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Uncovering the interplay between CD8, CD4 and antibody responses to complex pathogens

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Abstract

Vaccinia virus (VACV) was used as the vaccine strain to eradicate smallpox. VACV is still administered to healthcare workers or researchers who are at risk of contracting the virus, and to military personnel. Thus, VACV represents a weapon against outbreaks, both natural (e.g., monkeypox) or man-made (bioterror). This virus is also used as a vector for experimental vaccine development (cancer/infectious disease). As a prototypic poxvirus, VACV is a model system for studying host–pathogen interactions. Until recently, little was known about the targets of host immune responses, which was likely owing to VACVs large genome (>200 open reading frames). However, the last few years have witnessed an explosion of data, and VACV has quickly become a useful model to study adaptive immune responses. This review summarizes and highlights key findings based on identification of VACV antigens targeted by the immune system (CD4, CD8 and antibodies) and the complex interplay between responses.

Keywords

adaptive immunity; epitopes; immunodominant; protection; vaccinia virus

Structure & taxonomy of poxviruses, & their relevance to human health

Amongst the viruses of relevance to human health, members of the poxvirus family have some of the largest viral genomes (ranging from 130 to 300 kb), with as many as 260 open reading frames (ORFs). All poxviruses replicate exclusively in the cytoplasm of their hosts and have an enveloped viral particle that carries the single, linear dsDNA genome. In general, the genes located in the center of the genome are relatively conserved among poxviruses and have essential molecular functions for replication and survival. By contrast, terminally located genes are more variable and encode proteins that interfere with the host response to infection (virulence factors) and determine host-range restriction [1].

The poxviruses that infect vertebrate hosts comprise eight genera and, of these, the orthopox genus has the best-known members and also those most relevant to human health. The most prominent is variola virus (VARV), the causative agent of smallpox, and vaccinia virus (VACV), the vaccine used to prevent and eradicate this once dreaded disease. With smallpox conquered in the 1980s, attention was focused on the use of recombinant VACV as a vector for protein expression in the study of cancer and infectious disease vaccines [2]. A variety of strains of VACV have been used for these studies. The high rates of severe adverse events associated with the traditional smallpox vaccine led to the development of attenuated strains, exemplified by modified vaccinia virus Ankara (MVA). MVA was generated by extensive passaging of VACV Ankara on chicken embryo fibroblasts, resulting

in the loss of several genes compared with the parental genome and loss of the ability to replicate in most mammalian cells, including primary human cells [3]. By contrast to the extreme attenuation of MVA, the most commonly used VACV strain for laboratory experiments, namely Western Reserve (WR), is amongst the most virulent of strains, having been selected by serial passage in suckling mouse brain. Smallpox vaccine strains such as Dryvax, ACAM2000 (a clone from Dryvax) and Lister are less pathogenic than WR in animal models [4,5], but remain fully replicating viruses and are associated with unacceptably high rates of adverse events in vaccines [6,7].

In the past few years, the threat of accidental or intentional release of VARV, as well as concerns about the emergence of zoonotic poxviruses such as monkeypox, which can also cause fatal disease in humans, has intensified interest in studying VACV. In this context, VACV serves not only as a vaccine, but as a surrogate model of virulent poxvirus infection in humans owing to the high sequence similarity with VARV (~90% identical) [8]. VACV expresses approximately 200 genes that have classically been divided into early (0–1.5 h), intermediate (1–3 h), early/late and late (>3 h) classes, but recent data suggests that early genes can be subdivided into two groups, the earliest being called immediate-early [1,9]. In general, VACV transcription proceeds in three phases [1]:

- The immediate-early- and early-expressed proteins include enzymes and factors needed for DNA replication, intermediate gene expression and host defense modulators (virulence factors);
- In the intermediate phase mainly *trans*-activating factors for late gene transcription are expressed;
- The late genes encode for major membrane and core proteins that are contained within the viral particle (virion).

Immunization with VACV confers protection against smallpox owing to a multispecific humoral and cellular immune response. Both antibody and T-cell immunity remain detectable for decades following VACV immunization [10–15]. The general consensus is that cellular and humoral immunity are both key for poxvirus clearance [16,17], while humoral immunity is key in conferring protection from reinfection.

Genome tiling array analysis of VACV ORF expression during infection

Vaccinia virus gene expression at both RNA and protein levels has been intensely investigated [1]. Methods commonly utilized for transcriptional analyses of VACV genes include northern blot [18], 5′-end extension analysis [19] and RNA protection assays [20]. There are several advantages of these methods – in particular, their high sensitivity of detection. On the other hand, these methods tend to be very labor-intensive and therefore impractical for measuring large sets of genes.

Until recently, experimental results on expression kinetics were available for only about two thirds of the annotated ORFs in the VACV WR genome. A study based on canonical promoter motifs, performed by Lefkowitz and colleagues, predicted the kinetic expression for as many as 46 additional ORFs [201]. Predictions were not possible for another 23 ORFs lacking prototypic promoter sequences. Despite this wealth of data in the literature, the experimental settings and methodologies used were highly diverse, making comparisons between the studies difficult. For example, some ORFs, such as *I3L* [21,22], *D5R* [23,24] and *A36R* [25,26], have been denoted as early/late because different studies have suggested different temporal ORF expression.

More recently, the availability of microarray technology has offered an opportunity to experimentally address, in a comprehensive and systematic fashion, the levels and kinetics of VACV ORF transcription [9]. This analysis yielded, for the first time, a complete map of VACV gene expression, including experimental expression data for 69 ORFs that had not been previously characterized. To group expression profiles as a function of time postinfection in an unbiased manner, a hierarchical clustering analysis based on relative expression levels was performed. It was found that segregation into four clusters provided the most reproducible grouping of genes. Most strikingly, we found a clear division of early gene expression into two previously unreported discrete clusters, which we denoted as immediate-early and early genes. More than half of these genes were of unknown function. The largest fraction of the immediate-early genes with known functions was associated with immune evasion/virulence. The early class was the largest by number, containing 73 genes. A late class of 60 genes was also clearly identified. Finally, an early/late class of 26 genes exhibited onset of transcription typical of early genes, but with sustained expression at late times, similar to the late genes (Figure 1A).

Proteomic analysis of VACV antigens

At the protein level, immunohistochemistry [27] and western blot [28] approaches have been used to analyze the expression of many VACV-derived proteins. Alternatively, when specific antibodies are unavailable, viruses containing genes linked with a protein tag (e.g., GFP) can be generated, which makes protein detection possible [26,29,30]. These approaches are highly specific and sensitive, and immunohistochemical analysis can, in addition, provide information on the cellular location of the proteins studied. However, neither a panel of antibodies specific for all VACV ORFs, nor and a systematic comparison of all ORFs at the protein level, is currently available.

Several independent studies examined the composition of the VACV virion by using mass spectrometry analysis, resulting in the identification of a total of 93 VACV proteins [31–33]. The majority of the proteins were membrane proteins, structural/core proteins and proteins involved in regulating transcription. Interestingly, several host proteins associated with vaccinia virus intracellular mature virion particles (C22L, E3L and N1L) were also identified. A subset of 51 proteins was identified by all three studies. In terms of relative abundance within the virion [31,32], the majority of the most abundant proteins are core proteins (F17R, A4L, A3L, A10L, A27L, A14L, D8L, H3L and A13L) and only one is a transcription protein (L4R).

With these data one can examine the correlation between protein abundance in the virion and mRNA expression levels. Specifically, we compared the average relative abundance, as described by Chung *et al.* [31] and Resch *et al.* [32], with average mRNA expression levels for each ORF identified in the microarray analysis [9]. Of the 21 (top 10% of all ORFs) most abundant proteins in the virion (Supplementary Table 1, see online www.futuremedicine.com/ toc/fmb/5/2), five viral proteins (H5R, A10L, A13L, A17L and F17R) were also found within the top 21 (10%) expressed ORFs mRNAs at the 4 or 24 h time point.

