Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines

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The large size of poxvirus genomes has stymied attempts to identify determinants recognized by CD8+ T cells and greatly impeded development of mouse smallpox vaccination models. Here, we use a vaccinia virus (VACV) expression library containing each of the predicted 258 open reading frames to identify five peptide determinants that account for approximately half of the VACV–specific CD8+ T cell response in C57BL/6 mice. We show that the primary immunodominance hierarchy is greatly affected by the route of VACV infection and the poxvirus strain used. Modified vaccinia virus ankara (MVA), a candidate replacement smallpox vaccine, failed to induce responses to two of the defined determinants. This could not be predicted by genomic comparison of viruses and is not due strictly to limited MVA replication in mice. Several determinants are immunogenic in cowpox and ectromelia (mousepox) virus infections, and immunization with the immunodominant determinant provided significant protection against lethal mousepox. These findings have important implications for understanding poxvirus immunity in animal models and bench–marking immune responses to poxvirus vaccines in humans.

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Abbreviations used: CPXV, cowpox virus; ECTV, ectromelia virus; ICS, intracellular cytokine staining; ID, immunodominance; IDD, immunodominant determinant; MVA, modified vaccinia virus ankara; ORF, open reading frame; SDD, subdominant determinant; VACV, vaccinia virus; VARV, variola virus.

In the late 1700s, Edward Jenner published the seminal observation that infection with animal poxviruses protects humans against smallpox (1). Though similar practices had been followed for centuries by other societies around the world, Jenner was the first to apply the scientific method to evaluating efficacy. In so doing, he laid the foundations for both the eradication of smallpox and for the discipline of immunology.

Despite its historic origins, current understanding of the molecular basis for cross-protection between heterologous poxviruses is woefully incomplete. This is particularly the case for antigens recognized by T cells, although they are assumed to play an important role in immunity (2–4). The neglect stems partly from the daunting size of poxvirus genomes and from the lack of a pressing need to understand poxvirus immunology after the

eradication of smallpox (2). Recent improvements in molecular methods and determinant prediction have made genome size less of an obstacle. The specter of variola virus (VARV), the causative agent of smallpox, being used as a bioweapon has eliminated complacency about human poxvirus immunity (5). The current live vaccinia virus (VACV)-based smallpox vaccine, while remarkably effective, is associated with mild to severe complications and safer alternatives are required.

As a consequence, identification of human T cell determinants of VACV, the current smallpox vaccine, is now underway. To date, papers have been limited to a few determinants presented by a single restricting class I molecule (6–8). Although this work is important for characterizing human responses to safer vaccines, the biological relevance of responses to these determinants cannot be tested. Fortu-
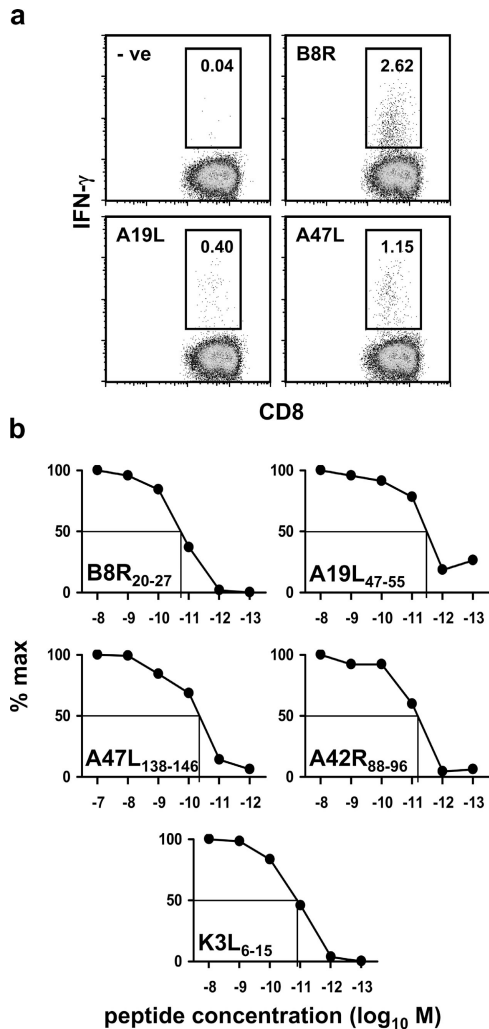
a

b

Figure 1. Mapping and characterizing CD8$^+$ T cell determinants. Mice were infected with 10^6 PFU VACV i.p. and 7 d later, purified CD8$^+$ T cells (a) or splenocytes (b) were used in ICS assays. (a) Examples taken from the original screening assays used to identify VACV genes with K^b-restricted CD8$^+$ T cell determinants. 293KbC2 cells transfected with VACV genes (top left) as part of a 96-well array were used as stimulators. IFN-γ^+, CD8$^+$ events as a percent of all CD8$^+$ events is shown in the gates drawn around the IFN-γ^+ population. (b) Antigenic potency of synthetic peptides. DC2.4 cells pulsed with the peptides at the indicated concentrations were used as stimulators in ICS assays. Data are expressed as the percent of maximum stimulation; lines are drawn to indicate peptide concentrations inducing half-maximal CD8$^+$ T cell activation. Peptide titrations were repeated.

nately, there are animal models to shed light on the problem of cross-protection between heterologous poxviruses. For example, just as VACV protects humans against smallpox, this same virus protects mice against mousepox (9, 10).

