Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 23 (2005) 4647–4657
Vaccine
www.elsevier.com/locate/vaccine

Development of a synthetic consensus sequence scrambled antigen HIV-1 vaccine designed for global use

Scott A. Thomson [a,*], Angel B. Jaramillo [b], Maryanne Shoobridge [a], Kerrie J. Dunstan [b,c], Beth Everett [b], Charani Ranasinghe [a], Stephen J. Kent [d], Ke Gao [a], Jill Medveckzy [a], Rosemary A. Ffrench [b,e], Ian A. Ramshaw [a]

[a] *Division of Immunology and Genetics, John Curtin School of Medical Research (JCSMR), Australian National University, P.O. Box 334, Canberra, ACT 2601, Australia*
[b] *School of Women's and Children's Health, University of New South Wales, and Sydney Childrens Hospital, Sydney, NSW, Australia*
[c] *Centre for Virus Research, Westmead Millennium Institute, Sydney, NSW, Australia*
[d] *Department of Microbiology and Immunology, University of Melbourne, Parkville, Vic., Australia*
[e] *Viral Immunology, Burnet Institute, Melbourne, Vic., Australia*

Received 20 January 2005; received in revised form 18 March 2005; accepted 28 April 2005
Available online 6 June 2005

Abstract

Induction of high levels of broadly reactive cytotoxic T lymphocytes (CTL) remains a promising approach for an effective HIV-1 vaccine. We have developed a novel genetic-based vaccine strategy that encodes consensus overlapping peptide sets from all HIV-1 proteins scrambled together. This synthetic scrambled antigen vaccine (SAVINE) strategy has significant advantages, e.g. capacity to encode more antigens safely and is very flexible compared to traditional isolate-based strategies. The SAVINE vaccine strategy is clearly immunogenic, being able to restimulate a range of human HIV-1 specific responses in vitro and induce HIV-1 specific immunity in vivo in mice. Interestingly, different in vivo delivery strategies affected the resulting immunity and immunodominance pattern in mice. This platform strategy could be used for other infections and cancers where T cell responses are important for protection.

Keyword: Synthetic HIV vaccine

1. Introduction

An inexpensive, safe and effective HIV-1 vaccine capable of protecting the majority of the population from the major subtypes of HIV-1 is desperately required. Current evidence suggests the importance of T cell responses, particularly CTL responses, in controlling HIV-1 replication and the development of AIDS [1,2]. Unlike antibodies, T cells recognize peptide epitopes presented on the surface of infected cells by major histocompatibility complexes (MHC) called human leukocyte antigens (HLA) in humans. In the human population, polymorphism in HLA is the reason why CTL generally recognize different epitopes in different individuals and ethnic groups. Identifying and incorporating sufficient HIV-1 T cell epitopes to provide adequate coverage of the population represents a significant challenge for any T cell-based HIV-1 vaccine strategy.

The subtype diversity and mutation rate of HIV-1 is another significant challenge for vaccine design [3]. Although eight or so major subtypes occur globally, the majority of the HIV-1 infections are caused by C, A/E, B and D subtypes. Considerable diversity exists between and within these subtypes making selection of the final antigenic sequences to be incorporated into a vaccine critically important [4]. Furthermore, the high mutation rate of HIV-1 means that the sequence of the subtypes that might exist in the future are likely to be quite different, making the design of vaccines

* Corresponding author. Tel.: +61 2 61252627; fax: +61 2 61252595.
E-mail address: Scott.Thomson@anu.edu.au (S.A. Thomson).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.04.045

that often take years to manufacture even more difficult. This has led to the notion that conserved regions of the virus should be targeted.

Conserved regions in HIV-1 are potentially useful targets for T cell based vaccines because they are often involved in maintaining viral fitness and hence more difficult for the virus to mutate. It is also interesting that these regions often appear to contain significant concentrations of CTL epitopes, suggesting that there may be an immunological advantage if T cells in persistently infected individuals target such regions [5]. Finally the link with viral fitness also means that conserved epitopes are also are more likely to generate cross-reactive T cell responses and hence be effective against multiple HIV-1 subtypes [6]. Vaccines dependent on just a small number of conserved T cell epitopes, however, should be developed with caution. The relative ease with which HIV-1 has become resistant to some antiretroviral therapies, despite such therapies targeting reasonably well-conserved regions, suggests that a global HIV-1 vaccine may ultimately have to incorporate large numbers of such epitopes so as to reduce the probability of vaccine resistant subtypes emerging in the future.

