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CD4 T Cells Specific for a Latency-Associated γ-Herpesvirus Epitope Are Polyfunctional and Cytotoxic

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A majority of people worldwide are infected with the oncogenic human γ-herpesviruses (γHVs) EBV and Kaposi sarcoma–associated herpesvirus (KSHV), making these viruses a considerable public health concern. After an initial acute infection, γHVs are maintained lifelong in a latent state within cells of the immune system. Under conditions of immune suppression, such as following transplantation or HIV infection, γHVs can reactivate from latency, leading to recrudescence disease and the development of cancers. Cytotoxic CD4 T cells play an important role in immune control of the γHVs in part because viral immune-evasion mechanisms impair CD8 T cell recognition by downregulating MHC class I (MHC-I) molecules (1), and the major viral latency reservoir is within MHC class II (MHC-II)* B cells (2). Decline of CD4 T cell immunity to EBV correlates with the development of EBV-associated cancers, including Hodgkin disease, nasopharyngeal carcinoma, and Burkitt lymphoma, and CD4 T cells have also been used therapeutically for treatment of EBV-associated malignancies (3–5). In this study, we have infected mice with murine γHV68 to study antiviral CD4 T cell responses to acute and latent γHV infections, as the human γHVs are highly species specific, making detailed in vivo kinetic studies of the host immune response difficult. We identify 16 new epitopes during acute infection that promote cytokine-producing CD4 T cell responses. These responses exhibit differential kinetics during the early stages of latency establishment, with some responses expressed only transiently and others maintained throughout stable latency. Infection with a latency-deficient virus shows that the long-term maintenance of epitope-specific CD4 T cell responses, but not their initial generation, is dependent on latency establishment. Expression of an additional epitope from the latency-associated M2 protein is unique in that it does not stimulate cells during acute infection, but only after the establishment of latency. M2-specific CD4 T cells sustain IL-2 production in addition to IFN-γ and TNF-α and exhibit potent killing of MHC-II–expressing cells in vivo. This study substantially broadens our understanding of the CD4 T cell response to γHV68 infection and identifies a valuable latency-associated target for rational vaccine design.

Materials and Methods
Mice and virus infections
C57BL/6 and B6.SJL-Ptprc<sup> Pepc<sup>-</sup></sup>/Boy mice were bred at Trudeau Institute and kept under specific-pathogen free conditions. Mice were anesthetized with 2,2,2-tribromoethanol and infected intranasally with 400–800 PFU γHV68 (strain WUMS) or AC-RTA (6) (a gift from R. Sun and T.-T. Wu, University of California Los Angeles). All experiments were approved by the Institutional Animal Care and Use Committee of the Trudeau Institute.

Peptide prediction
Protein sequences were analyzed using previously described algorithms (7) that predict the affinity of 15-mer peptides for I-A<sup>κ</sup> class II molecules.
Briefly, all 15-mer peptides that are encoded in open reading frames (ORFs) of the \( \gamma H V 68 \) genome (GenBank NC_001826) were predicted for binding to H-2 I-A\(^b\). Two independent algorithms [ARB (8) and SMM-Align (9)] based on positional scoring matrices were used to assign predicted IC\(_{50}\) binding affinities to all peptides. For each method, peptides were ranked by their predicted binding affinity, and the median of the two ranks was used to select the top 680 out of 34,008 peptides (top 2%). Peptides overlapping by nine or more residues were excluded. Peptides were synthesized by Mimotopes (Clayton, Victoria, Australia).

**T cell function assays**

Intracellular cytokine staining, functional avidity analysis, and ELISPOT assay for IFN-\( \gamma \) expression were performed as described previously (10). For the combined intracellular cytokine staining and BrdU incorporation assay, mice were treated in vivo with a single i.p. injection of 250 \( \mu \)g BrdU and 0.8 mg/ml BrdU in the drinking water for 4 d (11). In vivo cytotoxicity assay was performed essentially as described previously (10). Experimental peptide-pulsed splenocytes were labeled with differential concentrations of CFSE, and control peptide (influenza NP\(_{11-32}\)-pulsed cells were left unlabeled. Cells were mixed in a 1:1:1 ratio (M2 124–138/ M2124–138-specific tetramers/CFSE+) and analyzed using FlowJo software (Tree Star). Specific lysis was calculated using the formula: \((1 - [\text{ratio uninfected/ratio infected}]) \times 100\).

