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Diverse recognition of conserved orthopoxvirus CD8+ T cell epitopes in vaccinated rhesus macaques

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Abstract

Vaccinia virus (VACV) induces a vigorous virus-specific CD8+ T cell response that plays an important role in control of poxvirus infection. To identify immunodominant poxvirus proteins and to facilitate future testing of smallpox vaccines in nonhuman primates, we used an algorithm for the prediction of VACV peptides able to bind to the common macaque MHC class I molecule Mamu-A*01. We synthesized 294 peptides derived from 97 VACV ORFs; 100 of these peptides did not contain the canonical proline at position three of the Mamu-A*01 binding motif. Cellular immune responses in PBMC from two vaccinia-vaccinated Mamu-A*01+ macaques were assessed by IFN γ ELISPOT assays. Vaccinated macaques recognized 17 peptides from 16 different ORFs with 6 peptides recognized by both macaques. Comparison with other orthopoxvirus sequences revealed that 12 of these epitopes are strictly conserved between VACV, variola, and monkeypoxvirus. ELISPOT responses were also observed to eight epitopes that did not contain the canonical P3 proline. These results suggest that the virus-specific CD8+ T cell response is broadly directed against multiple VACV proteins and that a subset of these T cell epitopes is highly conserved among orthopoxviruses.

Keywords

Vaccinia virus; T cell epitopes; non-human primates

INTRODUCTION

Although variola virus, the causative agent for smallpox, was eradicated in 1978, recent events have reawakened concerns that variola could be used as a biological weapon [1,2]. Over 100

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million Americans have been born since universal immunization ceased [1], and the waning immunity of the estimated 150 million Americans who were remotely vaccinated means that reintroduction of smallpox in the United States could lead to catastrophic consequences [3]. The smallpox vaccine used in the United States up until 2008, Dryvax, while highly effective in preventing disease caused by smallpox [1], has a significant incidence of adverse effects, which have prompted efforts to develop safer smallpox vaccines [4,5]. Rational development of improved smallpox vaccines will require a significant expansion of our understanding of what immune responses play a role in mediating protection induced by vaccinia vaccination, the specific virus proteins recognized by the host immune response, as well as testing of candidate vaccines in a relevant animal model.

Several observations suggest that cell-mediated immune responses play a significant role in the host response to poxvirus infection. Individuals with defects in cell-mediated immunity have suffered fulminant disease following vaccinia vaccination [6-8] whereas children with hypogammaglobulinemia could be vaccinated safely and effectively [9]. Cytotoxic T lymphocyte (CTL) activity and interferon- γ (IFN- γ) production by peripheral blood mononuclear cells (PBMC) have been described in response to VACV administration and have been found to persist for decades after vaccination [10-12]. In murine models, cellular immune responses also play an important role in survival from primary ectromelia challenge [13] and in the containment of sublethal [14] and lethal [15,16] vaccinia virus infection.

The macaque-monkeypoxvirus (MPV) model of human variola infection has emerged as a leading model to test novel vaccines and therapeutics against variola [17,18]. While there are significant differences between the manifestations of monkeypox in macaques and variola in humans, in the absence of circulating variola, there is no way to test efficacy of any new vaccine or biological in humans. We therefore sought to identify CD8+ T cell epitopes derived from VACV using rhesus macaques. Rhesus macaques (*Macaca mullata*) have been used in many vaccine [19] and immunological studies [20], and there are several well-described MHC-peptide binding motifs known [21]. Using a bioinformatics approach, we screened the entire predicted VACV strain Western Reserve (VACV-WR) proteome for peptides likely to bind the common rhesus macaque MHC I allele Mamu-A*01 and then tested 294 peptides for recognition by PBMC from two Mamu-A*01+ macaques that had been vaccinated with vaccinia virus. Our bioinformatic screening and *in vitro* binding assays suggested that a P3 proline was not required for binding to Mamu-A*01 [21], despite prior evidence that the P3 proline is an essential feature of the binding motif [22-24]. In order to test this hypothesis, we also included in our 294 peptides a subset of 100 predicted Mamu-A*01-binding peptides that did not contain a canonical P3 proline. Both animals developed a strong cellular immune response to VACV and recognized a considerable number of epitopes contained in a broad range of VACV ORFs. These results underscore the diversity of cellular immune responses against a large and complex pathogen.

MATERIALS AND METHODS

Cells and viruses

The New York City Board of Health (NYCBH) strain of vaccinia was obtained from Therion Biologics (Cambridge, MA) and replicated and titered in CV-1 cells maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 50 IU/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 10% non-heat-inactivated fetal bovine serum (FBS, Invitrogen). The modified vaccinia Ankara (MVA) strain was a gift of Dr. Mark Feinberg (Emory University, Atlanta, GA and Merck, West Point, PA) and grown and titered in DF-1 cells maintained in DMEM supplemented as above.

Rhesus macaques

All animals were housed at the New England Primate Research Center in a centralized animal biosafety containment facility and maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and the Guide for the Care and Use of Laboratory Animals [25]. Two adult rhesus macaques were studied. Both animals were tested and found to be free of simian retrovirus type D, SIV, simian T lymphotropic virus type 1, and herpes B virus prior to assignment. The animals were vaccinated by scarification in the interscapular region with Dryvax (Wyeth, Marietta, PA) using a bifurcated needle.

