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Clearance of Herpes Simplex Virus Type 2 by CD8$^+$ T Cells Requires Gamma Interferon and either Perforin- or Fas-Mediated Cytolytic Mechanisms

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The T-cell-mediated resolution of herpes simplex virus type 2 (HSV-2) genital infections is not fully understood. In these studies, the mechanisms by which CD8$^+$ T cells clear virus from the genital epithelium were examined. Ovalbumin (OVA)-specific CD8$^+$ T cells from OT-I transgenic mice cleared a thymidine kinase-deficient, ovalbumin-expressing HSV-2 virus (HSV-2 tk$^-$ OVA) from the genital epithelium of recipient mice, and clearance was abrogated by in vivo neutralization of gamma interferon (IFN-γ). Further, CD8$^+$ OT-I T cells deficient in IFN-γ were unable to clear HSV-2 tk$^-$ OVA from the vaginal epithelium. The requirement for cytolytic mechanisms in HSV-2 tk$^-$ OVA clearance was tested in radiation chimeras by adoptive transfer of wild-type or perforin-deficient OT-I T cells to irradiated Fas-defective or wild-type recipients. Although a dramatic decrease in viral load was observed early after challenge with HSV-2 tk$^-$ OVA, full resolution of the infection was not achieved in recipients lacking both perforin- and Fas-mediated cytolytic pathways. These results suggest that IFN-γ was responsible for an early rapid decrease in HSV-2 virus titer. However, either perforin- or Fas-mediated cytolytic mechanisms were required to achieve complete clearance of HSV-2 from the genital epithelium.

Herpes simplex virus type 2 (HSV-2) infects epithelial cells in the genital mucosa, spreads to the sensory ganglia via retrograde transport, and establishes a lifelong latent infection in sensory neurons (50). The virus periodically reactivates and descends sensory neurons via anterograde transport, resulting in development of recurrent lesions at or near the site of primary infection or in shedding of infectious virus in the absence of disease symptoms. The primary and recurrent lesions of immunocompetent individuals are generally self limiting and are resolved primarily by cell-mediated immune mechanisms. Recurrent disease is less well controlled in immunocompromised individuals, resulting in more frequent recurrences and sometimes severe mucocutaneous disease manifestations. Studies of HSV infection in human immunodeficiency virus (HIV)-infected individuals suggested that the severity of HSV disease could be inversely correlated with the number of HSV-specific CD8$^+$ T cells (39).

Studies of recurrent HSV lesions in immunocompetent humans have demonstrated the early infiltration of CD4$^+$ T cells and macrophages, local production of IFN-γ, and late arrival of CD8$^+$ T cells at the site of HSV infection. Both CD4$^+$ and CD8$^+$ T lymphocytes capable of IFN-γ secretion and HSV-specific cytolysis have been isolated from human herpetic lesions (10) and clearance of infectious virus, and resolution of lesions has been correlated with the detection of HSV-specific cytolytic T-lymphocyte activity (10, 22–23). However, the role for these cytolytic and noncytolytic immune mechanisms in resolution of HSV-2 genital infections is not well understood.

Murine models of HSV-2 genital infection have also demonstrated the importance of cell-mediated immunity in clearance of HSV and have provided evidence for both cytolytic and noncytolytic mechanisms in resolution of vaginal HSV-2 infections. Mice depleted of T cells are unable to resolve a genital HSV infection, but mice depleted of either CD4$^+$ or CD8$^+$ subset can ultimately resolve the infection, although clearance is delayed (24, 28, 30, 34–35). HSV-specific T cells exhibiting ex vivo cytolytic function have been isolated from the vaginal lumen of HSV-2-inoculated mice at a time concomitant with virus clearance (32). T-cell-produced IFN-γ has also been detected in vaginal secretions within 24 h of HSV-2 challenge of HSV-immune mice, and neutralization of IFN-γ by treatment with specific antibody has been shown to severely impair resolution of a primary genital HSV-2 infection as well as rechallenge infections of HSV-immune mice (30, 35). In contrast, other studies utilizing an HSV-1 model failed to detect a dominant role for IFN-γ and instead suggested that a mechanism involving major histocompatibility complex (MHC) class I was more important for resolution of HSV infection (15).

We examined the requirement for IFN-γ and cytolytic mechanisms in CD8$^+$ T-cell-mediated virus clearance using an adoptive transfer strategy. Ovalbumin (OVA)-specific CD8$^+$ T cells transferred to irradiated recipients cleared an engineered thymidine kinase-deficient, OVA-expressing virus (HSV-2 tk$^-$ OVA) from the genital mucosa. Virus clearance was abrogated by treatment of recipients with anti-IFN-γ antibody or by transfer of OT-I CD8$^+$ T cells from mice genetically deficient in IFN-γ. To examine the requirement for cytolytic mechanisms to clear HSV-2 from the genital epithelium, mice deficient in perforin and Fas-Fas ligand interactions were challenged with HSV-2 tk$^-$ OVA. While a significant decrease in

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virus titer was observed soon after viral challenge, mice lacking both perforin- and Fas-mediated cytolytic mechanisms were unable to completely clear the infection. These data suggest that both IFN-γ and T-cell-mediated cytolytic mechanisms are required for complete clearance of HSV-2 from the genital epithelium.

MATERIALS AND METHODS

Virus. HSV-2 tk$^-$ OVA was constructed by inserting the OVA gene under the control of the immediate-early cytomegalovirus promoter into the tk locus of HSV-2 strain 333. Resulting tk$^-$ mutant virus plaques were picked under acyclovir selection, and OVA expression was assured by Western blot and fluorescence microscopy of virus-infected Vero E6 monolayers (data not shown). HSV-2 333 tk$^-$ was obtained originally by Mark McDermott (McMaster University Ontario, Canada) (26). Virus stocks were prepared by infection of Vero E6 monolayers at a multiplicity of infection of 0.01 in the presence of 20 μg/ml acyclovir as described previously (28). Virus was released by three cycles of freeze-thaw, cell debris were removed by centrifugation, and virus-containing supernatant was stored at −80°C.