Currently there are only two T cell-based vaccine approaches that might provide sufficient coverage to contend with the high level of HIV-1 sequence variation [3]. The first is to develop a number of HIV-1 isolate based vaccines that reflect as closely as possible the viral isolates prevalent in the major geographic regions. This means, however, that significant resources need to be committed to multiple vaccine development efforts. The second approach is to develop a vaccine that uses a synthetic HIV-1 consensus sequence designed to match as closely as possible the major circulating subtypes. With this approach there is an increased likelihood of directing immunity towards potentially cross-reactive and/or conserved T cell epitopes. It is also more likely to provide protection in the population for a longer period of time and hence would be more cost effective.

Vaccine safety is another important design consideration. This is particularly so for whole gene-based HIV-1 vaccines incorporating a large proportion of the virus genome or indeed all the open reading frames (ORFs). Besides the obvious risk of reconstituting an intact virus, individual proteins can have undesirable functions for example; Pol (integrase and reverse transcriptase functions); Tat (transcription effects); Rev (translational effects); and Vpu, Env and Nef (affect development of immune responses). Many of these proteins contain reasonably conserved T cell epitopes that are often expressed soon after cell infection, making them potentially important targets for CTL recognition and viral control. Mutation and/or deletion of critical functional amino acids are two strategies often used to remove the unwanted protein functions but which may also disrupt or delete potentially important conserved T cell epitopes. Clearly an ideal vaccine strategy therefore is one that can deliver synthetic consensus sequences for all HIV-1 proteins safely without having to delete or mutate key conserved sequences.

Here we describe a novel scrambled antigen vaccine (SAVINE) strategy that we have used to construct a unique HIV-1 vaccine candidate. This vaccine encodes the overlapping peptides sets spanning all of the proteins in HIV-1 randomly joined together. The vaccine was also designed using a consensus sequence developed from ranked HIV-1 subtype consensus sequences to expand its global potential. The synthetic HIV-1 SAVINE cDNA was constructed and inserted into DNA vaccine, vaccinia and fowlpox virus delivery vectors for testing. We show that T cells from HIV-1 infected individuals can recognize the SAVINE vaccine and that different delivery combinations can have a significant impact on the T cell immunity and immunodominance pattern induced by such synthetic antigens in mice.

2. Methods

2.1. Consensus sequence design for each HIV-1 protein

An overall consensus sequence for each HIV protein was determined using a biased ranking of the subtype consensus sequences in the Los Alamos HIV sequence database [7]. The following ranking was used based on knowledge about the prevalence rates: subtype A/E > subtype C > subtype B > others. Subtype E and subtype A are different mainly in their envelope sequences and so were considered virtually equal in the ranking. Two isolate sequences were utilized where consensus sequences were unavailable at the time for some of the E and C subtype HIV proteins (Genbank accession U51189 and U46016, respectively)[8,9]. The hypervariable regions (HVR) of the HIV envelope protein (start HVR1 to end HVR2 plus start HVR3 to end HVR5) were also excluded from the vaccine design due to extreme sequence variability in these regions. Degenerate sequences were also incorporated into the consensus sequence to extend the subtype coverage according to the following: (1) subtype ranking; (2) no more than eight combinations possible in any nine amino acid stretch; and (3) their amino acid similarity. Eight constructs are theoretically necessary for the vaccine to contain all the possible designed amino acid combinations, however, only two were constructed to determine if using degenerate sequence provided any immunological benefit. These two constructs were selected randomly because the incorporation of degenerate sequences was also random, due to the construction approach used outlined below.

2.2. HIV-1 scrambled antigen vaccine design

Two spacer alanine amino acids were added to the start and end of each degenerate protein sequence and envelop fragments so that the 30AA fragments at the ends of each would be separated in the final SAVINE from amino acids in adjacent

fragments that might effect epitope processing [10]. Each consensus protein sequence was fragmented into 30 amino acid peptides that overlapped each other by 15 amino acids. A cDNA sequence was designed for each peptide sequence using the first and second most commonly utilized mammalian codons alternating with each other out of phase in the corresponding overlap regions. To cater for the degenerate protein sequence more than one base was assigned to some positions using the IUPAC DNA codes without exceeding more than three base variations (maximum eight possible combinations) in any group of 27 bases. Where a codon could not be designed to encode the degenerate amino acids within the 27 base rule described above, the least preferred degenerate amino acid was removed from the design. All the cDNA sequences encoding each peptide were then rearranged randomly and joined together. Due to size constraints the final scrambled cDNA design (∼17000 bp) was then separated into three open reading frames (ORFs). Each designed HIV-1 SAVINE ORF sequence included restriction enzyme sites at each end for subsequent cloning into DNA vaccine or recombinant virus shuttle plasmids (XbaI and BamHI at the 5′ end of all 3 ORFs and XhoI in ORF1 and SalI in ORFs 2 and 3 at the 3′ end). The additions also included Kozak (ccacc) plus translation start codon (atg) and stop codon (tga) at the 5′ and 3′ end of each ORF, respectively. The ORFs were separated into 22 subcassettes at the nearest appropriate 30AA fragment boundary. ORF1 and ORF2 sequences were each separated into seven subcassettes approximately 840 bp each and ORF3 separated eight subcassettes with subcassette eight being approximately 340 bp. To facilitate subcassette joining, each subcassette had one of the following cohesive restriction enzymes sites included at each end, SpeI, AvrII, NheI or XbaI, in such an arrangement that when joined to the next subcassette alternate cohesive sites remained at the ends to allow for the next joining step and the internal site disappeared. For example, the subcassettes comprising ORF1 (SCs1-7) were flanked with the following restriction enzyme sites: -SC1-SpeI, XbaI-SC2-NheI, SpeI-SC3-AvrII, NheI-SC4-XbaI, SpeI-SC5-AvrII, NheI-SC6-XbaI, NheI-SC7, respectively.