Mousepox is caused by ectromelia virus (ECTV), a mouse pathogen closely related to VACV and VARV (11, 12). Current understanding of smallpox pathogenicity owes much to pioneering work with mousepox (13, 14) and more recent work has taught us much about immunity in systemic poxvirus disease (15–17). Alternative models for lethal poxvirus disease in mice have also been exploited, including in-

tranasal VACV (6, 8, 18–20) and cowpox virus (CPXV; reference 21) infection. Although the specificities of T cells that contribute to protection of humans against smallpox and mice against various poxviruses will not be identical due to differences in antigen processing and presentation and T cell receptor repertoires, the basic principles underlying protection are likely to be similar. Due to the sophistication of the mouse model for immunity, many important questions about the basic immunology of poxvirus protection are best addressed at this time in mouse models. These include assessment of the importance of individual effector mechanisms and the degree and mechanism of protection afforded by smallpox vaccine candidates.

A critical step in refining the mouse model is to define poxvirus determinants recognized by CD8$^+$ T cells and determine their role in protective immunity. Here, we provide the original description of poxvirus determinants recognized by mouse CD8$^+$ T cells, and their biological relevance in protection against lethal poxvirus infections.

RESULTS
Identification of VACV CD8$^+$ T cell determinants in mice
To identify immunogenic VACV gene products, we generated an expression library of 258 predicted open reading frames (ORFs) and highly transfectable cells (human 293 cells) expressing H-2K^b or D^b. Cells transfected with individual plasmids were tested for their ability to induce IFN-γ expression in CD8$^+$ T cells derived from C57BL/6 mice infected i.p. with VACV 7 d previously (Fig. 1 a). Five antigenic VACV ORFs were discovered, three restricted by H-2K^b and two by H-2D^b (Table I). For two ORFs, A19L and A47L, this is the first empirical evidence that they are expressed during infection. Ironically, of the known genes, two (B8R and K3L) are involved in virus evasion of host interferon responses (22). Whether this apparent targeting of immune modulators by CD8$^+$ T cells is a general phenomenon or a quirk of the mouse model used requires more determinants to be mapped in mice and other species.

Antigenic VACV ORFs were analyzed in silico for peptides matching the relevant class I peptide-binding motif. Synthetic peptides for predicted determinants were tested for their ability to bind the relevant class I allomorph and to activate CD8$^+$ T cells from VACV-infected mice (Table I). None of the predicted A42R peptides were immunogenic, which led to testing subclones expressing gene fragments (residues 1–48, 1–82, 75–end, and 107–end) and ultimately a second round of in silico prediction resulting in identification of YAPVSPIVI as the determinant.

Synthetic peptides for each of the five mapped determinants were antigenic at $<10^{-10}$ M (Fig. 1 b) when tested for their ability to activate primary VACV-specific CD8$^+$ T cells. Peptide-specific CD8$^+$ T cell cultures were generated by in vitro stimulation of splenocytes from VACV-infected mice over several weeks. Cultures stimulated with the five peptides were found to recognize VACV-infected DC2.4 cells and VACV-infected 293 expressing the correct restric-

Table I. Immunogenic proteins, peptides tested, and CD8^{+} T cell epitopes

Gene	H–2	Function	Time[a]	Peptides tested[b]	IC_{50} (nM)[c]	Determinant[d]	Name[e]
B8R	K[b]	soluble IFN-γ receptor	early	TSYKFESV	0.19	TSYKFESV	$B8R_{20-27}$
				WQTMYTNV	6.6		
				ISLKNYGI	16		
				LAVLFINSI	28		
				KVEEYCTGL	129		
A19L	K[b]	unknown	–	VSLDYINTM	0.77	VSLDYINTM	$A19L_{47-55}$
				SDITKVSL	1,261		
				VKISDITKV	4,930		
A47L	K[b]	unknown	–	TIQRFSSL	1.1		
				AAFEFINSL	1.3	AAFEFINSL	$A47L_{138-146}$
				ATIQRFSSL	1.3		
				VSDLYTSM	7.0		
				LTPIFSDLL	7.3		
A42R	D[b]	profilin homologue	late	YAPVSPIVI	0.24	YAPVSPIVI	$A42R_{88-96}$
				FAKINPGEI	54		
				LMDENTYAM	767		
				MYAPVSPIVI	1,266		
				FAKINPGEII	2,071		
K3L	D[b]	IFN resistance	early	YSLPNAGDVI	0.22	YSLPNAGDVI	$K3L_{6-15}$
				YSLPNAGDV	102		
				YIDVNYKRM	5,138		

[a]Class of VACV gene as published: early, late, or unknown.
[b]Top five peptides (by H-2 binding) predicted for each gene. Total peptides tested: B8R, 28; A19L, 3; A47L, 45; A42R, 7; and K3L, 3.
[c]Peptide binding to relevant H-2 molecule.
[d]CD8^{+} T cell determinant as determined by T cell assays.
[e]Name used for determinant is gene name followed by amino acid position.

tion element (unpublished data). Similar cultures recognized 293 cells expressing the appropriate restriction element transfected with the source VACV gene, but not another gene, or the source gene where the wrong restriction element was present (unpublished data). This strongly suggests that the peptides identified represent bona fide virus-encoded determinants generated by antigen processing pathways and not spuriously cross-reacting peptides. We note that the odds of this latter possibility increase with increasing genome size of the immunogen and might prove to be particularly relevant to poxviruses and other large DNA viruses.

