T Cell Receptor–γ/δ Cells Protect Mice from Herpes Simplex Virus Type 1–induced Lethal Encephalitis

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Summary

Increased numbers of T cell receptor (TCR)-γ/δ cells have been observed in animal models of influenza and sendai virus infections, as well as in patients infected with human immunodeficiency virus and herpes simplex virus type 1 (HSV-1). However, a direct role for TCR-γ/δ cells in protective immunity for pathogenic viral infection has not been demonstrated. To define the role of TCR-γ/δ cells in anti–HSV-1 immunity, TCR-$\alpha^{-/-}$ mice treated with anti–TCR-γ/δ monoclonal antibodies or TCR-γ/δ × TCR-α/β double-deficient mice were infected with HSV-1 by footpad or ocular routes of infection. In both models of HSV-1 infection, TCR-γ/δ cells limited severe HSV-1–induced epithelial lesions and greatly reduced mortality by preventing the development of lethal viral encephalitis. The observed protection resulted from TCR-γ/δ cell–mediated arrest of both viral replication and neurovirulence. The demonstration that TCR-γ/δ cells play an important protective role in murine HSV-1 infections supports their potential contribution to the immune responses in human HSV-1 infection. Thus, this study demonstrates that TCR-γ/δ cells may play an important regulatory role in human HSV-1 infections.

Herpes simplex virus type 1 (HSV-1) is a neurotrophic virus that infects mucosal or abraded skin surfaces of nonimmune individuals (1). The virus replicates and destroys cells at the portal of entry. In addition, the virus infects nerve endings and is transported by retroaxonal flow to the nucleus of autonomic nervous system neurons in which it establishes a latent infection. Immunocompromised individuals develop viral encephalitis due to an inability to limit the spread of virus (2). Numerous studies have demonstrated that both cellular and humoral arms of the immune system contribute to the recovery from infection; however, T cells are ultimately required to protect the host (3).

The discovery of TCR-γ/δ cells a decade ago generated a great deal of interest in this novel T cell subset since it might manifest a unique role in immune responses. Significant progress towards understanding the development, antigen reactivity, and immunobiology of TCR-γ/δ cells has been made (4). Multiple studies have demonstrated that elevated numbers of TCR-γ/δ cells exist at inflammatory sites of a variety of human autoimmune disorders and infections (4, 5). In addition, these cells display an activated phenotype suggesting an important role for these cells during the immune response. In fact, the study of various in vivo animal models of bacterial and parasitic infections have revealed a critical role for TCR-γ/δ cells in regulating infection (4–11). In a bacterial model of infection using *Listeria monocytogenes*, TCR-γ/δ cells have a profound impact on reducing the pathogenic load in the spleen early in the infection, before TCR-α/β–mediated clearance (9). Similarly, in a parasitic model of *Plasmodium falciparum* infection, TCR-γ/δ cells are critical in regulating the parasitic burden in the liver (10). In contrast, the role of TCR-γ/δ cells in host immunity to viral infections is less clear (12). Increased numbers of TCR-γ/δ cells have been observed in animal models of influenza and sendai infection, as well as in patients infected with HIV (13–15). Furthermore, in these animal models, distinct subsets of TCR-γ/δ cells are recruited to the sites of viral replication. However, a direct role for TCR-γ/δ cells in regulating these viral infections has not been demonstrated.

Several reports have shown that HSV-1 seropositive individuals contain elevated numbers of TCR-γ/δ cells in their peripheral blood that are specific for infected cells (16, 17). In addition, we studied a murine TCR-γ/δ cell clone

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from an infected animal that is specific for the HSV-1 glycoprotein, gI (18, 19). These findings suggested that TCR-γ/δ cells may play an important role in HSV-1 immunity. To test this hypothesis, we assessed the role of TCR-γ/δ cell-immune responses to HSV-1 in both a footpad and ocular model of HSV-1 infection (20, 21). These studies used TCR-specific mAbs, TCR-α/β– and TCR-α/β × TCR-γ/δ–deficient mice to specifically target the TCR-γ/δ cell population. In both models of infection, the virus replicates at the site of infection and is transmitted to sensory ganglia where it establishes latency and, if not regulated, to the central nervous system where it can cause lethal encephalitis. Our results demonstrate that TCR-γ/δ cells regulate HSV-1 infections by controlling the viral replication and spread, thus preventing viral induced lethal encephalitis.

