Comparable Polyfunctionality of Ectromelia Virus- and Vaccinia Virus-Specific Murine T Cells despite Markedly Different *In Vivo* Replication and Pathogenicity

Adam R. Hersperger, Nicholas A. Siciliano, and Laurence C. Eisenlohr

Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, USA

Vaccinia virus (VACV) stimulates long-term immunity against highly pathogenic orthopoxvirus infection of humans (smallpox) and mice (mousepox [ectromelia virus {ECTV}]) despite the lack of a natural host-pathogen relationship with either of these species. Previous research revealed that VACV is able to induce polyfunctional CD8⁺ T-cell responses after immunization of humans. However, the degree to which the functional profile of T cells induced by VACV is similar to that generated during natural poxvirus infection remains unknown. In this study, we monitored virus-specific T-cell responses following the dermal infection of C57BL/6 mice with ECTV or VACV. Using polychromatic flow cytometry, we measured levels of degranulation, cytokine expression (gamma interferon [IFN- γ], tumor necrosis factor alpha [TNF- α], and interleukin-2 [IL-2]), and the cytolytic mediator granzyme B. We observed that the functional capacities of T cells induced by VACV and ECTV were of a similar quality in spite of the markedly different replication abilities and pathogenic outcomes of these viruses. In general, a significant fraction ($\geq 50\%$) of all T-cell responses were positive for at least three functions both during acute infection and into the memory phase. *In vivo* killing assays revealed that CD8⁺ T cells specific for both viruses were equally cytolytic ($\sim 80\%$ target cell lysis after 4 h), consistent with the similar levels of granzyme B and degranulation detected among these cells. Collectively, these data provide a mechanism to explain the ability of VACV to induce protective T-cell responses against pathogenic poxviruses in their natural hosts and provide further support for the use of VACV as a vaccine platform able to induce polyfunctional T cells.

Ectromelia virus (ECTV) (“mousepox”) is a natural murine orthopoxvirus that causes pathogenesis and clinical manifestations in mice that are strikingly similar to those of variola virus (VARV) (“smallpox”) infection of humans (14, 19). In nature, ECTV is thought to be transmitted primarily through abrasions of the skin (20), resulting in the widespread dissemination of the virus (19, 22). Following initial infection, ECTV multiplies locally before spreading to the regional draining lymph node. Additional viral replication in the lymphatics precedes spread to the bloodstream, followed by infection of many visceral organs, such as the spleen and liver. A high degree of tissue necrosis in these organs liberates virus into the bloodstream, which is then responsible for the seeding of the skin. Infection of the skin results in the characteristic rash and pock lesions that resemble those found after VARV infection of humans. Also reminiscent of smallpox is the observation that some mouse strains are resistant to lethal infection (e.g., C57BL/6), while other strains (e.g., BALB/c) are susceptible and succumb to mousepox at very high rates (4).

Many studies have shown that various components of the innate immune system, including natural killer cells, interferons, Toll-like receptors, and macrophages, are essential for resistance to ECTV (14, 40). With respect to adaptive immunity, early studies by Blanden and colleagues pointed to an important role of T cells in combating mousepox infection (5, 6, 30). Subsequent work confirmed and extended those initial reports through examinations of specific T-cell subsets. The findings of some groups highlighted the essential role of ECTV-specific CD8⁺ T cells in limiting viral replication (16, 29, 55), largely through the action of perforin and granzymes (36–39). Other reports have shown that the presence of CD4⁺ T cells is also critical for the clearance of ECTV, especially from the skin (29), and major histocompatibility

complex (MHC) class II-deficient C57BL/6 mice ultimately succumb to ECTV infection. Additionally, the helper capacity of CD4⁺ T cells to stimulate antigen-specific B cells and antibody class switching through CD40/CD40L interactions has been shown to be important for resistance to ECTV (16).

Shortly after the initial discovery of ECTV from infected stocks of laboratory mice (33), it became clear that the causative agent of “infectious ectromelia” was related to vaccinia virus (VACV) (8, 9), the vaccine strain used historically to combat smallpox infection of humans. As with VARV, cross-immunity exists between ECTV and VACV (9, 21). However, despite the high degree of homology between these two viruses (25), the courses of infection are quite distinct. While mice may be incidental hosts of VACV or may even serve as vectors of the virus in the wild (1), VACV infection is fatal only under certain experimental conditions and routes of infection. In contrast, ECTV infection of BALB/c mice typically results in death. Moreover, unlike ECTV, the replication of VACV is restricted to the site of infection after cutaneous inoculation (53). These stark differences in pathogenesis and *in vivo* replication highlight the potential value of utilizing ECTV for the

Received 7 January 2012 Accepted 11 April 2012

Published ahead of print 24 April 2012

Address correspondence to Laurence C. Eisenlohr, laurence.eisenlohr@jefferson.edu.

A.R.H. and N.A.S. contributed equally to this work.

Supplemental material for this article may be found at <http://jvi.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.00038-12

examination of host defense mechanisms. Therefore, in this study, we investigated whether the functional capacities of T cells, previously shown to be important for resistance against mousepox, were as distinct as the pathogenic outcomes of ECTV and VACV.

The presence of polyfunctional T_H1-type T-cell responses was previously correlated with pathogen control and improved clinical outcomes in several settings, including *Leishmania major* infection of mice (12) and nonprogressive HIV infection of humans (3, 27). These T-cell responses are typically characterized by a high degree of coexpression of gamma interferon (IFN- γ), tumor necrosis factor alpha (TNF- α), and interleukin-2 (IL-2). Precopio and colleagues performed one of the most comprehensive functional characterizations of poxvirus-specific T cells using multiparameter flow cytometry (44). Those authors assessed CD8⁺ T-cell functionality in humans during the course of a vaccine trial, comparing modified vaccinia virus Ankara (MVA) and Dryvax. They reported that these two VACV-based vaccination platforms induced highly functional CD8⁺ T cells that displayed significant levels of degranulation and the coexpression of several cytokines and the chemokine MIP1 β . However, since the last reported case of smallpox occurred several decades ago, and VACV is not a natural human pathogen (although humans can be incidental hosts [49]), it could not be determined whether the functional profile of T cells induced by VACV is similar to that of smallpox-specific T cells in the context of a truly natural infection. Therefore, in the present study, we used the mouse model of smallpox to compare the functional capacities of CD8⁺ and CD4⁺ T cells that arise following natural infection (ECTV) and nonnatural infection (VACV) of mousepox-resistant C57BL/6 mice. Using multiparameter flow cytometry and intracellular cytokine staining assays, we observed that both poxviruses induced polyfunctional CD8⁺ and CD4⁺ T cells of a highly similar nature in multiple organs during acute and memory time points.

MATERIALS AND METHODS

Ethics statement. Experimental procedures involving mice were carried out in strict accordance with regulations from the National Institutes of Health, the Association for Assessment and Accreditation of Laboratory Animal Care International, and the U.S. Department of Agriculture. The protocol (protocol number 177P) was approved by the Institutional Animal Care and Use Committee (Office of Laboratory Animal Welfare assurance A3085-01) at Thomas Jefferson University (Philadelphia, PA). All mouse work was carried out in a humane manner, and virus infections were performed under isoflurane anesthesia.