These results emphasize how the highest-ranking ORFs, in terms of mRNA levels, do not necessarily correspond to the highest-ranking proteins in terms of expression within virions. Several investigators have previously reported a similar lack of correlation [34], and attributed this result to differences in RNA transcription rates and protein stability. This finding is of relevance in the analysis of the antigen targets of CD8, CD4 and antibody responses, described in the present review, as discussed in more detail in the following sections.

Inventory of the epitopes recognized by adaptive immune responses to VACV antigens

The past few years have witnessed a quantum leap in our understanding of the molecular targets of adaptive immune responses against poxviruses. More than 40 papers defining the targets of immune responses in mice and humans have been published in the last 5 years, and additional papers are forthcoming [35–76]. A broad picture has started to emerge that defines which protein antigens are targeted by $CD8^+$ and $CD4^+$ T cells and antibody (Ab) responses and their interconnection with mRNA and protein expression, as described in more detail in the following sections [9,31–33,77]. For $CD8^+$ T cells, in most cases, the minimal peptide that is immunogenic and the restriction element have been defined (Supplementary table 2A) [41,46,47,50,52–54,58–62,66,67,71–75]. In the case of class II epitopes, a number of studies have defined targets of immune responses [40,49,51,56,57,68,70]. In some cases, while the protein antigens recognized by immune responses were identified, the exact epitopes and their restriction were not mapped (Supplementary Table 3B). However, many class II restricted $CD4^+$ epitopes derived from poxviruses have been identified and mapped (Supplementary Table 3A) [40,49,51,56,57,68].

The large size of many poxvirus genomes has precluded a systematic search of all potential epitopes using comprehensive series of overlapping peptides and blood samples from exposed donors (or HLA-transgenic mice), an approach used successfully in characterization of CD8 and CD4 epitopes from viruses with smaller genomes, such as HIV [78–80] and influenza [81]. Instead, some poxvirus investigators have relied on bioinformatic prediction tools to reduce the number of potential epitopes to be tested [8,56,57], or have limited their studies to particular proteins already known or suspected to be immunogenic [68]. Whole-genome expression library studies of the human T-cell response to vaccinia proteins have appeared, but these generally have not included fine mapping at the epitope level [49,51]. A different approach has been used in a few studies, in which mass spectrometry was employed to identify peptide antigens naturally processed and loaded onto MHC proteins in poxvirus-infected cells [68].

The Immune Epitope Database and Analysis Resource (IEDB) [82–86,202] is an online freely accessible resource that catalogs immune epitope data relating to infectious diseases and allergies and, in the future, transplant antigens and autoimmunity. Meta-analyses of epitope data were recently performed for influenza A [87], mycobacteria [88,89], anthrax/botulinum toxins [90] and *Plasmodium* [91]. Herein, we present a similar analysis of poxvirus-derived epitopes. We utilized the IEDB, which has inventoried all immune epitope data found in the scientific literature, and then reviewed the original papers.

According to the data assembled in the IEDB, 246 distinct epitopes restricted by MHC class I and 61 distinct epitopes restricted by class II have been identified following viral infection (data following DNA vaccination were not included). By contrast, only nine different antibody epitopes have been mapped (Table 1). In terms of the hosts of the immune responses from which the epitopes were defined, a total of 145 class I epitopes were identified in humans and/or HLA-transgenic mice (six were recognized by both human and transgenic mice) and 103 epitopes were identified in nontransgenic mice (two were recognized by both transgenic and wild-type mice) (Supplementary Table 2A). Regarding the MHC class II epitopes, a total of 43 were identified in humans and 18 in mice (Supplementary Table 3A). Of the nine antibody epitopes, five were identified in mice and four were unknown, as the host utilized to raise the monoclonal antibodies was not described in the relevant publication (Table 2). Most, if not all, defined epitopes were derived from VACV and, accordingly, in the analysis described herein we have focused on VACV epitopes. It should be noted that there are sequence differences between VACV strains and,

while mouse work has been done largely with the WR strain, mapping in humans is based on Dryvax immunization. We estimate that less than 10% of mapped epitopes differ between these strains. Having said this, there is also evidence that, even if an epitope is conserved in two strains, its immunogenicity is not guaranteed [74].

Identification of epitopes & antigens targeted by CD8+ T-cell responses

Initially, $CD8^+$ T-cell epitopes were identified for the purpose of tracking T-cell responses following immunization and to evaluate new and/or safer smallpox vaccines. Owing to the high prevalence of HLA-A*0201 in donor cohort populations, these studies were focused on the identification of HLA-A2-specific epitopes by selecting and testing peptides based on HLA-A*0201 binding motifs, leading to the identification of six unique HLA-A*0201 restricted epitopes [3,67,72].

However, while these studies shed some light on the VACV $CD8^+$ T-cell response, they covered only a fraction of the VACV genome. Several approaches were used to identify the complete $CD8^+$ T-cell response against VACV. More elaborate studies identified a total of 16 epitopes in humans using polypeptide fragments from a library of VACV genomic DNA to screen cloned and bulk VACV-specific $CD8^+$ T cells [50]. A series of studies compared the epitope prediction approach with expression library approaches. Initial studies utilizing an expression library approach to study $CD8^+$ T-cell epitopes from VACV WR in the H-2b mouse model led to the identification of five different epitopes that accounted for approximately 40% of the response [74]. A subsequent study in the same system, using the bioinformatic approach, identified 49 different epitopes, including the five epitopes identified with the expression library approach, which account for approximately 95% of the total response to VACV infection *in vivo*, as judged by comparing the fraction of $CD8^+$ T-cells derived from VACV-infected mice that recognized *in vitro* either a pool of the 49 epitopes or VACV-infected antigen-presenting cells (APCs) [58]. Similar results were obtained in the H-2d system [60,75]. Furthermore, independent studies by Mathew *et al.* and Cornberg *et al.* identified three more unique mouse epitopes restricted by Db and Kd molecules [41,53], resulting in a total of 103 unique murine $CD8^+$ T-cell epitopes.

Based on these results, the epitopes restricted by human HLA molecules were investigated further. More than 6000 peptides from putative vaccinia ORFs predicted to bind the common molecules of the HLA-A1, -A2, -A3, -A24, -B7, and -B44 supertypes were screened with peripheral blood mononuclear cells of 31 vaccinees. A total of 49 epitopes derived from 35 different VACV antigens were identified [59]. These antigens were expressed predominantly early during infection, although some late antigens were also recognized. This finding may be a result of limited viral gene expression (mostly early genes) in APCs (and lymphocytes), as VACV infection of dendritic cells is abortive [92–95], suggesting that recognition of early VACV antigens maybe be an effect of direct priming. Most epitopes were conserved in the genomes of VACV strains WR and MVA as well as VARV, supporting their potential use in vaccine and diagnostic applications. Screening this peptide panel for cytotoxic T lymphocytes from VACV-infected HLA transgenic mice, we identified 14 HLA-A*0201-, four HLA-A*1101- and three HLA-B*0702-restricted epitopes from over 20 distinct proteins [62], further enhancing the number of HLA-restricted epitopes available for study.

Several independent studies identified another nine HLA-A*0201 epitopes [46,61,66,71], as well as two HLA-B7 epitopes [71], by using different binding prediction algorithms and testing human donors. Also, mass spectrometry has been used to identify VACV-derived peptides from among the pool of peptides eluted from MHC class I molecules isolated from VACV-infected B-lymphoblastoid cell lines. In an early study, a single VACV-derived

peptide bound to HLA-A*0201 was identified, but no immunological characterization was described [46]. A recent study by another group used a novel stable-isotope tagging strategy to identify VACV-derived peptides, and described a total of 12 HLA-A*0201-bound naturally processed VACV peptides, including the one found earlier, and three HLA-B*0702-bound VACV peptides [48]. All 12 HLA-A*0201 peptides, including seven that had not been previously described, were demonstrated to be recognized by vaccinated donors and, thus, represent bonafide $CD8^+$ T-cell epitopes.

In summary, these studies show that the $CD8^+$ T-cell response to VACV is broad and diverse, rather than focused on a few antigenic targets.