Materials and Methods

Media. TCR-γ/δ cell cloning experiments were performed in complete media which consisted of DMEM media containing 10% FCS, 25 μM Hepes, 2 mM glutamine, 100 U penicillin, 100 μg/ml streptomycin, 2 mM nonessential amino acids, and 5×10^{-5} M 2-mercaptoethanol.

Mice. All mice used in this study were bred in the University of Chicago (Chicago, IL) animal barrier facility under specific pathogen-free conditions. TCR-$\alpha^{-/-}$ mice bred to the BALB/c background were provided by Adrian Hayday (Yale University, New Haven, CT). A breeding pair of TCR-$\beta^{-/-}$ mice bred to the C57BL/6 background were obtained from Jackson Labs. (Bar Harbor, ME). The TCR-$\delta^{-/-}$ mice were provided by Susumu Tonegawa (Massachusetts Institute of Technology, Boston, MA) and were bred to the C57BL/6 background at the University of Chicago. Mutant mice generated in our breeding were identified by cell-surface immunofluorescence staining of peripheral blood cells using anti–TCR-α/β (H57-597) and anti–Thy-1 (53-2.1) mAbs (PharMingen, San Diego, CA) and analyzed on a FACScan® (Becton Dickinson, Mountain View, CA). In addition, PCR analysis of genomic tail DNA was used to determine the presence of TCR-constant-δ and neomycin genes.

Virus. Two different virus strains were used. The F strain of HSV-1 was used for the footpad infections and the RE strain of HSV-1 was used for the ocular infections. Both viral stocks were grown in monolayer cultures of Vero cells overlayed with 199V medium (22). The stocks were stored frozen at 10^8–10^9 PFU/ml concentrations. The virus was diluted into PBS just before infection.

Infections. Mice were infected at 5–6 wk of age. Footpad infections were performed by injecting 50 μl of inoculum containing 10^6 or 10^7 PFU into a single hind footpad. Corneal infections of anesthetized mice were performed by scarifying the cornea in a crisscross pattern using a 30-gauge needle. An inoculum of 3 μl containing 5×10^4 or 10^5 PFU of HSV-1 was added and gently massaged into the cornea. Mice were visually examined for disease progression and survival over the course of the experiments.

Antibodies. Anti–TCR-γ/δ mAbs were produced in our laboratory from the GL3 hybridoma (23). The antibody was purified on protein A–sepharose (Pharmacia, Uppsala, Sweden) and stored frozen in PBS. Purified control hamster Ig (Cappel, Malvern, PA) or anti–TCR-γ/δ mAbs were administered to mice (intraperitoneally) at least 1 d before infection and continued every 7 d throughout the study at a dose of 250 μg/mouse. Some experiments used PBS treatments instead of hamster Ig. A human serum with a high titer of anti–HSV-1 antibody was used for immunohistochemical detection of viral coat proteins. The biotinylated secondary antibody used for immunohistochemistry was Fcγ-specific goat anti–human IgG (Jackson Immunoresearch Labs. Inc., West Grove, PA).

Assays of Viral Replication in the Brain. Corneal-infected mice were killed at day 35 after infection. The trigeminal ganglia and brains were aseptically removed and stored frozen in 1 ml of 199 media. Samples were homogenized in a mechanical tissue grinder, titrated in medium, and plated on Vero cells (22). Plaques were counted 2 d later.

