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Identification of Murine Poxvirus-Specific CD8⁺ CTL Epitopes with Distinct Functional Profiles

Anuja Mathew, Masanori Terajima, Kim West, Sharone Green, Alan L. Rothman, Francis A. Ennis, and Jeffrey S. Kennedy

Murine T cell epitopes against vaccinia virus (VV) have not been characterized to date in part due to the large and complex genome of VV. We have identified and characterized two CD8⁺ T cell epitopes on the A47L (modified VV Ankara strain (MVA)-029) and J6R (MVA-043) proteins of VV that are D₈ and K₉ restricted, respectively. Following i.p. immunization with VV New York City Board of Health (NYCBH) strain, MVA-029 peptide-stimulated splenocytes secreted IFN-γ from 7 days to 7 mo postimmunization, and virus-stimulated effectors were also able to lyse MVA-029-pulsed target cells at the same time points. In contrast, MVA-043 peptide-stimulated splenocytes secreted very low levels of IFN-γ only at day 7 but maintained the ability to lyse target cells up to 2 mo postimmunization. Both MVA-029 and MVA-043 peptide-stimulated lymph node cells degranulated similarly as assessed by Ag-induced CD107 expression. T cell responses to whole-virus stimulation remained robust and steady during the acute and memory T cell response to VV. Identification of T cell epitopes on VV will enable further studies to increase our understanding of the role of CD8⁺ T cells in VV infection and assist in the design of new protective strategies. The Journal of Immunology, 2005, 174: 2212–2219.

Vacci

nora virus (VV) is a large DNA virus and is a member of the Poxviridae family. It has been used as an effective vaccine against variola virus, the causative agent of smallpox. VV has also been used extensively as an expression vector for foreign genes, as a recombinant vaccine, and has been the most widely studied member in its family (1). Despite the use of VV in numerous experimental systems, study of the immune response to the parent vector has been limited.

Recently, the immunologic correlates of protection following infection with different strains of VV have begun to be examined. Both cellular and humoral immunity are thought to play a role in protection against orthopoxviruses (2, 3). NK cells and γδ T cells play a role in innate resistance to infection (4, 5). In a study performed by Xu et al. (2), CD4 T cells and neutralizing Abs (nAb) were important for clearing acute VV infection, whereas CD8 T cells seemed to play a less dominant role during the acute phase of infection. However, Karupiah et al. (6) have shown that CD8⁺ T cells are essential for recovery from infection with the poxvirus ectromelia virus. Additionally, in humans, the presence of nAb does not prevent the development of progressive vaccinia if cell-mediated immunity is defective (7). Xu et al. have also shown that, in the absence of Ab and CD4 T cells, CD8 T cells were necessary for protection, because skin lesions and adrenal necrosis were exacerbated in MHC class II⁻/- CD8-depleted mice. Adoptive transfer experiments have shown that CD8⁺ T cells can mediate control of VV infection (2).

Recently, we defined two HLA-A*0201-restricted poxvirus cross-reactive CD8⁺ T cell epitopes designated 74A (C16L aa 79–87) and 165 (C7L aa 74–82) (8). HLA-A*0201 transgenic mice immunized with peptide 165 were shown by Snyder et al. (9) to protect mice from a lethal dose of VV WR strain intranasally, indicating that CD8⁺ CTLs directed against a single epitope are protective. These data indicate that multiple arms of the effector immune response are likely involved in the acute and memory phase of VV infection and point to an important role for CD8 T cells in mediating protection and recovery from infection.

Following immunization of mice with VV, a robust T cell response is elicited with activated VV-specific CD8 T cells and CD4 T cells (10). By 2–3 wk, a very stable but lower virus-specific response is maintained long term in both C57BL/6 as well as BALB/c mice (2, 10). Although numerous proteins that elicit nAb responses have been mapped, no murine T cell epitopes have been defined against VV. The absence of defined murine CD8 T cell epitopes against VV has limited functional studies to date.

