Published in final edited form as:
Vaccine. 2009 December 30; 27(Suppl 6): G21–G26. doi:10.1016/j.vaccine.2009.10.011.

Definition of epitopes and antigens recognized by vaccinia specific immune responses: Their conservation in variola virus sequences, and use as a model system to study complex pathogens

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Abstract

In the last few years, a wealth of information has become available relating to the targets of vaccinia virus (VACV)-specific $CD4^+$ T cell, $CD8^+$ T cell and antibody responses. Due to the large size of its genome, encoding more than 200 different proteins, VACV represents a useful model system to study immunity to complex pathogens. Our data demonstrate that both cellular and humoral responses target a large number of antigens and epitopes. This broad spectrum of targets is detected in both mice and humans. $CD4^+$ T cell responses target late and structural antigens, while $CD8^+$ T cells preferentially recognize early antigens. While both $CD4^+$ and $CD8^+$ T cell responses target different types of antigens, the antigens recognized by T_H cells are highly correlated with those recognized by antibody responses. We further show that protein abundance and antibody recognition can be used to predict antigens recognized by $CD4^+$ T cell responses, while early expression at the mRNA level predicts antigens targeted by $CD8^+$ T cells. Finally, we find that the vast majority of VACV epitopes are conserved in variola virus (VARV), thus suggesting that the epitopes defined herein also have relevance for the efficacy of VACV as a smallpox vaccine.

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Conflict of Interest: The authors state that they have no conflict of interest.

Keywords

Vaccinia; Smallpox; Epitope

1. Materials and methods

All methods utilized for the experiments described herein, including peptide synthesis, MHC binding assays, and immunization of HLA transgenic mice, have been detailed elsewhere [1].

2. Introduction

Over the last few years our group has been interested in defining the antigens and epitopes recognized following infection and/or vaccination with vaccinia virus (VACV). VACV has been utilized for vaccination against variola virus (VARV), the causative agent of smallpox, as part of the worldwide campaign leading to eradication of smallpox. Our studies were initiated on a relatively small scale in order to examine vector-specific responses to VACV-based recombinant vaccines. However, in the aftermath of 9–1, and in the context of the widespread concern that variola virus stocks still in existence could be utilized for bioterrorist attacks, these studies were substantially expanded and re-directed.

As a result, our studies have provided a model of immune responses to complex pathogens, and allowed us to pose fundamental questions related to the nature of immunodominance. Specifically, we were able to examine how immunodominance was regulated at the epitope and/or antigen level, and if and how, MHC class I, class II and antibody responses were interconnected. At the same time, our results allowed for a better definition of the immune responses elicited by VACV, thus facilitating the evaluation in animal models and humans of new, and potentially safer or more effective, vaccines. In this context, we were specifically able to analyze the implications of our results in terms of breadth of responses, and of projected crossreactivity between VACV and VARV.

3. Identification of epitopes restricted by common H-2 and Mamu MHC molecules

As a first step in our studies to map VACV-specific responses, we sought to identify epitopes recognized in various animal models. Accordingly, we focused our efforts on analyzing class I restricted responses in murine systems, where we studied the common C57BL/6 (H-2b) and Balb/c (H-2d) laboratory mouse strains, and the specific rhesus macaque class I allele, Mamu A*01. Several papers published in the last few years have summarized our results from these studies. These papers described epitopes recognized in H-2d mice and restricted by the Ld, Kd, Dd molecules [2,3] and in H-2b mice and restricted by the Kb and Db molecules [4,5]. More recently, epitopes recognized by murine class II (H-2 IAb) restricted responses [6] have also been described. Finally, a study detailing responses restricted by Mamu A*01 is currently in press [7].

The approach our group has taken is primarily based on the bioinformatic selection of candidate peptide sequences predicted to bind specific MHC alleles with high affinity, followed by experimental testing and validation. In general, a relatively large number of potential epitopes is predicted and synthesized. These potential epitopes are tested in pools, and specific IFNγ producing T cells are enumerated by ELISPOT and/or ICS assays. Positive pools are subsequently deconvoluted to identify the individual epitopes recognized.