Mice. C57BL/6J (B6), perforin-deficient C57BL/6-Pfp$^{-/-tm1Sdz}$ (Pfp$^{-/-}$), IFN-γ-deficient B6.129S7-Ifng^{tm1Ts} (IFN$^{-/-}$), and Fas-defective B6.MRL-TNFR5F6LPR (Fas$^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). A breeding pair of OT-I mice was obtained from Raphael Hirsch (Children's Hospital of Pittsburgh, Pittsburgh, PA), and a breeding colony was maintained at the University of Texas Medical Branch animal facilities. OT-I mice were bred with IFN-γ$^{-/-}$ or Pfp$^{-/-}$ mice. Pups screening positive for the transgene by flow cytometric analysis for the expression of Vβ$_5$ were screened for the knockout genotype by PCR using primers located on either side of the inserted neomycin cassette. For the perforin gene, the primers 5′-GAAGAA CAGAACCGGAAGC-3′ (sense) and 5′-GACACCGGTCCTGAACTC-3′ (antisense) were designed to amplify DNA flanking the neomycin cassette inserted at the BstEII restriction site in exon III of the perforin gene as described by Kägi et al. (19). The wild-type gene gave an amplified product of 293 bp, and the disrupted gene yielded a gene product of 6.4 kb in size. Screening for the disrupted IFN-γ gene was performed as described by Sisto et al. (48). Mice screening positive for both the OT-I transgene and the IFN-γ or perforin knockout genotype were then bred back against IFN-γ$^{-/-}$ or Pfp$^{-/-}$ knockout mice, respectively. Screened mice from the third and fourth backcross generations were used in these experiments. All mice were housed in sterile microisolater cages under specific-pathogen-free conditions in the American Association for the Accreditation of Laboratory Animal Care-approved University of Texas Medical Branch animal facility. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee.

Virus inoculation and titration. Mice were inoculated intravaginally as described previously (27). Mice were treated with 3 mg of methyl-17-hydroxyprogesterone acetate (UpJohn Company, Kalamazoo, MI) 1 week prior to challenge. Hormonal pretreatment was necessary to induce susceptibility to genital HSV-2 inoculation in mice (26, 28), most likely reflecting the induction of the HSV entry receptor, nectin-1, on vaginal epithelial cells (25). For virus challenge, mice were anesthetized with sodium pentobarbital and swabbed with a sterile calcium alginate swab prior to instillation of 5×10^3 PFU of HSV-2 tk$^-$ OVA or HSV-2 333 tk$^-$ virus into the vagina. Virus titers were obtained by titration of vaginal swab samples on Vero E6 cells as described previously (28).

Adoptive transfer. CD8$^+$ T cells were purified using a CD8$^+$ T-cell purification kit and passage over magnetic columns (Miltenyi Biotec Inc., Auburn, CA). OT-I CD8$^+$ T cells were activated by 4-day culture with mitomycin-C-treated B6 spleen cells pulsed with 1 μM of the OVA-derived SIINFEKL peptide. Recipient mice were irradiated (650 rad) 24 h before intravenous (i.v.) reconstitution with 3×10^6 activated OT-I T cells or CD8$^+$ T cells from normal B6 mice. Recipient mice were then challenged intravaginally with 5×10^3 PFU of HSV-2 tk$^-$ OVA or HSV-2 333 tk$^-$. For IFN-γ neutralization, mice received daily intraperitoneal injections of 2 mg anti-IFN-γ antibody (XMG 1.2) for the duration of the experiment beginning 2 days prior to challenge. An isotype-matched antibody of irrelevant specificity (SFR8.B6) was used for control treatments.

Cytotoxic T-lymphocyte (CTL) assays. In vivo cytolytic assays were performed by a modification of the procedure described by Coles et al. (8). OT-I or OT-IFN$^{-/-}$ cells activated for 4 days in culture were adoptively transferred into irradiated syngeneic recipients followed by intravaginal inoculation with 5×10^3 PFU HSV-2 tk$^-$ OVA. In vivo lytic function was assessed on days 4, 6, and 8 postinoculation following injection of target cells. Target splenocytes from B6 mice were prepared in two fractions. One fraction was pulsed with 1 μM SIINFEKL peptide for 45 min at 37°C and labeled with 2.5 μM carboxy-fluorescein succinimydl ester (CFSE; Molecular Probes, Eugene, OR) (CFSE$_{high}$). The second fraction was incubated with media only as a control and labeled with 0.25 μM CFSE (CFSE$_{low}$). Equal numbers of CFSE$_{high}$ and CFSE$_{low}$ cells were mixed and injected i.v. into infected recipient mice (2×10^7 cells/mouse). After 4 h, the mice were euthanized and spleens and iliac lymph nodes were harvested. Single-cell suspensions were prepared and fixed with 1% formaldehyde. Data were acquired on a Becton Dickson FACS Canto flow cytometer at the University of Texas Medical Branch Flow Cytometry Core Facility and analyzed using FlowJo Software (Treestar Inc., Ashland, OR). The percent specific lysis was calculated as percent specific lysis = (1 − ratio naïve cell recipient/ratio activated CD8$^+$ T-cell recipient) × 100, where the ratio is %CFSE$_{low}$/%CFSE$_{high}$.

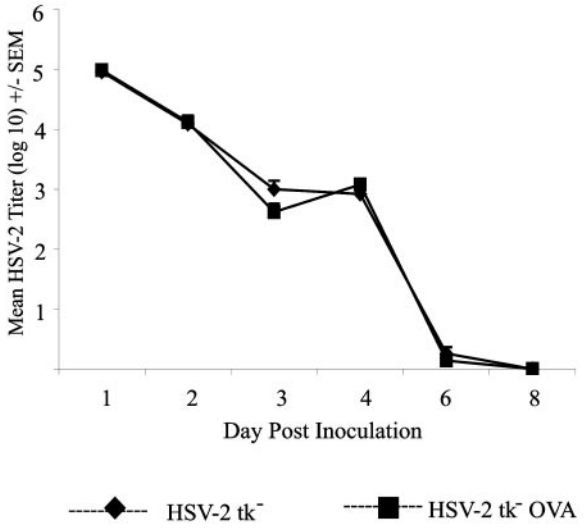

FIG. 1. Intravaginal inoculation of mice with HSV-2 tk$^-$ OVA or HSV-2 333 tk$^-$. Groups of 8 B6 mice were inoculated intravaginally with 2×10^5 PFU of HSV-2 tk$^-$ OVA or HSV-2 333 tk$^-$ and swabbed on the indicated days for quantification of virus in the genital epithelium. SEM, standard errors of the means.

Cytokine quantification. Cytokines in cell culture supernatants were quantified as described previously (11). Purified CD8$^+$ T cells were cultured with mitomycin-treated B6 spleen cells in the presence or absence of 1 μM SIINFEKL peptide. Supernatants were collected at 24 h and added in duplicate to enzyme-linked immunosorbent assay plates coated with capture antibody specific for IFN-γ, tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), or IL-5 (BD Pharmingen, San Diego, CA). Serial dilutions of recombinant cytokines were plated to generate standard curves. Plates were developed using biotinylated detection antibodies (BD Pharmingen) and streptavidin peroxidase (Sigma-Aldrich), and the optical density at 490 nm (OD$_{490}$) was measured on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Cytokines were quantified by comparison to OD$_{490}$ values obtained from standard curves using SoftMax Pro software (Molecular Devices, Sunnyvale, CA). The limit of detection for each cytokine was defined as the mean OD$_{490}$ plus 3 standard deviations of media-only control wells that were developed identically to experimental wells.