We identified a D₈- and a K₉-restricted CD8 T cell epitope on the A47L and J6R proteins of VV. Following immunization with VV New York City Board of Health (NYCBH), virus-stimulated effectors were able to lyse virus-infected as well as peptide-pulsed targets efficiently during both the acute and memory phases of infection. Although peptide MVA-029 stimulated large numbers of IFN-γ-secreting cells through all phases of the immune response, peptide MVA-043-stimulated splenocytes showed significantly lower numbers of IFN-γ-producing cells at 7 days postimmunization, and by day 14 IFN-γ-secreting cells were undetectable. However, Ag-induced CD107a expression did not differ between the two epitopes.

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Materials and Methods

Cells

Target cell lines were the EL-4 murine lymphoma line (H-2b), RMA, and RMA-S cells were obtained from Dr. R. Welsh at University of Massachusetts Medical School. Freshly isolated bone marrow cells were incubated in petri dishes with RPMI 1640/10% FBS overnight. The nonadherent cells were collected the next day and treated with IL-4 (5 ng/ml) and GM-CSF (10 ng/ml) every other day to generate bone marrow-derived dendritic cells (BMDCs). On day 6, adherent cells were assessed to be BMDCs because >80–90% of the cells were CD11c+

Peptides

Peptides of VV were based on published sequences of the modified VV Ankara strain (MVA) because the human vaccine NYCBH has not been cloned and is likely to be a quasispecies. In addition, MVA is considered to be a safer VV strain and is under development as a smallpox vaccine. Peptides were synthesized at the Protein Chemistry Core Facility at the University of Massachusetts Medical School using an automated Rainin Symphony peptide synthesizer.

Immunization of mice and preparation of splenocytes

Male C57BL/6 (4–8 wk old) mice were purchased from The Jackson Laboratory. Mice were immunized i.p. with 1 × 10^6 PFU of NYCBH. Splenocytes were collected at the indicated time points postimmunization, lysed with RBC lysis buffer (Sigma-Aldrich), and resuspended in RPMI 1640 medium with 10% heat-inactivated FBS and 5 × 10^−3 M 2-ME. All mice were maintained in the Animal Facility at the University of Massachusetts Medical School, which is regulated by AWA-1995, PHS-1986, and MA140-1985, and following the American Association for the Accreditation of Laboratory Animal Care 1965 guidelines.

Bulk cultures

For bulk cultures, 10% splenocytes (stimulators) were infected with NYCBH at a multiplicity of infection (MOI) of 5 for 1 h, washed three times, and added to 90% immune splenocytes (responders) in T25 flasks. Bulk culture 51Cr release assays were performed between days 7 and 9 of culture.

51Cr release assay

EL4 cells were infected with MVA or NYCBH (MOI = 5) for 18–20 h. Uninfected or virus-infected EL4 target cells were then labeled with 0.25 mCi of 51Cr for 60 min at 37°C. Following labeling, the cells were washed three times and then resuspended in RPMI 1640 containing 10% FBS. Effector cells were then added to virus-infected or peptide-pulsed EL4 cells in 96-well round-bottom plates at various E:T ratios. Plates were incubated for 4 h at 37°C, supernatants were harvested (Skatron Instruments), and specific lysis was calculated as follows: [(experimental release — spontaneous release)/(maximum release — spontaneous release)] × 100(%). All assays were performed in triplicate. All experiments were performed at least twice. Spontaneous lysis was <15% in all assays.

Peptide stabilization assays

A total of 1 × 10^5 RMA-S cells was pulsed with increasing concentrations of MVA-029C peptide or MVA-043 peptide in 96-well U-bottom plates and incubated at 28°C overnight. Cells were washed in FACS buffer (PBS/2% FBS/0.1% sodium azide), blocked for 15 min at 4°C with anti-CD16 Ab (24G2; BD Pharmingen), and stained with Abs directed against K0 (AF6-88.5) and D0 (KH95) (BD Pharmingen).