This general approach, combining both bioinformatic and experimental techniques, was rigorously validated in a study by Moutaftsi et al. [5]. It was previously known that at the time of peak responses in C57BL/6 mice infected with VACV, about one-third of the splenic $CD8^+$ T cells were specific for VACV. This was demonstrated by purifying splenocytes from infected mice and assessing the fraction of $CD8^+$ T cells that specifically recognized *in vitro* VACV infected target cells. Using the bioinformatic approach described above, we have defined a set of 49 different VACV-derived $CD8^+$ T cell epitopes. These epitopes were pooled and utilized as an *in vitro* antigen to similarly stimulate the splenocytes from infected mice. We found that the number of $CD8^+$ T cells recognizing these pooled epitopes was nearly identical to the total VACV response, as defined by the total number of $CD8^+$ T cells recognizing target cells infected *in vitro* with whole VACV. More specifically, we estimated that about 95% of the VACV-specific $CD8^+$ T cells could be attributed to the 49 epitopes defined by our studies. These results demonstrate that the approach we developed and utilized can identify the epitopes recognized by a large majority of the VACV-specific $CD8^+$ T cells.

4. Identification of HLA-restricted VACV-derived epitopes

In a related but independent series of studies, we targeted the identification of HLA-A2-, A3-, and B7-restricted epitopes utilizing transgenic mice [1], as well as the epitopes recognized by recent vaccinees in the context of six major HLA class I supertypes [8] and HLADR[9]. In general, these studies demonstrated that a very broad repertoire of epitopes is recognized by human vaccinees, and in a diverse pattern of HLA contexts. For example, about 30 different VACV epitopes were restricted by HLA A*0201. However, when a panel of A*0201 positive individuals was studied in detail, it was found that no particular individual recognized all epitopes, and no particular epitope was recognized in all individuals. The relevance of this finding for vaccine efficacy and potential bioterrorism concerns is discussed in more detail below.

In conclusion, the studies alluded to in this and the preceding section led to the identification of nearly 250 different VACV epitopes (Table 1). Additional epitopes have been identified by other scientists, thus resulting in a total of over 300 VACV epitopes being now available to the scientific community (a complete listing can be obtained at the IEDB website; http://www.immuneepitope.org).

5. How is immunodominance regulated?

In the next series of experiments, we investigated how immunodominance may be regulated in VACV infection. To tackle this sizeable topic, we had to address several separate sub-questions. We first asked, within a given ORF known to be targeted by $CD8^+$ T cell responses, what determines immunodominance? A number of studies and reviews have highlighted how different variables are all likely to contribute to the final outcome of immunodominance [10]. These include MHC binding affinity, efficiency of cellular processing capable of generating the relevant MHC binding peptides, availability of TCRs capable of recognizing complexes between the processed MHC binders and MHC, as well as somewhat ill-defined immunoregulatory mechanisms. However, the relative quantitative role of these variables in shaping the immunodominance pattern in the case of VACV in particular, and complex antigens in general, was not known.

We addressed this question with an in-depth analysis of 18 different ORFs recognized in the HLA A*0201 system [11]. Of a total of 10,660 possible 9-mer and 10-mer peptides derived from these 18 ORFs, 263 (2.5%) bound purified A*0201. Hence, MHC binding restricts the choices available to the immune system by about 40-fold.

Next, the effect of TCR repertoire was examined. All 15 known epitopes recognized in A*0201 transgenic mice following VACV infection, and a sample of 91 additional A*0201 binding peptides, were tested by direct peptide immunization. It was found that about 60% of these peptides were immunogenic. Hence, availability of a suitable TCR repertoire restricts the choices available to the immune system by a factor of 2-fold.

To evaluate the impact of cellular processing, all peptides recognized in A*0201 transgenic mice following peptide immunization were considered. Epitope specific T cells were tested for their ability to recognize VACV-infected cells to assess whether the peptide was generated in the course of natural infection. Of the 141 epitopes examined, it was found that approximately 14% were generated by natural processing. Hence, limitations in peptide-processing restrict the choices available to the immune system by a factor of seven.

In conclusion, within ORFs targeted by $CD8^+$ T cell responses, MHC binding restricts the potential choices for immunodominant epitopes by a factor of 40, processing by a factor of 7, and TCR repertoire by a factor of 2 (Table 2). Obviously, other variables such as immunoregulation or immunodomination can also influence the final outcome in terms of immunodominance.

