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Basic Research

Identification of protective T-cell antigens for smallpox vaccines

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ABSTRACT

Background aims: E3L is an immediate-early protein of vaccinia virus (VV) that is detected within 0.5 h of infection, potentially before the many immune evasion genes of vaccinia can exert their protective effects. E3L is highly conserved among orthopoxviruses and hence could provide important protective T-cell epitopes that should be retained in any subunit or attenuated vaccine. We have therefore evaluated the immunogenicity of E3L in healthy VV-vaccinated donors.

Methods: Peripheral blood mononuclear cells from healthy volunteers (n = 13) who had previously received a smallpox vaccine (Dryvax) were activated and expanded using overlapping E3L peptides and their function, specificity and antiviral activity was analyzed. E3L-specific T cells were expanded from 7 of 12 (58.3%) vaccinated healthy donors. Twenty-five percent of these produced CD8+ T-cell responses and 87.5% produced CD4+ T cells. We identified epitopes restricted by HLA-B35 and HLA-DR15.

Results: E3L-specific T cells killed peptide-loaded target cells as well as vaccinia-infected cells, but only CD8+ T cells could prevent the spread of infectious virus in virus inhibition assays. The epitopes recognized by E3L-specific T cells were shared with monkeypox, and although there was a single amino acid change in the variola epitope homolog, it was recognized by vaccinia-specific T-cells.

Conclusions: It might be important to include E3L in any deletion mutant or subunit vaccine and E3L could provide a useful antigen to monitor protective immunity in humans.

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Importance

Despite the eradication of smallpox (variola virus) in 1979, it remains a bioterrorism threat due to the retention of variola stocks. Vaccinia virus, the current vaccine, is considered unsafe for individuals who are immunosuppressed, very young, elderly or pregnant, and thus alternative, safer subunit or gene-deleted viral vaccines are sought. Vaccinia encodes approximately 250 genes, many of which elicit T-cell immunity, but not all viral antigen-specific T cells are protective. The E3L gene of vaccinia virus is expressed within 30 min of infection, and should be presented to T cells before the expression of the viral immune evasion genes. We show that E3L elicits CD8+ cells that recognize and kill vaccinia-infected cells and prevent virus spread in infected cultures. We propose that this protein, among

other immediate early genes should be retained in modified or subunit vaccines.

Introduction

Although smallpox (variola) has been eradicated by vaccination programs that ceased in the 1980s [1,2], stocks of variola still exist, and its potential as a biological weapon has become an international concern warranting the continued availability of a smallpox vaccine [3–5]. Outbreaks of animal poxviruses, particularly monkeypox [6,7], in human populations further highlight the continued need for a vaccine, and sequence homology between vaccinia virus (VV), variola, and monkeypox (all members of the *Orthopoxvirus* genus) means that VV is an effective vaccine for all [8].

Although the current NYCBH strain of VV (Dryvax) [9] is highly effective, it is a live, nonattenuated virus and is contraindicated in young children and the elderly, as well as in individuals who are immunosuppressed, are pregnant or have skin or heart conditions—altogether, approximately 25% of the population [10]. Hence, there is significant interest in a less pathogenic, but equally immunogenic, vaccine. The highly attenuated modified vaccinia Ankara (MVA) has

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proved safe in HIV-infected persons and is a promising alternative vaccine [11]. However, having lost up to 15% of its genome after extensive passage in chicken embryo fibroblasts [12,13], it cannot replicate in human cells and therefore requires doses more than 100-fold higher than for Dryvax and booster vaccination to provide equivalent protection in animal models [14,15].

E3L is an immediate early protein that inhibits the innate immune response to viral double stranded RNA [16]. An E3L deletion mutant provided a promising attenuated vaccine that was safe and effective in murine and rabbit infection models, but it did not prevent lethal infections in a monkeypox model [17]. Because E3L is expressed within 0.5 h of infection by VV [18], it should be processed and presented to the cellular immune responses before immune evasion genes of VV are expressed and allow T-cell killing before newly replicated virus is released from infected cells. If so, this may explain the lack of efficacy of E3L deletion mutant vaccines. Hence, E3L and other immediate early genes, might provide important, protective T-cell epitopes that should be preserved in any live-attenuated or subunit vaccine. The identification of viral proteins that induce protective T cells and are recognized by a majority of immune humans would be useful for the development of subunit or deletion mutant vaccines, might determine the relative importance of each arm of the immune response and assist in the monitoring and evaluation of effective T-cell responses to vaccination.

We therefore asked whether E3L contained immunodominant epitopes for T cells and evaluated the ability of E3L-specific T cells to kill VV-infected cells and prevent infectious virus spread in a tissue culture model. E3L-specific CD8⁺ T cells could recognize and kill VV-infected cells before they were able to replicate new virus. Hence, it might be important to retain E3L in any deletion mutant or subunit vaccine and E3L would provide a useful antigen to monitor protective immunity in humans.

Methods

Donors and cell lines

Peripheral blood mononuclear cells (PBMCs) were obtained with informed consent on Baylor College of Medicine Institutional Review Board–approved protocols from healthy volunteers who had previously received the VV vaccine Dryvax. PBMCs were used to generate VV antigen-specific T cells (VVSTs) as well as dendritic cells (DCs) and activated T cells (ATCs) for use as antigen-presenting cells (APCs).

Activated T-cells

ATCs for use as autologous target cells were generated by stimulation of PBMCs (5×10^5 cells per well) in 24-well non-tissue-culture-treated plates coated with a CD3 antibody produced by the OKT3 hybridoma (ATCC #CRL 8001, Manassas, VA, USA) and CD28 antibody (Becton Dickinson BD, Franklin Lakes, NJ, USA; each at 1 $\mu\text{g}/\text{mL}$) (CD3/28 MAbs). ATCs were maintained in T-cell medium (RPMI 1640; Hyclone, Waltham, MA, USA) supplemented with 45% Click's medium (Irvine Scientific, Santa Ana, CA, USA), 2 mmol/L GlutaMAX TM-1 (Invitrogen, Carlsbad, CA, USA) and 5% Human AB Serum (Valley Biomedical, Winchester, VA, USA) and supplemented with interleukin (IL)-2 (50 U/mL) (R&D Systems, Minneapolis, MN, USA), which was replenished every 3 to 4 days. Two days before antigen-specific T-cell re-stimulation, ATCs were reactivated on CD3/28 MAb-coated plates to upregulate costimulatory molecules [19].

Isolation of CD8 and CD4 T cells

CD8 and CD4 T cells were enriched from fresh or frozen PBMCs by magnetic selection using MACS Beads (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

DC generation

Monocytes were isolated from fresh or frozen PBMCs by CD14 selection using MACS Beads (Miltenyi) and cultured in DC medium (CellGenix, Portsmouth, NH, USA; supplemented with 2 mmol/L GlutaMAX TM-1) with 400 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems) and 400 U/mL IL-4 (R&D Systems) for 5 days. IL-4 and GM-CSF (R&D Systems) were replenished on day 3. On day 5, DCs were matured in DC medium using a cytokine cocktail containing 100 ng/mL IL-6 (R&D Systems), 10 ng/mL IL-1 β (R&D Systems), 10 ng/mL tumor necrosis factor (TNF)- α (R&D Systems), 1 $\mu\text{g}/\text{mL}$ PGE2 (Sigma-Aldrich, St. Louis, MO, USA), 800 U/mL GM-CSF and 1000 U/mL IL-4 for 48 h [20,21].

