Impact of distinct poxvirus infections on the specificities and functionalities of CD4+ T cell responses.

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Siciliano, Nicholas A; Hersperger, Adam R; Lacuanan, Aimee M; Xu, Ren-Huan; Sidney, John; Sette, Alessandro; Sigal, Luis J; and Eisenlohr, Laurence C., "Impact of distinct poxvirus infections on the specificities and functionalities of CD4+ T cell responses." (2014). *Department of Microbiology and Immunology Faculty Papers*. Paper 71.

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Impact of Distinct Poxvirus Infections on the Specificities and Functionalities of CD4⁺ T Cell Responses

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Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, USA; Fox Chase Cancer Center, Immune Cell Development and Host Defense Program, Philadelphia, Pennsylvania, USA; Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, California, USA; Department of Biology, Albright College, Reading, Pennsylvania, USA

ABSTRACT
The factors that determine CD4⁺ T cell (TCD4⁺) specificities, functional capacity, and memory persistence in response to complex pathogens remain unclear. We explored these parameters in the C57BL/6 mouse through comparison of two highly related (>92% homology) poxviruses: ectromelia virus (ECTV), a natural mouse pathogen, and vaccinia virus (VACV), a heterologous virus that nevertheless elicits potent immune responses. In addition to elucidating several previously unidentified major histocompatibility complex class II (MHC-II)-restricted epitopes, we observed many qualitative and quantitative differences between the TCD4⁺ repertoires, including responses not elicited by VACV despite complete sequence conservation. In addition, we observed functional heterogeneity between ECTV- and VACV-specific TCD4⁺ at both a global and individual epitope level, particularly greater expression of the cytolytic marker CD107a from TCD4⁺ following ECTV infection. Most striking were differences during the late memory phase where, in contrast to ECTV, VACV infection failed to elicit measurable epitope-specific TCD4⁺ as determined by intracellular cytokine staining. These findings illustrate the strong influence of epitope-extrinsic factors on TCD4⁺ responses and memory.

IMPORTANCE
Much of our understanding concerning host-pathogen relationships in the context of poxvirus infections stems from studies of VACV in mice. However, VACV is not a natural mouse pathogen, and therefore, the relevance of results obtained using this model may be limited. Here, we explored the MHC class II-restricted TCD4⁺ repertoire induced by mousepox (ECTV) infection and the functional profile of the responding epitope-specific TCD4⁺, comparing these results to those induced by VACV infection under matched conditions. Despite a high degree of homology between the two viruses, we observed distinct specificity and functional profiles of TCD4⁺ responses at both acute and memory time points, with VACV-specific TCD4⁺ memory being notably compromised. These data offer insight into the impact of epitope-extrinsic factors on the resulting TCD4⁺ responses.

Received 23 April 2014 Accepted 13 June 2014
Published ahead of print 25 June 2014
Editor: G. McFadden
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doi:10.1128/JVI.01150-14
VACV cause no discernible long-term effects. Thus, a comparative analysis of ECTV and VACV infection in mice provides an excellent opportunity to reveal the character of the ensuing virus-specific T<sub>CD4</sub> responses through the examination of specificity and functionality.

The primary aim of the present study was to compare the reactivity, magnitude, and functionality of ECTV- and VACV-specific T<sub>CD4</sub>. By screening a large number of 12- to 15-mer peptides, we identified a total of 14 ECTV-specific T<sub>CD4</sub>-epitopes and observed both quantitative and qualitative differences between the T<sub>CD4</sub>-epitope repertoires elicited by ECTV and VACV. Subsequently, we probed differences in virus-mediated imprinting on T<sub>CD4</sub> function and found that the resulting profiles of epitope-specific T<sub>CD4</sub> are distinct and that long-term T<sub>CD4</sub>-memory to ECTV is substantially stronger. In total, these data offer insight into the degree to which the alignment of host and pathogen can affect the specificity and functionality of responding virus-specific T<sub>CD4</sub>.

MATERIALS AND METHODS

Ethics statement. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University (Philadelphia, PA) and carried out in a humane manner.

Viruses. The VACV WR strain was obtained from Bernard Moss (National Institute of Allergy and Infectious Diseases) and grown in 143 TK<sup>+</sup> cells. The ECTV Moscow strain was grown in BSC1 cells. The Δevm0158 ECTV (Fox Chase Cancer Center) was generated by homologous recombination, like other mutant viruses, (32, 33) and grown in BSC1 cells.