Detection of OT-I T cells in the genital epithelium. Activated OT-I and OT-IFN$^{-/-}$ cells were adoptively transferred i.v. into groups of 10 irradiated mice followed by intravaginal inoculation with 5×10^3 PFU HSV-2 tk$^-$ OVA. Seven days postinoculation, recipients were euthanized and spleens, iliac lymph nodes, and vaginal tracts were harvested. Single-cell suspensions were prepared from pooled spleens and pooled lymph nodes. The vaginal tracts were pooled, minced into 1-mm^2 pieces, and incubated in Ca^{2+}Mg^{2+}-free PBS (Invitrogen Corporation, Grand Island, NY) for 30 min with stirring. The tissue was further dispersed by pushing through a stainless steel mesh screen. The resulting cells were resuspended in 30% Percoll (Sigma-Aldrich, St. Louis, MO), layered over an 80% Percoll cushion, and centrifuged at 500 × *g* for 15 min at ambient temperature. Cells at the interface were collected for analysis, stained with antigen-presenting cell-conjugated anti-CD8α and fluorescein isothiocyanate-conjugated Vβ$_{5.1/5.2}$ (BD Pharmingen), and fixed in 1% formaldehyde prior to flow cytometric analysis.

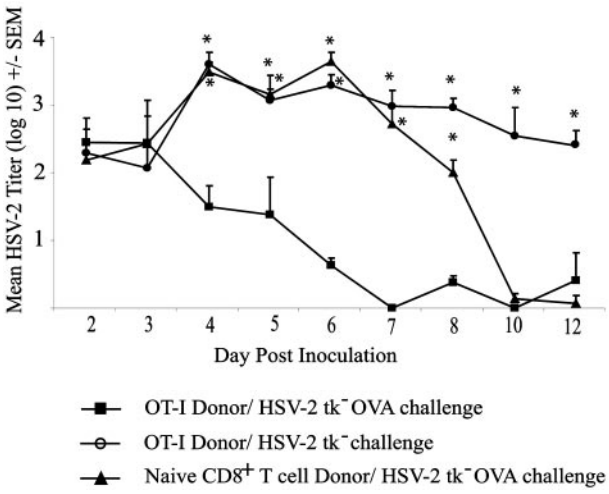

FIG. 2. Virus clearance by OT-I T cells requires viral expression of OVA. Groups of 8 irradiated B6 mice were repopulated with either naive B6 $CD8^+$ T cells or activated OT-I T cells and inoculated intravaginally with 5×10^3 PFU of HSV-2 333 tk^- or HSV-2 tk^- OVA. Infectious virus in the vaginal mucosa on the indicated days after virus challenge was quantified by plaque assay. Values marked with an asterisk are significantly different compared to same-day values for OT-I T-cell recipients challenged with HSV-2 tk^- OVA. ($P < 0.001$). SEM, standard errors of the means.

Statistical analysis. Data were analyzed by either student's *t* test or one-way analysis of variance (ANOVA) with the Bonferroni correction for multiple groups as appropriate.

RESULTS

Clearance of HSV-2 by $CD8^+$ OT-I T lymphocytes requires viral expression of OVA. We used an adoptive transfer approach to examine the mechanisms by which $CD8^+$ T cells clear HSV-2 from the genital epithelium. OVA-specific $CD8^+$ T cells from OT-I transgenic mice were utilized as the source of donor T lymphocytes to ensure functional homogeneity and minimize variability among experiments. A challenge virus was constructed by inserting the OVA gene into the thymidine kinase locus of HSV-2 strain 333. To demonstrate the utility of the resulting tk^- virus (HSV-2 tk^- OVA) as a challenge virus, immunocompetent C57BL/6 (B6) mice were inoculated intravaginally with either HSV-2 tk^- OVA or a thymidine kinase-deficient HSV-2 strain 333 virus that does not express OVA (HSV-2 333 tk^-) (26). As shown in Fig. 1, HSV-2 tk^- OVA and HSV-2 333 tk^- replicated to similar titers after intravaginal inoculation and were cleared in a similar manner from the genital epithelium ($P > 0.05$ on all days; Student's *t* test). To test the ability of $CD8^+$ OT-I T cells to resolve a vaginal infection with HSV-2 tk^- OVA, B6 mice were irradiated (650 rad) and repopulated intravenously with 3×10^6 activated OT-I T cells or naïve B6 $CD8^+$ T cells and challenged with either 5×10^3 PFU HSV-2 tk^- OVA or HSV-2 333 tk^-. Mice receiving OT-I T cells cleared the HSV-2 tk^- OVA virus by day 7 postinoculation (Fig. 2). Resolution of the genital infection required antigen-specific T-cell recognition, as OT-I T-cell recipients challenged with the HSV-2 333 tk^- virus failed to clear the virus and exhibited significantly higher virus titers on days 4 to 12 ($P < 0.001$; ANOVA) compared to HSV-2 tk^- OVA-challenged