6. Is immunodominance also regulated at the ORF level?

The experiments described above do not evaluate which variable(s) dictate which ORFs will be recognized by immune responses, and which ones will not. We performed further experiments to investigate this subject. We hypothesized that expression levels, kinetics, and cellular localization of the ORF/antigen of origin might, for example, play a role in determining which antigens (as opposed to which epitopes) would be immunodominant. If this were to be the case, we would expect certain antigens to be repeatedly recognized, in the context of different MHC loci and alleles, and even perhaps in different species. We propose to define such antigens as *immunoprevalent* antigens.

The studies, presented by Oseroff et al. [3], demonstrate that immunoprevalent antigens do indeed exist. Compilation of available data revealed that certain antigens are repeatedly recognized by different MHC alleles, across different loci and even in different species, more often than expected in a random distribution ($p < 0.01$). For example, the D1R antigen is recognized in the context of H-2 Kd, Kb, Db, HLA-A1, A3, B7 and B44. Interestingly, the major immunodominant antigens or epitopes, constituting the major targets of the immune response by a specific haplotype, are distinct from the immunoprevalent ones. This implies that different mechanisms govern the two phenomena, although the reason(s) for the dissociation between immunodominance and immunoprevalence is unclear. Indeed, these sets of proteins cannot be distinguished on the basis of biological function or time of expression. While it is likely that protein length is an important factor in determining immunoprevalence, it has also been noted that several relatively small proteins also appear to be immunoprevalent.

Evidence regarding the possibility that immunodominance, like immunoprevalence, is also regulated at the ORF level was provided by the comparison of the type of antigens recognized from $CD4^+$ versus $CD8^+$ T cell responses. In a study by Moutaftsi et al., it was found that, in general, these two different classes of epitopes are derived from antigens with different characteristics [6]. $CD4^+$ T cells tend to recognize structural antigens, produced late in the course of infection. By contrast, $CD8^+$ T cells mostly recognize early antigens, and show a bias in terms of the function and/or structure class of antigens recognized.

7. Extensive correlation between CD4+ T cell and antibody response specificities

At about the same time the above studies were being conducted, the technique of protein microarrays [12,13] began to reveal the exact pattern of VACV antigens recognized by antibody responses. Most relevant to the topic discussed herein, we found that there was a highly significant association between the antigens recognized by antibody responses and the antigens from which the epitopes recognized by $CD4^+$ T cells were derived [14]. $CD4^+$ T cells more frequently recognized antigens prominently recognized by antibody responses and similarly antigens recognized by $CD4^+$ T cells were also more frequently recognized by antibody responses. These results were further developed to demonstrate a deterministic linkage between helper T cell and antibody antigens, the molecular mechanism of which is currently under investigation [14].

Regardless of mechanism, we could demonstrate that preselection of antigens on the basis of their recognition by antibody responses was an effective method to predict which antigens might also be targeted by $CD4^+$ T cells, and thereby predicting immunodominance at the antigen level for $CD4^+$ T cell responses. In conclusion, the available data suggest that immunodominance is partially regulated at the antigen level, and that while antibody and $CD4^+$ T cells tend to recognize similar antigens, $CD8^+$ T cells recognize a different set of antigens. These data suggest that immunodominance at the antigen level is differentially regulated for $CD4^+$ and $CD8^+$ T cell responses.

8. Structural correlates of immunodominance

In the next series of experiments we sought to define structural correlates of immunodominance. First, the impact of mRNA expression levels was investigated. To accomplish this we utilized the technique of tiling arrays. This technique is fundamentally similar to conventional expression arrays, but allows for a much higher resolution, an unbiased analysis and other desirable features such as definition of complete transcriptional maps, the determination of the transcriptional onset and termination, and the identification of novel transcripts. Utilizing this technique we were able to derive a complete map of the VACV transcriptome, and to identify four broad classes of mRNA defined on the basis of expression kinetics (immediate early, early, early/late and late). A detailed account of the results has been presented elsewhere [15].

Interestingly, it was found that ranking $CD8^+$ T cell antigens in terms of immunoprevalence was well correlated with the ranking of mRNA expression at 4 h post-infection. Furthermore, we found that the mRNA expression level could be used to predict immunoprevalent $CD8^+$ T cell antigens. Specifically, about 90% of all immunoprevalent antigens were ranked in the top 50% in terms of mRNA expression (Fig. 1). In contrast, the expression ranks of non-immunoprevalent antigens, and antigens not recognized at all by $CD8^+$ T cell responses, were not significantly different from random. $CD4^+$ T cell and antibody responses were also not correlated with the 4 h mRNA levels.

