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Design and evaluation of multi-gene, multi-clade HIV-1 MVA vaccines

Patricia L. Earl^{a,*}, Catherine Cotter^{a,b}, Bernard Moss^a, Thomas VanCott^{b,c}, Jeffrey Currier^b, Leigh Anne Eller^b, Francine McCutchan^{b,d}, Deborah L. Birx^{b,e}, Nelson L. Michael^b, Mary A. Marovich^b, Merlin Robb^b, and Josephine H. Cox^{b,f}

^a Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD, USA

^b Military HIV Research Program, Walter Reed Army Institute of Research, Rockville, MD, USA

Abstract

Recombinant modified vaccinia virus Ankara (rMVA) expressing HIV-1 genes are promising vaccine candidates. Toward the goal of conducting clinical trials with one or a cocktail of recombinant viruses, four rMVAs expressing *env* and *gag-pol* genes from primary HIV-1 isolates representing predominant subtypes from Kenya, Tanzania, Uganda, and Thailand (A, C, D, and CRF01_AE, respectively) were constructed. Efficient expression, processing, and function of Env and Gag were demonstrated. All inserted genes were shown to be genetically stable after repeated passage in cell culture. Strong HIV-specific cellular and humoral immune responses were elicited in mice immunized with each individual vaccine candidate. The MVA/CMDR vaccine candidate expressing CRF01_AE genes has elicited HIV-specific T-cell responses in two independent Phase I clinical trials. Further testing of the other rMVA is warranted.

Keywords

MVA; HIV vaccine; immune response

1. Introduction

Since the beginning of the AIDS pandemic more than 20 years ago, HIV infections have been responsible for more than 20 million deaths and an estimated 33.2 million people are currently infected worldwide [1]. While antiviral therapies can control viral load and slow disease progression, an effective vaccine will be necessary to curb the spread of the virus. Numerous vaccines have either been tested or are in the pipeline for clinical testing [2]. Primary among these are viral vectors expressing multiple HIV-1 antigens either administered alone or in a prime-boost protocol with DNA or protein [3,4]. Modified vaccinia virus Ankara (MVA) offers many advantages as a vehicle for delivery of HIV antigens. MVA was derived by repeated

*Corresponding author. 33 North Drive, MSC 3210, National Institutes of Health, Bethesda, MD 20892-3210. Phone: (301) 402-4112. FAX: (301) 480-1535. pearl@niaid.nih.gov.

^cPresent address, Advanced BioScience Laboratories Inc., Kensington, MD, USA

^dPresent address, Bill & Melinda Gates Foundation, Seattle, WA, USA

^ePresent address, CDC Global AIDS Program, Atlanta, GA, USA

^fPresent address, International AIDS Vaccine Initiative, New York, NY, USA

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passage in chicken embryo fibroblasts (CEF) [5] during which several large deletions and small mutations arose resulting in the loss of immune defense genes and restriction of replication to a few cell lines including CEF and baby hamster kidney cells [6–10]. Because the block in morphogenesis in non-permissive cells occurs in a late stage of viral development, efficient expression of foreign genes is obtained both early and late after infection with the use of appropriate promoters [8,11]. MVA can accommodate multiple foreign genes in the same or different sites in the genome of a single virus and can be administered by a variety of routes [12–14]. Importantly, MVA and recombinant MVA (rMVA) have excellent safety records in immunocompromised mice and monkeys [15–17] as well as in humans [13,18–21].

Pre-existing vector immunity is a potential problem for recombinant viral vaccines and may have contributed to the failure of the STEP trial with a rAd5 expressing HIV gag, pol and nef [22,23]. Pre-existing vector immunity is less likely to present problems with poxvirus-based vaccines since worldwide smallpox vaccination ceased in the 1970s. Studies in non-human primates have shown that rMVA generates cellular and humoral responses to foreign antigens from a variety of sources [24–28]. Immunization of macaques with rMVA expressing HIV-1 genes has demonstrated significant protection from SHIV or SIV challenge as measured by decreased viral loads and prolonged survival [29–32]. Importantly, recent clinical trials have shown that HIV-specific immune responses are induced in humans vaccinated with rMVAs expressing *env* and *gag-pol* [13,33].

Because of the wide diversity of HIV-1 sequences, it may be necessary to match genes in a vaccine with those from the circulating subtype in targeted areas [34,35] or use more antigens and different subtypes to increase breadth of the immune response [13]. Thus, our strategy was to incorporate genes from viral isolates representative of the Military HIV Research Program (MHRP) international vaccine cohorts in East Africa and Thailand into rMVA. We selected HIV-1 isolates representative of circulating strains from subtypes A (Kenya), C (Tanzania), D (Uganda), and CRF01_A/E (Thailand) [36–38]. Gag and Pol are the primary targets of immune responses in humans and have demonstrated partially protective immunity in non-human primate challenge studies [39–43]. While no Env-based vaccines have currently been able to induce antibodies capable of neutralizing primary HIV isolates, Env is an important immunogen for vaccine design as it is the first virion associated protein to engage the CD4 receptor and chemokine co-receptors on susceptible target cells and contains many T cell helper epitopes [44]. Thus we chose to express Env, Gag and Pol in our vaccines.