**M2\(_{124-138}\)-specific tetramers**

MHC-II-restricted tetramers expressing M2\(_{124-138}\) peptide (NSEPYYIQ-PISTRSL) were generated by the National Institutes of Health tetramer core facility. To identify M2\(_{124}\)-specific CD4 T cells ex vivo, cells were incubated with 6 \( \mu \)g/ml tetramer at 37°C for 1.5 h. MHC-I-restricted tetramers expressing ORF61\(_{24-31}\) peptide (TSINFVKI) were generated and used as previously described (10).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA). Differences were considered significant at \( p \) values <0.05.

**Results**

**Identification of novel \( \gamma H V 68\)-specific CD4 T cell epitopes**

CD4 T cells are important in controlling persistent viral infections, not just as “helpers” for CD8 T cells, but also as cytokine-secreting and cytotoxic effector cells. Our understanding of the breadth of the virus-specific CD4 T cell response to \( \gamma H V 68\) in C57BL/6 mice has been largely limited to two epitopes, from gp150 (gp150\(_{67-83}\)) and ORF11 (ORF11\(_{68-180}\)) (12, 13). To identify additional CD4 T cell epitopes, we used an MHC-II-binding prediction algorithm to identify and synthesize 680 15-mer peptides from \( \gamma H V 68\) genome coding sequences \((7, 14, 15)\). As we observed previously when we used a similar algorithm to predict CD8 epitopes (10), there was a strong correlation between protein size and the number of predicted epitopes \( r^2 = 0.6909, p \leq 0.0001, \text{Pearson correlation} \). Each peptide was screened for its ability to stimulate IFN-\( \gamma \) by splenic CD4 T cells from mice 12 d after WT \( \gamma H V 68\) infection. We initially used peptide pools (up to eight peptides per pool) to narrow down the number of candidate peptides, then deconvoluted the positive pools, and tested each individual peptide. We identified 16 peptides that reproducibly elicited IFN-\( \gamma \) responses at 12 d postinfection (p.i.) (Fig. 1A, top panel). The 16 peptides were derived from 13 proteins, mainly encoded by early-late and late genes (Table I) (16), consistent with recent reports on the specificity of CD4 T cells to EBV and KSHV (17–19). As has been observed in KSHV infection (18), several proteins that elicit \( \gamma H V 68\)-specific CD8 T cell responses, including ORF6, ORF8, ORF17, ORF48, and ORF61, also stimulated CD4 T cell responses; the ORF17\(_{310-324}\) (ITNHAASFPGAGLS) sequence overlapped with a known CD8 epitope, ORF17\(_{308-316}\) (SAINSHTAAP) presented by H-2\(^D\) (10, 20). Eight of the 16 peptides elicited sustained (\( \geq 70\% \) of the day 12 production) or enhanced IFN-\( \gamma \) responses at 21 d p.i., suggesting there is differential regulation of epitope-specific CD4 T cell responses during the latency amplification phase, similar to antiviral CD8 T cell responses (21). Most of the responses were markedly reduced at 60 d p.i., consistent with memory CD4 T cells having undergone substantial contraction.

