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Human Cytotoxic CD4⁺ T Cells Recognize HLA-DR1-Restricted Epitopes on Vaccinia Virus Proteins A24R and D1R Conserved among Poxviruses¹

Shibani Mitra-Kaushik,* John Cruz,* Lawrence J. Stern, † Francis A. Ennis,* and Masanori Terajima**

We previously demonstrated that vaccinia virus (VV)-specific CD4⁺ cytolytic T cells can persist for >50 years after immunization against smallpox in the absence of re-exposure to VV. Nevertheless, there have been few studies focusing on CD4⁺ T cell responses to smallpox vaccination. To ensure successful vaccination, a candidate vaccine should contain immunodominant CD4⁺ T cell epitopes as well as CD8⁺ T cell epitopes. In the present study, we established cytotoxic CD4⁺ T cell lines from VV-immune donors, which recognize epitopes in VV proteins D1R and A24R in association with HLA-DR1. Comparisons of sequences between different members of the poxvirus family show that both epitopes are completely conserved among VV, variola viruses, and most mammalian poxviruses, including monkeypox, cowpox, and ectromelia. The CD4⁺ T cell lines lysed VV-infected, Ag- and peptide-pulsed targets, and the lysis was inhibited by concanamycin A. We also detected these peptide-specific cytolytic and IFN-γ-producing CD4⁺ T cells in short-term bulk cultures of PBMC from each of the three VV-immune donors tested. These are the first VV-specific CD4⁺ T cell epitopes identified in humans restricted by one of the most common MHC class II molecules, HLA-DR1, and this information may be useful in analyzing CD4⁺ T cell responses to pre-existing or new generation VV vaccines against smallpox. The Journal of Immunology, 2007, 179:1303–1312.

Worldwide immunization has successfully eradicated smallpox infections. Bioterrorism threats have brought a renewed interest in smallpox and its significant potential as a biological weapon. Of concern is the fact that a large majority of the population would be susceptible to smallpox infection, because routine smallpox vaccination was eliminated in the 1970s, when the disease was eradicated worldwide (1). The standard smallpox vaccines were effective, but had rare severe side effects, including myocarditis and encephalitis (2–4). A new generation of safer smallpox vaccines is being developed, and it is important to test and establish the safety and robustness of immunological responses to these new vaccines.

Both humoral and cellular immunity have been thought to play a role in protection against and recovery from orthopoxvirus infections as evidenced in individuals with either humoral or cellular immune defects who were unable to control vaccinia virus (VV)³ infections (5, 6). A number of studies indicate Ab responses are long-lived and essential for protection against orthopoxviruses (7–11). Several studies have analyzed the role of different cytokines during infection with VV: some cytokines increased virulence, as with IL-4 (12, 13), whereas others attenuated the infection, as with IFN-γ or IL-2 (14–20). Both NK cells and γδT cells have been shown to play a role in innate resistance to VV infection (21, 22). VV elicits a robust and long-lasting cellular immune response that includes large numbers of CD4⁺ and CD8⁺ T cells (23). CD8⁺ CTLs are crucial in controlling primary and secondary VV infections in mice and humans (24). VV-specific CD4⁺ T cells play an important role in expanding CD8⁺ T cell populations, as evidenced by the failure of control of virus replication in a mouse model with CD4⁺ T cell-depleted and MHC class II-knockout mice (25). Several laboratories including ours have identified an increasing number of CD8⁺ CTL epitopes in human (26–34) and murine systems (35–38).

