T Cell Reactivity during Infectious Mononucleosis and Persistent Gammaherpesvirus Infection in Mice

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Intranasal infection of mice with murine gammaherpesvirus 68 causes a dramatic increase in numbers of activated CD8\(^+\) T cells in the blood, analogous in many respects to EBV-induced infectious mononucleosis in humans. In the mouse model, this lymphocytosis has two distinct components: an early, conventional virus-specific CD8\(^+\) T cell response, and a later response characterized by a dramatic increase among CD8\(^+\) T cells that bear V\(\beta4\) TCRs. We previously demonstrated that V\(\beta4\)-CD8\(^+\) T cells recognize an uncharacterized ligand expressed on latently infected B cells in an MHC-independent manner. The frequency of V\(\beta4\)-CD8\(^+\) T cells increases dramatically following the peak of viral latency in the spleen. In the current studies, we show that elevated V\(\beta4\)-CD8\(^+\) T cell levels are sustained long-term in persistently infected mice, apparently a consequence of continued ligand expression. In addition, we show that V\(\beta4\)-CD8\(^+\) T cells can acquire effector functions, including cytotoxicity and the capacity to secrete IFN-\(\gamma\), although they have an atypical activation profile compared with well-characterized CD8\(^+\) T cells specific for conventional viral epitopes. The characteristics of V\(\beta4\)-CD8\(^+\) T cells (potential effector function, stimulation by latently infected B cells, and kinetics of expansion) suggested that this dominant T cell response plays a key role in the immune control of latent virus. However, Ab depletion and adoptive transfer studies show that V\(\beta4\)-CD8\(^+\) T cells are not essential for this function. This murine model of infection may provide insight into the role of unusual populations of activated T cells associated with persistent viral infections. *The Journal of Immunology*, 2004, 172: 3078–3085.

The murine gammaherpesvirus 68 (\(\gamma\)HV68)\(^1\) has striking biological and structural similarities to the human gammaherpesviruses, Kaposi’s sarcoma-associated herpesvirus, and EBV, and is emerging as an important in vivo experimental model for the gammaherpesviruses (1–3). Intranasal inoculation of mice with \(\gamma\)HV68 results in an acute lytic infection, which is cleared by CD8\(^+\) T cells (4, 5). The virus persists in latent form predominantly in germinal center and memory B lymphocytes (6), although there are also reservoirs of latent virus in macrophages, dendritic cells, and perhaps epithelial cells (7–9).

Primary infection with EBV during adolescence frequently results in infectious mononucleosis. One of the hallmarks of this syndrome is the presence of large numbers of activated CD8\(^+\) T cells in the peripheral blood, which are composed largely of clonal or oligoclonal expansions of EBV-specific T cells (10, 11). Whereas EBV Ag-specific CD8\(^+\) T cells have a potent cytotoxic effector function, many are hyporesponsive with respect to cytokine or chemokine production (11). In addition, EBV infection trans activates expression of an endogenous human retroviral superantigen encoded by the human endogenous retrovirus-K18, resulting in a V\(\beta\)-specific T cell activation (12, 13). The functional capacity and role of these activated CD8\(^+\) T cells are unclear. It has been postulated that superantigen-induced T cell expansion plays a critical role in providing T cell help required for persistent EBV infection of B cells (13).

We have previously described an EBV-like infectious mononucleosis syndrome in mice that develops subsequent to the resolution of acute \(\gamma\)HV68 infection (2, 14, 15). During EBV- and \(\gamma\)HV68-induced infectious mononucleosis, there are polyclonal B cell activation and associated autoantibody production, splenomegalia and enlarged lymph nodes, and a T cell lymphocytosis in the peripheral blood (14, 16–18). A striking feature of the T cell activation induced by \(\gamma\)HV68, in both the spleen and peripheral blood, is the selective increase in the percentage of CD8\(^+\) T cells using V\(\beta4\) chains in their \(\alpha\beta\)TCR (14). This expansion of V\(\beta4\)-CD8\(^+\) T cells increases from ~6% in naïve C57BL/6 mice to as high as 80% within 30 days after infection. The V\(\beta4\)-CD8\(^+\) T cell response does not result from an outgrowth of cells responding to known viral epitopes expressed during the acute infection (19, 20), but rather is a response to an as yet uncharacterized ligand expressed on latently infected B cells with a germline center phenotype (8, 21). The V\(\beta4\)-CD8\(^+\) T cell response is unusual, in that it is not dependent on classical MHC class I or II molecules, CD1, nor on the expression of TAP1 or \(\beta_2\)-microglobulin (21). Hallmarks of superantigen reactivity are V\(\beta\) specificity and MHC class II dependence (22). Thus, despite the V\(\beta\) bias, V\(\beta4\)-CD8\(^+\) T cell reactivity does not appear to be a response to a conventional superantigen.