Next, we examined the correlation between the various types of responses observed and protein abundance in the virion, as measured by mass spectrometry [16–18] and mRNA abundance at late time points after infection. We found that $CD4^+$ T cell responses, unlike $CD8^+$ T cell responses, preferentially targeted antigens with late mRNA expression and high abundance in the virion. Furthermore, we also found that antibody responses correlate with late expression and virion abundance. Thus, in conclusion, the existence of immunoprevalent antigens suggests that immunodominance is (also) regulated at the ORF level. $CD8^+$ and $CD4^+$ T cell responses tend to focus on different set of antigens. In contrast, there is extensive overlap between

antibody and $CD4^+$ T cell protein targets. Immunogenicity at the ORF level for $CD8^+$ T cell responses correlates with mRNA expression levels early after infection, while late expression and protein abundance predicts antigens targeted by antibody responses and $CD4^+$ T cell responses.

9. Conservation of the VACV epitopes in VARV

As part of our goal to determine the crossreactivity of identified human CTL and HTL VACV epitopes with the homologous VARV sequences, we focused our effort on VARV strain Bangladesh. For our analysis we considered a total of 109 HLA-restricted VACV epitopes identified by both Koelle's and our group. Epitopes identified in HLA transgenic mice were also included in these experiments. As a starting point we identified VARV orthologs of the VACV proteins from which the epitopes were derived, based on homology with the epitope-containing protein (i.e. BLAST *E*-value < 0.001). For 76 of the epitopes (69.7%), the corresponding VARV-Bangladesh peptides were 100% homologous with VACV. The total sequence conservation, and the fact that the sequences are derived from a protein ortholog, suggests that a large fraction of these epitopes would also be recognized following VARV infection, even though exceptions have been reported [4].

For an additional 14 (12.8%) epitopes, the homology was very high, as the sequences varied by only one amino acid. Of the remaining epitopes, 4 (3.7%) varied from the VARV sequence by 2 amino acids, 5 (4.6%) varied by 3 amino acids and 10 (9.2%) varied by 4 amino acids. Where corresponding HLA binding assays were available, variant variola peptides were synthesized and tested for *in vitro* binding to the appropriate restricting MHC molecules (Table 3). It was found that in 10 of 29 (34.5%) cases where the restricting HLA allele was defined, the homologous VARV sequences still bound the relevant MHC molecule with an affinity within 5-fold of the corresponding VACV peptide. This suggested that T cell responses against the VACV epitope might still be cross-reactive with the VARV variants.

10. Occasional crossreactivity of homologous VACV and VARV sequences

As shown above, a large majority of the VACV epitopes are found totally conserved in VARV, thus potentially mediating/contributing to the crossreactive immunity afforded against the heterologous VARV by VACV vaccination. To test whether crossreactivity between non-identical, but homologous, sequences could also contribute to heterologous immunity, the crossreactivity of HLA-A1-, A11-, A2-, and B7-supertype epitopes with the corresponding VARV homolog was directly examined in the appropriate HLA transgenic mice. For these experiments, mice were immunized with 2×10^6 VACV-WR. Responses were determined by enumerating $CD8^+$ T cells in *ex vivo* IFNγ ELISPOT assays for crossreactivity with the appropriate VARV epitopes (Table 4). No crossreactivity was detected for two A1 restricted epitopes. In the case of A11 restricted epitopes, significant crossreactivity was noted for 1 of the 5 epitopes. Finally, in the case of the A2- and B7-restricted epitopes, crossreactivity was seen for 1 of 1, and 1 of 2 of epitopes tested, respectively. Thus, overall crossreactivity was seen for 3 of the 10 epitopes tested.

In conclusion, the available data suggests that most of the epitopes defined in VACV, and representing the bulk of the reactivity induced by VACV vaccination, are also conserved in VARV. In addition, some epitopes that are different, but homologous, between the two viruses can also be crossreactive. This suggests that the protection induced by VACV vaccination will be broadly crossreactive with VARV, thus affording a large breadth of anti-VARV responses, comparable to those observed against VACV itself. Of course the ideal experiment would be to measure crossreactivity upon infection *in vivo*. However, this is clearly not possible to do with VARV. To work around this obstacle, we have recently begun to examine the crossreactivity between VACV and ectromelia virus (ECTV) $CD8^+$ T cells. Surprisingly, we

find that splenocytes from VACV-immunized mice are relatively poor (responses are ~25%) at recognizing ECTV-infected cells when compared to splenocytes from ECTV-infected mice.