Loading DCs or ATCs with pepmixes

Mature DCs or ATCs were pelleted and pulsed with pepmixes spanning E3L or other VV antigens for 30 min at 37°C in 5% CO₂. Pepmix sequences were derived from the Western Reserve strain of vaccinia. The E3L pepmix (Genemed Synthesis, San Antonio, TX, USA) is an overlapping peptide library consisting of thirty-five 20-mer peptides overlapping by 15 amino acids covering the complete sequence of E3L. These peptides were aliquoted into 12 pools containing five to six peptides per pool such that each peptide was uniquely present in two pools (see Figure 2B in Results) [22]. Pepmixes spanning A10L, H3L, C7L, G5R and B22R were obtained off the shelf from JPT Technologies (Berlin, Germany).

E3L-specific T-cell generation

Whole PBMCs, CD8 or CD4 T cells were used as responder cells and stimulated with pepmix-pulsed DCs at a stimulator: responder (S:R) ratio of 1:20 in T-cell medium supplemented with 400 U/mL IL-4 and 10 ng/mL IL-7 (PeproTech, Rocky Hill, NJ, USA), which we have previously shown to increase the expansion and repertoire of T cells specific for a range of other viruses [23,24]. T cells specific for G5R, C7L and H3L (early genes) and or A10L and D8L (late genes) were activated and expanded in the same way. On day 9, responder T cells were harvested, restimulated with pepmix-pulsed DCs at a S:R ratio of 1:10 and cultured for an additional 7 days with IL-4 and IL-7 on the day of restimulation then supplemented with IL-2 (50 IU/mL) from day 12. On day 16, cells were harvested, counted and analyzed in IFN- γ ELISpot and cytotoxicity assays or restimulated with irradiated (30 Gy), pepmix-pulsed ATCs at a S:R ratio of 1:1. On day 23, cells were harvested, counted and analyzed in tetramer staining and virus inhibition assays.

IFN- γ ELISpot assay

Ninety-six-well filter plates (MSIPS4W; Millipore, Billerica, MA, USA) were coated with 10 $\mu\text{g}/\text{mL}$ of an anti-IFN- γ monoclonal antibody (mAb), 1-D1K (Mabtech, Stockholm, Sweden). T cells were added to duplicate wells at cell numbers ranging from 2.5×10^4 to 1×10^5 in the presence of single or pooled peptides at a final concentration of 2 $\mu\text{mol}/\text{L}$ for each peptide. HLA class II blocking was performed with 2 $\mu\text{g}/\text{well}$ anti-HLA-DR, -DP and -DQ antibodies (clone Tu39, BD Biosciences, Franklin Lakes, NJ). The plates were incubated overnight at 37°C in 5% CO₂. The cells were discarded the following day, and a biotinylated anti-IFN- γ mAb, 7-B6-1 (Mabtech Inc., Mariemont, OH, USA) was added at 1 $\mu\text{g}/\text{mL}$ and left for 2 h at 37°C, followed by the addition of streptavidin-horseradish peroxidase (Mabtech) for 1 h at room temperature. Spot color was developed by adding 3-amino-9-ethylcarbazol substrate diluted in acetate buffer containing H₂O₂. Plates were sent to Zellnet Consulting (New York, NY, USA) for quantification. The results are shown as the number of spot-forming cells (SFC)/10⁵ cells.

Cytotoxicity assay

The cytotoxic activity of E3L-specific T cells was tested in a standard 6-h ^{51}Cr release assay using autologous or allogeneic (HLA-mismatched or matched at a single allele) OKT3 blasts. OKT3 blasts were pulsed with E3L pepmixes (50 ng) at 37°C for 30 min. Target cells were labeled with ^{51}Cr sodium chromate (MP Biomedicals, Solon, OH, USA) at 37°C for 1 h, then washed three times and seeded in triplicate in 96-well V-bottomed plates (Falcon) at 5×10^3 cells per well. Effector cells were added at 40:1, 20:1, 10:1 and 5:1 effector:target (E:T) ratio. Spontaneous and maximum release were determined by incubating target cells without effectors in medium alone or in 1% Triton X-100, respectively. After centrifugation at 1500 rpm for 3 min, the plates were incubated at 37°C for 6 h. Supernatants were harvested and analyzed using gamma counter. The percent specific lysis was calculated as specific lysis [experimental release – spontaneous release] / (maximum release – spontaneous release) \times 100. HLA class I blocking was performed with 2 $\mu\text{g}/\text{well}$ anti-HLA-A,-B,-C antibodies (BD Biosciences) at 20:1 of E:T. HLA class II blocking was performed as described earlier.

Tetramer staining

A phycoerythrin (PE)-labeled HLA-B35 tetramer complexed with the NPVTVINEY peptide (aa 117–125) was generated by the MHC Tetramer Production Facility of the Dan L. Duncan Cancer Center Proteomics Core (BCM, Houston, TX, USA). Cells were incubated with a PE-labeled HLA-B35 tetramer, fluorescein isothiocyanate (FITC)-labeled anti-CD3 antibody (BD Biosciences) and allophycocyanin (APC)-labeled anti-CD8 antibody (BD Biosciences) for 30 min at 4°C. After incubation, the cells were washed and fixed in PBS with 1% paraformaldehyde. Samples were analyzed by FACS and at least 20 000 events were collected for each sample.

Vaccinia virus infection of target cells

Target cells used in functional assays to determine the cytotoxicity activity and virus inhibition assay were Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines (LCLs) derived from PBMC using EBV isolate B95-8 in the presence of cyclosporin A as previously described [25–27]. A recombinant kinase deletion mutant (TK-VV) derived from the Western Reserve strain was used at a multiplicity of infection (MOI) of 1 for the infection of LCLs. LCL pellets were incubated with virus for 1 h, washed three times, diluted to 1×10^6 cells/mL and incubated at 37°C, 5% CO_2 . As cytotoxicity assay targets we used LCLs that had been infected with VV for 2, 4 and 8 h, respectively.

Virus inhibition assay

Two hours after infection with VV, LCLs were cocultured with autologous VVSTs for 48 h at 37°C in 5% CO_2 . To measure T-cell inhibition of virus replication in the LCLs, the cocultures were harvested and lysed by three freeze-thaw cycles and stored at -80°C . We then added 10-fold dilutions of cell lysate (from 10^{-1} to 10^{-7}) to confluent MRC-5 fibroblasts (ATCC) for 1 h at 37°C, then added fresh medium. Cells were incubated at 37°C, 5% CO_2 for 24 h. Virus plaques were quantified after washing wells with PBS and adding 0.5 mL of crystal violet (Sigma-Aldrich) solution to each well for 5 min, followed by washing with PBS. Plaques were counted and virus titer estimated.

Results

Response of healthy vaccinated donors to E3L

PBMCs from 12 previously VV vaccinated healthy donors were stimulated with E3L pepmix-loaded DCs in the presence of IL-4 and

IL-7 on days 0 and 9. Although responses to E3L were not directly detectable in IFN- γ ELISpot assays after overnight stimulation, the specific responder T cells could be detected on days 9 and 16 of culture. Seven of 12 donors responded to E3L with a median of 54.3 SFC per 1×10^5 cells (range 49.3–139.5 SFC) on day 16. Five of the 12 donors did not respond, with a median of 6.3 SFC per 1×10^5 cells (range 4.7–14.5 SFC) (Figure 1).