Contribution of mapped determinants to total response

We determined the contribution of these determinants to the total acute (day 7; Fig. 2 a) and memory (day 21; Fig. 2 b) CD8^{+} T cell response to VACV by intracellular cytokine staining (ICS) of splenocytes tested ex vivo. Uninfected mice failed to detectably respond to any of the peptides (unpublished data). 7 d after i.p. infection, $B8R_{20-27}$ elicited a response from 10–15% of all splenic CD8^{+} T cells, amounting to a staggering 10^7 cells in the spleen alone. Other determinants elicited a more modest response; in general, $K3L_{6-15}$ was the next strongest followed by $A47L_{138-146}$, $A42R_{88-96}$, and $A19L_{47-55}$. Occasionally, in individual mice, the order of $K3L_{6-15}$ and $A47L_{138-146}$ was swapped in the immunodomi-

nance (ID) hierarchy, but $A19L_{47-55}$ was always at the bottom. The stability of the VACV ID hierarchy in individual mice is similar to that seen with influenza virus (23). Comparing determinant-specific responses with the total VACV-specific response shows that $B8R_{20-27}$, the immunodominant determinant (IDD), alone stimulates >25% of the total response. When combined with the four subdominant determinants (SDDs), this accounts for up to 40% of the total response.

As expected, CD8^{+} T cell numbers decreased in memory CD8^{+} T cells, which were also measured ex vivo by ICS. The ID hierarchy was slightly altered, due to a less pronounced decrease in $A47L_{138-146}$-specific CD8^{+} T cells. Similar relative sparing of CD8^{+} T cells specific for SDDs in memory has been observed previously (24). Altogether, the CD8^{+} T cells specific for the defined determinants account for ∼30% of the total anti-VACV memory CD8^{+} T cell response.

Route of infection affects ID

Smallpox vaccination has been performed for centuries by dermal scarification, whose simplicity probably contributed to smallpox eradication. To our surprise, after scarification with VACV, $B8R_{20-27}$-specific responses were enhanced, whereas responses to SDDs were depressed (Fig. 3 a). Indeed, in most mice, responses to the two weakest SDDs were reduced to the limit of detection. The enhanced re-
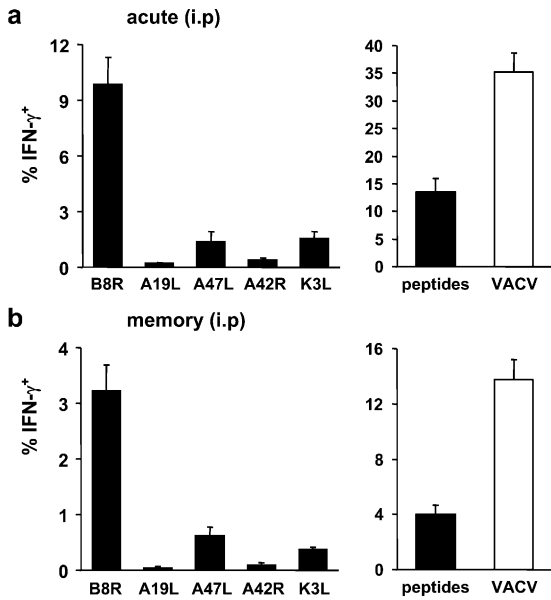
a acute (i.p)

b memory (i.p)

Figure 2. CD8^{+} T cell responses to VACV determinants and VACV-infected cells. (a and b) Mice were immunized i.p. with 10^6 PFU of VACV strain WR and responses measured by ICS 7 (a) and 21 (b) d later. (left) Graphs show the percent of splenic CD8^{+} T cells that produce IFN-γ in ex vivo stimulations with the individual peptides indicated (name of gene shown). (right) Graphs compare a summation of the peptide data with the percent of splenic CD8^{+} T cells that produce IFN-γ in ex vivo stimulations with VACV-infected DC2.4 cells. Data are means and SEM from groups of three mice. Experiments have been repeated with similar results.

sponse to $B8R_{20-27}$ is strikingly demonstrated by comparison to the total VACV-specific response; after dermal scarification, >50% of CD8^{+} T cells are specific for $B8R_{20-27}$. To test this apparent suppression of SDD responses more rigorously, the ratio of the sum of the SDD-specific responses to the IDD-specific response was calculated using data from individual mice from several experiments to provide a larger sample size (Fig. 3 b). The mean of this ratio was significantly different (P = 0.0013) between mice infected by the i.p. and dermal routes. Finally, responses to all determinants contribute nearly 60% of the total primary CD8^{+} T cell response induced by scarification.
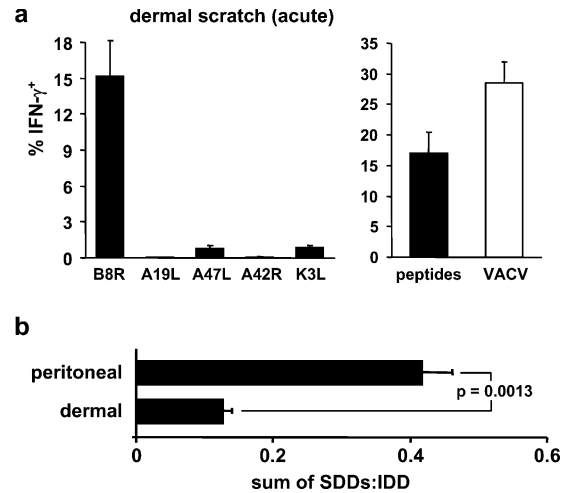
a dermal scratch (acute)

b

Figure 3. CD8^{+} T cell responses after infection by dermal scarification. (a) Mice were immunized by tail scarification and application of VACV at 10^7 PFU/ml, and responses were measured by ICS 7 d later. (left) Graph shows the percent of splenic CD8^{+} T cells that produce IFN-γ in ex vivo stimulations with the individual peptides indicated (name of gene shown). Graph on right compares a summation of the peptide data with the percent of splenic CD8^{+} T cells that produce IFN-γ in ex vivo stimulations with VACV-infected DC2.4 cells. Data are means and SEM from groups of three mice; the experiment was repeated with similar results. (b) Total response to SDDs as a ratio of the response to the IDD in acute infection by i.p. (peritoneal) and dermal scarification (dermal) routes. SDDs:IDD ratio was calculated for individual mice from several experiments (i.p., n = 19; dermal scarification, n = 6), and means and SEM were plotted. p-value was determined by Students' t test.