Some VACV proteins are recognized more frequently than others by $CD8^+$ T-cell responses

Parallel studies have investigated the mechanisms that determine which epitopes within an antigen are recognized by class I responses [37]. A comprehensive analysis of 18 viral proteins recognized by $CD8^+$ T cells demonstrated that approximately 1/40 of all possible 9- and 10-mer peptides were high-affinity HLA-A*0201 binders. Peptide immunization and T-cell recognition data from 90 peptides indicated that approximately half of the HLA binding peptides could elicit T-cell responses, and that 1/7 of immunogenic peptides are generated by natural processing. Based on these results, it has been estimated that VACV encodes approximately 150 HLA-A*0201 dominant and sub-dominant epitopes. However, only 15 are actually recognized following VACV infection of HLA transgenic mice. Thus, additional factors restrict the epitope repertoire by a factor of at least ten.

Do differences in expression of different VACV ORFs influence immunodominance? If different patterns and magnitude of ORF expression or transcription influence immunogenicity and immunodominance, these influences would not be expected to depend on the MHC type expressed in the infected host. Accordingly, we defined as immunoprevalent those antigens that were frequently recognized (prevalent) in the context of different MHC molecules, haplotypes or even different species. A recently published analysis of nearly 200 $CD8^+$ T-cell epitopes from H-2d, H-2b and HLA-transgenic mice, as well as humans, revealed that $CD8^+$ T-cell epitopes are not randomly distributed across the VACV proteome [60]. Ten different VACV antigens were described as immunoprevalent, which is defined as being recognized in the context of three or more different MHC class I molecules. By contrast, a set of different antigens was recognized infrequently or not at all.

This analysis is repeated here, encompassing all data from the 20 studies available to date that mapped VACV-specific $CD8^+$ T-cell epitopes (to the best of our knowledge) (Supplementary Table 2A) and further compiled the antigens and the frequency of $CD8^+$ T-cell responses (Supplementary Table 2B). To date, a total of 114 antigens (~50% of the total ORFs) have been shown to be recognized by $CD8^+$ T cells. Once again, it is apparent that this is a nonrandom distribution, with many antigens not being recognized and others being immunoprevalent or frequently recognized across different MHC types, loci and even species. The top 23 (~10% of all ORFs) VACV antigens that represent the most prevalent are listed in Table 3, and are associated with more than three MHC restrictions.

Thus, specific features of the VACV antigens, distinct from the characteristics of the epitopes contained within them, also contribute to rendering them more or less immunogenic. In general, prevalent $CD8^+$ T-cell responses preferentially recognize antigens that are expressed early during the viral lifecycle, although some virus late proteins are also recognized (82.6 vs 13%; Figure 1A). This prevalence is surprising because the established paradigm for VACV has been that late genes are poorly immunogenic for $CD8^+$ T cells [96].

The representation of recognized antigens within each functional category did not differ significantly from their representation within the viral genome. However, proteins regulating the viral life cycle seem to be recognized slightly more, and structural proteins slightly less frequently, as compared with their representation in the viral genome (Figure 1B). These data are in agreement with previously published results [34,58,59,62].

Identification of murine & HLA class II restricted CD4+ antigens & epitopes

Fewer studies have been conducted to identify the targets of VACV-specific $CD4^+$ T cells. In the murine system (C57Bl/6), using a random peptide library of 2146 different peptides, a total of 14 $CD4^+$ T-cell epitopes were identified [57]. Subsequent screening of five selected VACV proteins (B5, A4, A27, B2 and A33), with overlapping peptides covering the entirety of each protein, led to the identification of four more $CD4^+$ T-cell epitopes restricted by H-2b [65].

Several studies in humans have started to highlight a number of antigens and antigenic regions that are targeted by human HLA class II restricted responses [40,49,51,56,68,70]. In some cases the restriction was defined, and the epitope was mapped to a discrete peptide, while in other cases the epitopes were mapped to relatively large antigenic regions, and/or the exact HLA restriction was left undefined.

Tang *et al.* screened vaccinees for responses to four VACV proteins known to induce protective antibody responses in animals (B5R, A27L, A33R and L1R), and used overlapping peptides to identify three distinct $CD4^+$ T-cell epitopes within A27L [70]. Mistra-Kaulik *et al.* and Calvo-Calle *et al.* used an HLA-DR1 binding prediction algorithm covering the complete MVA genome to identify 25 additional $CD4^+$ T-cell epitopes recognized by VACV vaccinated or infected donors [40,56]. The epitopes derived predominantly, but not exclusively, from proteins present in the virion. Seven of the epitopes were shown to be restricted by HLA-DRB1*0101, with the remainder presented by other HLA-DRB gene products [40].

Mass spectrometry of naturally processed peptides eluted from VACV-infected cells has been used to identify $CD4^+$ T-cell epitopes. Three HLA-DRB1*0101 bound peptides, including one novel epitope, were characterized by Strug *et al.* [68]. As with the $CD8^+$ T-cell epitopes, each of the eluted peptides was shown to represent an actual T-cell epitope recognized by $CD4^+$ T cells from VACV-exposed donors.

Expression libraries have also been used to characterize the targets of the $CD4^+$ T-cell response to VACV. Initial studies by Koelle's group examined VACV-reactive $CD4^+$ *in vitro* T-cell lines with VACV protein fragments expressed from a genomic library, and a panel of membrane proteins [49]. $CD4^+$ T cells from three vaccinees reacted with 44 separate antigenic regions in 35 vaccinia proteins, with eight to 20 proteins being recognized per person. The expression of protein in virions was strongly associated with $CD4^+$ antigenicity, as was the case for the bioinformatic studies. A subsequent study [51] used a whole-proteome approach to study 180 predicted ORFs in 11 vaccinees. The most frequently recognized ORFs were present in virions, including A3L and A10L (core proteins), WR148/A25L, H3L (a membrane protein), D13L (a membrane scaffold protein) and L4R (a nucleic acid-binding protein). Serum immunoglobulin G profiling demonstrated a diverse response directed to membrane and nonmembrane antigens. These seminal results are consistent with the general assumption that abundantly expressed structural proteins are immunodominant for $CD4^+$ T cells.

A compilation of all antigens recognized in humans and mice on the basis of the data from several published reports [40,49,51,56,57,65,68,70] is summarized in Supplementary Table

3B and demonstrated that a total of 133 antigens are recognized by $CD4^+$ T cells. This illustrates that the $CD4^+$ T-cell response against VACV is directed against a large number of viral proteins. The top 21 antigens (approximately 10% of all ORFs), were recognized by more than 40% of all tested donors (Table 4). Analysis of their structural and functional characteristics revealed a significant preference for late antigens (Figure 1A), as well as structural proteins, compared with their representation in the viral genome (Figure 1B). This is in contrast to $CD8^+$ T-cell responses as described previously (early, regulatory proteins) and highlights fundamental differences in immune responses of $CD4^+$ and $CD8^+$ T-cell responses to complex pathogens.

Antigens recognized by Ab responses in humans & mice

The human Ab response to the smallpox vaccine is highly diverse and redundant [39]. Owing to the large size and existence of two distinct virion surface forms, antibody responses target numerous VACV antigens. These have been comprehensively identified using protein microarrays [42,45] and primary vaccinated donors [44]. Supplementary Table 4 lists all targets of Ab responses identified by all published studies to date. Several studies focusing on selected viral proteins (B5R, A33R, A56R, A27L, H3L and L1R) screened large cohorts of vaccinated donors and confirmed their importance as targets of the Ab responses [55,63,64]. While there is significant variability in the profile of targets recognized, a subset of 19 antigens is frequently recognized both in donors receiving primary immunizations and in those receiving boost immunizations [39,44,45]. These antigens are listed in Table 5 and were recognized by more than 20% of tested donors. Not surprisingly, Ab responses recognize mainly late-expressed viral genes and structural proteins (Figure 1A & B), most likely because they target virions, the main components of which are core proteins [31–33].