In this paper we describe the design, construction, and characterization of four rMVA vaccine candidates, henceforth called MVA/HIV. *Env* and *gag-pol* genes were inserted in separate locations in the MVA genome, both under control of the strong mH5 promoter. Specific mutations were engineered to improve expression, stability and safety. Proteins were expressed in the native form and were shown by *in vitro* assays to be correctly folded and processed. In addition, the inserted genes were stably and accurately maintained in the rMVAs after repeated passage in cell culture, as required for large-scale vaccine manufacturing. *In vivo* cellular and humoral immunogenicity of the four MVA/HIV was demonstrated in a mouse model.

2. Materials and Methods

2.1. Cells and viruses

Culture protocols for chicken embryo fibroblast (CEF) and BS-C-1 cells have been previously described [45]. MVAp579 [15] and MVA 1974/NIH Clone 1 [17] were used as the parental viruses for construction of rMVAs. For cell-cell fusion and reverse transcriptase (RT) assays, the following recombinant VACV were used: vTF7-3 [46], vCBYFI [47], vCCR5 [48], vCB3 [49], vCB21R [50], vSC60 [51], vSC43 [52], and vVK7 (Karacostas and Moss, unpublished).

2.2. HIV-1 *env* and *gag-pol* gene selection and mutagenesis

Env and *gag-pol* genes were amplified from HIV-1 isolates from Thailand and East Africa [36–38]. If not already present, Kozak sequences were generated at the initiating ATGs. The cytoplasmic tail of the *env* genes was truncated by 113–122 amino acids to generate the C-terminal sequence GGEQD(G). This deletion did not affect the transmembrane domain of the protein. The *gag-pol* genes were truncated to eliminate RNase H and integrase and yield the C-terminal amino acid sequence GAETF. Silent mutations were made in *env* genes (Quik Change kit, Stratagene) to eliminate naturally occurring vaccinia virus early transcription termination signals (T₅NT) [53]. To abolish reverse transcriptase (RT) activity, two mutations were made in the active site of *pol* genes [54]. The resulting amino acid changes were: VLDV to VLEV and YMDD to YMHD. Since the *pol* gene from the CRF01_A/E isolate, CM240, already had the latter mutation no changes were made in this gene. Table 1 shows the names of MVA/HIV viruses and accession numbers of the HIV-1 isolates from which genes were derived.

2.3. Construction of MVA/HIV-1 viruses

In all MVA/HIV viruses, the *gag-pol* gene was recombined into deletion II of MVA. For MVA/KEA, MVA/TZC, and MVA/CMDR the *env* gene was recombined into deletion III. For MVA/UGD, the *env* gene was recombined between the I8R and G1L genes in the central conserved region of MVA [55]. Plasmids pLW17 [56] and pLW9 [57] were used for insertion of *env* and *gag-pol* genes, respectively, into parental virus, MVAp579 [15], to generate MVA/CMDR. Positive isolates were selected by live immunostaining with polyclonal sera. MVA-1974 clone 1 [17] was the parental virus used for construction of MVA/KEA, MVA/TZC, and MVA/UGD and a novel transient EGFP selection procedure was employed for plaque selection, obviating the need for live immunostaining. For construction of these viruses, the *gag-pol* gene was cloned into the XmaI site of pLAS-1 [58]. The *env* genes for MVA/KEA and MVA/TZC were cloned between the XmaI and XhoI sites in pLAS-2 [58]. For MVA/UGD, the *env* gene was cloned into the XmaI site of pLW-73. The modified H5 (mH5) promoter [57] was chosen for all genes because it drives strong early and late gene expression. Virus recombination and amplification were performed in CEF cells using standard techniques [59]. After construction of each virus, gene expression, sequence of inserted DNA, and viral purity were verified.

2.4. In vitro expression and function

For analysis of expression from MVA/HIVs, BS-C-1 cells were infected at a multiplicity of infection of 5. Cell lysates and virus-like particles (VLP) were prepared [60] and proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and incubated with HIV+ sera and mAb 183-H5C to p24 followed by secondary R-Dye-800CW conjugated donkey anti-human and anti-mouse IgGs (Rockland Immunochemicals, Inc., Gilbertsville, PA). Blots were scanned using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Co-receptor specificity of Env proteins was measured with a receptor-dependent cell-cell fusion assay [61]. Reverse transcriptase activity of infected cell lysates and VLPs was assayed by incorporation of ³²P-TTP into acid-insoluble material as described previously [62].

2.5. Plaque immunostaining

Plaques were visualized by immunostaining as described before [59]. Env- or Gag-specific antibodies were used separately to determine the percent non-staining plaques. Between 160 and 600 plaques were enumerated for determination of the percent non-expressing plaques. For live immunostaining, monolayers were not fixed prior to staining.

2.6. Antibodies

For plaque immunostaining the following antibodies were used: gp120-specific mAbs T8 (MVA/CMDR), T24 (MVA/KEA and MVA/UGD), and T43 (MVA/TZC); gp41-specific mAb T32 (all viruses) [63]; p24 Gag-specific mAb 183-H12-5C [64], obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). For Western blots, mAb 183-H12-5C, and a pool of sera from HIV-infected individuals (gift of V. Polonis, MHRP, Rockville, MD) were used. For the human T cell assay, TW2.3 mAb to the VACV E3L protein was used [65].