In the current studies, we have compared the kinetics, effector function, and activation phenotype of V\(\beta4\)-CD8\(^+\) T cells with conventional virus-specific CD8\(^+\) T cells elicited in response to
open reading frame (ORF) 6387–499, a lytic epitope derived from the γHV68 ssDNA-binding protein. Because the ligand recognized by Vβ4+CD8+ T cells is exclusively expressed on latently infected B cells, it is possible that Vβ4+CD8+ T cells are produced during the mononucleosis phase against latently infected B cells to control their numbers during the establishment of latency or during persistent infection. It is also possible that they are a virally induced diversion strategy to consume immunological resources and favor the maintenance of viral latency. To test the impact of this unconventional population of CD8+ T cells, we have analyzed the in vivo role of Vβ4+CD8+ T cells during γHV68 infection using Ab depletion and adoptive transfer strategies.

Materials and Methods

Mice, viral infection, and tissue sampling

Female C57BL/6, C57BL/6-J-Igh-Thy1.1Pgp1+ mice (Thy-1.1) and B6.SJL-Pipr+Pep3+Boys mouse (Ly-5.1 or CD45.1), 6–12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY), or obtained from the Trudeau Institute breeding facility. Mice were housed under specific pathogen-free conditions until γHV68 infection, and in BL3 containment after infection. All animal procedures in these experiments were approved by the Institutional Animal Care and Use Committees at St. Jude Children’s Research Hospital and the Trudeau Institute.

The original stock of γHV68 (clone WUMS) was propagated and titered by plaque assay on monolayers of NIH-3T3 fibroblasts. Mice were anesthetized with 2,2,2-triethanolamine, then infected intranasally with 400 PFU of γHV68. Blood, spleen, lung, bronchoalveolar lavage (BAL), and lung tissues were obtained at various times after infection. BAL cells were collected by lavage of the lungs three times, each with 1 ml of PBS. Single cell suspensions were obtained from spleens, liver, and lung parenchyma by passage through cell strainers. Samples were additionally depleted of erythrocytes by treatment with Gey’s solution. Cells derived from lung and liver were purified by an isotonic Percoll gradient (23).

Quantitation of viral titers

The frequency of latently infected lymphocytes in spleen was determined by an infective center assay, based upon spontaneous reactivation during culture with NIH-3T3 fibroblasts, as previously described (24, 25). Briefly, spleen cell suspensions were incubated overnight on monolayers of NIH-3T3 cells, and overlaid with carboxymethyl cellulose. Following 6 days of culture, the overlay was removed, and plaques were quantitated after methanol fixation and Giemsa staining. Duplicate samples were simultaneously freeze thawed or disrupted with a bead beater and assayed for infectious virus.

T cell depletion

Female mice 9 wk of age were thymectomized according to standard procedures. Animals were rested for 3 wk before infection with γHV68. Thymectomized or intact mice were depleted of Vβ4+ T cells by i.p. injection of concentrated culture supernatant of anti-Vβ4 (KT-4) mAb before and every 2 days after viral infection, for the duration of the experiment. Control mice received anti-Vβ8 (F23.1) Ab. The efficiency of the depletion was monitored in PBLs and spleen cells by staining with anti-Vβ4 and CD8 mAbs.

Flow cytometry

Approximately 106 cells per sample were stained with combinations of the following Abs: Vβ2, Vβ3, Vβ4, Vβ5, Vβ6, Vβ7, Vβ8, Vβ9, Vβ10, Vβ11, Vβ12, Vβ13, Vβ14, CD44, CD45.1, CD45.2, CD25, CD69, CD4, and CD8 (BD Pharmingen, San Diego, CA), and the ORF66387–499/D7 tetrameric reagent. Samples were washed and resuspended in 1% paraformaldehyde (Ted Pella, Redding, CA) diluted in PBS before analysis. Annexin V-FITC (Roche Diagnostics, Mannheim, Germany) and 7-aminomethylcoumarine (7-AAC; Sigma-Aldrich, St. Louis, MO) at 10 μg/ml were used for the detection of apoptotic cells. To analyze cell transfer experiments, Fc receptors were blocked using purified anti-mouse CD16/CD32 Fc receptor (BD Pharmingen). Cells were stained with Thy-1.1 (BD Pharmingen), CD8, and Vβ4 or Vβ8. The MHC-class I peptide tetramer ORF66387–499/D7 was generated by the Molecular Biology Core Facility at the Trudeau Institute. Flow cytometric data were acquired on a FACScan or FACScalibur and analyzed using CellQuest software (BD Pharmingen).

Bromodeoxyuridine labeling

Mice were fed 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) in the drinking water at 0.8 mg/ml. The water containing BrdU was exchanged from light and changed every 1–2 days. Cells were surface stained with fluorochrome-conjugated Abs specific for Vβ4 or Vβ8 and CD8 before staining of incorporated BrdU using BrdU-FITC (BD Pharmingen), as described (26).