MHC class I sequence-based genotyping

The animals were typed by SSP-PCR [26,27] for the following MHC class I alleles: *A*01*, *A*02*, *A*03*, *A*04*, *A*05*, *A*06*, *A*07*, *A*08*, *A*11*, *A*13*, *NA4*, *NA7*, *B*01*, *B*03*, *B*04*, *B*07*, *B*12*, *B*17*, *NB2*, *NB4*, and *NB5*. Expanded MHC genotyping was performed at the Wisconsin National Primate Research Center (WNPRC) for confirmation. Total cellular RNA isolation, cDNA synthesis, MHC class I PCR, and bacterial cloning were performed as previously described [28,29]. Forty-eight *Mamu-A* locus and 144 *Mamu-B* locus colonies were selected, for a total of 192 per animal. Purified plasmid DNAs were sequenced unidirectionally using the primer 5'Refstrand, capturing sequence of the most polymorphic region (exons 2 and 3) of MHC class I transcripts [28]. Resulting sequences were compared to known *Mamu* MHC class I sequences.

Peripheral blood mononuclear cells

Heparinized venous blood was obtained by phlebotomy at serial time points after ketamine anesthesia. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over a Ficoll-sodium diatrizoate (Ficoll-Paque Pharmacia, San Diego, CA) gradient and washed twice in phosphate buffered saline (PBS, Ca^{2+} / Mg^{2+} -free, Cellgro/Mediatech, Fisher Scientific, Federal Way, WA). Residual erythrocytes were lysed in hypotonic ammonium chloride and fresh PBMC were washed in R-10 medium. For most experiments, freshly isolated PBMC were used. Where indicated, however, freshly isolated PBMC were cryopreserved in 90% FCS and 10% DMSO (Sigma-Aldrich, St. Louis, MO), and stored in liquid nitrogen. Immediately prior to use, cryopreserved PBMC were thawed rapidly in a 37°C water bath, gently mixed, washed with 37°C RPMI 1640 (Invitrogen) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 10% non-heat-inactivated fetal bovine serum (R-10 medium), washed again, and processed as described below.

Bioinformatic screening

The genomic sequence of VACV Western Reserve strain (VACVWR, Genbank accession number AY243312 [30]) was used to predict potential Mamu-A*01 epitopes. VACV-WR was derived from VACV-NYCBH [31] which is the strain of VACV found in the Dryvax vaccine; since the genomic sequence of VACV-NYCBH is not available, VACVWR provides the closest sequence and has been used in prior epitope mapping studies of humans vaccinated with Dryvax [32]. Each predicted ORF of VACV-WR was analyzed by using previously described algorithms [21,23,33]. Peptides predicted to bind with an $\text{IC}_{50} \leq 100$ nM were selected for study. These peptides were further screened manually for overall representation of the VACV-WR proteome, and 7 peptides with a predicted IC_{50} between 100-500 nM added, such that the 294 peptides chosen for *ex vivo* analysis of cellular immune responses represented 97 ORFs. These peptides were divided into those which contained the canonical proline at position 3 (P3, n=194, mean PIC_{50} =30 nM) and those which did not (non-P3, n=100, mean PIC_{50} =20 nM). The 100 non-P3 peptides were selected strictly on the basis of low PIC_{50} with no manual curation. Comparisons of identified Mamu-A*01 epitopes with orthologs in

vaccinia virus strain Copenhagen (Genbank accession number M35027 [34]), modified vaccinia Ankara (MVA, Genbank accession number U94848 [35]), variola virus strain Bangladesh (Genbank accession number L22579 [36]), and monkeypox virus strain Zaire (Genbank accession number AF380138 [37]) were performed using the Poxvirus Bioinformatics Resource Center (www.poxvirus.org [38]).

Peptides

Lyophilized peptides were synthesized by Pepscan (Amsterdam, Netherlands) using standard fluoronylmethyloxycarbonyl solid phase methods and reconstituted at a concentration of 10 mg/ml in 100% DMSO or 90% DMSO + 10% water with 1mM dithiothreitol for those peptides containing cysteine or methionine residues. The peptides were combined into pools containing 14 peptides each (P3 peptides) or 10 peptides each (non-P3 peptides) in sequential order such that each peptide was present in two pools and results could be analyzed via a matrix approach. For example, pool A for the P3 peptides contained peptides 1P through 14P, while matrix cross-pool AA contained 1P, 15P, etc. Peptide pools were diluted in R-10 media to a final concentration of 2 µg/ml and used in the initial screening for cellular immune responses in an IFN-γ ELISPOT assay.

MHC peptide binding assays

Quantitative assays to measure the binding affinity of both peptides containing the canonical P3 proline (P3 peptides) and those lacking this residue (non-P3 peptides) to purified MHC class I molecules are based on the competitive inhibition of binding of a radiolabeled standard peptide, and were performed as previously described [39-41]. Briefly, 0.1-1 nM of radiolabeled peptide was incubated at room temperature with 1 nM to 1 µM purified MHC class I molecule (Mamu-A*01, -A*02, -A*11, -B*01, or -B*17) in the presence of 1-3 µM human γ2-microglobulin (Scripps Laboratories, San Diego, CA) and a mixture of protease inhibitors. After a 2-day incubation, binding of the radiolabeled peptide to the given MHC I class molecule was determined by capturing MHC/peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-One, Monroe, NC) coated with the W6/32 monoclonal antibody, and measuring bound cpm using the TopCount microscintillation counter (PerkinElmer, Waltham, MA). For competition assays, the concentration of peptide yielding IC₅₀ of the binding of the radiolabeled peptide was calculated. Peptides were typically tested at six different concentrations covering a 100,000-fold dose range, and in three or more independent assays. Under the conditions used, where [label] < [MHC] and IC₅₀ ≥ [MHC], the measured IC₅₀ values are reasonable approximations of the true K_d values.