This list includes the six antigens known to be targets of neutralizing or protective Abs described in more detail in the following sections. Ab-mediated protection is thought to occur both by neutralizing the initial virus inoculum and by limiting the spread of virus particles within the host after infection is initiated [97]. Presence on the surface of the virion is a prerequisite for being a neutralizing Ab target. There are five known neutralizing Ab targets: L1R, H3L, A27L, D8L and B5R [97]. Interestingly, antibodies against the A33R surface protein are protective, but not neutralizing [97,98]. Abs to these antigens are very frequently observed in vaccinated humans, but the antigens are not present on the virion surface and do not seem to contribute to protective immunity. Studying such antigens would be of interest, since their frequent recognition makes them good candidates for diagnostics. There are additional surface proteins, for which it is yet unclear whether they are neutralizing and/or protective Ab targets. Also included in Table 5 is A56R, which is a target of Ab responses in smallpox vaccine-immunized mice and humans [42,44]. However, immunization against A56R was not protective [98], in contrast to immunization against B5R and A33R [98]. Finally, the A14L antigen is also of note; this is an abundant surface protein [31] that has only 14 amino acids exposed as a loop between two transmembrane domains [99]. It has been identified as an antigenic target in vaccinated humans [39,44], and we have shown that IgG responses to A14L correlate with neutralizing Ab levels in vaccinees [39]. Despite the wealth of information available regarding the targets of Ab responses, little is known about the actual epitopes recognized, which are listed in Table 2. This is most likely owing to the fact the most B cell epitopes may not be linear and can encompass secondary and tertiary structures and, therefore, are more difficult to identify.

Correlation between CD4+ T-cell, CD8+ T-cell & antibody responses versus mRNA & protein abundance & previously known gene functions

As described in the previous sections, a substantial body of knowledge is now available relating to the antigens and epitopes recognized by CD4, CD8 and Ab responses. Based on this knowledge, we started to examine whether any significant correlation existed between the types of antigens recognized by the various arms of adaptive immune responses and expression patterns.

In the case of $CD8^+$ T-cell responses, we detected the most significant correlation between antigens containing epitopes and mRNA levels at the relatively early 4 h time point. A mean mRNA expression ranking (MER) was calculated by taking the average of individual mRNA rankings for each ORF, as reported by Assarsson *et al.* [9]. Hence, a low number corresponding to high expression levels (e.g., a ranking of ten corresponds to the tenth highest expression level). The MER of the 23 most prevalently recognized ORFs was significantly higher (MER = 80) compared with the remaining antigenic ORFs (MER = 99) or nonantigenic for $CD8^+$ (MER = 126) (Figure 2A). At 24 h, the correlation was less pronounced, with MERs of 95, 93 and 126 for the prevalent, recognized and nonantigenic groups, respectively. Performing a similar analysis by correlating antigenicity with median ranking of the protein abundance in the virion, as detected by the proteomic analysis, we found that the MER of $CD8^+$ T-cell responses did not vary significantly between prevalent (MER = 145), recognized (MER = 128) and nonantigenic (MER = 145) ORFs. No correlation was observed between $CD8^+$ T-cell targets and abundance in virions, as determined by proteomic analysis. These data are in agreement with the finding that, in general, early proteins that are not found in virions are the best source of $CD8^+$ T-cell epitopes [57].

By contrast, a similar analysis with the ORFs recognized by $CD4^+$ T cells revealed a different pattern. Median mRNA expression levels at 4 h of the top 21 protein antigens recognized by $CD4^+$ T cells (MER = 112) [57,65] did not differ from the remaining antigenic proteins recognized by $CD4^+$ T cells (MER = 107) or nonantigenic ORFs (MER = 112). By contrast, a good correlation was detected with mRNA levels at the 24 h time point, with a MER of 74 for the most prominent CD4 antigens, 103 for the remaining CD4 antigens and 117 for the non-CD4 antigenic ORFs (Figure 2B). CD4 responses also demonstrated a similarly high correlation with protein abundance in the virion, with the top 21 ORFs recognized by $CD4^+$ T-cell responses being associated with a much lower ranking (MER = 71) compared with remaining (MER = 109) or nonantigenic (MER = 145) ORFs. Again, the analysis based on expression and virion protein levels was in broad agreement with that done using previously published data on the function and timing of VACV gene expression [9]. Specifically, $CD4^+$ T cells tend to recognize structural proteins that are expressed later during infection.

The ORFs recognized by Ab were associated with a pattern similar to that for $CD4^+$ T cells. The mRNA rankings of ORFs encoding proteins recognized by Ab responses [39] demonstrated little correlation at the 4 h time point (shown in Figure 2C). Specifically, mRNAs encoding these antigens were expressed at 4 h at levels (MER = 117) comparable to the remaining (MER = 98) or nonantigenic ORFs (MER = 107). By contrast, a good correlation was detected with mRNA levels at the 24 h time point with a MER of 73 for the most prominent CD4 antigens, 102 for the remaining CD4 antigens, and 117 for the nonantigenic CD4 ORFs (Figure 2B). Ab responses also demonstrated a rather dramatic correlation with virion protein abundance, with the top 19 ORFs being associated with a high ranking (MER = 44) compared with either the remaining (MER = 107) or nonantigenic (MER = 145) ones.

In conclusion, we found that the general pattern of VACV antigens recognized by $CD4^+$ and $CD8^+$ T-cell responses is different. $CD8^+$ T-cell responses correlate with early mRNA levels of the specific antigens recognized and not protein levels in virions. By contrast, the antigens recognized by $CD4^+$ T cells and Ab tend to correspond to antigens whose mRNAs are expressed at late time points, and also with antigens expressed in high amounts in viral particles [57].

Correlation between serologic reactivity & antigens recognized by $CD4^+$ & $CD8^+$ T-cell epitopes

At the same time as the above studies were done, an analysis of the potential correlation between Abs and $CD4^+$ T-cell responses was carried out. A simple model of $CD4^+$ T-cell–B-cell linkage would predict that T cells could deliver help to B cells specific for any protein antigen present in the viral particle. Unexpectedly, in a recent study, analysis of the Ab and $CD4^+$ T-cell response specificities to VACV revealed that many of the $CD4^+$ T-cell responses identified in an unbiased screen were specific for proteins that were also targets of Ab responses, consistent with a deterministic linkage ($p < 0.001$) [65].

This linkage was demonstrated by direct peptide immunizations with $CD4^+$ VACV epitopes. Mice vaccinated with a VACV-specific $CD4^+$ T-cell epitope ($I1L_{21–35}$), demonstrated a tenfold increase in the total anti-VACV Ab response as measured by a standard VACV ELISA. Unexpectedly, virus-neutralizing Ab titers were unimproved in VACV-infected mice preimmunized with $IlL_{21–35}$ when compared with unprimed mice. While I1L is a viral virion core protein and, therefore, not itself a neutralizing Ab target, I1L–specific $CD4^+$ T cells were expected to provide intermolecular help to all B cells specific for VACV viral particle proteins and, thereby, boost neutralizing Ab titers. Surprisingly, when serum samples were probed for the detailed antigen specificities of the Ab response using VACV protein microarrays, we found the increased Ab response was exclusively against I1L and not other VACV. These data led us to the conclusion that purified $CD4^+$ T cells specific for a given antigen (I1L, for example), only provided help for Ab responses to that same antigen. In contrast to the standard model, these data indicate that individual proteins are the primary unit of immunological recognition for VACV and suggest that while $CD4^+$ and $CD8^+$ T-cell responses recognize different antigens, $CD4^+$ and Ab responses tend to recognize the same structures.

This data led to a revision of the classic model of antigenic bridge in cognate recognition, at least in the case of VACV. It was also demonstrated that the knowledge of the serological targets of VACV responses could be used to predict $CD4^+$ reactivity. Specifically, four of five antigens predicted to have $CD4^+$ T-cell epitopes on the basis of recognition by serum from VACV infected mice were indeed found to have these epitopes. By contrast, of two antigens not recognized by antibody, neither was found to have a $CD4^+$ T-cell epitope [65], demonstrating that knowledge of the serological response can be used to support $CD4^+$ T-cell epitope identification.