CFSE labeling and cell transfer

Splenic and mesenteric lymph nodes were harvested from donor Thy-1.1 mice, passed through a steel seive, and washed. RBC were lysed, cells were counted, the suspension was diluted to 107 cells/ml, and CFSE (5 mM in DMSO; Molecular Probes, Eugene, OR) was added at a dilution of 1/8000. Cells were stained at room temperature for 20 min, and washed three times in FCS/PBS. The cells were resuspended in PBS and injected into the tail vein of recipient mice (~0.5 × 108 in a volume of 200 μl). Injection of CFSE-labeled Thy-1.1 cells was performed 2–3 consecutive days, such that each recipient received a total of ~1 × 108 cells.

Cytotoxic T cell assay

Vβ4+CD8+ T cell cytotoxic activity was determined by a redirected chromium release assay on suspensions of spleen cells from naive mice or mice infected with γHV68 23 days before infection. B15Fcr− targets were labeled with 51Cr (New England Nuclear, Boston, MA) overnight. After washing, ×106 targets were incubated with graded numbers of spleen cells in the presence of 0.5 μg/ml mAbs specific for Vβ4, CD3, CD8, B220, or rat IgG (BD Pharmingen) for 6 h at 37°C. After centrifugation, the supernatants were harvested for gamma counting. Total release (T) was determined by adding Triton X-100 (2%) to the targets. Spontaneous release (S) was determined from targets incubated in the absence of effector cells. The level of specific CFC lysis was calculated from the formula: percent specific lysis = [E-S/(T-S)], where E is the experimental Cr release in the presence of cytotoxic cells.

Intracellular IFN-γ staining

Splenic lymphocytes were stimulated in vitro with Abs specific for CD3 and CD28 (BD Pharmingen) or with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 10 μg/ml brefeldin A and 10 μM IL-2 for 5 h.Fc binding was blocked using anti-mouse CD16/CD32, and the cells were stained with fluorochrome-conjugated Abs specific for CD8 and Vβ4. After fixation with 2% paraformaldehyde, cells were permeabilized with 0.5% saponin and stained with anti-mouse IFN-γ or an isotype control IgG1 Ab (BD Pharmingen).

Cell sorting and adoptive transfer

Pooled splenocytes from C57BL/6 mice at 3–4 mo after infection were Fc blocked and stained with fluorochrome-conjugated Abs specific for CD8 and Vβ4. The cells were sorted twice using a FACSVantage SE/Diva sorter to obtain a final purity of 99.9%. The purified cells were resuspended in PBS, and 1.5 × 106 or 1 × 106 cells per mouse were injected i.v. in 200 μl of PBS in two independent experiments in C57BL/6 or congenic Ly-5.1 mice. The recipient and control mice were infected no later than 12 h. The efficiency of the cell transfer was monitored by staining of PBLs with anti-CD45.1, CD45.2, Vβ4, and CD8 mAbs during the duration of the experiment.

Results

Conventional virus-specific CD8+ T cells and Vβ4+CD8+ T cells have distinct kinetics and nonoverlapping TCR repertoires

The conventional CD8+ T cell response to lytic epitopes of γHV68 has been described. For example, T cells specific for ORF66387–499/D7 peak between 10 and 20 days after infection, and subsequently decline to levels characteristic of a memory response (19). In addition, there is a high turnover rate of γHV68-specific T cells in persistently infected mice compared with CD8+ T cells specific for influenza virus, which is cleared. This latter observation is consistent with the possibility of continual low levels of stimulatory lytic epitope expression as a consequence of viral reactivation during long-term latency (27). In contrast, we have previously shown that Vβ4+CD8+ T cells, which recognize a molecularly uncharacterized ligand on latently infected B cells, have delayed kinetics that correlate with the infectious mononucleosis
response rather than the acute lytic infection. Thus, the increased frequency of the Vβ4+ set of CD8+ T cells is not yet apparent at 14 days postinfection, and increases rapidly in blood and spleen between 14 and 30 days after yHV68 infection (14). This response is subsequent to the clearance of lytic virus from the lungs and correlates with the establishment of peak levels of latency in the spleen between 14 and 21 days postinfection. Interestingly, Vβ4+CD8+ T cell expansion was not observed in peripheral or draining lymph nodes, but the response has not been examined in the respiratory tract, which is not only the site of the acute lytic infection, but is also a reservoir of long-term latency (9, 25). Therefore, we determined the frequency of Vβ4+ T cells in lung parenchyma, lung airways, liver, and spleen, and compared them with T cells specific for a well-studied yHV68 epitope derived from a lytic cycle protein, ORF6487–495. The data (Fig. 1) illustrate the distinct kinetics of Vβ4+CD8+ T cells and the ORF6487–495/D^d-specific T cells. The data show that the kinetics of Vβ4+CD8+ T cells previously described for the blood and spleen (14), shown in this work only for the spleen, are mirrored in the lung parenchyma, lung airways, and liver. The levels of Vβ4+CD8+ T cells at all sites increase steadily between 14 and 35 days postinfection and remain elevated at 100 days after infection.