2.7. Genetic stability of MVA/HIV viruses

At least 5 individual plaque isolates from each MVA/HIV were serially passaged in CEF cells in 6-well tissue culture plates using a multiplicity of infection and propagation time similar to that used for GMP production. After 8–10 serial passages, genetic stability was assessed by plaque immunostaining as described above. Low-passage stocks used for GMP production were assessed for genetic stability, sequence fidelity of inserts, and protein expression by Western blotting.

2.8. Immunogenicity Studies

Immunizations of 10–12 week old BALB/c mice (Charles River Laboratories Inc., Wilmington, MA) were performed at Biocon, Inc. (Rockville, MD), in compliance with the Animal Welfare Act and other federal statutes and regulations. Groups of 10 mice each were immunized by the intraperitoneal route with either 10^6 or 10^7 infectious units of MVA/KEA, MVA/TZC, MVA/UGD, or MVA/CMDR diluted in PBS. Groups of 5 mice were similarly immunized with parental MVA-1974. Mice were immunized at weeks 0 and 3 and bled at weeks 0, 3, and 5. Spleens were harvested at week 5.

2.9. Peptides

Peptides for the mouse immunogenicity studies were as follows: Gag p24 CD8 restricted AMQMLKETI (abbreviated KE) [66] and the subtype C variant AMQMLKDTI (abbreviated KD) were used separately. Env peptides, DTEVHNWATHACVP and QQQSNLLRAIEAQH were used as a pool [67]. A pool of 8 Pol peptides representing subtypes A, B, C, D and CRF01_A/E with single amino acid variants of ELRQHLLRWGLTT and HGVVYDPSKDLIAE were used [68]. In the human CTL experiment, a peptide from the HLA-B27-restricted HIV gag epitope KRWILGLNK (KK10) was used. Peptides were synthesized at the Henry M. Jackson Foundation with free amino termini using Fmoc chemistry and standard solid-phase techniques (Excel automated synthesizer; Waters, Milford MA). MVA-specific responses were determined using peptides SPYAAGYDL and VGPSNSPTF from the F2L and E3L genes, respectively [69] (provided by J. Bennink, Laboratory of Viral Diseases, NIAID).

2.10. Intracellular cytokine staining (ICS) of mouse splenocytes

P815 target cells, suspended at 10^7 cells/ml in RPMI containing 10% FBS (RPMI-10), were either stimulated with peptides at 10 μ g/ml or infected with 10 pfu/cell of VACV strain WR. After 90 min incubation at 37°C, RPMI-10 was added to achieve 10^6 cells/ml. Incubation was continued for 5–6 h at 37°C after which the cells were washed twice with RPMI-10, suspended at 2.5×10^6 cells/ml, and irradiated at 10,000 RAD. Splenocytes were prepared from individual mice. For stimulation, 1.5×10^6 splenocytes were mixed with 2.5×10^5 peptide-stimulated or VACV-infected P815 cells. Brefeldin A was added to 10 μ g/ml and cells were incubated at 37°C for 10–15 h. Cells were incubated for 10 min with anti-CD16/32, clone 2.4G2 (gift of K. Grebe, Laboratory of Viral Diseases) then stained at room temperature for 30 min with peridinin chlorophyll-a protein (PerCP) conjugated anti-CD8 (clone 53.6–7). After fixation

and permeabilization, cells were stained with allophycocyanin (APC) conjugated anti-interferon gamma (IFN- γ) (clone XMG1.2) and either fluorescein isothiocyanate (FITC) conjugated anti-interleukin 2 (IL2) (clone JES6-5H4) or anti-tumor necrosis factor (TNF) (clone MP6-XT22), washed and resuspended in 2% paraformaldehyde (all stains were purchased from BD Pharmingen, San Jose, CA). Approximately 100,000 cells were acquired on a FACSCalibur cytometer using Cell Quest software (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Cupertino, CA).

2.11. ELISPOT of mouse splenocytes

96-well nitrocellulose plates were coated overnight with anti-mouse-IFN- γ mAb clone AN-18 (Mabtech, Cincinnati, OH) at room temp. The plates were washed and blocked with media. Dilutions of splenocytes were mixed with 1×10^5 irradiated P815 target cells and peptide (1 μ g/ml) and plates were incubated at 37°C, 5% CO₂ for 24–28 h. Production of IFN- γ by CD8 T cells was detected by addition of biotinylated anti-IFN- γ mAb Clone R4-6A2 (Mabtech, Cincinnati, OH). ELISPOT development consisted of a one h incubation with avidin horseradish peroxidase complex (Vectastain® ABC kit, Vector Labs, CA) in PBS/0.05% Tween-20 buffer followed by washing six times with PBS, and incubation with peroxidase substrate AEC for five minutes (AEC substrate Kit, Vector Labs, CA). ELISPOT plates were evaluated with an automated ELISPOT reader system and KS 4.3 software (Carl Zeiss, Thornwood, NY). The results are expressed as the number of IFN- γ spot forming cells (SFC)/ 10^6 spleen cells.

2.12. Pentamer Staining of mouse splenocytes

Two million spleen cells were incubated for 10 min at room temperature with 10 μ l of APC-labeled Pentamer bound to the HIV-1 Gag p24 peptide AMQMLKETI (ProImmune, Oxford, UK). After washing, cells were incubated for 20 min on ice with FITC conjugated anti-CD8, PECy5 conjugated anti-CD19 (ProImmune, Oxford, UK) and PE conjugated anti-CD49b (BD Biosciences, San Jose, CA). Cells were then fixed with 1% formaldehyde. CD19+, CD49b+ B cells, and NK cells were gated out and a 2-color plot of CD8+/pentamer+T cells was generated using a FACSCaliber cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Cupertino, CA).