We earlier demonstrated that VV-specific CD4⁺ T cells can persist for up to 50 years after immunization of humans against smallpox in the absence of re-exposure to VV (39), and that several CD4⁺ CTL clones were able to recognize and kill VV-infected targets (40). There have been a few studies focusing on CD4⁺ T cell responses to smallpox vaccination (41, 42). Hammarlund et al. (8) measured T cell immunity against VV in 306 vaccinees, up to 75 years after their last vaccination. Within the first 7 years after vaccination, CD4⁺ and CD8⁺ T cell responses remained high and then declined slowly over decades. The role of CD4⁺ T cell in protection in VV-immunized individuals is still poorly characterized. Studies of VV-specific T cell responses in BALB/c mice have shown that VV infection induces a potent primary CD8⁺ T cell response as well as long-term memory (43). However, β₂-microglobulin⁻/⁻ mice almost completely lacking CD8⁺ T cells survived high-dose s.c. infection with similar lesion development as control mice (44). In addition, studies in mice

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³Abbreviations used in this paper: VV, vaccinia virus; BLCL, B lymphoblastoid cell line; FasL, Fas ligand; ICS, intracellular cytokine staining; moi, multiplicity of infection; MVA, modified vaccinia Ankara.

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show that the absence of either perforin- or Fas-dependent cytotoxicity did not affect clearance of a primary VV infection (45). However, the roles of perforin-and Fas-dependent cytotoxicity have not been defined in human subjects. Xu et al. (25) have shown that, in acute infection, the CD4+ T cell-dependent Ab response is more important for clearing VV using Ab-depleted and gene knockout mice. Recently, Tang et al. (32) identified CD8+ and CD4+ responses to VV envelope proteins A27L, B5R, L1R, and A33R. They studied four VV envelope proteins expressed from mRNA in autologous dendritic cells and reported CD4+ T cell responses. Although they localized three peptide epitopes on the A27L protein of VV using 15-mer peptides, the epitopes were not well characterized in terms of MHC restriction and definition of minimal epitopes.

To analyze human CD4+ T cell responses against VV, it is important to identify CD4+ T cell epitopes, especially the ones restricted by common MHC class II molecules. Because we identified cytotoxic CD4+ T cells specific to VV previously (40), we decided to use cytotoxic CD4+ T cell lines for epitope mapping. We identified two peptide epitopes that are recognized by VV-specific CD4+ T cell lines restricted by HLA-DR1 that were isolated from vaccinated individuals and are encoded by the vaccinia genes A24R and D1R, respectively.

Materials and Methods

PBMC donors

PBMC donors in this study were healthy VV-naive volunteers who received smallpox vaccine, standard Dryvax, by multiple cutaneous punctures made with a bifurcated needle, as recommended by the Centers for Disease Control and Prevention for laboratory personnel working with VV (46). They were enrolled under an approved University of Massachusetts Medical School Institutional Review Board human study protocol. Blood was collected postvaccination at or 2 wk postvaccination. The postvaccination PBMC demonstrated VV-specific T cell proliferation and CTL activity postvaccination (data not shown). The HLA types of these donors were determined using Biotest HLA-class II SSPtray (Biotest Diagnostics), and HLA DR0101-positive donors’ PBMCs were used in this study.

Cells and cell lines

PBMC were purified by Ficoll-Hypaque density gradient centrifugation. Buffy coats were recovered, and mononuclear cells were resuspended at 5 × 10⁸ to 8 × 10⁹/ml in RPMI 1640-20% FBS-10% DMSO and cryopreserved until needed. B lymphoblastoid cell lines (BLCL) were generated from each donor, as previously described (26), and cultured in RPMI 1640 supplemented with penicillin, streptomycin, t-glutamic acid, HEPES, and 10% FBS. T2-DR1 is a T × B hybrid cell line transfected with the cDNA for DR1 (DRA*0101/DRB1*0101) and was cultured in RPMI 1640 medium containing 10% FBS (47).

Virus and virus Ag preparation

VV New York City Board of Health strain, the same strain used to produce the licensed vaccine, Dryvax, was provided by G. Mazzara and D. Panicali (American Type Culture Collection CCL-70). Virus stock was prepared by thawing and thawing infected CV-1 cell lysate. The lysate was then sonicated and cleared by centrifugation at 1000 rpm for 10 min. The supernatant was titrated by plaque assay using CV-1 cells and stored at −80°C until use, as previously described (40, 48). To prepare VV Ag, the same cell lysate was placed in boiling water for 30 min to inactivate the virus after freezing and thawing. Plaque assays were performed to confirm the absence of live VV.