ELISPOT

ELISPOT assays were performed essentially as described with minor modifications [10,42, 43]. Briefly, PBMC were resuspended in R-10 medium at a density of 6×10^6 cells/ml and used as effector cells. For analysis of responses to virally infected cells, aliquots of pre-immune PBMC were infected with either NYCBH or MVA at a multiplicity of infection (MOI) of 1 pfu/cell. After 60 minutes incubation at 37°C, these cells were washed with RPMI 1640 to remove excess virus, resuspended in R-10, and incubated overnight and used as target cells. For analysis of responses to peptide pools, effector cells were stimulated overnight with pools diluted in R-10 to a final concentration of 2 µg/ml per peptide. For analysis of responses to individual peptides, peptides were diluted in R-10 to a final concentration of 2 µg/ml, and used to stimulate effector cells overnight. Effector cells were plated in duplicate at 3×10^5 and 1.5×10^5 cells per well in a 96-well ELISPOT plate (Millipore, Bedford, MA) that was pre-coated with an anti-IFN-γ monoclonal antibody (clone GZ-4, Mabtech, Cincinnati, OH). Target cells were added at effector:target cell ratios of 2:1 and 10:1 in duplicate wells. Cells were incubated for 16 hours at 37°C after which time the cells were removed, the plate washed 6 times with

PBS, and a biotinylated detector anti-IFN- γ monoclonal antibody (clone 7-B6-1, Mabtech) added. After two hours of incubation at room temperature, the plate was washed 6 times with PBS, and a streptavidin-alkaline phosphatase complex (Mabtech) added. After one hour incubation at room temperature, the plate was washed 6 times, and AP developing solution (Bio-Rad, Hercules, CA) was added. The plates were washed three times with distilled water after spots appeared in positive control wells (PBMC stimulated with 5 μ g/ml Concanavalin A, Sigma) and allowed to dry at room temperature overnight in the dark. Spots were counted on an automated ELISPOT counter (Zellnet Consulting, Fort Lee, NJ) and quantified as spot-forming cells (SFC) per 1×10^6 PBMC. Background IFN- γ secretion was measured in wells containing effector cells and R-10 medium only and subtracted from all experimental wells. SFC are reported based on wells with 3×10^5 effector cells and wells with an E:T ratio of 2:1 as these conditions had the best sensitivity; wells with lower effector cell concentrations and E:T ratios of 10:1 were used to confirm reactivity.

Thresholds for the evaluation of positive ELISPOT responses were established by examining responses to vaccinia-derived peptides in naïve animals. For P3 proline-containing peptides, responses in 5 naïve macaques (2 Mamu-A*01+, 3 Mamu-A*01-) were 6.1 ± 9.6 SFCs/ 10^6 PBMC (mean $\pm$ standard deviation). As the mean plus three times the standard deviation was 35 SFCs/ 10^6 PBMC, we set our threshold at 40 SFCs/ 10^6 PBMC. Similarly, ELISPOT responses to the non-P3 proline-containing peptides in 4 naïve animals (1 Mamu-A*01+, 3 Mamu-A*01-) were 6.2 ± 10.7 SFCs/ 10^6 PBMC. As the mean plus three times the standard deviation was 38 SFCs/ 10^6 PBMC, we set our threshold at 40 SFCs/ 10^6 PBMC.

Statistics

Data are reported as the mean $\pm$ standard error of the mean. Chi-square (χ^2) analysis of the predicted or known function of the VACV ORFs which were recognized by our vaccinated animals compared with the ORFs which were not recognized was performed in Prism 5.01 (GraphPad Software, San Diego, CA)

RESULTS

Vigorous and sustained cellular immune responses to vaccination

Two macaques (353.99 and 415.01) expressing the *Mamu-A*01* class I allele were selected for this study; approximately 24% of captive rhesus macaques in American primate research colonies express this allele [21]. Typing of both animals by SSP-PCR using a panel of 20 other rhesus macaque MHC alleles revealed that both of the macaques also expressed *Mamu-A*02* and one macaque (353.99) expressed *A*1302*. Expanded MHC genotyping performed at WNPRC revealed that animal 415.01 expressed *Mamu-B*4002*, *B*460101*, *B*5002*, *B*5101*, *B*6002*, *B*6501*, and *B*6901* while animal 353.99 expressed *B*010101*, *B*0702*, *B*3001*, *B*5201*, *B*5501*, *B*5802*, and *B*7201*; other than *Mamu-A*01* and *A*02*, these animals shared no other A or B alleles. Both vaccinated animals developed typical “Jennerian take” reactions with papules progressing to pustules followed by rupture and healing over a 21 day period. At day 14 following vaccination, *ex vivo* IFN γ ELISPOT responses to the vaccine strain of virus (NYCBH) and the attenuated MVA strain were quite strong in both animals (Figure 1), with a mean response of 2153.5 ± 706.5 SFCs/ 10^6 PBMC when target cells were infected with NYCBH and 2610 ± 910 SFCs/ 10^6 PBMC when target cells were infected with MVA. Background responses in six naïve macaques (including baseline pre-vaccination responses in both our vaccinated animals) were 33 ± 21 and 119 ± 71 SFCs/ 10^6 PBMC for NYCBH and MVA respectively. Both animals maintained relatively strong cellular immune responses to NYCBH and MVA over a period of 60 weeks following vaccination (Figure 1).