ELISPOT assay for single-cell IFN-γ secretion

ELISPOT assays were performed according to the manufacturer’s protocol (Mabtech). Briefly, 96-well Multiscreen IP plates (Millipore) were coated with 15 μg/ml rat anti-mouse IFN-γ mAb (AN-18) overnight at 4°C. Then, freshly isolated splenocytes (1–2.5 × 10^7/well as indicated) were incubated with MVA (MOI = 1), NYCBH (MOI = 1), peptide (10 μg/ml), or Con A (5 μg/ml) at 37°C for 18–20 h in RPMI 1640 containing 10% FBS. In some ELISPOT assays, virus-infected EL4 cells, virus-infected BMDCs, peptide (10 μg/ml) plus EL4 cells, Con A plus EL4 cells, or EL4 cells alone were added to day 7 immune splenocytes and incubated for 18–20 h. Biotinylated rat anti-mouse IFN-γ mAb (R4-6A2) was then added and incubated for 2 h at room temperature, followed by addition of streptavidin-HRP for 1–2 h at room temperature. Spots were stained with Vector NovaRED Substrate kit for peroxidase (Vector Laboratories). The precursor frequency was calculated as follows: [(number of spots in medium control well)/(total number of cells per well)] × 10^6. Experiments were performed in duplicate or triplicate wells.

Intracellular cytokine assay

A total of 1 × 10^6 splenocytes was incubated with peptides (10 μg/ml) for 6 h. Cells were also incubated with medium alone as a negative control or with PMA (50 ng/ml) plus ionomycin (500 ng/ml) as a positive control. In some assays, 2 × 10^4 EL4 cells infected with NYCBH or MVA 24 or 48 h prior (MOI = 5) were used to stimulate 1 × 10^6 splenocytes for 6 h. Recombinant human IL-2 (10 U/ml) was also added during the incubation. For the CD107a assays, the Ab (1D4B) (BD Pharmingen) was added to the cells during the stimulation, and 2 μl of Golgi Stop was added to the tubes. The cells were washed and stained with mAb directed at surface phenotypic markers CD3 (BD Pharmingen) and CD8 (53-67; eBiosciences). The cells were then fixed and permeabilized (Cytofix/Cytoperm) and stained with anti-IFN-γ mAb (XMG1.2; eBiosciences). The stained cells were analyzed by flow cytometry in the University of Massachusetts Medical School Flow Cytometry Core.

Results

Identification of VV-specific murine CTL epitopes using i.p. immunization of C57BL/6 mice

Following i.p. immunization with 10^6 PFU of NYCBH, cytolytic T cell responses were detected after in vitro stimulation of splenocytes with NYCBH virus (Fig. 1, Table I). Effectors were able to efficiently lyse target cells that were infected with either MVA or NYCBH. In addition, these effectors were able to lyse targets pulsed with two (MVA-029A-19-mer and MVA-043A-13-mer) of a panel of peptides that were used in a screening assay (Fig. 1, Table I, and data not shown). The data indicate that VV infection of C57BL/6 mice elicits a strong virus-specific T cell response that is stable. We have identified two murine T cell epitopes on VV that contribute to this robust immune response in mice.

Identification of the minimal epitope required for CTL recognition and IFN-γ secretion

To define the minimal epitope that was recognized by virus-stimulated effectors, we made a series of truncations of both the MVA-029 (19-mer) and MVA-043 (13-mer) peptides. MVA-029E appears to be the minimal epitope recognized by day 7 effectors, because only targets pulsed with two (MVA-029A-19-mer and MVA-043A-13-mer) of a panel of peptides that were used in a screening assay (Fig. 1, Table I, and data not shown). The data indicate that VV infection of C57BL/6 mice elicits a strong virus-specific T cell response that is stable. We have identified two murine T cell epitopes on VV that contribute to this robust immune response in mice.