Analysis of the protective capacity of VACV $CD8^+$ T-cell epitopes

The identification of a large array of different VACV-derived $CD8^+$ T-cell epitopes offered a unique opportunity to systematically analyze the correlation between protective efficacy and variables such as kinetics of expression and function of viral proteins, binding affinity to MHC molecules, immunogenicity and viral antigen processing/presentation. In a recent study, 49 different H-2b restricted epitopes were tested for their ability to protect peptide-immunized C57Bl/6 mice from lethal intranasal challenge with VACV [100]. The epitopes varied greatly in their ability to confer protection, ranging from complete protection with

minimal disease to no protection at all (Table 6). The function or kinetics of the viral antigen expression did not correlate with protective efficacy. However, binding affinity partially predicted protection efficacy and ultimately epitope immunogenicity and recognition of infected cells offered the best correlation. Thus, while antigen expression, both at the mRNA and protein levels, greatly influence the antigenic focus of the immune response, the intrinsic protective capacity of each epitope is directly dependent on its MHC binding affinity, the efficiency of its generation by antigen processing, and the number of T cells that can recognize it in the naive repertoire. To date, no protective capacity has been associated with reported $CD4^+$ T cell or antibody epitopes.

Conclusions: gaps in knowledge & directions for future research

Owing to the rapid progress in knowledge in the last few years, VACV has become a useful model to study adaptive immune responses against complex pathogens, both in murine systems and in humans. This review summarizes and highlights key findings based on identification of VACV antigens targeted by the immune system (CD4, CD8 and Ab) and the complex interplay between these responses. More specifically, recent studies provided a first complete inventory of the mRNA expression patterns, and proteomic studies provided data relating to expression at the protein level. In parallel, additional studies provided an inventory of the epitopes recognized by CD8 and CD4 T cells, and protein array studies also mapped the antigens recognized by antibody responses. The data in the VACV system have started to shed light on the mechanisms that determine which antigen(s) will be targeted by immune responses. CD8 responses, on the one hand, and CD4/Ab responses, on the other hand, tend to recognize different antigens associated with discrete characteristics, both at the functional and expression kinetic level. While CD8 responses focus on early antigens, CD4 and Ab responses focus on late and structural proteins. When this data was correlated with the mRNA and proteomic data, it was found that $CD8^+$ T-cell responses correlate with early mRNA levels and not protein levels in virions. By contrast, the antigens recognized by $CD4^+$ T cells and Ab tend to correspond to antigens whose mRNAs are expressed at late time points, and also with antigens expressed in high amounts in viral particles. While the mechanisms underlying this apparent dichotomy have not been elucidated, it is likely that direct priming of CD8 responses by infected cells might contribute to the effect, especially since dendritic cells are thought to be only abortively infected [92–95] and, thereby, are likely to best present early antigens. Conversely, the recognition of late and structural antigens by CD4 and Ab responses might reflect antigen uptake and presentation by noninfected APCs. Finally, VACV and poxviruses in general are known to express many systems for evasion of immune responses [101], including those that affect antigen presentation pathways [102], and it is possible that these may play a role in shaping the spectrum of T cell and antibody epitopes recognized.

These studies illustrate that immune responses to complex pathogens are highly diverse, broad and multispecific, and that a narrow immunodominance with responses directed against only one or a few epitopes does not exist. Indeed, a recent study of lymphocytic choriomeningitis virus infection in inbred mice [103] has also suggested that the narrowness of immunodominance has been overestimated. Having said this, at least for $CD8^+$ T cells, some epitopes are far more immunogenic than others. The $CD8^+$ T-cell immunodominance hierarchies found in two strains of inbred mice after intraperitoneal VACV infection look superficially similar, with both having one dominant epitope that accounts for around 25% of the antiviral response [58,60]. There is also evidence that immunization route can sharpen this inequity between epitopes: in C57Bl/6 mice cells that recognize the most dominant epitope increase in proportion to around 50% of all anti-VACV $CD8^+$ T cells if mice are immunized by a dermal route [74]. Recent work carried out with VACV and lymphocytic choriomeningitis virus has also started to outline two different paths that shape

immunodominance hierachies and select the determinants of immune responses. Within a particular antigen, the choice of the epitope(s) that will be recognized is dictated by, in decreasing order of importance, MHC binding, ease of cellular processing and T-cell receptor repertoire [37]. Nevertheless, complex cellular regulatory events, such as immunodomination, might further decrease the epitopes actually recognized, but direct evidence for this in the case of VACV has not yet been found [104].

Of practical relevance, the targets of CD8, CD4 and Ab responses could be, to a certain extent, retrospectively predicted by a combination of tiling array and proteomic analysis. As our knowledge grows, it might be reflected in an increase in predictive accuracy. It is possible that, in the not so distant future, we may be able to combine algorithms predicting which antigens have a high likelihood of being recognized by adaptive responses with algorithms capable of predicting, within a given antigen, which particular epitopes (particularly in the cases of CD8 and CD4) might be recognized and induce protective immunity. In the end, this would translate in an unprecedented accuracy in the prediction of the targets of immune responses to complex pathogens.

While these hypotheses are reasonable, it still remains to be investigated how occasional CD8 responses against late antigens, and CD4 responses against early antigens, are primed and develop, perhaps because of cross-priming of CD8 responses [105–108], and direct priming by infected APCs of CD4 responses. Furthermore, if the pathways that lead to priming of CD4 and CD8 responses are largely nonoverlapping, it will be important to clarify how these patterns intersect, and how CD4 help is effectively provided to CD8 T cells. Another issue relating to the intersection between different effector pathways is the deterministic linkage between Ab responses and CD4 responses. Thus far, several possible mechanisms have been proposed and are currently under investigation. The fundamental components of this proposed linkage include antigen presentation to B cells in the underlying follicles by professional APCs bridging the subcapslar sinus, followed by the interaction of B cells with $CD4^+$ T cells at the T/B cell boundary [65]. Taken together, these data suggest that a revision of the classic model of antigenic bridge (direct presentation) might be in order, at least for certain antigenic systems.

In conclusion, the VACV system continues to expand in sophistication. While many important questions remain unanswered and will provide challenges for future research, the detailed knowledge of the nature of the targets of immune responses provided an invaluable tool for researchers to continue to expand our knowledge of the mechanisms at the root of adaptive responses against complex pathogens.

Future perspective

The extensive map of immune epitope reactivity for poxviruses, including the VACV, makes this one of the most well-understood model systems to study human and murine immune responses. Findings from these studies will initially greatly increase our basic understanding of the immunobiology of complex host–virus interactions, including a clearer understanding of antigen selection and effector cell interplay. This knowledge can then be utilized for further development of safe and efficacious vaccines, tailored toward different target populations, and in the development of more sophisticated poxvirus vectors as transgene delivery systems.

Executive summary

- Due to the rapid progress in knowledge in the last few years, vaccinia virus (VACV) has become a useful model to study adaptive immune responses against complex pathogens, both in murine systems and in humans.