Identification of T cell responses to predicted VACV epitopes

Using previously described algorithms [21,23,33], we selected a total of 194 predicted Mamu-A*01-restricted T cell epitopes containing the canonical Mamu-A*01 proline at position 3 in the VACV-WR proteome. Peptides were chosen based on having a predicted $IC_{50} \leq 100$ nM and for overall representation of the VACV-WR proteome as described in more detail above. The sequences of these peptides, along with predicted and measured IC_{50} binding values to Mamu-A*01, are available as Supplemental Table 1. At 14 days following infection, freshly isolated PBMC from the vaccinated animals were screened for $IFN\gamma$ secretion in response to overnight stimulation with 28 pools of P3 proline-containing peptides. Figure 2A illustrates the breadth and diversity of responses amongst the animals to pools of peptides containing a P3 proline. Of the 28 pools tested, four pools were recognized by both macaques, and ten pools were recognized by cells isolated from one of the macaques. These responses accounted for a sizable fraction of the response to the whole virus, ranging from 3.7% to 10.6% of the total response to target cells infected with NYCBH.

All pools which resulted in a positive $IFN\gamma$ ELISPOT response in at least one macaque were deconvoluted, and responses to the individual P3 peptides were assessed in freshly isolated PBMC from both vaccinated animals at day 21 post-vaccination. Figure 2B illustrates that nine individual peptides were recognized above the threshold frequency (accounting for between 1.5% and 11.0% of the total response to NYCBH-infected target cells), and two of these epitopes (50P and 150P) were recognized by both macaques. For these two immunodominant peptides, both animals maintained relatively strong cellular immune responses to peptides 50P and 150P over at least 52 weeks following vaccination.

Predicted Mamu-A*01-binding peptides that do not contain the canonical P3 proline are presented *in vivo* and are immunogenic

Based on *in vitro* and *in silico* evidence that peptides lacking the canonical proline at position 3 could bind to Mamu-A*01 [21], we tested the hypothesis that the Mamu-A*01-restricted cellular immune response to VACV vaccination included non-P3 proline epitopes. A total of 100 predicted non-P3 proline epitopes were selected; the peptide sequences and predicted and measured IC_{50} binding values are shown as Supplemental Table 2. Freshly isolated PBMC from both vaccinated animals were screened for $IFN\gamma$ secretion in response to overnight stimulation with 20 pools of peptides that did not contain the canonical proline of Mamu-A*01-restricted epitopes at position 3. Both macaques 353.99 and 415.01 were tested 15 months following vaccination. Nine of twenty pools were recognized by PBMC from at least one animal (Figure 3A) and one pool was recognized by cells from both macaques. These responses again accounted for a considerable fraction of the response to the whole virus, ranging from 3.1% to 8.9% of the total response to target cells infected with NYCBH.

All non-P3 proline peptide pools that resulted in a positive ELISPOT response in at least one macaque were deconvoluted, and responses to individual peptides were assessed in freshly isolated PBMC from both vaccinated animals 15 months post-vaccination. Eight individual non-P3 peptides were recognized above the threshold frequency by at least one macaque. All eight of these non-P3 peptides were recognized by macaque 353.99 and four of these were also recognized by animal 415.01. As with the P3 proline-containing epitopes, the epitope-specific responses accounted for a significant percentage of the total cellular immune response, ranging from 5.4% to 13.2% of the $IFN\gamma$ ELISPOT response to NYCBH-infected target cells.

In vitro binding assays confirm that peptides predicted to bind Mamu-A*01 by a bioinformatic algorithm generally bind with high avidity

To confirm that the peptides identified by our bioinformatic algorithm bound to Mamu-A*01, we performed *in vitro* binding assays. The measured IC_{50} values for the 29 VACV-derived

epitopes we identified *ex vivo* are presented in Table 1, and the values for the remaining peptides are available as Supplemental Tables 1 and 2. To evaluate the specificity of binding, we also measured *in vitro* binding to Mamu-A*02, -A*11, -B*01, and -B*11. As shown in Table 1, most peptides containing the canonical P3 proline bound very poorly to MHC class I molecules other than Mamu-A*01. Surprisingly, three of the P3 peptides (5P, 39P, and 51P) also bound to Mamu-A*02 with an $IC_{50} < 500$ nM, although all three bound to Mamu-A*01 with higher affinity. None of the peptides tested bound to Mamu-A*11 or -B*17 *in vitro* and only 86P bound to Mamu-B*01 with an $IC_{50} < 500$ nM; neither of our animals expressed *Mamu-A*11*, *-B*01*, or *-B*17*, however these data demonstrate that the *in silico* binding predictions are specific, as overall the peptides have a much higher affinity for A*01 than for any other allele for which *in vitro* binding assays were available. Several of the peptides lacking the P3 proline bound to Mamu-A*02 *in vitro* with an $IC_{50} < 500$ nM; however, most of these peptides bound to Mamu-A*01 more avidly than Mamu-A*02.