To determine whether Vβ4+CD8+ T cells make a significant contribution to the ORF6487–495 response, complicating the analysis of the two distinct populations, we analyzed the Vβ usage of ORF6487–495-specific CD8+ T cells (Fig. 2). The data show that the ORF6487–495 response is biased toward Vβ2+ and Vβ8+ T cells, and that Vβ4+ T cells do not make a major contribution to this response. Together, the kinetics and repertoire data support the idea that there are two distinct phases of T cell reactivity, conventional CD8+ effector T cells and Vβ4+CD8+ T cells, initiated during the acute and infectious mononucleosis stages of the infection, respectively.

Sustained levels of Vβ4+CD8+ T cells reflect continual ligand stimulation

We asked next whether the sustained high frequencies of Vβ4+CD8+ T cells present during the latency phase of the infection were dependent on the continuous export of thymic emigrants into the peripheral T cell pool. Therefore, we monitored the frequencies of CD8+ T cells and Vβ4+CD8+ T cells over time in the peripheral blood of mice that had been thymectomized as adults and then infected with yHV68. As shown in Fig. 3, the elevated levels of CD8+ T cells in the peripheral blood characteristic of the lymphocytosis had returned to normal by ~15 wk postinfection (Fig. 3A). In contrast, analysis of individual mice (Fig. 3B) showed that, although the degree of Vβ4+CD8+ T cell expansion varied, the elevated levels of Vβ4+CD8+ T cells are maintained for at least 34 wk, well after numbers of latently infected B cells had dropped to low, persistent levels ~4 wk after infection (6). Maintenance of elevated levels of Vβ4+CD8+ T cells in the absence of a thymus is consistent with two possibilities. Either

FIGURE 1. Time course analysis of the T cell reactivity during yHV68 infection in lung parenchyma, liver, spleen, and BAL. A, Vβ4+CD8+ T cells. B, ORF6487–495/D^d+ CD8+ T cells. Data show the mean ± SD of three mice per time point. Data are from one of three independent experiments with similar results.

FIGURE 2. TCR Vβ chain usage in naive or ORF6487–495/D^d+ CD8+ T cells isolated from spleen (A), lung (B), and BAL (C) 13 days after yHV68 infection. Data are expressed as the percentage of epitope-specific CD8+ T cells expressing each Vβ in infected mice (■) and the percentage of total CD8+ T cells expressing each Vβ in naive mice (□). Data are from one of two independent experiments with similar results.

FIGURE 3. Time course analysis of the frequencies of CD8+ T cells (A) and Vβ4+CD8+ T cells (B) in peripheral blood of thymectomized mice after yHV68 infection. A, Shows mean ± SD; n = 6; B, shows analysis of individual mice. Data are from one of two independent experiments with similar results.
the Vβ4⁺CD8⁺ T cells induced by γHV68 are long-lived, or the cells are a cycling, self-renewing population, maintained either by bystander activation or in response to continual expression of the stimulatory ligand.

To distinguish between these possibilities, mice were given BrdU in the drinking water at 3, 4, 9, and 19 wk after infection, and the frequency of proliferating, BrdU-positive Vβ4⁺CD8⁺ cells in the spleen and peripheral blood of naive or γHV68-infected mice was assessed by flow cytometry. Vβ8⁺CD8⁺ cells were analyzed as a control. Proliferation of Vβ4⁺CD8⁺ T cells was maximal at 3 wk after infection, and dropped rapidly, but was still detectable over background levels up to 19 wk after infection (Fig. 4A). Consistent with the transient, generalized activation of CD8⁺ T cells during the infectious mononucleosis phase (14, 28), there was clear proliferation among CD8⁺ T cells expressing Vβ8 at 3–4 wk postinfection. In contrast, long-term proliferation was confined to the Vβ4 set. The data are even more striking when analyzed in terms of absolute numbers of proliferating cells (Table I). At 3 and 4 wk postinfection, there were 70- and 24-fold, respectively, more BrdU⁺ Vβ4⁺CD8⁺ cells in infected vs naive mice. Although the absolute number of proliferating Vβ4⁺CD8⁺ cells dropped significantly at later times, the numbers of proliferating cells were 13-fold greater in infected mice at 19 wk. In contrast, even though a significant proportion of Vβ8⁺CD8⁺ T cells was cycling at 3 and 4 wk postinfection (Fig. 4), there were generally negligible increases in the number of BrdU⁺ cells in this population. These results indicate that Vβ4⁺CD8⁺ T cells undergo sustained proliferation during long-term γHV68 latency.