MHC restriction of MVA-029 and MVA-043D epitopes

To determine whether MVA-029C and MVA-043D are K0 or D0 restricted, we performed peptide stabilization assays using the TAP mutant cell line RMA-S. Cells were then stained with Abs...
directed against K\textsuperscript{b} or D\textsuperscript{b}. Peptide MVA-029C stabilized D\textsuperscript{b} and not K\textsuperscript{b} molecules, whereas peptide MVA-043D stabilized K\textsuperscript{b} and not D\textsuperscript{b} molecules on the surface of RMA-S cells (Fig. 3). The data indicate that peptide MVA-029C binds to D\textsuperscript{b} and is most likely D\textsuperscript{b} restricted, whereas MVA-043D binds to K\textsuperscript{b} and therefore is most likely K\textsuperscript{b} restricted.

**Kinetics of the immune response to VV and peptides MVA-029 and MVA-043**

Effectors generated between 1 wk and 4 mo postimmunization with NYCBH were able to efficiently lyse EL-4 targets infected with either NYCBH or MVA virus (Table I). Although lytic activity against MVA-029-pulsed targets was detected both early and late in the memory response at different E:T ratios, CTL killing against MVA-043-pulsed targets was not detected at time points beyond 2 mo (Table I).

We also quantitated the number of virus-specific IFN-\(\gamma\)-secreting cells in splenocytes at multiple time points postimmunization using ELISPOT. At days 7 and 9 postimmunization, responses to both NYCBH and MVA viruses were robust, and by day 14, the response to whole virus was lower but then remained relatively stable up to 7 mo (Fig. 4). The frequency of IFN-\(\gamma\)-producing cells was consistently higher in response to MVA even though the mice were immunized with NYCBH virus (Fig. 4).

To assess the effector responses to the two newly identified T cell epitopes, we determined whether peptide-stimulated effectors would secrete IFN-\(\gamma\). At day 7 postimmunization, \(\sim 500–800\) IFN-\(\gamma\)-producing spots/\(10^6\) splenocytes were detectible after stimulation with MVA-029 (Fig. 4). The response to MVA-029 is long-lasting, because peptide-specific IFN-\(\gamma\) secretion was detected up to 7 mo postinfection (Fig. 4). In contrast, IFN-\(\gamma\) secretion by MVA-043-stimulated splenocytes was very modest at days 7 and 9 (50 spots/\(10^6\) splenocytes), and by day 14, levels of IFN-\(\gamma\) were almost below detection (Fig. 4).

Because the ELISPOT assays were performed on ex vivo splenocytes and the CTL assays were performed on virus-stimulated bulk culture cells, we further characterized the IFN-\(\gamma\) response following stimulation with these two peptides on bulk cells that were generated for the CTL assays. Even at the bulk culture level when effectors were able to lyse both MVA-029- and MVA-043-pulsed targets, the number of IFN-\(\gamma\)-producing cells by MVA-043 stimulation was much lower than MVA-029-stimulated IFN-\(\gamma\)-producing cells (data not shown).

The data indicate that, although the IFN-\(\gamma\) response to VV is vigorous during the acute infection, there is a differential response to the two epitopes with a higher number of IFN-\(\gamma\)-producing cells specific for MVA-029 and a much lower number of IFN-\(\gamma\)-producing cells specific for MVA-043.

**IFN-\(\gamma\) secretion by Ag-specific T cells**

When NYCBH or MVA was added directly to day 7 immune splenocytes, we were able to detect between 900 and 2000 IFN-\(\gamma\)-producing cells per \(10^6\) splenocytes that responded to virus in ELISPOT assays (Fig. 4 and Table II). Additionally, when virus-infected EL4 cells or virus-infected primary BMDCs were added
to day 7 immune splenocytes, 2800–3500 IFN-γ-producing cells per 10^6 splenocytes were found to be VV specific (Table II).