We next analyzed IFN-\( \gamma \) production p.i. with AC-RTA, a recombinant \( \gamma H V 68\) that induces robust peak lytic titers in the lungs (similar to or greater than WT virus) but cannot establish latency (6, 10). Only 11 of the 16 peptides elicited IFN-\( \gamma \)-responses above background 12 d p.i. with AC-RTA (Fig. 1A, bottom panel, Table I). AC-RTA does not reliably traffic to the spleen and so would result in negligible systemic viral loads after acute infection. Therefore, these data suggest the initial breadth of the CD4 T cell response may be governed at least in part by Ag presentation during latency amplification in the spleen. Accordingly, none of the CD4 T cell responses were sustained or increased at 21 d or maintained 60 d after AC-RTA infection (Fig. 1A, bottom panel). These results also suggest that antiviral CD4 T cell responses generated during acute infection are more reliant on prolonged Ag presentation than their antiviral CD8 T cell counterparts (10). We then tested the peptide pools at later times p.i. to identify epitopes that might arise during latency. Notably, one peptide (M2\(_{124-138}\)) that did not elicit IFN-\( \gamma \)-production above background 12 d p.i. induced robust IFN-\( \gamma \)-production at 21 and 60 d p.i. with WT \( \gamma H V 68\), but not AC-RTA (Fig. 1B). Extrapolating from the ELISPOT data, the M2\(_{124-138}\)-specific response in WT \( \gamma H V 68\)-infected mice accounted for 0.65% of the total CD4 T cell response at 12 d, 7.0% at 21 d, and 17.5% at 60 d p.i. Expression of the M2 gene is temporally restricted and associated with latent infection (22, 23). The M2 protein is key in the establishment of, and reactivation from, viral latency and drives the differentiation of infected B cells (24–26). M2 also encodes an MHC-I-restricted epitope that induces a latency-associated CD8 T cell response in BALB/c mice (22). Thus, we have identified a novel, latency-associated CD4 T cell epitope within M2 that stimulates robust IFN-\( \gamma \) expression as late as 60 d p.i.

**\( \gamma H V 68\)-specific CD4 T cells are polyfunctional cytokine producers**

Early studies described an IFN-\( \gamma \)-mediated role of CD4 T cells in maintaining \( \gamma H V 68\) latency (27). Therefore, we set out to analyze the cytokine secretion profile of epitope-specific CD4 T cells throughout infection. We observed robust ex vivo IFN-\( \gamma \)-production by splenic CD4 T cells from \( \gamma H V 68\)-infected mice 12 d p.i. regardless of whether the cells were stimulated with positive control gp150\(_{67-83}\) peptide or negative control influenza NP\(_{11-32}\) peptide (Fig. 2A). These data confirm the previous observation that CD4 T cells isolated from \( \gamma H V 68\)-infected mice produce substantial IFN-\( \gamma \) in a nonspecific manner when assessed by intracellular cytokine staining (28). When cells were stimulated with the newly identified peptides ORF8\(_{112-206}\), ORF48\(_{25-269}\), ORF61\(_{491-705}\), or ORF7b\(_{1020-1034}\), however, we detected significantly greater IFN-\( \gamma \) production than the nonspecific or gp150\(_{67-83}\) peptide-induced levels (Fig. 2A, 2B). These data strongly suggest that at least some Ag-driven stimulation of \( \gamma H V 68\)-specific CD4 T cells elicits IFN-\( \gamma \) production above the nonspecific “background” level. Notably, stimulation of CD4 T cells with each peptide induced substantially less IFN-\( \gamma \)-after AC-RTA infection than WT infection (Fig. 2A, 2B). It is unclear why the gp150\(_{67-83}\) peptide does not stimulate more robust IFN-\( \gamma \)
expression in this assay, given it was originally identified by its ability to stimulate IFN-γ production (12).

We also observed robust ex vivo coproduction of IFN-γ and TNF-α by lung CD4 T cells from γHV68-infected mice 12 d p.i. (Fig. 3A, 3B). Stimulation of cells from AC-RTA–infected mice with the M2124–138 peptide p.i. with WT γHV68 or AC-RTA. The dotted lines are at 30 spots above background. Samples were run in duplicate and are from at least two experiments per time point.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Robust IFN-γ production by novel epitope-specific CD4 T cell responses. (A) IFN-γ production by CD4 T cells at 12, 21, and 60 d p.i. with WT γHV68 (top panel) or AC-RTA (bottom panel). (B) IFN-γ production by CD4 T cells after stimulation with the M2124–138 peptide p.i. with WT γHV68 or AC-RTA. The dotted lines are at 30 spots above background. Samples were run in duplicate and are from at least two experiments per time point.

(CD4 T cell polyfunctionality (i.e., expression of IFN-γ, TNF-α, and IL-2) is associated with improved protection after vaccination or secondary challenge (29, 30), so we next tested whether IFN-γ[TNF-α]CD4 T cells were capable of producing IL-2 after peptide-specific stimulation (Fig. 3C, 3D). We observed considerable IL-2 production by ORF48255–269 and ORF75b1020–1034–specific IFN-γ[TNF-α]CD4 T cells in the lungs 12 d p.i. IL-2 expression declined rapidly, and by 21 d p.i., these cells produced very little IL-2 (Fig. 3D). IL-2 production also declined in two
responses, ORF6593–607 and ORF61343–357 (Fig. 3E), that had sustained IFN-γ production by ELISPOT analysis (Fig. 1A).