Dryvax immunized mice respond to all mapped determinants
Until this point, we had exclusively used the WR strain of VACV, which was derived from the New York City Board of Health (NYCBH) vaccine strain by serial passage in mice and tissue culture (25). Dryvax (Wyeth Laboratories), the smallpox vaccine currently available in the USA, is the NYCBH strain propagated on the skin of calves. All determinants were immunogenic in mice infected i.p. with Dryvax. The ID hierarchy was similar to that found with WR; the only determinant with responses that differed significantly was $K3L_{6-15}$ (P = 0.0158; Fig. 4 a). A screen of the VACV ORF library

Table II. Conservation of immunogenic proteins and determinants across poxvirus species and strains

VACV-WR		VACV-MVA[a]		CPXV-BR		ECTV-Mos		VARV-Bsh[b]	
Gene	Determinant	Gene[c]	Determinant[d]	Gene	Determinant	Gene	Determinant	Gene	Determinant
B8R	TSYKFESV	NA/176	Y	202	Y	158	Y	B8R	Y
A19L	VSLDYINTM	130	Y	152	Y	122	Y	A20L	Y
A47L	AAFEFINSL	160	Y	185	Y	146	A[e]	J1L	Y
A42R	YAPVSPIVI	154R	Y	179	Y	141	Y	A45R	Y
K3L	YSLPNAGDVI	024	Y	043	Y	NA	Y	C3L	A[f]

[a]VACV-MVA: two available sequences (U94848 and AY603355) agree except for B8R. CPX-BR: CPXV-Brighton red, ECTV-Mos: ECTV-Moscow, VARV-Bsh: VARV-Bangladesh.
[b]VARV does not infect mice; determinant conservation is shown only for completeness.
[c]Gene name given where annotated; NA: gene not included in annotation.
[d]Y: Determinant conserved in genomic sequence; gene may or may not be intact or expressed.
[e]A: Determinant is altered to ATFEFINSL.
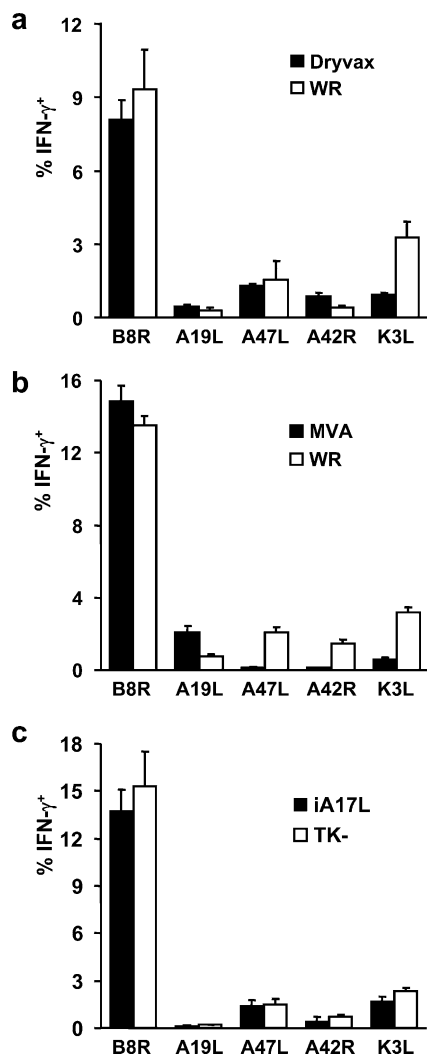[f]A: Determinant is altered to YSLPNVGDVL.

Figure 4. CD8$^+$ T cell responses to VACV determinants after immunization with different strains. Mice were immunized i.p. with 10^6 WR (a and b), 10^6 PFU of Dryvax (a), 5 × 10^7 FFU of MVA (b), and 5 × 10^6 PFU of iA17L and 10^6 PFU Vsc8 (TK$^-$) (c), and CD8$^+$ T cell responses to peptides were measured by ICS after 7 d. Data are percent of splenic CD8$^+$ T cells that produce IFN-γ in ex vivo stimulations with the peptides derived from the VACV genes shown. Means and SEM of four (a and b) and three (c) mice are plotted; all data have been independently replicated.

performed with splenocytes from Dryvax immunized mice failed to identify novel determinants (unpublished data).

Conservation of immunogenic proteins and determinants across poxvirus strains and species

We searched databases for the presence of immunogenic ORFs in VACV strain modified vaccinia virus ankara (MVA); ECTV strains Naval and Moscow; CPXV strains Brighton red and GRI-90; and VARV strains Bangladesh, India, and VARV minor. Results for different strains from the same species (with the exception of VACV) were identical, so only data from a representative strain of each are included in Table II. All poxviruses have a range of broken, truncated, or otherwise incomplete ORFs, whose annota-

tions are inconsistent. Therefore, where immunogenic ORFs were not annotated (e.g. B8R in the original MVA sequence), we looked for conservation of the sequence encoding the determinants in the genome. In the case of B8R in MVA, the determinant is encoded upstream of the inactivating truncation of the ORF and a more recent sequence has included this ORF in the annotation. Because the promoter sequences are conserved, the determinant should be expressed. On the other hand, K3L function is not conserved in ECTV and while the determinant encoding sequence is present, there are sequence changes in the promoter region that could interfere with expression.