Generation of cytotoxic CD4+ T cell lines

A total of 3 × 10⁶ PBMC was dispensed per well in 24-well plates (Corning Glass) with 2 × 10⁶ (stimulator cells) autologous PBMC infected with VV at a multiplicity of infection (moi) of 1 or pulsed with VV Ag (the same volume of infected cell lysate used for the infection at a moi of 1, but inactivated by boiling for 30 min) in 1 ml of AIM-V medium containing 20% FBS and 25 ng/ml human rIL-7 (PeproTech). After 2 days, human rIL-2 (BD Biosciences) was added to the wells at 50 U/ml final concen-

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Cytotoxic CD4+ T cell lines kill VV-infected, VV Ag-pulsed, and peptide-pulsed autologous BLCL targets in a 51Cr release assay. The IF2 (A) or 3F3 (B) cells were cocultured with 51Cr-labeled autologous BLCL targets, which were untreated, infected with VV at a moi of 1, pulsed with VV Ag (the same volume of infected cell lysate used for the infection at a moi of 1, but inactivated by boiling for 30 min), or pulsed with 2.5 μg/ml specific peptide in a U-bottom plate in triplicates. Cells were incubated at the indicated E:T cell ratio for 4 h, after which the percent lysis of target cells was calculated. The mean 51Cr release of the CTL lines ± SD is shown.
Synthetic peptides and screening

HLA-DR1 (DRB*0101)-binding epitopes were predicted using genomic sequences of the VV strain modified vaccinia Ankara (MVA; U94848) (50). MVA was chosen as a prototype for epitope prediction because it is a potential smallpox vaccine for the future, and most open reading frames in MVA strain are conserved in WR, Copenhagen, and the Dryvax strains. All 193 MVA open reading frames were subjected to the scan using a prediction algorithm (L. Stern, J. Calvo-Calle, I. Strug, M. Nastke, S. Baker, and L. Stern, unpublished data). Briefly, matrices of predicted peptides were derived using two different algorithms (P9 and SYFPEITHI). P9 modified from a published algorithm (51) predicts HLA-DR1 peptide binding based on inhibition-binding assays of a set of minimal peptides (available at http://rcdev.umassmed.edu/nwpredict.php), and SYFPEITHI predicts HLA-DR1-restricted epitopes based on analysis of naturally processed endogeneous peptides (52) (available at www.syfpeithi.de). The top 45 predicted binding peptides were selected for synthesis as 21-mer peptides, of which 36 peptides were successfully synthesized by Genemed Synthesis and purified to >70% purity. Each peptide has an HLA-DR1-binding motif (9 aa) flanked by 6 aa on each side. Peptide pools containing 4 μg/ml each of six peptides were used to screen the T cell lines. The 21-mer peptides from positive pools were then tested individually with the CTL lines in ELISPOT assays at 4 E:T ratios for a standard 4-h Cr release assay. The graphs are representative of the values of specific lysis at an E:T ratio of 30:1. Bars, Mean of triplicate samples ± SD. B. Dose-dependent reactivity of the MVA peptides with the cell lines in ELISPOT assay. The 1F2 or 3F3 cells were cocultured with autologous BLCL on ELISPOT plates with different dilutions of cognate peptide or VV, VV Ag, or PHA (as positive controls) for 16 h. Graph shows the number of IFN-γ-secreting cells. Bars, Mean of triplicate wells ± SD.

Peptide-binding assays

A competition assay was used to determine binding affinities of peptides to the HLA-DR1 molecule. Peptide-free HLA-DR1 produced in Escherichia coli (25 nM) was mixed together with biotinylated H2-A9 (306–318) peptide probe (HαB2M 25 nM) and varying concentrations of unlabeled competitor peptide (10⁻¹²–10⁻⁵ M). The mixtures were incubated for 3 days at 37°C in 100 mM sodium phosphate buffer (pH 5.5), containing protease inhibitors and 0.5 mg/ml octylglucoside, followed by detection of bound biotinylated peptide using an immunoassay that used anti-DR1 capture Ab LB3.1 and alkaline phosphatase-labeled streptavidin. IC₅₀ values were obtained by fitting a binding curve to the plots of absorbance vs concentration of competitor peptide.