2.3. Construction of the synthetic HIV-1 SAVINE vaccine

Each of the 22 subcassettes (that collectively make up the three ORFs) was constructed using in most cases ten long oligonucleotides (100 mers PAGE gel purified, 6% Urea TBE) and two short oligonucleotides (20mer)(Invitrogen, USA). Construction of each subcassette was begun at both ends simultaneously using multiple steps of asymmetric PCR extension to progressively add on the long oligonucleotides [11]. This was done using standard PCR reagents and conditions. The two halves of each subcassette were then joined using standard splicing by overlap extension using both short primers and standard PCR reagents and conditions. Each subcassette fragment was digested with BamHI and EcoRI restriction enzymes and ligated into the general cloning plasmid pBluescript II KS- (Stratagene, USA) cut with the same enzymes and checked by sequencing (Biomolecular Resource Facility, JCSMR, ANU). Due to the degenerate design of the HIV-1 SAVINE two clones for each subcassette were selected so we could construct a second version of the HIV-1 SAVINE.

To complete the ORFs for each version of the HIV-1 SAVINE, the appropriate subcassettes were joined together in multiple steps of restriction enzyme digestion (SpeI, NheI, AvrII or XbaI, New England Biolabs, USA), ligation (T4 ligase, New England Biolabs, USA) and PCR amplification using Elongase (Invitrogen, USA). Once each full length ORF fragment was amplified, it was restriction enzyme digested with BamHI/EcoRI (New England Biolabs, USA) and ligated into pBluescript II KS- (Stratagene, USA) cut with the same enzymes and checked again by sequencing (Biomolecular Resource Facility, JCSMR, ANU). Each ORF was then subcloned into the plasmids pDNAVacc [12] (using enzymes XbaI/XhoI or XbaI/SalI where appropriate), pTK-7.5A [13] (using enzymes BamHI/SalI) and pAF09 [14] (using enzymes BamHI/SalI). The pTK-7.5A derived plasmids were used to generate recombinant vaccinia viruses by marker rescue recombination as described previously [13]. The plasmids based on pAF09 were used to generate recombinant Fowlpox viruses also by marker rescue recombination as described previously [14].

2.4. PBMCs from HIV-1 Infected Subjects

HIV infected blood donors were enrolled in an unselected manner from an outpatient clinic at Albion Street Clinic, Sydney, Australia. Approval to perform the experiments was given by the relevant institutional ethics committee and informed consent obtained from the subjects prior to study commencement. HIV-negative controls were healthy laboratory workers. PBMC were isolated by Ficoll-Hypaque gradient density centrifugation and cryopreserved in FCS/10% DMSO for later use.

2.5. Analysis of HIV-1 specific responses using cytotoxicity lysis assays

For cytotoxicity lysis (CTL) assays, thawed cryopreserved PBMCs were restimulated before being mixed with target cells. Briefly PBMC were thawed quickly and washed twice in RPMI containing 10% FCS/glutamine/penicillin + streptomycin (R10). A fifth of the thawed PBMCs were incubated with the rVV-HIV-SAVINE pool (MOI = 1) or with the rFPV-HIV-SAVINE pool (MOI = 5) for 90 min, washed twice in R10 and added back to the remaining cells. The total PBMC culture (2×10^6/ml) was incubated for 7 days in R10 containing 300 U/ml IL-7. CTL target cells used were autologous BLCL infected with rVV-HIV-1 SAVINE, rVV-gag, rVV-pol, rVV-env or rVV-lac (MOI = 1) (rVV-gag, MRC; rVV-pol, VVTG1132, Transgene; rVV-env, vPE16; rVV-lac, VSC8; NIH AIDS

Reference and Reagent program). Target cells were infected overnight and labelled with ^{51}Cr (100 uCi), washed three times and resuspended in R10. Effector and target cells were plated out in triplicate at ratios of 50:1, 25:1, 12.5:1 and 6.25:1 in a total volume of 200 μl and incubated for 5 h before measuring release of ^{51}Cr.