ID hierarchy is altered in MVA–immunized mice

MVA is a highly attenuated strain of VACV being developed as a smallpox vaccine replacement (26) and is a potential vector for recombinant vaccines (27). To determine if conservation of genes/determinants in published poxvirus genomes equates with conservation of CD8$^+$ T cells' immunogenicity and dominance hierarchy, we measured CD8$^+$ T cell responses to i.p. infection with MVA (Fig. 4 b). Though B8R$_{20-27}$ maintained its IDD status, responses to the SDDs were greatly altered relative to WR. Responses to all SDDs were significantly different in MVA compared with WR–infected mice (P < 0.0006 for A47L$_{138-146}$, A42R$_{88-96}$, and K3L$_{6-15}$ and P = 0.014 for A19L$_{47-55}$). A19L$_{47-55}$, usually the weakest determinant, was the second strongest after MVA immunization at ∼2% of all CD8$^+$ T cells, whereas responses to K3L$_{6-15}$ were lower and those to A47L$_{138-146}$ and A42R$_{88-96}$ were at background levels. The lack of response to the latter two determinants cannot be attributed to genetic variation in the determinants in the MVA strain we used as determined by DNA sequencing. The total VACV-specific response was slightly lower after MVA infection than WR infection, but higher than in Dryvax-immunized mice (unpublished data). We also screened the VACV ORF library with splenocytes from MVA-infected mice, but no new CD8$^+$ T cell determinant–bearing gene products were identified (unpublished data).

These data demonstrate that first, the presence of conserved determinants in conserved ORFs predicted to be expressed provides no guarantee of immunogenicity and, second, the ID hierarchy can be affected by genomic differences in poxviruses not predicted to effect the expression of determinants.

Responses to mapped determinants are found in mice immunized with attenuated VACVs

The striking difference in ID hierarchies between WR and MVA could stem from the attenuation of MVA, which, in contrast with WR/Dryvax, is capable of only marginal replication in mammalian cells in vitro and in vivo (27, 28). To examine the relationship between virus attenuation and ID hierarchy, we infected mice with a VACV that only expresses the essential viral structural gene A17L in the presence of IPTG (iA17L; reference 29) and, therefore, cannot

replicate in vivo. This virus, like many recombinant VACVs, also lacks thymidine kinase and so a thymidine kinase–deficient VACV (VACV-TK⁻; reference 30) is shown as a control for this experiment. VACV-TK⁻ is replication competent but less pathogenic than WR in vivo (31), and several thymidine kinase–deficient VACVs have been found to have similar ID hierarchies to WR (unpublished data). Despite being unable to replicate in vivo, iA17L generated an ID hierarchy highly similar to TK⁻ (Fig. 4 c) or WR (Figs. 2 a and 4, a and b). We conclude that attenuation per se does not lead to significant alterations in ID hierarchy to poxviruses and cannot account for the alterations between CD8⁺ T cell responses to VACV and MVA.

Identification of VACV–CD8⁺ T cell determinants by ECTV and CPXV

The protection afforded by the smallpox vaccine relies on cross-reactivity between VACV and VARV. To examine cross-reactivity of poxvirus-specific CD8⁺ T cell determinants in a mouse system, we extended our characterization of CD8⁺ T cell responses to include ECTV and CPXV (Fig. 5). $B8R_{20-27}$ maintained IDD status in responses to both viruses but generated weaker responses when compared with VACV, raising the possibility of other dominant determinants in these viruses. ECTV failed to induce reliable responses to $A42R_{88-96}$ or $K3L_{6-15}$. The latter was expected from genomic analysis (Table II). The anti-ECTV response to $A47L_{138-146}$ was of interest due to a predicted sequence change in the determinant in the second residue (A to T). Using a synthetic version of the altered peptide we confirmed its cross-reactivity with ECTV and VACV-specific CD8⁺ T cells (unpublished data).

For CPXV infection, $B8R_{20-27}$, $A47L_{138-146}$, and $K3L_{6-15}$ consistently generated responses in individuals, whereas $A19L_{47-55}$ responses were at the limits of detection. The difficulty to demonstrate $A42R_{88-96}$ immunogenicity in these

experiments may be a reflection of the subcutaneous route of infection, as responses to this determinant were also equivocal after VACV infection by dermal scarification.

Vaccination with the immunodominant VACV determinant leads to partial protection against mousepox

Finally, we examined the ability of CD8⁺ T cells primed by immunization with DC exposed to the IDD $B8R_{20-27}$, to protect against lethal ECTV challenge. In a first experiment, we found that $B8R_{20-27}$–pulsed DCs induced low but measurable CD8⁺ T cell responses (unpublished data) and protection from intranasal challenge with ECTV that was significant (P = 0.0279), but incomplete (Fig. 6 a). This protection was confirmed in a second experiment controlled with groups of mice immunized with TK⁻ VACV and DCs pulsed with an irrelevant peptide (P = 0.0196; Fig. 6 b). These data demonstrate that the IDD, $B8R_{20-27}$, not only cross-reacts amongst poxvirus species but is also cross-protective.