11. Implications of epitope breadth and conservation for VACV as a smallpox vaccine and as a viral vector

In conclusion, the available data demonstrates that antibody, and both $CD4^+$ and $CD8^+$ T cell, responses elicited by VACV are extremely broad and target a wide range of antigens. This observation is of great significance, as it implies that the generation, either by natural processes, or by deliberate engineering, of poxviruses lacking those antigens and epitopes would likely be impossible. In other words, generation of a stealth virus capable of evading the immune response elicited by vaccination is extremely unlikely. Indeed, it is apparent that immune evasion by epitope mutation is a strategy utilized by natural viral evolution for RNA viruses with genomes of limited dimensions, but not feasible for large viruses such as pox or herpes viruses, which are both DNA viruses with high fidelity polymerases encoding hundreds of different viral products.

The high degree of conservation of the epitopes and antigens between VACV and VARV, suggest that measuring responses against the various epitopes defined in the literature could be utilized to evaluate immune responses elicited by new and improved vaccines. Finally, these same epitopes can also be utilized to monitor responses against VACV itself, either as a viral vector, or in basic studies investigating immune responses against complex pathogens.

Acknowledgments

The authors thank Maya Kotturi, Josie Babin, Matthew Maybeno, Lori Giancola, and Jean Glenn for technical support. This research was supported by NIH contract HHSN266200400024C.

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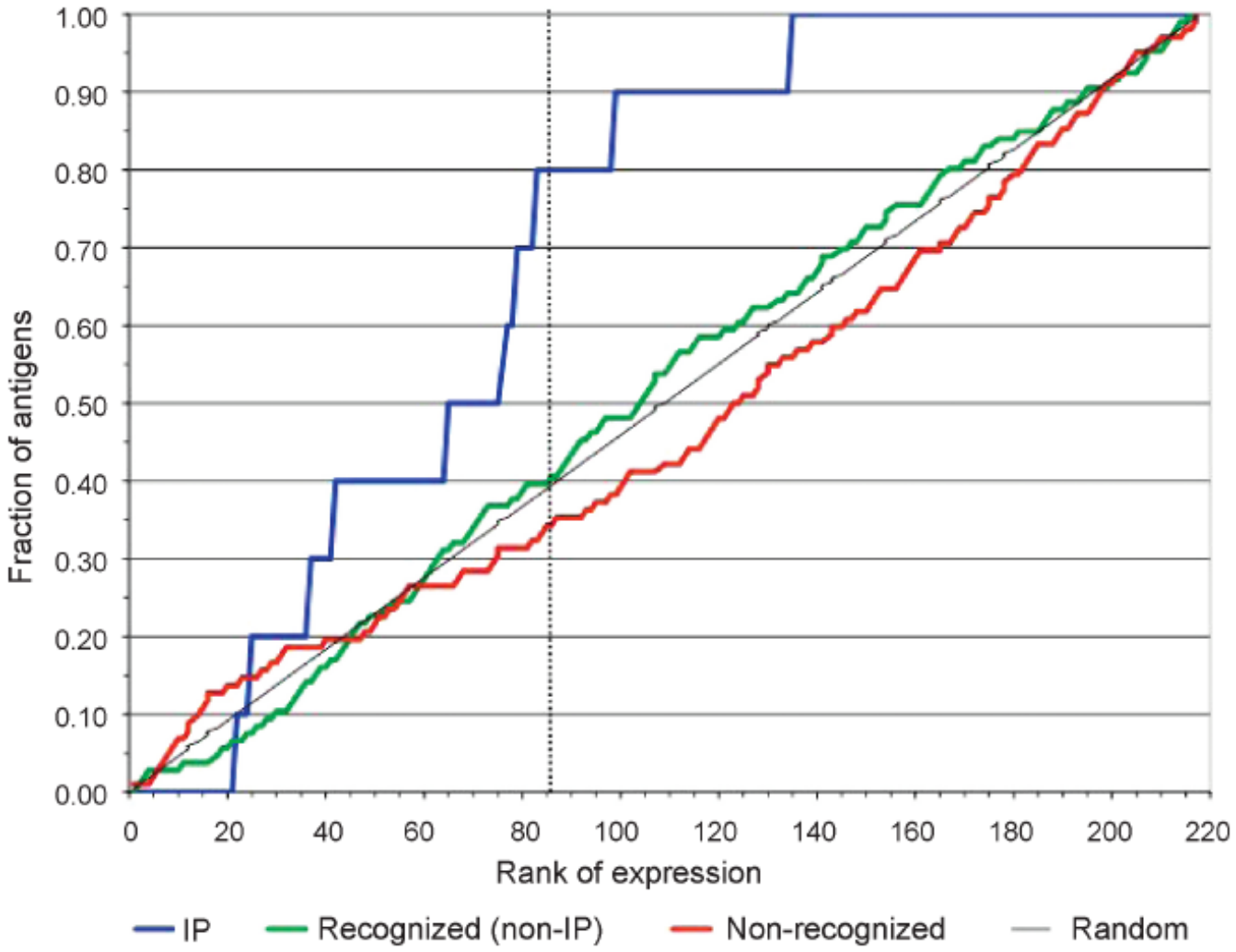