2.6. Analysis of HIV-1 specific responses using IFN-gamma ELISPOT assays

Cryopreserved PBMCs were also used in an IFN-gamma ELISPOT assays. Briefly PBMCs were resuspended in R10 and seeded at 2×10^5 and 1×10^5 cells/well in triplicate into 96-well plates (Cat. S4510 Multiscreen-IP, Millipore Corp., Bedford, MA) coated with 5 μg/ml anti-IFN-gamma monoclonal antibody (1-DK-1; Mabtech). Cells were stimulated either with the rVV-HIV-SAVINE pool, or control rVV-lac by adding virus to the bulk PBMC culture at MOI = 1. Cells were cultured for 20 h after which the cells were removed from the plate. Following removal of cells, and between each subsequent reagent step, plates were washed three times with PBS/0.05% Tween20, then three times with PBS. Plates were then incubated with 1 μg/ml of biotin-conjugated anti-human IFN-gamma antibody (Clone 7-B6-1; Mabtech) for 2 h at room temperature followed by incubation for 1 h at room temperature with streptavidin-alkaline phosphatase at 1 μg/ml. The plates were developed by addition of 100 ul of BCIP/NBT substrate (Sigma Chemicals) and incubated for 30 min at room temperature. The plates were analysed by first washing the plates six times with tap water and then using an AID ELISPOT Reader System (Autoimmn Diagnostika GmbH, Strassberg, Germany) and the AID ELISPOT software (AutoImmun Diagnostika) to count the spots. A value of spot-forming cells/10^6 PBMC was calculated by averaging the spot count per well of replicate wells and adjusting for cell number. A positive response was defined where the calculated value was >20 SFC/10^6 PBMC and greater than twice the mean value for control wells (medium or rVV-lac, which ever was greatest). IFN-gamma ELISPOT assays were also done using HIV-peptide restimulated cryopreserved PBMCs. Specific restimulations were performed as for the CTL assays but using pools of HIV peptides corresponding to HIV gag or pol (15 mers overlapping by 11 amino acids, NIH AIDs Research and Reference Reagent program) at a final concentration of 1 μg/ml of each peptide for three days. Following restimulation, the bulk PBMC population was harvested, washed twice in R10 and used in an IFN-gamma ELISPOT assay as above.

2.7. Mouse immunisations

Specific pathogen-free mice (6–8-week-old) female BALB/c and C57BL/6J mice were obtained from the Animal Breeding Facility, John Curtin School of Medical Research, Canberra, Australia. The mice were immunised twice 2–4 weeks apart with the pDNAVacc-HIV-SAVINE plasmid pool (100 μg total, 50 μg /leg, i.m. in PBS) and boosted with the rFPV-HIV-SAVINE pool 2–4 weeks later (3×10^7 pfu, i.p. or i.m). For the improved prime-boost experiments BALB/c mice were immunised intramuscularly with the rFPV-HIV-SAVINE pool followed by the rVV-HIV-SAVINE pool two weeks later or with these same vector pools in the opposite order. ANU institutional ethics committee approval was obtained prior to commencement of the experiments.

2.8. Murine cytotoxicity lysis assays

Splenocytes from immunised mice ($n = 3$ for BALB/c, $n = 6$ C57BL/6J) were restimulated for 5 days before being used in in vitro assays to measure HIV-1 specific CTL killing of rVV infected ^{51}Cr labelled target cells [12]. Briefly red blood cells in the splenocytes were lysed using red blood cell lysis buffer and the remaining cells washed 3 times in media. A fifth of the cells were then washed in PBS once and infected with 0.25 MOI of the rVV HIV-1 SAVINE pool in a small volume for 1 h before being added back to the remaining cells. Media was added to the cell cultures until the cell concentration was 5×10^6/ml and the cells plated into six-well tissue culture plates. Seven days later effector cells were harvested washed once and mixed with either uninfected or infected ^{51}Cr labelled P815 cells for BALB/c derived splenocytes or MC57G cells for C57BL/6J splenocytes at the ratios 100:20:1 and 4:1. The target cells were infected either with control (CTR) virus (VV-TK-), the rVV-HIV-SAVINE pool, rVV-HIV-1 Gag, rVV-HIV-RT, rVV-HIV-Nef, rVV-HIV-Env. The latter four viruses were as described previously [15]. P815 cells were infected for 1 h at a MOI of 20:1 after a PBS wash and cultured in media overnight before being labelled with ^{51}Cr. The adherent MC57G cells were rinsed once with PBS infected at a MOI of 10:1 for 1 h and cultured for 3 h before being trypsinised to resuspend and label them with ^{51}Cr. 6 h later supernatants (25 μl) from the combined effector/target cell cultures were harvested and the amount of released ^{51}Cr measured. Percent lysis was calculated as previously [12].