2.13. Env and p24 ELISA

Oligomeric gp140 proteins homologous to the Env in each of the four MVA/HIV were produced from recombinant MVA viruses (PE & BM, unpublished) as previously described [70]. Env ELISAs were performed as described [12] except that the times of incubation of sera and secondary antibodies were increased to overnight and 5 h, respectively. For p24 ELISAs Immulon-2-HB 96-well microtiter plates (Thermo Labsystems, Franklin, VA) were coated overnight with p24 (Immunodiagnostics, Woburn, MA) at 0.3 μ g/ml in CB1 bicarbonate buffer (Immunochemistry Technologies, Bloomington, MN). The assay was continued as for the Env ELISA.

2.14. VACV ELISA

Sucrose gradient purified VACV strain WR was used to coat 96-well microtiter plates (Thermo Labsystems, Franklin, VA) at 10^7 pfu/ml in CB1 bicarbonate buffer at 37°C overnight. Virus was inactivated by incubation with 2% paraformaldehyde for 10 min at 4°C. The assay was continued as described above for Env ELISA except that incubation times with sera and secondary antibody were for 1 h.

2.15. Human CTL effector cell generation and intracellular cytokine staining (ICS) assay

Cells were isolated from leukapheresis samples obtained from healthy, HIV seropositive adult volunteers recruited after providing informed consent into an IRB approved protocol (RV149) at the Walter Reed Army Medical Center (WRAMC, Washington, DC, USA). Human peripheral blood mononuclear cells (PBMC) were isolated by Ficol-hypaque (Pharmacia Biotech, NJ, USA) gradient centrifugation and cryopreserved in liquid nitrogen. Effector cells were prepared by *in vitro* stimulation (IVS) of thawed cryopreserved PBMC. 20×10^6 PBMC were washed three times with complete medium (CM: RPMI-1640, 10% FBS, 2 mM L-glutamine) and then cultured with KK10 peptide (10 μ g/ml) in 10 ml CM supplemented with 5 ng rhIL-7/ml (R&D Systems, Minneapolis, MN) for 7 days. 5 ng rhIL-2/ml (R&D Systems, Minneapolis, MN) in 10 ml of CM was then added to the cultures, which were maintained and split with fresh CM and rhIL-2 (5 ng/ml) for up to 28 days. Homologous B-lymphoblastoid cell lines (BLCL) targets were either pulsed overnight with KK10 peptide (10 μ g/ml) or infected overnight with MVA/HIV (10 pfu/cell).

For the ICS assay, effector cells (0.5 to 1.0×10^6) were mixed with target cells (1×10^5) in 96 well polypropylene tissue culture trays and incubated for 6 h at 37°C (5% CO_2) in the presence Brefeldin A (10 μ g/ml; Sigma, St.Louis, MO). The assay was interrupted by transfer to reduced temperature (either 4°C or 18°C) until the following day. Cells were stained for surface markers and intracellular IFN γ expression as follows. Cells were washed once with flow buffer (DPBS/0.1% BSA/0.1% sodium azide) and incubated in the 96-well tissue culture tray wells for 10 minutes in 200 μ l flow buffer at room temperature (same volume, temperature and base buffer used for all subsequent washings and incubations) containing 1 mM EDTA. Cells were washed once, fixed in 2% formaldehyde for 30 min and washed again. Fixed cells were permeabilized with 0.5% saponin (Sigma, St. Louis, MO) for 30 min, washed and resuspended in 0.5% saponin containing the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ (clone 25723.11); phycoerythrin (PE)-conjugated anti-TNF α (clone Mab11); PerCP-Cy5.5-conjugated anti-CD8 (clone SK1); and APC-conjugated anti-CD3 (clone SK7)(BD Biosciences, San Jose, CA). After 30 min, cells were washed three times and re-suspended in 200 μ l flow buffer. Stained cells were stored at 4°C and analyzed by flow cytometry within 24 hours using a FACSCaliber cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Cupertino, CA).

3. Results

3.1. Selection of genes and construction of MVA/HIV vaccines

Prevalent HIV-1 subtypes were chosen from Thailand and East Africa where the MHRP has established clinical trial sites. Where necessary, panels of genes were screened to ensure the selection of genes that expressed full-length, functional products. Then mutations were introduced to improve the quality and safety of the vaccines. Thus, VACV early transcription termination signals were eliminated by silent mutation [53] and *env* genes were truncated to enhance cell surface expression and confer genetic stability [58]. In addition, the integrase and RNase H genes were deleted from *gag-pol* and the active site of RT was mutated [54]. The structure of the MVA/HIV viruses is shown in Fig 1A.

