

## CD4<sup>+</sup> T Cells Are Required for the Priming of CD8<sup>+</sup> T Cells following Infection with Herpes Simplex Virus Type 1<sup>†</sup>

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**The role of CD4<sup>+</sup> helper T cells in modulating the acquired immune response to herpes simplex virus type 1 (HSV-1) remains ill defined; in particular, it is unclear whether CD4<sup>+</sup> T cells are needed for the generation of the protective HSV-1-specific CD8<sup>+</sup>-T-cell response. This study examined the contribution of CD4<sup>+</sup> T cells in the generation of the primary CD8<sup>+</sup>-T-cell responses following acute infection with HSV-1. The results demonstrate that the CD8<sup>+</sup>-T-cell response generated in the draining lymph nodes of CD4<sup>+</sup>-T-cell-depleted C57BL/6 mice and B6-MHC-II<sup>-/-</sup> mice is quantitatively and qualitatively distinct from the CD8<sup>+</sup> T cells generated in normal C57BL/6 mice. Phenotypic analyses show that virus-specific CD8<sup>+</sup> T cells express comparable levels of the activation marker CD44 in mice lacking CD4<sup>+</sup> T cells and normal mice. In contrast, CD8<sup>+</sup> T cells generated in the absence of CD4<sup>+</sup> T cells express the interleukin 2 receptor  $\alpha$ -chain (CD25) at lower levels. Importantly, the CD8<sup>+</sup> T cells in the CD4<sup>+</sup>-T-cell-deficient environment are functionally active with respect to the expression of cytolytic activity *in vivo* but exhibit a diminished capacity to produce gamma interferon and tumor necrosis factor alpha. Furthermore, the primary expansion of HSV-1-specific CD8<sup>+</sup> T cells is diminished in the absence of CD4<sup>+</sup>-T-cell help. These results suggest that CD4<sup>+</sup>-T-cell help is essential for the generation of fully functional CD8<sup>+</sup> T cells during the primary response to HSV-1 infection.**

Infection due to herpes simplex virus type 1 (HSV-1) results in a wide spectrum of clinical presentations depending on the host's age, the host's immune status, and the route of inoculation (47). HSV-1 typically causes mild and self-limited lesions on the orofacial areas or genital sites. However, the disease can be life-threatening, as in the case of neonatal and central nervous system infections (18). The host's immune responses, particularly CD8<sup>+</sup> T cells, play an important role in determining the outcome of HSV infections in both the natural human host (18, 19, 28) and experimental murine models (11, 43). Immunodepletion and adoptive transfer studies have demonstrated the role of CD8<sup>+</sup> T cells in reducing viral replication, resolving cutaneous disease, and providing overall protection upon rechallenge (6, 25, 26). CD8<sup>+</sup> T cells play a particularly important role in preventing infection of the peripheral nervous system (PNS) and the reactivation of latent virus from neurons in the sensory ganglia of infected mice (21, 24, 36). The mechanisms that CD8<sup>+</sup> T cells employ include gamma interferon (IFN- $\gamma$ ) production and functions associated with cytolytic granule content at the sites of primary infection (23, 31, 38). In the PNS of infected mice, the mechanisms primarily

involve IFN- $\gamma$  secretion (16, 20, 29), particularly against infected neurons expressing surface Qa-1 (41). Histopathological evidence from HSV-1-infected human ganglion sections show a large CD8<sup>+</sup>-T-cell infiltrate and the presence of inflammatory cytokines, suggesting that the presence of activated, effector memory cells within the PNS is important for maintaining HSV-1 latency in the natural human host (10, 42).

The generation of a robust CD8<sup>+</sup>-T-cell response is essential for the control of various infectious pathogens. Some studies suggest that a brief interaction with antigen-presenting cells (APCs) is sufficient for CD8<sup>+</sup>-T-cell activation and expansion into functional effectors (44). However, the magnitude and quality of the overall CD8<sup>+</sup>-T-cell response generated may be dependent on additional factors (49). Recent evidence suggests that CD4<sup>+</sup> T cells facilitate the activation and development of CD8<sup>+</sup>-T-cell responses either directly through the provision of cytokines or indirectly by the conditioning of dendritic cells (DC) (8, 48, 51). Those studies suggested that the latter mechanism is the dominant pathway, wherein CD4<sup>+</sup> T cells assist CD8<sup>+</sup>-T-cell priming via the engagement of CD40 ligand (CD154) on CD4<sup>+</sup> T cells and CD40 expressed on DC (4, 30, 33). This interaction results in the activation and maturation of DC, making them competent to stimulate antigen-specific CD8<sup>+</sup>-T-cell responses (35, 37).

The requirement for CD4<sup>+</sup>-T-cell help in the generation of primary and secondary CD8<sup>+</sup>-T-cell responses to antigen varies. Primary CD8<sup>+</sup>-T-cell responses to infectious pathogens, such as *Listeria monocytogenes*, lymphocytic choriomeningitis virus (LCMV), influenza virus, and vaccinia virus, can be mounted effectively independently of CD4<sup>+</sup>-T-cell help (3, 12, 22, 34). In contrast, primary CD8<sup>+</sup>-T-cell responses to nonmicrobial antigens display an absolute dependence on CD4<sup>+</sup>-T-

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<sup>†</sup> We dedicate this paper to our late colleague, Patrick Mitchell Smith (1956 to 2007).

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cell help (4, 5, 30, 33, 46). This observed difference in the requirement for CD4<sup>+</sup>-T-cell help may ultimately be a product of the initial inflammatory stimulus generated following immunization (49). Microbial antigens trigger an inflammatory response that can lead to the direct activation and priming of APCs, such as DC, thereby bypassing the need for CD4<sup>+</sup>-T-cell help. Nonmicrobial antigens, however, trigger an attenuated inflammatory response that does not directly activate and prime DCs. In the absence of this inflammation, CD4<sup>+</sup> T cells are thought to condition and license DC functions through CD154/CD40 interactions, which leads to the subsequent activation of antigen-specific CD8<sup>+</sup>-T-cell responses (5, 49). Even in the case of pathogens where primary CD8<sup>+</sup>-T-cell responses were independent of CD4<sup>+</sup>-T-cell help, the secondary responses to these pathogens were found to be defective in the absence of CD4<sup>+</sup>-T-cell help (3, 12, 34, 40).

The requirement for CD4<sup>+</sup>-T-cell help in priming CD8<sup>+</sup>-T-cell responses against HSV-1 infection is not well defined. Earlier studies with HSV-1 suggested that CD4<sup>+</sup> T cells play an important role in the generation of primary CD8<sup>+</sup>-T-cell responses, detected *in vitro*, to acute infection with HSV-1 (14), principally through the provision of interleukin 2 (IL-2) for optimal CD8<sup>+</sup>-T-cell differentiation and proliferation. Subsequent studies, utilizing an *in vivo* approach, indicated that CD4<sup>+</sup> T cells were not required for CD8<sup>+</sup>-T-cell-mediated cytolytic function (23). CD4<sup>+</sup> T cells are thought to provide help by conditioning DC in a cognate, antigen-specific manner, thereby making them competent to stimulate HSV-1-specific CD8<sup>+</sup>-T-cell responses (37). By contrast, findings from other studies show that CD4<sup>+</sup>-T-cell-depleted mice were able to fully recover from acute infection with HSV-1 (38). These studies imply that the absence of CD4<sup>+</sup> T cells does not prevent priming of CD8<sup>+</sup> T cells *in vivo*.

Studies from this laboratory have identified two distinct HSV-1-specific CD8<sup>+</sup>-T-cell subpopulations generated during the primary response, based upon the ability to synthesize IFN- $\gamma$  following antigenic stimulation *in vitro* (1). To better understand the need for CD4<sup>+</sup>-T-cell help, we examined the functional characteristics and phenotypes of these CD8<sup>+</sup>-T-cell populations generated during a primary response to acute infection with HSV-1 in mice lacking CD4<sup>+</sup> T cells. Our findings show that primary CD8<sup>+</sup>-T-cell responses to HSV-1 are compromised in the absence of CD4<sup>+</sup>-T-cell help. Specifically, the HSV-1 gB-specific CD8<sup>+</sup> T cells produced in the absence of CD4<sup>+</sup> T cells were found to be active with regard to cytolysis *in vivo* but were functionally impaired in the production of IFN- $\gamma$  and TNF- $\alpha$  compared with intact C57BL/6 mice. Virus-specific CD8<sup>+</sup> T cells were also reduced in number in CD4-depleted mice and in B6 mice lacking major histocompatibility complex (MHC) class II expression (B6-MHC-II<sup>-/-</sup>) compared to wild-type (WT) mice. In addition, our data showed higher virus burdens in the infectious tissues obtained from mice lacking CD4<sup>+</sup> T cells than in those from intact mice. Collectively, these findings demonstrate that CD4<sup>+</sup>-T-cell help is essential for the generation of primary CD8<sup>+</sup>-T-cell responses following acute cutaneous infection with HSV-1.

#### MATERIALS AND METHODS

**Mice.** Three- to five-week-old male C57BL/6 mice and B6-MHC-II<sup>-/-</sup> (C57BL/6-A<sup>b</sup> $\beta$ <sup>-/-</sup>) were purchased from Jackson Laboratories (Bar Harbor,

ME) and Taconic (Germantown, NY), respectively. The animals were maintained in pathogen-free animal facilities at the Louisiana State University Health Sciences Center (LSUHSC), Shreveport, LA, and Drexel University College of Medicine, Philadelphia, PA. Mice were used between 6 and 10 weeks of age.

**Virus.** HSV-1 strain Patton, originally obtained from Richard Tenser, Pennsylvania State University College of Medicine, Hershey, PA, was plaque purified four times on Vero cell monolayers and established as a stock by infection of Vero cells at a multiplicity of infection of 0.01. Virus present in culture supernatant and virus released from infected cells by a freeze-thaw cycle were pooled, titrated on Vero cell monolayers, and stored at -80°C before use.

**HSV-1 immunization of mice.** Mice were anesthetized by intraperitoneal (i.p.) injection of 60 mg/kg of sodium pentobarbital (Butler, Columbus, OH). Mice were then injected subcutaneously in each hind footpad (FP) with 2  $\times$  10<sup>6</sup> PFU HSV-1 in 50  $\mu$ l of phosphate-buffered saline (PBS). Mice were sacrificed 5 days following infection, and the draining popliteal lymph nodes (PLN) were isolated for analysis.

**Quantification of virus in the FP tissue.** Levels of infectious virus were determined in FP tissues as described previously (38). Briefly, mice were sacrificed 5 days postinfection (p.i.) with HSV-1, the FP surface was cleaned with 70% alcohol, and the tissue was removed with a 21-gauge scalpel. Tissues were homogenized in 1-ml glass tissue grinders (Wheaton, Millville, NJ) and centrifuged, and the cell-free homogenate was assayed at various dilutions on Vero cell monolayers in 12-well tissue culture plates overlaid with 0.5% methylcellulose. Plaques were visualized following fixation of the monolayers with 10% buffered formalin and staining with 0.5% crystal violet.

