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

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ABSTRACT

The factors that determine CD4$^+$ T cell (T_{CD4^+}) 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 T_{CD4^+} repertoires, including responses not elicited by VACV despite complete sequence conservation. In addition, we observed functional heterogeneity between ECTV- and VACV-specific T_{CD4^+} at both a global and individual epitope level, particularly greater expression of the cytolytic marker CD107a from T_{CD4^+} 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 T_{CD4^+} as determined by intracellular cytokine staining. These findings illustrate the strong influence of epitope-extrinsic factors on T_{CD4^+} 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 T_{CD4^+} repertoire induced by mousepox (ECTV) infection and the functional profile of the responding epitope-specific T_{CD4^+}, 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 T_{CD4^+} responses at both acute and memory time points, with VACV-specific T_{CD4^+} memory being notably compromised. These data offer insight into the impact of epitope-extrinsic factors on the resulting T_{CD4^+} responses.

Through their recognition of pathogen-derived peptides presented by major histocompatibility complex class II (MHC-II), CD4$^+$ T cells (T_{CD4^+}) play important roles in shaping cellular (1, 2) and humoral immunity (3, 4) and in establishing immunological memory (5–7). Additionally, T_{CD4^+} can suppress viral replication through the secretion of antiviral cytokines, such as gamma interferon (IFN-γ), and less frequently, through cytotoxic granule-mediated killing of infected cells (5, 8, 9).

Smallpox, caused by the *Variola virus* poxvirus, plagued mankind for millennia and continues to be a concern due to the threat of weaponization (10–13). Other poxviruses are equally lethal to their natural hosts, including ectromelia virus (ECTV), a poxvirus that causes smallpox-like symptoms in mice. Due to the threat it poses to mouse colonies, ECTV has not been widely investigated, and our understanding of host-poxvirus interplay and the resulting T_{CD4^+} response stems mainly from studies in mice with vaccinia virus (VACV), a poxvirus of unknown origin and the centuries-old vaccine against smallpox. Moreover, these poxviruses have distinct courses of infection after intradermal infection in mice. ECTV multiplies rapidly at the site of infection before disseminating into the lymphatics and bloodstream, where it leads to a systemic infection that affects both the liver and spleen (14, 15), whereas VACV remains relatively localized after intradermal infection and does not lead to systemic infection (16). Importantly, because VACV is not a natural mouse pathogen, despite a high degree of homology with ECTV, the relevance of results from the widely studied VACV murine infection model may be limited. For example, distinct innate responses (17–21) that can alter the array of immunogenic peptides (22), which can profoundly affect T_{CD4^+} responses, can differ substantially even with highly related viruses due to host cell tropism and host-specific immunomodulatory factors, such as viral cytokine mimics and/or receptors (23–30). These epitope-extrinsic factors can dramatically alter the course of infection and the resulting host immune response. For instance, it has been previously reported that Toll-like receptor 9 (TLR9) is critical for resistance against ECTV but not VACV (31). Indeed, low-dose footpad infection of C57BL/6 mice with ECTV usually results in loss of the infected limb, while much higher doses of

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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_{CD4^+} 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_{CD4^+}. By screening a large number of 12- to 15-mer peptides, we identified a total of 14 ECTV-specific T_{CD4^+} epitopes and observed both quantitative and qualitative differences between the T_{CD4^+} epitope repertoires elicited by ECTV and VACV. Subsequently, we probed differences in virus-mediated imprinting on T_{CD4^+} function and found that the resulting profiles of epitope-specific T_{CD4^+} are distinct and that long-term T_{CD4^+} 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_{CD4^+}.

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^- cells. The ECTV Moscow strain was grown in BSC1 cells. The Δ*evm0158* 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 primed via footpad with 3,000 PFU ectromelia virus (Moscow strain), 3,000 PFU or 3×10^6 PFU vaccinia virus (Western Reserve), or 3,000 PFU Δ*evm0158* 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.

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 3×10^6 PFU vaccinia virus (Western Reserve), or 3,000 PFU Δ*evm0158* ECTV. At various time points postinfection, the mice were observed and/or sacrificed and the inguinal lymph nodes, liver, and spleen were harvested. The splenocytes were used in either *ex vivo* IFN-γ enzyme-linked immunosorbent spot assay (ELISpot) or intracellular cytokine staining (ICS) assay as described below.

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_2. Peptide-primed splenocytes were then coincubated overnight with either ECTV-specific whole splenocytes or T_{CD4^+} isolated from splenocytes (Dynal mouse CD4 negative isolation kit; Invitrogen). IFN-γ-positive T cell responses were assayed by IFN-γ ELISpot (BD). Spots were counted using ImmunoSpot software (Cellular Technology Limited). To account for varying signal-to-noise ratios, we also calculated a stimulation index (SI), defined as (SFC [spot-forming cell] experiment results)/(SFC background) (34). Peptides with average spot numbers of ≥20, means of 1×10^6 effector T cells, P values of <0.05, and SI values of >2 in three independent experiments were considered positive.

ICS assay. ICS assays were performed as previously described (15). Briefly, bone marrow-derived dendritic cells (1×10^6) generated using previously published methods (35) were either pulsed with peptides (3 μg/ml) for 1 h in a 96-well plate or were infected with VACV WR (multiplicity of infection [MOI] of 5) for between 10 and 18 h before the addition of 1×10^6 to 2×10^6 splenocytes (pooled from two to five mice that were immunized with VACV WR for 10 days). Two hours later, brefeldin A (10 μg/ml) was added, and cells were cultured for another 6 h before staining according to the protocol of the BD Fix/Perm solution kit (BD Biosciences). At least 1.5×10^6 to 2×10^6 events per sample were collected using an LSRII fluorescence-activated cell sorting (FACS) system (BD Biosciences) and were analyzed with FlowJo software (Tree Star). Background values were determined from samples pulsed with dimethyl sulfoxide (DMSO) only (no peptide) and were subtracted from the experimental values. At least three independent experiments were performed for each peptide or peptide pool. A peptide was considered positive if the average of the individual experiments was at least 1 standard deviation above the background.