3.2. In vitro production, processing, and function of HIV-1 proteins

Protein expression and processing were demonstrated by Western blotting of lysates of cells infected with MVA/HIVs (Fig 1B). Cell associated gp150 Env was produced by all viruses although more extensive processing to gp120 was seen with MVA/CMDR. We have noted that the intensity of Env bands observed in Western blots is antibody-dependent, most likely reflecting the divergence in amino acid sequences between different clades rather than differences in absolute amount of protein made. Thus, the apparently weaker band seen with

MVA/CMDR in Fig 1B does not reflect lower expression but rather less efficient reactivity with the antibody used for that protein. All viruses produced p55 gag but exhibited varying degrees of processing to p41 and p24 as seen both in cell lysates and VLPs. In addition, MVA/UGD and MVA/CMDR exhibited less budding of VLPs into the medium than did the other two viruses (Fig 1B). However, this did not impair immunogenicity of the Gag protein. Correct folding of Env and transport to the cell surface were demonstrated in a cell-cell fusion assay in which production of β -galactosidase is dependent on fusion of cells expressing cleaved Env on the surface with cells expressing both CD4 and either CCR5 or CXCR4. Recombinant VACVs expressing either BaL or HXB2 Env were used as positive controls for fusion with CCR5 and CXCR4-expressing cells, respectively. As shown in Fig 1C, all four MVA/HIV viruses induced CCR5- but not CXCR4-specific fusion. To verify that the engineered mutations in RT abrogated enzymatic activity, lysates and VLPs from infected cells were assayed for RT activity by incorporation of ^{32}P -TTP into acid insoluble material. The positive control, expressing HXB2 Gag-Pol (vTF7-3 + vVK7), exhibited high levels of enzymatic activity while none was detected in samples from the four MVA/HIVs (Fig 1D). In summary, all the selected genes demonstrated the desired properties of efficient expression, accurate processing, folding and transport, and function.

3.3. Genetic stability of MVA/HIV viruses

Because genetic instability has been observed with some rMVA viruses expressing high levels of foreign antigens [57,71] and because extensive passage in cell culture is necessary to amplify viruses in sufficient quantity for clinical trials, we carefully assessed the genetic stability of our MVA/HIV vaccines. For each virus, at least 5 individual plaque isolates were selected and serially passaged 8–10 times in CEF cells on a small scale that mimicked the method used for vaccine production. Insert stability was evaluated in the high passage viral stocks in several ways. First, to determine the percent of viral progeny expressing Env or Gag, we employed a plaque immunostaining assay using Env or Gag-specific mAbs. Plaques that did not express the specific protein were visualized as non-staining areas of cytopathic effect in the cell monolayer. Greater than 95% of the virions expressed Env and Gag in all high-passage stocks (Table 2). Second, the regions of the MVA genome into which foreign genes were recombined were PCR-amplified and separated by agarose gel electrophoresis to verify the absence of parental MVA. Third, production of Env and Gag proteins in infected cells was demonstrated by Western blotting. No change in the quantity or quality of the expressed proteins was observed in comparison to the low-passage stocks. Finally, DNA sequencing of the inserts as well as 1 kb of flanking MVA verified that no changes had occurred during passage (data not shown).

3.4. Immunogenicity of MVA/HIV viruses

To evaluate and compare the immunogenicity of the four MVA/HIV vaccines, we immunized groups of mice with two doses of each virus (10^6 and 10^7 pfu). In addition, 5 mice were immunized with non-rMVA at each dose. Mice were bled after each immunization and splenocytes were prepared 2 weeks after the second immunization. Cellular responses to the immunodominant CD8 restricted Gag peptide, AMQMLKETI (KE), were measured in fresh splenocytes from individual animals. Because the TZC Gag has one amino acid mismatch (E to D), we also evaluated responses to the TZC sequence, AMQMLKDTI (KD). Cells were stained for surface expression of CD4 and CD8 and for intracellular expression of IFN- γ and either IL-2 or TNF. As shown in Fig 2A, all MVA/HIV viruses induced strong responses to the Gag peptide with those from MVA/KEA and MVA/UGD being higher (average of 5.4 and 5.0 %, respectively) than those from MVA/CMDR (average of 2.2%). In the MVA/TZC group, stimulation with the homologous KD peptide gave higher responses (average of 1.0%) than did the heterologous KE peptide (average of 0.3%). The relatively low responses found with this group were likely due to the fact that the sequence in the immunogen was a variant of the

immunodominant sequence. Importantly, we found co-expression of IFN- γ and either TNF or IL-2. Up to 1.9% of CD8+/IFN- γ + cells expressed TNF and up to 0.2% of CD8+/IFN- γ + cells expressed IL-2.

Cellular responses to predominant epitopes in Pol and Env were measured using pools of several peptides from each protein. Vaccination with MVA/HIV viruses induced 0.02–0.12% Pol-specific CD8+/IFN- γ + cells and 0.07–0.33% Env-specific CD4+/IFN- γ + cells. The rank order of responses to both Env and Pol peptides was MVA/UGD > MVA/TZC > MVA/CMDR > MVA/KEA (Fig 2B&C).

Uniformly robust cellular responses to MVA were raised in all groups of animals (Fig 2D). With immunization doses of 10^6 and 10^7 , 19–32% and 28–32%, respectively, of the CD8+/IFN- γ + cells were specific for MVA. Very high responses to the exogenously-added E3L peptide, VGPSNSPTF, were observed. This was not surprising as this is an immunodominant epitope and peptide was added at supra-optimal concentrations to the P815 cells.