**Antibodies and reagents used for staining.** The following panel of monoclonal antibodies (MAbs) and reagents was used for phenotypic analysis of lymphocytes in the PLN: phycoerythrin (PE)-conjugated anti-CD8 $\alpha$  (clone 53-6.7; eBioscience, San Diego, CA), PE-TXR anti-CD8 $\alpha$  (5H10; Caltag, Burlingame, CA), fluorescein isothiocyanate (FITC)-conjugated anti-CD44 (IM7; eBioscience), biotin-conjugated anti-CD25 (PC 61; eBioscience), and allophycocyanin-Cy7-conjugated anti-CD25 (PC61; BD Biosciences, San Diego, CA). Flow-cytometric analysis was performed on a FACSVantage SE (Becton Dickinson [BD], San Jose, CA) in the research Core Facility at LSUHSC, Shreveport, LA, and a FACSCalibur (BD, San Jose, CA) in the Flow Cytometry Core Facility of the Department of Microbiology and Immunology, Drexel University College of Medicine. The data were analyzed using Flow Jo software (Tree Star, Ashland, OR).

**CD4<sup>+</sup>-T-cell depletion.** B6 mice were depleted of CD4<sup>+</sup> T cells by administering 300  $\mu$ g of anti-CD4 GK1.5 MAb i.p. at 3 days and 1 day before infection with HSV-1. Depletion of CD4<sup>+</sup> T cells was confirmed using flow-cytometric analysis (data not shown).

**CD8<sup>+</sup>-T-cell depletion.** B6 mice were depleted of CD8<sup>+</sup> T cells by administering 300  $\mu$ g of anti-CD8 $\alpha$  2.43 MAb i.p. at 3 days and 1 day before infection with HSV-1. Depletion was confirmed using flow-cytometric analysis (data not shown).

**Intracellular staining.** Lymphocytes were cultured for 5 h in 96-well U-bottom microtiter plates (Costar, Cambridge, MA) at a concentration of 1  $\times$  10<sup>6</sup> cells/well in 200  $\mu$ l of Iscove's modified Dulbecco's medium (American Type Culture Collection) supplemented with 10% fetal calf serum, 20 mM HEPES, 2 mM L-glutamine, 50  $\mu$ M  $\beta$ -mercaptoethanol, 20  $\mu$ g gentamicin sulfate (complete medium), and 1  $\mu$ l/ml brefeldin A (Golgi Plug; BD) in the presence of 10<sup>-5</sup> M HSV-1 glycoprotein B (gB) (<sup>498</sup>SSIEFARL<sup>505</sup>) peptide or 10<sup>-5</sup> M vesicular stomatitis virus (VSV) nucleoprotein (<sup>52</sup>RGYVYVYGL<sup>59</sup>) (LSUHSC Core Laboratories, New Orleans, LA). After 5 h, the cells were washed and surface stained with PE-conjugated anti-CD8 $\alpha$ , FITC-conjugated anti-CD44, and allophycocyanin-Cy7-conjugated anti-CD25 MAbs. After the unbound antibodies had been washed, intracellular cytokine staining was performed using a Cytotfix/CytoPerm kit as per the manufacturer's instructions (BD). For IFN- $\gamma$ /TNF- $\alpha$  staining, the cells were stained with allophycocyanin-conjugated rat anti-mouse IFN- $\gamma$  MAb (XMG 1.2; BD) and FITC-conjugated rat anti-mouse TNF- $\alpha$  MAb (MP6-XT22; BD). For perforin and granzyme B intracellular staining, lymphocytes were surface stained for CD8 expression. Cells were then washed, fixed, permeabilized, and stained for perforin and granzyme B using PE-conjugated rat anti-mouse perforin (JAW246; eBioscience) and FITC-conjugated rat anti-mouse granzyme B (16G6; eBioscience).

**In vivo cytolytic assay.** To prepare target cells for measurement of *in vivo* cytolysis, erythrocytes were removed from spleen cell suspensions from naive B6 mice using a lysis buffer (Pharm Lyse; BD, San Diego, CA). The cells were then washed and split into two populations. One population was pulsed with 10<sup>-5</sup> M HSV-1 gB viral peptide for a period of 75 min at 37°C and then labeled with 5.0  $\mu$ M carboxy fluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) (CFSE<sup>hi</sup>). The other population was pulsed with a nonspecific control VSV peptide and labeled with 0.5  $\mu$ M CFSE (CFSE<sup>lo</sup>). For intravenous (i.v.) injection, equal

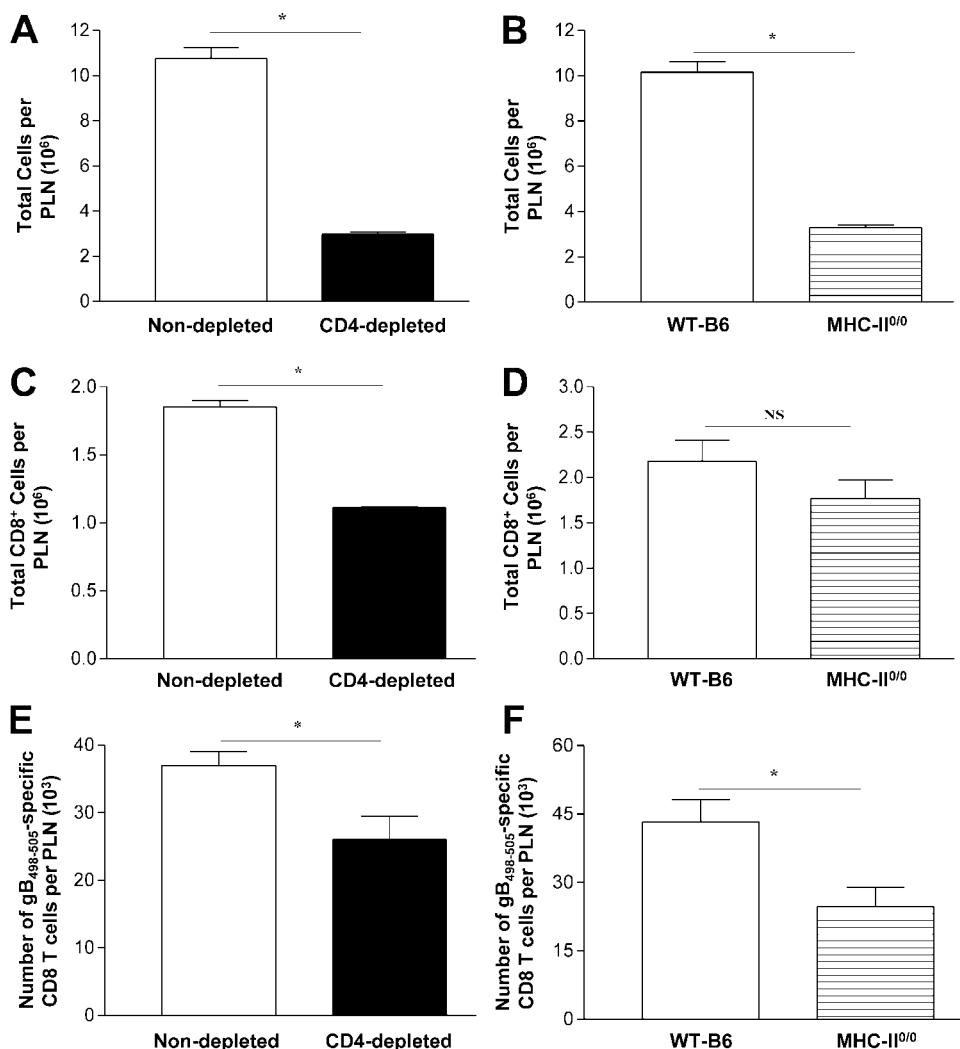


FIG. 1. Lymphocyte cellularity in the draining PLN following acute infection with HSV-1. C57BL/6 (WT) mice, CD4-depleted B6 mice, or B6-MHC-II<sup>-/-</sup> mice were infected in each hind FP with  $2 \times 10^6$  PFU HSV-1. Mice were sacrificed 5 days p.i., and the draining PLN cells were analyzed by flow cytometry. (A) Absolute number of HSV-1 gB-specific CD8<sup>+</sup> T cells (four mice/group; \*,  $P = 0.03$ ). (B) Absolute number of HSV-1 gB-specific CD8<sup>+</sup> T cells (three mice/group; \*,  $P = 0.04$ ). (C) Absolute number of total cells per PLN (four mice/group; \*,  $P < 0.0001$ ). (D) Absolute number of total cells per PLN (three mice/group; \*,  $P = 0.0001$ ). (E) Absolute number of CD8<sup>+</sup> T cells per PLN (four mice/group; \*,  $P < 0.0001$ ). (F) Absolute number of CD8<sup>+</sup> T cells per PLN (three mice/group;  $P = 0.2$ ). For panels A and B, lymph node cells were simultaneously stained with anti-CD8 $\alpha$  and H-2K<sup>b</sup>-gB<sub>498-505</sub> tetramers to identify HSV-1-specific CD8<sup>+</sup> T cells. Data are representative results from one of two independent experiments for each group (means  $\pm$  SEM).

numbers of cells from each population were mixed together such that each mouse received a total of  $15 \times 10^6$  cells in 300  $\mu$ l of PBS. Cells were injected into mice that had been infected 5 days earlier with HSV-1, as described above. The mice were sacrificed 4 h after adoptive transfer for their PLN. Lymph node cell suspensions were analyzed by flow cytometry, and each population was detected by its differential fluorescence intensities. The percent specific lysis was calculated by the following formula, as previously described (38):  $[1 - (\text{ratio uninfected}/\text{ratio infected})] \times 100$ , where ratio is percent CFSE<sup>lo</sup> cells/percent CFSE<sup>hi</sup> cells.

**Quantitative in vivo cytolytic assay.** To prepare target cells to measure in vivo cytotoxicity, erythrocytes were removed from spleen cell suspensions from naive B6 mice using a lysis buffer (Pharm Lyse; BD, San Diego, CA). Cells were washed and separated into four populations. Cells were pulsed with either  $10^{-6}$  M gB peptide or  $10^{-10}$  M gB peptide for 60 min at 37°C and then labeled with 5.0  $\mu$ M of either red fluorescent dye PKH26 (Sigma) or CFSE (Molecular Probes, Eugene, OR), respectively. The other populations were pulsed with a nonspecific control VSV peptide at  $10^{-6}$  M or  $10^{-10}$  M, and labeled with 0.5  $\mu$ M of PKH26 or CFSE. For i.v. injection, equal numbers of cells from each population were mixed together such that each mouse received a total of  $20 \times 10^6$  cells in 300  $\mu$ l

of PBS. Cells were injected into mice that had been infected 5 days earlier with HSV-1. The mice were sacrificed 3.5 h after adoptive transfer for their PLN. Lymph node cell suspensions were analyzed by flow cytometry and specific lysis was calculated as given above. This experiment was repeated at  $10^{-14}$  M and  $10^{-18}$  M peptide concentrations as well.

**Statistical analysis.** Statistical analyses were performed using unpaired Student's *t* test unless otherwise mentioned. Analyses were made using Prism 3 software, (Graph Pad, San Diego, CA). Probability (*P*) values of  $<0.05$  are considered significant. Unless otherwise stated, plotted data are means  $\pm$  standard errors of the mean (SEM).