Immunohistochemical Analysis of Virus Replication in the Trigeminal Ganglia. Mice were killed at the indicated time points after corneal infection. Ipsilateral trigeminal ganglions were excised and processed for frozen sectioning as previously described (21). Frozen and fixed sections were blocked with normal goat serum for at least 20 min and then incubated with anti–HSV-1 antibody at 37°C for 1 h (or at 4° overnight). The biotinylated secondary antibody was incubated for 30 min at room temperature after extensive washing. The avidin–biotin complex developing reagent (Vectastain ABC kit; Vector Labs., Inc., Burlingame, CA) was used to detect antiviral antibody binding. Sections were counterstained with eosin and mounted with a coverslip using Permount. No positive cells were observed in uninfected trigeminal ganglia. Statistical differences were assessed by a one-way ANOVA with Tukey's post test.

Isolation of HSV-1 gI–reactive TCR-γ/δ Cells. TCR-$\alpha^{-/-}$ splenocytes were enriched for T cells by antibody- and complement-mediated depletion of MHC class II^+ cells with a mixture of anti–heat stable antigen (J11D) and anti–class II culture supernatants (25-9-3) plus rabbit complement. This mixture was incubated for 45 min at 37°C and was then subjected to Ficoll-Hypaque gradient centrifugation to remove dead cells. The resultant cells were plated in 24-well Linbro plates (ICN Biomedicals, Lisle, IL) at a concentration of 4×10^6 cells/well in the presence of 6×10^5 mitomycin C (40 μg/ml; Sigma Chemical Co., St. Louis, MO)–treated gI-transfected L cells (19), 5×10^6 irradiated (20Gy) BALB/c splenic feeder cells, 1 μg/ml purified anti-CD28 mAb, 2,000 U/ml recombinant human (*r*h)[1] IL-6 (Immunex, Seattle, WA), and 10 U/ml IL-12 (24). This culture was incubated at 37°C in a 7.5% CO_2 incubator for 5 d at which time they were harvested, washed once, and replated in the same conditions as above except the growth factors were changed to include 50 U/ml *r*hIL-2, and 10 ng/ml *r*hIL-7 (Immunex). After 7 d, the cells were assayed for specificity. Immunofluorescence analysis shows that the expanded cells were 100% TCR-γ/δ positive.

HSV-1 gI Stimulation of Expanded TCR-γ/δ Cells. Cells were tested for antigen specificity by assaying IFN-γ production. Soluble gI was constructed by fusing the ectodomain of HSV-1 gI and the Ig Fc domain of human IgG as previously described (19). Soluble gIIg stimulation was performed by immobilizing 5 μg/ml gIIg antigen on plastic wells at 4°C overnight. Wells were washed three times with 1× PBS, and TCR-γ/δ cells (10^5 cells/well) were incubated at 37°C in 7.5% CO_2 incubator for 48 h. IFN-γ production was detected by an ELISA (19).

Results

TCR-γ/δ Cells Mediate Host Protection After HSV-1 Footpad Infections. The ability of TCR null mice to respond to

[1]*Abbreviations used in this paper:* HSK, herpetic stromal keratitis; *r*h, recombinant human.