The effect of immunization dose on induction of Gag-specific IFN- γ producing cells as measured by ELISPOT and pentamer staining is shown in Fig 3A and C, respectively. Although there was a trend towards a dose-response, the differences between the average responses at 10^6 and 10^7 were not consistently significant. The hierarchy of Gag-specific responses was the same as that measured by ICS (MVA/UGD = MVA/KEA > MVA/CMDR > MVA/TZC). As with ICS, responses in the MVA/TZC group were higher with the homologous (KD) peptide than with the heterologous (KE) peptide (Fig 3A, inset). Vector specific responses, as measured either by infection with VACV (Fig 3B) or stimulation with VACV peptides SPYAAGYDL and VGPSNSPTF (not shown), were robust and equivalent in all MVA/HIV groups. These responses also did not show a significant dose-response.

Antibody responses to Env, Gag, and VACV were measured after one and two immunizations with 10^7 MVA-HIV (Fig 4). Binding antibodies to Env, as measured with soluble gp140 proteins homologous to those in each MVA/HIV, were induced by one and boosted by the second immunization (Fig 4A). Unlike the cellular responses, a dose-response was found with the 10^7 compared with 10^6 immunization dose (not shown). The increase in titer ranged from 9–28 fold (at 3 weeks) and 2.4–12 (at 5 weeks). Importantly, cross-reactive Env antibodies were generated by each MVA/HIV (Fig 4B). Each immunogen induced the strongest titer against the homologous Env (indicated by *above bar), but also induced antibodies to each of 3 heterologous Env proteins. Antibodies to p24 were also elicited by one and boosted by a second immunization (Fig 4C). In addition, vector immunity was elicited after a single immunization and boosted by the second immunization (Fig 4D).

3.5. Human T cell recognition of MVA/HIV viruses

The ability of the four MVA/HIV constructs to express Gag protein in sufficient quantities to be naturally processed and presented to human CD8⁺ T cells was verified using an *in vitro* model system. PBMC from an *HLA-B*2705*-positive subject with a defined response to the HLA-B27-restricted HIV gag epitope KRWILGLNK (KK10) were seeded into an IVS using the 15-mer peptide IYKRWILGLNKIVR as the stimulus. The epitope KK10 was selected for IVS because this sequence is absolutely conserved in the Gag protein expressed all four of the MVA/HIV constructs. Fig 5A shows that CD8⁺ effector cells derived in the peptide-driven IVS could recognize autologous BLCL infected with each of the four constructs. The magnitude of recognition, as measured by IFN γ /TNF α expressing CD8⁺ T cells, was virtually identical among the constructs (boxes e–h; range = 4.54–5.58%) and was similar to that detected against autologous BLCL incubated with the original peptide that was used to expand the effector cells (box c; 6.09%). In contrast, few responding cells were detected in the unstimulated effector cell population (box a; 0.34%), against uninfected non-peptide pulsed

BLCL (box b; 0.60%), or against the control MVA backbone construct infected BLCL (box d; 0.56%). In addition, the MVA/HIV-infected BLCL were assessed for efficiency of MVA-infection and Gag protein expression after the over night incubation prior to use as stimulator cells in the ICS assay. As shown in Fig 5B virtually all of the cells were infected, as measured by TW2.3 mAb binding to the E3L protein of MVA (range = 98.2–99.0%). Subsequent gating on the MVA-infected cells demonstrated a small yet consistent amount of intracellular Gag protein. The number of p24 positive cells ranged from 26.7% to 85.9%. Therefore, each of the MVA/HIV constructs is capable of infecting human target cells and expressing sufficient amounts of the Gag protein, which can be processed and presented to human T cells.

4. Discussion

Recombinant poxviruses expressing HIV-1 antigens are promising vaccine candidates as demonstrated by results from several on-going clinical trials [13,72–74]. Because of the wide sequence diversity, our goal was to design and test MVA/HIV vaccines expressing *env* and *gag-pol* genes from prevalent strains in Thailand and East Africa at MHRP clinical sites. All proteins were efficiently produced and maintained proper function as demonstrated by Env/coreceptor-dependent fusion and budding of VLPs. Since increased levels of protein production correlates with increased induction of both antibody and CTL in small animals [58], we chose to use the strong early/late mH5 promoter. However, we and others [57,71] have found that high levels of foreign antigen production can occasionally lead to genetic instability in rMVAs. Thus, it was of primary importance to demonstrate the stability of the inserted genes in our MVA/HIV vaccine candidates. To this end, each virus was serially passaged in CEF cells in a manner designed to mimic GMP production. The genes from our clade A, C, and CRF01_A/E isolates proved to be stable in deletions II (*env*) and III (*gag-pol*). However, insertion of the clade D *env* into deletion II resulted in loss of expression in up to 20% of the progeny at passage 7. Our work with SIV *env* (Earl and Moss, unpublished) as well as that of Burgers et al with a clade C HIV *env* [71] showed that such instability can be circumvented by use of a weaker promoter. Rather than compromise promoter strength, we inserted the gene between two essential VACV genes thus preventing propagation of viruses with large deletions [55]. In the resulting rMVA, both genes were expressed to high level while maintaining insert stability and good virus yields.