2.9. Murine IFN-gamma ELISPOT assays

Initially primary ex vivo splenocytes and splenocytes that were restimulated in vitro for 5 days with the VV-HIV-SAVINE as above, from six mice, were assessed in an IFN-gamma ELISPOT assay after being pulsed with the HIV-1 HXB2 Gag (15 mers overlapping by 11 mer, NIH AIDs Research and Reference Reagent Program). In later experiments, using primary ex vivo splenocytes only ($n = 5$ mice), the peptide pools used included HIV-1 A/E isolate (93TH253) Gag, Pol, Rev or Tat (Australian Thai HIV NIH Consortium), Consensus Gag A subtype, B subtype or C subtype, Consensus B subtype Pol (first 125 peptides, Cat no. 5461–5585) overlapping peptide pools (15 mers overlapping by 11 mer, NIH AIDs Research and Reference Reagent Program) and a 9 mer peptide corresponding to a known

BALB/c Gag epitope (AMQMLKETI)[23]. 96-well plates (Millipore Corp., Bedford, MA, Cat. S4510 Multiscreen-IP) were coated with a purified mouse IFN-gamma capture antibody (PharMingen) spleen cells were added to the plate and restimulated with the overlapping peptides (1 μg/ml of each peptide) for 24 h. After removing the cells, a detection antibody, biotinylated rat antimouse IFN-gamma (PharMingen), was added followed by Streptavidin-Alkaline Phosphatase (Amersham Pharmacia) and a premixed substrate solution of BCIP/NBT (Sigma). Spots were counted and expressed as spot forming cells (SPC)/10^6 cells.

2.10. Murine in vivo CTL assay

An in vivo analysis to assess CTL activity was also done on HIV-1 SAVINE immunised mice using a method as described previously [16]. Briefly, splenocytes from naive mice were labelled with SNARF or CFSE (Molecular Probes, Eugene, OR) at 2.5 mM as described elsewhere [17]. The CFSE-labelled cells were separated into two aliquots one was pulsed with a single pool of HIV-1 HXB2 Gag overlapping peptides (15 mers overlapping by 11mer)(NIH Reagent Program) for 90 min at 37 °C in PBS and the other not exposed to peptides. CFSE aliquots were washed twice in media and twice in PBS, then added to an equal number of washed SNARF-labelled cells (internal control). The cell mixture (4×10^7 cells in 400 ul PBS) was injected i.v. into prime-boost (DNA/FPV) immunised mice (total four mice, $n = 2$ for peptide labelled CSFE cells, $n = 2$ CSFE cells with no peptide as an external control). Splenocytes were harvested 24 h later and analysed by flow cytometry analysis.