RESULTS

T_{CD4^+} 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_{CD4^+} responses could be discriminately 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_{CD4^+} responses following poxvirus infection.

The global T_{CD4^+} 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_{CD4^+} responses by examining several T cell functional outputs (Fig. 1B). Heterologous stimulation produced approximately 62% (VACV T_{CD4^+} to ECTV BMDC) to 67% (ECTV T_{CD4^+} to VACV BMDC) of the numbers of activated T_{CD4^+} 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_{CD4^+} responses to the two poxviruses extending beyond sequence heterogeneity, a possibility that was first investigated by assessing responses to individual epitopes.

T_{CD4^+} 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

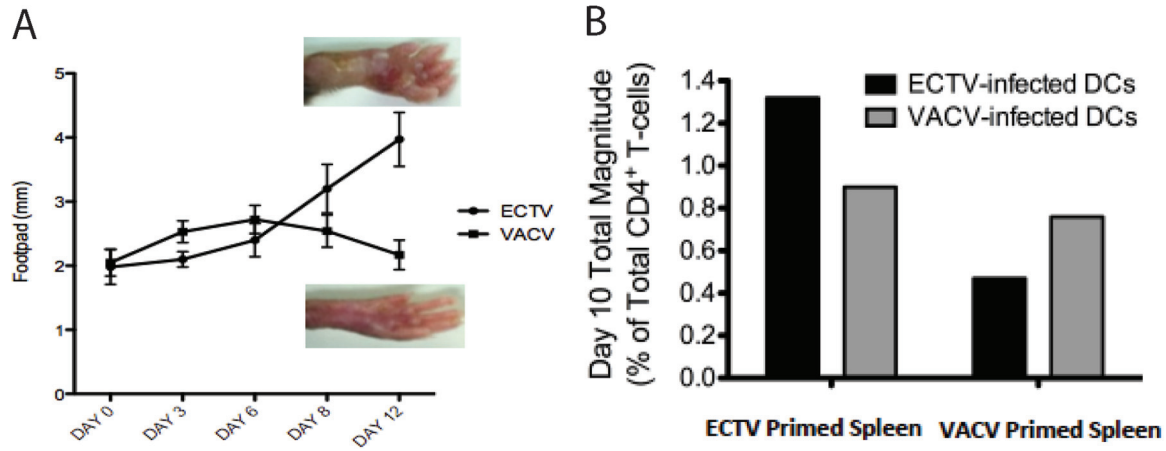

FIG 1 Divergent pathogenesis and magnitude of T_{CD4^+} response to ECTV and VACV. (A) C57BL/6 mice were infected with either VACV or ECTV (3,000 PFU per footpad). Footpad swelling was measured using a caliper and recorded in millimeters at the indicated days postinfection. Representative photos of footpads at day 12 postinfection with either ECTV or VACV are shown. (B) T_{CD4^+} response magnitude, represented as the frequency of total responding T_{CD4^+} from mice primed with either VACV and ECTV (3,000 PFU per footpad). Responses were measured by intracellular cytokine staining after splenocytes were stimulated with BMDCs infected with ECTV or VACV (MOI = 1). Total magnitude was calculated after background subtraction by summing across all combinations of cells producing at least one of the following functions: CD107a, IFN-γ, IL-2, and TNF-α.

(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 and C and Table 1). Eleven of these had been previously identified in the VACV screen, with two common epitopes (residues 46 to 60 of A18R [$A18R_{46-60}$] and $I1L_{7-21}$) differing by a single, apparently neutral, amino acid (Table 2). Thus, three epitopes ($I4L_{632-646}$, $B13R_{14-28}$, and $E2L_{426-440}$) were novel (Table 3). Interestingly, when we screened VACV-specific T_{CD4^+} (Fig. 2, solid bars), these three specificities were also elicited, although $B13R_{14-28}$ and $E2L_{426-440}$ were just above the limit of detection. Three other specificities identified in the original VACV screen ($D8L_{238-252}$, $A28L_{10-24}$, and $A24R_{399-413}$) were not elicited by ECTV. This could not be attributed to sequence heterogeneity for any of the three because $A28L_{10-24}$ and $A24R_{399-413}$ are 100% conserved. And while the ECTV sequence differs from $D8L_{238-252}$ by two residues ($A_{244}\rightarrow V_{244}$ and $A_{249}\rightarrow V_{249}$), the ECTV counterpart was also nonreactive (Fig. 4).

At the same time, we expanded the screen to include $CD8^+$ T cell (T_{CD8^+}) specificities and identified a novel and relatively potent MHC class I (MHC-I)-restricted epitope, $M1L_{424-438}$, that elicited T_{CD8^+} responses after both ECTV and VACV infections (Fig. 2 and 3C). The minimal H2-K^b-restricted epitope within the $M1L_{424-338}$ peptide was defined via a series of truncated peptides as $M1L_{426-434}$ (IIIPFIAYF) (data not shown).

The sum of the differences in T_{CD4^+} 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. Particularly striking are the unequal responses to homologous epitopes. For example, despite the sequence identity, ECTV elicits significantly greater responses to the $A20R_{230-244}$, $E2L_{426-440}$, and $F15L_{41-55}$ epitopes than VACV. Conversely, VACV elicits far greater responses to the $I1L_{7-21}$, $I1L_{18-33}$, and $L4R_{173-187}$ epitopes. These differences in both directions indicate qualitative differences in the T_{CD4^+} responses to the two viruses that are independent of epitope sequence. This led us to assess additional attributes of the resulting T_{CD4^+} responses.

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 (B8R in VACV). The T_{CD4^+} responses to WT and Δ*evm0158* ECTV were generally quite similar, with only a few significant differences (Fig. 5). For example, the $E2L_{426-440}$ and $F15L_{41-55}$ responses were reduced and the $I1L_{18-33}$ response elevated in comparison to the responses of these epitopes to WT ECTV. Collectively, these data indicate that Δ*evm0158* ECTV does not significantly affect virus-specific T_{CD4^+} reactivity or IFN-γ production. Compatible with this finding, Δ*evm0158* ECTV was not appreciably attenuated *in vivo* in our hands (unpublished data).