## RESULTS

**The absence of CD4<sup>+</sup> T cells reduces the overall HSV-1-specific CD8<sup>+</sup>-T-cell response.** The primary response to cutaneous HSV-1 infection results in the activation and expansion

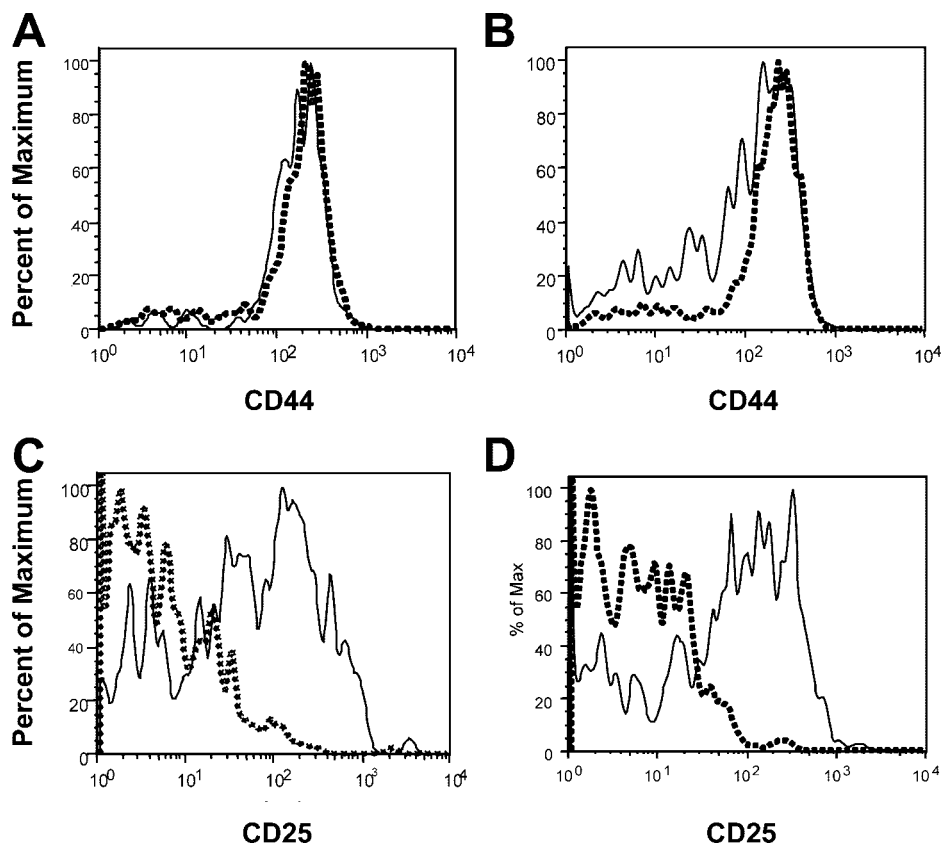


FIG. 2. Analysis of expression of activation markers on HSV-1 gB-specific CD8<sup>+</sup> T cells in CD4-depleted, B6-MHC-II<sup>-/-</sup>, and WT mice in response to acute infection with HSV-1. Draining PLN cells were obtained from WT, CD4-depleted, or B6-MHC-II<sup>-/-</sup> mice 5 days following infection of each hind FP with HSV-1 and stained using anti-CD8 $\alpha$ , anti-CD44, anti-CD25, and H-2K<sup>b</sup>-gB tetramers. (A and B) Expression of CD44 on HSV-1 gB-specific CD8<sup>+</sup> T cells in WT compared to CD4-depleted B6 mice (A) and WT compared to B6-MHC-II<sup>-/-</sup> mice (B). (C and D) Expression of CD25 in WT compared to CD4-depleted B6 mice (C) and WT compared to B6-MHC-II<sup>-/-</sup> mice (D). Solid lines, WT; dotted lines, CD4-depleted or B6-MHC-II<sup>-/-</sup> mice. Data are representative of two independent experiments for each group.

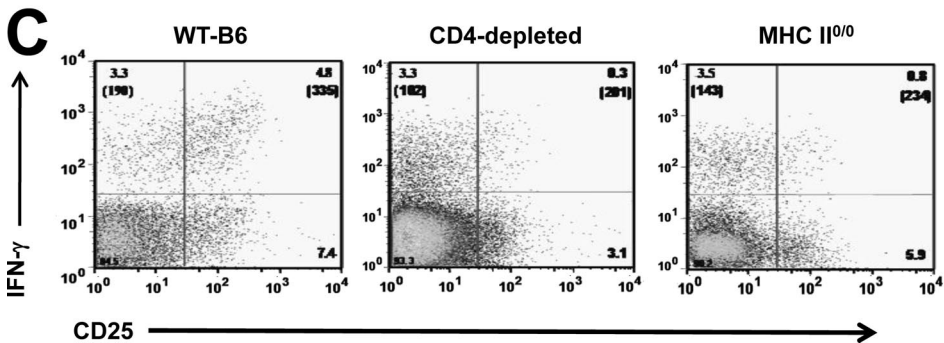
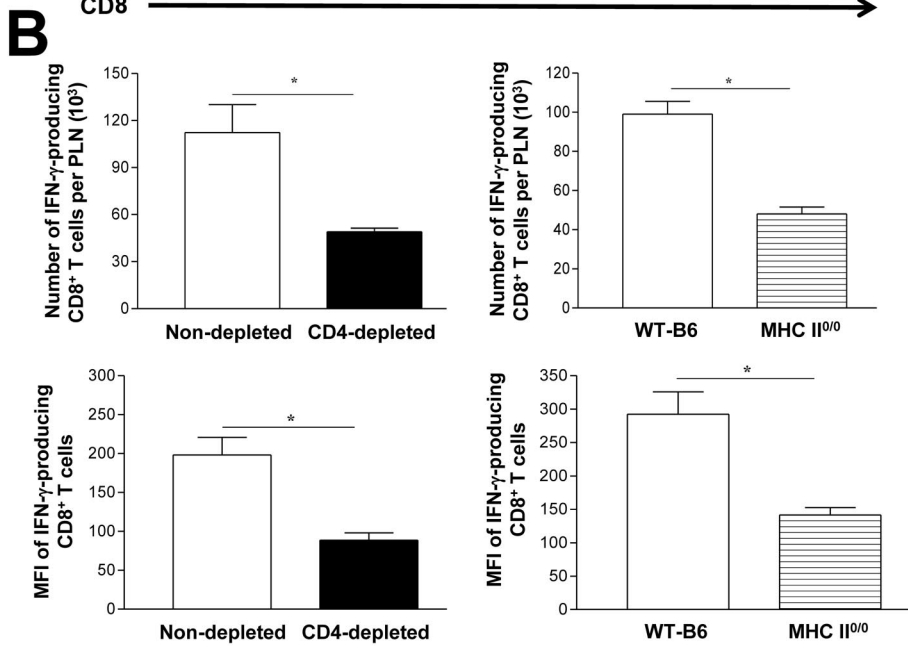
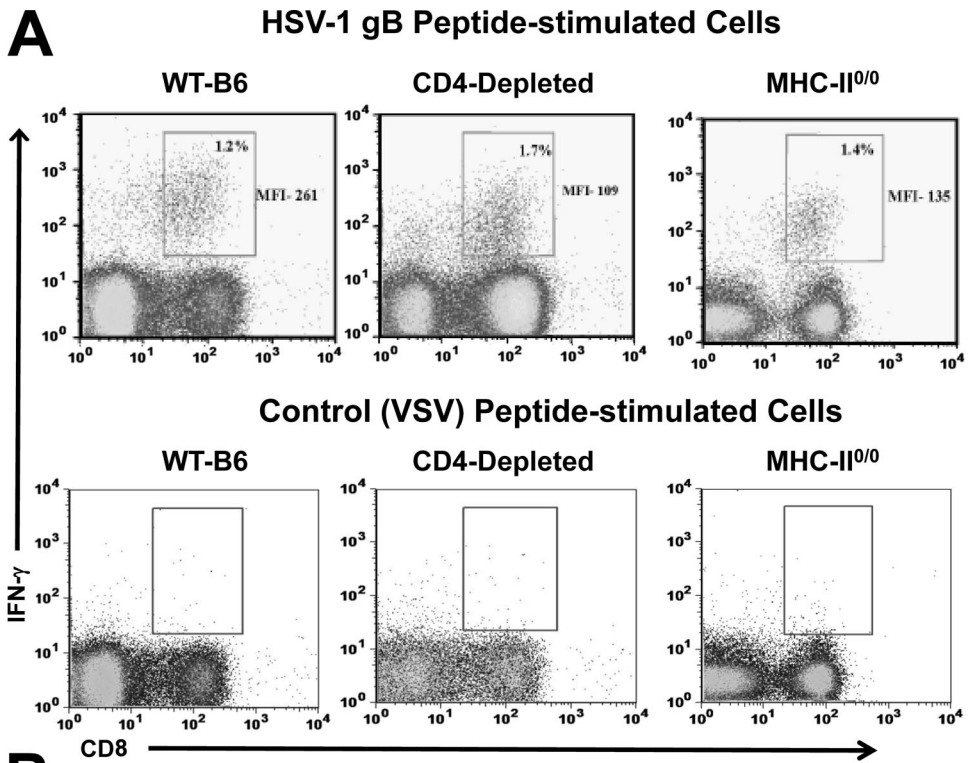
of two phenotypically defined CD8<sup>+</sup>-T-cell subpopulations, based upon the surface expression of CD25 (1). To determine the impact of the absence of CD4<sup>+</sup> T cells on each of the subpopulations, the characteristics of the CD8<sup>+</sup>-T-cell response in B6 mice subjected to depletion of CD4<sup>+</sup> T cells prior to infection and in B6 mice lacking class II MHC expression due to genetic knockout of the H-2A $\beta$  gene within the class II MHC locus (B6-MHC-II<sup>-/-</sup>) were examined. The draining PLN of CD4-depleted B6 mice, harvested on day 5 of infection, which corresponds to the peak of the response in WT B6 mice (9), were reduced in total cellularity compared to control HSV-1-infected B6 mice (Fig. 1C;  $P < 0.0001$ ). Essentially identical results were obtained for B6-MHC-II<sup>-/-</sup> mice (Fig. 1D;  $P < 0.0001$ ). The reduced cellularity likely reflected not only the absence of CD4<sup>+</sup> T cells themselves but also a reduction in the expansion of CD8<sup>+</sup> T cells. A comparison of absolute CD8<sup>+</sup>-T-cell numbers in CD4-deficient mice to those in WT B6 control mice confirmed a significant reduction in CD4-depleted mice (Fig. 1E;  $P < 0.0001$ ). While there was a reduction of total CD8<sup>+</sup> T cells in B6-MHC-II<sup>-/-</sup> mice, this was not significant compared to WT B6 mice (Fig. 1F;  $P < 0.2$ ). However, when the absolute numbers of CD8<sup>+</sup> T cells specific for the immunodominant H-2K<sup>b</sup>-binding epitope derived from gB (498<sup>SSIEFARL</sup>505) recognized in B6 mice were determined, a

significant reduction was observed in both CD4-depleted (Fig. 1A;  $P < 0.04$ ) and B6-MHC-II<sup>-/-</sup> (Fig. 1B,  $P < 0.03$ ) mice. These results indicated that the primary expansion of HSV-1 gB-specific CD8<sup>+</sup> T cells was reduced in the absence of CD4<sup>+</sup>-T-cell help.

**The phenotype of HSV-1-specific CD8<sup>+</sup> T cells in the CD4-deficient environment.** The expression of CD44 and CD25 on HSV-1 gB-specific CD8<sup>+</sup> T cells was quantified to determine the role of CD4<sup>+</sup> T cells in the generation of each subpopulation. CD44 expression was similar in CD4-depleted B6 and B6-MHC-II<sup>-/-</sup> mice, compared to control B6 mice (Fig. 2A and B). In contrast, the expression of CD25 was substantially reduced in both CD4-depleted B6 and B6-MHC-II<sup>-/-</sup> mice compared to control B6 mice (Fig. 2C and D). These findings indicated that the absence of CD4<sup>+</sup>-T-cell help shifted the primary HSV-1 gB-specific CD8<sup>+</sup> T cells toward the CD8<sup>+</sup> CD25<sup>lo</sup> T-cell response as a consequence of a pronounced reduction in the CD8<sup>+</sup> CD25<sup>hi</sup> T-cell subpopulation.