Mice. Female C57BL/6 mice aged 6 to 8 weeks were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were infected with either ECTV (3,000 PFU) (Moscow strain) or VACV (3,000 PFU) (Western Reserve strain). All infections were carried out via the injection of ECTV or VACV (in a 30- μ l total volume) into the left hind footpad. On the indicated days postinfection, spleens and inguinal lymph nodes (I-LNs) (ipsilateral to the site of infection) from all mice were isolated and processed to create single-cell suspensions by using standard protocols (15). Three mice were used for each condition and time point; cells were pooled prior to stimulation.

Generation of BMDCs. Bone marrow-derived primary dendritic cells (BMDCs) were generated by using methods similar to those previously reported (48). Briefly, bone marrow cells were flushed from the femurs of naive, female C57BL/6 mice using sterile 1 $\times$ phosphate-buffered saline (PBS), counted, and then seeded at 2 $\times$ 10⁶ cells per 10-cm plate in 10 ml of medium (RPMI supplemented with 10% fetal calf serum [HyClone], 100 IU/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-mercaptoetha-

TABLE 1 Description of all individual peptides that comprised each pool

Peptide	Amino acid sequence	VACV protein
CD4 ⁺ T-cell/MHC class II epitopes		
5B11	GDNIFIPSVITKSGK	A20R (VACWR141)
12F8	EFQVVPNPHLLRVLTE	I4L (VACWR073)
F15L	TPRYPSTSISSNI	F15L (VACWR054)
11C9	VLTIKAPNVISSKIS	E1L (VACWR057)
I1LA	ARALKAYFTAKINEM	I1L (VACWR070)
I1LQ	QLVFNSISARALKAY	I1L (VACWR070)
11F10	RLMFEYPLTKEASDH	E2L (VACWR058)
14B1	PKIFFRPTTITANVS	D13L (VACWR118)
E9L	PSVFNPISHTSYCY	E9L (VACWR065)
H3L	PGVMYAFTTPLISFF	H3L (VACWR101)
L4R	ISKYAGINILNVYSP	L4R (VACWR091)
CD8 ⁺ T-cell/MHC class I epitopes		
10G2	KSIIPFIAYFVLMH	M1L (VACWR030)
B8R	TSYKFESV	I4L (VACWR190)

nol, 2 mM L-glutamine, and 20 ng/ml murine granulocyte-macrophage colony-stimulating factor [GM-CSF; Peprotech, Rocky Hill, NJ]). Medium changes were performed on days 3, 6, and 8. BMDCs were harvested on day 10.

Cell stimulation. Splenocytes and I-LN cells were stimulated with either (i) virus-infected BMDCs or (ii) uninfected BMDCs pulsed with peptide pools. For stimulation with virus-infected BMDCs, after 10 days of culture, BMDCs were washed twice in 1 $\times$ PBS before being transferred into antibiotic-free medium (RPMI supplemented with 5% fetal calf serum and 20 ng/ml GM-CSF). BMDCs were then infected with either ECTV (Moscow strain) (multiplicity of infection [MOI] of 1) or VACV (Western Reserve strain) (MOI of 1) for 8 to 10 h. The cells were subsequently washed twice in 1 $\times$ PBS prior to being cocultured with splenocytes or lymph node cells at a 6:1 effector/T-cell ratio. Cells from ECTV-infected mice were stimulated with ECTV-infected BMDCs, whereas cells from VACV-infected mice were stimulated with VACV-infected targets. For stimulation with uninfected BMDCs pulsed with peptide pools, two separate peptide pools were generated (Table 1). One pool was comprised of ECTV-specific (N. A. Siciliano et al., unpublished data) and previously identified VACV-specific CD4⁺ T-cell/MHC class II-restricted epitopes (34). A second pool was comprised of two CD8⁺ T-cell/MHC class I-restricted epitopes (35, 52). It should be noted that the class I epitope 10G2 from the M1L protein of VACV was not found to be immunodominant by Moutafsi et al. (35), but we found a large population of 10G2-specific CD8⁺ T cells following dermal infection with both VACV and ECTV (Siciliano et al., unpublished). All individual peptides (New England Peptide, Gardner, MA) in both pools were used at a final concentration of 2 μ g/ml; the amino acid sequences of all peptides were 100% conserved between ECTV and VACV at the epitope level.

ICS assays. Intracellular cytokine staining (ICS) assays were carried out in a fashion similar to that described in a previous work (27). Briefly, cells were resuspended in RPMI supplemented with 10% fetal bovine serum at a concentration of $\sim$ 2 $\times$ 10⁶ cells/ml in a total volume of 1 ml under each condition. Monensin (1 μ g/ml; BD Biosciences) and brefeldin A (BFA) (1 μ g/ml; BD Biosciences) were added under each condition at time zero. Anti-CD107a fluorescein isothiocyanate (FITC) was also included at the start of all stimulations to measure levels of degranulation (2). Cells were then incubated at 37°C in 6% CO₂ for 6 h. At the end of the stimulation period, cells were washed once with 1 $\times$ PBS and stained with Aqua amine-reactive dye (Invitrogen) for 15 min in the dark at room temperature (RT) in order to later identify viable cells during flow cytometric analyses (42). A cocktail of antibodies was then added to stain for surface markers for an additional 20 min at RT. Following

staining for cell surface molecules, cells were permeabilized by using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. A cocktail of antibodies against intracellular markers was added and allowed to incubate for 1 h in the dark at RT. Finally, cells were fixed in $1 \times$ PBS containing 2% paraformaldehyde before being stored at 4°C.

Antibody reagents. The following fluorochrome-conjugated antibodies were employed for this study: anti-CD8a Texas Red-phycoerythrin (PE) (clone 5H10), anti-CD4 Pacific Blue (clone RM4-5), anti-granzyme B (grz B) PE (clone GB12; this antibody cross-reacts [54] with murine and human granzyme B [Invitrogen/Molecular Probes, Carlsbad, CA]), anti-TNF- α PE-Cy7 (clone MP6-XT22), anti-IFN- γ Alexa-700 (clone XMG1.2), anti-CD3e allophycocyanin (APC)-Cy7 (clone 145-2C11), anti-CD14 peridinin chlorophyll protein (PerCP)-Cy5.5 (clone rmC5-3), anti-CD19 PerCP-Cy5.5 (clone 1D3), anti-CD107a FITC (clone 1D4B), and anti-IL-2 APC (clone JES6-5H4; BD Pharmingen, San Jose, CA) antibodies.