DISCUSSION

The large coding capacity of poxvirus genomes presents a challenge for determinant mapping. Consequently, it is tempting to use various criteria to winnow the number of
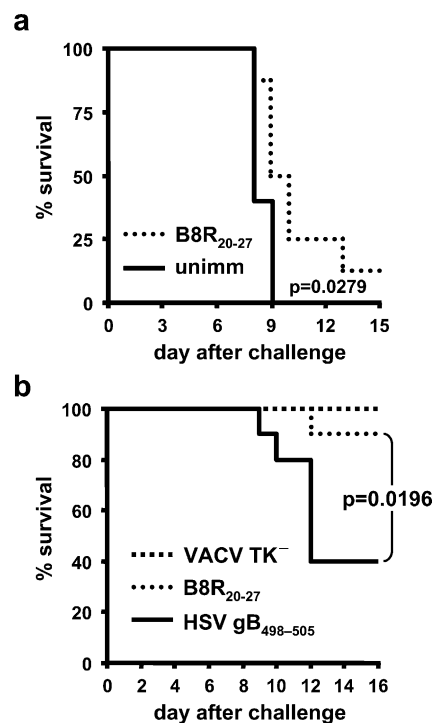
Figure 6. Immunization with peptides and mousepox challenge. (a) Unimmunized mice ($n = 5$) or mice immunized with DCs pulsed with $B8R_{20-27}$ 3 wk previously ($n = 8$) were challenged with 300 PFU ECTV intranasally and monitored for survival. (b) Groups of 10 mice immunized with VACV TK⁻ or DCs pulsed with $B8R_{20-27}$ or herpes simplex virus $gB_{498-505}$ were challenged as in panel a. In both experiments, splenic DCs were matured with LPS overnight before pulsing with peptides and 5×10^5 cells were administered i.v. per mouse. After challenge mice killed when moribund were counted as mortality events, p-values were generated by comparison of survival curves using the log-rank test.
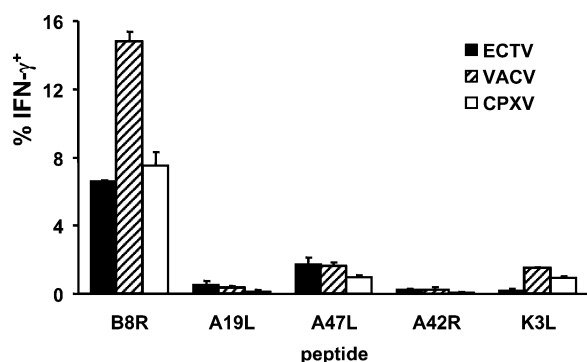
Figure 5. Responses to VACV CD8⁺ T cell determinants after ECTV and CPXV infection. Mice were immunized s.c. in the rear leg shank with 10^3 PFU of ECTV (black bar), VACV strain WR (diagonally striped bar), and CPXV (white bar) and CD8⁺ T cell responses to peptides were measured by ICS after 7 d (VACV and CPXV) and 8 d (ECTV). Data are percent of splenic CD8⁺ T cells that produce IFN-γ in ex vivo stimulations with the individual peptides shown and are means and SEM of groups of four mice. Experiment was repeated with similar results.

genes to be considered. Virus late genes are often excluded in determinant screens (6, 7). VACV-determinant mapping projects also use conservation between orthopoxviruses as a determinant selection criteria (6–8). Our results show that both criteria are seriously flawed. We discovered an immunogenic determinant in A42R, considered to be a late gene product (32). In addition, we provide several examples in which the immunogenicity of determinants in poxviruses cannot be predicted from the conservation of their sequence and integrity of their source ORFs according to sequences in the available databases. Furthermore, conserved determinants may easily be missed because fragments of known genes are not always included in annotations of poxvirus genome, as was the case for the $B8R_{20-27}$ determinant from B8R in the original MVA sequence. Finally, practical considerations aside, a complete understanding of cross-protection requires knowledge not only of the determinants two viruses have in common, but the ones that are unique to each infection.

The identification of the immunogenic determinants in poxviruses should greatly facilitate development of the mouse model for smallpox vaccination and also the utility of mouse poxvirus infections for understanding viral immunity. The latter point is underscored by our observation that the ID hierarchy varies with the route of infection, the first observation of its kind to our knowledge. It will be of great interest to determine the underlying mechanism. It could reflect route-dependent differences in the identity of antigen-presenting cells that activate $CD8^+$ T cells, the nature of antigen presented (e.g., direct vs. cross-priming), or the repertoire of $CD8^+$ T cells that interact with antigen-presenting cells in the locale where priming occurs. Understanding route-dependent effects on immunogenicity is critical to interpreting vaccine trials such as the recent paper comparing intramuscular immunization with MVA to standard scarification with Dryvax (33).

We found that the ID hierarchy amongst SDDs was greatly altered in mice immunized with MVA compared with WR. Furthermore, responses to two VACV determinants that were always detectable in mice infected by the i.p. route with WR were at background levels when MVA was used. Experiments with another highly attenuated VACV (iA17L) demonstrate that the results found with MVA cannot be attributed per se to decreased replication in vivo. Sequence database searches found these two determinants and their genes intact and expected to be expressed in two separate isolates of MVA, one of which is derived from the same source as our MVA stock. Furthermore, DNA sequencing of our virus stock revealed no alterations in the determinants. It is more likely that the altered ID hierarchy is related to one or more of the many genomic deletions and other changes in MVA compared with WR (34). It is possible that MVA encodes a novel IDD that suppresses other responses via immunodomination (23). Alternatively, genes encoding the cryptic determinants may not be expressed at immunogenic levels by MVA in vivo. Irrespective of the cause, the finding that responses were not predictable on the basis of

sequence information challenges assumptions about conservation of $CD8^+$ T cell responses between related viruses and underscores the need for empirical data. It also raises the important issue of the degree of overlap between cellular immune responses to Dryvax and potential smallpox vaccine replacements.

Using the VACV/ECTV model, we identify the first VACV-$CD8^+$ T cell determinant that may mediate cross-protection against a lethal systemic disease with a heterologous poxvirus. This extends prior studies with individual $CD8^+$ T cell determinants using VACV challenge models in HLA-A2 transgenic mice (6, 8) and shows the potential of $CD8^+$ T cells to mediate protection against poxvirus disease. Immunization with $B8R_{20-27}$ synthetic peptide provided incomplete protection in our challenge model, but we note that the number of $CD8^+$ T cells induced by peptide vaccination was at least 50-fold less than after VACV infection, and inducing higher $CD8^+$ T cell responses could well provide better protection. Furthermore, although our data show a potential basis for cross-protection between orthopoxviruses, more work is needed to understand the roles of $CD8^+$ T cells and other effector mechanisms in the context of current VACV-based vaccines.