Conversely, M2124–138-specific CD4 T cells exhibited marked expression of IL-2 that persisted at least 83 d. Thus, early in infection, virus-specific CD4 T cells demonstrate polyfunctionality, the combined production of IFN-γ, TNF-α, and IL-2. These data are in contrast to previous observations showing that γHV68-specific CD4 T cells that secreted IFN-γ did not make TNF-α or IL-2 (28). After the latency-amplification phase, only M2124–138-specific CD4 T cells exhibit sustained IL-2 production, suggesting that polyfunctional M2124–138-specific CD4 T cells may play an important role in protection from viral reactivation.

We next sought to determine whether there were appreciable differences in the cell cycling or proliferation of lytic or latent epitope-specific CD4 T cells, using a strategy of combining in vivo BrdU treatment with ex vivo stimulation and intracellular cytokine staining (11). Two months p.i., we observed considerable enrichment of epitope-specific CD4 T cells in the BrdU-positive (proliferating) population (Fig. 4A), but we did not observe differential BrdU incorporation in IFN-γ+TNF-α+ CD4 T cells specific for ORF48255–269, ORF75b1020–1034, or M2124–138 (Fig. 4B). Notably, we also did not observe any differences in the functional avidity of lytic or latent Ag-specific CD4 T cells. Each population synthesized IFN-γ at similarly low concentrations of peptide stimulation (Fig. 4C).

M2124–138-specific CD4 T cells are cytotoxic in vivo

CD4 T cell cytotoxicity has been described after γHV68 infection, although no Ag specificity for this effect was identified (28, 31).

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**Table I. Novel CD4 T cell epitopes**

<table>
<thead>
<tr>
<th>ORF</th>
<th>Amino Acids</th>
<th>Sequence</th>
<th>Function</th>
<th>Gene Expression</th>
<th>Stimulated by AC-RTA (12 d p.i.)</th>
</tr>
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<tr>
<td>M2</td>
<td>124–138</td>
<td>NSEPVYIQPISTRSL</td>
<td>Immune regulation/latency</td>
<td>E-L</td>
<td>No</td>
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<td>M4</td>
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<td>Immune regulation</td>
<td>IE</td>
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<td>ORF6</td>
<td>593–607</td>
<td>CNTNWLPFCPHHNL</td>
<td>ssDNA binding protein</td>
<td>E-L*</td>
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<td>ORF8</td>
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<td>gp B</td>
<td>L</td>
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<tr>
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<td>gp B</td>
<td>L</td>
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<td>ORF8</td>
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<td>ITNHAFASTPGAGLS</td>
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<td>ORF25</td>
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<td>ORF61</td>
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<td>ESGAYAEAVPIKSVM</td>
<td>Ribonucleotide reduce large subunit</td>
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<tr>
<td>ORF61</td>
<td>691–705</td>
<td>LFLNEDYASSASNIK</td>
<td>Ribonucleotide reduce large subunit</td>
<td>L</td>
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<td>ORF68</td>
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<td>MLQYAGFLEIVHSS</td>
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</tbody>
</table>

*ORF6 gene expression has been characterized as E (41), E-L (42), L (16), and kinetic cluster II (mostly corresponding to E and E-L genes) (43). E, early; E-L, early-late; IE, immediate-early; L, late (16).