Mice. Six- to 8-week-old female C57BL/6 mice were obtained from Jackson Laboratory and were used between 6 and 8 weeks of age according to the National Institutes of Health guidelines and Institutional Animal Care and Use Committee-approved animal protocols.

Infection and immunizations. Six- to 8-week-old female C57BL/6 female mice were infected in the left hind footpad with either 3,000 PFU ectromelia virus (Moscow strain), 3,000 PFU or 3 × 10<sup>6</sup> PFU vaccinia virus (Western Reserve), or 3,000 PFU Δevm0158 ECTV. C57BL/6 mice were obtained from The Jackson Laboratory and were used between 6 and 8 weeks of age according to the National Institutes of Health guidelines and Institutional Animal Care and Use Committee-approved animal protocols.

Epitope mapping. The 1,022 peptides used for mapping were a subset of a previously described library (34). Briefly, peptides were synthesized as crude material by Pepscan Systems and mimotopes ranging from 12 to 15 amino acids in length were used previously to identify VACV epitopes (34). The peptides were screened for reactivity against splenocytes from ECTV-primed mice at various time points postinfection. Splenocytes from naive mice were used as antigen-presenting cells. Naive splenocytes were incubated with peptide (final concentration, 2 μg/ml) at 37°C and 5% CO<sub>2</sub>. Peptide-primed splenocytes were then coincubated overnight with either ECTV-specific whole splenocytes or T<sub>CD4</sub> isolated from splenocytes (Dynal mouse CD4 negative isolation kit; Invitrogen). IFN-γ-positive T cell responses were assessed by IFN-γ ELISPOT (BD). Spots were counted using ImmunoSpot software (Cellular Technology Limited).

RESULTS

T<sub>CD4</sub>-response magnitude after infection with ECTV or VACV. The distinct pathogenesis of ECTV compared with that of VACV in C57BL/6 mice, despite >92% genetic identity (36), could be appreciated following footpad inoculation of these viruses (Fig. 1A). VACV-induced inflammation was often detectable by day 3 postinfection, while the onset of ECTV-induced swelling was typically not appreciable until day 6. This can be ascribed, at least in part, to greater subversion of early immune responses by ECTV than by VACV (14, 37). However, while VACV-induced inflammation remained relatively mild and eventually subsided, ECTV-associated swelling became considerable, ultimately leading to necrosis and loss of the limb within ~21 days of infection in most cases, consistent with previous reports (38). In both infections, we found day 10 postinfection to be the time point at which virus-specific T<sub>CD4</sub>-responses could be discriminatingly measured, providing an optimal signal-to-noise ratio (data not shown). The divergent pathogenesis of ECTV and VACV in mice, despite a high degree of genetic similarity, provides an ideal experimental system to explore the influence of virulence and infectivity on the resulting T<sub>CD4</sub>-responses following poxvirus infection.

The global T<sub>CD4</sub>-responses to ECTV and VACV were initially compared to determine differences in the overall magnitudes of the responses to these two distinct poxviruses. We primed C57BL/6 mice with ECTV or VACV, and 7 days later, cocultured splenocytes with bone marrow-derived dendritic cells (BMDCs) infected with either ECTV or VACV. Using flow cytometry, we measured the magnitude of the T<sub>CD4</sub>-responses by examining several T cell functional outputs (Fig. 1B). Heterologous stimulation produced approximately 62% (VACV T<sub>CD4</sub> to ECTV BMDC) to 67% (ECTV T<sub>CD4</sub> to VACV BMDC) of the numbers of activated T<sub>CD4</sub>-produced by homologous stimulation. This was 25 to 30% lower than what would be anticipated based upon the degree of sequence identity shared by the proteomes of VACV and ECTV (36, 39). This suggested a disparity in the T<sub>CD4</sub>-responses to the two poxviruses extending beyond sequence heterogeneity, a possibility that was first investigated by assessing responses to individual epitopes.