Fig. 1.

mRNA expression predicts immunoprevalence for CD8 responses. The fraction of antigens in each class, to include immunoprevalent (IP) antigens, antigens recognized by CD8 responses but that are not immunoprevalent, and non-recognized antigens, is plotted as a function of the rank of antigen mRNA expression.

Table 1

Summary of epitopes identified in studies from our group.

MHC supertype	# of epitopes
A1	10
A2*	28
A3*	15
A24	3
B7*	8
B44*	4
Kb	29
Db	22
Kd	39
Dd	3
Ld	12
Mamu	17
DR*/DP/DQ	53
IAb	18

The number of epitopes identified per HLA supertype is shown. For specificities highlighted by an asterisk, the total includes epitopes identified in HLA transgenic mice.

Table 2

Summary of factors that contribute to antigen selection.

Antigen selection	
Cellular processing	1 of 7
MHC binding	1 of 40
T cell receptor recognition/immunogenicity	1 of 2
Other (immunoregulation)	1 of 10

Several specific processes involved in the determination of the final specificity of T cell responses during an immune response are listed in the left column. The frequency of which antigen-derived peptides successfully progress through each respective process is shown in the right column. One out of seven peptides are naturally processed in VACV-infected DCs. Of these, 1 of 40 nonamer and decamer peptides bind to the restricting MHC with high affinity. Of the high affinity binders, one-half can elicit $CD8^+$ T cell responses upon peptide immunization. Additional factors, including immunoregulatory mechanisms, reduce the number of VACV epitopes by at least a factor of 10.

Table 3

Comparison of homologous vaccinia and variola epitopes.