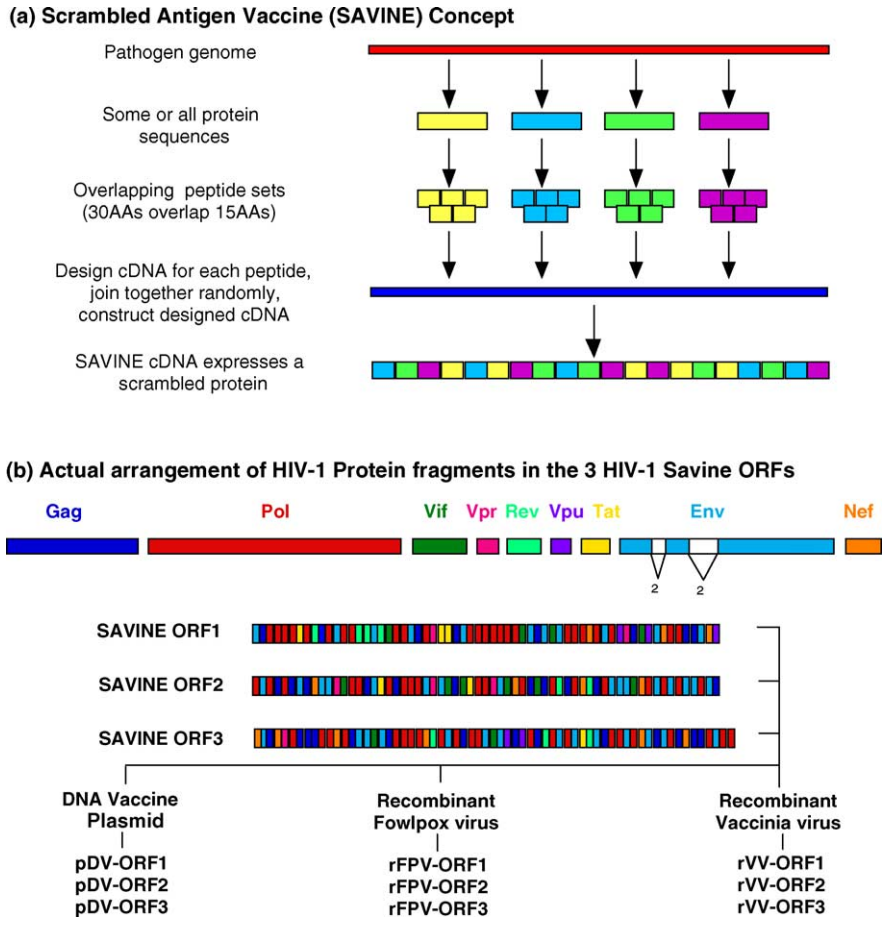


Fig. 1. Scrambled antigen vaccine (SAVINE) concept and HIV-1 SAVINE. (a) Schematic representation of the SAVINE concept. (b) Colour coded HIV-1 proteins and the actual arrangement of the overlapping fragments from these proteins in the three HIV-1 SAVINE ORFs approximately to scale. The cDNA to encode each initial overlapping peptide was designed by alternating, where possible, the first and second most commonly used mammalian codons. In the overlapping regions codon assignment was out of phase with the assignment for the same sequence in the next peptide. This was done primarily to keep DNA sequence duplication to a minimum so as to avoid cDNA instablity. Replacing the original HIV-1 DNA sequence with optimised codons has been shown elsewhere to enhance protein expression [17a]. The cDNA and corresponding peptide sequences were then joined randomly to create a full length HIV-1 SAVINE cDNA and protein design, respectively. To keep the size of the final DNA vaccine plasmids below 10 kb, the full length designed HIV-1 SAVINE cDNA sequence was separated into three open reading frame (ORF) fragments (each approximately 5700 bp, actual arrangement and approximate scale shown in (b). To each ORF appropriate restriction enzyme cloning sites and a Kozac sequence, start and stop codons were added to enable expression. Each ORFs was inserted into a DNA vaccine plasmid, fowlpox and vaccinia viral vector. The three recombinant constructs for each vector were then pooled for subsequent experiments.

3. Results

3.1. Design and construction of the HIV-1 SAVINE

A novel synthetic vaccine strategy referred to as scrambled antigen vaccines (SAVINEs) (Fig. 1a), was used to design and construct a HIV-1 candidate vaccine that encodes all HIV-1 proteins, excluding the envelope hypervariable regions (Fig. 1b). The scrambled nature of the vaccine ensures that the functions of HIV-1 proteins were destroyed. Virtually all of the potential CTL epitopes (8–12AAs) remain intact in this vaccine because the 30 amino acid protein fragments overlap each other by 15 amino acids. The 15 amino acid overlap also ensures that unnatural flanking amino acids are unlikely to block epitope processing.

The starting HIV-1 sequence used to design the SAVINE cDNAs above was a degenerate consensus HIV-1 sequence that was generated using a combination of ranked isolate (where consensus sequences were unavailable) and subtype consensus sequences [7]. The subtype ranking used was A/E > C > B > D > others. At some positions additional criteria were used to design the consensus sequence including the amount of amino acid similarity and in difficult positions the actual amount of conservation within each subtype consensus sequence. Degeneracy was also included in this sequence at a rate of no more than eight different combinations in any stretch of nine amino acids. Degenerate amino acids were incorporated to enable us to construct a second set of recombinant constructs to examine if the SAVINE strategy could be further enhanced, by the addition of approximately 350 common subtype amino acid substitutions. To date, however, the second set of constructs has not demonstrated sufficient immunological benefit to outweigh the disadvantage of significantly increasing the number of vaccine components involved (data not shown).

We constructed the HIV SAVINE ORFs synthetically (Fig. 2). The synthetic SAVINE cDNAs were transferred by standard techniques, three DNA vaccination plasmids, three recombinant fowlpox (rFPV) viruses and three recombinant vaccinia (VV) viruses, which were then pooled to make the final DNA and virus-vectored vaccines (Fig. 1b). The ability of each synthetic ORF to be fully expressed was demonstrated by subcloning each separately into an expression plasmid in frame and ahead of the gene for enhanced green fluorescence protein (EGFP) and fluorescence detected following transfection into 293 cells (data not shown).