Mamu-A*01-restricted vaccinia virus-derived epitopes are generally conserved across the pathogenic orthopoxviruses

We compared these VACV WR-derived peptides with their orthologs in the reference strain vaccinia Copenhagen as well as the pathogenic viruses monkeypox virus strain Zaire and variola virus strain Bangladesh and the candidate vaccine MVA (Table 2). Overall, twelve of 17 epitopes are strictly conserved between vaccinia, variola, and monkeypox. Examination of the expression pattern of the identified ORFs (Table 2) revealed that ten are definitely or likely expressed early in the viral life cycle while 16 are definitely or likely expressed late in the viral life cycle (totals exceed 16 ORFs as several ORFs are expressed throughout the VACV life cycle). Interestingly, none of the immunogenic ORFs that we identified appear to be expressed exclusively during the early phase of viral replication in contrast to previous findings in humans [32,44] and mice [45,46]. Notably, nine of 16 identified ORFs are found in VACV virions, five encode transcription or replication proteins, one is thought to encode an immunomodulatory protein, and one has no known function. This predominant recognition of virion proteins is significant ($p=0.0266$ by χ^2) compared with the composition of the entire VACV proteome. Of the 17 Mamu-A*01-restricted epitopes we identified, six were recognized by both vaccinated macaques. In total, we identified 16 VACVWR-derived ORFs as immunogenic; two of these ORFs have not previously been reported to be targeted by either humoral or cellular immune responses in other models of orthopoxvirus infection (Table 2). The total IFN γ ELISPOT response to these 17 epitopes accounted for a sizable percentage of each animal's total response to NYCBH infected target cells, ranging from 51.7% to 58.8%.

Avidity of Mamu-A*01-restricted epitopes

Analysis of T cell specificity using relatively high concentrations of peptides can occasionally lead to identification of cross-reactive epitopes of uncertain significance [47]. Moreover, animal model experiments suggest that higher avidity T cells may have greater *in vivo* antiviral activity than lower avidity T cells [48]. Dilution experiments were therefore performed with a subset of the immunodominant epitopes, and representative data are shown in Figure 4. The 50% maximum response for peptides #50P and #150P was approximately 20 ng/ml (approximately 20 nM), consistent with other reported Mamu-A*01-restricted epitopes [22].

DISCUSSION

This report highlights three important findings. First, the cellular immune response to vaccinia vaccination recognizes a diverse range of T cell epitopes and thus targets a large number of viral proteins. Second, a bioinformatic approach can quickly and efficiently identify a large number of CD8+ T cell epitopes in a large, complex pathogen such as vaccinia virus. Third, many of the proteins recognized by the cellular immune response in vaccinia-vaccinated

macaques have previously been identified as targets of the immune response in other model systems.

Recently, several groups have identified a number of CD8+ T cell epitopes induced by vaccination with VACV [16,32,41,44-46,49-55]. Approaches used have included bioinformatic prediction of likely epitopes based on known MHC-peptide binding motifs [32,41], expression library [44,50] approaches and mass spectrometry [56]. These efforts have identified over 100 CD8+ T cell epitopes in mice and more than 75 epitopes in humans; most of these epitopes have tended to be relatively conserved across the orthopoxviruses. While the first eight epitopes identified in inbred strains of mice accounted for a considerable fraction of the total response to VACV vaccination, suggesting that the cellular immune response might be focused on a few immunodominant epitopes [45,50], a follow-up study identified a total of 49 epitopes in C57BL/6 mice, which accounted for nearly all the total response [57]. In contrast, the targets of VACV-specific CD8+ T cells in humans appear to be quite diverse, with at least 47 different ORFs identified as immunogenic thus far [32,49,51,53,58,59].

Our work suggests that the CD8+ T cell response to vaccinia vaccination in macaques closely resembles that of humans and is similarly broadly directed against multiple viral proteins. In light of recent findings that the human neutralizing antibody response to VACV vaccination is heterogeneous and apparently multiply redundant with respect to antigenic targets [60], it is likely that the total T cell response to VACV in humans and macaques is also quite diverse and not directed against a narrow set of immunodominant proteins. This diversity is underscored by the recent identification of 122 ORFs recognized by CD4+ T cells using a whole-proteome approach in vaccinated humans [61]. It has been suggested that this vast breadth of antigenic targets may be a key attribute of VACV vaccination and may be responsible for the potent vaccine efficacy against disease caused by pathogenic orthopoxviruses [60].