Identification of CD8+ T-cell epitopes within E3L

To identify CD8+ T-cell epitopes in E3L, CD8+ T cells were stimulated on days 0 and 9 with E3L pepmix-pulsed DCs in the presence of IL-4 and IL-7. On day 16, they were challenged with peptide pools of 5 to 6 E3L peptides, in which each peptide was uniquely represented just two pools (Figure 2B) [22]. Donor 1 responded to pools 4 and 10 (Figure 2A), indicating specificity for peptide 22 that is common to both pools (Figure 2C). To validate the response to peptide 22 we tested the ability of donor 1 E3L-specific CD8+ T cells to kill autologous activated T cells alone (negative control) or pulsed with peptide 22, in a 6 h ^{51}Cr release assay (Figure 2D). At an effector:target (E:T) ratio of 20:1, T cells killed peptide 22-pulsed target cells (45.5% specific ^{51}Cr release), with minimal recognition of control target cells (2% specific ^{51}Cr release). Cytotoxicity was blocked by Class I MABs (14.6% specific killing) (Figure 2D). To determine the HLA-restricting allele of peptide 22, we evaluated killing of partially HLA class I-matched, peptide-pulsed target cells. At an E:T ratio of 20:1, we observed specific lysis of autologous and HLA-B35 matched but not other partially matched OKT3 blasts pulsed with peptide 22 (57.2% ^{51}Cr release for autologous peptide-pulsed ATCs, 57.5% for HLA-B35, 5% for HLA A24, 16% for A26 and 29% for B40) (Figure 2E). Specific killing was blocked by HLA class I MABs (32.1% and 37.5% specific lysis) (Figure 2F). We fine mapped the E3L epitope within peptide 22, by testing every possible 9mer for its ability to mediate recognition of HLA-B35 positive target cells. Using T cells from HLA B35 positive donors 1 and 2, we identified a novel VV epitope aa 117-125 - NPVTVINEY (NPV) (Figure 2G). We then generated an HLA B35-NPV tetramer that recognized 18.1% and 1.9% of CD8+ VZV-specific T cells from donors 1 and 2.

Identification of CD4+ T-cell epitopes within E3L

To identify CD4 epitopes within E3L, we activated and expanded CD4+ T cells from donor 3 with E3L pepmix-loaded DCs in the presence of IL-4 and IL-7, then challenged them with E3L peptide pools in

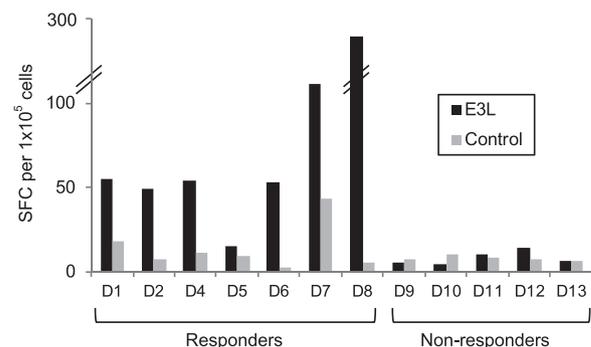


Figure 1. Response of healthy vaccinated donors to E3L. PBMCs were stimulated with autologous DCs loaded with E3L pepmixes on days 0 and 9 in the presence of IL-4 and IL-7. The frequency of E3L-specific T cells generated from 12 donors was measured on day 16 by IFN- γ ELISpot in response to stimulation with the E3L pepmix (black bars) is shown. Results are expressed as spot-forming cells (SFC) per 1×10^5 cells. Control was IFN- γ release in response to stimulation with irrelevant pepmix (gray bars). The data shown represent repeated experiments with all responders at least three times, whereas experiments for non-responders were repeated twice.

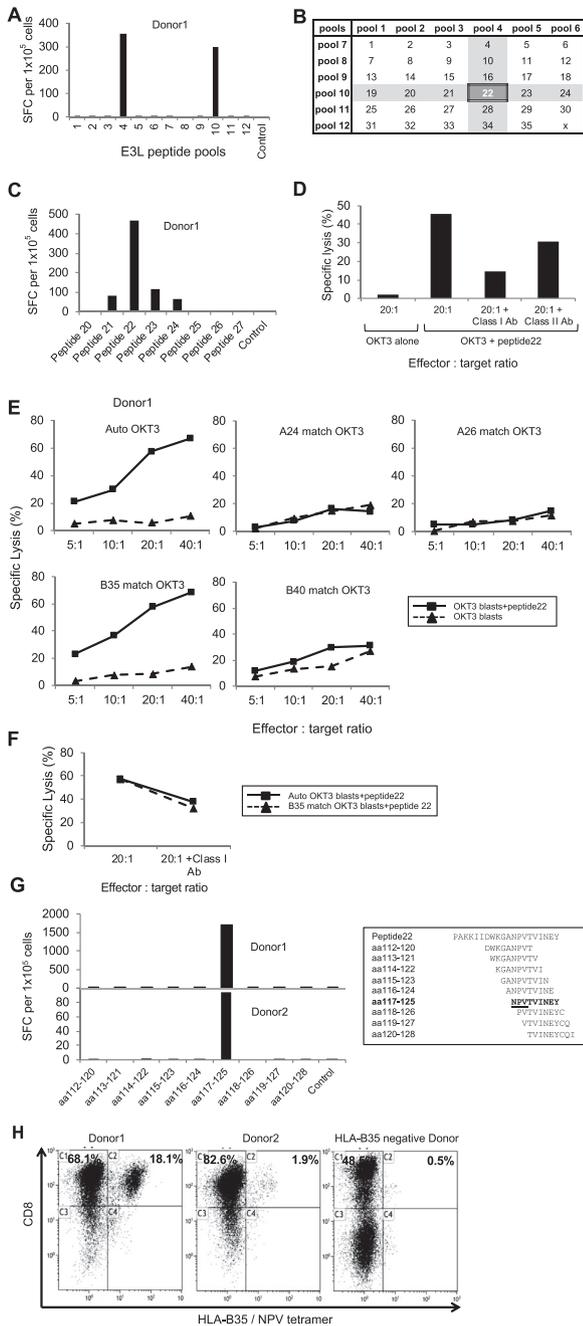


Figure 2. Identification of CD8+ T-cell epitopes within E3L from HLA-B35 donors. (A) CD8 PBMCs from donor 1 were stimulated with autologous DCs loaded with E3L pepmix in presence of IL-4 and IL-7. E3L-specific CD8 T-cells were measured by IFN- γ ELISpot in response to stimulation with E3L peptide pools on day 16. Results are expressed as spot-forming cells (SFC) per 1×10^5 cells. (B) Design of peptide pools. Each pool contained 5 to 6 peptides and was assigned a number from 1 to 12. The numbers of the pools (top low and left column) are shown in bold. Individual peptides (n = 35) in these 12 pools correspond to the numbers in the respective columns and row. Each peptide was uniquely present in two pools. (C) E3L-specific CD8+ T-cells were stimulated with individual E3L peptides 20 to 27, and IFN- γ production measured by ELISpot. (D) The cytotoxic activity of E3L-specific CD8+ T-cells from donor 1 was measured in a 6 hours ^{51}Cr release assay using E3L peptide 22-loaded autologous OKT3 blast target cells with or without blocking antibodies to HLA class I and II at an E:T ratio of 20:1. (E) Specific lysis of autologous or allogeneic OKT3 blasts matched at single HLA alleles and pulsed with E3L peptide 22 was evaluated in a 6-h Cr^{51} release assay, at E:T ratios of 40:1, 20:1, 10:1 and 5:1. (F) Specific lysis of autologous and HLA-B35 matched allogeneic OKT3 blast pulsed with E3L peptide 22 the presence or absence of a HLA class I blocking antibody was evaluated in a 6-h ^{51}Cr release assay in at E:T ratio of 20:1. (G) Fine mapping of E3L 9-mer peptide within peptide 22 (20-mer). E3L-specific CD8+ T cells from donor 1 and donor 2 (HLA-B35 donors) were stimulated with individual 9-mer peptides and IFN- γ production measured in ELISpot assays. (H) E3L-specific CD8+ T-cells from donors 1 and 2 were stained with an HLA-B35/NPV tetramer and anti-CD8 antibody and analyzed by FACS.