Preparation of APC for CTL, ELISPOT, and intracellular cytokine staining (ICS) assays

Virus-infected targets. Autologous BLCL, T2, or T2-DR1 cells were infected with VV at a moi of 10 for 60 min at 37°C. The cells were then diluted in 1 ml of RPMI 1640-10% FBS for an additional 16 h. Target cells were then radiolabeled or used directly.

Ag-pulsed targets. Autologous BLCL, T2, or T2-DR1 cells were incubated with VV Ag preparation (the same volume of infected cell lysate used for the infection at a moi of 10, but inactivated by boiling for 30 min) for 60 min at 37°C. The cells were then diluted in 1 ml of RPMI 1640-10% FBS for an additional 16 h. Target cells were then radiolabeled or used directly.

Peptide-pulsed targets. BLCL, T2, or T2-DR1 cells were pulsed or used unlabeled in the assays. Cells were incubated with 25 μg of peptide/ml in 96-well round-bottom plates at 1 × 10³ to 2 × 10⁵/well for 30 min at 37°C before addition of effector cells. The peptides remained in the wells for the duration of the assay.

⁵¹Cr release assay

T cell lines or bulk culture effector cells were added to 2 × 10⁵ ⁵¹Cr-labeled target cells at various E:T ratios. For inhibition of the perforin pathway, effector T cells were preincubated with concanamycin A (Sigma-Aldrich) at various concentrations (100, 50, 20, and 10 nM) for 2 h at 37°C, washed, and added to labeled targets on 96-well plates at a ratio of 30:1 (54–56). Similarly, to inhibit the Fas-Fas ligand (FasL) pathway of
cytotoxicity, efforts were treated with anti-FasL Ab (clone 100419; R&D Systems) at 50, 10, and 5 g/ml for 16 h or peptide pulsed for 30 min, then used as targets for 1F2 or 3F3. Cytotoxicity was determined using a 31Cr release assay in the presence or the absence of anti-Fas Ab (50, 10, and 5 mg/ml) or concanamycin A (100, 50, 20, and 10 nM)-treated CD4+ T cell lines. Assays were performed in triplicate.

ELISPOT assay for single-cell IFN-γ secretion

ELISPOT assays were performed, as described previously (58–60). Briefly, 96-well filtration plates (MSIPS4W; Millipore) were coated with 15 µg of mouse anti-human IFN-γ mAb (clone D1K; Mabtech) per ml at 4°C overnight. Plates were blocked with RPMI 1640-10% FBS for at least 2 h. A total of 2.5 x 10^5 APC and 2 x 10^5 T cells was added in RPMI 1640-10% FBS. Peptides were added at 2.5 µg/ml, PHA at 10 µg/ml, VV at a moi of 10 or VV Ag (the same volume of infected cell lysate used for the infection at a moi of 10, but inactivated by boiling for 30 min), and plates were incubated for 18–20 h at 37°C. The cells were removed by washing, and biotinylated mouse anti-human IFN-γ mAb (clone 7-B6-1; Mabtech) was then added and left for 2 h at room temperature, followed by a 1/4000 dilution of streptavidin HRP for 30 min. Substrate (Novared; Vector Laboratories) was added and left for 10 min at room temperature. The frequency of IFN-γ-producing T cells was calculated as number of spots/total number of cells/well. Experiments were performed in triplicate.