**The absence of CD4<sup>+</sup> T cells reduces HSV-1-specific CD8<sup>+</sup>-T-cell cytokine synthesis.** The functional capacity of HSV-1-specific CD8<sup>+</sup> T cells, generated in the presence or absence of CD4<sup>+</sup> T cells in vivo, was examined through the determination of IFN- $\gamma$  (Fig. 3) and TNF- $\alpha$  (Fig. 4) synthesis in response to stimulation in vitro with a synthetic peptide corresponding to





the immunodominant HSV-1 gB peptide, compared to a control, H-2K<sup>b</sup>-binding peptide derived from VSV NP. It was found that the absence of CD4<sup>+</sup> T cells did not prevent IFN- $\gamma$  synthesis by HSV-1 gB-specific CD8<sup>+</sup> T cells (Fig. 3A) but resulted in a 50 to 60% downward shift in the mean fluorescence intensity (MFI) of the IFN- $\gamma$  signal in both CD4-deficient environments (Fig. 3B, lower panels). The absolute number of HSV-1 gB-specific CD8<sup>+</sup> T cells able to synthesize IFN- $\gamma$  was also significantly reduced in CD4-depleted and B6-MHC-II<sup>-/-</sup> mice compared to control B6 mice (Fig. 3B, upper panels). Importantly, the synthesis of IFN- $\gamma$  by HSV-1 gB-specific CD8<sup>+</sup> T cells was almost entirely due to CD8<sup>+</sup> CD25<sup>lo</sup> T cells in both CD4-deficient environments, compared to control B6 mice (Fig. 3C). These results indicated that the absence of CD4<sup>+</sup> T cells resulted not only in a reduction in the absolute numbers of IFN- $\gamma$  synthesizing HSV-1 gB-specific CD8<sup>+</sup> T cells, but also in a pronounced shift in the phenotype of the major CD8<sup>+</sup>-T-cell subpopulation responsible for IFN- $\gamma$  synthesis. The analysis was expanded to include TNF- $\alpha$  synthesis by HSV-1 gB-specific CD8<sup>+</sup> T cells. These results indicated that CD4 depletion caused a profound reduction in the detection of TNF- $\alpha$  synthesis by CD8<sup>+</sup> T cells (Fig. 4A), which corresponded to a decrease in both the absolute numbers of TNF- $\alpha$  synthesizing CD8<sup>+</sup> T cells and the MFI of the TNF- $\alpha$  signal detected in HSV-1 gB-specific CD8<sup>+</sup> T cells (Fig. 4B). In control B6 mice, the majority of HSV-1 gB-specific CD8<sup>+</sup> T cells synthesized both IFN- $\gamma$  and TNF- $\alpha$  (Fig. 4C, left). Depletion of CD4<sup>+</sup> T cells appeared to have a greater impact upon TNF- $\alpha$  synthesis by CD8<sup>+</sup> T cells than upon IFN- $\gamma$ , resulting in a higher percentage of CD8<sup>+</sup> T cells producing IFN- $\gamma$  alone and a pronounced reduction in TNF- $\alpha$  synthesis, compared to control B6 mice (Fig. 4C, right). Taken together, these findings demonstrated an important role for CD4<sup>+</sup> T cells in the optimal cytokine synthetic response to acute cutaneous HSV-1 infection.

**The absence of CD4<sup>+</sup> T cells does not impair CD8<sup>+</sup>-T-cell-mediated CTL function in vivo.** In addition to cytokine synthesis, CD8<sup>+</sup> T cells utilize direct target cell recognition and lysis to control virus infection. Therefore, the *in vivo* cytolytic capacity of CD8<sup>+</sup> T cells from CD4-depleted and B6-MHC-II<sup>-/-</sup> mice was assessed using a CFSE-based assay to determine the requirement of CD4<sup>+</sup> T cells for the induction of the primary HSV-1-specific cytotoxic-T-lymphocyte (CTL) responses. In these experiments, B6 spleen cells pulsed with HSV-1 gB peptide expressed high levels of CFSE (CFSE<sup>hi</sup>), while B6 spleen cells pulsed with control H-2K<sup>b</sup>-binding VSV peptide expressed low levels of CFSE (CFSE<sup>lo</sup>). In an assay assessing *in vivo*

lysis 4 h after injection of the labeled target cells, it was found that little target cell lysis was observed in uninfected B6 mice, while HSV-1 gB-pulsed targets were completely lysed in HSV-1-infected B6 mice (Fig. 5A). Similar levels of HSV-1 gB-pulsed target lysis were observed in both CD4-depleted and B6-MHC-II<sup>-/-</sup> mice (Fig. 5A). Depletion of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells eliminated the lysis of HSV-1 gB-pulsed target cells (Fig. 5B), which confirmed that the lysis of the peptide-pulsed target cells was mediated entirely by the CD8<sup>+</sup>-T-cell subpopulation. These findings indicated that the expression of HSV-1-specific CTL activity *in vivo* was independent of functional CD4<sup>+</sup> T cells. While this finding is consistent with other studies that have analyzed the lysis of HSV-1 gB-pulsed target cells *in vivo* (37), our results differ from previous studies analyzing the cytolytic activity of HSV-1-specific CTL cultured *in vitro* (14). Because target cell lysis was essentially 100% by 4 h after introduction of the target cells into the infected animals, differences between the control and CD4-deficient mice may have been overlooked. However, while the lysis of HSV-1 gB-pulsed target cells was lower in both control and CD4-depleted B6 mice at 1 and 2 h compared to 4 h posttransfer (Fig. 6), there was no significant difference in the levels of lysis between the two groups. Therefore, it was concluded that CD4<sup>+</sup> T cells were not required for the optimal expression of HSV-1-specific CTL function *in vivo*.

A second possibility is that high surface density of the H-2K<sup>b</sup>-binding HSV-1 gB peptide may mask a potential role for CD4<sup>+</sup> T cells in the generation of the optimal HSV-1-specific CD8<sup>+</sup>-T-cell response. In this scenario, even if the CD4-independent CTL response is qualitatively inferior, this would be offset by the potential of a lower-avidity CTL to interact with the target cells due to the artificially high surface density of the MHC-peptide target structure. To address this question, the CFSE-based *in vivo* CTL assay was modified to include two distinct lipophilic dyes. This method enabled the simultaneous examination of target cells pulsed with two peptide concentrations. Briefly, target cells were pulsed with different concentrations of the immunodominant HSV-1 gB peptide. Pulsed cells were then labeled with the lipophilic dye CFSE or the PKH26 red fluorescent cell linker dye (gB-CFSE<sup>hi</sup> and gB-PKH26<sup>hi</sup>, respectively). VSV-pulsed control target populations were labeled with a lower concentration of the appropriate dyes (VSV-CFSE<sup>lo</sup> and VSV-PKH26<sup>lo</sup>). Target cells were then mixed at a ratio of 1:1:1 and injected into B6 mice such that each animal received a total of  $2 \times 10^7$  cells. Using this approach, no difference in killing capacity was detected between CD4-depleted and nondepleted mice at any of

FIG. 3. Assessment of IFN- $\gamma$ -producing HSV-1 gB-specific CD8<sup>+</sup> T cells during acute infection with HSV-1 in CD4-depleted, B6-MHC-II<sup>-/-</sup>, and WT mice. Mice were infected with HSV-1 in the hind FP, and the draining PLN were harvested on day 5 p.i. The lymph node cells were stimulated *in vitro* with gB peptide or control (VSV) peptide in the presence of brefeldin A for 5 h. The stimulated lymphocytes were then surface stained for the expression of CD8 and CD25, fixed, permeabilized, and stained for intracellular expression of IFN- $\gamma$ . (A) Representative dot plots show the frequency of CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells in the draining lymph nodes. Numbers in the gated regions are the percentages of CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells. Numbers beside the boxed area indicate the MFI for IFN- $\gamma$  staining within the gated region. (B) (Top panels) Absolute numbers of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells (four mice/group; \*,  $P = 0.01$  [left]; three mice/group; \*,  $P = 0.002$  [right]). (Bottom panels) MFI of IFN- $\gamma$  staining by gB-specific CD8<sup>+</sup> T cells in the gated region (four mice/group; \*,  $P = 0.004$  [left]; three mice/group; \*,  $P = 0.01$  [right]). (C) Representative histograms showing expression of IFN- $\gamma$  and CD25 on gated CD8<sup>+</sup> T cells. Numbers in the quadrants are percentages of CD8<sup>+</sup> cells which are IFN- $\gamma$ <sup>+</sup> CD25<sup>-</sup> (quadrant 1) or IFN- $\gamma$ <sup>+</sup> CD25<sup>+</sup> (quadrant 2). Numbers in parentheses are the MFI for IFN- $\gamma$  staining. Data are representative of two independent experiments (means  $\pm$  SEM).  $P$  values of  $<0.05$  were considered significant.