ELISpot assays. Donor 3 T cells produced IFN- γ in response to pools 1, 2 and 12 (Figure 3A) that contained peptides 31 and 32 in common (Figure 3B). We validated the response to peptides 31 and 32 individually, and the HLA class II restriction by demonstrating a decreased response in the presence of HLA-DR MABs (Figure 3C). Three other donors (donors 4, 5 and 6) also responded to peptides 31 and 32 and were also inhibited by HLA-DR MAb (Figure 3D). All peptide 31/32-responsive donors shared HLA-DR15. The minimal E3L epitope in peptides 31 and 32, identified a new VV epitope aa156-170-GRVFDKADGKSKRDA (GRV) (Figure 3E). CD4+ T cells from HLA-DR15 negative donors did not respond to peptides 31 and 32 (data not shown).

The HLA-A, -B, -DRB1 and -DQB1 alleles of all donors screened is shown in Table 1. In summary, CD8 VVSTs were induced from PBMC of donors 1 and 2. We found a VV epitope aa 117-125-NPV in these CD8 VVSTs as shown in Figure 2. By contrast, CD4 VVSTs were induced from donors 2–8. As we showed in Figure 3, a new VV class II epitope was identified in donors 3 to 6. CD4 T cells from donors 2, 4, 6, 7 and 8 responded to different peptides. Neither CD4 VVSTs nor CD8 VVSTs were induced from donors 9–13.

Variant E3L epitope in variola and modified vaccinia Ankara (MVA)

The new HLA DR15-restricted epitope, GRVFDKADGKSKRDA was conserved between vaccinia, variola, MVA and monkeypox. However, the class I restricted epitope NPVTVINEY was modified in variola from valine 121 to isoleucine (NPV V→I). Nevertheless, VV-NPVTVINEY-specific CD8+ T-cells from donors 1 and 2 recognized the variola epitope in IFN- λ ELISpot assays (Figure 4A). At an E:T ratio of 40:1, donor 1 T cells killed NPV-pulsed target cells (89.9% ^{51}Cr release), NPV V→I pulsed target cells (100% ^{51}Cr release) and control target cells (10.1% ^{51}Cr release), while donor 2 T-cells induced 59.2%, 59.2% and 2.1% killing of the same target cells, respectively (Figure 4B). To validate this cross-reactivity, we generated an HLA-B35/NPV V→I (variola) tetramer that recognized 7.6% and 0.5% of the E3L stimulated CD8 T-cells from donors 1 and 2. Of note, this compared with a frequency of 1712.5 SFC (1.7%) and 85 SFC (0.085%) per 1×10^5 cells as measured in the ELISpot assay, showing that the ELISpot assay underestimates the true T-cell frequency of antigen-specific T cells by at least 10-fold (Figure 4C).

Vaccinia E3L-specific T cells can kill VV-infected LCLs and prevent production of infectious virus particles

We predicted that E3L-specific T cells would be among the most rapid killers of newly infected cells because E3L is not only expressed early after infection (transcript detected within 30 minutes) but is also packaged into the virion [28], from whence it can be processed and presented to specific T cells. To test this hypothesis, E3L-specific T cells from donors 1 and 2 were co-cultured with autologous VV-infected LCLs at increasing times after infection, and the ability of the virus to become amplified in target cells was measured. Due to their low frequency, donor 2 CD3+/CD8+/NPV tetramer+ cells were first enriched using a FACS cell sorter (BD FACSAria II). At an E:T ratio of 20:1, donor 1 T cells killed both LCLs pulsed with peptide 22 (96.6% specific lysis) and VV-infected LCLs, whether added at 2, 4 or 8 h after infection (44.6%, 31.6% and 39.3% specific lysis, respectively), whereas unpulsed, uninfected LCLs were not killed (8.6% specific lysis). Donor 2 T cells also killed infected LCLs after 2, 4 and 8 h target cells (66.2%, 46.5% and 53.9% specific lysis, respectively) (Figure 5A). To determine if CD8+ E3L-specific T cells could inhibit virus replication, they were added to autologous LCLs 2 h after infection with VV at a T cell to LCL ratio of 5:1 and cultured for 2 days. Co-culture lysates were then added to MRC-5 fibroblasts at limiting dilution to measure the amount of virus produced by the infected LCLs using a plaque assay. Figure 5B shows that E3L-specific CD8+ T-cells from both donors