ICS for production of IFN-γ by epitope-specific CD4+ T cells

Bulk culture cells at day 28 (donors A, B, and C) or day 14 (donors D and E) of in vitro culture were resuspended at 10^6/ml in RPMI 1640 medium supplemented with 10% FBS. For in vitro stimulation, peptides were used at 2.5 µg/ml final concentration. For each sample, a negative control containing only medium and a positive control (PHA; final concentration 10 µg/ml) was used to ensure that cells were responsive. The cultures were incubated for 2 h at 37°C in a 5% CO2 incubator, followed by an additional 5 h in the presence of GolgiPlug (BD Biosciences). Stimulated cells were washed, fixed/permeabilized (Cytofix/Cytoperm; BD Biosciences), and stained for surface markers and IFN-γ. Cells were then washed and resuspended in Permwash buffer for flow cytometric analysis. Responses are reported after background correction, using the unstained and single-stained controls to define nonspecific responses. FITC, PECy7, PerCPCy5.5, PE, Pacific blue, and allophycocyanin were used as the fluorophores for these experiments. Multiparameter flow cytometric analyses were performed using a FACSCalibur or ARIA flow cytometer. The list-mode data files were analyzed using FlowJo (version 6.3; Tree Star). Graphs were plotted as biexponential transformed dot plots of CD19, CD3+CD8+, T cells in the live cell population of each bulk culture tested.
**Results**

*Generation of VV-specific human CD4⁺ CTL lines using vaccinia-immune donor PBMC*

Bulk culture lines were generated by incubating PBMC (drawn at 28 or 45 days after vaccination) from four VV-immune donors with either VV-infected or VV Ag-pulsed autologous stimulator cells. The bulk cultured cells were tested for CTL activity after 7 days of culture and specifically lysed autologous BLCL target cells that were either infected with VV or pulsed with VV Ag. The lines were subjected to limiting dilution, and a total of 14 cell lines

![Graph showing CTL activity](image)
was found to possess substantial VV-specific CTL activity. The cell lines were then stained with Abs to CD3, CD8, and CD4, and the 12 cell lines that were >98% CD4+ T cells were further studied.

Mapping of CD4+ CTL epitopes using predicted HLA-DR1-binding peptides from VV

The CD4+ CTL lines were tested for their ability to secrete IFN-γ when incubated with T2-DR1 cells (T2 cells expressing only HLA DR1) that were pulsed with each of 6 peptide pools comprising a total of 36 peptides in a screening ELISPOT assay. These peptides were selected based on an HLA-DR1-binding and epitope prediction algorithm (L. Stern, J. Calvo-Calle, I. Strug, M. Nastke, S. Baker, and L. Stern, unpublished data). Two cell lines showed positive reactivity to different peptide pools. T cell lines 1F2 from donor B and 3F3 from donor C recognized cells pulsed with peptides MVA 344 and MVA 302, respectively. The cell lines were tested for their ability to lyse peptide-pulsed APC in addition to VV-infected and VV Ag-pulsed targets and, as shown in Fig. 1, A and B, they lysed targets pulsed with the respective peptide in a specific manner. The ELISPOT assay was repeated with autologous BLCL as APC-testing peptides at different concentrations, and we found that both cell lines generated IFN-γ responses to the respective epitopes in a peptide dose-dependent manner (Fig. 2B). The cell lines lysed T2-DR1 cells presenting cognate peptides, but not untransfected T2 cells in a CTL assay, indicating that they are HLA-DR1-restricted CD4+ CTLs (Fig. 2A).

We also confirmed that these cytotoxic CD4+ T cell lines recognized cells pulsed with cognate peptides, VV-infected and VV Ag-pulsed APC (T2-DR1 cells), and secrete IFN-γ in an ICS assay (data not shown). MVA 344 is encoded by the VV gene, A24R (DNA-dependent RNA polymerase subunit rpo132) gene, and MVA 302 is encoded by the VV gene, D1R (mRNA capping enzyme large subunit).

We tested the ability of these CD4+ T cell lines to lyse specific targets after pretreatment with inhibitors of the Fas-FasL and the perforin pathways. The 1F2 and 3F3 effector T cell lines were preincubated with anti-Fas Ab or concanamycin A, an inhibitor of the perforin-mediated pathway. Although inhibition of the Fas pathway had no effect on their killing, pretreatment with concanamycin A significantly decreased CTL activity in a dose-dependent manner, indicating that both CTL lines lyse epitope-specific cells via a perforin-mediated pathway (Fig. 3). The cell lines were stained with anti-perforin Ab for intracellular perforin. However, we did not see any detectable differences between anti-perforin Ab (perforin-FITC kit, clone 6G9; BD Biosciences) and the isotype-matched control Ab (data not shown).