T<sub>CD4</sub>-specificities elicited by VACV and ECTV. Previous mapping of the MHC-II-restricted C57BL/6 response to intraperitoneal VACV infection was accomplished with a library of synthetic peptides, comprising ~30% of the predicted transcriptome of VACV.
(34). That study identified 14 specificities, with late-phase antigens predominating among the list of parent proteins (34). Utilizing 1,022 of those peptides (comprising ~15% of the predicted transcriptome of VACV), we compared the reactivities elicited by ECTV and VACV in C57BL/6 mice by ELISpot analysis (Fig. 2) (34). Since the common route of entry for ECTV is through abrasions on the skin (38, 40–42), we performed footpad injection at the standard dose (3,000 PFU) as the route of infection for this study. Importantly, for the ELISpot screening, both the route and dose of VACV were matched to allow for direct comparison with the results obtained following ECTV priming. Spleens were harvested 10 days after infection, and the frequency of reproducible ECTV epitopes (Fig. 3A) measured was relatively low, on the order of 1 to 2% (Fig. 3B), consistent with the previous VACV screen (34).

The majority of peptides screened were fully conserved between VACV and ECTV, consistent with the high degree of homology. Fourteen distinct and reproducible MHC-II-restricted specificities were identified in response to ECTV infection (Fig. 3A). Fourteen distinct and reproducible MHC-II-restricted specificities were identified in response to ECTV infection (Fig. 3A). Attempts to generate footpads were recorded in millimeters at the indicated days postinfection. Representative photos of footpads at day 12 postinfection with either ECTV or VACV are shown. (B) T

As M1L426 – 434 (IIIPFIAYF) (data not shown).

The sum of the differences in TCD4+ specificities depicted in Fig. 2, 3, and 4 do not, on their own, account for the unexpected deficit in cross-reactivity shown by the results in Fig. 1. Particular striking are the unequal responses to homologous epitopes. For example, despite the sequence identity, ECTV elicits significantly greater responses to the A20R230 –244, E2L426 – 440, and F15L41–55 epitopes than VACV. Conversely, VACV elicits far greater responses to the I1L7–21, I1L18–33, and L4R73–87 epitopes. These differences in both directions indicate qualitative differences in the T

Minimal influence of the ECTV IFN-γ binding protein on assay results. One factor we needed to address at an early stage was the IFN-γ binding protein expressed by both ECTV and VACV, since only the ECTV version has specificity for murine IFN-γ (43). Thus, the comparative ELISpot assays might have been compromised by this selective activity. To address this, we compared the responses of mice to wild-type (WT) ECTV and a recombinant strain that lacks the soluble IFN-γ receptor (VACV). The T

Comparative functional profiles of epitope-specific T

At the same time, we expanded the screen to include CD8+ T cell (TCD8+) specificities and identified a novel and relatively potent MHC class I (MHC-I)-restricted epitope, M1L424–438, that elicited TCD8+ responses after both ECTV and VACV infections (Fig. 2 and 3C). The minimal H2-Kb-restricted epitope within the M1L424–438 peptide was defined via a series of truncated peptides as M1L426–434 (IIIPFIAYF) (data not shown).

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FIG 2 Comparative epitope specificities and magnitudes of responses to VACV- and ECTV-primed splenocytes. Naive splenocytes were incubated with synthetic poxvirus peptides ranging from 12 to 15 amino acids in length and screened for reactivity against splenocytes from ECTV-primed mice 10 days postinfection. Naive splenocytes were incubated with peptide (final concentration, 2 μg/ml). Peptide-primed splenocytes were coincubated overnight with whole splenocytes from ECTV-primed mice (3,000 PFU per footpad (f.p.)) 10 days postinfection. IFN-γ-positive T cell responses were assayed by IFN-γ ELISpot. To determine the level of statistical significance, Student’s t test was performed using the mean of triplicate values of the response. #, number; *, P < 0.05; limits, means ± standard deviations (SD); APC, antigen-presenting cell. Data are representative of 3 independent experiments.
being completely conserved between ECTV and VACV. Three cytokines were measured: IFN-γ, interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α). In general, these molecules play well-described roles in antiviral immunity (44–47), and both IFN-γ and TNF-α have been found to be particularly important for protection following poxvirus infection (48, 49). In order to identify T_{CD4^+} with cytotoxic potential, we also assessed degranulation by measuring the surface expression of CD107a after stimulation (50). In agreement with our prior study (15), the average T_{CD4^+} response profile across all six epitopes was comparable between the two viruses (Fig. 6A). However, there were significant differences in the overall frequency of cytokine production when