In intracellular cytokine staining (ICS) assays, when EL4 cells that were infected with MVA or NYCBH were used to stimulate day 7 immune splenocytes, 3.8–4.1% of total CD8 T cells secreted IFN-γ in response to virus-infected EL4 cells (Fig. 5A). Peptide MVA-029C stimulated ~0.5% of total CD8 T cells from day 7 splenocytes to secrete IFN-γ with lower levels at later time points (Fig. 5B). MVA-043 stimulation of splenocytes using ICS were barely above background levels at days 7 and 19, and 7 mo postimmunization (Fig. 5B). The data indicate that MVA-029-specific IFN-γ-producing T cells are a component of the virus-specific T cell response because the measured response to whole virus (either NYCBH or MVA) stimulated only 2- to 3-fold higher levels of IFN-γ-producing cells by ELISPOT and 7- to 8-fold higher levels of IFN-γ secretion by ICS.

**Degranulation and cytokine secretion upon Ag encounter**

To compare the capacity of CD8 T cells from splenocytes and lymph nodes (LN) of acutely infected mice to degranulate and secrete cytokines upon Ag encounter, we measured IFN-γ and degranulation by cell surface modulation of CD107a (LAMP-1) (11). When virus-infected EL4 cells (infected 24 or 48 h prior) were used to stimulate day 7 splenocytes, the majority of IFN-γ-producing cells also expressed CD107 (Fig. 6A). However, there were cells that expressed CD107 but did not secrete IFN-γ following stimulation with either MVA or NYCBH. A significant number of CD8 T cells from LN during acute infection (day 7 after i.p. infection) up-regulated CD107a on their cell surface upon Ag stimulation with either MVA-029 or MVA-043 with no difference between the two peptide stimulations (Fig. 6B). However, IFN-γ levels were increased, with MVA-029 stimulation resulting in higher numbers of IFN-γ-producing T cells from the LN than MVA-043 (Fig. 6B). Our data indicate that, although peptides MVA-029 and MVA-043 stimulate a similar percentage of effector cells from the LN to degranulate, they have a distinct difference in their ability to induce IFN-γ CD8^+^ T cells.

### Table I. Recognition by CTL of VV-infected and peptide-pulsed target cells

<table>
<thead>
<tr>
<th>Time Post-immunization</th>
<th>E/T</th>
<th>% Specific Lysis of Target Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NYCBH</td>
<td>MVA</td>
</tr>
<tr>
<td>Day 7</td>
<td>50</td>
<td>67.3</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>58.6</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>50.2</td>
</tr>
<tr>
<td>Day 9</td>
<td>50</td>
<td>35.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>31.1</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>25.3</td>
</tr>
<tr>
<td>2 wk</td>
<td>50</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>14.5</td>
</tr>
<tr>
<td>4 wk</td>
<td>50</td>
<td>25.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>16.7</td>
</tr>
<tr>
<td>8 wk</td>
<td>50</td>
<td>37.1</td>
</tr>
<tr>
<td></td>
<td>15 wk</td>
<td>34.7</td>
</tr>
</tbody>
</table>

^a Mice were immunized i.p. with 1 × 10^6 PFU of NYCBH virus. At the indicated time points postimmunization, splenocytes were collected and stimulated with NYCBH. After 7 days of in vitro stimulation, CTL assays were performed using EL4 cells infected with NYCBH (MOI = 5) or MVA (MOI = 5) or pulsed with 10 μg/ml MVA-029 or MVA-043 peptide at different E:T ratios.

^b Numbers in bold indicate specific lysis of target cells (9% or more over medium controls).

^c nt, Not tested.

**FIGURE 2.** Dose response of MVA-029 and truncated peptides in CTL assays. Bulk cultures established from mice immunized 15 wk prior with NYCBH were tested in CTL assays against MVA-029 and truncated peptides 029B, 029C, 029E, and 029F at the indicated peptide concentrations.

**Discussion**

In this study, we have examined VV-specific T cell responses following i.p. immunization of C57BL/6 mice with NYCBH virus. Using a panel of synthetic peptides based on the MVA strain, we were able to identify two CD8 T cell epitopes on VV. To our knowledge, this is the first report characterizing peptide-specific murine CD8 T cell responses directed against VV. We further characterized the effector functions of the two CD8^+^ epitope-specific T cell populations using CTL, ELISPOT, and ICS assays. MVA-029- and MVA-043-specific T cells were both able to lyse peptide-pulsed targets efficiently during the acute and memory phase of the immune response to VV. However, there was a marked difference in the ability of these two peptides to induce VV-specific IFN-γ-producing T cells.