- Studies have shown that immune responses to VACV are highly diverse, broad and multispecific; narrow immunodominance does not exist.
- The data in the VACV system has started to shed light on the mechanisms that determine which antigen(s) will be targeted by immune responses.
- CD8 and CD4/antibody (Ab) responses tend to recognize different antigens associated with discrete characteristics, both at the functional and expression kinetic level – CD8 recognize early antigens; CD4 and Abs recognize later antigens.
- VACV, and poxviruses in general, are known to express many systems for evasion of immune responses, including those that affect antigen presentation pathways. Thus, it is possible that these may play a role in shaping the spectrum of T cell and antibody epitopes recognized.
- It is as yet unclear as to how CD8 responses against late antigens, and CD4 responses against early antigens, are primed and develop; perhaps due to cross-priming of CD8 responses and direct priming by infected antigen-presenting cells of CD4 responses.
- Another issue relating to the intersection between different effector pathways is the deterministic linkage between Ab responses and CD4 responses – characterizing these relationships should be a future priority.
- In the future, antigenic targets of CD8, CD4 and antibody responses may be retrospectively predicted by a combination of tiling array and proteomic analysis; as our knowledge grows, so too should the predictive accuracy of these tools.
- It is possible to combine computational algorithms predicting which antigens have a high likelihood of being recognized by adaptive responses with algorithms capable of predicting which epitopes might be recognized and induce protective immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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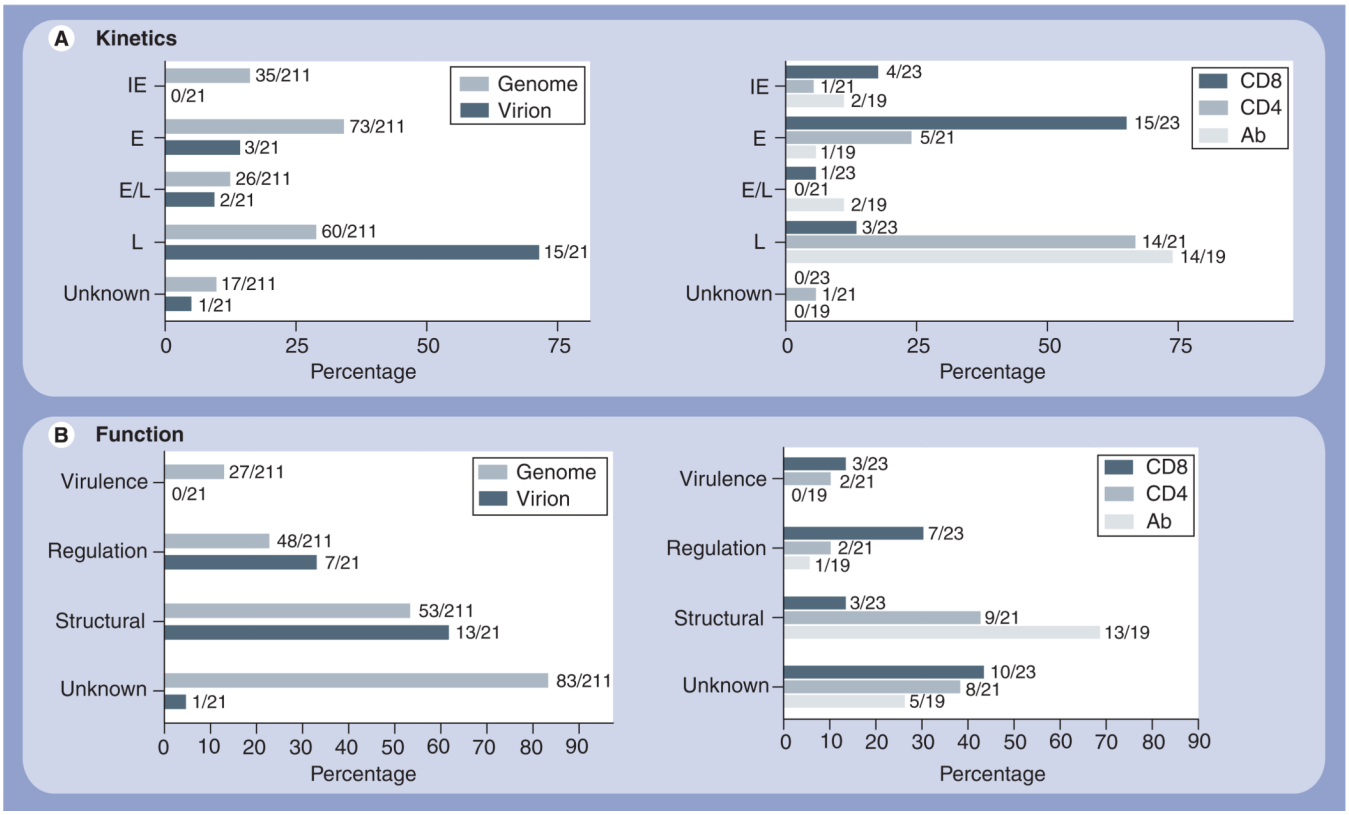

Figure 1. Kinetic and functional categories of vaccinia virus antigens either contained within vaccinia virus genome and virion or targeted by adaptive immunity: CD8 and CD4/antibody target different sets of viral antigens

Left panels show the distribution of vaccinia virus (VACV) proteins within the viral genome (grey; total of 211 unique VACV genes) and top 21 (10% of all open reading frames) most abundant proteins contained within virion (black) based on **(A)** expression kinetics (IE, E, E/L, L, unknown) or **(B)** functional category (virulence, regulation, structural, unknown).

Right panels show the distribution of VACV proteins identified as targets of CD8 T cells (black; top 23 most prevalent antigens), CD4 T cells (grey; top 21 antigens, >40% of tested donors) or antibodies (white; top 19 antigens, >20% of tested donors) based on **(A)** expression kinetics (IE, E, E/L, L, unknown) or **(B)** functional category0 (virulence, regulation, structural, unknown).

Ab: Antibody; E: Early; E/L: Early/late; IE: Immediate-early; L: Late.

Data were compiled from published literature based on experimental data as described in the text.

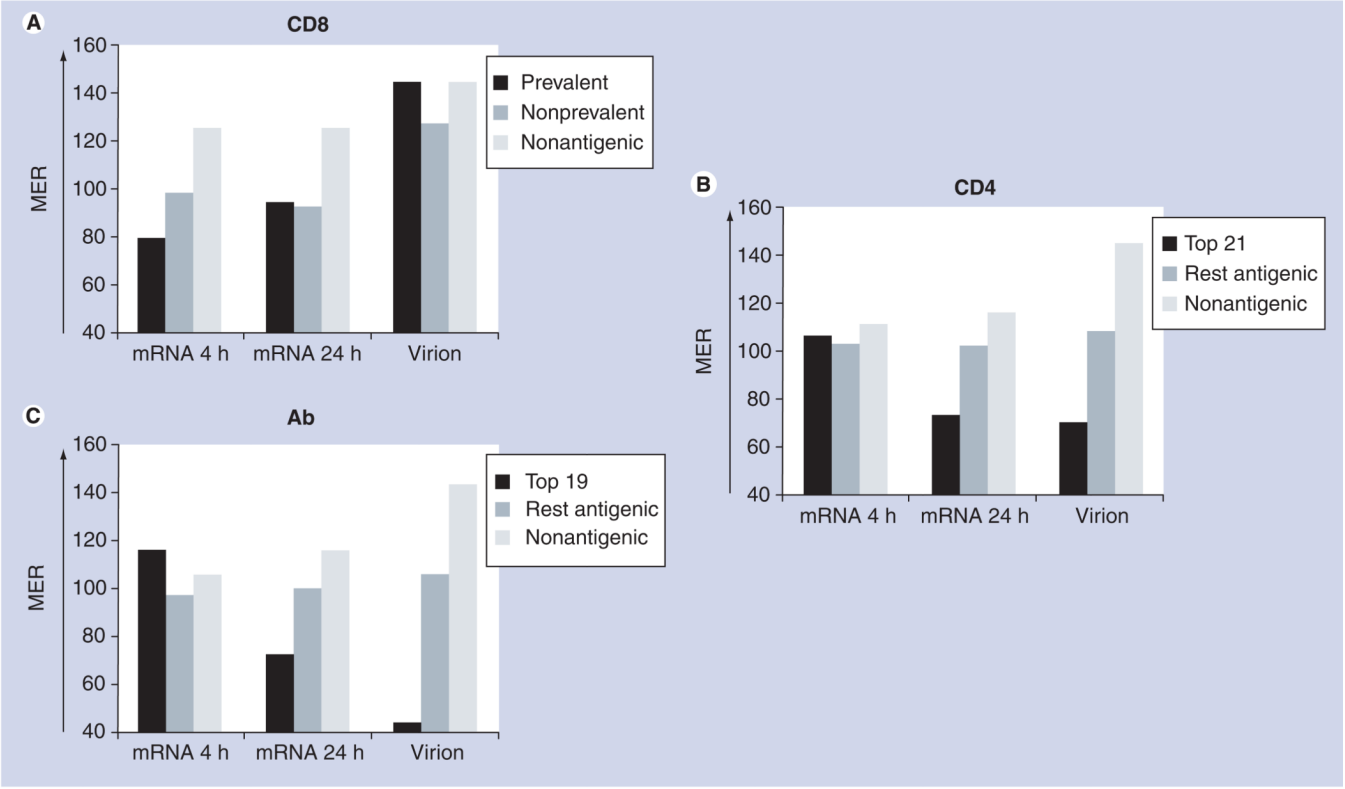

Figure 2. Vaccinia virus mRNA early expression pattern correlates with CD8 T-cell responses, whereas late expression correlates with CD4 and antibody responses

The MER was calculated by taking the average of individual mRNA rankings of each open reading frame at 4 h and 24 h postinfection based on [9]. Low MER numbers correspond to high expression levels. Similar analysis was performed with median ranking of protein abundance in the virion based on studies by Chung *et al.* [31] and Resch *et al.* [32]. **(A)** MER of the 23 most prevalently (black), nonprevalent (grey) or nonantigenic (nonrecognized; white) recognized ORFs by CD8 T cells of mRNA levels at 4 h and 24 h and protein abundance in virion. **(B)** MER of the top 21 (black), remaining antigenic (grey) or nonantigenic (nonrecognized; white) recognized ORFs by CD4 T cells of mRNA levels at 4 h and 24 h and protein abundance in virion. **(C)** MER of the top 19 (black), remaining antigenic (grey) or nonantigenic (non-recognized; white) recognized ORFs by antibodies of mRNA levels at 4 h and 24 h and protein abundance in virion.