Flow cytometric analysis. Under each stimulation condition, between 500,000 and 1 million total events were acquired by using an LSRII flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data analysis was performed by using FlowJo (version 9.3.2; TreeStar, Ashland, OR), Spice (version 4.3; Mario Roederer, NIH, Bethesda, MD), and GraphPad Prism software (version 5.0a). Our gating strategy (see Fig. S1A in the supplemental material) was similar to that employed in prior reports (3, 27). Briefly, we gated on singlets, CD14^{neg} CD19^{neg} cells, viable cells (Aqua Blue^{neg}), lymphocytes, CD3⁺ cells, and then either CD8⁺ CD4^{neg} or CD4⁺ CD8^{neg} cells before finally gating on antigen-responsive T cells (see Fig. S1B in the supplemental material). Boolean gating analysis was carried out once positive gates were established for each functional parameter to determine the frequency of each permutation based on all possible combinations of cytokine, CD107a, or granzyme B expression. Background responses under the negative-control condition (no stimulation/dimethyl sulfoxide [DMSO]) were subtracted from those detected under the various stimulation conditions for every specific functional combination. Importantly, two combinations were ignored in all analyses: (i) events negative for any measured functional parameters and (ii) granzyme B single-positive cells. By analyzing the data in this manner, we examined granzyme B production resulting from antigen-specific activation and ensured that its expression was considered only within T cells expressing at least one other functional parameter following stimulation (i.e., resting levels of granzyme B from nonresponding/unstimulated T cells were disregarded). All reported functional profile data represent the findings of two independent experiments that yielded similar results.

In vivo cytotoxicity assay. The *in vivo* killing ability was determined by using peptide-pulsed, carboxyfluorescein succinimidyl ester (CFSE)-labeled whole splenocytes from naïve C57BL/6 mice as targets. Splenocytes were differentially labeled with CFSE (Invitrogen), with 0.5 or 2.5 μ M dye. Cells of the lower CFSE concentration (peak 1) were left untreated, while peak 2 cells were pulsed with the CD8/class I peptide pool for 45 min at 37°C. Next, all cells were washed several times, and equal numbers were combined ($\sim 5 \times 10^6$ cells for each peak) and then injected intravenously (i.v.) into mice that were either uninfected ($n = 2$) or infected 7 days prior with VACV ($n = 3$) or ECTV ($n = 3$). (Note that virus infections were carried out as described above.) Four hours after the injection of CFSE-labeled target cells, splenocytes were isolated and collected by using a BD FACSCalibur flow cytometer. The degree of specific killing was calculated by using the following formula: $(1 - [(naïve^{unpulsed} / naïve^{pep\ pool}) / (infected^{unpulsed} / infected^{pep\ pool})]) \times 100$. All reported *in vivo* killing data represent the findings from two independent experiments that yielded similar results.

RESULTS

Examination of T-cell functionality upon poxvirus infection. In this study, we infected C57BL/6 mice with ECTV or VACV via

footpad inoculation. Infection with ECTV resulted in significant inflammation and loss of limb, as previously reported (33), while considerable swelling of the primary site among VACV-infected mice ultimately resolved (data not shown). These observations, which highlight the *in vivo* differences between ECTV and VACV, led us to investigate whether the functional nature of T cells induced by distinct poxviruses also differed following infection. In the experiments that followed, we compared the functional capacity of CD4⁺ and CD8⁺ T cells specific for both viruses using standard intracellular cytokine staining (ICS) assays, polychromatic flow cytometry, as well as *in vivo* killing assays. We developed a flow cytometric staining panel and gating strategy (see Fig. S1A and S1B in the supplemental material) that allowed for the simultaneous detection of T-cell lineage markers (CD3, CD4, and CD8) along with various functional parameters (an example of staining is shown in Fig. S1 in the supplemental material), including degranulation (surface expression of CD107a [2]), cytokine expression (IFN- γ , TNF- α , and IL-2), and the presence of the cytolytic mediator granzyme B (grz B). We assessed the magnitude and functional characteristics of virus-specific T cells from the spleen and inguinal lymph node (I-LN) following stimulation with either virus-infected or peptide-pulsed bone marrow-derived dendritic cells (BMDCs). We chose the I-LN due to previous studies that identified this site as an important location for the priming of virus-specific T cells following cutaneous poxvirus infection (28). In addition, the examination of poxvirus-specific T cells from both the I-LN and the spleen allows for a comprehensive functional analysis of various T-cell populations that reside in or traffic to distinct lymphoid organs and thus may be subject to diverse priming conditions or other stimuli.

Polyfunctional ECTV- and VACV-specific CD8⁺ T cells predominated at acute and memory time points. We first assessed the degranulation ability of and cytokine production by CD8⁺ T cells stimulated with virus-infected BMDCs or a mixture of two class I-restricted epitopes (Table 1). We gated on cells positive for each functional molecule (similar to data shown in Fig. S1B in the supplemental material) and performed a Boolean analysis. The average proportion of all poxvirus-specific CD8⁺ T-cell responses comprised of each individual function from two independent experiments is presented in Table 2. We also calculated the magnitude of each CD8⁺ T-cell response by summing across all combinations that were positive for at least one measured functional marker (CD107a, IFN- γ , TNF- α , and/or IL-2). As shown in Fig. 1A, the response magnitudes, represented as percentages of total cells, were higher in the spleen than in the I-LN during the acute phase of infection at day 10. (Note that day 10 postinfection was chosen for this analysis due to lower background cytokine expression levels observed at this time than at earlier time points, such as day 7 [see Fig. S2 in the supplemental material].) We also typically observed a higher frequency of ECTV-specific CD8⁺ T cells in the spleen than those specific for VACV (Fig. 1A), but the absolute numbers of responding cells were similar between the two infections (see Fig. S3A in the supplemental material).

We next examined the functional profile of ECTV- and VACV-specific CD8⁺ T cells from both the spleen and I-LN; the major contributing functional permutations are depicted in Fig. 1B for each response to the CD8/class I peptide pool. Approximately 40 to 60% of the various responses were comprised of degranulating cells coexpressing IFN- γ and TNF- α . The second most prominent population consisted of degranulating cells that also expressed

TABLE 2 Contribution of each individual functional parameter to poxvirus-specific CD8⁺ T-cell responses

Infection	Stimulation	Organ	dpi ^a	% of cells expressing:			
				CD107a	IFN- γ	IL-2	TNF- α
ECTV	Peptide pool	I-LN	10	78	79	17	68
	Peptide pool	I-LN	75	73	63	41	66
	Peptide pool	Spleen	10	95	98	19	71
	Peptide pool	Spleen	75	93	89	33	83
	ECTV-DCs ^b	I-LN	10	68	81	11	78
	ECTV-DCs	I-LN	75	89	59	28	69
	ECTV-DCs	Spleen	10	89	89	13	65
	ECTV-DCs	Spleen	75	95	69	23	67
VACV	Peptide pool	I-LN	10	72	95	25	77
	Peptide pool	I-LN	75	66	64	51	70
	Peptide pool	Spleen	10	92	98	29	74
	Peptide pool	Spleen	75	93	91	50	87
	VACV-DCs ^c	I-LN	10	62	87	33	85
	VACV-DCs	I-LN	75	77	68	47	72
	VACV-DCs	Spleen	10	90	94	32	80
	VACV-DCs	Spleen	75	95	82	44	86

^a dpi, day postinfection.^b ECTV-infected BMDCs.^c VACV-infected BMDCs.