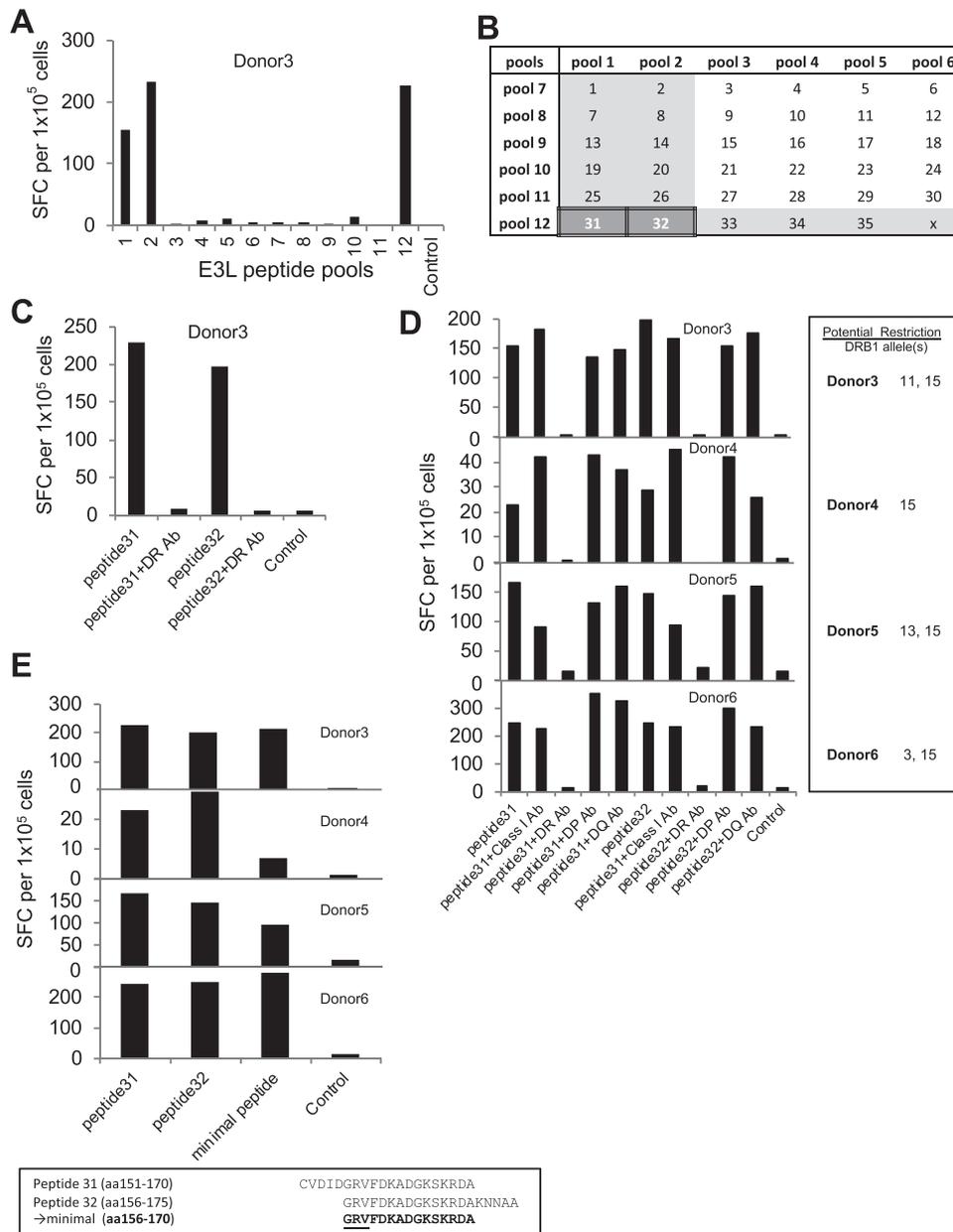


Figure 3. Identification of CD4+ T-cell epitopes within E3L from HLA-DR15 donors. (A) CD4+ T-cells from donor 3 were stimulated with autologous DCs loaded with E3L pepmixes in presence of IL-4 and IL-7, and the response to stimulation with E3L peptide pools was measured on day 9. Results are expressed as spot-forming cells (SFC) per 1×10^5 responder cells. (B) Identification of reactive peptides 31 and 32 within the peptide pools. (C) The γ -IFN response of E3L-specific CD4+ T cells to single peptides 31 and 32 with or without blocking antibodies to HLA class II DR. (D) E3L-specific CD4+ T cells from donors 3 to 6 (HLA-DR15 donors) were stimulated with peptides 31 and 32, with or without HLA class II DR, DP and DQ blocking antibodies and the response was measured by IFN- γ ELISpot assay. (E) To fine map the minimal HLA class II-restricted epitope within peptides 31 and 32 (20-mers), E3L-specific CD4+ T cells from donors 3 to 6 were stimulated with 15-mer peptides and the responses measured by IFN- γ ELISpot.

could reduce the titer of VV harvested from LCLs by 5 logs from 10^7 pfu/ml to 10^2 pfu/ml.

Protective capacity of VV-specific T cells

To determine whether T cells specific for other vaccinia proteins were able to prevent the spread of VV within infected cell cocultures, we stimulated CD4+ and CD8+ T cells from donors 2, 4 and 5 with DCs loaded with vaccinia pepmixes from G5R (early/core), C7L (early) and B22R (early), A10L (late/virion) and H3L (late/membrane) that were available off the shelf from JPT Peptide Technologies (Table 2) [29–35]. CD8+ VVSTs were elicited by C7L and E3L in donor 2, by B22R in donor 4 and by A10L in donor 5, whereas CD4+ T cells were elicited by A10L, H3L and E3L in donor 4 and by A10L, H3L, G5R and E3L in donor 5.

A10L, C7L and E3L-specific CD8 CTLs killed both pepmix-pulsed LCLs and VV infected LCLs. However, only E3L-specific T-cells prevented VV replication in co-culture virus inhibition assay (Figure 6A). B22R-specific CD8+ T cells from donor 4 neither killed VV infected target LCLs nor prevented VV replication (Figure 6B). E3L and C7L-specific CD8+ T cells both killed VV-infected LCLs, whether cocultured at 2, 4 or 8 h after infection with VV. A10L-specific CD8+ T cells killed VV infected LCL 8 h after infection, but not if co-cultured 2 and 4 h after infection with VV, despite its presence in the virion [28], perhaps reflecting its late gene expression (Figure 6C).

CD4+ T cells specific for A10L-, H3L-, G5R- and E3L did not killed VV infected target cells, nor could they prevent VV replication in cocultures (Figure 6D,E). Table 2 shows the function, time of transcription of each VV gene and presence in virion as well as

Table 1
Listing of CD8 and CD4 T cell peptides in E3L

Donor	HLA type				CD8 responder		CD4 responder	
	HLA-A	HLA-B	HLA-DRB1	HLA-DQB1	CD8 peptide number (HLA type)	Cytotoxicity % Lysis / % Lysis using class I Ab	CD4 peptide number (HLA type)	ELISpot % inhibition using class II Ab
D1	A24, A26	B35 , B40(61)	8, 12	3(8), 3(9)	22 (B35)	57.2% / 32.1% (20:1)	—	—
D2	A2, A3	B14, B35	ND	ND	22 (B35)	39.9% / 4.1% (100:1)	8 (DR), 35 (DR)	97.0%, 95.2%
D3	A2, A3	B7, B51	11, 15	3(7), 6	—	—	31,32 (DR)	96.10%
D4	A24	B7	15	6	—	—	23,24 (DR), 31,32 (DR)	96.4%, 97.8%
D5	A2, A11	B7, B8	3, 15	1, 2	—	—	31,32 (DR)	91.90%
D6	A2, A3	B7, B41	13, 15	3(7), 6	—	—	9 (DR), 20,21 (DR), 31,32 (DR)	95.8%, 77.4%, 97.9%
D7	A2, A26	B15(61), B15(71)	4	3(7), 3(8)	—	—	1 (DR), 4,5 (DQ)	90.0%, 81.5%
D8	A2, A3	B35 , B57	7, 17	2, 9	—	—	34,35 (DR)	72.40%
D9	A2, A29	B7, B38	10, 15	5	—	—	—	—
D10	A11, A28	B35 , B53	ND	ND	—	—	—	—
D11	A11, A33	B35 , B39	4, 8	3(8), 6	—	—	—	—
D12	A11, A24	B35 , B55	8, 9	ND	—	—	—	—
D13	A2, A11	B15(62), B40(60)	9, 15	3(9), 5	—	—	—	—

