypothesis that the underlying mechanism for increased neurological pathology was an inability to maintain latency effectively in CD8 KO mice. However, when trigeminal ganglia from parental, C-CD4 KO and C-CD8 KO mice were compared for kinetics of virus production following infection, reactivation rates or viral loads as determined by limiting dilution analysis, no significant differences were noted. Because we used more than one virus/mouse strain combination in these studies, we feel more confident in our conclusion that the increased disease we observed in CD8 KO mice is not due to persistent virus production, increased rates of reactivation or increased viral load in trigeminal ganglia.

Regulatory T cells have experienced a renaissance in their importance in controlling immune responses (Shevach, 2000). While most of the recent literature has focused on CD4^+ CD25^+ T cells with regulatory activity (Field et al., 2001; Piccirillo & Shevach, 2001; Skelsey et al., 2003), there also exist CD8^+ T cells that express regulatory functions (Cosmi et al., 2003; Ferguson et al., 2002, 2003). These CD8^+ T regulatory cells are found in both humans (Cortesini et al., 2002; Filaci & Suciu-Foca, 2002) and mice (Ferguson et al., 2002; Jiang & Chess, 2000; Jiang et al., 2001) and operate in a variety of antigen-specific responses (Ferguson et al., 2002; Nakamura et al., 2003) and diseases (Jiang et al., 2001; Taneja et al., 2002; Zhang et al., 2002). They have also been implicated in viral and parasitic infections (Hafalla et al., 2003). Finally, there is evidence that CD8^+ T regulatory cells mediate antigen-specific tolerance in models of anterior chamber-associated immune deviation (Nakamura et al., 2003; Skelsey et al., 2003), as well as controlling T cells that infiltrate the eyes of mice with autoimmune anterior uveitis (Zhang et al., 2002).

Concomitant with resolution of HSK lesions associated with the activity of CD4^+ Th1 cells is an increase in cytokines associated with immunosuppression (Niemialkowski & Rouse, 1992). We hypothesize that the cells that mediate resolution of corneal disease are most likely CD8^+ T regulatory cells. While it is possible that CD4^+ T cells may also be involved, as has been suggested recently (Suvas et al., 2003), we have no evidence to support their involvement in regulating HSK. We do know from this work that, in the absence of CD4^+ T cells, a population of CD8^+ T cells is generated that protects mice from disease and that this is not by more efficient clearance of virus. Thus, we propose that these cells act by regulating the CD4^+ Th1 cells.

In conclusion, our data best support the hypothesis that primary HSK is mediated by T cells bearing the CD4^+ phenotype and that CD8^+ cells protect the mice from HSK by a mechanism that does not involve more efficient virus clearance or an inability to maintain latency, but likely involves their ability to regulate the function of disease-mediating CD4^+ T cells. Future studies will focus on further defining these CD8^+ T cells and the specific means that they use to regulate corneal disease.

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REFERENCES


Role of CD8+ T cells in herpetic stromal keratitis