every measured cytokine, including IL-2. Comparable results were also obtained for the functional profiles of CD8⁺ T cells activated using infected BMDCs (data not shown). As shown in Fig. 1B, we grouped the functional combinations by the degree of positivity (i.e., cells producing four functions simultaneously [4+ {red}], three functions [3+ {orange}], two functions [2+ {blue}], and one function [1+ {gray}]). In general, we observed similar profiles between ECTV- and VACV-specific CD8⁺ T cells responding to stimulation with peptide (Fig. 1C) or infected BMDCs (Fig. 1D) from both spleen and I-LN at day 10. A similar functionality between virus-specific CD8⁺ T cells was also seen at day 7 postinfection (see Fig. S4A in the supplemental material). VACV-specific CD8⁺ T cells displayed slightly higher levels of IL-2 expression than did ECTV-specific CD8⁺ T cells (Table 2), which accounts for the higher proportion of 4+ cells seen in Fig. 1C and D. Upon quantification, our analysis revealed that CD8⁺ T cells from both compartments displayed significant levels of multifunctional cells (Fig. 1E).

As shown in Fig. 1F, poxvirus-specific CD8⁺ T cells from the spleen and I-LN remained readily detectable at day 75, which we chose as the time point to examine memory T-cell responses. Spleen-derived CD8⁺ T cells specific for both poxviruses maintained their polyfunctional characteristics and also demonstrated an increased presence of 4+ cells compared to those at day 10, which was most prominent in VACV-infected mice (Fig. 1G). CD8⁺ T-cell responses from the I-LN at day 75 maintained a high proportion of 3+ and 4+ cells but also displayed a greater contribution of monofunctional cells to the overall functional profile than in acute infection (Fig. 1G). The functional profiles of peptide-responsive CD8⁺ T cells are shown in Fig. 1G, but stimulation with infected BMDCs produced similar findings (data not shown). The most prominent functional combinations present during acute infection (Fig. 1B) were similar to those found at day 75 postinfection (data now shown). The quantification of the percentage of each memory CD8⁺ T-cell

response simultaneously producing three or four functions revealed similar levels of functionality between CD8⁺ T cells specific for ECTV and those specific for VACV (Fig. 1H). In summary, infection with both poxviruses induced polyfunctional CD8⁺ T-cell responses in multiple organs during both acute and memory time points.

Polyfunctional ECTV- and VACV-specific CD4⁺ T cells predominated at acute and memory time points. We next measured degranulation and cytokine production by CD4⁺ T cells stimulated with either infected BMDCs or a peptide pool comprised of class II-restricted epitopes (Table 1) and subsequently performed a Boolean analysis as described above. The average proportion of all poxvirus-specific CD4⁺ T-cell responses comprised of each individual function from two independent experiments is presented in Table 3. We then calculated the magnitude of each CD4⁺ T-cell response by summing across all combinations that were positive for at least one measured functional marker. As shown in Fig. 2A, the response magnitudes, represented as percentages of total cells, were higher in the spleen than in the I-LN during the acute phase of infection. Additionally, we typically observed a higher frequency of ECTV-specific CD4⁺ T cells in the spleen than of VACV-specific CD4⁺ T cells (Fig. 2A), but the absolute numbers of responding cells were similar between the two infections (see Fig. S3B in the supplemental material).

We then compared the functional profiles of ECTV- and VACV-specific CD4⁺ T cells from both compartments; the major contributing functional permutations are depicted in Fig. 2B for each response to the CD4/class II peptide pool. Similar results were also obtained for the functional profiles of CD4⁺ T cells activated using infected BMDCs (data not shown). In general, we observed similar functional profiles between ECTV- and VACV-specific CD4⁺ T cells responding to stimulation with peptide (Fig. 2C) or infected BMDCs (Fig. 2D) from both spleen and I-LN at day 10. A similar functionality between virus-specific CD4⁺ T cells was also seen at day 7 postinfection (see Fig. S4B in the supplemental material). Interestingly, VACV induced a slightly higher fraction of multifunctional CD4⁺ T cells than did ECTV (Fig. 2C to E), which was due to higher IL-2 expression levels among VACV-specific CD4⁺ T-cell responses (Table 3). Overall, our analysis revealed that CD4⁺ T cells from both compartments displayed significant levels of polyfunctional cells (Fig. 2E).

As shown in Fig. 2F, poxvirus-specific CD4⁺ T cells remained readily detectable in both compartments into the memory phase. CD4⁺ T cells specific for both poxviruses from the I-LN and spleen maintained their polyfunctional quality but with an increase in the contribution of monofunctional cells (Fig. 2G). The profiles of the responses to the CD4 peptide pool are shown in Fig. 2G, but stimulation with infected BMDCs produced comparable findings (data not shown). Additionally, the most prominent functional combinations present during acute infection (Fig. 2B) were also found at day 75 postinfection (data now shown). The quantification of the percentage of each response producing three or four functions again revealed the presence of multifunctional CD4⁺ T cells (Fig. 2H). In summary, infections with both ECTV and VACV induced polyfunctional CD4⁺ T-cell responses in multiple organs during both acute and memory time points.

Polyfunctional T cells expressed more cytokine per cell than did monofunctional cells. Relative amounts of cytokine production at the level of single cells can be assessed by examining the

FIG 1 CD8⁺ T cells specific for ECTV or VACV are functionally similar. (A) The magnitude, represented as the frequency of total CD8⁺ T cells, of each response was calculated by summing across all combinations of CD107a, IFN- γ , IL-2, and TNF- α . (B) CD8⁺ T-cell responses to the CD8/class I peptide pool at day 10 were divided into relative contributions of each functional combination. Permutations that did not contribute significantly to the functional profile are not shown due to space constraints. (C and D) Responses are grouped according to the degree of positivity and matched to the colors in the pie graphs for responses at day 10 to the peptide pool (C) or virus-infected BMDCs (D) from the spleen and I-LN. (E) The proportions of poxvirus-specific CD8⁺ T cells at day 10 that coexpressed three or four functions from the spleen and I-LN after stimulation are shown. (F) Total magnitude of poxvirus-specific CD8⁺ T-cell responses at day 75, determined as described above for panel A. (G) The functional profiles of CD8⁺ T cells responsive to the CD8/class I peptide pool at day 75 are shown for ECTV- and VACV-infected mice. (H) The proportions of poxvirus-specific CD8⁺ T cells at day 75 that coexpressed three or four functions from the spleen and I-LN after stimulation are shown. All depicted data were obtained from pooled cells of three mice at each time point and represent the averages of data from two independent experiments. Bars represent the means, and error bars indicate the standard deviations.

median fluorescence intensity (MFI) of the fluorescent signal associated with each functional molecule (12, 47). An examination of poxvirus-specific CD4⁺ and CD8⁺ T cells in our study revealed that activated cells of a greater functional capacity produced more cytokine than did less functional cells on a per-cell basis (representative data are shown in Fig. 3A). The quantification of the MFIs of IFN- γ , TNF- α , and IL-2 within the various functional permutations showed that multifunctional T cells expressed higher per-cell levels of all measured cytokines (Fig. 3B). Interestingly, the difference in IL-2 MFIs, but not IFN- γ or TNF- α MFIs, between polyfunctional and monofunctional cells was more prominent among CD4⁺ T cells than among CD8⁺ T cells (Fig. 3B). We found no difference in the MFIs of CD107a among the

various combinations of functionality (data not shown). In general, our findings are in agreement with previous reports of polyfunctional CD8⁺ (13, 44) and CD4⁺ (12, 13) T cells in the context of different infection settings. However, in contrast to our results, a previous MFI analysis of TNF- α and IL-2 among VACV-specific CD8⁺ T-cell responses in humans by Precopio et al. (44) did not yield the association between polyfunctionality and the per-cell production of these cytokines that we observed. In summary, multifunctional poxvirus-specific T cells in mice produced several-fold more cytokine per cell than did less functional T-cell populations.