Cell numbers are controlled by the relative rates of cell division and apoptosis. We therefore determined levels of apoptosis among Vβ4⁺CD8⁺ T cells in the spleen and peripheral blood using annexin-V and 7-AAD staining. High frequencies of apoptotic cells (annexin-V⁺7-AAD⁻) were present in the Vβ4⁺CD8⁺ subpopulation, peaking at 3 wk in the spleen and 4 wk in the blood (Fig. 4B), compared with low frequencies in Vβ8⁺CD8⁺ T cells at all time points analyzed. These results and the BrdU incorporation data indicate that the Vβ4⁺CD8⁺ T cells are continuously proliferating and undergoing apoptosis after resolution of the mononucleosis phase of the infection.

Although the proliferation and cellular death data indicate that Vβ4⁺CD8⁺ T cells in γHV68-infected mice are continuously turning over, the data do not distinguish between activation due to sustained expression of the stimulatory ligand, and bystander proliferation (29, 30). A more rigorous test was to determine whether Vβ4⁺CD8⁺ T cells isolated from naive mice and adoptively transferred into latently infected mice could be activated and proliferate at late time points after infection, well after resolution of the acute respiratory infection and the generalized activation associated with the mononucleosis. Therefore, pooled lymphocytes harvested from the lymph node and spleens of naive Thy-1.1 mice were stained with CFSE and transferred into mice that had been infected with γHV68 either 2 or 10 wk earlier. The 2-wk time point was chosen because it coincides with the time of peak ligand expression in splenic B cells (8, 21), whereas the 10-wk time point represents a time after resolution of the mononucleosis and well past the peak of Vβ4-stimulatory activity of splenocytes in vitro (14, 21). Recipient mice were sacrificed 4 days after transfer, and the outgrowth of donor-derived Vβ4⁺CD8⁺ T cells was assessed by the loss of CFSE in the Thy-1.1⁺CD8⁺ T cells. The results clearly show that upon transfer into mice infected 2 wk earlier, there was significant proliferation of both Vβ4⁺CD8⁺ and Vβ8⁺CD8⁺ T cells, consistent with the generalized activation characteristic of the mononucleosis stage of infection (Fig. 5). Importantly, cells that were transferred into mice 10 wk postinfection showed proliferation predominantly among the Vβ4⁺ subset. Comparable results were obtained from analysis of spleen and blood lymphocytes. These data suggest that long after clearance of lytic virus and resolution of the generalized activation associated with γHV68 infection at 2 wk postinfection, the ligand that stimulates Vβ4⁺CD8⁺ T cells is still expressed.

In conclusion, the data show that the elevated frequencies of Vβ4⁺CD8⁺ T cells are sustained in long-term latently infected mice, even in the absence of a thymus. Combined with the data showing ongoing proliferation and apoptosis of Vβ4⁺CD8⁺ T cells, these observations are suggestive of sustained expression of the stimulatory ligand.

Vβ4⁺CD8⁺ T cells have cytotoxic and IFN-γ-secreting capacity CD8⁺ T cells express a range of effector molecules that mediate defense against pathogens by direct cytolysis of the cells or by secretion of cytokines (31). The functional capacity of Vβ4⁺CD8⁺ T cells during latent γHV68 infection has not been determined. Because the stimulatory ligand has not been identified, the effector function of Vβ4⁺CD8⁺ T cells was assessed indirectly.
First, we assessed cytolytic function using a redirected lysis assay, in which the Vβ4+CD8+ T cells were artificially bridged to target cells via Ab specific for the effector cells and Fc receptors on the target cells. As shown in Fig. 6A, splenocytes from mice infected 3 wk previously with γHV68 were able to specifically carry out cytotoxic activity when bridged to P815 targets using anti-Vβ4 Ab. The specificity of the killing by Vβ4+CD8+ T cells is validated by the lack of nonspecific cytolytic activity in the γHV68-infected samples with anti-rat IgG2b and anti-Vβ11 Abs (Fig. 6A) and the lack of cytotoxicity by splenocytes from naive mice (data not shown). The difference between specific lysis when bridged with anti-CD3 vs anti-Vβ4 may reflect the residual CTL activity of the conventional virus-specific (non-Vβ4+) CD8+ T cells and/or differences between the CD3- and Vβ4-specific Abs.

The capacity of Vβ4+CD8+ T cells to secrete IFN-γ, a key cytokine in antiviral defense, was assessed using intracellular cytokine staining. Spleen cells isolated from mice infected 4 wk previously were restimulated in vitro with anti-CD3/CD28 Abs or with PMA/ionomycin, and analyzed for IFN-γ production by flow cytometry (Fig. 6B). The data show that the Vβ4+CD8+ T cells are capable of making IFN-γ after either CD3/CD28 or PMA/ionomycin stimulation. These results show that Vβ4+CD8+ T cells are functional ex vivo in terms of their ability to secrete IFN-γ and to mediate cytolytic activity after TCR engagement.