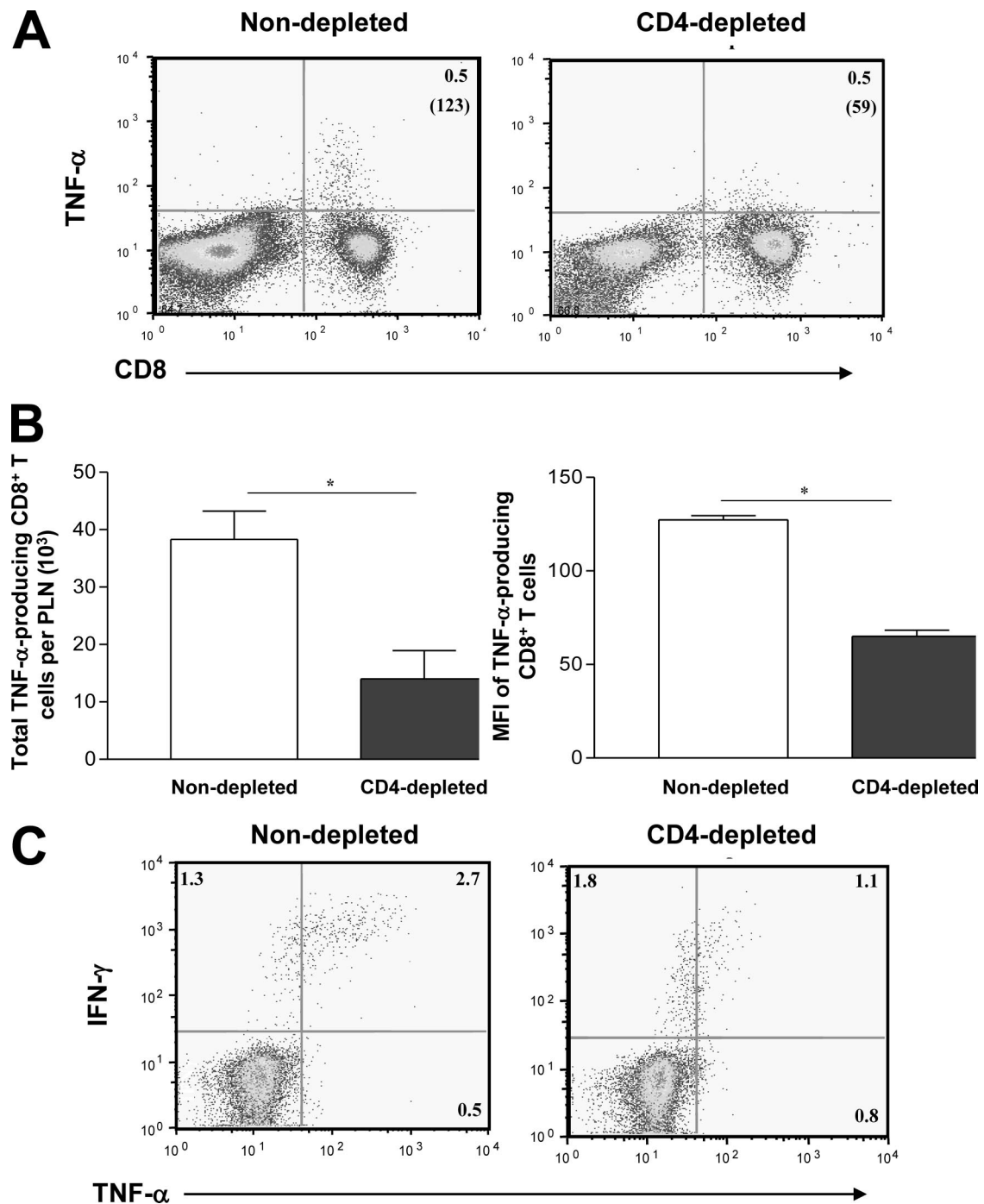


FIG. 4. Analysis of TNF- $\alpha$ -producing HSV-1 gB-specific CD8<sup>+</sup> T cells following acute infection with HSV-1. WT and CD4-depleted mice were infected with HSV-1 in the FP, and draining PLN cells were harvested on day 5 p.i. and stimulated *in vitro* with gB peptide in the presence of brefeldin A for 5 h. The stimulated lymphocytes were then surface stained for the expression of CD8 and CD25, fixed, permeabilized, and stained for intracellular expression of TNF- $\alpha$  and IFN- $\gamma$ . (A) Representative dot plots show the frequency of CD8<sup>+</sup> TNF- $\alpha$ <sup>+</sup> T cells in the draining lymph nodes. Numbers are the percentages of CD8<sup>+</sup> TNF- $\alpha$ <sup>+</sup> T cells. Numbers in parentheses are the MFI for TNF- $\alpha$  staining. (B) (Left) Absolute number of TNF- $\alpha$ -producing CD8<sup>+</sup> T cells (three mice/group; \*,  $P = 0.02$ ). (Right) MFI of TNF- $\alpha$  staining by CD8<sup>+</sup> T cells (three mice/group; \*,  $P < 0.0001$ ). Data shown are representative of two independent experiments (means  $\pm$  SEM). (C) Representative histograms show percentages of gated CD8<sup>+</sup> T cells which express IFN- $\gamma$  only (upper left quadrant), IFN- $\gamma$  and TNF- $\alpha$  (upper right quadrant), or TNF- $\alpha$  only (lower right quadrant).  $P$  values of  $<0.05$  were considered significant.

the peptide concentrations examined (Fig. 7A and B), which further supported the conclusion that CD4<sup>+</sup>-T-cell help was not necessary for optimal CTL functions. Notably, it was observed that killing of cells pulsed with HSV-1 gB-peptide was detected at

concentrations of  $10^{-6}$  M and  $10^{-10}$  M. Lysis was reduced at a gB peptide concentration of  $10^{-14}$  M, and no lysis was observed at  $10^{-18}$  M. (Fig. 7B). Therefore, it was possible to modify the surface density of H-2K<sup>b</sup>-binding gB peptide to demonstrate that

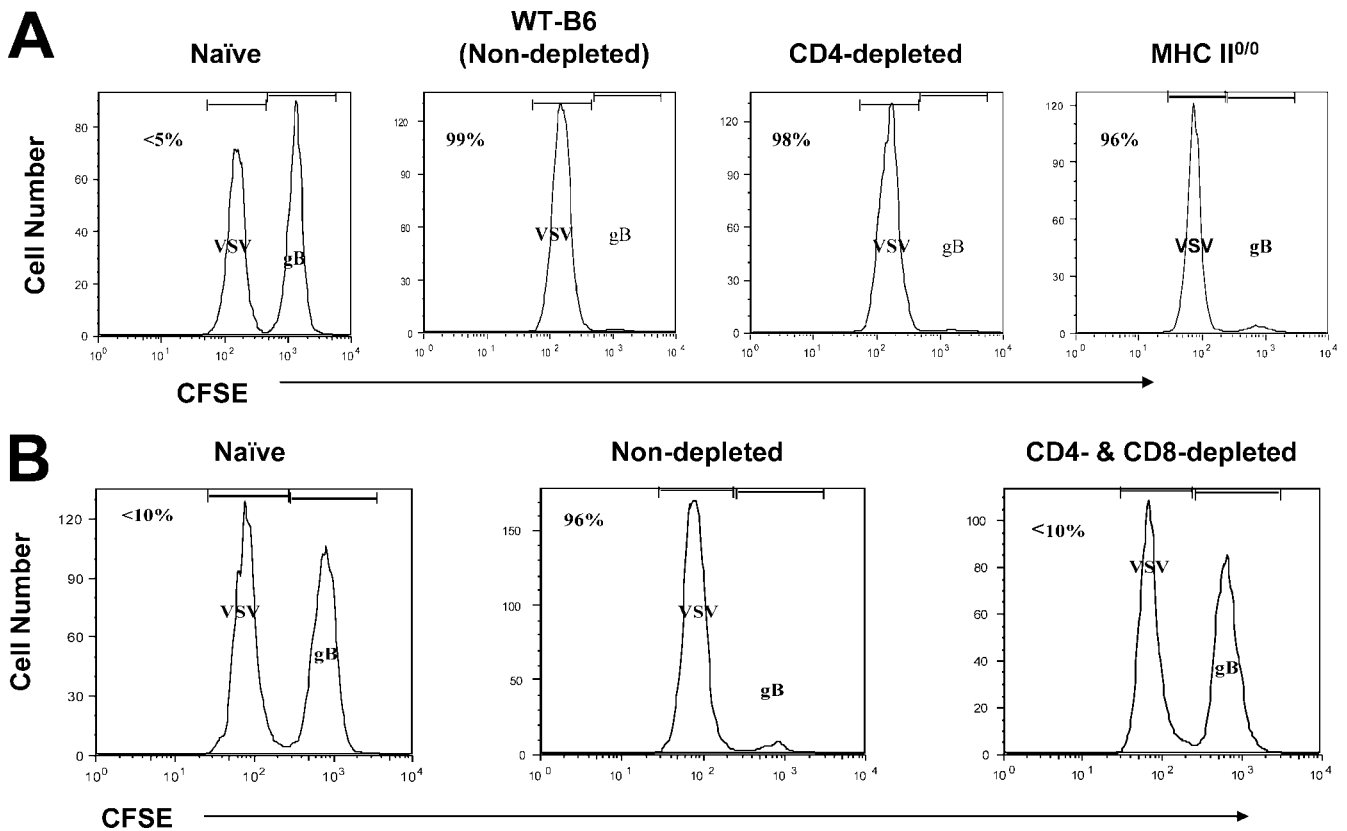


FIG. 5. Analysis of in vivo cytolytic activity in WT, CD4-depleted, and B6-MHC-II<sup>-/-</sup> mice. WT, CD4-depleted, and B6-MHC-II<sup>-/-</sup> mice were infected in each hind FP with  $2 \times 10^6$  PFU HSV-1 and examined for HSV-1-specific cytolytic function in vivo using a 4-h CFSE-based assay as described in Materials and Methods. For acute responses to HSV-1 infection, cytolytic activity was measured on day 5 p.i., which corresponds to the peak of the response in the draining PLN. (A) Representative histogram plots of lymph node cells obtained from naive control and day 5 infected WT, CD4-depleted, and B6-MHC-II<sup>-/-</sup> mice 4 h after transfer of CFSE-labeled target cells. Numbers in the plots are percent specific lysis of target cells. (B) Histograms show lysis of CFSE-labeled target cells in naive control and day 5 infected WT and CD4/CD8-depleted mice 4 h after transfer. Numbers in the plots are percent specific lysis of target cells.

a critical threshold of target density was required for recognition and lysis of the target cells, but no difference was detected whether CD4<sup>+</sup> T cells were present or absent.

**Synthesis of perforin and granzyme B is not affected in CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup>-T-cell help.** The perforin-granzyme B pathway is considered the primary lytic mechanism utilized by CD8<sup>+</sup> T cells to kill virus-infected cells. Therefore, intracellular straining was employed to determine the influence of CD4<sup>+</sup> T cells on the synthesis of lytic granules by CD8<sup>+</sup> T cells from WT mice, CD4-depleted B6 mice and MHC class II<sup>-/-</sup> mice infected with HSV-1 (5 days p.i.). The results showed a comparable level of perforin (Fig. 8A) and granzyme B (Fig. 8B) expression in the CD8<sup>+</sup>-T-cell populations from CD4-depleted, MHC class II<sup>-/-</sup> and intact WT mice. These results further confirm that CD8<sup>+</sup>-T-cell cytolytic activity is not dependent on CD4<sup>+</sup>-T-cell help.

**Higher virus levels in B6-MHC-II<sup>-/-</sup> and CD4-depleted mice.** Because efficient HSV-1 clearance from the infected tissues is dependent on competent CD8<sup>+</sup>-T-cell responses (38), the levels of the infectious virus in the FP tissues of mice were determined 5 days following cutaneous infection with HSV-1. The results showed significantly higher levels of infectious virus in the FP tissues in CD4-depleted ( $P < 0.01$ ) and B6-MHC-II<sup>-/-</sup> ( $P <$

0.03) mice than in control B6 mice (Fig. 9). Taken together, these findings suggest that the ability of HSV-1-specific CD8<sup>+</sup> T cells to produce optimal levels of antiviral cytokines is more important than their ability to recognize and kill infected cells in the control of HSV-1 infection in the skin.

## DISCUSSION

In this study, we examined the functions and phenotypes of CD8<sup>+</sup> T cells generated in the absence of CD4<sup>+</sup>-T-cell help following acute infection with HSV-1. The findings show that CD4<sup>+</sup>-T-cell help is required for the efficient induction of primary CD8<sup>+</sup>-T-cell responses to HSV-1. Our results suggest that phenotypically distinct populations of CD8<sup>+</sup> T cells develop in the absence of CD4<sup>+</sup>-T-cell help. CD8<sup>+</sup> T cells produced in CD4-depleted or CD4-deficient mice (B6-MHC-II<sup>-/-</sup>) expressed levels of CD44 similar to those in WT B6 mice. In contrast, expression of CD25 (the IL-2 receptor alpha [IL-2R $\alpha$ ]) was significantly reduced in CD4-depleted and B6-MHC-II<sup>-/-</sup> mice compared to WT mice. The lower expression of CD25 on CD8<sup>+</sup> T cells in CD4-depleted mice and B6-MHC-II<sup>-/-</sup> mice is likely a reflection of decreased levels of IL-2 (51) or the absence of optimal DC activation (30, 37).