Poxvirus-specific CD8⁺ T cells demonstrated a high cytolytic potential during acute infection. Following the analysis of

TABLE 3 Contribution of each individual functional parameter to poxvirus-specific CD4⁺ T-cell responses

Infection	Stimulation	Organ	dpi ^a	% of cells expressing:			
				CD107a	IFN- γ	IL-2	TNF- α
ECTV	Peptide pool	I-LN	10	34	82	42	60
	Peptide pool	I-LN	75	19	52	40	76
	Peptide pool	Spleen	10	42	83	43	77
	Peptide pool	Spleen	75	32	50	53	85
	ECTV-DCs	I-LN	10	22	52	27	56
	ECTV-DCs	I-LN	75	16	34	41	80
	ECTV-DCs	Spleen	10	35	83	37	63
	ECTV-DCs	Spleen	75	23	54	57	81
VACV	Peptide pool	I-LN	10	23	65	61	80
	Peptide pool	I-LN	75	21	27	60	63
	Peptide pool	Spleen	10	29	75	59	87
	Peptide pool	Spleen	75	26	47	60	68
	VACV-DCs	I-LN	10	16	51	49	66
	VACV-DCs	I-LN	75	27	26	37	77
	VACV-DCs	Spleen	10	20	58	44	67
	VACV-DCs	Spleen	75	25	53	66	83

^a dpi, day postinfection.

cytokine production by poxvirus-specific CD8⁺ T cells, we went on to assess the cytolytic nature of these cells by measuring grz B levels. This serine protease is a major component of cytotoxic granules in many CD8⁺ T cells and cooperates with perforin to induce the lysis of target cells (46). During the acute phase, total resting levels of grz B among CD8⁺ T cells from the I-LN and spleen varied substantially between the two infections: ECTV induced both a higher frequency and a higher total number of grz B-positive (grz B⁺) cells than did VACV (Fig. 4A). It should be noted that a previous study (16) reported higher levels of grz B in the spleen at day 7, especially after VACV infection, than what we observed at day 10. However, it is known that grz B expression in poxvirus-specific CD8⁺ T cells can be rapidly modulated during the course of acute infection (17). In addition, the authors of that study (16) inoculated mice with a higher dose and by a different route (5×10^6 PFU of VACV intraperitoneally [i.p.]), either of which may influence grz B levels. Finally, we did not detect the presence of grz B in memory CD8⁺ T cells from any organ (data not shown); therefore, the analysis of grz B expression was limited to the acute phase.

As shown in Fig. 4B, we calculated the proportions of all responding poxvirus-specific CD8⁺ T cells that were positive for granzyme B after stimulation. This analysis revealed 40 to 60% coexpression of grz B with other functions at day 10 (Fig. 4B) and >90% coexpression at day 7 (data not shown). We did not observe any difference in the fractions of grz B between responses to peptide or infected BMDCs, or between ECTV- and VACV-specific CD8⁺ T cells, within each compartment (Fig. 4B). After including grz B in the analysis of functional profiles following activation with class I peptides, we determined that approximately 70 to 90% of the CD8⁺ T-cell responses from ECTV- or VACV-infected mice were positive for three, four, or five functions (Fig. 4C); the major contributing functional combinations are shown in Fig. 4D. About 5 to 10% of poxvirus-specific CD8⁺ T cells simultaneously expressed all five functional markers, but the highest-frequency permutation (25 to 35% of the response) consisted of cells positive for all markers except IL-2 (Fig. 4D).

In general, we did not detect a high frequency of cells that coexpressed IL-2 and grz B, which is analogous to data from prior reports showing a dichotomous relationship between perforin and IL-2 production among virus-specific CD8⁺ T cells in humans (32).

Upon a closer examination of grz B that was coexpressed with other measured functions, such as IFN- γ (Fig. 4E), we found that a substantial fraction (~90%) of grz B was found within CD107a⁺ CD8⁺ T cells that had released cytotoxic granules after activation (Fig. 4F). These cells that coexpressed CD107a and grz B likely upregulated a large fraction of the detectable grz B levels from new transcription and/or translation. A CD107a⁺ CD8⁺ T cell has presumably lost all (or nearly all) of its preformed, granule-associated grz B, which would no longer be detectable in an ICS format after 6 h. Support for this conclusion is shown in Fig. 4G. ECTV-specific CD8⁺ T cells that degranulated but did not also produce cytokine were found to express very low levels of grz B. In comparison, cells that expressed cytokine but did not release their granules had substantially higher levels of grz B, which is most likely due to the presence of preformed stores of grz B in these cells. Conversely, cells that were positive for both degranulation and cytokine expression displayed intermediate levels of grz B (Fig. 4G). In summary, by including grz B in our functional analysis, we found that poxvirus-specific CD8⁺ T cells displayed a significant cytolytic nature by expressing and upregulating grz B within a large fraction of responding cells.

VACV- and ECTV-specific CD8⁺ T cells demonstrated equivalent *in vivo* cytolytic abilities. The data described above showed that virus-specific CD8⁺ T cells from both VACV- and ECTV-infected mice displayed similar levels of degranulation and grz B expression following stimulation, suggesting comparable cytolytic potentials. To test this supposition, we performed *in vivo* cytotoxicity assays with CD8/class I peptide-pulsed targets labeled with CFSE dye and injected them into mice at day 7 postinfection (example data are shown in Fig. 5A). In line with the flow cytometric data, we found that acute-phase CD8⁺ T cells induced by VACV and ECTV demonstrated equivalently high capacities to eliminate targets that were pulsed with the CD8/class I peptide pool (Fig. 5B). We chose to examine the cytolytic capacity at day 7 postinfection because the grz B content within CD8⁺ T cells is highest at this time (data not shown), which maximized the possibility of observing class I-restricted target cell lysis. Nevertheless, comparable results were also obtained at day 10 postinfection under similar experimental conditions (data not shown). Therefore, CD8⁺ T cells induced by either VACV or ECTV were equally capable of target cell lysis during acute time points.