The two epitopes that we have identified are in genes that are expressed early after VV infection. J6R is a subunit of the DNA-dependent RNA polymerase and the MVA-043 epitope is conserved among variola and vaccinia viruses, suggesting that MVA-043-specific T cells will recognize cells infected with several different poxviruses. The MVA-029 epitope is also conserved among VV and is present in the A47L gene whose function is not known.

Based on the peptide stabilization assay with RMA-S cells, the CD8 T cell epitopes MVA-029 and MVA-043 that we have identified are D^b^ and K^b^ restricted, respectively. Ags recognized by CTL are typically 8–11 aa in length and contain two to three dominant anchor residues (12). In the case of H-2K^b^, aromatic side residues in positions 3 and 5, and aliphatic side residues like leucine (L) or isoleucine (I) in position 8 constitute defined anchor residues. The two epitopes that we have identified are in genes that are D^b^ and K^b^ restricted, respectively. Ags recognized by CTL are typically 8–11 aa in length and contain two to three dominant anchor residues (12). In the case of H-2K^b^, aromatic side residues in positions 3 and 5, and aliphatic side residues like leucine (L) or isoleucine (I) in position 8 constitute defined anchor residues. The two epitopes that we have identified are in genes that are D^b^ and K^b^ restricted, respectively. Ags recognized by CTL are typically 8–11 aa in length and contain two to three dominant anchor residues (12). In the case of H-2K^b^, aromatic side residues in positions 3 and 5, and aliphatic side residues like leucine (L) or isoleucine (I) in position 8 constitute defined anchor residues.

Our data concur with those of other groups, which show that the immune response following VV infection is robust and long-lasting (2, 10). The peak response of VV-specific IFN-γ-producing T cells occurs between days 7 and 9, which then wanes but remains steady at this lower level for at least 7 mo postimmunization. Using ICS, the measured response to whole virus (3.8–4.1% of total CD8) in our hands is lower than has been reported by Harrington et al. (30% of total CD8) (10). At day 7, we were able to detect
cytes incubated with 5

with NYCBH: C57BL/6J mice were immunized i.p. with 1
db. The mean fluorescence intensity (MFI) of RMA-S cells incubated with

are performed. 

5. Splenocytes collected from at least two mice and pooled. A total of 2.5

expressed on the surface of RMA-S cells: 1 × 10^5 RMA-S cells were

expressed. 

10^6 PFU of NYCBH virus, and 7 days later, a

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TABLE II. IFN-γ response to VV using virus-infected APC^a^

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Stimulation Concentration</th>
<th>APC</th>
<th>IFN-γ-Producing Cells per 10^6 Splenocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MVA MOI = 1</td>
<td></td>
<td>915</td>
</tr>
<tr>
<td></td>
<td>NYCBH MOI = 1</td>
<td></td>
<td>980</td>
</tr>
<tr>
<td></td>
<td>MVA-029C 10 µg/ml</td>
<td></td>
<td>515</td>
</tr>
<tr>
<td></td>
<td>MVA-043C 10 µg/ml</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>2b</td>
<td>Medium</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>MVA MOI = 5</td>
<td>EL4</td>
<td>3487</td>
</tr>
<tr>
<td></td>
<td>NYCBH MOI = 5</td>
<td>EL4</td>
<td>2900</td>
</tr>
<tr>
<td></td>
<td>MVA-029C 10 µg/ml</td>
<td>EL4</td>
<td>1033</td>
</tr>
<tr>
<td></td>
<td>MVA-043C 10 µg/ml</td>
<td>EL4</td>
<td>240</td>
</tr>
<tr>
<td>3c</td>
<td>Medium</td>
<td>DC</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>NYCBH MOI = 1</td>
<td>DC</td>
<td>2926</td>
</tr>
<tr>
<td></td>
<td>NYCBH MOI = 0.25</td>
<td>DC</td>
<td>2860</td>
</tr>
</tbody>
</table>

^a^ Mice were immunized with 1 × 10^6 PFU of NYCBH virus, and 7 days later, a total of 1 × 10^6 freshly isolated splenocytes was added to wells in triplicate with the indicated stimuli.