Ab: Antibody; MER: Median mRNA expression ranking.

Table 1

Defined poxvirus epitopes*.

Restriction	Unique epitopes	Host species		
		Human‡	*Mouse*	*Other/ND*
MHC class I/CD8	246	145§	103¶	0
MHC class II/CD4	61	43	18	0
B cell/Ab	9	0	5	4

*Based on data from the Immune Epitope Database and reviewing original papers.

‡Includes epitopes recognized by HLA-transgenic mice.

§Includes six epitopes recognized by both human and transgenic mice.

¶Includes two epitopes recognized both by transgenic and wild-type mice.

Ab: Antibody; ND: Not determined.

Table 2

Vaccinia virus-specific B-cell epitopes as described in the literature.

COP Ag Name*	VACWR Ag Name‡	Sequence	Species	Ref.
B5R (256–275)	VACWR187	DLSKLSKDVVQYEQEIESLE	Mouse	[35]
B5R (56–75)	VACWR188	DPNAVCETDKWKYENPCKKM	ND	[35]
B5R (65–75)	VACWR189	KWKYENPCKKM	Mouse	[35]
B5R (65–84)	VACWR190	KWKYENPCKKMCTVSDYISE	Mouse	[35]
L1R (118–128)	VACWR088	SSAVVDNKLKI	ND	[36]
14-Kd protein (30–46)	ND	AKREAIVKADEDDNEET	Mouse	[109]
14-Kd protein (21–35)	ND	STKAAKKPEAKREAI	ND	[109]
H4L (18–20)	VACWR102	YLL	ND	[110]
E3L (164–183)	VACWR059	GKSKRDAKNNAAKLAVDKLL	Mouse	[76]
ND	ND	D56, P57, N58, A59, V60, C61, E62, T63, D64, K65	Mouse	[35]
ND	ND	K65, W66, K67, Y68, E69, N70, P71, C72, K73, K74	Mouse	[35]
L1R or A17L	VACWR088 or VACWR137	D35		[111]
ND	ND	L118, S120		[48]
L1R	VACWR088	N27, Q31, T32, K33, D35, S58, A59, D60, A61, D62	Mouse	[69]

*Nomenclature according to COP.

‡Nomenclature according to VACWR.

Ag: Antigen; COP: Copenhagen strain of vaccinia virus; ND: Not determined; VACWR: Western Reserve strain of vaccinia virus.

Table 3

Prevalent Ag* recognized by CD8 T cells.

COP Ag name‡	VACWR Ag name§	Kinetics¶	Function	MHC restriction	No. of MHC types#	Positive/ tested**	Positive (%)‡‡	Ref.§§
D1R	VACWR106	E	Regulation	HLA A*2601, HLA A*2902, HLA B*4403, HLA B*0702, HLA A*0301, H-2 Kb, H-2 Db, H-2 Kd	8	24/183	13.1	[50,58–62]
B8R	VACWR190	E	Virulence	HLA A*0101, HLA A*2601, HLA A*2902, HLA B*4001, H-2 Kb, HLA A*0201	6	13/120	10.8	[54,58,59,74]
A47L	VACWR173	IE	Unknown	HLA A*1101, HLA A*0201, H-2 Kb, H-2 Db, H-2 Kd	5	8/44	18.2	[53,58,60,62,71,74]
D5R	VACWR110	E	Regulation	HLA A*2402, HLA A*2301, HLA B*4403, HLA A*3303, H-2 Kd	5	21/154	13.6	[50,59–61]
C12L	VACWR205	E	Virulence	HLA A*0101, HLA A*3002, HLA B*4403, HLA A*0301, HLA A*2902	5	9/79	11.4	[50,59]
D12L	VACWR117	E	Regulation	HLA A*0101, HLA A*3002, HLA A*0201, H-2 Kb	4	18/81	22.2	[54,58,59,61,62]
J6R	VACWR098	E	Regulation	HLA B*0702, HLA B7, H-2 Kb, H-2 Kd, HLA A*0301	4	11/80	13.8	[53,54,58–60,71]
M1L	VACWR030	E	Unknown	HLA A*0201, H-2Kb, H-2 Db, H-2 Ld	4	4/30	13.3	[58,60,62]
A3L	VACWR122	L	Structural	HLA B*4403, H-2 Db, H-2 Kb, H-2 Kd	4	6/66	9.1	[50,58,60]
F11L	VACWR050	E	Unknown	HLA A*2601, HLA A*2902, HLA A*0201, H-2 Kd	4	4/47	8.5	[59,60,62]
A26L	VACWR149	L	Unknown	HLA-A*0201, H2-Db, H-2 Kb, H-2 Ld	4	6/72	8.3	[60,61,67]
B5R	VACWR187	E/L	Structural	HLA B35, HLA B55, HLA A2, HLA B44	4	4/63	6.3	[70]
C7L	VACWR021	E	Unknown/ virulence	HLA-B*1801, HLA A*0301, HLA A*0201	3	22/119	18.5	[54,59,62,64,67,72]
A8R	VACWR127	IE	Regulation	HLA A*0301, HLA A*0201, H-2 Kb	3	11/68	16.2	[58,59,61]
O1L	VACWR068	IE	Unknown	HLA A*0201, HLA B*0702, H-2 Ld	3	12/75	16.0	[54,59,60]
C10L/no ortholog	VACWR010/ VACWR209	E	Unknown	HLA A*0101, HLA A*3002, H-2 Kd	3	9/71	12.7	[59,60]
B6R	VACWR188	E	Unknown	HLA A*0201, H-2 Kb, HLA A*1101	3	5/45	11.1	[58,59,62]
I8R	VACWR077	E	Structural/ regulation	HLA A*0201, H-2 Ld, H-2 Kd	3	9/70	10.0	[60,61]
No ortholog	VACWR148	L	Unknown	HLA A*0201, H-2 Kd, H-2 Ld	3	4/44	9.1	[58,60]

COP Ag name‡	VACWR Ag name§	Kinetics¶	Function	MHC restriction	No. of MHC types#	Positive/ tested**	Positive (%)‡‡	Ref.§§
E9L	VACWR065	E	Regulation	HLA A *0201, H-2 Ld, H-2 Db	3	5/65	7.7	[58–61]
N2L	VACWR029	IE	Unknown	H-2 Kd, H-2 Db, HLA A *0201	3	3/43	7.0	[58–60]
A23R	VACWR143	E	Regulation	HLA A *0201, HLA B *4403, H-2 Db	3	3/51	5.9	[50,54,58]
C19L/B25R	VACWR008/ VACWR211	E	Unknown	HLA A *2601, HLA A *2902, H-2 Kb	3	3/69	4.3	[58,59]

*Prevalence was defined as being frequently recognized (>3) in the context of different MHC molecules, haplotypes or different species.

‡Nomenclature according to COP.

§Nomenclature according to VACWR.

¶Based on [9].

#Each mouse strain tested was counted as one MHC type.

**Number of donors responding positive to antigen versus total number of donors that were tested compiled from all published studies.