DISCUSSION

The study of a natural pathogen within its endemic host reveals the evolutionary adaptations that fundamentally influence pathogenesis and/or immunity. For example, both ECTV and VACV express a number of immune modulators (14), but in most strains of VACV, many of these host response modifiers are absent or fail to interact productively with the host target proteins (51). Therefore, the usefulness of VACV in mice as a model for smallpox is limited because its pathogenesis, disease progression, and outcome of infection are unlike those of VARV. In contrast, the clinical course of ECTV in mice closely resembles that of VARV infection of humans (7, 18). Accordingly, the study of ECTV infection

FIG 2 CD4⁺ T cells specific for ECTV or VACV are functionally similar. (A) The magnitude, represented as the frequency of total CD4⁺ T cells, of each response was calculated by summing across all combinations of CD107a, IFN-γ, IL-2, and TNF-α. (B) CD4⁺ T-cell responses to the CD4/class II peptide pool at day 10 were divided into relative contributions of each functional combination. Permutations that did not contribute significantly to the functional profile are not shown due to space constraints. (C and D) Responses are grouped according to the degree of positivity and matched to the colors in the pie graphs for responses at day 10 to the peptide pool (C) or virus-infected BMDCs (D) from the spleen and I-LN. (E) The proportions of poxvirus-specific CD4⁺ T cells at day 10 that coexpressed three or four functions from the spleen and I-LN after stimulation are shown. (F) Total magnitude of poxvirus-specific CD4⁺ T-cell responses at day 75, determined as described above for panel A. (G) The functional profiles of CD4⁺ T cells responsive to the CD4/class II peptide pool at day 75 are shown for ECTV and VACV mice. (H) The proportions of poxvirus-specific CD4⁺ T cells from the spleen and I-LN at day 75 that coexpressed three or four functions after stimulation are shown. All depicted data were from pooled cells of three mice at each time point and represent the averages of data from two independent experiments. Bars represent the means, and error bars indicate the standard deviations.

of mice provides a powerful platform for exploring the interplay between a poxvirus and its natural host.

The high degree of homology among orthopoxviruses (25) has allowed VACV to serve as a highly effective vaccine against smallpox. Similarly, VACV provides protection against lethal mousepox challenge (21). A previous report that analyzed the response of immunized humans using multiparameter flow cytometry showed that VACV induces long-lasting polyfunctional CD8⁺ T cells (44). (Note that those authors did not report on the functional responses of VACV-specific CD4⁺ T cells in that study.) However, VACV is not a native human pathogen, and the degree to which the functional profile of T

cells induced by VACV is similar to that of T cells induced by smallpox, the natural human poxvirus, remains unknown. Therefore, we used the mouse model of smallpox to compare the functional capacities of poxvirus-specific T cells that arise following both natural infection (ECTV) and nonnatural infection (VACV). In general, we find that significant fractions of T-cell responses specific for both poxviruses are positive for at least three or more measured functions. Additional observations can also be made from these data: (i) the cytokine expression patterns and functional capacities of VACV- and ECTV-specific T cells are similar, (ii) polyfunctional T cells are found very early during acute infection and persist after viral clear-

FIG 3 Polyfunctional T cells produce more cytokine per cell than do monofunctional cells. (A) Representative examples of the relative degrees of IFN- γ and TNF- α production from poxvirus-specific T cells of various of functional capacities. The depicted data are from splenocytes of ECTV-infected mice at day 10. The various functional subsets were overlaid onto a density plot (black shading) of total T cells. (B) Compiled cytokine MFIs within the various functional subsets among all T-cell responses to the peptide pools and virus-infected BMDCs are shown. Only functional permutations comprised of at least 10 events were used for this analysis. Each red line represents the median. Statistical analysis was carried out by using one-way analysis of variance (ANOVA) tests (nonparametric; Kruskal-Wallis), followed by a Dunn's test for multiple comparisons. * denotes a P value of <0.05 , ** denotes a P value of <0.01 , and *** denotes a P value of <0.001 . All depicted data are from day 10 responses, but similar results were also obtained at day 75.

ance in multiple organs, and (iii) CD8⁺ T cells induced by either poxvirus readily degranulate, express high levels of grz B, and demonstrate equivalent cytolytic abilities.

The presence of polyfunctional T_H1-type T-cell responses was

previously correlated with improved clinical outcomes in several infection settings (3, 12, 27). Our results here provide further support for the precedent that multifunctional T-cell responses may be important in the context of controlled viral infections (47). Of

FIG 4 Poxvirus-specific CD8⁺ T cells express large amounts of granzyme B during acute infection. (A) Each plot shows the total frequency of grz B within unstimulated CD8⁺ T cells during acute infection in the spleen and I-LN. The values in parentheses represent the total numbers of grz B⁺ events within the CD8⁺ T-cell gate. SSC, side scatter. (B) The proportions of poxvirus-specific CD8⁺ T cells that coexpressed grz B with at least one additional function in the spleen and I-LN at day 10 are shown. Bars represent the means, and error bars indicate the standard deviations. (C) The functional profiles of CD8⁺ T cells stimulated with the class I peptide pool, constructed similarly to those shown in Fig. 1 and 2, were analyzed to include grz B such that five functions are now shown. The relative amounts of grz B positivity within each functional group (i.e., each pie slice) are depicted as black arcs. (D) The major functional combinations of CD8⁺ T cells responding to peptide stimulation after grz B was included in the overall response profile, as described above for panel C, is shown. Permutations that did not contribute significantly to the functional profile are not shown due to space constraints. The depicted data are from pooled cells of three mice and represent the averages of data from two independent experiments. (E) Flow cytometric plots of grz B versus IFN- γ from representative poxvirus-specific CD8⁺ T-cell responses are shown. Percentages denote the proportions of IFN- γ ⁺ cells that are positive or negative for grz B. (F) All grz B⁺ cells expressing at least one other function (red) are overlaid onto a plot of total CD8⁺ T cells (black density plots), showing CD107a versus IFN- γ from representative peptide-specific responses. Percentages represent the fractions of overlaid cells that fall within each quadrant. (G) MFIs of grz B within CD8⁺ T cells that demonstrate a differential ability to degranulate and/or express cytokine (IFN- γ) are shown for a representative response. The indicated values denote the grz B MFIs for each respective population of cells.

FIG 5 Poxvirus-specific CD8⁺ T cells from the spleen are highly cytolytic *in vivo* during acute infection. (A) *In vivo* cytotoxic-T-lymphocyte (CTL) assays were performed to investigate the cytotoxic functionality of poxvirus-specific CD8⁺ T cells during acute infection. Representative data comparing specific killing in a naïve control mouse to that in an immunized mouse are shown. Further details on the experimental setup can be found in Materials and Methods. (B) Quantification of the results from all infected mice ($n = 3$ for each group) is shown in a graphical format. Bars represent the means, and error bars indicate the standard deviations. The Mann-Whitney test (nonparametric; two tailed) revealed no statistically significant difference in CD8⁺ T-cell-mediated lytic abilities between ECTV and VACV, as indicated (N.S.). Statistical analysis was performed by using GraphPad Prism software (version 5.0a). All depicted data are representative of two independent *in vivo* CTL assays.

the prototypical type 1 cytokines, the actions of IFN- γ and TNF- α have been shown to be essential for full control over the replication of ECTV and the survival of normally resistant mouse strains (11, 45). Notably, we find that both ECTV and VACV induce a high frequency of polyfunctional T cells that produce up to 10-fold-higher levels of several type 1 cytokines than do less functional T cells. Therefore, during natural poxvirus infection, ECTV-specific T cells, due to their polyfunctional nature, express significant quantities of cytokines that are known to be crucial for combating ECTV and likely help establish and/or maintain a resistant cytokine environment in C57BL/6 mice. However, the importance of antibodies in protective immunity against ECTV should not be understated (10, 40, 41) and are complementary to the action of T cells (16).