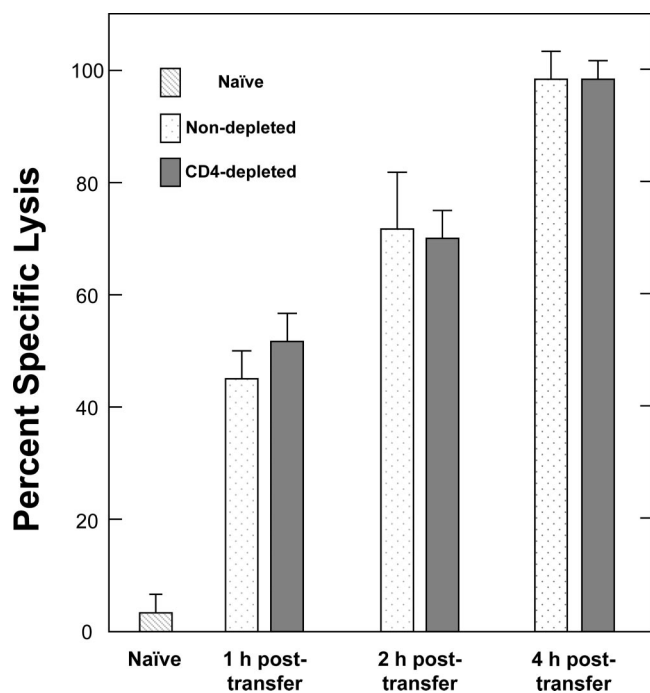


FIG. 6. Kinetic analysis of in vivo cytolytic activity in WT and CD4-depleted mice. WT, CD4-depleted, and B6-MHC-II<sup>-/-</sup> mice were infected in each hind FP with  $2 \times 10^6$  PFU HSV-1 and examined for HSV-1-specific cytolytic function in vivo using a CFSE-based assay as described in Materials and Methods. The presence of CFSE<sup>hi</sup> and CFSE<sup>lo</sup> cells remaining 1, 2, and 4 h after i.v. transfer of target cells was determined. No significant difference was apparent between the two experimental groups at any time point examined.

Specifically, studies using short-term in vitro assays suggest that DCs isolated from HSV-1-infected B6-MHC-II<sup>-/-</sup> mice are compromised in their ability to stimulate CD8<sup>+</sup>-T-cell proliferation and induce the upregulation of IL-2R (37). It is entirely possible that the reduction of the CD25<sup>hi</sup> CD8<sup>+</sup> T cells within the lymphoid tissues of mice lacking CD4<sup>+</sup> T cells, as defined in this study, may be due to an enhanced migration of this subpopulation into the infected cutaneous tissues or to enhanced apoptosis of the subpopulation as it develops in the absence of sufficient IL-2. These possibilities will be addressed in future studies.

At the peak of the primary response to HSV-1, the numbers of HSV-1-specific CD8<sup>+</sup> T cells were lower in mice lacking CD4<sup>+</sup> T cells compared with WT mice. Furthermore, the absence of CD4<sup>+</sup> T cells reduced the frequency of IFN- $\gamma$  producing HSV-1 gB-specific CD8<sup>+</sup> T cells during the primary response. These results are consistent with a recent study (37). Importantly, our studies revealed that CD8<sup>+</sup> T cells from CD4<sup>+</sup>-T-cell-depleted mice and B6-MHC-II<sup>-/-</sup> mice produced much less IFN- $\gamma$  on a per-cell basis than their WT counterparts. Our findings also indicate lower levels of TNF- $\alpha$ -producing CD8<sup>+</sup> T cells in CD4-depleted B6 mice than in WT mice. These results show that CD4<sup>+</sup>-T-cell help is crucial for the proliferation and function of virus-specific CD8<sup>+</sup> T cells during the primary phase of response to HSV-1. This is in sharp contrast to acute infection with infectious pathogens such as *Listeria* and LCMV, where a significant proportion of

antigen-specific CD8<sup>+</sup> T cells retained their capability to produce IFN- $\gamma$  in the absence of CD4<sup>+</sup>-T-cell help during the primary phase of infection (12, 40, 45). It is likely that additional factors or stimulatory signals provided by CD4<sup>+</sup> T cells are required for optimal IFN- $\gamma$  synthesis in the case of HSV-1 infection. It is interesting to note that a higher percentage of CD8<sup>+</sup> CD25<sup>-</sup> cells produced IFN- $\gamma$  in CD4-depleted and B6-MHC-II<sup>-/-</sup> mice than in WT mice. Although CD8<sup>+</sup> CD25<sup>-</sup> T-cell populations are capable of producing IFN- $\gamma$  (1), our findings suggest that they have a lower capacity to produce this cytokine than the CD8<sup>+</sup> CD25<sup>+</sup>-T-cell subpopulation. These results suggest a close correlation between CD25 expression and IFN- $\gamma$  production on a per-cell basis.

An interesting point arising from these and other studies (37) is the apparent dichotomy between the CD4<sup>+</sup>-T-cell independence of HSV-1-specific CTL function in vivo and the CD4<sup>+</sup>-T-cell dependence of HSV-1-specific CTL function in vitro (14). This suggests the possibility of two distinct CTL populations being measured in each of these circumstances. It has long been known that the expression of HSV-1-specific CTL activity in vitro requires ex vivo culture for 2 to 3 days (27). However, CD8<sup>+</sup> T cells from immunologically intact B6 mice expressing high levels of CD25 mediate CTL activity directly ex vivo (23), indicating that this T-cell subpopulation has become fully activated in vivo. It was argued that CD8<sup>+</sup> T cells, programmed to become cytolytic, were likely to emigrate rapidly from the lymph node (23). The in vitro culture period may have retained these cells and allowed them to complete their activation, and the completion of the activation process was dependent upon functional CD4<sup>+</sup> T cells, presumably through the continued availability of IL-2 in culture (23). It is possible that the latter population is identical to the "armed" CD8<sup>+</sup> T cells that leave the lymph node and take up residence in the spleen following cutaneous HSV-1 infection (9). This population is likely to retain the CD25<sup>-</sup> phenotype within the spleen (1). The fact that CD4<sup>+</sup> T cells are not an absolute requirement for HSV-1-specific CTL function in vivo suggests that alternative sources of IL-2, such as NK cells, play a more important role in vivo. Alternatively, HSV-1-specific CD8<sup>+</sup> T cells may respond to cytokines other than IL-2 in vivo to achieve optimal CTL activity. Finally, the in vivo CTL assay may measure a wider range of cytolytic functions than the in vitro assay. With respect to the latter point, our studies have demonstrated that the in vitro assay detected perforin-dependent cytolytic functions only, while the in vivo assay detected both perforin-dependent and -independent cytolytic functions (N. K. Rajasagi et al., unpublished data). It should be noted that the measurement of cytokine synthesis is performed upon lymphocytes present within the regional lymph nodes, while the cytolytic activity is determined by the ability of effector cells to recognize the target cells that have migrated into the lymphoid tissues. Therefore, the apparent requirement for CD4<sup>+</sup> T cells in the generation of cytokine synthesis in CD8<sup>+</sup> T cells, as opposed to their requirement for cytolytic function in vivo, may reflect differences in the location of the effector cells at the time of assay and their ability to traffic in vivo.

A key observation from these studies is that low expression of IL-2R did not affect cytolysis in vivo or the intracellular expression of perforin and granzyme B, suggesting that the expression of cytolytic function in vivo may not be tightly reg-

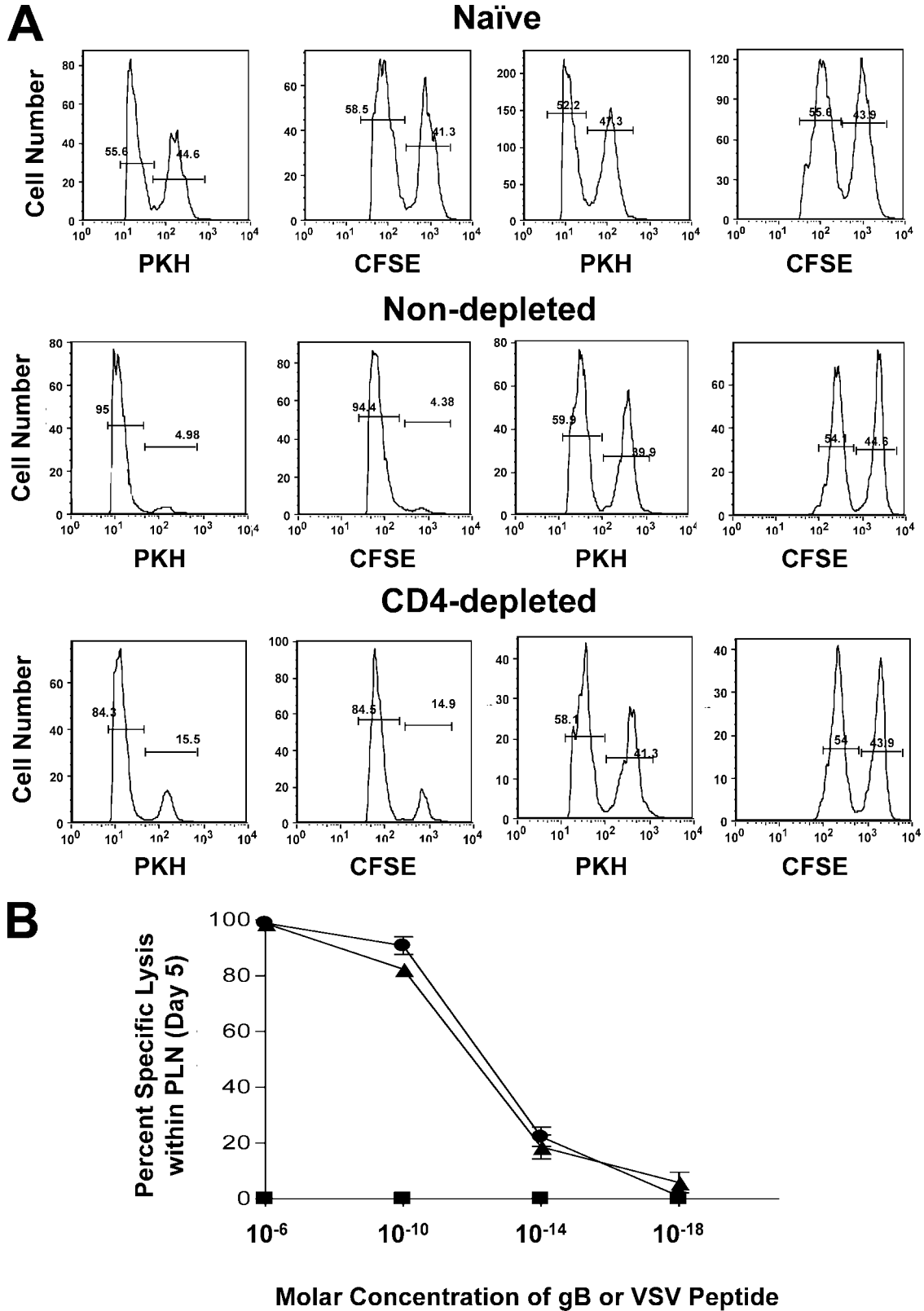


FIG. 7. CD4 depletion does not affect CTL activity in vivo. (A) Representative histograms from the PLN of naïve, CD4-depleted, and nondepleted B6 mice, 5 days post-HSV-1 infection. (B) Percent specific lysis as a function of peptide concentration (means  $\pm$  SEM). Percent specific lysis was calculated as described in the text. Squares, naïve mice; triangles, CD4-depleted mice; circles, nondepleted mice. Statistical analysis using the Wilcoxon sum of ranks test revealed no significant differences in the percentage of HSV-1-specific lysis between CD4-depleted and nondepleted mice at any of the concentrations examined.