MATERIALS AND METHODS

Viruses and cell lines. VACV strains WR, MVA, Vsc8 (30), and iA17L (29); ECTV strain Moscow; and CPXV strain Brighton were grown and titrated using standard methods. Dryvax smallpox vaccine was obtained from the Centers for Disease Control, Atlanta. Titred stocks of MVA and titration of Dryvax were provided by L. Wyatt (National Institute of Infectious Diseases [NIAID], Bethesda, MD). 293A cells were maintained in Dulbecco's Modified Eagle Medium with 10% FBS (D10). DC2.4 (35) and EL-4 were maintained in D10 or RPMI 1640 medium with 10% FBS (R10). All media was from Invitrogen. For use as stimulators in $CD8^+$ T cells assays, $1–5 \times 10^6$ DC2.4 were infected with VACV at 5–10 PFU/cell in <200 μl of PBS at 37°C for 30–60 min with occasional shaking. After this initial incubation, 9 ml R10 was added, and the incubation continued until a total time after infection of at least 4 h. Infected cells were spun and washed, and the appropriate number was added to T cell assays.

Synthetic peptides. Peptides were synthesized as described previously (36) by A & A Labs, at the John Curtin School of Medical Research Biomolecular Resources Facility (Australian National University, Canberra, Australia) or purchased as crude material from Mimotopes or Pepscan Systems B.V. Peptides were resuspended at 4–20 mg/ml in 100% DMSO and diluted to required concentrations in PBS, PBS with 0.05% NP-40, or RPMI 1640. Peptides for use as radiolabeled probes were purified to >95% homogeneity by reversed phase HPLC, and composition was ascertained by amino acid analysis, sequencing, and/or mass spectrometry analysis. Radiolabeling was done using the chloramine T method (37).

Enrichment of splenic DCs. DCs were enriched from C57BL/6 mouse spleens essentially as described previously (38). In brief, spleen fragments were digested for 20 min at room temperature with collagenase-DNase and treated for 5 min with EDTA. Low-density cells (including DCs) were selected by centrifugation in a Hepes-buffered balanced salt solution, pH 7.2, with 3% FCS and Nycodenz at a density of 1.077 g/cm^3 (Nycomed). The DC-enriched fraction was cultured overnight at 37°C with 5% CO_2 in DMEM (high glucose) with 10% FBS and 100 ng/ml LPS. The nonadherent population from this culture was collected. Enriched DCs were incubated with peptide at 1 μM for 2 h before washing and resuspension in PBS.

Mice, infections, and immunizations. Specific pathogen-free C57BL/6 mice were obtained from Taconic, Animal Resource Centre (Perth, Australia), and Animal Services Division, John Curtin School of Medical Research. Mice were housed, and experiments were done according to the relevant ethics requirements. In most experiments, mice were infected i.p. with 10^6–5×10^7 PFU of VACV, depending on virus strain. For dermal scarification, 10 scratches were made at the base of the tail in a crosshatch pattern using a 21 gauge needle and a drop of VACV at 10^7 PFU/ml applied for 1 min before the excess was removed with a tissue. For subcutaneous infections, 10^3 PFU ECTV and 10^6 PFU CPXV and VACV were injected in the shanks of mice. Except where stated, mice were euthanized 7 d after infection and spleens taken for analysis of CD8$^+$ T cell responses. For DC immunization, 2.5×10^5 peptide-coated, spleen-derived DCs suspended in PBS were injected intravenously. For ECTV challenge, DC-immunized or control mice were anesthetized and infected intranasally with 300 PFU of ECTV in 20 μl of PBS. Mice were monitored for signs of illness twice daily, and moribund animals euthanized.

It was noted that C57BL/6 mice available in Australia (from two sources) made very high VACV-specific responses (Figs. 2–5). Mice available in the USA had lower total VACV-specific responses in the spleen (20–25% of CD8$^+$ T cells; unpublished data), similar to other published studies (20, 39). The relative contribution of the mapped determinants to the total CD8$^+$ T cell response was very similar irrespective of country or source of mice.

Generation of VACV ORF library. Primary PCR products (and associated information) representing 266 predicted ORFs of VACV, made for a project investigating VACV protein–protein interactions by yeast 2-hybrid (40), were a gift from B. Moss (NIAID, Bethesda, MD). These were reamplified by PCR using Platinum Pfx (Invitrogen) and the primers 5′-CACCGAATTCCAGCTGACCACC-3′ and 5′-TATTCGGATCCCCGGGAATTGC-3′ and cloned into the eukaryotic expression vector pcDNA3.1D/V5-His-TOPO (Invitrogen) according to the manufacturer's instructions. Clones were screened by cleavage of miniprep DNA (Wizard SV 96; Promega) with BamHI (site underlined in the aforementioned primer sequence) and separation on 1.2% agarose gels. This initial library was edited, with 12 duplicated ORFs being removed and four ORFs (known to exist but missing) being added (A13L, A14.5L, A44L, and A48R); ORFs were PCR amplified directly from crude virus stocks and cloned as described before. The complete library comprised 258 unique clones each representing a single predicted VACV ORF. The 5′ end (∼600 bp) of each ORF clone was sequenced using ABI BigDye Terminators reagents and an ABI PRISM 3100 genetic analyzer (Applied Biosystems). Clones with early terminations or frame shifts were removed and remade. Transfection grade (low endotoxin) DNA for each clone was produced by Aldevron.