The degree to which VACV is able to induce protective immunity against mousepox is remarkable considering its comparatively inefficient replication and limited dissemination following dermal infection (53). For a human vaccine trial, it was reported previously that both an attenuated VACV strain (MVA) and the fully replication-competent strain Dryvax induced detectable virus-specific CD8⁺ T cells (44). However, it took multiple immunizations with MVA for Precopio and colleagues to observe VACV-specific CD8⁺ T cells in immunized humans (44). We made use of the Western Reserve (WR) strain of VACV, which, like Dryvax, is replication competent. Consequently, we detected virus-specific T cells after only one inoculation in the footpad with VACV-WR. Moreover, we find that the overall magnitudes of the T-cell responses are similar after VACV and ECTV infections. Other groups reported higher-magnitude CD8⁺ T-cell responses (16, 52) in the spleen during acute infection than what we observed in this study. However, a number of factors can influence the total magnitude and detection of virus-specific T cells, including the dose

and route of primary infection, the day at which responses are sampled, the choice of target cells, and the MOI at which these targets are infected (16, 17, 52). In general, our data show that poxviruses are able to induce robust polyfunctional T-cell responses, even in nonnatural hosts, in the absence of a high degree of replication or spread. Future work will determine whether a minimum level of VACV replication is needed for the induction of polyfunctional T cells.

In addition to measuring cytokine expression, we also assessed the cytolytic nature and capacity of poxvirus-specific T cells. In the context of ECTV infection, it was shown previously that the granule exocytosis pathway of cytotoxicity is an indispensable component of a protective immune response (36). In this study, we evaluated the cytolytic nature of responding CD8⁺ T cells by staining for the presence of grz B to detect this protein by flow cytometry (54). We found that the relative proportions of grz B were roughly equivalent within CD8⁺ T-cell responses against both ECTV and VACV, suggesting that these cells have similar cytolytic capacities. Indeed, we observed that the *in vivo* abilities to lyse targets labeled with class I epitopes were equivalent among ECTV- and VACV-infected mice during acute infection. Additionally, poxvirus-specific CD8⁺ T cells displayed a marked ability to increase the synthesis of grz B molecules following cellular activation. It was recently reported that perforin upregulation following target cell recognition potentiates the killing capacity of human CD8⁺ T cells and promotes sustained cytotoxicity (31). Since perforin alone cannot induce target cell death (43), it is possible that the upregulation of grz B may also be important for the continuous cytolytic capability after initial degranulation. Further experimentation is necessary to understand the mechanism(s) underlying this aspect of effector functionality and the consequences for pathogen control.

It is known that CD8⁺ T-cell-deficient mice survive high-dose dermal inoculation of VACV-WR at the base of the tail (50). However, the dispensability of CD8⁺ T cells under these circumstances is not altogether surprising given that BALB/c mice survive cutaneous VACV infection, whereas these same mice are highly susceptible to death after an equivalent exposure to ECTV. Conversely, CD8⁺ T cells are absolutely required for recovery from primary ECTV infection in otherwise resistant mice (16, 29). Furthermore, the adoptive transfer of VACV-immune CD8⁺ T cells protects BALB/c mice from a lethal dose of mousepox (55). Therefore, previously reported results in combination with our data suggest that VACV-mediated protection against natural poxvirus infections (ECTV and VARV) is due, at least in part, to the generation of polyfunctional T cells of a similar character as that of natural infection. In future studies, we plan to examine the functional characteristics of VACV-specific T cells following the immunization of mousepox-susceptible strains, such as BALB/c.

In summary, the functional profile of T cells generated by VACV closely mimics the functionality of ECTV-specific T cells, which are known to be essential for protection against mousepox, at both acute and memory time points in C57BL/6 mice. Collectively, our results provide one explanation for the ability of VACV to induce protective T-cell responses against lethal ECTV infection. Therefore, by extension, the ability of VACV to induce polyfunctional T cells in humans likely contributes to its ability to stimulate highly effective immune responses against smallpox in immunized individuals. Ultimately, our findings provide further

support for the use of VACV as a vaccine platform able to induce durable polyfunctional T cells (23, 24, 26).

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant U19-AI083008. Research in this publication includes work carried out within the Kimmel Cancer Center Flow Cytometry Facility, which is supported in part by NCI Cancer Center support grant P30 CA56036.