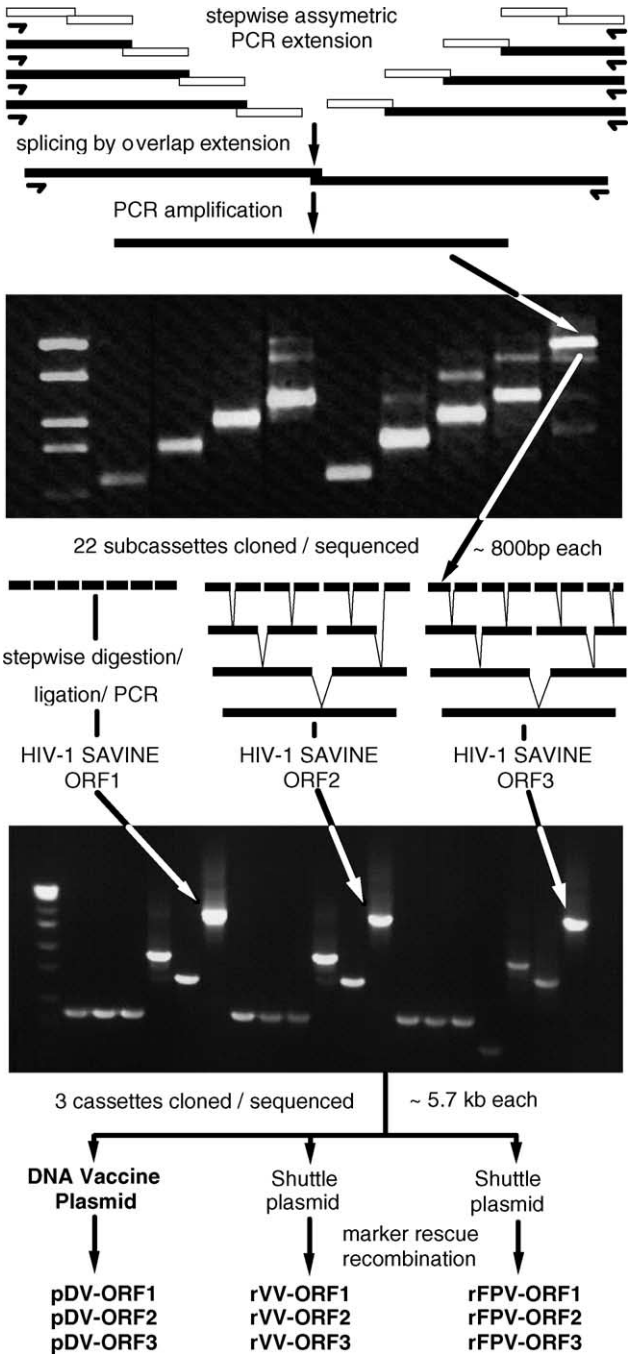


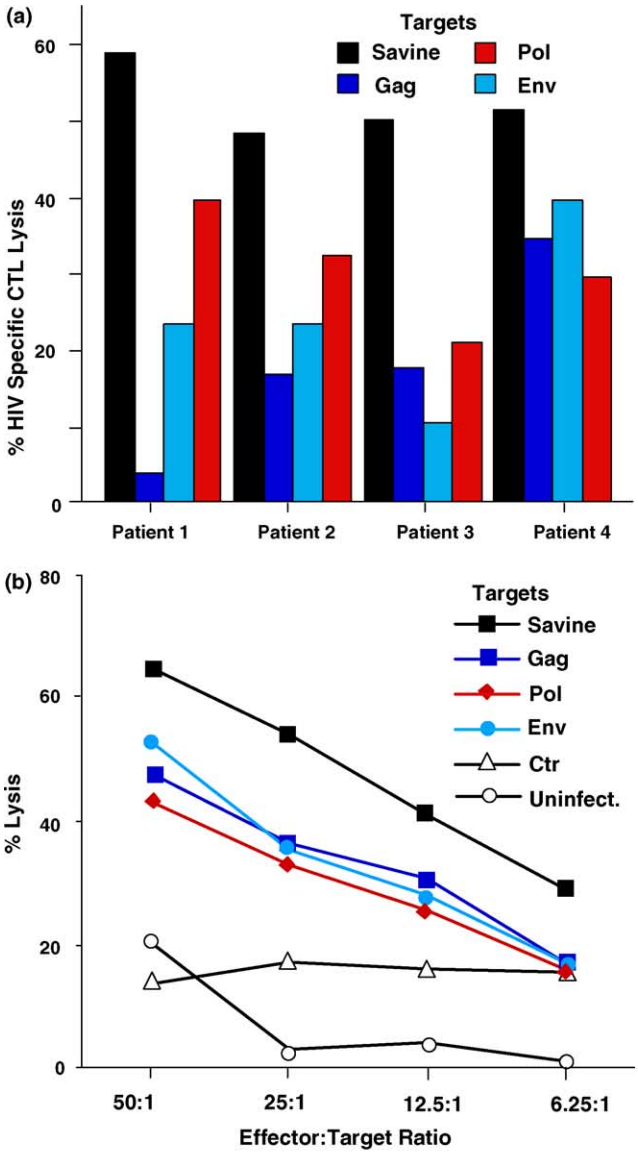
Fig. 2. Construction of the synthetic HIV-1 SAVINE cDNA and vaccine vectors. The three designed HIV-1 SAVINE ORF sequences were constructed in a stepwise fashion (ORF1 and ORF2 sequences were each separated into seven subcassettes approximately 840 bp each and ORF3 separated into eight subcassettes, the last subcassette being 340 bp). The 22 subcassettes were constructed from long (generally 100 mers) and short (generally 20 mers) oligonucleotides using stepwise asymmetric PCR extension followed by standard splicing using overlap extension, restriction digestion, ligation into a general cloning plasmid and checking by sequencing. The subcassettes were joined by stepwise restriction enzyme digestion, ligation and PCR amplification. The synthetic ORFs were restriction enzyme digested and ligated into a general cloning plasmid and checked by sequencing. A single set (due to designed degeneracies) of three ORFs that together comprise the HIV-1 SAVINE were used in the assays described here (Genbank Accession HIVSAVINEORF1 AY787839, HIVSAVINEORF2 AY787840 and HIVSAVINEORF3 AY787841). Each ORF was subcloned into pDNAVacc and the virus recombination plasmids, pTK-7.5A and pAF09. The latter two plasmid sets were then used to make three recombinant vaccinia viruses and three Fowlpox viruses, respectively, by marker rescue recombination.