As has been reported in humans, many of the targets of the rhesus macaque CD8+ T cell response are relatively conserved across the orthopoxviruses, and twelve of the 17 epitopes are strictly conserved across VACV, MVA, variola, and MPV. Of the 16 ORFs identified as immunogenic in our animals, two have not previously been shown to be the targets of either humoral or cellular immune responses. Of the 14 ORFs which have been previously found to be immunogenic in humans or mice, only one has been described as immunoprevalent [46], that is, frequently immunogenic in the context of different MHC alleles and across species. Interestingly, there was a dramatic excess of immunogenic ORFs which are predicted (by promoter sequence) or have been demonstrated experimentally to be expressed late in the viral life cycle (Table 2). Our results contrast strikingly with Oseroff *et al* [32,46] and Jing *et al* [44] who noted a preponderance of epitopes derived from VACV genes expressed early in the viral life cycle. It has been suggested that this seeming preference for early genes is due to efficient presentation of early viral peptides by VACV-infected dendritic cells (DC), while the apparent block in late viral gene expression in DC [62] prevents T cell priming [63,64]. As vaccinated macaques predominately respond to peptides derived either from late VACV genes or genes expressed throughout the viral life cycle, it may be that macaque DC are more permissive for late viral gene expression than human DC, although future studies with additional vaccinated macaques will be necessary to confirm this finding. This differential peptide presentation by DC from different species may also explain the apparent bias for late genes found in a study of VACV-vaccinated HLA transgenic mice [41]. Some of these ORFs may be differentially regulated amongst the orthopoxviruses: for example VACV-WR ORF 065 (the ortholog of E9L) is expressed early [65-67] while the monkeypox ortholog F8L was found to be expressed late [67]. The temporal expression patterns of orthopoxvirus genes are an ongoing area of investigation and, in some cases, studies have occasionally found discrepant results [66,67]. Also of note, over half the immunogenic ORFs (9 of 16) we identified are found

in VACV virions. The significance of this finding with respect to CD8+ T cell responses is not known, whereas an apparent bias toward CD4+ T cell recognition of virion proteins [61] might be expected if CD4+ T cell responses were linked to B cell recognition of viral antigens as has been reported [68].

As MVA has been proposed as a safer, new generation smallpox vaccine [4], it is notable that twelve of 17 epitopes elicited in our vaccinated animals are identical between VACV and MVA. There may, however, be important differences in the immune responses generated by traditional scarification with replication-competent VACV compared with replication-defective MVA, which is usually given by the intramuscular route. In some murine models, the route of poxvirus vaccination has had a significant impact on the hierarchy of immunodominance [45,50], although a subsequent study found little difference [46].

Our approach likely only identifies a small fraction of the total cellular immune response to VACV vaccination. We restricted our analysis to one rhesus macaque MHC class I molecule and only assessed responses in two vaccinated macaques to 294 potential epitopes identified by our bioinformatic algorithm. It is likely that there are additional Mamu-A*01-restricted epitopes, including subdominant epitopes as reported in a murine model [55], as well as a large number of epitopes presented by other macaque MHC molecules. Interestingly, however, when compared to the IFN γ ELISPOT response to NYCBH-infected target cells, the total response to these 17 epitopes accounted for approximately half the response to the virally-infected cells. Similar results were seen in a comprehensive study of human epitope-specific responses to Dryvax vaccination in which the 48 epitopes identified were responsible for a high fraction (up to 60.9%) of the response to VACV-WR-infected target cells [32]. Another group found that epitope-specific PBMC from one donor exceeded that individual's response to VACV-infected target cells [59]. This observation suggests that using live VACV-infected target cells is likely to underestimate the total orthopoxvirus-specific cellular immune response. In vaccinia-vaccinated humans and macaques, we have found that IFN γ ELISPOT responses to MVA-infected cells consistently exceed that observed to vaccinia-virus infected cells, despite the fact that the MVA genome has fewer predicted ORFs than VACV-WR (163 versus 223 respectively); this discrepancy may well be due to VACV immune evasion mechanisms (Walsh and Johnson, manuscript in preparation). Nonetheless, these epitopes could prove useful in future measurements of immunogenicity following administration of novel smallpox vaccines to Mamu-A*01+ rhesus macaques.

Since the identification of Mamu-A*01-restricted epitopes, several groups have highlighted the apparent necessity of a proline residue at position 3 [22,23]. Recently, the crystal structure of Mamu-A*01 complexed with the SIV Gag CM9 and Tat TL8 epitopes was resolved [24] and it was found that the P3 proline interacts with a hydrophobic pocket in the Mamu-A*01-peptide binding cleft (comprised of Tyr9, Arg97, Tyr159, Val99, and Met156). While these authors argue that this pocket requires a P3 proline for peptide binding [24], *in vitro* binding studies have demonstrated that peptides lacking a P3 proline can nonetheless bind to Mamu-A*01 molecules [21]. In fact of a set of 111 SIV-derived peptides predicted to bind Mamu-A*01 with a IC₅₀ < 500 nM, 31 of 59 that were found to bind *in vitro* did not have the canonical P3 protein [21].

Using the same bioinformatic algorithms, we identified a set of VACV-WR-derived peptides that were predicted to bind Mamu-A*01 and tested these for both *ex vivo* recognition using the IFN γ ELISPOT (Figures 2 and 3) and *in vitro* binding (Table 1 and Supplemental Tables 1 and 2). We tested 194 peptides with a canonical P3 proline and 100 peptides without this residue. The success rate of epitope identification was similar between the two sets (4.6% vs 8%), suggesting that a P3 proline is not strictly required for presentation by Mamu-A*01 *in vivo*. As we tested PBMC isolated from both our animals 15 months following vaccination, our *ex*

vivo ELISPOT assays may have missed a large number of low frequency non-P3 proline epitopes which might have been detectable soon after vaccination. Our *in vitro* binding assays suggest that predicted peptides that do not contain the canonical P3 proline do bind avidly to Mamu-A*01 (Table 1 and Supplemental Table 2), but may also bind other macaque MHC class I molecules, notably Mamu-A*02. It is possible that, *in vivo*, the VACV-derived peptides we have identified may, in some cases, be presented in the context of other MHC class I molecules in addition to Mamu-A*01. Recent findings suggest that promiscuous epitope presentation may be a widespread phenomenon, as Frahm *et al* identified an extensive number of HIV-1 derived epitopes which were presented by HLA molecules in addition to the originally described restricting molecule [69]. Additional experiments will be necessary to rigorously demonstrate the MHC restriction of these peptides, especially for the subset of peptides that do not contain the canonical P3 proline, as our dataset does not allow us to definitively assign MHC class I restriction with certainty.