Comparative functional profiles of epitope-specific T_{CD4^+} from ECTV- or VACV-primed mice. To assess functionality using intracellular cytokine staining (ICS) and polychromatic flow cytometry, we pooled six peptides (Table 4, boldface) that consistently elicited robust T_{CD4^+} responses in ELISpot assays, five

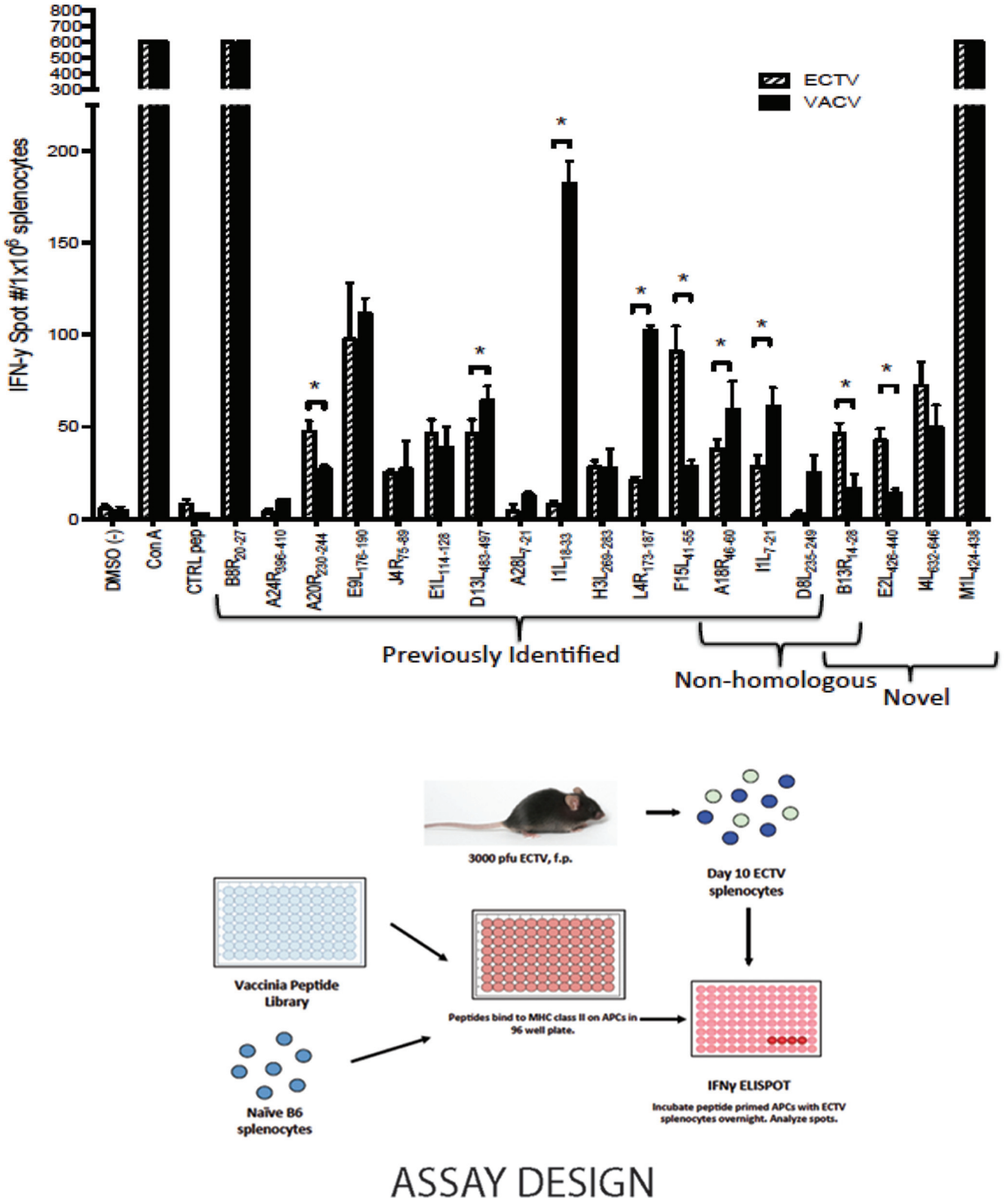

FIG 2 Comparative epitope specificities and magnitudes of responses to VACV- and ECTV-primed splenocytes. Naive splenocytes were incubated with synthetic poxvirus peptides ranging from 12 to 15 amino acids in length and screened for reactivity against splenocytes from ECTV-primed mice 10 days postinfection. 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.