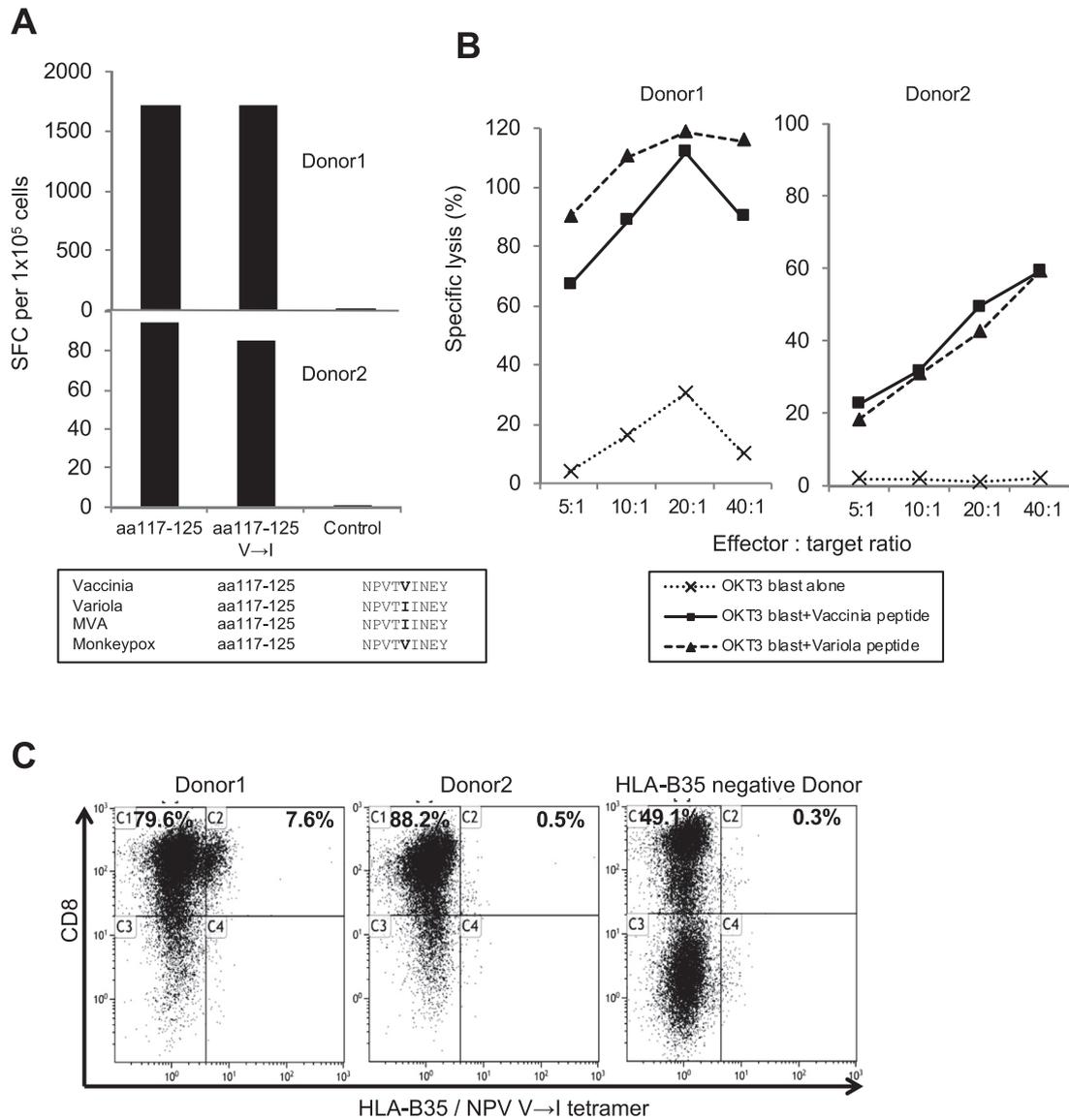


Figure 4. Variant E3L epitope in variola and modified vaccinia nkrara (MVA). (A) E3L-specific CD8+ T-cells from donor 1 and donor 2 were restimulated with vaccinia E3L peptide (aa 117-125:NPV) or the variola/MVA variant E3L peptide (aa117-125:NPV V→I). (B) Specific lysis of target cells alone or loaded with vaccinia and variola peptides in was evaluated in a 6-h Cr⁵¹ release assay at E:T ratios of 40:1, 20:1, 10:1 and 5:1. (C) E3L-specific CD8+ T cells from donors 1 and 2 were stained with the variola variant tetramer - HLA-B35/NPV V→I (and anti-CD8 antibody and analyzed by FACS.

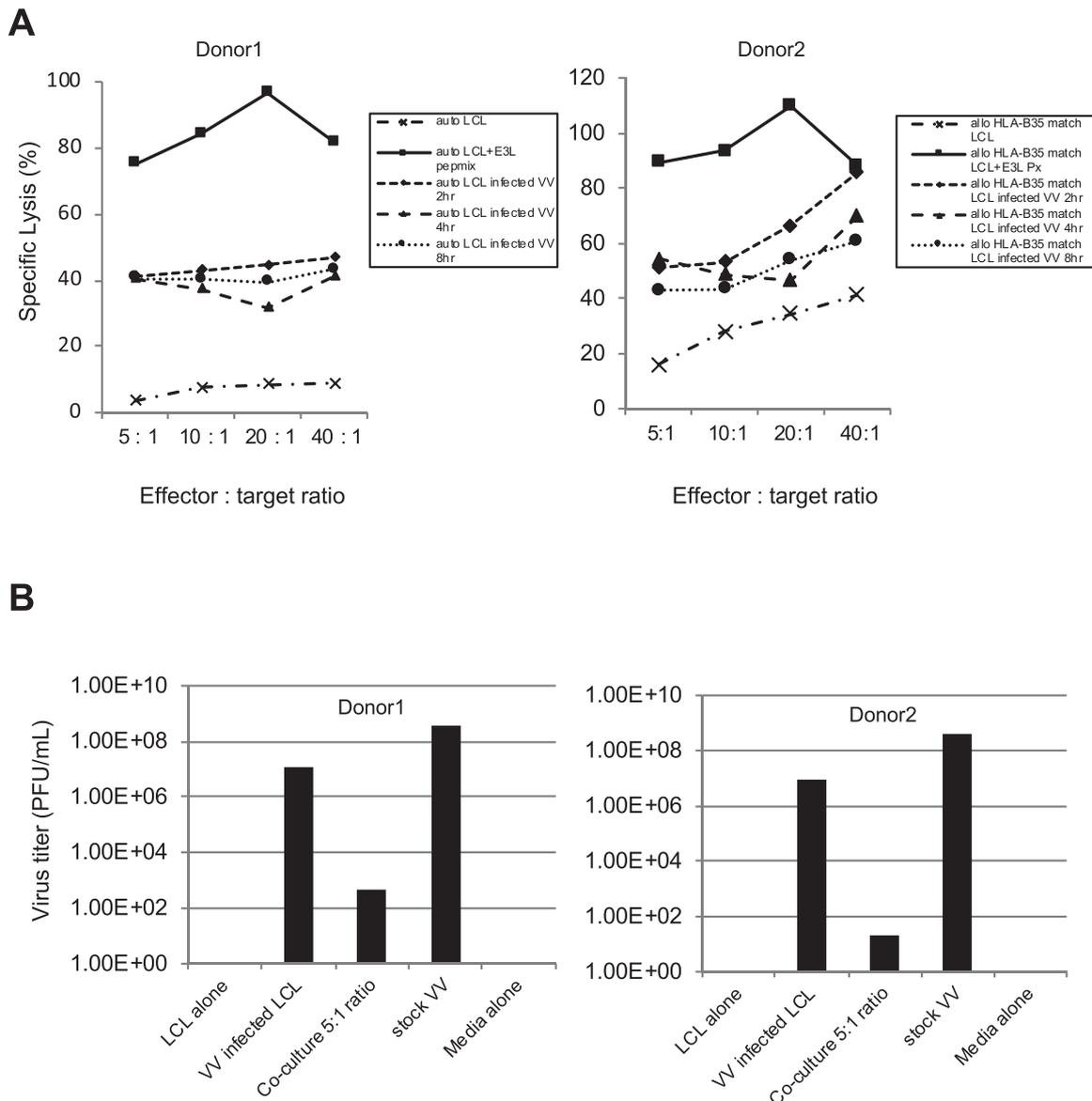


Figure 5. Vaccinia-specific T cells can kill VV-infected LCL and prevent VV replication. (A) E3L-specific CD8⁺ T cells from donors 1 and 2 were cultured with autologous or HLA-B35 matched allogeneic LCLs at 2, 4 and 8 h after infection with VV. Specific lysis was evaluated in 6-h Cr⁵¹ release assay at E:T ratio of 40:1, 20:1, 10:1 and 5:1. (B) VV-infected LCLs were cultured alone or with E3L-specific CD8⁺ T cells at a T-cell to VV-infected LCL ratio of 5:1, or with stock VV or medium alone. Virus titers were measured as described in material and methods.