Generation of 293 cells expressing mouse MHC genes. The complete coding sequences for H-2K^b and D^b were amplified by PCR from recombinant VACV encoding these products and cloned into pcDNA3.1D/V5-His-TOPO as described before. The following primers were used: K^b, 5′-CACCATGGTACCGTGCACGCTG-3′ and TCACGCTAGAGAATGAGGGTC; and D^b, 5′-CACCATGGGGGCGATGGCTC-3′ and 5′-TCACGCTTTACAATCTCGGAG-3′. All clones were verified by sequencing and comparison to GenBank/EMBL/DDBJ accession nos.: K^b, U47328; and D^b, U47325. To make stable cell lines, plasmids were cleaved with SmaI and transfected into a single well of a six-well dish of 293A cells using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). After overnight incubation (37°C, 9% CO$_2$), cells were harvested and six serial 1:5 dilutions were made in a fresh six-well plate in medium supplemented with 0.5 mg/ml G418 (Biofluids). After several weeks of selection and growth in G418-containing medium, transfectants were cloned by limiting dilution (H-2 K^b) or FACS followed by single cell deposition (H-2 D^b, FACStar Plus; BD Biosciences). Potential H-2–expressing clones were selected by staining with mAbs (HB176 supernatant followed by rabbit anti–mouse Ig-FITC for K^b and direct FITC conjugates

for D^b; BD Biosciences) and analysis on a FACSCalibur (BD Biosciences). The clones selected were 293KbC2 and 293DbA5 and were maintained in 0.5 mg/ml G418. Expression of H-2 antigens was found to be stable for many passages in nonselecting medium.

Transfections in 96-well format and harvesting transfectants. 293KbC2 or 293DbA5 cells were seeded in flat-bottom 96-well plates between 5 and 8×10^4 cells/well the night before transfection. Diluted Lipofectamine 2000 (25 μl/well of 1:50 dilution) was arrayed in 96-well plates and mixed with plasmid DNA (25 μl at 0.01 μg/ml). These transfection mixes were left at room temperature for 30 min before being used to replace medium on the 293Kb/Db cells. After 2 h at 37°C with 5% CO$_2$, 100 μl of fresh D10 was added to each well and the plates returned to 37°C overnight. For T cell stimulations, transfected cells were harvested by washing wells with 50 μl PBS and incubating them with 20 μl trypsin for several minutes. Cells were collected with 100 μl D10 and moved to U-bottom 96-well plates. Plates were spun, medium and trypsin were removed, and transfectants were resuspended with suspensions of splenocytes or CD8$^+$ T cells to begin stimulations.

Stimulations and ICS. Splenocytes (0.2–1×10^6) or CD8$^+$ T cells (10^5, CD8a$^+$ T Cell Isolation Kit; Miltenyi Biotec) were incubated with the following: (a) transfected cells (see previous paragraph), (b) synthetic peptides, (c) DC2.4 pulsed with synthetic peptides at various concentrations, or (d) DC2.4 infected with VACV (see Viruses and cell lines) in the wells of a 96-well plate at 37°C and 5% CO$_2$. Synthetic peptides (if used) were added to a final concentration of 0.5 μM; cells (except those transfected in 96-well format) were used at 1–2×10^5 cells per well. 10 μg/ml brefeldin A was added after 1 h, and the incubation continued for another 3–4 h. Plates were spun, medium was removed, and cells were resuspended in 50 μl of α-CD8-PE (clone 53–6.7; BD Biosciences; some experiments used FITC or PE-Cy5) and incubated on ice for 20 min. Cells were washed, resuspended in 50 μl of 1% paraformaldehyde, and incubated at room temperature for 20 min before another two washes and staining with α-IFN-γ–allophycocyanin (clone XMG1.2; BD Biosciences; some experiments used FITC or PE) overnight in PBS with 0.5% saponin at 4°C. Cells were washed once before acquisition and analysis of fluorescence using a FACSCalibur (BD Biosciences). Analysis was done using Flowjo software (Tree Star Inc.); events were gated for live lymphocytes on FSC × SSC followed by CD8$^+$ cells using CD8 × SSC and displayed as CD8 × IFN-γ. Data was recorded as IFN-γ$^+$, CD8$^+$ cells as a percentage of total CD8$^+$ cells. Backgrounds as determined using irrelevant peptides, cells transfected with irrelevant constructs or uninfected cells were usually in the order of 0.1% and were subtracted from the values presented for test samples.

MHC binding assays. Quantitative assays to measure the binding of peptides to soluble class I molecules are based on the inhibition of binding of a radiolabeled standard peptide (37). In brief, 1–10 nM radiolabeled peptide was coincubated at room temperature with 1 μM to 1 nM of purified class I molecules (from EL-4) in the presence of 1 μM of human β2-microglobulin (Scripps Laboratories) and a cocktail of protease inhibitors. The radiolabeled peptides were used, and their average IC50s were RGYVFQGL, 3.1 nM and SGPSNTYPEI, 4.4 nM for K^b and D^b, respectively (41). After a 2-d incubation, binding of the radiolabeled peptide to the corresponding MHC class I molecule was determined by capturing MHC–peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-one) coated with the W6/32 antibody, and measuring bound cpm using the TopCount microscintillation counter (Packard Instrument Co.). In the case of competitive assays, the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide was calculated (IC50). Peptides were typically tested in three or more independent experiments. Under the conditions used, where [label] < [MHC] and IC50 ≥ [MHC], the measured IC50 values are reasonable approximations of the true Kd values (42).

Sequence comparison. ORF and determinant conservation between poxviruses was done with the aid of poxvirus orthologous clusters (43)

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