^b^ EL4 cells were infected with MVA or NYCBH virus at MOI = 5 18 h prior, and 5 × 10^5 virus-infected, peptide-pulsed, or unpulsed EL4 cells were added to 1 × 10^6 splenocytes.

^c^ Primary BMDCs were infected with NYCBH at MOI = 0.25 or 1, 18 h prior, and 1 × 10^5 virus-infected BMDCs were added to 1 × 10^6 splenocytes. DC, Dendritic cell.

FIGURE 3. MVA-029C stabilizes Db molecules and MVA-043D stabilizes Kb molecules on the surface of RMA-S cells: 1 × 10^5 RMA-S cells were pulsed with increasing concentrations of MVA-029C peptide (A) or MVA-043D peptide (B) in 96-well U-bottom plates and incubated at 28°C overnight. Cells were washed and stained with Abs directed against Kb or Db. The mean fluorescence intensity (MFI) of RMA-S cells incubated with MVA-029 (A) or MVA-043 (B) is indicated. Data represent one of two experiments performed.

FIGURE 4. Time course of IFN-γ secretion following immunization with NYCBH: C57BL/6J mice were immunized i.p. with 1 × 10^6 PFU of NYCBH. At multiple time points after the immunization, splenocytes were collected from at least two mice and pooled. A total of 2.5 × 10^6 freshly isolated splenocytes was added to wells and stimulated with 10 µg/ml of each peptide, MVA (MOI = 1) or NYCBH (MOI = 1). The stimulations were performed in triplicate wells. Splenocytes incubated with medium were included as negative controls. The data are presented as the number of IFN-γ-producing cells/10^6 splenocytes, with medium control values subtracted. The number of spots in medium control wells was <5. Splenocytes incubated with 5 µg/ml Con A were included as positive controls. NYCBH, the dose of virus used to immunize mice (2 × 10^6 vs 1 × 10^6); the strain of mice (BALB/c vs C57BL/6), as well as the use of APCs (A20-infected cells vs virus added directly or virus-infected EL4/BMDC cells added to splenocytes) in the ELISPOT and ICS assays.

It is of interest that the response to MVA in the ELISPOT assay was consistently higher than the response to NYCBH, even though mice were immunized with NYCBH virus. MVA has recently been shown to induce T cell activation from PBMC of naive cattle and may therefore have mitogenic or superantigenic properties or in-duce γδ T cell activation (13). However, we have detected no IFN-γ secretion from MVA-stimulated naive murine splenocytes (data not shown) and therefore do not believe that this explains our results. We have also examined whether MVA infection of APCs could enhance MHC class I expression, thereby increasing the number of peptides potentially presented to Ag-specific T cells. Our preliminary data suggest that MVA infection of EL-4 cells results in increased Db expression compared with uninfected or NYCBH-infected EL-4 cells (data not shown). Kb expression did not differ following infection with either of these two strains.

The number of IFN-γ-secreting MVA-029-specific T cells was ~800/10^6 splenocytes during acute VV infection, which was then maintained at lower but significant levels in vivo for at least 7 mo. In striking contrast, the only time points where we were able to detect any IFN-γ secretion with MVA-043 stimulation were at days 7 and 9 postimmunization. Our bulk culture CTL results indicate that, despite the low numbers of MVA-043-specific IFN-γ-secreting T cells, we can detect CTL activity against MVA-043 peptide-pulsed targets at 2, 4, and 8 wk postimmunization with NYCBH. After 8 wk, MVA-043-specific CTL activity was not detected. MVA-029-specific CTL activity in contrast was long-lived, and there were high numbers of IFN-γ-secreting T cells at all of the time points. It is tempting to speculate that varying levels of IL-7R on these two distinct VV-specific T cells may explain why responses to peptide MVA-029 are stable and long-lasting, whereas responses to peptide MVA-043 are short-lived (14).