FIG 3 Determination of ECTV-specific T_{CD4^+} epitopes in C57BL/6 mice. A synthetic VACV peptide library of 1,022 peptides, ranging from 12 to 15 amino acids in length, was screened for reactivity against splenocytes from ECTV-primed mice (3,000 PFU per footpad) 10 days postinfection. Naive splenocytes were incubated with peptide (final concentration, 2 μg/ml). Peptide-primed splenocytes were coincubated overnight with ECTV-specific whole splenocytes. (A) IFN-γ-positive T cell responses were assayed by IFN-γ ELISpot. Stars indicate previously unreported poxvirus epitopes, and MHC class I or MHC class II epitopes are indicated below. (B) Frequency map of the positive peptides identified in panel A. (C) Confirmation that the epitopes identified in panel A are recognized by T_{CD4^+}. A screen was performed as described in the legend to panel A with splenocytes or purified T_{CD4^+} from ECTV-primed mice. Limits, means ± SD. MHC class I or MHC class II epitopes are indicated below.
individual functions were assessed (Fig. 6B), and it became clear that ECTV-specific T CD4 H11001 released significantly more cytotoxic granules after peptide stimulation.

Next, we examined the functional profiles of the individual specificities. Despite the functional similarity between ECTV- and VACV-specific T CD4 H11001 at a global (peptide pool) level, we observed heterogeneity in the individual response profiles (Fig. 6C). Of note, VACV-infected mice did not yield a response above the background for two specificities, I4L 632–646 and H3L269–283 (Fig. 6C). Of the specificities that could be compared, E9L176–190-specific T CD4 H11001 displayed the greatest functional divergence.

Additionally, due to the importance and relevance of long-lasting poxvirus immunization strategies (51, 52, 113), we examined epitope-specific TCD4/H11001 functionality and persistence into the memory phase (>100 days postinfection). We found that five of the six ECTV specificities were detectable (greater than or equal to 0.05% of total TCD4/H11001) at 100 days postinfection (reactivity to I4L632–646 was undetectable by ICS), with minor degradation of functionality over time in each case. In striking contrast, no individual VACV-specific epitopes were detectable at 100 days postinfection, even though a 1,000-fold high dose of VACV was used for priming (Fig. 6D). In the context of this study, these data suggest that ECTV and VACV infections mediate distinct priming and

<table>
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<tr>
<th>Table 1 Identification of ECTV-specific epitopes a</th>
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<tr>
<td>Peptide hit</td>
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<tr>
<td>B6R20–27</td>
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<tr>
<td>M11L424–438</td>
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<tr>
<td>A18R46–60</td>
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<tr>
<td>A20R230–244</td>
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<tr>
<td>E9L176–190</td>
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<tr>
<td>I4L632–646</td>
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<tr>
<td>J4R13–28</td>
</tr>
<tr>
<td>E11L114–128</td>
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<tr>
<td>D13L483–497</td>
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<tr>
<td>I1L21</td>
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<tr>
<td>I1L33–55</td>
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<tr>
<td>H3L269–283</td>
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<tr>
<td>L4R173–187</td>
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<tr>
<td>F15L41–56</td>
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a Boldface indicates previously unreported epitopes.

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<th>Table 2 T CD4 + poxvirus-specific epitopes</th>
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<tr>
<td>Peptide hit</td>
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<tr>
<td>ECTV &gt; VACV</td>
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<tr>
<td>A20R230–244</td>
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<tr>
<td>F15L41–56</td>
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<tr>
<td>VACV &gt; ECTV</td>
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<td>I1L21</td>
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<tr>
<td>I1L33–55</td>
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<td>L4R173–187</td>
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<tr>
<td>A24R399–413</td>
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<tr>
<td>A28L7–21</td>
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<tr>
<td>D8L235–249</td>
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<tr>
<td>ECTV &lt; VACV</td>
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<td>A18R46–60</td>
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<td>E9L27–60</td>
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<td>J4R13–28</td>
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<tr>
<td>E11L14–128</td>
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<tr>
<td>D13L483–497</td>
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<td>H3L269–283</td>
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a Stimulation index (SI) of >2.

b Includes 5 amino acid residues upstream and downstream from the epitope.
functional imprinting of epitope-specific T<sub>CD4</sub> that affect persistence into the memory phase.