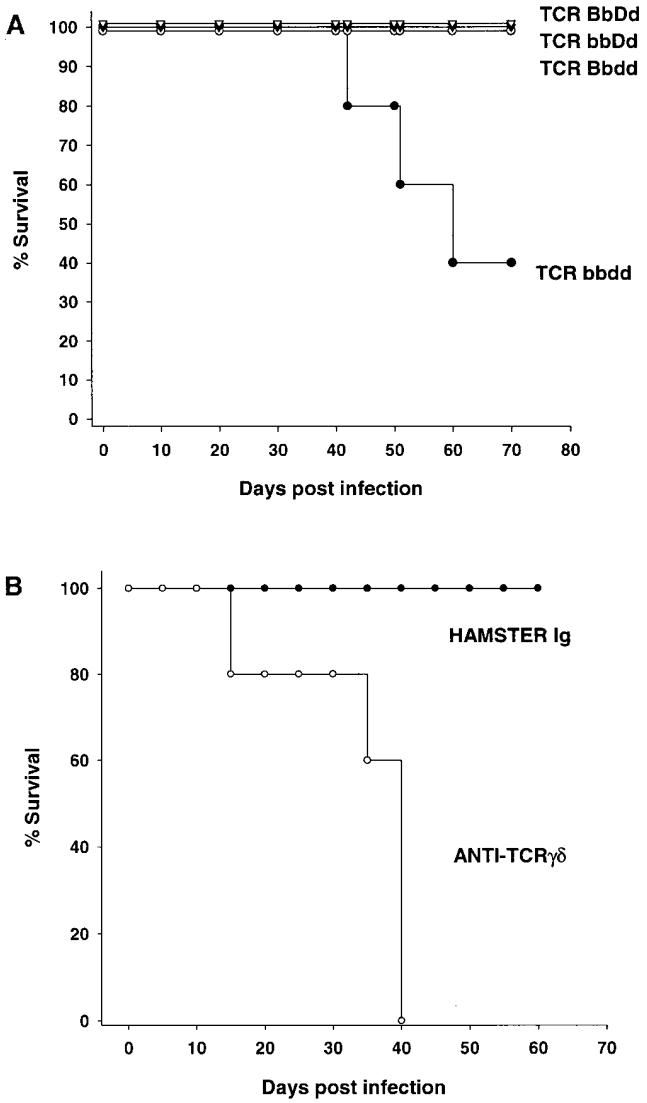

Figure 1. TCR-γ/δ cells regulate HSV-1 infection. (*A*) TCR null mice were infected with HSV-1 in the hind footpad and were monitored for survival. TCR-$\beta^{+/-}/\delta^{+/-}$ mice, *open triangles*; TCR-$\beta^{+/-}/\delta^{-/-}$ mice, *closed triangles*; TCR-$\beta^{-/-}/\delta^{+/-}$ mice, *open circles*; and TCR-$\beta^{-/-}/\delta^{-/-}$ mice, *closed circles*. All groups of mice contained at least three animals, and this plot is representative of two separate experiments. (*B*) TCR-$\alpha^{-/-}$ mice were infected with HSV-1 in the cornea and were monitored for survival. Control hamster Ig-treated TCR-$\alpha^{-/-}$ mice, *closed circles*; anti–TCR-γ/δ mAb–treated TCR-$\alpha^{-/-}$ mice, *open circles*. Both groups of mice contained five animals and this plot is representative of three separate experiments.

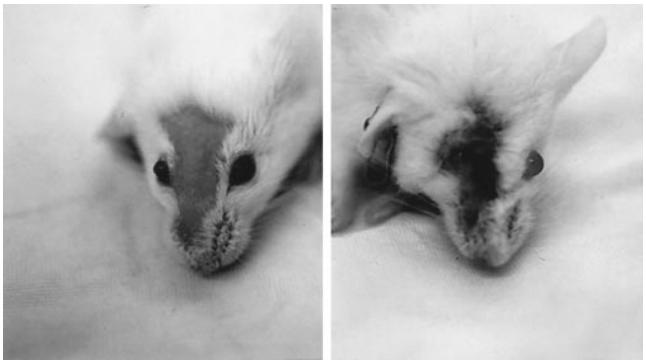

Figure 2. TCR-$\alpha^{-/-}$ mice recover from HSV-1 infection and heal their lesions. Elimination of viral-induced vesicles and scabbing as well as initiation of new hair follicles is evident in the PBS-treated TCR-$\alpha^{-/-}$ mouse on the left. In contrast, the anti–TCR-γ/δ mAb–treated TCR-$\alpha^{-/-}$ mouse on the right has continued vesicle formation and scabbing that covers the eye. Photos were taken at day 37 after infection from the same experiment.