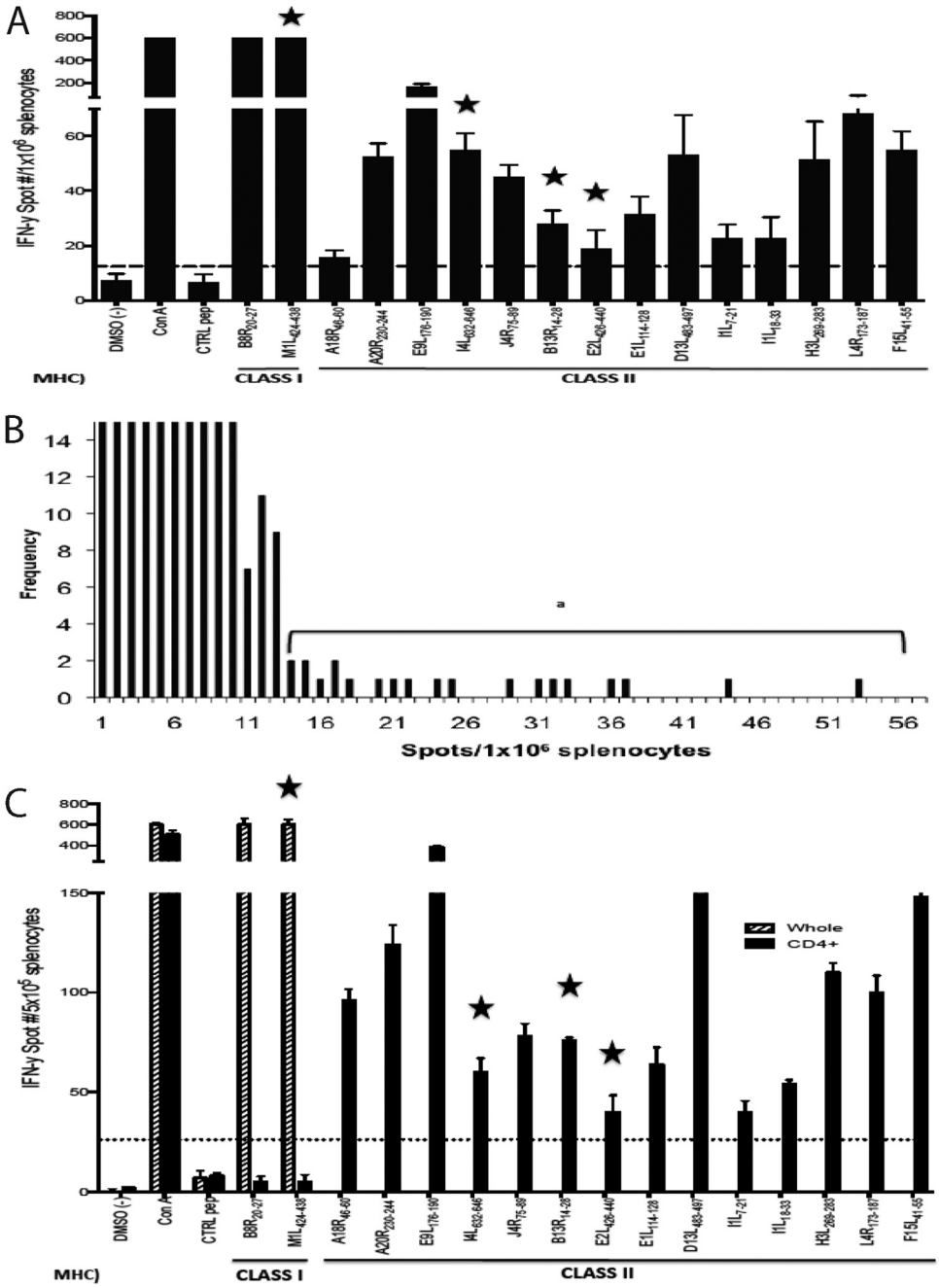

FIG 3 Determination of ECTV-specific T_{CD4^+}epitopes in C57BL/6 mice. A synthetic VACV peptide library of 1,022 peptides, ranging from 12 to 15 amino acids in length, was screened for reactivity against splenocytes from ECTV-primed mice (3,000 PFU per footpad) 10 days postinfection. Naive splenocytes 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.

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

TABLE 1 Identification of ECTV-specific epitopes[a]

Peptide hit	Sequence	VACV protein	Time of expression	Function	MHC class
$B8R_{20–27}$	TSYKFESV	B8R (VACWR190) [A190]	Early	Virulence	I
$M1L_{424–438}$	**KSIIIPFIAYFVLMH**	**M1L (VACWR030) [A030]**	**Early**	**Unknown**	**I**
$A18R_{46–60}$	PKGFYASPSVKTSLV	A18R (VACWR138)	Early	Regulation	II
$A20R_{230–244}$	GDNIFIPSVITKSGK	A20R (VACWR141) [A141]	Early	Regulation	II
$E9L_{176–190}$	PSVFINPISHTSYCY	E9L (VACWR065)	Early	Regulation	II
$I4L_{632–646}$	**EFQVVNPHLLRVLTE**	**I4L (VACWR073) [A073]**	**Early**	**Regulation**	**II**
$J4R_{75–89}$	DDDYGEPIIITSYLQ	J4R (VACWR096)	Early	Regulation	II
$B13R_{14–28}$	**ENVFISPASISSVLT**	**B13R (VACWR195) [A195]**	**Early**	**Virulence**	**II**
$E2L_{426–440}$	**RLMFEYPLTKEASDH**	**E2L (VACWR058) [A058]**	**Early**	**Unknown**	**II**
$E1L_{114–128}$	VLTIKAPNVISSKIS	E1L (VACWR057) [A057]	Late	Regulation	II
$D13L_{483–497}$	PKIFFRPTTITANVS	D13L (VACWR118) [A118]	Late	Structural	II
$I1L_{7–21}$	QLVFNSISARALKAY	VACWR070 (I1L)	Late	Structural	II
$I1L_{18–33}$	LKAYFTAKINEMVDE	I1L (VACWR070)	Late	Structural	II
$H3L_{269–283}$	PGVMYAFTTPLISFF	H3L (VACWR101)	Late	Structural	II
$L4R_{173–187}$	ISKYAGINILNVYSP	L4R (VACWR091) [A091]	Late	Structural	II
$F15L_{41–56}$	TPRYIPSTSISSSNI	F15L (VACWR054)	Late	Unknown	II

[a] Boldface indicates previously unreported epitopes.

individual functions were assessed (Fig. 6B), and it became clear that ECTV-specific T_{CD4^+} 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^+} 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 $H3L_{269–283}$ (Fig. 6C). Of the specificities that could be compared, $E9L_{176–190}$-specific T_{CD4^+} 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 T_{CD4^+} 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 T_{CD4^+}) at 100 days postinfection (reactivity to $I4L_{632–646}$ 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 2 T_{CD4^+} poxvirus-specific epitopes

Peptide hit	Reactivity[a]	% Conservation of:			Time of expression	MHC class
		Epitope (differing residue[s])	Flanking region[b]	Parent protein		
ECTV > VACV						
$A20R_{230–244}$	ECTV and VACV	100	100	97	Early	II
$F15L_{41–56}$	ECTV and VACV	100	100	99	Late	II
VACV > ECTV						
$I1L_{7–21}$	ECTV and VACV	93.3 ($V_9 \rightarrow I_9$)	100	99	Late	II
$I1L_{18–33}$	ECTV and VACV	100	100	99	Late	II
$L4R_{173–187}$	ECTV and VACV	100	100	99	Late	II
$A24R_{399–413}$	VACV only	100	100	99	Early	II
$A28L_{7–21}$	VACV only	100	100	99	Late	II
$D8L_{235–249}$	VACV only	86.7 ($A_{244} \rightarrow V_{244}$, $A_{249} \rightarrow V_{249}$)	100	95	Late	II
ECTV ≈ VACV						
$A18R_{46–60}$	ECTV and VACV	93.3 ($A_{54} \rightarrow S_{54}$)	100	97	Early	II
$E9L_{176–190}$	ECTV and VACV	100	100	98	Early	II
$J4R_{75–89}$	ECTV and VACV	100	100	99	Early	II
$E1L_{114–128}$	ECTV and VACV	100	100	99	Late	II
$D13L_{483–497}$	ECTV and VACV	100	100	99	Late	II
$H3L_{269–283}$	ECTV and VACV	100	100	96	Late	II

[a] Stimulation index (SI) of >2.
[b] Includes 5 amino acid residues upstream and downstream from the epitope.