Identification of the minimal epitopes required for IFN-γ secretion by the 1F2 and the 3F3 T cell lines

To define the minimal epitope recognized by the 1F2 and 3F3 cell lines, we made a series of truncations of the 21-mer MVA 344 and MVA 302 peptides. A 14-mer peptide, 177WPKFRVVKPNSFTF190, appeared to be the minimal epitope recognized by 1F2, because only 1F2 cells stimulated with T2-DR1 cells pulsed with MVA 344.03 and MVA 344.11 peptides and not MVA 344.04, MVA 344.06, and MVA 344.12 peptides secreted IFN-γ (Fig. 4A).

We also performed ELISPOT assays to determine the minimal epitope for the 3F3 cell line using truncations of the original 21-mer MVA 302 peptide. The data (Fig. 4B) indicate that both 10-mer peptides, MVA 302.02, 406VFRYMSSPEPIL15, and MVA 302.03, 406VFRYMSSPEPIL16, were minimal epitopes for the 3F3 cell lines. Comparisons of sequences between different members of the poxvirus family show that both epitopes are completely conserved among VV (WR, GenBank accession number NC 006998; Copenhagen, M35027; MVA, U94848; Dryvax, DQ777945, AY313847, and AY313848), variola viruses (India 1967, NC 001611; Bangladesh-1975, L22579; Garcia-1966, Y16780), and most mammalian poxviruses, including monkeypox (Zaire-96-I-16, NC_003310), cowpox (GRI-90, X94355), and ectromelia (Moscow, NC_004105).

VV-immune donors have T cell precursors specific to MVA 302 and MVA 344

We generated bulk cultures from PBMC derived from three different vaccine recipients, including the donors from whom the cell lines were originally generated. Two different bulk cultures were set up from each donor PBMC and stimulated with VV-infected...
autologous PBMC and VV Ag-pulsed PBMC. Bulk culture cells from all three donors were tested in a CTL assay at day 28 to determine whether there were any T cells specific to these two epitopes (Fig. 5). In donor A, we found significant lysis (>20%) of MVA 302-pulsed target cells by both the live VV- and VV Ag-stimulated bulk culture, and the lysis of MVA 344 peptide-pulsed targets was 5–8%. With donor B, there was significant killing ranging between specific lysis of 20–40% by the VV Ag-stimulated bulk culture. The specific target cell lysis by VV-stimulated cultures was lower (3–10%). In the third donor, C, we detected higher CTL lysis to both epitopes in VV-stimulated bulk culture (10–25%), and killing by the VV Ag-stimulated bulk culture cells of this donor was low. To determine whether the peptide-specific responses observed were indeed a product of vaccinia immunization, PBMCs obtained from five individuals, including donors A, B, and C, before vaccination were tested for the presence of precursors. Bulk cultures were set up using similar methods, and the cells were tested for VV-, VV Ag-, and cognate peptide-specific CTL lysis. None of the short-term bulk culture lines from the day 0 PBMC had any detectable levels of VV-, VV Ag-, or peptide-specific CTL activity.

An ICS assay was also performed to determine whether the bulk cultures contain CD3+CD4+ T cell precursors that secrete IFN-γ in response to specific epitope peptides. We tested bulk cultures set up from day 0 (prevaccination bleeds) PBMC for VV-, VV Ag-, or peptide-specific IFN-γ responses, but did not see any discernable precursor frequencies in any of the five donors. Among five vaccinia-immune donors, the postvaccination bulk lines stimulated with live VV (Fig. 6, A and C) or VV Ag (Fig. 6, B and D) from donors C and D had detectable IFN-γ-secreting CD4+ cells in response to VV, VV Ag, and peptides MVA 302 and MVA 344 (Fig. 6), whereas the bulk cultures from the three donors, A and B, after two rounds of in vitro restimulation, and donor E, after one round of in vitro restimulation, showed no responses to these two peptides (data not shown).
Discussion