A greater frequency of effector T<sub>CD4</sub> is elicited by ECTV during acute infection. Having examined CD107a expression among the poxvirus-specific responses (Fig. 6), we looked in more detail at the effector status of T<sub>CD4</sub> responses after infection. Our recent work points to the importance of cytolytic function by T<sub>CD4</sub> during acute ECTV infection of mice (9). Here, we asked whether cytolytic function is a poxvirus-specific phenomenon or unique to ECTV. Using flow cytometric analysis, we observed significant differences in the surface mobilization of cytotoxic granules by T<sub>CD4</sub> that were dependent upon both the epitope specificity and identity of the infecting poxvirus. The T<sub>CD4</sub> responses to two conserved epitopes, D13L483–497 and E9L176–190, serve as an illustration of these points, with ECTV infection inducing E9L176–190-specific T<sub>CD4</sub> with greater degranulation capacity than T<sub>CD4</sub> with other specificities (Fig. 7A). Additionally, compared with VACV, ECTV infection consistently elicited a higher proportion of responding T<sub>CD4</sub> with discernible CD107a expression after stimulation (Fig. 7A). The frequency of degranulation typically declined over time but was better maintained for some epitope specificities (Fig. 7B).

Granzyme B (gzmB) is a major proapoptotic mediator stored within cytotoxic granules. As an additional way to assess cytotoxic potential, we measured the global levels of this molecule within total T<sub>CD4</sub> at acute time points postinfection with both viruses in the liver, inguinal lymph nodes, and spleen. We found that total T<sub>CD4</sub> in all three locations within ECTV-infected mice expressed 2- to 3-fold-higher levels of gzmB than were observed with VACV (Fig. 7C and D). The enhanced gzmB expression observed from ECTV T<sub>CD4</sub> suggests that the induction of cytolytic T<sub>CD4</sub> is characteristic of the murine host response to ECTV and not generally associated with murine poxvirus infection, as it is absent in VACV-immunized animals.

To further explore differential effector phenotypes between ECTV and VACV T<sub>CD4</sub>, we examined the expression levels of macrophage inflammatory protein 1α (MIP1α) from ECTV- and VACV-specific T<sub>CD4</sub>. MIP1α is a proinflammatory chemokine involved in the recruitment of immune cells, and its expression by T cells is associated with a more robust effector profile (53–55). We found greater expression of MIP1α from ECTV-specific T<sub>CD4</sub> than from VACV T<sub>CD4</sub> (Fig. 8), offering additional evidence that, in general, ECTV-specific T<sub>CD4</sub> display a greater effector-like profile than their VACV counterparts.

**DISCUSSION**

This study has revealed several ways in which host responses to the natural murine poxvirus, ECTV, differ considerably from those to VACV, the predominant model for examining poxvirus virulence and immunity in mice (16, 56–59). The initial experiments revealed a level of cross-reactivity, ~62 to 67%, that was far lower than that expected by the degree of homology (92%). The basis for this became clear when we examined individual specificities with a 12- to 15-mer peptide library. Eleven of 14 previously identified VACV-induced specificities were elicited by ECTV (34), with the remaining three specificities (D8L238–252, A28L10–24, and A24R399–417) failing to develop in response to ECTV. The screen also uncovered four novel poxvirus epitopes for both ECTV and VACV, three of which were MHC class II (I4L332–346, B13R14–28, and E2L426–440) and one of which was MHC class I (M1L426–434, the precise boundaries being subsequently determined by a truncated-peptide series). Several factors may explain why the three MHC-II epitopes were not discovered in an earlier screen (33). Different routes of infection can affect T<sub>CD4</sub> differentiation, since they determine the initial cell types that interact with the virus (60), and prior screens utilized an intraperitoneal challenge (34), whereas here, we employed a dermal footpad challenge to mimic the natural infection route of ECTV (42). Additionally, prior VACV epitope screens utilized B cells to present synthetic peptides to T<sub>CD4</sub> (34), and differential costimulatory molecule expression by unique antigen-presenting cell types can alter signaling at the