TABLE 3 Previously unreported poxvirus-specific epitopes

Peptide hit	Reactivity[a]	% Conservation of:			Time of expression	MHC class
		Epitope (differing residue)	Flanking region[b]	Parent protein		
$M1L_{424–438}$	ECTV and VACV	100	96	96	Early	I
$I4L_{632–646}$	ECTV and VACV	100	100	97	Early	II
$B13R_{14–28}$	ECTV only	93.3 ($P_{20}\rightarrow S_{20}$)	100	95	Early	II
$E2L_{426–440}$	ECTV only	100	100	98	Early	II

[a] Stimulation index (SI) of >2.
[b] Includes 5 amino acid residues upstream and downstream from the epitope.

functional imprinting of epitope-specific T_{CD4^+} that affect persistence into the memory phase.

A greater frequency of effector T_{CD4^+} 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_{CD4^+} responses after infection. Our recent work points to the importance of cytolytic function by T_{CD4^+} 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_{CD4^+} that were dependent upon both the epitope specificity and identity of the infecting poxvirus. The T_{CD4^+} responses to two conserved epitopes, $D13L_{483–497}$ and $E9L_{176–190}$, serve as an illustration of these points, with ECTV infection inducing $E9L_{176–190}$-specific T_{CD4^+} with greater degranulation capacity than T_{CD4^+} with other specificities (Fig. 7A). Additionally, compared with VACV, ECTV infection consistently elicited a higher proportion of responding T_{CD4^+} 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_{CD4^+} at acute time points postinfection with both viruses in the liver, inguinal lymph nodes, and spleen. We found that total T_{CD4^+} in all three locations within ECTV-infected mice expressed 2- to 3-fold-higher levels of grzB than were observed with VACV (Fig. 7C and D). The enhanced grzB expression observed from ECTV T_{CD4^+} suggests that the induction of cytolytic T_{CD4^+} 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_{CD4^+}, we examined the expression levels of macrophage inflammatory protein 1α (MIP1α) from ECTV- and VACV-specific T_{CD4^+}. 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_{CD4^+} than from VACV T_{CD4^+} (Fig. 8), offering additional evidence that, in general, ECTV-specific T_{CD4^+} display a greater effector-like profile than their VACV counterparts.

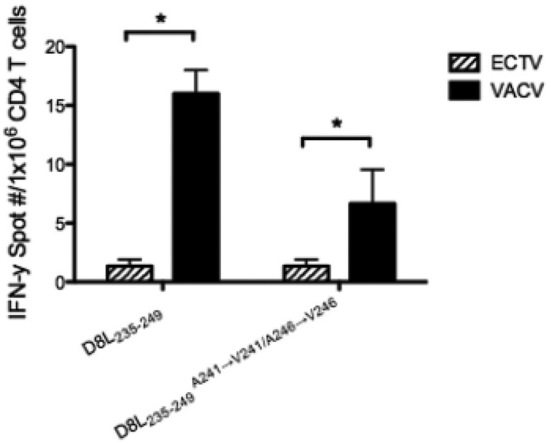

FIG 4 Comparative epitope reactivities to $D8L_{238–252}$ between VACV- and ECTV-primed splenocytes. Naive splenocytes were incubated with synthetic $D8L_{238–252}$ (VACV sequence) or $D8L_{238–252}^{A244\rightarrow V244/A249\rightarrow V249}$ (ECTV sequence) at a final concentration of 2 μg/ml. Peptide-primed splenocytes were then coincubated 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. *, $P < 0.05$.

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 ($D8L_{238–252}$, $A28L_{10–24}$, and $A24R_{399–413}$) 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 ($I4L_{632–646}$, $B13R_{14–28}$, and $E2L_{426–440}$) and one of which was MHC class I ($M1L_{426–434}$, 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_{CD4^+} 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_{CD4^+} (34), and differential costimulatory molecule expression by unique antigen-presenting cell types can alter signaling at the