Table 2
Comparison each of vaccinia proteins in this study

Protein	Length (aa)	Transcript	Function	Cytotoxic activity (Px pulsed LCL / VV infected LCL)		Virus inhibition	
				CD8	CD4	CD8	CD4
A10L	891	Late	Major Core Protein P4a	(+)/(+)	(-)/(-)	(-)	(-)
D8L	304	Late	Cell surface-binding protein	—	—	—	—
B22R	188	Early	Serpin	(+)/(+)	—	(-)	—
H3L	324	Late	Heparin binding protein	—	(-)/(-)	—	(-)
C7L	150	Early	Host range protein 2	(+)/(+)	—	(-)	—
C5R	434	Immediate early	Hypothetical protein	—	(-)/(-)	—	(-)
E3L	190	Immediate early	ds RNA-binding, inhibits IFN	(+)/(+)	(-)/(-)	(+)	(-)

comparison of vaccinia proteins using cytotoxicity and virus inhibition assay in this study. Hence, the only T cells that were able to prevent infectious virus spread were CD8⁺ E3L-specific T cells. The failure of CD4-E3L-specific T cells was likely because killing by CD4⁺ T cells is slower than that of CD8⁺ T cells [36].

Discussion

We have demonstrated that the immediate early E3L protein of VV provides T-cell epitopes that can be recognized in the context of HLA B35 and HLA DR15. E3L is highly conserved among poxviruses,

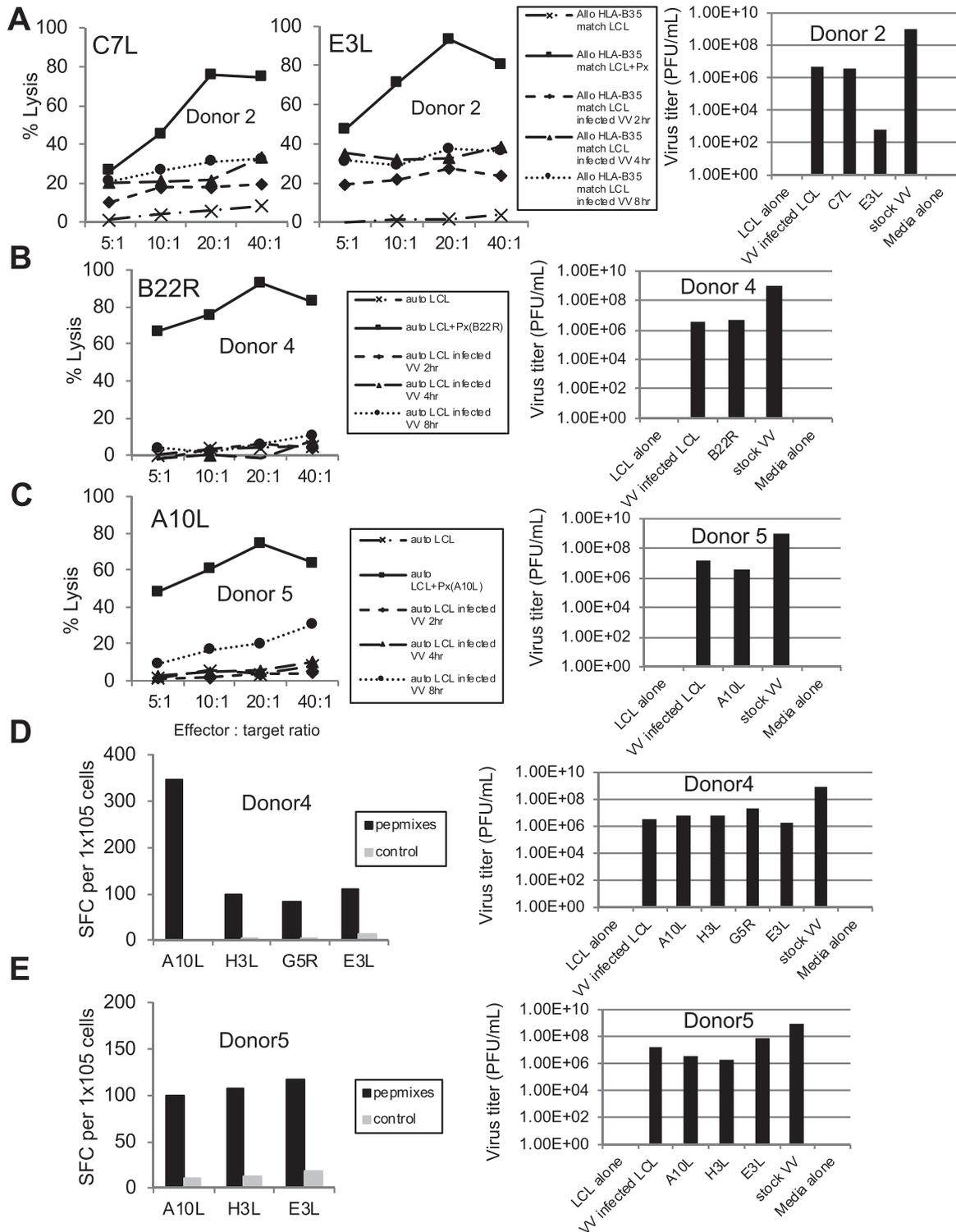


Figure 6. Comparative function of T cells specific for E3L and other vaccinia proteins using cytotoxicity and virus inhibition assay. (A) The cytotoxic activity of C7L- and E3L-specific CD8+ T cells from donor 2 against VV infected or pepmix-pulsed, HLA-B35 matched allogeneic LCLs plated 2, 4 and 8 hours after infection was evaluated in a 6-h Cr⁵¹ release assay at E:T ratio of 40:1, 20:1, 10:1 and 5:1. E3L- and C7L-specific CD8+ T cells both killed VV infected HLA-B35 matched allogeneic donor LCL in all the conditions of 2, 4 and 8 h after infection with VV. Only E3L prevented VV replication in co-culture virus inhibition assay (right panel). (B) B22R-specific CD8+ T cells from donor 4 were able to kill pepmix pulsed, but not VV-infected, LCLs in chromium release assay (left panel), and were unable to prevent infectious virus spread in cocultures (right panel). (C) The cytotoxic activity and viral inhibition assay of A10L-specific CD8+ T-cells from donor 5. A10L-specific CD8+ T cells killed pepmix-pulsed LCLs and VV infected LCLs if added 8 h after LCL infection, but they did not kill VV-infected LCL when added 2 or 4 h after infection with VV. A10L-specific T cells did not prevent infectious virus spread in infected LCLs. (D) CD4+ T cells from donor 4 that were specific for A10L-, H3L-, G5R- and E3L as measured by IFN-γ ELISpot (left panel) did not prevent VV replication in coculture with VV-infected autologous LCLs. (E) CD4+ T cells from donor 5 secreted IFN-γ in response to A10L-, H3L- and E3L in ELISPOT assays (left panel) did not inhibit VV replication in cocultures with VV-infected autologous LCLs.

but although the CD8 epitope we identified in VV is shared with monkeypox, there is a single amino acid change in the variola epitope (NPVTIINEY) that contains a V to I mutation in position 5. However, position 5 is a non-anchor position, and VV E3L-NPVTIINEY-specific T cells recognized the variola epitope and should therefore provide protection not only against VV but also variola and monkeypox. E3L-specific T cells could kill peptide-loaded target cells as well as VV-infected cells and could prevent the spread of infectious virus in a cell culture system. By contrast, although CD8+ T cells specific for an early VV protein, C7L and a late/virion protein A10L were able to kill vaccinia-infected cells, they were unable to prevent VV amplification and infectious virus spread. CD4+ T cells specific for E3L, H3L, G5R and A10L were detected but were unable to kill infected target cells or prevent infectious virus spread in our assays (Table 2). This suggests that simple induction of a T-cell immune response to a viral antigen by vaccination may not guarantee that the vaccine will be effective.