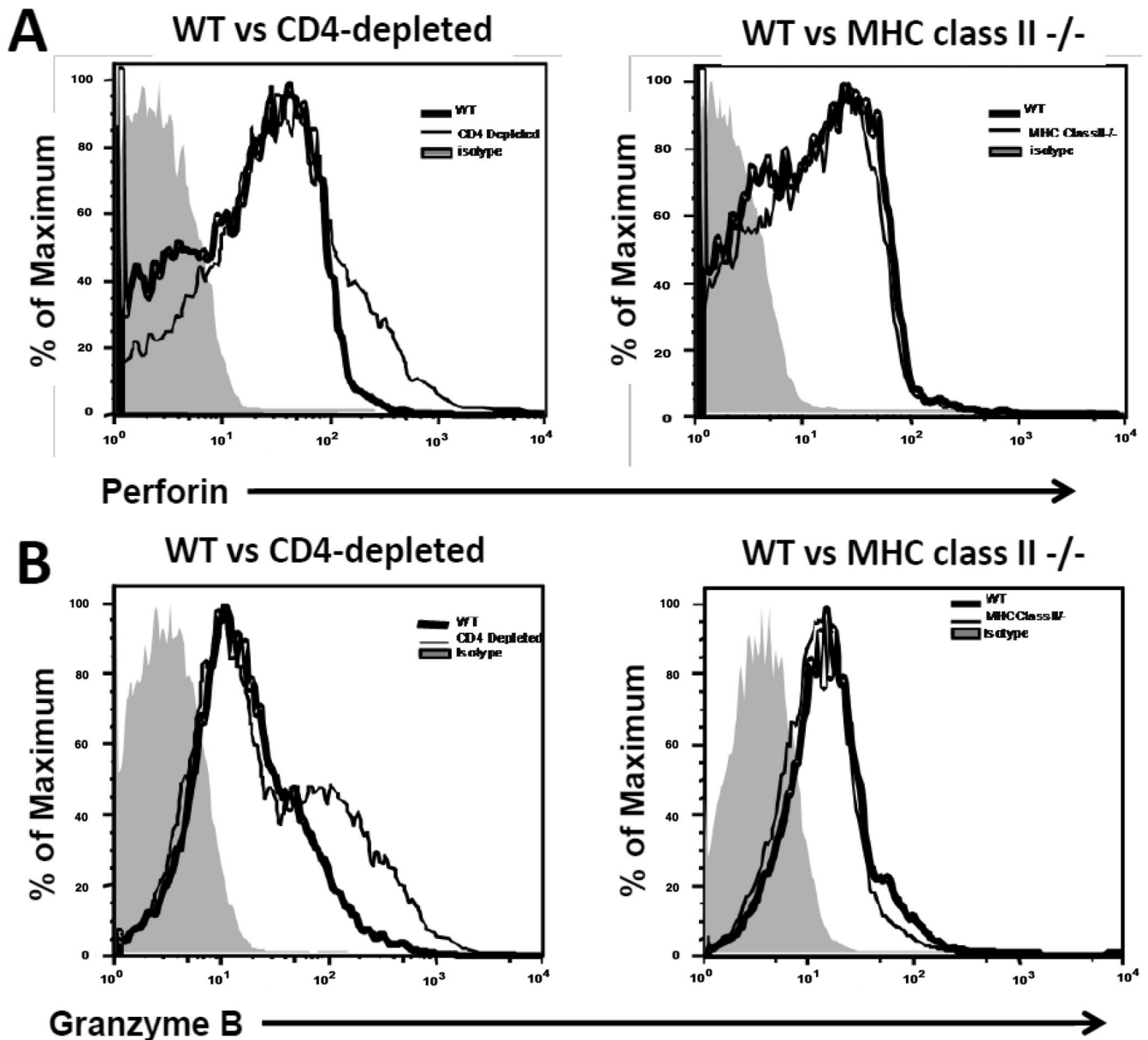


FIG. 8. Perforin and granzyme B levels in  $CD8^+$  T cells were not affected in mice lacking  $CD4^+$  T cells during primary responses to HSV-1 infection. Draining cells from CD4-depleted B6, MHC class II $^{-/-}$ , and WT mice were harvested on day 5 p.i. with HSV-1 and stained for expression of CD8, perforin, and granzyme B as described in Materials and Methods. Expression of perforin (A) and granzyme B (B) on  $CD8^+$  T cells is shown. Data are representative of two independent experiments for each group.

ulated by CD25 expression. This observation is supported by the fact that gB-specific cytolytic activity was found in the spleen in the absence of CD25 expression on HSV-1-specific  $CD8^+$  T cells, indicating that the continued expression of CD25 is not required for the expression of cytolytic function *in vivo* (9). By contrast, reports of *in vitro* studies advocate an important role for IL-2 and IL-2R signaling in the expression of perforin and granzyme B genes (13, 52). On the other hand, our data suggest the possible involvement of other mechanisms *in vivo* in the regulation of lytic granule levels in response to viral infections, which may become more important in the absence of  $CD4^+$ -T-cell help.

The findings also show higher viral loads in  $CD4^+$ -T-cell-depleted mice and B6-MHC-II $^{-/-}$  mice than in WT mice. It is important to note that mice selectively depleted of  $CD4^+$  T cells were able to recover from cutaneous HSV-1 infection, with a slight delay in the total clearance of the virus (38). These studies also suggest that the clearance of the virus in CD4-depleted mice is  $CD8^+$ -T-cell mediated, as *in vivo* depletion of  $CD4^+$  and  $CD8^+$  T cells resulted in the inability of these mice to clear the virus. Therefore, despite the reduced primary  $CD8^+$ -T-cell response to HSV-1 infection, the phenotypically different pools of HSV-1-specific  $CD8^+$  T cells that are generated in the absence of  $CD4^+$ -T-cell help seem to be sufficient

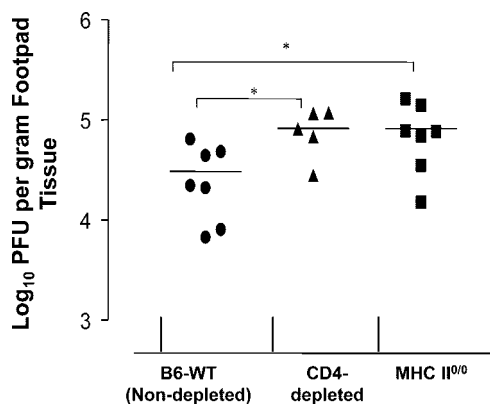


FIG. 9. Increased viral titers in CD4-depleted and B6-MHC-II<sup>-/-</sup> mice. Virus levels in the FP tissues of WT, CD4-depleted, and B6-MHC-II<sup>-/-</sup> mice were determined by virus plaque assay of samples from five to seven mice per group at 5 days after infection with HSV-1. \*,  $P = 0.01$  ( $n = 5$ ) for CD4-depleted versus WT mice and  $0.03$  ( $n = 7$ ) for B6-MHC-II<sup>-/-</sup> versus WT mice. Each symbol represents one individual animal. Horizontal bars indicate means. A  $P$  value of  $<0.05$  is considered significant.

to resolve the cutaneous infection, even though their functional quality is reduced. The expression of cytolytic function at the sites of infection must be considered an important mechanism in clearing the infectious virus. On the other hand, previous studies suggest that IFN- $\gamma$  plays an important role, both in the resolution of cutaneous infection (38) and preventing reactivation of the latent virus (21, 16). Therefore, it is important to address the potential effects of reduced IFN- $\gamma$  production in the recovery process, especially in preventing dissemination of the virus to the PNS.

It is not known why primary CD8<sup>+</sup>-T-cell responses to HSV-1 show a greater dependence on CD4<sup>+</sup>-T-cell help than responses to viruses such as LCMV, influenza virus, vaccinia virus, ectromelia virus, or murine gammaherpesvirus 68, which can generate robust primary CD8<sup>+</sup>-T-cell responses in the absence of CD4<sup>+</sup>-T-cell help (2, 3, 7, 12, 34). There are several plausible reasons for the differential requirement of CD4<sup>+</sup>-T-cell help, including the ability of the pathogen to directly activate APCs through pathogen-derived stimuli (5). Studies by Smith et al. suggest that in the case of HSV-1, CD4<sup>+</sup>-T-cell help is essential for the activation of DC, thereby making them competent to stimulate CD8<sup>+</sup>-T-cell responses (37). Nonetheless, as the bulk of expansion of the HSV-1-specific CD8<sup>+</sup> T cells takes place outside the lymphoid compartment (9), cytokines such as IL-2 produced by CD4<sup>+</sup> T cells may play an important role in improving the magnitude of the CD8<sup>+</sup>-T-cell response to viral infections (15, 51). Also, IL-2 was found to be important for promoting IFN- $\gamma$  production by CD8<sup>+</sup> T cells (15). In addition, a more recent study suggests that IL-2 signals during the priming stage are required for the secondary expansion of memory CD8<sup>+</sup> T cells (50). Data presented here suggest that CD4<sup>+</sup> T cells most likely provide help through the provision of IL-2, since they are the main source of IL-2 in vivo (32, 39, 50). Furthermore, our recent studies demonstrate an important role for IL-2 in the induction of primary CD8<sup>+</sup>-T-cell responses following infection with HSV-1 (Rajasagi et al., unpublished).

Overall, these findings suggest that CD8<sup>+</sup>-T-cell populations that develop in the absence of CD4<sup>+</sup>-T-cell help are phenotypically different from the CD8<sup>+</sup> T cells produced in normal WT mice during primary response to acute infection with HSV-1. The data show diminished primary CD8<sup>+</sup>-T-cell responses to HSV-1 in mice lacking CD4<sup>+</sup> T cells. Notably, CD8<sup>+</sup> T cells produced in the absence of CD4<sup>+</sup>-T-cell help expressed normal cytolytic activity in vivo but were functionally impaired in the production of IFN- $\gamma$  and TNF- $\alpha$ . Although the unhelped CD8<sup>+</sup> T cells appear to resolve acute HSV-1 infection (38), it is important to examine whether the CD8<sup>+</sup> T cells maintained in the absence of CD4<sup>+</sup>-T-cell help will be able to provide long-term protection in the case of a persistent virus like HSV-1. This aspect is particularly important, as recent studies suggest that the formation of memory CD8<sup>+</sup>-T-cell precursors is affected in the absence of CD4<sup>+</sup> T cells (37).