footpad infection with HSV-1 was analyzed. Fig. 1 *A* shows that TCR-$\beta^{-/-}$ (TCR-γ/δ cell$^+$) or TCR-$\delta^{-/-}$ (TCR-α/β cell$^+$) mice survived HSV-1 infection. In contrast, the majority of the footpad-infected T cell–deficient TCR-$\beta^{-/-}/\delta^{-/-}$ mice succumbed to a lethal infection as had previously been shown with T cell–deficient nude mice (25). In addition, the TCR-$\beta^{-/-}/\delta^{-/-}$ mice that eventually succumbed to the infection developed hind limb paralysis during the infection supporting the conclusion that, in the absence of TCR-γ/δ cells, the virus gains access to spinal cord tissue. All groups of mice developed lesions at the site of infection (the footpad); however, only the TCR-$\beta^{-/-}/\delta^{-/-}$ mice, including those that had survived, failed to resolve their lesions (data not shown). These data suggest that, under these conditions, both TCR-γ/δ and TCR-α/β cells were able to clear the infection in the absence of the other T cell subset. Therefore, under conditions of suboptimal TCR-α/β cell responses, TCR-γ/δ cells can provide a critical protective role in this infection. In fact, it is likely that TCR-γ/δ cells are involved in HSV-1 infections in normal mice as TCR-γ/δ cells are recruited to the infected ganglia as early as day 6 after infection (21).

TCR-γ/δ Cells Mediate Host Protection After HSV-1 Corneal Infection. To further define the role of TCR-γ/δ cells in HSV-1 pathogenesis, an ocular model of HSV-1 infection was examined. Corneal infection results in both a lytic infection in the cornea and in the surrounding skin tissues (periocular lesions) as well as migration of the virus to the trigeminal ganglia. The viral replicative cycle as well as the induced immune response are best characterized in the BALB/c strain (26–30). Therefore, since the double knockout mice were bred to the C57BL/6 background, studies using the corneal model required the use of TCR-$\alpha^{-/-}$ mice that had been bred to the BALB/c background. In this setting, it is impossible to generate double knockout mice. Therefore, the TCR-γ/δ cells were depleted using an anti–TCR-γ/δ mAbs. As seen in Fig. 1 *B*, TCR-$\alpha^{-/-}$ mice treated with control hamster Ig did not develop encephalitis, whereas anti-TCR-γ/δ mAb–treated TCR-$\alpha^{-/-}$ mice succumbed to a disseminated viral infection and lethal encephalitis. Both groups of mice developed periocular skin lesions around day 10–15 after infection that contained vesicles of HSV-1 (data not shown). These lesions spread as the infection proceeded, but the control group ultimately resolved the skin infection by clearing viral induced vesicles and initiating growth of new hair (Fig. 2). The anti–TCR-γ/δ mAb–treated mice died without resolving the skin lesions, similar to previous results in the footpad-infected mice.