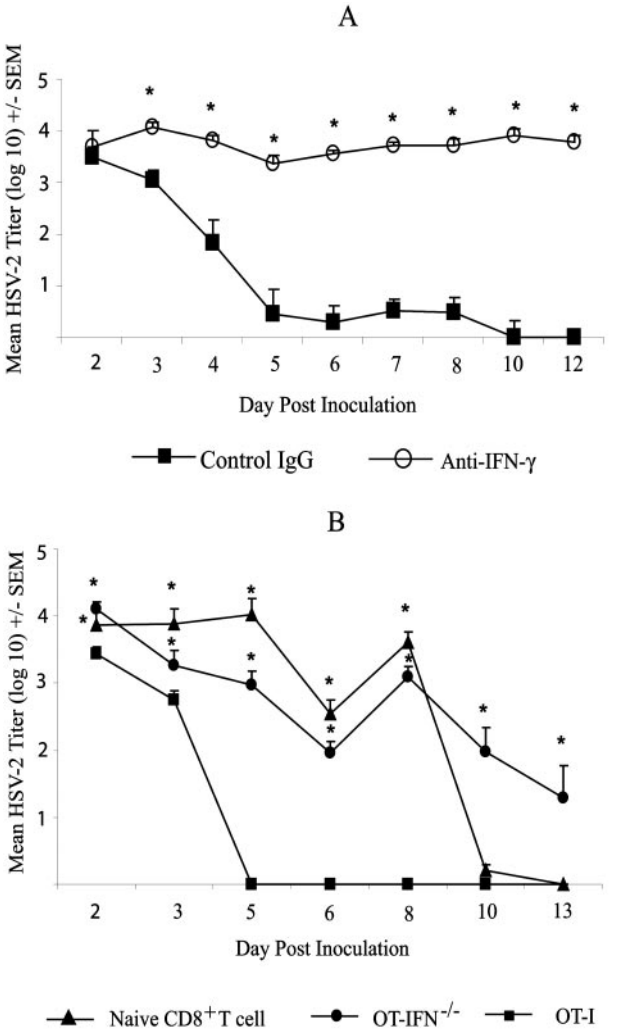

FIG. 3. IFN-γ is important for rapid clearance of HSV-2 tk^- OVA by $CD8^+$ T cells. (A) Neutralization of IFN-γ in vivo using specific antibody. Groups of 8 irradiated B6 mice received activated OT-I cells and were challenged with HSV-2 tk^- OVA. Mice were treated daily with anti-IFN-γ (XMG 1.2) or control antibody (SFR8.B6) beginning 2 days prior to adoptive transfer through the end of the experiment. Mice were swabbed on the indicated days for virus quantification. (B) Inability of OT-IFN $^{-/-}$ T cells to clear HSV-2 tk^- OVA from the vaginal epithelium. Irradiated B6 mice received naïve B6 $CD8^+$ T cells ($n = 6$), activated OT-I T cells ($n = 10$), or activated OT-IFN $^{-/-}$ T cells ($n = 10$) prior to intravaginal inoculation with HSV-2 tk^- OVA. Infectious virus was quantified on the indicated days. Values marked with an asterisk are significantly different ($P < 0.01$) compared to same-day values for OT-I T-cell recipients. SEM, standard errors of the means.

OT-I recipients. Mice receiving naive B6 $CD8^+$ T cells ultimately resolved the HSV-2 tk^- OVA infection at a later time point (day 10), although virus titers were significantly higher than those of OT-I recipients ($P < 0.001$; ANOVA) on days 4 to 8 after HSV-2 tk^- OVA inoculation. Lymphocytes isolated from naive B6 $CD8^+$ T-cell recipients at the conclusion of the experiment secreted IFN-γ following stimulation with mitomycin C-treated B6 spleen cells pulsed with an immunogenic peptide from HSV glycoprotein B, $gB_{498\text{-}505}$, but not cells pulsed with the OVA-derived SIINFEKL peptide (data not shown). These results suggest that

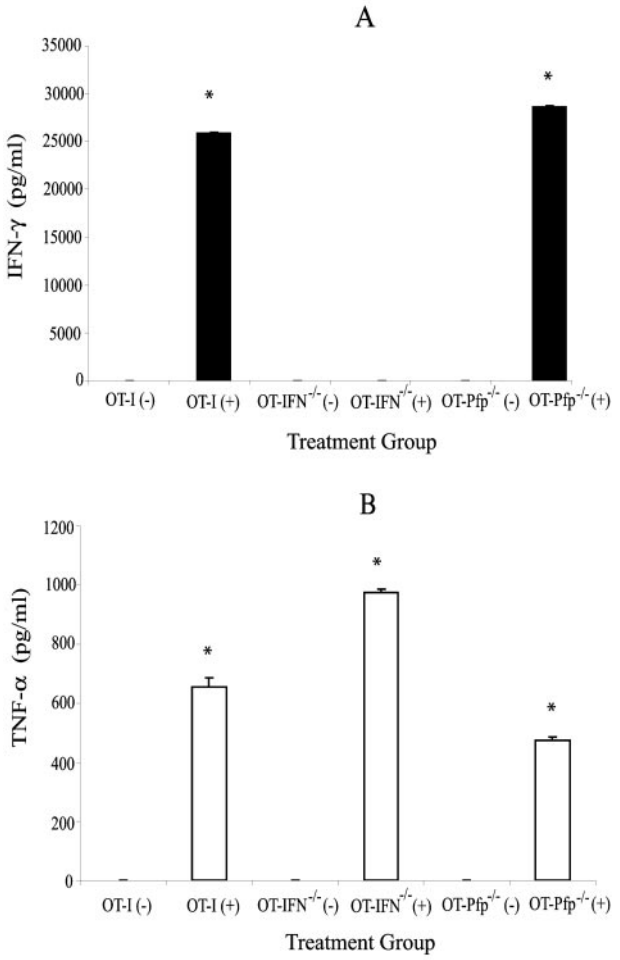

FIG. 4. Cytokine production by activated OT-I, OT-IFN$^{-/-}$, and OT-Pfp$^{-/-}$ T cells. OT-I, OT-IFN$^{-/-}$, and OT-Pfp$^{-/-}$ T cells were cultured with mitomycin C-treated B6 spleen cells in the presence (+) or absence (−) of the OVA-derived SIINFEKL peptide. Supernatants were analyzed for the presence of IFN-γ (A), TNF-α (B), IL-4, and IL-5 by enzyme-linked immunosorbent assay. Levels of IL-4 and IL-5 were below the limit of detection (<98 pg/ml). Significant differences in cytokine levels in the presence and absence of peptide are marked with an asterisk.