Vaccinia epitope	VACWR name	Restriction	Reference(s)	Binding (IC50 nM)	Variola peptide	Variant residues	Binding (IC50 nM)	Fold affinity reduction
FGDSKEPVPY	VACWR190	A*0101	Oseroff et al. [8]	134	FGDSEEPVTY	2	880	6.6
		A*2902	Oseroff et al. [8]	564			37945	>65
FTIDFKLKY	VACWR106	A*2601	Oseroff et al. [8]	21	SNIDFKIKK	4	-	>100
		A*2902	Oseroff et al. [8]	1.4			-	>100
QSDTVFDYY	VACWR209	A*0101	Oseroff et al. [8]	0.46	FNNTKFDYY	4	-	>100
		A*3002	Oseroff et al. [8]	322			1475	4.6
VTDTNKFDNY	VACWR013	A*0101	Oseroff et al. [8]	0.97	VTDTNKFAHY	2	3.4	3.5
		A*3002	Oseroff et al. [8]	256			545	2.1
VYINHPFMY	VACWR205	A*2902	Jing (2005) [19]	0.68	VYINHPFIY	1	6.5	9.6
NLWNGIVPT	VACWR149	A*0201	Snyder (2004) [20]	12	NLWNGIVPM	1	20	1.6
LLYAHINAL	VACWR173	A*0201	Terajima (2006) [22]	8.3	LLYTHINAL	1	41	5.0
MMLVPLITV	VACWR159	A*0201	Oseroff et al. [8]; Assarsson [11]	0.12	MILVPLITV	1	29	>100
AVFKDSFLRK	VACWR195	A*1101	Oseroff et al. [8]	6.1	AVFKNSFLGK	2	1.5	0.24
NQVKFYFNK	VACWR098	A*0301	Oseroff et al. [8]	2.2	RQGKFIKNK	4	67	31
VTSSGAIYK	VACWR154	A*1101	Oseroff et al. [8]	3.1	VTSSGVIYK	1	3.9	1.2
ATSLDVINY	VACWR019	A*1101	Oseroff et al. [8]	6.8	YTSLDVYGS	4	231	34
				6.8	KKGGDVINY	4	1274	>100
RYYDGNIYEL	VACWR022	A*2407b	Oseroff et al. [8]	9.6	RYYDGNIYDL	1	121	13
VWINNSWKF	VACWR110	A*2402	Oseroff et al. [8]	6.4	VDINRNNKF	4	-	>100
		A*2301	Oseroff (2005) [21]	5.3			2262	>100
KPKPAVRFAI	VACWR027	B*0702	Oseroff et al. [8]	0.18	KPKPAVRYAI	1	15	83
KPFNNILNL	VACWR094	B*0702	Pasquetto [1]	202	KPFNNILDL	1	362	1.8
HPRHYATVM	VACWR106	B*0702	Oseroff et al. [8]	0.87	HPRHYATIM	1	12	14
APNPNRFVI	VACWR043	B*0702	Oseroff et al. [8]	1.4	AKNPNRFVI	1	291	>100
WLKIKRDYL	VACWR176	B*0801	Jing (2005) [19]	0.77	VLSYKVDYL	4	445	>100
GESKSYCEL	VACWR025	B*4001	Oseroff et al. [8]	27	GEYKSYCKL	2	49	1.8
EEIPDFAFY	VACWR110	B*4403	Jing (2005) [19]	0.25	SELPDFACS	4	3529	>100
YEFRKVKSY	VACWR122	B*4403	Jing (2005) [19]	6.9	YEFRRVKSY	1	14	2.1

Vaccinia epitope	VACWR name	Restriction	Reference(s)	Binding (IC50 nM)	Variola peptide	Variant residues	Binding (IC50 nM)	Fold affinity reduction
LENGAIRIY	VACWR110	B*4403	Jing (2005) [19]	3.3	LENDAIRIY	1	20	6.1

The homology between vaccinia virus derived epitopes identified from our studies was compared with homologous sequences derived from variola virus. For each epitope the HLA restriction, source information, *in vitro* binding affinity, and sequence of the homolog are shown. The degree of homology between the vaccinia epitope and its variola homolog is represent as the number of variant amino acids. Conservation of binding capacity is indicated by fold reduction in binding affinity seen with the homolog compared to the corresponding vaccinia epitope. (a) A dash indicates IC50 > 10,000 nM. (b) Binding affinity shown is for A*2402.

Table 4

Crossreactivity of VACV epitopes with homologous VARV sequences.

Tg strain	VACV	VARV	AA variation	Copenhagen	IFNg (SFC × 10^6)	
					VACV	VARV
A1	QSDTVFDYY	FNNTKFDYY	4	C10L	**495.0**	0.0
A1	FTIDFKLKY	SNIDFKIKK	4	D1R	**21.7**	0.0
A11	NQVKFYFNK	RQGKFIKNK	4	J6R	**24.4**	0.0
A11	AVFKDSFLRK	AVFKNSFLGK	2	B13R	**43.3**	13.3
A11	VTSSGAIYK	VTSSGVIYK	1	A31R	**46.1**	0.0
A11	ATSLDVINY	YTSLDVYGS	4	C9L	**45.0**	**25.0**
A11	ATSLDVINY	KKGGDVINY	4	C9L	**45.0**	8.9
A2	MMLVPLITV	MILVPLITV	1	A36R	**20.6**	**25.6**
B7	HPRHYATVM	HPRHYATIM	1	D1R	**179.4**	**109.4**
B7	APNPNRFVI	AKNPNRFVI	1	F4L	**50.6**	8.3

Potential crossreactivity between vaccinia epitopes and their variola-derived homologs was assessed by IFNγ ELISPOT assays. The values shown in the “VACV” (vaccinia) and “VARV” (variola) columns represent average IFNγ *ex vivo* ELISPOT responses from 2 × 10^6 cells stimulated with the shown peptide. Bolded values represent responses that are considered positive by our in-house standard criteria ($p < 0.05$; SI > 2).