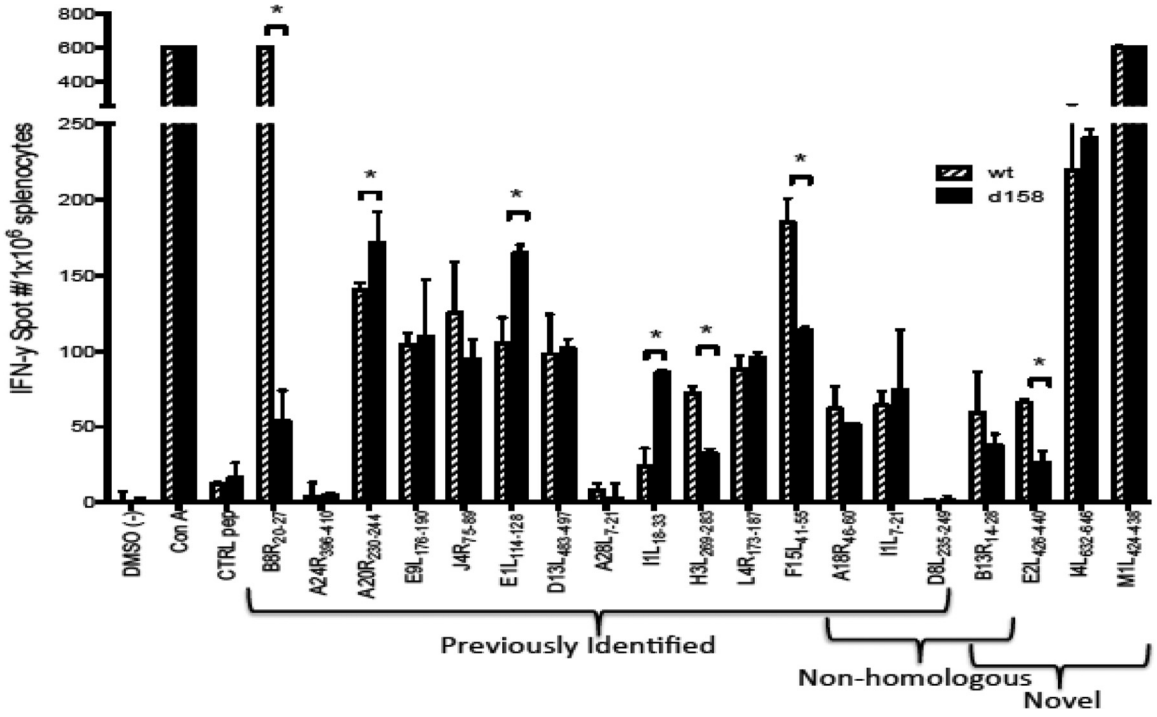

FIG 5 Comparative epitope specificities and magnitudes of responses between WT ECTV- and Δ*evm0158* ECTV-primed splenocytes. Naive splenocytes were incubated with synthetic poxvirus peptides ranging from 12 to 15 amino acids in length (final concentration, 2 μg/ml). Peptide-primed splenocytes were coincubated overnight with whole splenocytes from WT ECTV- or Δ*evm0158* ECTV-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. *, $P < 0.05$; limits, mean ± SD. Data are representative of 3 independent experiments.

immunological synapse, resulting in shifted T_{CD4^+} activation thresholds (61).

The novel MHC-I epitope, $M1L_{426-434}$, induced relatively equivalent responses from both ECTV and VACV splenocytes that were nearly as potent as the immunodominant $B8R_{20-27}$ 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-2D^b class I epitope (TSNVITDQTV/$M1L_{291-300}$) within the M1L parent protein (56) but not at the 426–434 location.

The lack of ECTV T_{CD4^+} reactivity to the three specificities identified in the original VACV screen could not be accounted for by sequence heterogeneity, as $A28L_{10-24}$ and $A24R_{399-413}$ are both 100% conserved. And although there were two amino acid differences in the $D8L_{238-252}$ epitope ($A_{244}\rightarrow V_{244}$ and $A_{249}\rightarrow V_{249}$), the possibility that these changes prevent proper processing of the antigen or prevent binding to the I-A^b molecule was discounted by the observation that VACV T_{CD4^+} 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_{CD4^+} magnitude or on the individual T_{CD4^+} reactivities, consistent with our observation that Δ*evm0158* is not attenuated *in vivo* (unpublished data).

Differences in participating antigen-presenting cells and anti-

TABLE 4 Selected T_{CD4^+}/MHC class II poxvirus-specific epitopes[a]

Peptide	Amino acid sequence
$A20R_{230-244}$	GDNIFIPSVITKSGK
$I4L_{632-646}$	**EFQVVNPHLLRVLTE**
$F15L_{41-55}$	TPRYIPSTSISSSNI
$E1L_{114-128}$	VLTIKAPNVISSKIS
$I1L_{7-21}$	**QLVFNSISARALKAY**
$I1L_{18-33}$	LKAYFTAKINEMVDE
$D13L_{483-497}$	**PKIFFRPTTITANVS**
$E9L_{176-190}$	**PSVFINPISHTSYCY**
$H3L_{269-283}$	**PGVMYAFTTPLISFF**
$L4R_{173-187}$	**ISKYAGINILNVYSP**

[a] Boldface indicates peptides that consistently elicited robust T_{CD4^+} responses in ELISpot assays and were pooled to assess functionality using intracellular cytokine staining (ICS) and polychromatic flow cytometry.