Use of our bioinformatic algorithm to probe the VACV-WR proteome generates more potential Mamu-A*01-restricted epitopes without a P3 proline than containing a proline residue at position three. We tested only the 100 non-P3 proline peptides with the highest predicted binding affinity; these peptides therefore have residues optimal for binding at all secondary anchor positions. Further testing of additional potential non-P3 proline epitopes derived from other viruses, such as SIV, lymphocryptovirus and rhesus cytomegalovirus, is required to confirm that this is a generalizable finding, and not a phenomenon unique to VACV vaccination, as well as confirm that non-P3 proline-containing peptides can be presented *in vivo* by Mamu-A*01.

In conclusion, a bioinformatic algorithm allowed us to efficiently screen the VACV proteome and identify a large number of immunogenic ORFs. Two of these immunogenic ORFs have not previously been shown to be the targets of either humoral or cellular immune responses in humans or in murine models of orthopoxvirus infection and twelve of the 17 epitopes identified herein are strictly conserved across the pathogenic orthopoxviruses. Our results suggest that the virus-specific CD8⁺ T cell response is broadly directed against multiple VACV proteins in vaccinated rhesus macaques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

IFN γ ELISPOT responses in VACV-vaccinated macaques. PBMC isolated from vaccinated macaques at indicated timepoints following Dryvax vaccination were stimulated overnight with autologous PBMC infected with either VACV strain NYCBH or MVA at an MOI of 1.0 pfu/cell at an effector:target ratio of 2:1. IFN γ -secreting cells were quantitated and background (secretion in medium alone) subtracted. Cryopreserved PBMC were used for all time points except day 14.

Figure 2.

Cellular immune responses to VACV-derived peptides containing the canonical Mamu-A*01 motif with a proline at position 3. (A) PBMC from macaques vaccinated 14 days previously were stimulated overnight with pools containing 10-14 peptides each at a final concentration of 2 $\mu\text{g/mL}$. The dashed line indicates the background threshold of 40 SFC per 10^6 PBMC. (B) PBMC freshly isolated from macaques vaccinated 21 days previously were stimulated overnight with single peptides corresponding to the positive pools in panel 2A. Each peptide was present at a final concentration of 2 $\mu\text{g/mL}$. (C) Longitudinal responses to peptides 50P (LSPETLVGV) and 150P (YLPLSVFII) were analyzed. Cryopreserved PBMC were used for all time points except day 21.

Figure 3.

Cellular immune responses to VACV-derived peptides that do not contain a canonical proline at position 3. **(A)** PBMC freshly isolated from macaques vaccinated 15 months previously were stimulated overnight with pools containing 10 peptides each at a final concentration of 2 $\mu\text{g}/\text{mL}$. The dashed line indicates the background threshold of 40 SFC per 10^6 PBMC. **(B)** 15 months previously were stimulated overnight with single peptides corresponding to the positive pools from macaque 313.97 in panel 3A. Each peptide was present at a final concentration of 2 $\mu\text{g}/\text{mL}$.

Figure 4.

Avidity of IFN γ ELISPOT responses to immunodominant VACV epitopes. PBMC isolated from macaque 353.99 12 months following vaccination were stimulated overnight with decreasing concentrations of peptide 50P (LSPETLVGV) and 150P (YLPLSVFII) to determine the 50% maximum response to these two P3 proline-containing epitopes.

Table 1

In vitro binding of Mamu-A*01 epitopes. VACV-derived peptides recognized by PBMCs in *ex vivo* IFN- γ ELISPOT assays were tested for *in vitro* binding to common rhesus macaque MHC class I molecules. IC₅₀ values are reported in nM. IC₅₀ < 500 nM are considered potential epitopes. (-) - no binding detected.

Peptide ¹	AA Sequence ²	VV-Cop ORF	P3	Recognized by	A*01 ³	A*02	A*11	B*01	B*17
47P	YDPVLMFLLF	E8R	Yes	353.99	0.29	528	-	-	-
50P	LSPETLVGV	E9L	Yes	both	1.35	9217	-	-	-
51P	SSPLELFML	E9L	Yes	415.01	0.94	70	15182	10193	11777
65P	LSPILAEEL	L1R	Yes	353.99	0.46	8495	-	-	-
73P	LTPEQKAYV	L1R	Yes	415.01	22	-	-	-	-
83P	KTPVIVVPV	H3L	Yes	353.99	0.65	-	44362	30067	-
86P	IIPKIKAYL	H4L	Yes	353.99	0.07	-	670	302	-
148P	SVPAAIMMI	A34R	Yes	415.01	229	-	-	-	-
150P	YLPLSVFII	A37R	Yes	both	0.08	1255	24491	-	-
9NP	QTIVHWFH	A14.5L	No	both	514	40	23993	7183	15454
63NP	VTVAIYISM	A13L	No	both	13	137	-	-	-
68NP	LTREMGLV	A29L	No	353.99	3217	68	5401	-	-
71NP	LTIEAIENYF	L4R	No	both	2.31	13	10512	975	8773
75NP	KTSTLIFFV	H2R	No	both	7.44	20	1204	6463	13271
81NP	PTSPLAYF	I7L	No	353.99	73	1257	-	-	-
83NP	RTIILVGYM	F12L	No	353.99	1673	9.43	-	24721	-
89NP	LTLDIFYLF	A39R	No	353.99	1.20	4.35	14033	7985	4461