**FIG 4** Comparative epitope reactivities to D8L238–252 between VACV- and ECTV-primed splenocytes. Naive splenocytes were incubated with synthetic D8L238–252 (VACV sequence) or D8L238–252-A244–246/A249–249 (ECTV sequence) at a final concentration of 2 μg/ml. Peptide-primed splenocytes were then cocultivated overnight with whole splenocytes from ECTV-primed mice or VACV-primed mice (3,000 PFU per footpad) 10 days postinfection. IFN-γ-positive T cell responses were assayed by IFN-γ ELISPot. To determine the level of statistical significance, Student’s t test was performed using the mean of triplicate values of the response. *, P < 0.05.

**TABLE 3** Previously unreported poxvirus-specific epitopes

<table>
<thead>
<tr>
<th>Peptide hit</th>
<th>Reactivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Epitope (% Conservation of: differing residue)</th>
<th>Flanking region&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Parent protein</th>
<th>Time of expression</th>
<th>MHC class</th>
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</thead>
<tbody>
<tr>
<td>M1L426–434</td>
<td>ECTV and VACV</td>
<td>100</td>
<td>96</td>
<td>96</td>
<td>Early</td>
<td>I</td>
</tr>
<tr>
<td>H2L32–44</td>
<td>ECTV and VACV</td>
<td>100</td>
<td>100</td>
<td>97</td>
<td>Early</td>
<td>II</td>
</tr>
<tr>
<td>B13R14–28</td>
<td>ECTV only</td>
<td>93.3 (P&lt;sub&gt;20&lt;/sub&gt;–S&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>100</td>
<td>95</td>
<td>Early</td>
<td>II</td>
</tr>
<tr>
<td>E2L426–440</td>
<td>ECTV only</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>Early</td>
<td>II</td>
</tr>
</tbody>
</table>

<sup>a</sup> Stimulation index (SI) of >2.

<sup>b</sup> Includes 5 amino acid residues upstream and downstream from the epitope.

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immunological synapse, resulting in shifted T<sub>CD4</sub> activation thresholds (61).

The novel MHC-I epitope, M1L<sub>426–434</sub>, induced relatively equivalent responses from both ECTV and VACV splenocytes that were nearly as potent as the immunodominant B8R<sub>20–27</sub> for both VACV and ECTV splenocytes (Fig. 2), independent of infection route and dose (data not shown). In silico analyses that utilized algorithm-based predictions for MHC-I binders within the VACV transcriptome identified a 10-mer H-2Db class I epitope (TSNVITDQTV/M1L<sub>291–300</sub>) within the M1L parent protein (56) but not at the 426–434 location. The lack of ECTV T<sub>CD4</sub> reactivity to the three specificities identified in the original VACV screen could not be accounted for by sequence heterogeneity, as A28L<sub>10–24</sub> and A24R<sub>399–413</sub> are both 100% conserved. And although there were two amino acid differences in the D8L<sub>238–252</sub> epitope (A244<sub>≠</sub>V244 and A249<sub>≠</sub>V249), the possibility that these changes prevent proper processing of the antigen or prevent binding to the I-A<sub>B</sub> molecule was discounted by the observation that VACV T<sub>CD4</sub> responds to the ECTV homolog. Thus, in all three cases, factors extrinsic to epitope composition are at work. One clear difference between the two viruses is the course of infection. ECTV productively infects a wider range of murine cell types, including dendritic cells, epidermal T cells, and keratinocytes (14, 41, 62). Thus, different sets of antigen-presenting cells with differing processing capabilities will be engaged. Furthermore, ECTV produces species-specific factors that allow for evasion and subversion of host responses and far greater replication in mice, as well as a greater antigen load, which will affect the levels of epitope display. Virulence factors and antigen load can also affect the cytokine milieu, which is markedly different in the two infections (15, 63–67) and which can strongly influence antigen-processing capabilities (68, 69). Chief among the cytokines of interest is IFN-γ, which, in addition to influencing the expression of antigen-processing components (68–71), drives upregulation of MHC-II (72). This was especially true for these investigations since ECTV but not VACV encodes a soluble IFN-γ receptor (B8R) that binds to murine IFN-γ (25, 73, 74). However, deletion of B8R did not have a substantial impact on overall T<sub>CD4</sub> magnitude or on the individual T<sub>CD4</sub> reactivities, consistent with our observation that Δevm0158 is not attenuated in vivo (unpublished data).