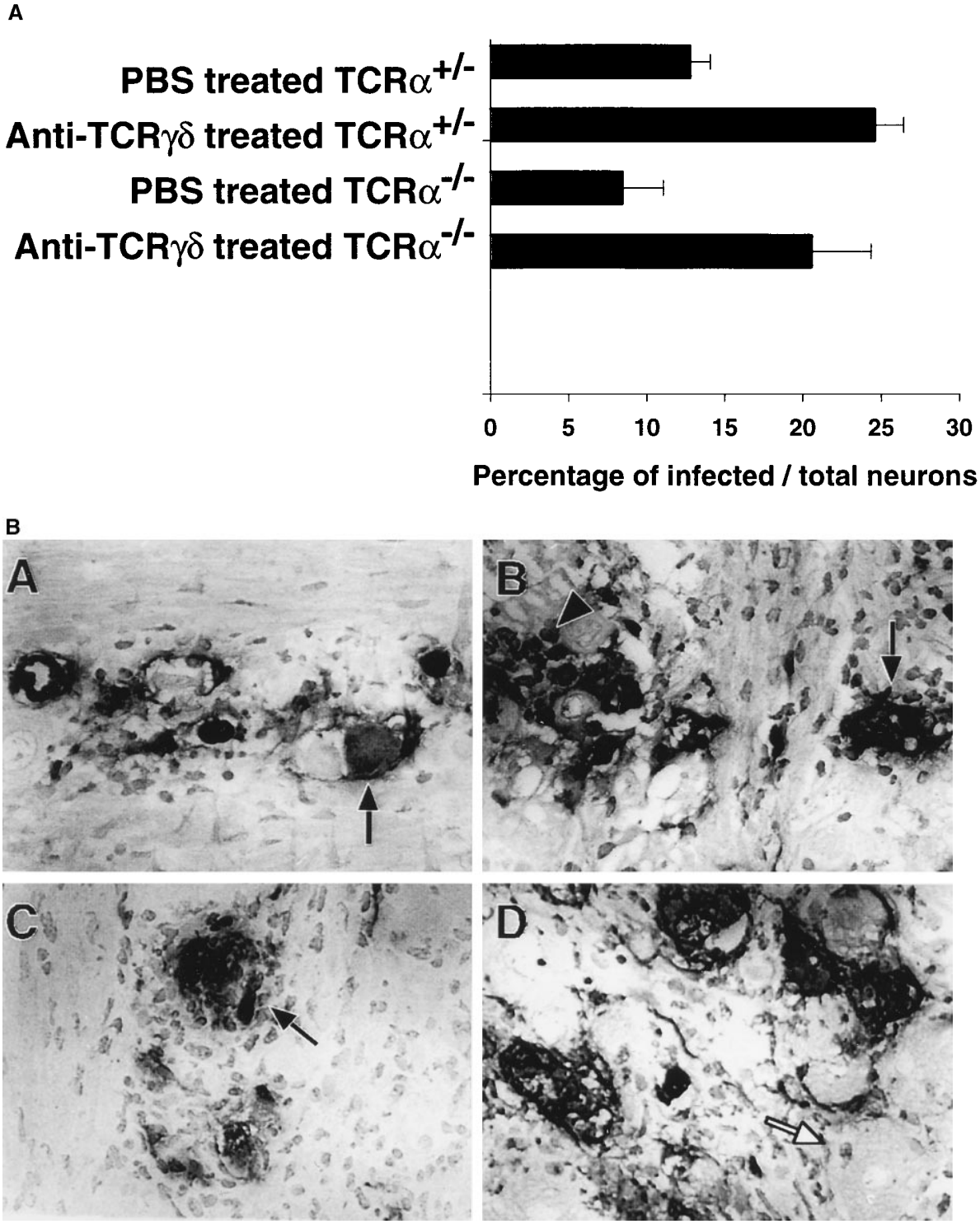

Figure 3. The dynamics of HSV-1 replication is different in TCR-$\alpha^{+/-}$ and TCR-$\alpha^{-/-}$ mice. (*A*) Quantitation of immunohistochemical staining of HSV-1 viral antigens in the trigeminal ganglia at day 6 after infection. Two mice per group and three representative sections of each ganglia (six sections per group) were prepared for immunohistochemical staining. An average of 1,200 neurons were counted for each group. Data are recorded as the percent of neurons that exhibited specific HSV-1 staining. The differences between the day 6 after infection control and anti–TCR-γ/δ mAb–treated TCR-$\alpha^{+/-}$ mice, or between the control and anti–TCR-γ/δ mAb–treated TCR-$\alpha^{-/-}$ mice, were statistically significant ($P < 0.05$). (*B*) Photomicrographs depicting immunohistochemical staining of HSV-1 antigens in the trigeminal ganglion. Trigeminal ganglia were obtained 6 d after corneal infection from TCR-$\alpha^{+/-}$ mice (*A* and *B*) and TCR-$\alpha^{-/-}$ mice (*C* and *D*). Mice received control (*A* and *C*) or anti–TCR-γ/δ mAb (*B* and *D*) treatments. Infected neurons, *black arrows*; uninfected neurons, *white arrows*; a cluster of infected inflammatory cells, *black arrowhead* in *B*. Original magnification: 100×.

Table 1. *TCR-γ/δ Cells Eliminate the Lytic HSV-1 Infection*

HSV-1–infected TCR-$\alpha^{-/-}$ mice (day 35 after infection)	Trigeminal ganglia	Brain
	PFU	*PFU*
PBS	0	0
Anti–TCR-γ/δ	75	>10,000

Trigeminal ganglion and brain tissue were harvested from treated or untreated ocularly infected TCR-$\alpha^{-/-}$ mice 35 d after infection and were used to quantitate the amount of replicating lytic virus using a viral plaque assay. PFU were counted and averaged from two mice and the data are representative of two separate experiments.