¹ Peptide number includes annotation for P3 (P) and non-P3 (NP) proline-containing peptides

² VV-WR sequence used to generate peptides

³ Measured IC₅₀ reported in nM

Comparison of Mamu-A*01-restricted epitopes identified with orthologous sequences from variola and monkeypoxviruses. Sequence data was obtained from www.poxvirus.org (27) and nomenclature of genes based on vaccinia strain Copenhagen (20), modified vaccinia Ankara (4), variola major strain Bangladesh (28), and monkeypox strain Zaire (37)

Table 2

Peptide ¹	AA Sequence ²	VV-Cop ORF	MVA	Variola	Monkeypox	Expression Pattern ³	ORF Immunogenic?		Reference
							Humans	Mice	
50P ⁴	LSPETLVGV	E9L	056L	E9L	F8L	EARLY/late	CD8 ⁺ & CD4 ⁺	CD8 ⁺ , CD4 ⁺ & Antibodies	[32,41,46,54,65-67,70,71]
71NP ⁴	LTTEAIENYF	L4R	083R	M4R	M4R	LATE	CD4 ⁺ & Antibodies	CD4 ⁺ & Antibodies	[61,66,68,70-73]
63NP ⁴	VTVAILYSM	A13L	124L - VTVAILYAL	A14L - VTVAILYAM	A14L - VTVAILYTL	LATE	Antibodies	Antibodies	[60,66,67,73]
150P ⁴	YLPLSVFII	A37R	148R - YSPLSVFII	truncated	A39R	IMMEDIATE	NR ⁵	NR	[66,67]
9NP ⁴	QTVFIWFI	A14.5L	125.5L	A14.5L	A15.5L	early/LATE	NR	NR	[67]
68NP ⁶	LTREMGLV	A29L	140L	A32L	A13L	EARLY/late	CD4 ⁺	NR	[61,66,67,74]
89NP ⁶	LTLDIFYLF ⁷	A39R	NO ⁸	A42R/A43R ⁹	NO	LATE	CD4 ⁺	NR	[61,66,67]
83NP ⁶	RTHLVGYM	F12L	042L	C16L	C18L	EARLY/LATE	CD8 ⁺	Antibodies	[32,41,44,54,66,67,75]
81NP ⁶	PTSIPLAYF	I7L	068L	K7L	I7L	LATE	CD4 ⁺	NR	[61,66,67,76]
75NP ⁴	KTSTLIFFV	H2R	092R	I2R	H2R	LATE	CD4 ⁺ , antibodies	NR	[60,61,71]
83P ⁶	KTPVIVVPV	H3L	093L	I3L	H3L	EARLY/LATE	NAbs ¹⁰ , CD4 ⁺ , & CD8 ⁺	NAbs & CD4 ⁺	[41,49,60,61,66-68,70,71,73,77,78]
148P ¹¹	SVPAAIMMI	A34R	145R - SVPV ₁ AIMMI	A37R	A36R: SVPAAIMM ₁ L	early/LATE	CD8 ⁺ & CD4 ⁺	NR	[41,61,66,67,71]
73P ¹¹	LTPEQKAYV	L1R	080R	M1R	M1R	LATE	NAbs, CD4 ⁺ , & CD8 ⁺	NAbs	[60,61,66,67,71,79-81]
51P ¹¹	SSPLELFML	E9L	056L	E9L	F8L	EARLY/late	CD8 ⁺ & CD4 ⁺	CD4 ⁺ & Antibodies	[32,41,46,54,65-67,70,71]
47P ⁶	YDPVLMFLLF	E8R	055R	E8R	F7R	EARLY/LATE	CD4 ⁺	NR	[67,76,82]
65P ⁶	LSPLAEEL	I8R	069R	K8R	074	EARLY/INTERMEDIATE/late	CD4 ⁺	NR	[46,61,66,67,71,76,83,84]
86P ⁶	IIPKIKAYL	H4L	094L	I4L	H4L: IIPKIKSYL	early/LATE	CD4 ⁺	NR	[46,61,66,67,76]

¹ Peptide number includes annotation for P3 (P) and non-P3 (NP) proline-containing peptides

² VV-WR sequence used to generate peptides

³ Expression pattern in capitalized letters when published experimental data exist, small letters when predicted based on promoter sequence

⁴ peptide recognized by PMBC from 415.01 and 353.99

⁵ NR - not reported

⁶ peptide recognized by PMBC from 353.99

⁷ Epitope found in leader sequence only present in VV-WR ORF 163

⁸ Ortholog fragmented into 2 genes in MVA strains Acambis 3000 (ORF 152 and ORF 153) and MVA-1721 (ORF 152 and ORF 153)

⁹ Ortholog fragmented into 2 genes in variola

¹⁰ NAb_s - neutralizing antibodies

¹¹ peptide recognized by PMBC from 415.01