Vβ4+CD8+ T cells express a nonconventional activation phenotype

Previous studies have shown that the expression of surface molecules on CD8+ T cells varies with the degree of cellular activation, with the effector/memory status and with localization in the periphery or in lymphoid organs (32, 33). To further compare Vβ4+CD8+ T cells with CD8+ T cells specific for ORF6487/Dβ, we next investigated their activation phenotype in spleen, lung parenchyma, lung airways, and liver both during the infectious mononucleosis and persistent phases of the infection, 21 and 100 days postinfection, respectively. The data for spleen show that both ORF6487/Dβ-specific and Vβ4+CD8+ T cells maintain an activated phenotype in terms of CD44 expression (Fig. 7), as well as a panel of other markers, including Ly-6c, CD62L, and peanut lectin (data not shown). Interestingly, however, Vβ4+CD8+ T cells do not up-regulate CD69 or CD25 expression to the same extent as their ORF6487/Dβ-specific counterparts. Similar results were found for cells from the lung parenchyma, lung airways, and liver (data not shown). Up-regulation of CD25, the low affinity IL-2R α-chain, and CD69, a marker of early activation, are both characteristic events associated with T cell activation (34). These results indicate that Vβ4+CD8+ T cells do not have the conventional activation phenotype of virus-specific CD8+ T cells from γHV68 or other viral infections. It is possible that the atypical
Adoptive transfer of $10^5-10^6$ VΔ4-depleted mice shows the efficacy of the Ab depletion in the experiment shown in A was monitored in PBLs and compared with the levels of VΔ4CD8+ T cells in control γHV68-infected mice. Data (A and B) show mean ± SD of at least three mice analyzed at each time point, and are representative of three separate experiments. C, Depletion of VΔ4CD8+ T cells has no impact on the frequency of total or virus-specific CD8+ T cells after γHV68 infection. Kinetic analysis of CD8+ T cells in blood of VΔ4-depleted (■) and control (□) mice after γHV68 infection. D, Depletion of VΔ4CD8+ T cells has no impact on the frequency of ORF6-agy/Db virus-specific CD8+ T cells after γHV68 infection. The panel shows peripheral blood analysis of VΔ4-depleted (■) and control (□) mice after γHV68 infection. E, The frequency of VΔ4CD8+ T cells in blood of VΔ4Ab-depleted and nondepleted mice shows the efficacy of the Ab depletion in the experiment shown in C and D. Data (C–E) show mean ± SD of three mice analyzed at each time point and are representative of two independent experiments. F, Adoptive transfer of $10^5-10^6$ VΔ4CD8+ T cells before γHV68 infection has no effect on the establishment of splenic latency 14 days after infection. VΔ4CD8+ T cells were FACS sorted from mice infected with γHV68 3 mo previously. Virus titers were assessed by an infective center assay. Data are pooled from two independent experiments analyzing a total of 9–14 individual mice in each experimental group. G, The data show the fate of the adoptively transferred VΔ4CD8+ T cells (squares) tracked in peripheral blood using the allelic distinction Ly-5.1 and Ly-5.2 to differentiate them from the host cells. Staining in the control mice, which did not receive transferred cells, is also shown (circles). Three individual mice per group were analyzed at each time point.

**FIGURE 8.** VΔ4 CD8+ T cells have no marked effect on the numbers of latently infected cells during γHV68 infection. A, Time course analysis of latent virus titers in the spleen of VΔ4-depleted (■) and control (□) thymectomized mice after γHV68 infection. Virus titers were assessed by an infective center assay, as described in Materials and Methods, B. The efficacy of the Ab depletion in the experiment shown in A was monitored in PBLs and compared with the levels of VΔ4CD8+ T cells in control γHV68-infected mice. Data (A and B) show mean ± SD of at least three mice analyzed at each time point, and are representative of three separate experiments. C, Depletion of VΔ4CD8+ T cells has no impact on the frequency of total or virus-specific CD8+ T cells after γHV68 infection. Kinetic analysis of CD8+ T cells in blood of VΔ4-depleted (■) and control (□) mice after γHV68 infection. D, Depletion of VΔ4CD8+ T cells has no impact on the frequency of ORF6-agy/Db virus-specific CD8+ T cells after γHV68 infection. The panel shows peripheral blood analysis of VΔ4-depleted (■) and control (□) mice after γHV68 infection. E, The frequency of VΔ4CD8+ T cells in blood of VΔ4Ab-depleted and nondepleted mice shows the efficacy of the Ab depletion in the experiment shown in C and D. Data (C–E) show mean ± SD of three mice analyzed at each time point and are representative of two independent experiments. F, Adoptive transfer of $10^5-10^6$ VΔ4CD8+ T cells before γHV68 infection has no effect on the establishment of splenic latency 14 days after infection. VΔ4CD8+ T cells were FACS sorted from mice infected with γHV68 3 mo previously. Virus titers were assessed by an infective center assay. Data are pooled from two independent experiments analyzing a total of 9–14 individual mice in each experimental group. G, The data show the fate of the adoptively transferred VΔ4CD8+ T cells (squares) tracked in peripheral blood using the allelic distinction Ly-5.1 and Ly-5.2 to differentiate them from the host cells. Staining in the control mice, which did not receive transferred cells, is also shown (circles). Three individual mice per group were analyzed at each time point.