We identified two HLA-DR1-restricted epitopes by screening cytotoxic CD4+ T cell lines against computer-predicted peptides encoded by the VV genes A24R and D1R, and both epitopes are completely conserved among vaccinia, variola, and monkeypox viruses. To our knowledge these are the first VV-specific cytotoxic CD4+ T cell epitopes restricted by HLA-DR1, one of the common MHC class II alleles. In a recent report, CD4+ T cell responses were demonstrated against four envelope proteins on VV, A27L, B5R, L1R, and A33R (32). The study analyzed three class II-restricted epitopes on A27L defined using overlapping 15-mer peptides, but did not define MHC restrictions of the epitopes, nor are the minimal epitopes outlined. We adopted a different criterion for our screening assays by selecting our peptides based on a HLA-DR1-binding score and not any particular proteins. The prediction algorithm used in this study (L. Stern, J. Calvo-Calle, I. Strug, M. Nastke, S. Baker, and L. Stern, unpublished results) is a combination of two previously approaches, as follows: one is modified from a published algorithm developed by Hammer et al. (51), which predicts HLA-DR1 peptide binding based on inhibition-binding assays of a set of minimal peptides, and one developed by Rammensee et al. (52), which predicts HLA-DR1-restricted Ag processing and presentation based on analysis of naturally processed endogenous peptides. Peptides from MVA open reading frames scoring highly in both algorithms were selected for further analysis. HLA-DR1 was selected for this analysis because it is one of the well-studied human MHC class II alleles. In Caucasoid lineage, average frequency of HLA-DR1 was 9.42% (range of frequency is from 4.50 to 26.00%); in Blacks, 5.46% (0.00–9.20%); and in Orientals, 2.98% (0.00–16.10) (61). Imanishi et al. (62) reported similar frequencies. Considering the promiscuity of HLA-DR1-restricted CD4+ T cell epitopes (63–65) and the presence of DR1 supermotic (66, 67), these epitope peptides may also be presented by HLA-DR molecules other than DR1.

We synthesized 36 21-mer peptides based on the predicted HLA-DR1-binding motifs. Each peptide has a 9-aa-long HLA-DR1-binding motif flanked by 6 aa at each side, which were added in case flanking amino acid sequences would influence the binding to HLA or interaction with TCR. The predicted binding scores for the two peptides identified in our screen are 3.3 (P9 score) and 32 (SYFPEITHI) for MVA 302, and 2.1 (P9) and 39 (SYFPEITHI) for MVA 344, respectively. They are in between the highest (3.8 (P9) or 38 (SYFPEITHI)) and the lowest (1.9 (P9) or 29 (SYFPEITHI)) predicted binders among the 45 predicted epitopes (the range of possible scores for the P9 algorithm is −16 to 23, with positive scores indicating predicted binding, and for the SYFPEITHI algorithm is 0–42, with scores >28 indicating predicted presentation). Measured IC50 values for the MVA 302 and MVA 244 peptides are 37 and 33 nM, respectively, as compared with 12 nM for the tight binding influenza hemagglutinin-derived peptide (68).

The minimal epitope for MVA 344 is a 14 mer and MVA 302 is a 10 mer (Fig. 4). Our results corroborate findings by other studies that the optimal epitope peptides for CD4+ T cells are sometimes longer than the binding motifs and a given T cell can tolerate peptides of variable length and sequence (61).

Several human virus infections, such as HSV, varicella-zoster virus, EBV, CMV, hepatitis B virus, Dengue virus, poliovirus, and HIV, induce CD4+ CTL responses, which may contribute to clearance of virus, as well as producing cytokines (69–77). In most reports, CD4+ CTL activity has been defined using T cell clones that lyse Ag-expressing cells (78). Direct ex vivo CD4+ CTLs have been described using T cells from patients for HIV (79).