REFERENCES

- Abraham JS, et al. 2009. One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil? *PLoS One* 4:e7428. doi:10.1371/journal.pone.0007428.
- Betts MR, et al. 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* 281:65–78.
- Betts MR, et al. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107:4781–4789.
- Bhatt PN, Jacoby RO. 1987. Mousepox in inbred mice innately resistant or susceptible to lethal infection with ectromelia virus. I. Clinical responses. *Lab. Anim. Sci.* 37:11–15.
- Blanden RV. 1970. Mechanisms of recovery from a generalized viral infection: mousepox. I. The effects of anti-thymocyte serum. *J. Exp. Med.* 132:1035–1054.
- Blanden RV. 1971. Mechanisms of recovery from a generalized viral infection: mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. *J. Exp. Med.* 133:1074–1089.
- Breman JG, Henderson DA. 2002. Diagnosis and management of smallpox. *N. Engl. J. Med.* 346:1300–1308.
- Burnet FM. 1945. An unsuspected relationship between the viruses of vaccinia and infectious ectromelia of mice. *Nature* 155:543.
- Burnet FM, Boake WC. 1946. The relationship between the virus of infectious ectromelia of mice and vaccinia virus. *J. Immunol.* 53:1–13.
- Chaudhri G, Panchanathan V, Bluethmann H, Karupiah G. 2006. Obligatory requirement for antibody in recovery from a primary poxvirus infection. *J. Virol.* 80:6339–6344.
- Chaudhri G, et al. 2004. Polarized type 1 cytokine response and cell-mediated immunity determine genetic resistance to mousepox. *Proc. Natl. Acad. Sci. U. S. A.* 101:9057–9062.
- Darrah PA, et al. 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat. Med.* 13:843–850.
- Duvall MG, et al. 2008. Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur. J. Immunol.* 38:350–363.
- Esteban DJ, Buller RM. 2005. Ectromelia virus: the causative agent of mousepox. *J. Gen. Virol.* 86:2645–2659.
- Fang M, Sigal L. 2010. Studying NK cell responses to ectromelia virus infections in mice. *Methods Mol. Biol.* 612:411–428.
- Fang M, Sigal LJ. 2005. Antibodies and CD8+ T cells are complementary and essential for natural resistance to a highly lethal cytopathic virus. *J. Immunol.* 175:6829–6836.
- Fang M, Sigal LJ. 2006. Direct CD28 costimulation is required for CD8+ T cell-mediated resistance to an acute viral disease in a natural host. *J. Immunol.* 177:8027–8036.
- Fenner F. 2006. Classic paper: Fenner on the exanthemata. *Rev. Med. Virol.* 16:353–363.
- Fenner F. 1949. Mouse-pox; infectious ectromelia of mice; a review. *J. Immunol.* 63:341–373.
- Fenner F. 1947. Studies in infectious ectromelia in mice; natural transmission; the portal of entry of the virus. *Aust. J. Exp. Biol. Med. Sci.* 25:275–282.
- Fenner F. 1947. Studies in infectious ectromelia of mice; immunization of mice against ectromelia with living vaccinia virus. *Aust. J. Exp. Biol. Med. Sci.* 25:257–274.
- Fenner F. 1948. The pathogenesis of the acute exanthems; an interpretation based on experimental investigations with mousepox; infectious ectromelia of mice. *Lancet* ii:915–920.
- Garcia-Arriaza J, Najera JL, Gomez CE, Sorzano CO, Esteban M. 2010. Immunogenic profiling in mice of a HIV/AIDS vaccine candidate (MVA-B) expressing four HIV-1 antigens and potentiation by specific gene deletions. *PLoS One* 5:e12395. doi:10.1371/journal.pone.0012395.
- Gomez CE, et al. 2011. The HIV/AIDS vaccine candidate MVA-B administered as a single immunogen in humans triggers robust, polyfunctional, and selective effector memory T cell responses to HIV-1 antigens. *J. Virol.* 85:11468–11478.
- Gubser C, Hue S, Kellam P, Smith GL. 2004. Poxvirus genomes: a phylogenetic analysis. *J. Gen. Virol.* 85:105–117.
- Harari A, et al. 2008. An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J. Exp. Med.* 205:63–77.
- Hersperger AR, et al. 2010. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. *PLoS Pathog.* 6:e1000917. doi:10.1371/journal.ppat.1000917.
- Hickman HD, et al. 2008. Direct priming of antiviral CD8+ T cells in the peripheral interfollicular region of lymph nodes. *Nat. Immunol.* 9:155–165.
- Karupiah G, Buller RM, Van Rooijen N, Duarte CJ, Chen J. 1996. Different roles for CD4+ and CD8+ T lymphocytes and macrophage subsets in the control of a generalized virus infection. *J. Virol.* 70:8301–8309.
- Kees U, Blanden RV. 1976. A single genetic element in H-2K affects mouse T-cell antiviral function in poxvirus infection. *J. Exp. Med.* 143:450–455.
- Makedonas G, et al. 2009. Rapid up-regulation and granule-independent transport of perforin to the immunological synapse define a novel mechanism of antigen-specific CD8+ T cell cytotoxic activity. *J. Immunol.* 182:5560–5569.
- Makedonas G, et al. 2010. Perforin and IL-2 upregulation define qualitative differences among highly functional virus-specific human CD8 T cells. *PLoS Pathog.* 6:e1000798. doi:10.1371/journal.ppat.1000798.
- Marchal J. 1930. Infectious ectromelia: a hitherto undescribed virus disease of mice. *J. Pathol. Bacteriol.* 33:713–728.
- Moutafsi M, et al. 2007. Vaccinia virus-specific CD4+ T cell responses target a set of antigens largely distinct from those targeted by CD8+ T cell responses. *J. Immunol.* 178:6814–6820.
- Moutafsi M, et al. 2006. A consensus epitope prediction approach identifies the breadth of murine T(CD8+)-cell responses to vaccinia virus. *Nat. Biotechnol.* 24:817–819.
- Mullbacher A. 2003. Cell-mediated cytotoxicity in recovery from poxvirus infections. *Rev. Med. Virol.* 13:223–232.
- Mullbacher A, et al. 1996. Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. *Proc. Natl. Acad. Sci. U. S. A.* 93:5783–5787.
- Mullbacher A, Hla RT, Musseteau C, Simon MM. 1999. Perforin is essential for control of ectromelia virus but not related poxviruses in mice. *J. Virol.* 73:1665–1667.
- Mullbacher A, et al. 1999. Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* 96:13950–13955.
- Panchanathan V, Chaudhri G, Karupiah G. 2008. Correlates of protective immunity in poxvirus infection: where does antibody stand? *Immunol. Cell Biol.* 86:80–86.
- Panchanathan V, Chaudhri G, Karupiah G. 2006. Protective immunity against secondary poxvirus infection is dependent on antibody but not on CD4 or CD8 T-cell function. *J. Virol.* 80:6333–6338.
- Perfetto SP, et al. 2006. Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. *J. Immunol. Methods* 313:199–208.
- Pipkin ME, Lieberman J. 2007. Delivering the kiss of death: progress on understanding how perforin works. *Curr. Opin. Immunol.* 19:301–308.
- Precopio ML, et al. 2007. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J. Exp. Med.* 204:1405–1416.
- Ruby J, Bluethmann H, Peschon JJ. 1997. Antiviral activity of tumor necrosis factor (TNF) is mediated via p55 and p75 TNF receptors. *J. Exp. Med.* 186:1591–1596.
- Russell JH, Ley TJ. 2002. Lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 20:323–370.
- Seder RA, Darrah PA, Roederer M. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* 8:247–258.
- Siciliano NA, Skinner JA, Yuk MH. 2006. Bordetella bronchiseptica

- modulates macrophage phenotype leading to the inhibition of CD4⁺ T cell proliferation and the initiation of a Th17 immune response. *J. Immunol.* 177:7131–7138.
49. Silva-Fernandes AT, et al. 2009. Natural human infections with vaccinia virus during bovine vaccinia outbreaks. *J. Clin. Virol.* 44:308–313.
 50. Spriggs MK, et al. 1992. Beta 2-microglobulin-, CD8⁺ T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. *Proc. Natl. Acad. Sci. U. S. A.* 89:6070–6074.
 51. Symons JA, Tscharke DC, Price N, Smith GL. 2002. A study of the vaccinia virus interferon-gamma receptor and its contribution to virus virulence. *J. Gen. Virol.* 83:1953–1964.
 52. Tscharke DC, et al. 2005. Identification of poxvirus CD8⁺ T cell determinants to enable rational design and characterization of smallpox vaccines. *J. Exp. Med.* 201:95–104.
 53. Tscharke DC, Smith GL. 1999. A model for vaccinia virus pathogenesis and immunity based on intradermal injection of mouse ear pinnae. *J. Gen. Virol.* 80(Pt 10):2751–2755.
 54. Wherry EJ, et al. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4:225–234.
 55. Xu RH, Fang M, Klein-Szanto A, Sigal LJ. 2007. Memory CD8⁺ T cells are gatekeepers of the lymph node draining the site of viral infection. *Proc. Natl. Acad. Sci. U. S. A.* 104:10992–10997.