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#### REFERENCES

- Andersen, H., D. Dempsey, R. Chervenak, and S. R. Jennings. 2000. Expression of intracellular IFN-gamma in HSV-1-specific CD8<sup>+</sup> T cells identifies distinct responding subpopulations during the primary response to infection. *J. Immunol.* **165**:2101–2107.
- Belz, G. T., H. Liu, S. Andreansky, P. C. Doherty, and P. G. Stevenson. 2003. Absence of a functional defect in CD8<sup>+</sup> T cells during primary murine gammaherpesvirus-68 infection of I-A (b<sup>-/-</sup>) mice. *J. Gen. Virol.* **84**:337–341.
- Belz, G. T., D. Wodarz, G. Diaz, M. A. Nowak, and P. C. Doherty. 2002. Compromised influenza virus-specific CD8<sup>+</sup>-T-cell memory in CD4<sup>+</sup>-T-cell-deficient mice. *J. Virol.* **76**:12388–12393.
- Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393**:478–480.
- Bevan, M. J. 2004. Helping the CD8+ T-cell response. *Nat. Rev. Immunol.* **4**:595–602.
- Bonneau, R. H., and S. R. Jennings. 1989. Modulation of acute and latent herpes simplex virus infection in C57BL/6 mice by adoptive transfer of immune lymphocytes with cytolytic activity. *J. Virol.* **63**:1480–1484.
- Buller, R. M., K. L. Holmes, A. Hugin, T. N. Frederickson, and H. C. D. Morse. 1987. Induction of cytotoxic T-cell responses *in vivo* in the absence of CD4 helper cells. *Nature* **328**:77–79.
- Carvalho, L. H., G. Sano, J. C. Hafalla, A. Morrot, M. A. Curro de Lafaille, and F. Zavala. 2002. IL-4-secreting CD4<sup>+</sup> T cells are crucial to the development of CD8<sup>+</sup> T-cell responses against malaria liver stages. *Nat. Med.* **8**:166–170.
- Coles, R. M., S. N. Mueller, W. R. Heath, F. R. Carbone, and A. G. Brooks. 2002. Progression of armed CTL from draining lymph node to spleen shortly after localized infection with HSV-1. *J. Immunol.* **168**:834–838.
- Derfuss, T., S. Segerer, S. Herberger, I. Sinicina, K. Hüfner, K. Ebel, H. G. Knaus, I. Steiner, E. Meinel, K. Dornmair, V. Arbusow, M. Strupp, T. Brandt, and D. Theil. 2007. Presence of HSV-1 immediate early genes and clonally expanded T-cells with a memory effector phenotype in human trigeminal ganglia. *Brain Pathol.* **17**:389–398.
- Holterman, A.-X., K. Rogers, K. Edelmann, D. M. Koelle, L. Corey, and C. B. Wilson. 1999. An important role for major histocompatibility complex class I-restricted T cells, and a limited role for gamma interferon, in protection of mice against lethal herpes simplex virus infection. *J. Virol.* **73**:2058–2063.
- Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4<sup>+</sup> T cells are required for secondary expansion and memory in CD8<sup>+</sup> T lymphocytes. *Nature* **421**:852–856.
- Janus, M. L., P. Groves, N. Kienzle, and A. Kelso. 2005. IL-2 regulates perforin and granzyme gene expression in CD8<sup>+</sup> T cells independently of its effects on survival and proliferation. *J. Immunol.* **175**:8003–8010.
- Jennings, S. R., R. H. Bonneau, P. M. Smith, R. M. Wolcott, and R. Chervenak. 1991. CD4-positive T lymphocytes are required for the generation of the primary but not the secondary CD8-positive cytolytic T lymphocyte



- response to herpes simplex virus in C57BL/6 mice. *Cell. Immunol.* **133**:234–252.
15. **Kaech, S. M., and R. Ahmed.** 2001. Memory CD8<sup>+</sup> T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol.* **2**:415–422.
  16. **Khanna, K. M., R. H. Bonneau, P. R. Kinchington, and R. L. Hendricks.** 2003. Herpes simplex virus-specific memory CD8<sup>+</sup> T cells are selectively activated and retained in latently infected sensory ganglia. *Immunity.* **18**: 593–603.
  17. Reference deleted.
  18. **Koelle, D. M., and L. Corey.** 2008. Herpes simplex: insights on pathogenesis and possible vaccines. *Annu. Rev. Med.* **59**:381–395.
  19. **Koelle, D. M., C. M. Posavad, G. R. Barnum, M. L. Johnson, J. M. Frank, and L. Corey.** 1998. Clearance of HSV-2 from recurrent genital lesions correlates with infiltration of HSV-specific cytotoxic T lymphocytes. *J. Clin. Investig.* **101**:1500–1508.
  20. **Liu, T., K. M. Khanna, B. N. Carriere, and R. L. Hendricks.** 2001. Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. *J. Virol.* **75**:11178–11184.
  21. **Liu, T., K. M. Khanna, X. Chen, D. J. Fink, and R. L. Hendricks.** 2000. CD8 (+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *J. Exp. Med.* **191**:1459–1466.
  22. **Marzo, A. L., V. Vezys, K. D. Klonowski, S. J. Lee, G. Muralimohan, M. Moore, D. F. Tough, and L. Lefrancois.** 2004. Fully functional memory CD8 T cells in the absence of CD4 T cells. *J. Immunol.* **173**:969–975.
  23. **McNally, J. M., H. Andersen, R. Chervenak, and S. R. Jennings.** 1999. Phenotypic characteristics associated with the acquisition of HSV-specific CD8 T-lymphocyte-mediated cytolytic function in vitro. *Cell. Immunol.* **194**: 103–111.
  24. **Nash, A. A.** 2000. T cells and the regulation of herpes simplex virus latency and reactivation. *J. Exp. Med.* **191**:1455–1458.
  25. **Nash, A. A., K. N. Leung, and P. Wildy.** 1985. The T-cell mediated immune response of mice to herpes simplex virus, p. 87–102. *In* B. Roizman and C. Lopez (ed.), *The herpesviruses*, vol. 4. Plenum Publishing Corp., New York, NY.
  26. **Nugent, C. T., R. M. Wolcott, R. Chervenak, and S. R. Jennings.** 1994. Analysis of the cytolytic T-lymphocyte response to herpes simplex virus type 1 glycoprotein B during primary and secondary infection. *J. Virol.* **68**:7644–7648.
  27. **Pfizenmaier, K., H. Jung, A. Starzinski-Powitz, M. Rollinghoff, and H. Wagner.** 1977. The role of T cells in anti-herpes simplex virus immunity. I. Induction of antigen-specific cytotoxic T lymphocytes. *J. Immunol.* **119**:939–944.
  28. **Posavad, C. M., D. M. Koelle, and L. Corey.** 1998. Tipping the scales of herpes simplex virus reactivation: the important responses are local. *Nat. Med.* **4**:381–382.
  29. **Prabhakaran, K., B. S. Sheridan, P. R. Kinchington, K. M. Khanna, V. Decman, K. Lathrop, and R. L. Hendricks.** 2005. Sensory neurons regulate the effector functions of CD8 (+) T cells in controlling HSV-1 latency *ex vivo*. *Immunity* **23**:515–525.
  30. **Ridge, J. P., F. Di Rosa, and P. Matzinger.** 1998. A conditioned dendritic cell can be a temporal bridge between a CD4<sup>+</sup> T-helper and a T-killer cell. *Nature* **393**:474–478.
  31. **Rouse, B. T., and H. Wagner.** 1984. Frequency of herpes simplex virus-specific cytotoxic T lymphocytes precursors in lymph node cells of infected mice. *Immunology.* **51**:57–64.
  32. **Schoenberger, S. P., and E. M. Janssen.** 2006. IL-2 gets with the program. *Nat. Immunol.* **7**:798–800.
  33. **Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Ofringa, and C. J. Melief.** 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**:480–483.
  34. **Shedlock, D. J., and H. Shen.** 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**:337–339.
  35. **Shedlock, D. J., J. K. Whitmire, J. Tan, A. S. MacDonald, R. Ahmed, and H. Shen.** 2003. Role of CD4 T cell help and costimulation in CD8 T cell responses during *Listeria monocytogenes* infection. *J. Immunol.* **170**:2053–2063.
  36. **Simmons, A., and D. C. Tschärke.** 1992. Anti-CD8 impairs clearance of herpes simplex virus from the nervous system: implications for the fate of virally infected neurons. *J. Exp. Med.* **175**:1337–1344.
  37. **Smith, C. M., N. S. Wilson, J. Waithman, J. A. Villadangos, F. R. Carbone, W. R. Heath, and G. T. Belz.** 2004. Cognate CD4<sup>+</sup> T cell licensing of dendritic cells in CD8<sup>+</sup> T cell immunity. *Nat. Immunol.* **5**:1143–1148.
  38. **Smith, P. M., R. M. Wolcott, R. Chervenak, and S. R. Jennings.** 1994. Control of acute cutaneous herpes simplex virus infection: T cell-mediated viral clearance is dependent upon interferon- $\gamma$  (IFN- $\gamma$ ). *Virology* **202**:76–88.
  39. **Su, H. C., L. P. Cousens, L. D. Fast, M. K. Slifka, R. D. Bungiro, R. Ahmed, and C. A. Biron.** 1998. CD4<sup>+</sup> and CD8<sup>+</sup> T cell interactions in IFN- $\gamma$  and IL-4 responses to viral infections: requirements for IL-2. *J. Immunol.* **160**:5007–5017.
  40. **Sun, J. C., and M. J. Bevan.** 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* **300**:339–342.
  41. **Suvas, S., A. K. Azkur, and B. T. Rouse.** 2006. Qa-1b and CD94-NKG2a interaction regulate cytolytic activity of herpes simplex virus-specific memory CD8<sup>+</sup> T cells in the latently infected trigeminal ganglia. *J. Immunol.* **176**: 1703–1711.
  42. **Theil, D., T. Derfuss, I. Paripovic, S. Herberger, E. Meinel, O. Schueler, M. Strupp, V. Arbusow, and T. Brandt.** 2003. Latent herpesvirus infection in human trigeminal ganglia causes chronic immune response. *Am. J. Pathol.* **163**:2179–2184.
  43. **van Lint, A., M. Ayers, A. G. Brooks, R. M. Coles, W. R. Heath, and F. R. Carbone.** 2004. Herpes simplex virus-specific CD8<sup>+</sup> T cells can clear established lytic infections from skin and nerves and can partially limit the early spread of virus after cutaneous inoculation. *J. Immunol.* **172**:392–397.
  44. **van Stipdonk, M. J. B., E. E. Lemmens, and S. P. Schoenberger.** 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* **2**:423–429.
  45. **Wallace, M. E., R. Keating, W. R. Heath, and F. R. Carbone.** 1999. The cytotoxic T-cell response to herpes simplex virus type 1 infection of C57BL/6 mice is almost entirely directed against a single immunodominant determinant. *J. Virol.* **73**:7619–7626.
  46. **Wang, J. C., and A. M. Livingstone.** 2003. Cutting edge: CD4<sup>+</sup> T cell help can be essential for primary CD8<sup>+</sup> T cell responses in vivo. *J. Immunol.* **171**:6339–6343.
  47. **Whitley, R. J.** 2002. Herpes simplex virus infection. *Semin. Pediatr. Infect. Dis.* **13**:6–11.
  48. **Whitmire, J. K., and R. Ahmed.** 2000. Costimulation in antiviral immunity: differential requirements for CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses. *Curr. Opin. Immunol.* **12**:448–455.
  49. **Williams, M. A., and M. J. Bevan.** 2007. Effector and memory CTL differentiation. *Annu. Rev. Immunol.* **25**:171–192.
  50. **Williams, M. A., A. J. Tzysnik, and M. J. Bevan.** 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8<sup>+</sup> memory T cells. *Nature* **441**:890–893.
  51. **Wodarz, D., and V. A. Jansen.** 2001. The role of T cell helps for anti-viral CTL responses. *J. Theor. Biol.* **211**:419–432.
  52. **Zhang, J., I. Scordi, M. J. Smyth, and M. G. Lichtenheld.** 1999. Interleukin 2 receptor signaling regulates the perforin gene through signal transducer and activator of transcription (Stat) 5 activation of two enhancers. *J. Exp. Med.* **190**:1297–1308.