T-cell immunity is important for control of viral replication [75,76] and most current vaccine candidates are aimed at stimulating this arm of the immune system. To demonstrate potency of our vaccine candidates, we immunized Balb/C mice with each MVA/HIV. The primary endpoint was measurement of responses to the immunodominant Gag epitope AMQMLKETI. Consistently strong CD8⁺/IFN- γ responses were found with MVA/KEA, MVA/UGD, and MVA/CMDR. Because the TZC gene has a single amino acid substitution in the epitope, we also performed ICS and ELISPOT assays with the TZC-matched peptide. Although responses were somewhat higher with the matched peptide, they were still significantly lower than that found with the other three viruses. We reasoned that this was likely due to sequence heterogeneity rather than poorer expression or processing as compared to the other Gag proteins. To address this, we searched the four gag sequences for conserved human gag CD8 epitopes. The epitope KRWIILGLNK (KK10), restricted by *HLA-B*270,5* was identical in all four of the MVA/HIV constructs. *In vitro*-expanded CD8⁺ T cells from an HIV positive, *HLA-B*2705*-positive subject with a known response to this epitope secreted IFN- γ and TNF- α in response to autologous BLCL expressing each of the four MVA-HIV recombinants demonstrating that the gag expressed from the 4 MVA/HIVs is processed and presented equally in human cells for presentation to CD8⁺ T cells.

Polyfunctional CD8⁺ T cells have been shown to correlate with viral control in HIV positive people [77–79]. In non-human primates polyfunctional CD8⁺ T cell responses induced by

vaccination are associated with control of SIV [75,76]. Importantly, our vaccine candidates induced Gag-specific CD8+ T cells expressing IFN- γ as well as either TNF or IL-2. Approximately 40% and 4% of the IFN- γ + cells also produced TNF or IL-2, respectively. In other pre-clinical studies performed while these vaccine candidates were being developed, we demonstrated robust cytotoxic T lymphocyte activity in mice immunized with all 4 MVA recombinants (data not shown) in a ^{51}Cr -release assay. Thus, in addition to release of cytokines, T cells from MVA-immunized mice were fully capable of lysing target cells pulsed with Gag peptides and Gag protein that was processed and presented through the MHC-class I pathway.

In addition to the immunodominant Gag epitope, T cell epitopes in Pol and Env have been described in Balb/C mice [67,68]. Our MVA/HIV-immunized mice demonstrated Pol-specific CD8 and Env-specific CD4 responses as measured by ICS and IFN- γ ELISPOT. Also, strong cellular responses to the MVA vector were demonstrated in all animals, with more than half of this response attributable to the immunodominant epitope VGPSNPTF [69]. At both immunization doses, in spite of the large anti-MVA vector response, robust HIV specific T cell responses were seen suggesting that anti-vector immunity does not impair the boosting effect of a second dose of virus.

Temporal development of antibodies to Env, Gag and MVA were assessed by ELISA. Antibodies to all three antigens were elicited after a single immunization and were boosted by the second immunization. Gag titers were equivalent in all groups but Env titers were consistently higher in the MVA/UGD group than any of the other groups. High titer cross-reactive Env antibodies were induced by each of the MVA/HIV with MVA/UGD showing the most potent cross-reactivity suggesting the possibility that the UGD env is a more potent immunogen.

The effect of immunogen dose and frequency is an important consideration for clinical trials. Thus, we immunized mice twice with two doses of each MVA/HIV. In agreement with other studies in mice [58,80], we saw only a modest increase in the T-cell responses with the higher dose. However, we did find a significant increase in antibody titers to Env, Gag, and VACV in animals immunized with 10^7 as compared to those immunized with 10^6 . At both immunization doses, we observed enhanced antibody titers after the second immunization suggesting that anti-vector immunity does not impair the boosting effect of a second dose of virus. In this regard, prior smallpox immunity did not significantly reduce HIV response rates induced by MVA/CMDR in a clinical trial though the overall magnitude of the responses was lower [13,81].

In summary, we have constructed four MVA/HIV vaccine candidates targeted for clinical trials in Kenya, Tanzania, Uganda, and Thailand. Each virus efficiently and stably expresses functional Env and Gag proteins and showed pre-clinical immunogenic potency in mice. In addition, MVA/CMDR has been GMP-produced and under a US FDA IND is currently in phase I trials in the US and Thailand (MHRP) as a single modality and will soon be evaluated as a boost to DNA priming in a MHRP phase I trial in the US, Africa, and Thailand. MVA/CMDR was also tested in Sweden and Tanzania (Karolinska Institute) as a boost to a DNA prime different than that to be used in the upcoming MHRP DNA prime-MVA boost trial. T-cell responses to both Env and Gag have been elicited in volunteers in all studies [13,72,73].

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Figure 1. MVA/HIV

A. Diagram of the orientation and location of HIV genes in MVA/HIV vaccines. mH5 promoters are indicated by arrowheads and insertion sites in MVA are shown above the diagrams.

B. Expression of Env and Gag by MVA/HIV. BS-C-1 cells were infected with each MVA/HIV with a moi of 5. At 24 h post-infection, cell lysates and VLPs were prepared and proteins were separated on 10% polyacrylamide gels. Western blotting was performed using pooled polyclonal sera from HIV-infected individuals (gift of V.Polonis, MHRP) and mAb 183-H12-5C to p24 [64].

C. Co-receptor-dependent fusion. BS-C-1 cells infected with viruses expressing CD4, either CXCR4 or CCR5, and T7 RNA polymerase were mixed with cells infected with each MVA/HIV and a virus expressing β -galactosidase under control of the T7 promoter. Fusion of cells resulted in expression of β -galactosidase. MVA-infected cells and media only were negative controls; vSC60 and vCB43 expressed HIV-1 IIIB and HIV-1 Bal Env proteins, respectively and served as positive controls for CXCR4 and CCR5-dependent fusion, respectively.