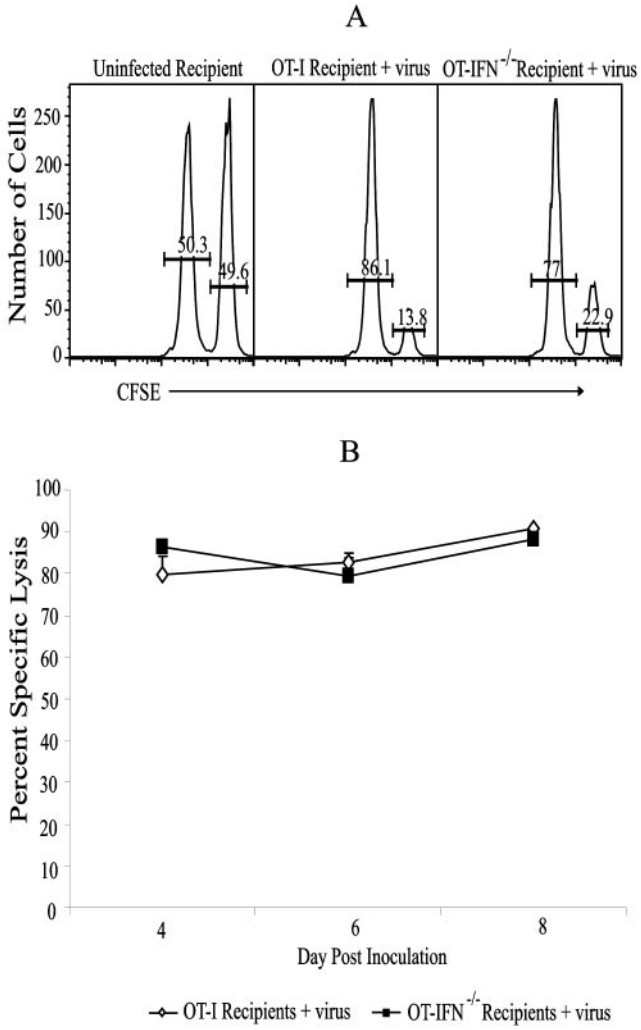

FIG. 5. Comparable cytolytic function in wild-type OT-I and OT-IFN$^{-/-}$ T cells. (A) OVA-specific cytolysis observed in recipients of OT-I and OT-IFN$^{-/-}$ T cells. Irradiated B6 mice receiving either OT-I or OT-IFN$^{-/-}$ T cells were challenged intravaginally with HSV-2 tk^{-} OVA. OVA-peptide-pulsed and control-treated CFSE-labeled target cells were injected into groups of 4 recipient mice on days 4, 6, and 8 after virus challenge. Spleens were removed, and the relative levels of peptide-pulsed and control targets were assessed by flow cytometry. Data shown are representative histograms from individual spleens on day 4 after challenge. (B) Kinetics of the OVA-specific cytolytic T-cell response. The percent specific lysis was calculated for spleen cells from four animals per group on the indicated days postinoculation as described in Materials and Methods. There was no statistical difference between groups on any day ($P > 0.05$).

the delayed resolution of the infection in normal CD8^{+} T-cell recipients reflected the time required for activation and expansion of an HSV-specific CD8^{+} T-cell response.

Important early role for IFN-γ in resolution of genital HSV-2 tk^{-} OVA infection. IFN-γ has been reported to play an important role in resolving HSV infections in some studies (30, 35) but not others (15). Two approaches were taken to determine the role of IFN-γ in clearance of HSV-2 tk^{-} OVA by OT-I T cells. First, IFN-γ was neutralized in recipient mice by daily injection of 2.0 mg specific antibody beginning 2 days prior to HSV-2 tk^{-} OVA challenge through the end of the experiment. Thymidine kinase-deficient strains of HSV-2 do not replicate well in neuronal tissue, and genital inoculation of such strains generally results in mild clinical disease (26). Consistent with these results, intravaginal inoculation of HSV-2 tk^{-} OVA only rarely resulted in clinical symptoms (erythema, swelling), even in the absence of T-cell-produced IFN-γ. However, anti-IFN-γ treatment affected clearance of infectious virus. As shown in Fig. 3A, HSV-2 tk^{-} OVA was not cleared through day 12, and virus titers were significantly higher ($P \leq 0.001$; ANOVA) on all days tested in anti-IFN-γ-treated mice compared to control immunoglobulin G-treated mice. In contrast, 75% of control immunoglobulin G-treated mice cleared virus by day 5 after challenge, and the remaining mice cleared virus on day 8 post-challenge. In an alternate approach, activated wild-type OT-I T cells or OT-I cells from mice genetically deficient in IFN-γ (OT-IFN$^{-/-}$) were adoptively transferred into irradiated recipients followed by intravaginal challenge with HSV-2 tk^{-}

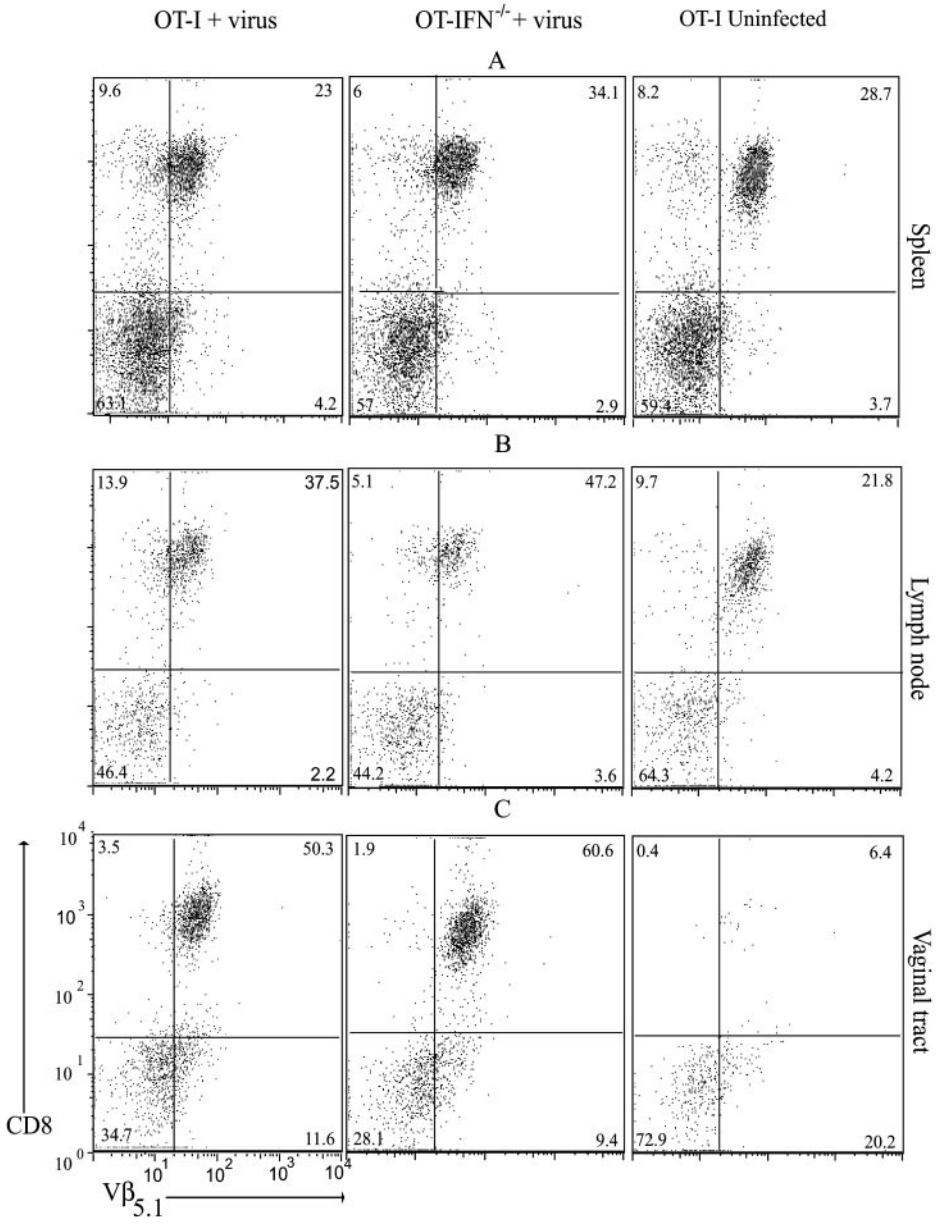

FIG. 6. Recruitment of OT-IFN$^{-/-}$ cells to the infected genital epithelium. Groups of 10 OT-I or OT-IFN$^{-/-}$ recipients were challenged intravaginally with HSV-2 tk$^-$ OVA, and one group of 10 OT-I recipients remained uninoculated as a control. On day 7 after inoculation, spleens, iliac lymph nodes, and vaginal tracts were harvested and stained for CD8 and Vβ_5 to detect donor transgenic T cells. Numbers listed on plots represent the percentage of cells in each quadrant.