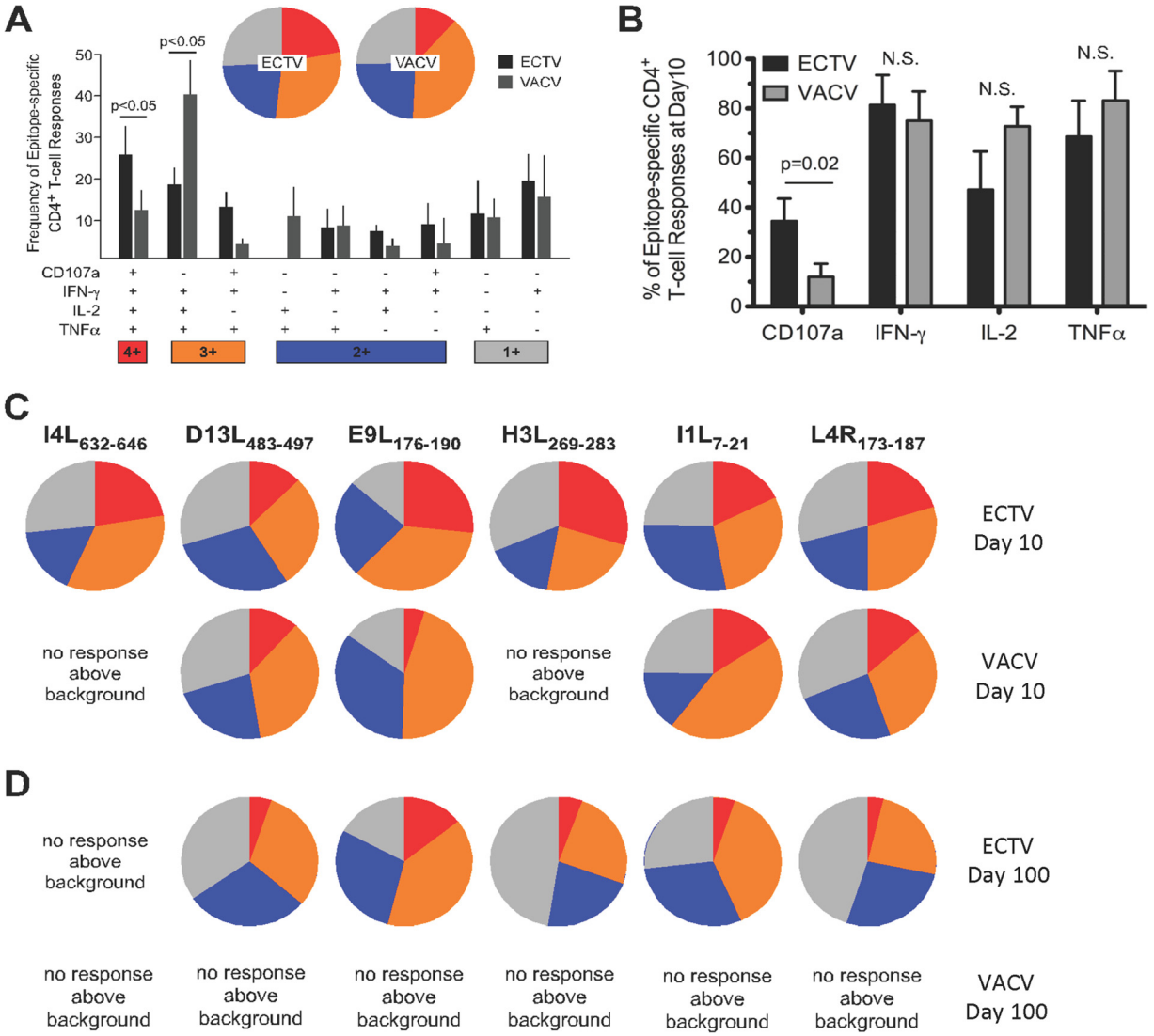

FIG 6 Comparative functional profiles of epitope-specific T_{CD4^+} from ECTV- or VACV-primed mice. (A) T_{CD4^+} responses to a poxvirus-specific class II peptide pool (Table 4) at day 10 were divided according to the relative contribution of each functional combination. Permutations that did not contribute significantly to the functional profile are not shown due to space constraints. Responses are grouped according to the degree of positivity and matched to the colors in the pie graphs for responses to the peptide pool at day 10. (B) Proportion of poxvirus-specific T_{CD4^+} at day 10 postinfection for each specific function measured after stimulation. (C and D) Functional profiles of T_{CD4^+} responsive to a selected cohort of poxvirus epitopes at day 10 (C) and day 100 (D) postinfection are shown for ECTV-infected (3,000 PFU per footpad) and VACV-infected (3,000 PFU per footpad or 3×10^6 PFU per footpad) mice. (A to D) All depicted data were from pooled cells of three mice at each time point and represent the average of two independent experiments. Bars represent the means, and error bars indicate the standard deviations. *P* values are defined where applicable; N.S., not significant. Data are representative of 3 independent experiments.

gen load can also affect the functional character of the T_{CD4^+} response (75–79). Indeed, we found that for the majority of the epitopes tested (5 of 6), ECTV-specific T_{CD4^+} demonstrated a higher effector capacity; most notably enhanced were cytolytic potential and MIP1α expression. Furthermore, VACV T_{CD4^+} exhibited responses above the background for just 4 of the 6 epitopes examined at 10 days postinfection, while ECTV-specific T_{CD4^+} 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_{CD4^+} 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_{CD4^+} in both acute and chronic viral infections (80, 81).

Previous work by Fang et al. demonstrated that cytolytic T_{CD4^+} 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