Differences in participating antigen-presenting cells and anti-

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**TABLE 4** Selected T<sub>CD4</sub> /MHC class II poxvirus-specific epitopes<sup>a</sup>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A20R&lt;sub&gt;230–244&lt;/sub&gt;</td>
<td>GDNIFPSVITKSGK</td>
</tr>
<tr>
<td>H4L&lt;sub&gt;832–646&lt;/sub&gt;</td>
<td>EFQVVNPHLLRVTLE</td>
</tr>
<tr>
<td>F15I&lt;sub&gt;51–55&lt;/sub&gt;</td>
<td>TPRYIPSTISSNNI</td>
</tr>
<tr>
<td>E1L&lt;sub&gt;114–128&lt;/sub&gt;</td>
<td>VLTIKAPVIISSKIS</td>
</tr>
<tr>
<td>H1L&lt;sub&gt;5–21&lt;/sub&gt;</td>
<td>QLVFNSISARALKAY</td>
</tr>
<tr>
<td>H1L&lt;sub&gt;18–35&lt;/sub&gt;</td>
<td>LKAYFTAKINEMVDE</td>
</tr>
<tr>
<td>D13L&lt;sub&gt;483–497&lt;/sub&gt;</td>
<td>PKIFFRPTITANVS</td>
</tr>
<tr>
<td>E9L&lt;sub&gt;176–190&lt;/sub&gt;</td>
<td>PSVFINPISHSYCY</td>
</tr>
<tr>
<td>H3L&lt;sub&gt;269–283&lt;/sub&gt;</td>
<td>PGVMYAFTPILSSF</td>
</tr>
<tr>
<td>L4R&lt;sub&gt;173–187&lt;/sub&gt;</td>
<td>ISKYAGINILVYYP</td>
</tr>
</tbody>
</table>

<sup>a</sup> Boldface indicates peptides that consistently elicited robust T<sub>CD4</sub> responses in ELSpot assays and were pooled to assess functionality using intracellular cytokine staining (ICS) and polychromatic flow cytometry.
gen load can also affect the functional character of the T<sub>CD4</sub><sup>+</sup> response (75–79). Indeed, we found that for the majority of the epitopes tested (5 of 6), ECTV-specific T<sub>CD4</sub><sup>+</sup> demonstrated a higher effector capacity; most notably enhanced were cytolytic potential and MIP1α expression. Furthermore, VACV T<sub>CD4</sub><sup>+</sup> exhibited responses above the background for just 4 of the 6 epitopes examined at 10 days postinfection, while ECTV-specific T<sub>CD4</sub><sup>+</sup> reacted strongly to all 6. We also identified higher percentages of grzB-positive cells from the tissues of ECTV-infected mice (Fig. 7C and D). This was true of all three tissues examined at acute time points postinfection. This result demonstrates that the generation of robust cytolytic T<sub>CD4</sub><sup>+</sup> is not a general property of all poxvirus infections and is consistent with earlier reports that increased antigen loads drive the development of cytolytic T<sub>CD4</sub><sup>+</sup> in both acute and chronic viral infections (80, 81).

Previous work by Fang et al. demonstrated that cytolytic T<sub>CD4</sub><sup>+</sup> contribute to the suppression and host control of ECTV replication (9), consistent with the well-documented pleiotropic antiviral effects of grzB expression. In addition to triggering apoptosis during ECTV infection, despite the production of antiapoptotic proteins such as B13R (82, 83), grzB has been shown to directly suppress VACV replication via cleavage of eukaryotic initiation factor 4 gamma 3 (eIF4G3), a protein essential for the initiation of protein translation (84). Increased grzB production may also play a role in the greater inflammation shown with ECTV at the site of infection (footpad) (Fig. 1) by inducing apoptosis of endothelial...
cells (anoikis) via granule exocytosis (85) and remodeling of extracellular matrix through cleavage of vitronectin, fibronectin, and laminin (86).