‡‡Percentage of donors responding (positive) to antigen divided by total number of donors tested.

§§Only references containing positive epitope identification are listed.

Ag: Antigen; COP: Copenhagen strain of vaccinia virus; E: Early; E/L: Early/late; IE: Immediate-early; L: Late; VACWR: Western Reserve strain of vaccinia virus.

Table 4

Top 21 antigens (>40% of tested donors) recognized by CD4 T cells.

COP Ag name*	VACWR Ag name‡	Kinetics§	Function	MHC restriction	Frequency		Ref.**
					Positive/tested¶	*Positive (%)*#	
D13L	VACWR118	L	Structural	IAb, DRB1*0405	14/16	87.5	[49,51,57]
H3L	VACWR101	L	Structural	DRB4*0101, IAb	13/16	81.3	[49,51,57]
L4R	VACWR091	L	Regulation	IAb	13/16	81.3	[49,51,57]
A2.5L	VACWR121	L	Structural	ND	10/16	62.5	[49,51,57]
B2R	VACWR184	IE	Unknown	IAb	9/17	52.9	[51,65]
A9L	VACWR128	L	Structural	ND	11/21	52.4	[40,49,51,57,65]
I1L	VACWR070	L	Structural	IAb, DR1 predicted, IAb	10/20	50	[51]
J6R	VACWR098	E	Regulation	DR	8/20	50	[49,51]
266	VACWR069.5	Not expressed	Unknown	ND	8/16	50	[40,51,57]
H7R	VACWR105	L	Unknown	ND	8/16	50	[51]
A26L	VACWR149	L	Unknown	DR	8/16	50	[49,51]
F13L	VACWR052	L	Structural	ND	8/16	50	[49,51]
D8L	VACWR113	L	Structural	IAb	8/16	50	[49,51,57]
A39R	VACWR163	L	Virulence	ND	7/16	43.8	[51]
A6L	VACWR125	L	Unknown	ND	7/16	43.8	[51]
F11L	VACWR050	E	Unknown	ND	7/16	43.8	[49,51]
F15L	VACWR054	E	Unknown	IAb	7/16	43.8	[57]
A3L	VACWR122	L	Structural	DP, DR, DQB1*0202	7/16	43.8	[49,51]
A46R	VACWR172	E	Virulence	DR1 predicted	8/20	40	[40;51]
F16L	VACWR055	E	Unknown	DR1 predicted	8/20	40	[40,51]
A17L	VACWR137	L	Structural	DR1 predicted	8/20	40	[40,51]

*Nomenclature according to COP.

‡Nomenclature according to VACWR.

[§] Based on [9]

[¶] Number of donors responding positive to antigen versus total number of donors that were tested compiled from all published studies.

[#] Percentage of donors responding (positive) to antigen divided by total number of donors tested.

[**] Only references containing positive responses are listed.

Ag: Antigen; COP: Copenhagen strain of vaccinia virus; E: Early; IE: Immediate-early; L: Late; ND: Not determined; VACWR: Western Reserve strain of vaccinia virus.

Table 5

Top 19 antigens (>20% of tested donors) recognized by B cells.

COP Ag name*	VACWR Ag name‡	Kinetics§	Function	Frequency		Ref.
				Positive/tested¶	*Positive (%)*#	
A10L	VACWR129	L	Structural	68/73	93.2	[43,44,45,51,65]
H3L	VACWR101	L	Structural	304/336	90.5	[39,43–45,51,55,63,65]
B5R	VACWR187	E/L	Structural	254/287	88.5	[43–45,51,55,63,64,65]
A33R	VACWR156	IE	Structural	113/155	72.9	[43–45,51,63,65]
A27L	VACWR150	L	Structural	227/336	67.6	[43–45,51,55,63,65]
A56R	VACWR181	E/L	Structural	99/155	63.9	[43–45,51,63,65]
No ortholog	VACWR148	L	Unknown	44/70	62.9	[44,45,51,65]
D8L	VACWR113	L	Structural	57/124	46	[39,43–45,51,64,65]
D13L	VACWR118	L	Structural	57/124	46	[39,43–45,51,64,65]
A13L	VACWR132	L	Structural	48/123	39	[39,43–45,51,65]
A11R	VACWR130	L	Unknown	28/74	37.8	[43–45,51,64,65]
I1L	VACWR070	L	Structural	46/124	37.1	[39,43–45,51,64,65]
B2R	VACWR184	IE	Unknown	27/74	36.5	[43–45,51,64]
L1R	VACWR088	L	Structural	64/205	31.2	[39,43–45,51,63,65]
A26L	VACWR149	L	Unknown	36/123	29.3	[39,43–45,51,65]
L4R	VACWR091	L	Regulation	21/73	28.8	[43–45,51,65]
F13L	VACWR052	L	Structural	20/73	27.4	[43–45,51,65]
A14L	VACWR133	L	Structural	33/124	26.6	[39,43–45,51,64,65]
E2L	VACWR058	E	Unknown	16/70	22.9	[44,45,51,65]

*Nomenclature according to COP.

‡Nomenclature according to VACWR.

§Based on [9]

¶Number of donors responding positive to antigen versus total number of donors that were tested compiled from all published studies.

#Percentage of donors responding (positive) to antigen divided by total number of donors tested.

Ag: Antigen; COP: Copenhagen strain of vaccinia virus; E: Early; E/L: Early/late; IE: Immediate-early; L: Late; VACWR: Western Reserve strain of vaccinia virus.

Table 6

Protection efficacy of $CD8^+$ epitopes

Epitope name	Epitope sequence	Survival (%)*
A23R (297–305)	IGMFNLTFI	100
A51R (78–85)	RISRFANL	100
A6L (265–272)	YTLIYRQL	100
A19L (47–55)	VSLDYINTM	100
A26L (257–264)	SIYQYVRL	93.3
L2R (53–61)	VIYIFTVRL	90
A8R (189–196)	ITYRFYLI	90
C4L (125–132)	LNFRFENV	90
J4R (24–32)	YAVINRNVL	90
B8R (20–27)	TSYKFESV	87
J3R (289–296)	SIFRFLNI	80
A8R (70–77)	IHYLFRCV	80
D13L (118–126)	NCINNTIAL	80
K2L (161–169)	WAIINTIYF	80
A3L (270–277)	KSYNYMLL	76.7
E7R (130–137)	STLNFNNL	73.3
F1L (200–207)	STREYLKL	70
G8R (34–41)	LMYIFAAL	70
D1R (578–586)	SMYCSKTFL	70
G6R (77–85)	YMLENIQVM	70
A47L (138–146)	AAFEFINSL	70
E9L (858–866)	RMNSNQVCI	66.7
A51R (310–318)	NLIRNRDYI	66.7
D1R (282–290)	LGYIIRYPV	60
B6R (108–116)	LMYDIINSV	53.3
A3L (191–199)	YSPSNHHIL	53.3
E11L (17–25)	FAIKNTDDV	53.3
A47L (93–101)	TMMINPFMI	53.3
A42R (88–96)	YAPVSPIVI	53.3
B2R (54–62)	YSQVNKRYI	50
B1R (92–99)	INVEYRFL	50
A18R (57–64)	TSLVFETL	50
K3L (6–15)	YSLPNAGDVI	46.7
F5L (279–287)	SAPMNVDNL	46.7
A10L (265–272)	FIYTYDRV	40
A38L (203–210)	KVFSFWLL	26.7

Epitope name	Epitope sequence	Survival (%)*
F13L (307–315)	FTIQNNTKL	26.7
J6R (993–1000)	INFEFVCL	20
N2L (60–68)	FLMMNKDEL	20
E8R (141–150)	FWFKNTQFDI	20
E ORF A (35–43)	STLSTQEAL	13.3
A2L (129–137)	EVVEIFKHL	10
C19L (77–85)	FNPSVLKIL	10
K ORF B (27–34)	IPLKYIVL	10
B16R (275–283)	ISVANKIYM	0
L2R (61–69)	LVSRNYQML	0
D12L (14–22)	VLLPFYETL	0
B12R (121–130)	VTMINTLEFI	0
M1L (291–300)	TSNVITDQTV	0

* Data modified from [100]