**Discussion**

Comparative analysis of the VΔ4 CD8+ T cell response and the conventional CD8+ T cell response to a well-characterized lytic viral epitope in γHV68-infected mice has revealed unique kinetics, turnover, and activation profiles of the VΔ4 CD8+ T cells. These differences can be explained by the distinct recognition properties and patterns of ligand expression for the two groups of CD8+ T cells. Whereas ORF6-agy/Db-specific T cells recognize a conventional viral peptide presented during the acute, lytic phase of the infection, the ligand for VΔ4 CD8+ T cells is unusual. Analysis of VΔ4 CD8+ T cell stimulation in knockout mice and in vitro Ab-blocking studies of hybridoma reactivity showed that expression of the stimulatory ligand is not dependent on the presence of MHC class I, MHC class II, TAP1, CD1, or β2-microglobulin (21, 28). In addition, the ligand for VΔ4 CD8+ T cells is expressed maximally at 14 days postinfection, during the peak of VΔ4CD8+ T cell activation phenotype may be a consequence of the unusual, MHC-independent recognition properties of these cells (21, 28). VΔ4 CD8+ T cells have no marked effect on the numbers of latently infected cells during γHV68 infection.

The profound expansion of VΔ4 CD8+ T cells that correlates with the precipitous drop in peak levels of splenic latency 2–4 wk after infection, taken together with our previous observations that the ligand driving the VΔ4 CD8+ T cell expansion is expressed on latently infected activated B cells (8), raise the possibility that VΔ4 CD8+ T cells function to control the number of latently infected cells. This might be accomplished directly, by VΔ4 CD8+ T cell-mediated effector function, or indirectly, by diversion of the immune response away from the virus and facilitating the establishment of latency. This question was examined in the context of a γHV68 infection in vivo by two different approaches, after infection of naive mice depleted of VΔ4 CD8+ T cells and after infection of mice into which VΔ4 CD8+ T cells from persistently infected mice had been adoptively transferred.

First, the effect of the absence of VΔ4 CD8+ T cells on the kinetics of establishment of latency was examined. Thymectomized mice were depleted of VΔ4 CD8+ T cells by in vivo injection of anti-VΔ4 Abs 2 days before infection and every other day for the duration of the experiment. The levels of splenic viral latency, as assessed by an infective center assay, showed that depletion of VΔ4 CD8+ T cells had no effect on the kinetics of establishment of latency, nor on the frequency of latently infected spleen cells (Fig. 8A). The number of VΔ4 CD8+ T cells in the Ab-depleted and control mice was monitored until day 90 (Fig. 8B). To examine the effect of VΔ4 depletion on the conventional effector response, VΔ4-depleted C57BL/6 mice were analyzed up to 35 days after infection for CD8+ T cell numbers (Fig. 8C) and conventional virus-specific CD8+ T cell response, as assessed by tetramer analysis. Data shown are for analysis of blood. Similar results (data not shown) were obtained for spleen. Taken altogether, our results indicate that the absence of VΔ4 CD8+ T cells does not impact the course of γHV68 latent infection.

Second, the impact of the presence of VΔ4 CD8+ T cells at the time of infection on the establishment of viral latency in the spleen was assessed. Thus, VΔ4 CD8+ T cells were FACS purified from mice infected with γHV68 3 mo previously and i.v. transferred into naive mice, which were subsequently infected intranasally. Fourteen days later, at the peak of viral latency in the spleen, the latent load was assessed by the infective center assay. As shown in Fig. 8F, the adoptive transfer of VΔ4 CD8+ T cells just before infection had no impact on the latent load in the spleen 14 days later. Two different doses of purified VΔ4 CD8+ T cells were transferred, $1.5 \times 10^5$ and $1 \times 10^6$, with no detectable impact on the viral load. The persistence of the transferred cells was regularly monitored using Ly-5 allelic distinctions (Fig. 8G). Altogether, our results indicate that neither depletion of VΔ4 CD8+ T cells nor the presence of FACS-purified VΔ4 CD8+ T cells at the time of γHV68 infection had any detectable impact on the latent load of virus in the spleen.
spleenic latency (21), on activated, latently infected B cells (8). Current data, discussed in more detail below, also suggest that there may be sustained expression of the stimulatory ligand during long-term, persistent infection.