HSV-1 infection of the cornea results in a widely studied inflammatory phenomenon termed herpetic stromal keratitis (HSK; 31) characterized by corneal opacity, necrosis, and ultimately blindness. HSK may be due to autoimmunity. Corneal infection exposes the immune system to a normally privileged corneal antigen that is cross-reactive with HSV-1–reactive T cells and results in an autoimmune mediated destruction of the cornea (32). The surviving TCR-$\alpha^{-/-}$ mice had no evidence of HSV-1–associated corneal opacity, destruction, or blindness, revealing that TCR-γ/δ cells do not participate in this autoimmune reaction. These results are consistent with earlier experiments using *nu/nu* mice or CD4- and CD8-depleted mice where HSK does not develop (25, 28). This experiment, however, proves that TCR-γ/δ cells have no role since the *nu/nu* mutation and CD4- and CD8-depletion can affect the TCR-γ/δ cell compartment.

The Dynamics of HSV-1 Replication are Different in TCR-$\alpha^{+/-}$ and TCR-$\alpha^{-/-}$ Mice. The virus load in the trigeminal ganglia from ocularly infected mice was analyzed both at early (day 6) and late (day 20) time points to determine whether the TCR-γ/δ cells regulate HSV-1 replication. Immunohistochemical analyses were done to determine the presence of viral antigen using an antiserum that is specific for viral structural proteins expressed during productive infections. Interestingly, normal mice treated with anti-TCR-γ/δ mAbs, but not PBS, exhibited an increased viral burden in the trigeminal ganglion at day 6 after infection (Fig. 3). These results suggested that TCR-γ/δ cells decreased viral replication early during the infection, when few TCR-α/β cells were found homing to the trigeminal ganglion (21). Examination of late time points showed that normal mice completely resolved the lytic virus infection in the trigeminal ganglion. In contrast, both TCR-$\alpha^{-/-}$ mice treated with control or anti-TCR-γ/δ mAbs continued to express viral antigens characteristic of a productive infection in the ganglia at day 21 after infection (data not shown). Importantly, using a viral plaque assay, the surviving control treated TCR-$\alpha^{-/-}$ mice eventually cleared the lytic infection (day 35 after infection) and had no detectable infectious virus in the brain or trigeminal ganglia. In contrast, the moribund (day 35 after infection) anti–TCR-γ/δ mAb–treated TCR-$\alpha^{-/-}$ mice contained high levels of systemic infectious virus (Table 1). The prolonged viral load in the trigeminal ganglia of TCR-$\alpha^{-/-}$ mice is consistent with the delayed resolution of skin lesions in these mice. It is not clear why the virus persists longer in TCR-$\alpha^{-/-}$ mice. However, clonal expansion of TCR-γ/δ cells to protective levels may take longer to occur because the total number T cells is reduced in the TCR-α null mice (33). Together, these results suggest that TCR-γ/δ cells are both limiting viral replication and restricting its progression into the brain.

TCR$\alpha^{-/-}$ Mice Contain HSV-1 gI–specific TCR-γ/δ Cells. Previous results have shown that a single TCR-γ/δ cell clone recognized a HSV-1–encoded glycoprotein, gI. To determine whether HSV-1 gI-specific TCR-γ/δ cells could be isolated from TCR-$\alpha^{-/-}$ mice, spleen cells were cultured with HSV-1 gI-transfected L cell fibroblasts. After 2 wk in culture in the presence of antigen and growth factors, gI-specific TCR-γ/δ cells could be detected based on their ability to secrete IFN-γ in response to antigen stimulation. Recognition was direct and specific for unprocessed gI (Fig. 4) since immobilized gIIg fusion protein could be recognized in the absence of antigen-processing cells, just as the TgI4.4 clone (19). TCR variable region repertoire of these expanded TCR-γ/δ cells shows that they were polyclonal (data not shown). Lastly, the expansion of TCR-γ/δ cells from unimmunized TCR-$\alpha^{-/-}$ mice suggests that there exists a circulating pool of HSV-1–specific TCR-γ/δ cells. This pool may account for the rapid TCR-γ/δ cell–mediated HSV-1 neutralization observed at day 6 after infection of normal mice (Fig. 3).