OVA. Figure 3B shows that wild-type OT-I recipients resolved the infection by day 5 and mice receiving naïve B6 CD8$^+$ T cells cleared virus by day 10 after challenge. However, mice receiving activated OT-IFN$^{-/-}$ T cells were unable to clear virus through day 13, and virus titers were significantly higher ($P \leq 0.01$; ANOVA) in OT-IFN$^{-/-}$ recipients on all days tested compared to wild-type OT-I recipients. Mice receiving naïve CD8$^+$ T cells from IFN-$\gamma^{-/-}$ mice also did not clear the virus through day 13 (data not shown).

It was possible that OT-IFN$^{-/-}$ T cells failed to clear HSV-2 tk$^-$ OVA due to the secretion of inappropriate cytokines such as IL-4 or IL-5, a lack of cytolytic activity, or a failure to home to the infected genital epithelium. To determine the effector phenotype of these cells, OT-I and OT-IFN$^{-/-}$ cells were analyzed for cytokine production and cytolytic capability. As expected, wild-type OT-I CD8$^+$ T cells produced high levels of IFN-γ in response to the OVA-derived SIINFEKL peptide, whereas no IFN-γ was detected in OT-IFN$^{-/-}$ cell supernatants (Fig. 4A). As shown in Fig. 4B, both types of cells produced TNF-α, but IL-4 and IL-5 were detected only at background levels following stimulation with SIINFEKL peptide. The cytolytic function of wild-type OT-I and OT-IFN$^{-/-}$ cells was compared in irradiated recipients following intravaginal inoculation with HSV-2 tk$^-$ OVA using an in vivo cytolytic

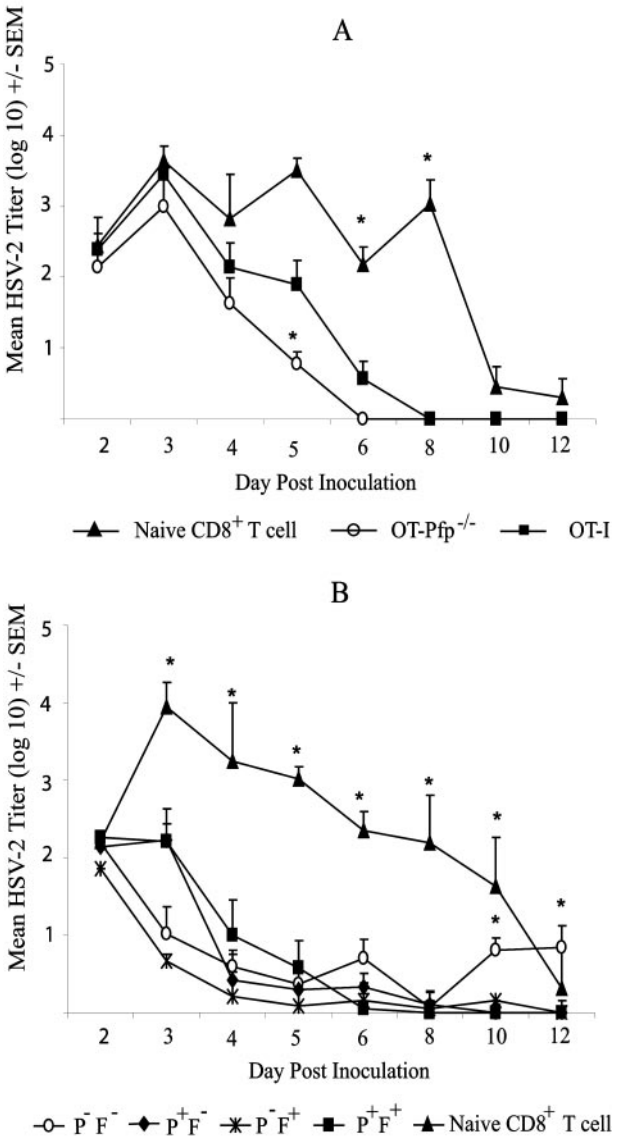

FIG. 7. Resolution of a genital infection with HSV-2 tk$^-$ OVA requires either perforin- or Fas-mediated cytolytic mechanisms. (A) OT-Pfp$^{-/-}$ cells clear an HSV-2 tk$^-$ OVA infection. Groups of at least 8 irradiated B6 mice were repopulated with naive B6 CD8$^+$ T cells, activated OT-Pfp$^{-/-}$ T cells, or activated OT-I T cells prior to inoculation with 5×10^3 PFU HSV-2 tk$^-$ OVA. Infectious virus was quantified on the indicated days. Data marked with an asterisk are significantly different compared to same-day values for OT-I recipients ($P < 0.01$). (B) Virus clearance in radiation chimeras. Groups of at least 8 irradiated Fas$^{-/-}$ or B6 (Fas$^+$) mice received activated OT-I or OT-Pfp$^{-/-}$ cells and were inoculated intravaginally with 5×10^3 PFU HSV-2 tk$^-$ OVA. Infectious virus was quantified by plaque assay on the indicated days after virus challenge. Values marked with an asterisk are significantly different ($P < 0.05$) compared to same-day values for P$^+$F$^+$ mice. Representative data from one of two identically designed studies performed are shown. SEM, standard errors of the means.

assay. As shown in Fig. 5A, OT-I and OT-IFN$^{-/-}$ T cells lysed SIINFEKL peptide-pulsed target cells but not antigen-free targets, and there were no significant differences in the level of OVA-specific lysis observed in the spleens of OT-I and OT-IFN$^{-/-}$ recipients on days 4, 6, and 8 following inoculation with HSV-2 tk$^-$ OVA (Fig. 5B).

It was also possible that the lack of T-cell-produced IFN-γ in OT-IFN$^{-/-}$ recipients affected the ability of effector T cells to home to and infiltrate the virus-infected vaginal epithelium. Following adoptive transfer of OT-I or OT-IFN$^{-/-}$ cells and inoculation of recipients with HSV-2 tk$^-$ OVA, secondary lymphoid organs and vaginal tracts were harvested, mechanically dissociated, and stained for the presence of CD8 and the Vβ_5 chain utilized in the OT-I T-cell receptor. Comparable percentages of OT-I and OT-IFN$^{-/-}$ cells were found in spleens and regional lymph nodes of recipient mice (Fig. 6A and B). Importantly, similar levels of OT-I and OT-IFN$^{-/-}$ cells were also found in the HSV-2 tk$^-$ OVA-infected vaginal tracts (Fig. 6C). In contrast, very few OT-I cells were detected in the vaginal epithelium of uninoculated OT-I recipients. Together, these data suggest that abrogation of clearance by CD8$^+$ T cells deficient in IFN-γ was due to loss of IFN-γ as an effector molecule and not due to a change in cytokine profile, deficient lytic function, or a lack of correct homing of these cells to the site of infection or secondary lymphoid tissues.