CTLs directed against both peptides MVA-029 and MVA-043 require high concentrations of peptide to lyse target cells and secrete IFN-γ (for MVA-029), suggesting that the responses of both
of the epitopes that we have characterized are low-affinity responses. However, bulk lines specific for MVA-029 and MVA-043 are able to lyse virus-infected targets, indicating that, following viral infection of EL4 cells, sufficient MVA-029 and MVA-043 peptide is expressed on the surface to trigger MVA-029- and MVA-043-specific CTLs to lyse virus-infected target cells (data not shown). Low-affinity epitopes directed against influenza A virus and lymphocytic choriomeningitis virus have been shown to contribute to protection against virus challenge (15, 16). Because VV is a very large virus that expresses >200 proteins, it is likely that higher affinity epitopes directed against other proteins exist.

Cytolysis and IFN-γ production have been used interchangeably for detection of virus-specific CD8+ T cells but appear to be independent functions of activated CTLs. Murray valley encephalitis peptide-immune T cells were found to require much lower concentrations of peptide to trigger cytolytic function than the production of IFN-γ and effector populations were unable to produce IFN-γ upon contact with virus-infected target cells even though cytosis occurred (17). In another study, at high concentrations of peptide, most of the primary CMV and HIV-specific CD8+ CTLs that were able to degranulate in response to peptide could secrete IFN-γ as well (18). At lower concentrations of peptide, far fewer cells produced IFN-γ while still maintaining the ability to degranulate. Thus, if a peptide is presented at very low levels, the predominant response may be cytolytic, whereas a peptide that is presented at a higher density may be able to stimulate Ag-specific T cells to secrete IFN-γ as well as lyse infected targets (17). Both the MVA-029 and MVA-043 peptides were used at relatively high concentrations, and even at these high concentrations, there was a significant difference in the ability of these two peptides to induce IFN-γ secretion.

CD107a expression was increased upon stimulation with either MVA-029 or MVA-043 day 7 post-VV infection in the LNs, indicating that both peptides could stimulate a similar percentage of
Ag-specific cells to degranulate during acute infection. Our bulk culture CTL results also indicate that, early in the memory phase of infection, VV-immune splenocytes lyse both MVA-029- and MVA-043-pulsed EL-4 cells. Therefore, our data may be explained by an inherent difference in intrinsic affinity between the two peptides, with MVA-029 being a higher affinity peptide. However, neither one of the peptides had a high predicted binding score using the “HLA Peptide Binding Predictions” to predict D<sup>B</sup> or K<sup>B</sup> affinity (http://bimas.dctr.nih.gov/molbio/hla_bind/), although MVA-029 did rank higher than MVA-043. In addition, the relative frequencies of MVA-029- and MVA-043-specific T cells may differ during the acute vs the memory phase T cell response. Use of tetramers to track Ag-specific cells in vivo and isolation of MVA-029- and MVA-043-specific T cell clones will allow further characterization of the distinct profiles of these two epitope-specific T cell populations.

We had previously identified novel human HLA-A*0201 VV CD8<sup>T</sup> CTL epitopes, and recently 165 peptide-immunized HLA-A2 transgenic mice were protected from lethal challenge by VV (8, 9). We plan to test the hypothesis that adoptive transfer of MVA-029 T cells may be protective upon a lethal challenge with VV, whereas transfer of MVA-043-specific T cells might not protect mice from a lethal infection due to their inability to secrete IFN-γ (19, 20). These interesting differences in effector function between MVA-043- and MVA-029-specific T cells may translate into functional differences in vivo and are worthy of further investigation.

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