**FIGURE 2.** Epitope-specific IFN-γ production. (A) Representative dot plots showing IFN-γ production and CD44 expression by splenic CD4 T cells measured by intracellular cytokine assay 12 d after WT γHV68 (top panel) or AC-RTA (bottom panel) infection (n = 5, representative of two experiments). (B) The percent of CD4 T cells that are IFN-γ+ (left panel) or the mean fluorescence intensity (MFI) of IFN-γ expression by CD4 T cells (right panel) in the spleen 12 d after WT γHV68 or AC-RTA infection (n = 5, representative of four experiments; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student t test, compared with control values; ns p ≤ 0.01, *p ≤ 0.001, Student t test, comparing AC-RTA to WT values).
To determine if any of the CD4 T cells specific for the newly identified γHV68 epitopes possessed cytotoxic function, we first measured CD107a expression after ex vivo Ag stimulation. CD107a expression correlates with the ability of antigenic stimulation to induce the release of lytic granules (32). At 12 d p.i., about half of the Ag-specific CD4 T cells in the lungs over time after WT γHV68 or AC-RTA infection (n = 5, representative of four experiments; *p ≤ 0.05, **p ≤ 0.01, one-way ANOVA). (C) Representative dot plots showing IFN-γ and IL-2 production by CD4 T cells at 12 or 35 d after WT γHV68 infection. Numbers in the plots indicate the percent of IFN-γ+TNF-α+ CD4 T cells expressing IL-2. (D and E) The percent of IFN-γ+TNF-α+ CD4 T cells that are IL-2+ in the lungs specific for the indicated Ags over time after WT γHV68 infection (n = 5, representative of four experiments; **p ≤ 0.01, ***p ≤ 0.001, one-way ANOVA).

To determine if any of the CD4 T cells specific for the newly identified γHV68 epitopes possessed cytotoxic function, we first measured CD107a expression after ex vivo Ag stimulation. CD107a expression correlates with the ability of antigenic stimulation to induce the release of lytic granules (32). At 12 d p.i., about half of the Ag-specific CD4 T cells in the lungs expressed CD107a after stimulation, and the percentage of IFN-γ+TNF-α+ cells specific for ORF48255–269 and ORF75b1020–1034 that expressed CD107a declined over time (Fig. 5A, 5B). In contrast, once M2124–138-specific CD4 T cells had substantially accumulated in the lungs, nearly all of them expressed CD107a on the cell surface following stimulation for up to 83 d p.i. (Fig. 5A, 5B).

FIGURE 3. Epitope-specific CD4 T cells are polyfunctional. (A) Representative dot plots showing IFN-γ and TNF-α production by lung CD4 T cells at 12 or 35 d after WT γHV68 or AC-RTA infection. Numbers in the plots indicate the percent of CD4 T cells in the gate. (B) The number of IFN-γ+TNF-α+ CD4 T cells in the lungs over time after WT γHV68 or AC-RTA infection (n = 5, representative of four experiments; *p ≤ 0.05, **p ≤ 0.01, one-way ANOVA). (C) Representative dot plots showing IFN-γ and IL-2 production by CD4 T cells at 12 or 35 d after WT γHV68 infection. Numbers in the plots indicate the percent of IFN-γ+TNF-α+ CD4 T cells expressing IL-2. (D and E) The percent of IFN-γ+TNF-α+ CD4 T cells that are IL-2+ in the lungs specific for the indicated Ags over time after WT γHV68 infection (n = 5, representative of four experiments; **p ≤ 0.01, ***p ≤ 0.001, one-way ANOVA).
Plots indicate the percent of IFN-\(\gamma\) were harvested and analyzed by flow cytometry. Left panel: Lung CD4 T cells at 12 or 35 d after WT ORF75\(_{b1020-1034}\) target cells (4–9, combined from two experiments). (D) Histograms showing granzyme B (GzmB) expression in CD44 hi or CD44 lo populations; ***\(p \leq 0.001\), one-way ANOVA.

Figure 5. M2\(_{124-138}\)-specific CD4 T cells are cytotoxic. (A) Representative dot plots showing IFN-\(\gamma\) production and CD107a expression by lung CD4 T cells at 12 or 35 d after WT \(\gamma\)HV68 infection. Numbers in the plots indicate the percent of IFN-\(\gamma\)-TNF-\(\alpha\)-\(\gamma\) CD4 T cells expressing CD107a. (B) The percent of IFN-\(\gamma\)-TNF-\(\alpha\)-\(\gamma\) CD4 T cells that are CD107a+ in the lungs over time after WT \(\gamma\)HV68 infection (n = 5, representative of four experiments). ***\(p \leq 0.001\), one-way ANOVA. (C) Specific lysis of peptide-pulsed target cells (SEM) in WT \(\gamma\)HV68-infected mice (n = 4–9, combined from two experiments). (D) Specific lysis of peptide-pulsed target cells (SEM) in AC-RTA-infected mice (n = 5–7, combined from two experiments). (E) Twenty-one days after WT \(\gamma\)HV68 infection, spleens were harvested and analyzed by flow cytometry. Left panel: Representative histograms showing granzyme B (GzmB) expression in CD44hi or CD44lo CD4 T cells (top panel) or CD8 T cells (bottom panel). Center panel: Representative dot plots showing expression of CD44 and either M2\(_{124-138}\)-I-A\(^b\) (top panel) or ORF61\(_{524-531}\)-K\(^b\) (bottom panel) tetramers. Right panel: Percent of cells expressing GzmB (n = 7, representative of three experiments). ***\(p \leq 0.001\), one-way ANOVA.