Role for lytic mechanisms in resolution of HSV-2 infection. We utilized CD8$^+$ T cells from OT-I mice lacking the perforin gene (OT-Pfp$^{-/-}$) to analyze the requirement for lytic mechanisms in clearance of HSV-2 from the vaginal epithelium by CD8$^+$ T cells. Similar to wild-type OT-I T cells, OT-Pfp$^{-/-}$ T cells produced IFN-γ and TNF-α, but IL-4 and IL-5 were not detectable above background levels (Fig. 4). Naïve B6 CD8$^+$ T cells or activated OT-I or OT-Pfp$^{-/-}$ T cells were adoptively transferred into irradiated B6 mice, and recipients were challenged intravaginally with HSV-2 tk$^-$ OVA. As shown in Fig. 7A, virus was not cleared by naïve B6 CD8$^+$ T cells until after day 12 postchallenge. OT-Pfp$^{-/-}$ CD8$^+$ T cells cleared HSV-2 tk$^-$ OVA from the vaginal epithelium on day 6 compared to day 8 by OT-I cells, and virus titers were significantly lower on day 5 in OT-Pfp$^{-/-}$ recipients ($P < 0.01$).

We used OT-Pfp$^{-/-}$ mice as donors in the construction of radiation chimeras to evaluate if virus clearance could be achieved in the absence of both perforin and Fas-FasL interaction. OT-I and OT-Pfp$^{-/-}$ cells were activated in culture and adoptively transferred into irradiated B6 (F$^+$) or Fas-defective (F$^-$) recipients, generating chimeric mice singly deficient in perforin (P$^-$F$^+$) or Fas (P$^+$F$^-$) or deficient in both perforin and Fas (P$^-$F$^-$). The resultant chimeras were then inoculated intravaginally with HSV-2 tk$^-$ OVA. A rapid reduction of virus

TABLE 1. Incidence of virus shedding in short-term radiation chimeras

Group	% Mice shedding virus on day postinoculation[a]:						
	2	4	6	8	10	12	15
P$^-$F$^-$	100	100	75	75	62.5	62.5	75
P$^-$F$^+$	100	50	12.5	25	12.5	12.5	0
P$^+$F$^-$	100	90	30	30	0	0	0
P$^+$F$^+$	100	100	62.5	12.5	0	0	0
B6 CD8$^+$ T-cell control	100	100	100	100	80	80	20

[a] Data are derived from the experiment described in Fig. 7B and are representative of two identical experiments performed. Incidence of virus shedding is expressed as the number of mice shedding virus in the vaginal epithelium on the indicated day divided by the total number of mice infected for each group.

titer was apparent on days 1 to 7 after challenge in all chimeric groups receiving activated T cells (Fig. 7B). Mice representing the wild-type phenotype (P^+F^+) cleared virus by day 8. P^-F^+ and P^+F^- mice were able to clear virus by day 10. In contrast, low levels of virus were still detectable in P^-F^- mice on day 15 after challenge. As shown in Table 1, the titers from these mice represented low-level shedding by the majority of recipients (75%) on day 15. Together, these data suggest that lytic mechanisms were required to completely resolve HSV-2 infection from the genital epithelium.

DISCUSSION

$CD8^+$ OT-I T cells were able to clear an engineered OVA-expressing HSV-2 virus in an antigen-specific manner from the genital epithelium. This clearance was abrogated by treatment of recipient mice with neutralizing anti-IFN-γ antibody or the use of donor $CD8^+$ T cells genetically deficient in IFN-γ. These results are consistent with our previous reports and those of others indicating an important role for IFN-γ in the resolution of herpes infections (6, 29–30, 35, 49, 54). It is possible that the insertion of OVA into the thymidine kinase locus diminished the pathogenicity of the HSV-2 tk^- OVA virus compared to the previously described HSV-2 tk^- virus (26). However, the results clearly demonstrate the antigen-specific nature of $CD8^+$ T-cell-mediated clearance of the OVA-expressing virus as well as the role of IFN-γ and lytic mechanisms in resolution of a vaginal infection.

IFN-γ has multiple functions that may be either directly or indirectly responsible for clearance of HSV-2. IFN-γ may promote processing and presentation of viral epitopes by up-regulating the expression of MHC class I proteins, proteasome subunits, and TAP1 and TAP2 proteins (2, 12, 33, 45). It is possible that the requirement for IFN-γ in the present studies solely reflected an IFN-γ-enhanced recognition and lysis of HSV-infected cells by $CD8^+$ T cells. However, this seems unlikely given that both P^-F^- and P^+F^+ chimeras exhibited a rapid and dramatic drop in virus titer during the first 6 days after virus challenge (Fig. 7B). This rapid decline in virus titer was not observed if IFN-γ was neutralized by specific antibody (Fig. 3A). Rather, these results are consistent with the possibility that IFN-γ acted as an effector molecule to clear HSV-2 from the genital epithelium either directly by inhibition of virus replication in epithelial cells or indirectly by activation of innate immune cells.

IFN-γ is known to activate multiple antiviral genes that inhibit viral replication. The IFN-inducible, RNA-dependent protein kinase R (PKR) has direct inhibitory effects on viral replication through the phosphorylation of the eukaryotic translation initiation factor eIF-2α, which in turn inhibits translation of host and viral mRNA (44, 46). IFN-γ can also promote the degradation of viral RNA through the induction of the enzymes 2′,5′ oligoadenylate synthetase and RNase L (37, 41) and can cause alterations in viral RNA by the replacement of adenosines with inosines by induction of double-stranded RNA-specific adenosine deaminase (4). As evidence of the importance of these antiviral mechanisms, HSV has evolved strategies to interfere with interferon-activated antiviral pathways. The HSV-1 $\gamma_1$34.5 and US11 gene products have been reported to block PKR-mediated phosphorylation of eIF-2α, allowing continued protein synthesis (13, 38). Further, HSV-1 has recently been shown to interfere with IFN-γ signaling pathways by decreasing intracellular levels of Jak 1 kinase (7). The extent to which these mechanisms serve as effective escape mechanisms in vivo during HSV-2 infection is uncertain, although the decreased virulence of HSV-1 $\gamma_1$34.5 deletion mutants in animals suggests at least some of these escape strategies are effective (5). IFN-γ has been shown to act synergistically with IFN-α/β to inhibit HSV replication (43). As these IFNs induce overlapping yet distinct sets of genes utilizing different signaling pathways, it is possible that the presence of both cytokines may overcome the virus-mediated interference. In this regard, it is possible that the requirement for IFN-γ in the present studies reflected a synergism between the innate IFNs and T-cell-produced IFN-γ, as suggested by Sainz and Halford in an HSV-1 model (43).