Discussion

This study provides direct evidence that TCR-γ/δ cells can respond to and suppress HSV-1 infection. Other viral infections, such as influenza (13) and sendai (14), induce increases in the TCR-γ/δ population after infection. However, little evidence exists for a direct role in regulating the infection. Therefore, the critical role of TCR-γ/δ cells in HSV-1 infection of TCR-$\alpha^{-/-}$ mice provides direct evidence that TCR-γ/δ cells are an important regulatory subset of immune cells.

The mechanism of the protective response, such as the nature of the antigenic ligands and the effector functions

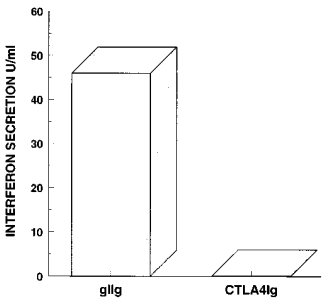

Figure 4. TCR-γ/δ cells isolated from TCR-$\alpha^{-/-}$ mice are specific for HSV-1 gI. Expanded TCR-γ/δ cells were stimulated with immobilized gIIg or control fusion CTLA4Ig and IFN-γ production was measured.

(cytokine production and/or cytolysis) used by the protective TCR-γ/δ cell population, remains to be elucidated. Importantly, TCR-γ/δ cells from TCR-$\alpha^{-/-}$ mice produced IFN-γ production in the trigeminal ganglion after HSV-1 infection (Kodukula, P., R. Sciammas, J.A. Bluestone, and R.L. Hendricks, unpublished observations). In addition, the isolation of cytolytic, IFN-γ–producing, HSV-1 gI-specific TCR-γ/δ cells, TgI4.4 and the expanded cells, suggests that TCR-γ/δ cells can directly recognize viral antigens and mount antiviral effector function. Finally, preliminary results suggest that the observed protective response is polyclonal since reverse transcriptase-PCR analysis shows that both TCR-Vγ 1.1 and TCR-Vγ2 transcripts are present in infected trigeminal ganglia (Sciammas, R., P. Kodukula, R.L. Hendricks, and J.A. Bluestone, unpublished observations).

Antiviral humoral responses have also been shown to confer important viral neutralizing activity in HSV-1 infections (34). However, we have not been able to detect any anti–HSV-1 IgG in the serum of infected TCR-$\alpha^{-/-}$ mice (Sciammas, R., P. Kodukula, R.L. Hendricks, and J.A. Bluestone, unpublished observations). These results suggest that, in contrast to other disease models (35, 36), TCR-γ/δ cells are not protecting HSV-1–infected mice by providing B cell help. Therefore, it is striking that the TCR-γ/δ cells in TCR-$\alpha^{-/-}$ mice are able to regulate HSV-1 pathology in light of a nonexistent humoral response and a reduced number of TCR-γ/δ cells.

TCR-γ/δ cells, including TgI4.4, recognize antigen directly in a MHC-independent manner (19, 37). Intriguingly, this mode of recognition may be useful in regulating the viral life cycle by interacting with envelope glycoproteins on the surface of infected cells or on the virus itself. In addition, it has been reported that HSV-1 has the ability to specifically intervene with efficient MHC class I expression by inhibiting the TAP complex (38, 39). Therefore, direct antigen recognition by TCR-γ/δ cells may circumvent viral intervention of MHC presentation. Secondly, since HSV-1 is a neurotrophic virus, TCR-γ/δ cells could be adept at recognizing antigens directly on central nervous system neurons that are poor at processing and presenting antigens in an MHC-restricted manner (40, 41). Thus, these data suggest that under circumstances where TCR-α/β cell function is compromised, such as in human acquired immunodeficiency syndromes, TCR-γ/δ cells may be essential to protect the infected individual.

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