A safe and effective viral vaccine should induce both neutralizing antibodies that neutralize free infectious virus and protective T cells that kill virus-infected cells and prevent virus spread. The protective function of antibodies can readily be determined in virus neutralization assays, but no such assay exists for T cells, and although T cells specific for a range of viral antigens may be detected, they are not necessarily protective. CD4+ and CD8+ T cells specific for any viral antigen may be elicited by professional antigen-presenting cells that have phagocytosed dying or lysed infected cells and presented viral peptides on HLA class II molecules or cross-presented them on HLA class I. However, if the antigens they recognize are not processed and presented by infected cell before the expression of the many immune evasion genes encoded by poxviruses, they may never be presented on infected cells and will have little biological relevance [37–40]. In our assay, CD8+ T cells specific for C7L and A10L—expressed early and late, respectively—were able to kill virus-infected LCLs, but whereas T cells specific for B22R, expressed early, were able to kill pepmix-pulsed LCLs, they could not kill VV-infected LCLs. B22R may not be expressed early enough in the viral replicative cycle to escape viral inhibition of antigen processing, whereas recognition of the late protein A10L may be explained by its abundant presence in the virion. Thus, A10L core antigens enter the cell at the time of infection and will be among the first proteins processed and presented to the immune response.

CD4+ T cells, whether specific for E3L, H3L, G5R or A10L, did not kill VV-infected LCLs, regardless of the timing of their expression. This may be because these T cells lacked cytolytic activity, because the frequency of CD4+ T cells was too low or because viral antigens were not presented by infected cells on HLA class II. Indeed Li *et al.* showed that the window of opportunity for CD4+ T-cell recognition of VV-infected cells diminished from 1 h after infection, and Rehm *et al.*, suggested that this resulted from decreased loading of peptides into the MHC II cleft [40–42].

Even if VV-infected cells are killed by VV-specific CD8+ T cells, their antiviral activity depends on their ability to prevent the release of infectious virus. VV has a rapid replication cycle, and new virus particles can be produced in some cell types within 4 h of infection. Therefore, killing of newly infected cells must occur rapidly to prevent virus replication and spread. E3L-specific CD8+ T cells were able to prevent virus replication in infected cells only if added within 2 h of infection, whereas CD8+ T cells specific for the early protein, C7L, that is not expressed until 2 h post-infection [18], were unable to prevent infectious spread despite their ability to kill infected target cells. These observations suggest that rapid killing is required for virus control and indicates the importance of T cells specific for rapidly presented proteins. Our “inhibition of virus spread” assay can therefore test for protective T cells and may be helpful for the screening of potential VV vaccines before testing in long, complex and expensive studies in animals. It will be important to test other immediate early antigens as well as abundant virion proteins for their abilities to

activate protective T cells because in our small study of 13 donors, CD8 responses to E3L appeared restricted to HLA B35 donors.

Although IFN- λ alone is able to inhibit the replication of VV in murine fibroblasts, it has no such effect on human fibroblasts due to the effect of several vaccinia immune evasion genes including E3L [43], therefore local production of IFN- λ by T cells specific for viral proteins cross presented on APCs would not be expected to control virus spread. This emphasizes the importance of protective T cells [44].

T-cell recognition of VV antigens is broad and Sette *et al.* [45] reported T-cell recognition of 250 epitopes in 23 VV antigens. As a result of these studies the NIAID compiled a bank of potential HLA class I-restricted VV epitopes that were identified using the SYTHPE-THI and Bioinformatics and Molecular Analysis Section of the National Institutes of Health BIMAS (<http://www-bimas.cit.nih.gov>) algorithms [46]. However, in our hands, when tested in VV-immune individuals with appropriate HLA alleles, only one of these epitopes elicited a T-cell response (not shown). We therefore took a different approach to the identification of protective epitopes and hypothesized that the viral proteins first presented to the immune system after infection were most likely to induce protective T cells. These antigens would include virion proteins carried into the infected cell during infection as well as immediate early proteins expressed rapidly after infection. Of immediate early proteins, the most abundant are likely to be most immunogenic. E3L is abundantly detected within 0.5 h of infection, potentially before the many immune evasion genes of VV take their effect, and is also carried in the virion so that it can exert its immune evasion functions promptly. E3L inhibits innate immunity to poxvirus infection by inhibiting interferon responses to dsRNA and downstream activation of MAP kinase and NF- κ B pathways [16,47,48]. We predicted that because of its abundance and rapid expression, E3L would induce T cells that could protect against virus infection. Indeed, CD8+ E3L-specific T cells were able to kill newly infected cells and prevent infectious VV spread in autologous LCLs.

E3L encodes a relatively short protein of 190 amino acids that induced CD8 T-cell responses only in HLA-B35-positive donors. The SYFPEITHI score for our E3L epitope (NPVTIINEY) is 22. By comparison, the score for the immunodominant HLA A2-restricted epitopes of CMV (NLVPMVATV) and influenza A (GILGFVFTL) are 31 and 30, respectively. There are other potential epitopes within E3L with higher (better) scores, restricted by other HLA alleles. However, these epitopes were not detected by our T-cells even though our donor panel carried the predicted HLA alleles.

VVs deleted for E3L showed early promise as vaccines and induced Th1 responses to VV that protected against lethal challenge with wildtype virus in mice and rabbits [17,49,50]. However, the same vaccine evaluated in cynomolgus macaques provided only partial protection against monkeypox compared with wildtype virus [17]. If the E3L protein induces protective T cells in monkeys, then elimination of important T-cell epitopes could have a detrimental effect on the protective immune response and explain the failure of this virus in the monkey model. However, T cells were not evaluated in this system. This principle may be applied to other viral vaccines, such as SARS-CoV-2, if protective T cells are to be produced. In this virus, the first proteins to be expressed immediately after virus entry are the polyproteins pp1a and pp1b, whereas the SPIKE protein, which is the target of neutralizing antibodies as well as most vaccines, is not expressed until much later, after the negative strand is produced, transcribed and translated.

VV is also used as a vector for heterologous vaccine antigens and immediate early expression of vaccine antigens is likely to be important for optimal transgene expression and direct induction of a CD8+ immune response by infected cells [51,52]. Deletion of E3L in VV vaccine vectors may prolong the persistence of VV by removing important T-cell epitopes and prolonging the vaccine effect. However, because E3L deletion from wildtype VV may

increase pathogenicity, this strategy should be tested in an attenuated strains, such as MVA [53].

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Declaration of Competing Interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: JA, AL and CMR. Acquisition of data: JA and MCN. Analysis and interpretation of data: JA and CMR. Drafting or revising the manuscript: JA, MA and CMR. All authors have approved the final article.

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