Mouse CD4+ T cells specific for lymphocytic choriomeningitis virus were shown to be cytotoxic in vivo (80). Recent reports highlight the evidence for roles played by the effector CD4+ T cells in virus clearance in influenza in mice (81) and by a distinct subset of a mature CD4+ subpopulation in CMV infections in humans (82). It will be interesting to find the role(s) that VV-specific cytotoxic CD4+ T cells play in VV infection.

Both proteins encoding the epitopes described in this study are enzymes involved in mRNA synthesis. A24R is a DNA-dependent RNA polymerase subunit rpo132, and D1R is an mRNA capping enzyme large subunit (DIR). We have observed specific cell lysis of up to 80% of target cells infected with VV, pulsed with VV Ag or peptides by the VV-specific cytotoxic CD4+ T cell lines, 1F2 and 3F3, in vitro cytotoxicity assays suggesting potent cytotoxicity of these cells in vivo (Fig. 1). We characterized the cytolytic mechanism used by the VV-specific cytotoxic CD4+ T cell lines. We found the lysis by these cell lines was inhibited by concanamycin A, an inhibitor of perforin, not by anti-Fas Ab (Fig. 3), although we were not able to detect intracellular perforin in the cell lines; therefore, there is a possibility that concanamycin A may inhibit a pathway other than perforin, which might be involved in cytotoxicity by CD4+ T cells.

Bulk culture cell lines generated from PBMC from three VV-immune donors demonstrated MVA 302- and MVA 344-specific CD4+ CTLs (Fig. 5). Interestingly, VV Ag- and live VV-stimulated bulk cultures performed differently in these donors. In donor C, the bulk culture stimulated with VV Ag had higher cytotoxicity than the bulk culture stimulated with live VV. In donor B, the bulk culture stimulated with live VV had a higher level of cytotoxicity. In donor A, the VV Ag- and live VV-stimulated bulk cultures induced similar levels of cytotoxicity against VV in in vitro CTL assays.

As shown in Fig. 6, among five vaccinia-immune donors, the postvaccination bulk lines stimulated with live VV or VV Ag from two donors had detectable IFN-γ-secreting CD4+ cells in response to VV, VV Ag, and peptides MVA 302 and MVA 344. In both donors’ bulk lines, the sum of the epitope-specific T cell frequencies was higher than the T cell frequencies for whole live virus or viral Ag. We and others reported that total numbers of IFN-γ-secreting cells responding to individual peptides (in these cases the peptides were 9-mer or 10-mer CD8+ T cell epitopes) were higher than the number of IFN-γ-secreting cells responding to whole VV in some human donors’ PBMC (26, 29, 83).

CD4+ T cells can survey for presentation of extracellular proteins by MHC class II molecules. At the same time, class II proteins can present peptides that are derived from endogenous or intracellular proteins. In fact, most peptides that are extracted from class II molecules are derived from endogenous membrane proteins that traffic into exocytic and endocytic pathways (84, 85). Several studies have described class II presentation of endogenous viral Ags (86). Measles virus proteins are presented by TAP-dependent or -independent pathways, although the cytoplasmic matrix protein required high and sustained levels of expression compared with class I presentation (87, 88). Certain epitopes derived from influenza virus membrane proteins were presented in a proteasome- and TAP-dependent class II pathway, whereas other epitopes were presented only as exogenous Ags (89, 90). There may be donor to donor differences in determining which pathway is dominant in presenting VV epitopes to CD4+ T cells.

In this study, we were unable to directly detect MVA 302- and MVA 344-specific CD4+ T cells using IFN-γ ELISPOT assays without in vitro restimulation (data not shown). Because the PBMC samples analyzed in the IFN-γ ELISPOT assays were from either 28 days (donor A and C) or 45 days (donor B, D, and E)
after immunization, we probably did not catch the early highest peak of the responses. A relatively low frequency of CD4+ T cells specific for virus is also not unexpected (91–93). The two HA-LR1-restricted epitopes described in this work may be useful to monitor and analyze CD4+ T cell responses to the licensed smallpox vaccine or new poxvirus vaccines. To ensure successful vaccination, a candidate vaccine should contain immunodominant CD4+ T cell epitopes as well as CD8+ T and B cell epitopes.

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Disclosures

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