We also assessed the functional profiles of several peptide-specific T<sub>CD4+</sub> at memory (day 100) time points using flow cytometry (Fig. 6D). While ECTV infection stimulated robust long-term T<sub>CD4+</sub> memory, none of the 6 epitopes examined yielded a response above the background at day 100 postinfection with VACV, even when the input dose was 1,000-fold-greater for VACV than for ECTV. This was unexpected, since prior studies have shown persistence of VACV-specific T<sub>CD4+</sub> in both humans (52) and mice (15, 66). In our own earlier study (15), we detected both T<sub>CD4+</sub> memory responses at day 75 postinfection, utilizing a more comprehensive peptide pool than the one examined here, and VACV-infected presenting cells. Thus, while long-term T<sub>CD4+</sub> memory to VACV may not be completely absent, it is significantly compromised.

The considerably greater difference between ECTV- and
FIG 8 ECTV-specific TCD4+ express larger amounts of MIP1α during acute infection. (A) Representative staining showing higher expression of MIP1α by ECTV-specific TCD4+ compared to VACV-specific TCD4+ cells. ECTV- or VACV-primed mice (3,000 PFU per footpad) at 10 days postinfection are shown. (B) Quantification of MIP1α expression among several different peptide-specific TCD4+ populations from four independent experiments. *P value was determined using the Mann-Whitney test. Bars and whiskers represent the means and SD.

VACV-specific TCD4+ at the memory phase is consistent with the earlier demonstration that differences in the initial antiviral TCD4+ character can be exaggerated as populations transition to central and/or effector memory cells (87, 88). Several factors might contribute to this amplification effect. Early expression of IL-15 by phagocytes and innate immune cells has been found to induce both a cytolytic TCD4+ character and TCD4+ effector memory cells (89–91) and has also been associated with NK cell recruitment, shown to be essential for natural resistance to ECTV (92). In addition, the above-mentioned parameters of antigen exposure and persistence can greatly affect the initial and long-term effector properties of responding TCD4+ (77, 87, 93). At the same time, excessive antigen exposure can also lead to exhaustion in some settings (94). A greater understanding of the factors that set the balance between long-lasting TCD4+ effector-memory and exhaustion has been elusive (87, 94) but will likely be critical for insight into the profound differences in TCD4+ memory to two such homologous viruses.

The long-term protection against smallpox conferred by VACV is well known (52, 95–105). In a recent study of smallpox vaccinees, the levels of VACV-specific antibody, generally held to be the measure of protection (52), remained stable over many years postinmunization. In contrast and in line with our findings, VACV-specific TCD4+ memory declined over time (52). Due to the complexity of poxviruses, which clearly contain an abundance of MHC-II-restricted epitopes (this study and reference 34), this decline may not meaningfully impact protection. Alternatively or in addition, memory TCD4+ may not play a critical role in protection from smallpox. The importance of robust TCD4+ memory populations for protection from agents such as influenza virus and hepatitis A and C viruses is more apparent (106–111). Whether this is due to their relative simplicity and the limited number of TCD4+ epitopes they encode or other factors remains to be determined.

ECTV is proving to be an excellent model both in terms of providing an opportunity to study a natural host-pathogen relationship under defined conditions and in being highly relevant to other poxviruses (112), including smallpox and monkeypox (11, 12). The overall results of this study highlight interesting distinctions between the TCD4+ epitope reactivity profiles of ECTV and VACV. A better understanding of the mediators that influence TCD4+ reactivity and function is critical to designing better vaccines and antiviral therapeutics. Factors such as tropism and replication efficiency are likely major reasons for the functional differences observed between ECTV and VACV TCD4+ responding to common epitopes. Moreover, distinct host-pathogen relationships may ultimately play a predominant role in both shaping the TCD4+ repertoire and influencing the functional imprinting and differentiation of poxvirus-specific TCD4+. The striking cytolytic character of TCD4+ induced by ECTV and the inability of VACV to drive the development of this protective TCD4+ subset (89) or a substantial memory TCD4+ population demonstrate the impact of epitope-extrinsic mediators on TCD4+ repertoire, function, and persistence. Further study in this comparative poxvirus model may yield additional insights into the design of vaccine strategies that lead to more robust and long-lived TCD4+ responses.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant U19-AI083008.

Research in this publication includes work carried out at the Kimmel Cancer Center Flow Cytometry Facility, which is supported in part by NCI Cancer Center Support grant P30CA56036.

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