Following vaginal inoculation with HSV-2, large numbers of innate immune cells are recruited to the vaginal mucosa (27). Depletion of $Gr\text{-}1^+$ monocytes and neutrophils from either normal or HSV-immune mice resulted in a prolonged disease course, including shedding of significantly higher virus titers. These results suggest an important role for innate cells, in addition to T cells, in virus clearance and resolution of the infection (27, 31). Together with the results of the current study, these results also suggest that IFN-γ activation of recruited innate immune cells might be required to promote clearance of virus in addition to its effects on the vaginal epithelium. If so, virus clearance may be achieved, in part, by the induction of enzymes such as the inducible isoform of nitric oxide synthase (21) or indoleamine-2,3-dioxygenase (1, 3) in recruited monocytes and polymorphonuclear leukocytes. Alternatively, IFN-γ may be necessary only for the recruitment of innate immune cells to the vaginal vault. However, preliminary studies suggest there is no difference in recruitment of neutrophils and macrophages to the vaginal epithelium in the absence of IFN-γ (data not shown).

It may be possible that IFN-γ is necessary for homing of effector T cells. Secretion of IFN-γ by memory T cells has been shown to result in rapid recruitment of B and T lymphocytes to the vaginal mucosa (35). Further studies demonstrated an IFN-γ-dependent up-regulation of V-CAM and ICAM-1 on vascular endothelium and vaginal epithelium following intravaginal inoculation of immune mice, suggesting a possible mechanism for recruitment of T lymphocytes to the vagina during HSV-2 infection (36). In the current studies, OT-$IFN^{-/-}$ cells were observed to migrate to the secondary lymphoid tissues and more importantly to the infected vaginal epithelium similar to wild-type OT-I cells, suggesting that recruitment of T cells to the site of infection did not involve an absolute requirement for T-cell-produced IFN-γ. However, it is possible that NK cells surviving the nonlethal radiation dose used in these studies may have secreted sufficient IFN-γ to facilitate lymphocyte recruitment.

The involvement of specific T-cell-mediated cytolytic mechanisms in resolution of infection has been examined in a number of virus systems. Neither perforin- nor Fas-mediated cytolysis was required to clear vaccinia virus, vesicular stomatitis virus, or Semliki virus (18). Studies with lymphocytic choriomeningitis virus (19) and Theiler's virus (42) demonstrated that perforin-mediated cell lysis, but not Fas-FasL interaction,

was required for clearance of virus. Our previous studies demonstrated the presence of T cells with HSV-specific cytolytic function in the vaginal epithelium of HSV-2-infected mice at a time coincident with virus clearance (32). Consistent with the results of the current study, virus was cleared in these previous studies by either perforin-deficient or Fas-defective mice, suggesting that the presence of one mechanism could compensate for the loss of the other (32). In the present study, OT-Pfp$^{-/-}$ T-cell recipients cleared virus more rapidly than recipients of wild-type OT-I T cells, perhaps reflecting an increased proliferation of perforin-deficient T cells compared to wild-type cells in response to antigen (20). In the present experiments utilizing radiation chimeras lacking both perforin- and Fas-mediated lytic mechanisms, we were able to provide evidence for a role of T-cell-mediated cytolysis in resolution of the genital infection. These results are similar to those of Topham et al. (51), in which clearance of influenza virus from the lung was abrogated in short-term chimeras lacking both perforin- and Fas-mediated cytolytic mechanisms.

CD8$^+$ T-cell-dependent cytolysis can also be mediated through the production of TNF-α (40, 52). While TNF-α has been shown to inhibit HSV replication in vitro (1), it seems unlikely that this cytokine was responsible for resolution of the HSV-2 genital infection in the current studies, since the P$^-$F$^-$ chimeras were unable to clear virus completely, yet the donor OT-Pfp$^{-/-}$ cells used in the construction of this chimera were fully capable of TNF-α production. Additionally, neutralization of TNF-α by treatment of normal mice or irradiated OT-I recipients with specific antibody does not significantly alter clearance of virus from the genital epithelium (32 and data not shown).

Evidence that cytolytic mechanisms are an important immune component necessary for resolution of genital herpes infections can be inferred from the mechanisms that HSV has accumulated to prevent cell lysis. HSV-1, but not HSV-2, is able to block CTL-induced apoptosis through the production of glycoprotein J, which inhibits granzyme B and Fas-mediated cell lysis in vitro (16, 17). It is possible that the utility of Fas-mediated cytolysis to clear virus in the current studies may reflect an inability of HSV-2 to interfere with Fas-mediated killing. Studies have suggested that interaction of either HSV-1- or HSV-2-infected cells with HSV-specific CD8$^+$ T cells may interfere with T-cell release of cytotoxic granules or cytokine synthesis following signaling through the T-cell receptor (47). HSV also impairs the loading of viral peptides on MHC class I proteins, and thus recognition by HSV-specific CD8$^+$ T cells, by binding of the HSV ICP47 protein to the TAP molecule (14).

In summary, these data suggest a model requiring both IFN-γ- and T-cell-mediated cytolysis for complete clearance of HSV-2 from the vaginal epithelium. In the murine model of HSV-2 genital infection, innate immune mechanisms mediated by infiltrating monocytes and neutrophils and including release of IFN-γ by NK cells would arise early after challenge, contribute to virus clearance, and limit virus spread. Local IFN-γ levels would continue to increase following the recruitment and infiltration of antigen-specific T cells into the vagina, resulting in a rapid decrease in HSV-2 titers due to the induction of antiviral genes in vaginal epithelial cells and activation of infiltrating neutrophils and monocytes. Ultimately, the complete elimination of HSV-2-infected cells from the vaginal mucosa would require the action of antigen-specific cytotoxic T lymphocytes. The action of these T cells would be enhanced by augmented recognition of HSV-infected cells and increased cellular expression of Fas and Fas ligand in the presence of IFN-γ (53). In human herpetic lesions, local IFN-γ levels rise concurrently with the recruitment of CD4$^+$ T cells into the lesion (9, 22). In addition to direct antiviral effects, it is thought the local production of IFN-γ overcomes the inhibition of antigen presentation due to ICP47 binding to the TAP molecule (14), thus allowing recognition of HSV-infected cells by CD8$^+$ T lymphocytes. Resolution of herpetic lesions has been correlated with the presence of cytolytic T lymphocytes (10, 22); however, the role for cytolytic mechanisms in this process is not clear. The results of the current study support the idea that lesion resolution involves cognate recognition and cytolysis of HSV-infected cells by HSV-specific lymphocytes.

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