The data showing Vβ4+CD8+ ligand expression during peak levels of latency have raised the possibility that the ligand is encoded by, or its expression is dependent on, M2, M2 is the only γHV68-encoded latency-restricted gene identified to date (35, 36). Analysis of γHV68-infected BALB/c mice has shown that CD8+ T cells specific for M2 are detected transiently, between 14 and 21 days after infection. In addition, adoptive transfer of an M2-specific CD8+ T cell line and DNA vaccination with the M2 epitope reduced the initial load of latently infected cells, consistent with their role in controlling the early peak of latency (37). We have shown that expression of M2 is B cell dependent (37), and restricted to latently infected B cells (not macrophages or dendritic cells) at 14 days postinfection (38). Thus, the kinetics and cell-type specificity of M2 expression correlate with that of the Vβ4 ligand. However, analysis of an M2-deficient virus showed normal expansion of the Vβ4+ subset of CD8+ T cells, indicating that the ligand was not encoded by M2 (38). The sustained kinetics of the Vβ4+CD8+ T cells contrasts sharply with the transient expression of M2-specific CD8+ T cells (37), also arguing against an M2-specific ligand.

Our data show that transfer of Vβ4+ T cells from naive mice into persistently infected mice as late as 10 wk after infection resulted in significant proliferation of the Vβ4+CD8+ T cells. Whereas transfer at 2 wk showed activation of both Vβ4+ and Vβ8+ T cells, at 10 wk after infection, the stimulation was specific to Vβ4+CD8+ T cells. A reasonable interpretation of these results is that at early time points after infection, in addition to expression of the stimulatory ligand, the generalized high state of activation and associated cytokine-rich milieu associated with the mononucleosis phase of the infection (14) cause generalized T cell activation. However, at 10 wk postinfection, the generalized stimulation has resolved, and activation of transferred T cells reflects a ligand-specific event. Therefore, we favor the interpretation that there is sustained expression of the stimulatory ligand, although our data do not rule out the formal, less likely, possibility that Vβ4+CD8+ T cells are preferentially susceptible to bystander activation. Sustained expression of the ligand is consistent with it being an Ag associated with latency or reactivation from latency. At the peak of latency in the spleen, latently infected cells can reactivate efficiently, as measured ex vivo. In addition, although the sensitivity of the assay is insufficient to detect reactivation during long-term latency, it is thought that there is constant, low-level virus reactivation that is held in check by effective immune control (25, 27). This scenario is consistent with the sustained expression of the stimulatory ligand.

The suggestion that Vβ4+CD8+ T cells recognize an Ag associated with latency or reactivation from latency, coupled with the demonstration that these cells mediate effector function ex vivo, raised our expectation that depletion or early expression of Vβ4+CD8+ T cells would impact the kinetics or magnitude of latency during the first few weeks following infection. However, we found that Vβ4+CD8+ T cells have no impact on the course of early latency. These data are consistent with our previous analysis of the course of γHV68 infection in two inbred strains of mice with dramatically different levels of Vβ4+CD8+ T cells (28). Comparative analysis of various parameters of γHV68 infection in DBA/2 mice, which have virtually no Vβ4+CD8+ T cell expansion, and C57BL/6 mice showed no differences in clearance of lytic virus from the lung, levels, and kinetics of latent virus in the spleen, or the development of the virus-specific Ab response. Together, these data suggest either that Vβ4+CD8+ T cells have no role in controlling the establishment and early maintenance of latency or there are redundant mechanisms in the absence of Vβ4+CD8+ T cells.

In conclusion, the dramatic expansion of Vβ4+CD8+ T cells that occurs following γHV68 infection of mice demonstrates kinetics consistent with reactivity to a latent Ag or an Ag expressed by latently infected cells that are reactivating. However, the Vβ4+CD8+ T cells, despite their predominance, play no essential role in the early control of latency. There are three possible explanations for this surprising observation. First, the absence of a conventional role in immune control of the virus may be consistent with the unusual recognition and activation characteristics of these cells. For example, although they can mediate effector functions in vitro when artificially stimulated via their TCR, it is possible that ligand recognition in vivo fails to elicit bona fide effector functions. Thus, the Vβ4+CD8+ T cell response may represent an epiphenomenon, a consequence of the infection, but of no relevance for the virus or immune protection. As has been demonstrated in other systems, caution should be taken in assuming that a large expansion of T cells necessarily fulfills an important effector function (39). A second possibility is that the Vβ4+CD8+ T cells may play a redundant role in controlling the latent infection. This could either be as a redundant effector mechanism or as an immune diversion mechanism. In either case, however, it might have been expected that the introduction or depletion of Vβ4+CD8+ T cells early during the infection would have impacted the viral load or the conventional CD8+ T cell response, although this was not seen. A third possibility is that they may play a role during long-term latency, which would be consistent with the observation that the ligand is expressed long after resolution of the infectious mononucleosis phase of infection. The ability to test this third, intriguing possibility awaits the development of molecular assays to monitor early events in viral reactivation in an immunocompetent host.

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References