Discussion

We have identified a panel of \(\gamma\)HV68-specific CD4 epitopes and followed the expression of epitope-specific CD4 T cells throughout the course of natural \(\gamma\)HV68 infection. Previously, we identified a panel of \(\gamma\)HV68-specific CD8 T cell epitopes and followed their expression (10). The combined results show that antiviral CD4 and CD8 T cells have very different kinetic patterns. Comparative analysis with WT virus and a recombinant virus incapable of establishing latency allowed us to examine the influence of latency on differential expression of epitope-specific responses. Whereas the CD8 T cells exhibited two basic patterns of expression apparently dependent on epitope expression during lytic and latent infection, the kinetics of CD4 T cells were more complex. One group of epitopes was expressed predominantly during the acute infection, a second group was expressed both during the acute infection and early stages of latency, and an additional epitope was expressed exclusively during latency. These data represent the first kinetic analysis, to our knowledge, of a panel of CD4 epitopes during \(\gamma\)HV68 infection and illustrate the importance of latency in initiating and sustaining CD4 T cells specific for some but not all epitopes. We did not detect any evidence for inflation of the M2\(_{124-138}\)-specific response (33), consistent with the temporally restricted expression of the M2 gene (22).

The newly identified epitopes will facilitate analysis of Ag presentation during \(\gamma\)HV68 infection. We recently demonstrated that B cells and dendritic cells from latently infected mice could each stimulate \(\gamma\)HV68-specific CD4 T cells, but it is not yet known whether they present the same epitopes (34). Notably, EBV-specific cytolytic CD4 T cells recognize a variety of both lytic and latent epitopes on B cell lymphoma cells (35). Cytotoxic CD4 T cells specific for latent epitopes may be important because the majority of tumor cells sustain a latent infection, whereas noncytotoxic, cytokine-secreting lytic epitope-specific CD4 T cells may be crucial for targeting cells harboring reactivating virus to prevent full recrudescence. As CD4 T cells are capable of mediating protection in two models of \(\gamma\)HV68-associated tumors (36, 37), it will be of particular interest to investigate the protective efficacy of latency-specific cytotoxic CD4 T cells in these systems.

In our hands, immunization with M2\(_{124-138}\) peptide in CFA induced a strong cytokine-producing epitope-specific CD4 T cell response, but did not induce antiviral T cells that were protective...
from viral challenge or in reducing latent viral loads (data not shown). We believe this is at least in part due to a dearth of cytotoxic CD4 T cells generated after immunization, as our initial results showed little or no M2-ultrathin-specific cytotoxicity (data not shown). Our data are consistent with the findings of Smith and colleagues (38), who did not observe protection from challenge in an engineered latency epitope vaccination strategy even though they induced robust epitope-specific CD4 T cells. Previously, vaccination to induce gp150_83-specific CD4 T cells has resulted in the generation of protective responses, although whether the T cells exhibited cytotoxicity is unclear (12).

Understanding how to induce and sustain a strong cytolytic CD4 T cell response by vaccination and how to generate CD4 effectors therapeutically are important challenges. It will be important to determine if other prophylactic vaccination strategies can induce virus-specific CD4 T cells capable of reducing acute infection or latency establishment and whether therapeutic vaccination designed to boost M2-specific CD4 T cells reduces latent viral loads, thereby lessening the risk for oncogenesis. Of note, transgenic mice designed to boost M2-specific CD4 T cells reduces latent viral loads (data not shown). We believe this is at least in part due to a dearth of cytolytic CD4 T cells defined by IFN-gamma.

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Disclosures

The authors have no financial conflicts of interest.

References