D. Reverse transcriptase activity. BS-C-1 cells were infected with each MVA/HIV. RT activity was measured in cell lysates and VLPs. MVA and vTF7-3 plus vVK7 (expressing HXB2 *gag-pol*) were negative and positive controls, respectively.

Figure 2. Intracellular cytokine responses induced by MVA/HIV. Mice were immunized twice with either 10^6 or 10^7 pfu of each MVA/HIV or with non-recombinant MVA. Two weeks after the second immunization, splenocytes were prepared and mixed with P815 cells pulsed with peptide(s) or infected with VACV. Cells were stained for surface expression of CD8 and CD4 and for internal expression of IFN- γ , TNF, and/or IL-2

A. P815 target cells were pulsed with Gag peptide, mixed with splenocytes from individual mice (10^6 dose), and the %CD8⁺/IFN- γ ⁺/TNF⁺ or %CD8⁺/IFN- γ ⁺/IL-2⁺ cells were enumerated. Top panel: CD8⁺/IFN- γ ⁺ responses to the immunodominant AMQMLKETI peptide (white bars) or the TZC variant AMQMLKDTI (gray bars) are shown. Bottom panel: The % CD8⁺/IFN- γ ⁺ splenocytes that also express either TNF or IL-2 following stimulation with AMQMLKETI are shown.

B. P815 target cells were pulsed with a pool of Pol peptides. Splenocytes from each immunization group (10^7 dose) were pooled prior to mixing with target cells.

C. P815 target cells were pulsed with a pool of Env peptides. Splenocytes from each immunization group (10^6 dose) were pooled prior to mixing with target cells.

D. P815 target cells were either infected with VACV or pulsed with the immunodominant peptide VGPSNSPTF from the VACV E3L protein. For the VACV stimulation, splenocytes from individual animals (10^6 dose) were assayed (white bars); for the peptide stimulation, splenocytes from each group were pooled (gray bars).

Figure 3. Dose-response to Gag and VACV. Mice were immunized twice with either 10^6 or 10^7 infectious units of each MVA/HIV or with non-rMVA. Two weeks after the second immunization, splenocytes were prepared from individual animals. White bars = 10^6 dose, gray bars = 10^7 dose. A. Gag-specific IFN- γ ELISPOT. IFN- γ -producing splenocytes were enumerated after stimulation with P815 target cells pulsed with Gag peptide AMQMLKETI. The inset shows the responses in the MVA/TZC group to the both the immunodominant peptide (KE) and the TZC peptide (KD) (10^6 dose). B. VACV-specific IFN- γ ELISPOT. IFN- γ -producing splenocytes were enumerated after stimulation with VACV-infected P815 target cells.

C. Splenocytes from individual animals were with incubated with APC-labeled pentamer bound to the Gag peptide AMQMLKETI. CD19+ and CD49b+ B and NK cells, respectively, were gated out and a 2-color plot of CD8+ pentamer+ T cells was generated.

Figure 4. Serum IgG ELISA responses in mice immunized with MVA/HIV. Mice were immunized with 10^7 infectious units of each MVA/HIV or non-rMVA at weeks 0 and 3 and bled at weeks 3 and 5

A. Temporal Env antibody responses were measured using gp140 proteins homologous to those in each MVA/HIV. Lower limit of detection is indicated by dashed line.

B. Cross-reactive Env antibody responses to homologous (denoted with *) and heterologous gp140 proteins were determined using sera pooled from each group after the second immunization.

C. Temporal antibody responses to p24 Gag were determined using sera pooled from each group.

D. Temporal responses to VACV were determined with purified MV protein as antigen. Data from animals in all immunization groups were combined in this panel.

Figure 5. Human T cell recognition of MVA/HIV viruses

A. Dot-plots show the IFN γ and TNF α response of CD3⁺CD8⁺ gated T cells in response to stimulation with autologous BLCL treated as described above each dot-plot box. Peptide (box c); *HLA-B*2705*-restricted HIV-Gag peptide KK10.

B. Histograms show the level of MVA infection in the target cells. VACV E3L positive cells and Gag p24 positive cells are shaded in the top and bottom rows, respectively. In each histogram the unfilled thick black line is the uninfected autologous BLCL.

Table 1

MVA/HIV vaccines and origin of inserted genes

MVA virus name	Origin	Subtype	<i>gag-pol</i> gene		<i>env</i> gene	
			HIV-1 isolate	Genbank accession #	HIV-1 isolate	accession #
MVA/KEA	Kenya	A	KER2008	AF457052	KNH1144	AF457066
MVA/TZC	Tanzania	C	TZA246	AY253308	TZA125	AY253304
MVA/UGD	Uganda	D	AO3349	AF484518	AO7412	AF484477
MVA/CMDR	Thailand	CRF_A/E	CM240	U54771	CM235	AY736837

Table 2

Genetic stability of MVA/HIVs

MVA/HIV	passage #	% staining plaques	
		Env	Gag
MVA/CMDR	vaccine lot	98.8	99.8
MVA/KEA	10	99.2	96.7
MVA/TZC	8	95.8	96.5
MVA/UGD	8	99.2	95.8