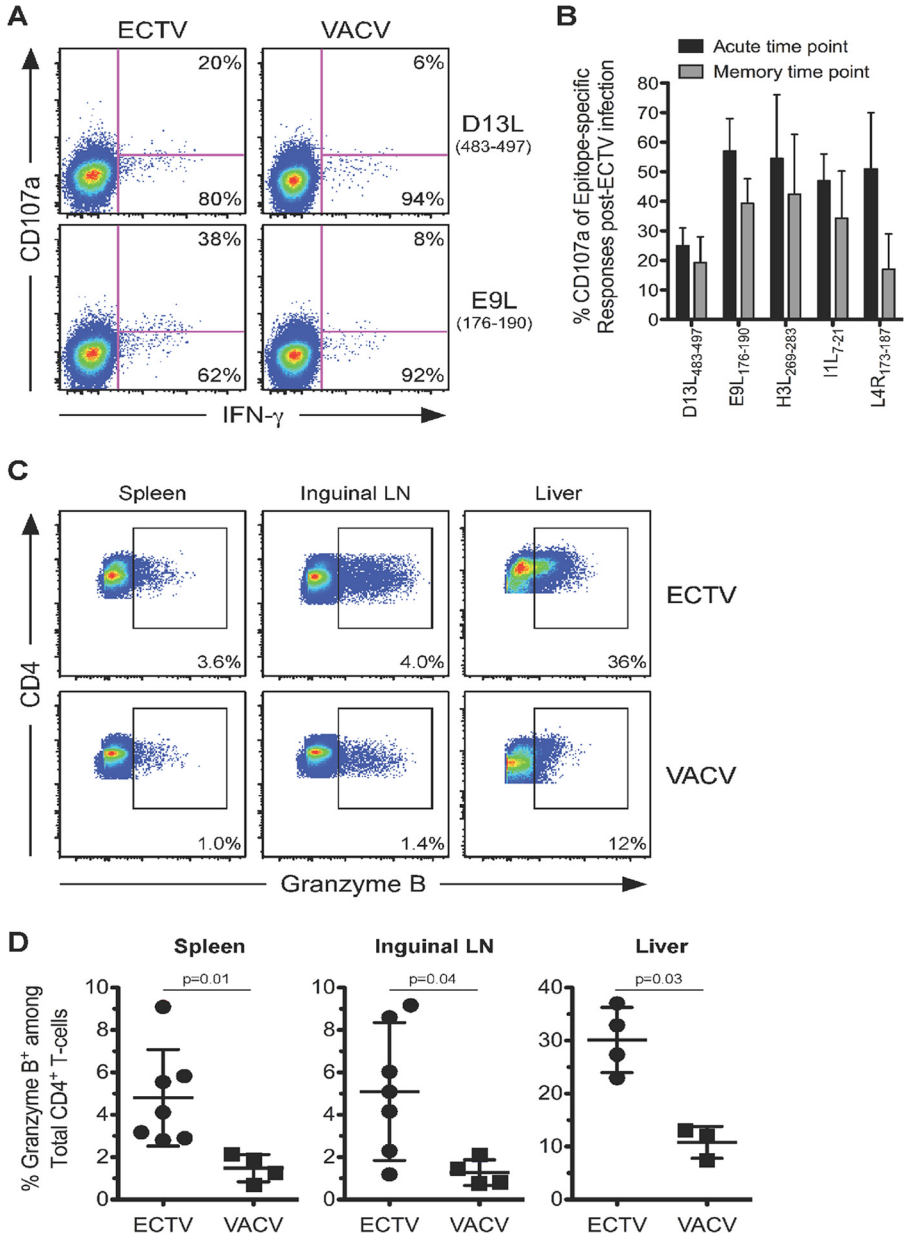

FIG 7 ECTV-specific T_{CD4^+} degranulate and express larger amounts of granzyme B during acute infection. (A) Proportions of D13L- or E9L-specific T_{CD4^+} splenocytes coexpressing CD107a and IFN-γ from ECTV- or VACV-primed mice (3,000 PFU per footpad) at 10 days postinfection. Percentages represent the fraction of overlaid cells that fall within each quadrant. (B) Percentages of D13L-, E9L-, H3L-, I1L-, or L4R-specific T_{CD4^+} splenocytes expressing CD107a from ECTV-primed mice (3,000 PFU per footpad) at acute (10 days postinfection) and memory (75 to 100 days postinfection) time points. (C) Percentages of grzB-positive ($grzB^+$) cells from bulk T_{CD4^+} collected from the spleen, inguinal lymph nodes (LN), or liver of ECTV- or VACV-primed mice (3,000 PFU per footpad) at 10 days postinfection. (D) Quantification of the average percentages of $grzB^+$ from bulk T_{CD4^+} collected from the spleen, inguinal LN, or liver of ECTV- or VACV-primed mice (3,000 PFU per footpad) at acute time points postinfection. Depicted data are from pooled cells of three mice and represent the average and SD of two independent experiments. *P* values are defined where applicable. Data are representative of 2 independent experiments.

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_{CD4^+} at memory (day 100) time points using flow cytometry (Fig. 6D). While ECTV infection stimulated robust long-term T_{CD4^+} 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_{CD4^+} in both humans (52) and mice (15, 66). In our own earlier study (15), we detected both T_{CD4^+} 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_{CD4^+} memory to VACV may not be completely absent, it is significantly compromised.

The considerably greater difference between ECTV- and

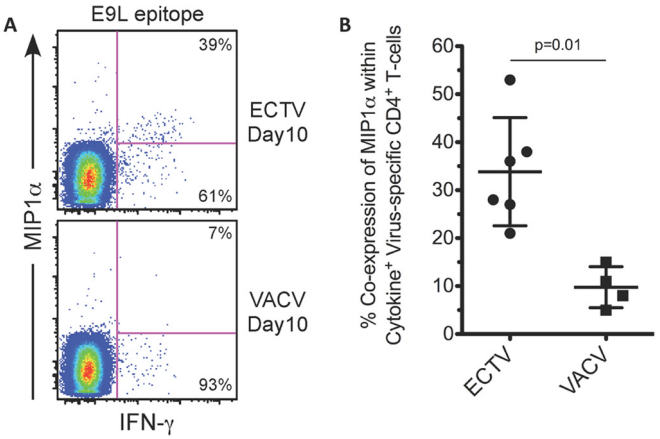

FIG 8 ECTV-specific T_{CD4^+} express larger amounts of MIP1α during acute infection. (A) Representative staining showing higher expression of MIP1α by ECTV-specific T_{CD4^+}. E9L-specific T_{CD4^+} responses from ECTV- or VACV-primed mice (3,000 PFU per footpad) at 10 days postinfection are shown. Percentages represent the fractions of IFN-γ^+ cells that were either positive or negative for MIP1α after peptide stimulation. (B) Quantification of MIP1α expression among several different peptide-specific T_{CD4^+} responses. Total percentages of virus-specific T_{CD4^+} splenocytes coexpressing MIP1α and at least one additional cytokine from ECTV- or VACV-primed mice (3,000 PFU per footpad) at 10 days postinfection are shown. Depicted data are representative of four independent experiments. *P* value was determined using the Mann-Whitney test. Bars and whiskers represent the means and SD.

VACV-specific T_{CD4^+} at the memory phase is consistent with the earlier demonstration that differences in the initial antiviral T_{CD4^+} characters can become 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 T_{CD4^+} character and T_{CD4^+} 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 effectorlike properties of responding T_{CD4^+} (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 T_{CD4^+} effector-memory and exhaustion has been elusive (87, 94) but will likely be critical for insight into the profound differences in T_{CD4^+} 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 postimmunization. In contrast and in line with our findings, VACV-specific T_{CD4^+} 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 T_{CD4^+} may not play a critical role in protection from smallpox. The importance of robust T_{CD4^+} 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 T_{CD4^+} 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 T_{CD4^+} epitope reactivity profiles of ECTV and VACV. A better understanding of the mediators that influence T_{CD4}+ 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 T_{CD4^+} responding to common epitopes. Moreover, distinct host-pathogen relationships may ultimately play a predominant role in both shaping the T_{CD4^+} repertoire and influencing the functional imprinting and differentiation of poxvirus-specific T_{CD4^+}. The striking cytolytic character of T_{CD4^+} induced by ECTV and the inability of VACV to drive the development of this protective T_{CD4^+} subset (89) or a substantial memory T_{CD4^+} population demonstrate the impact of epitope-extrinsic mediators on T_{CD4^+} 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 T_{CD4^+} responses.

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