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

Stephen R. Walsh[a,b], Jacqueline Gillis[b], Björn Peters[c], Bianca R. Mothé[d], John Sidney[c], Alessandro Sette[c], R. Paul Johnson[a,b,*]

[a] Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114, United States
[b] Division of Immunology, New England Primate Research Center, Harvard Medical School, One Pine Hill Drive, Southborough, MA 01772, United States
[c] La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121, United States
[d] California State University, 333 South Twin Oaks Valley Road, San Marcos, CA 92096, United States

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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 non-human 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 IFNγ 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.

1. 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 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 (IFNγ) 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

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* Corresponding author at: New England Primate Research Center, Harvard Medical School, Southborough Campus, Pine Hill Drive, Southborough, MA 01772, United States. Tel.: +1 508 624 8148.
E-mail address: paul_johnson@hms.harvard.edu (R.P. Johnson).

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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.

2. Materials and methods

2.1. 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 μg/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.

2.2. 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 inter-scapular region with Dryvax (Wyeth, Marietta, PA) using a bifurcated needle.

2.3. 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.

2.4. Peripheral blood mononuclear cells

Heparinized venous blood was obtained by phlebotomy at serial time points after ketamine anesthesia. Peripheral blood mononuclear cells 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.

2.5. Bioinformatic screening

The genomic sequence of VACV Western Reserve strain (VACV-WR, 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, VACV-WR 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 $IC_{50} \leq 100$ 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 and 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]).

2.6. 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 1 mM 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 IFNγ ELISPOT assay.