3.2. Restimulation of human HIV-1 specific responses in vitro

To test whether naturally generated human HIV-1 specific T cell responses could recognise the HIV-1 SAVINE, PBMCs from B subtype HIV-1 positive individuals were restimulated in vitro using the rVV-HIV- SAVINE pool and the resulting effectors used in CTL lysis assays. Fig. 3a clearly shows that the HIV-1 SAVINE stimulated effector cells recognised target cells infected with either the VV-HIV-SAVINE or VVs encoding natural B subtype HIV-1 antigens. Fig. 3b shows the dilution of lytic activity and controls for one subject, showing decreased recognition with decreasing input effector numbers. As expected the pattern of HIV-1 specific responses was different in each individual. Also, recognition of the HIV-1 SAVINE expressing target cells was consistently higher than target cells expressing the whole Gag, Pol and Env in all four subjects most likely reflecting the sum of multiple HIV-1 specific CTL responses. Significant IFN-gamma production was observed when PBMCs from B subtype HIV-1 positive subjects were restimulated overnight with rVV-HIV-SAVINE in ELISPOT assays confirming the results of the CTL lysis assays (Fig. 3c). Similar results were obtained if PBMCs were stimulated with the rFPV-HIV-SAVINE pool or if PBMCs were initially restimulated with Gag or Pol overlapping peptides before being exposed to rVV-HIV-SAVINE pool in an IFN-gamma ELISPOT assay (data not shown).

3.3. Immunogenicity of the HIV-1 SAVINE in mice

To initially examine the immunogenicity of HIV-1 SAVINE strategy in vivo, mice were immunized by the DNA-prime/rFPV-boost vaccination regime that has been previously shown to be able to generate large numbers of high avidity T cells [16,18]. Groups of BALB/c ($n = 3$) and C57BL/6J ($n = 6$) mice were immunised by prime-boosting (DNA and rFPV-HIV-SAVINE vaccine pools) and their splenocytes restimulated in vitro with the rVV-HIV-SAVINE pool. The resulting effectors were then used in CTL lysis assays against P815 or MC57G target cells, respectively, infected with the rVV-HIV-1 SAVINE, VV-CTR or rVVs encoding native B subtype HIV-1 proteins. In both mouse strains significant



(c) In vitro IFN- ELISPOT assay using PBMCs from HIV-1 infected subjects. Spot-forming cells/10^6 cells generated following the indicated stimulation. Values in bold represent significant responses.

	Stimulus		
Subject	**Medium**	**CTR virus**	**HIV-1 SAVINE**
1	70	15	**275**
2	60	100	**435**
3	130	145	**315**
4	105	90	**523**

Fig. 3. In vitro recognition of the rVV-HIV-1 SAVINE by restimulated T cell responses from HIV-1 B subtype infected subjects. (a) HIV specific lysis of autologous target cells infected with rVV-HIV-1 SAVINE or rVVs expressing native B subtype HIV-1 proteins by peripheral blood mononuclear cells (PBMCs) restimulated with the rVV-HIV-1 SAVINE pool from four B subtype HIV-1 infected patients. Lysis of uninfected or control infected target cells was generally below 20%. (b) Titration of restimulated effector cells demonstrating decreasing cytotoxic acitvity for one of these subjects. (c) IFN-γ ELISPOT assays showing SFC/10^6 cells using PBMCs from four B subtype HIV-1 infected patients unstimulated or restimulated with the rVV-HIV-1 SAVINE pool or control virus at different cell densities per well overnight.

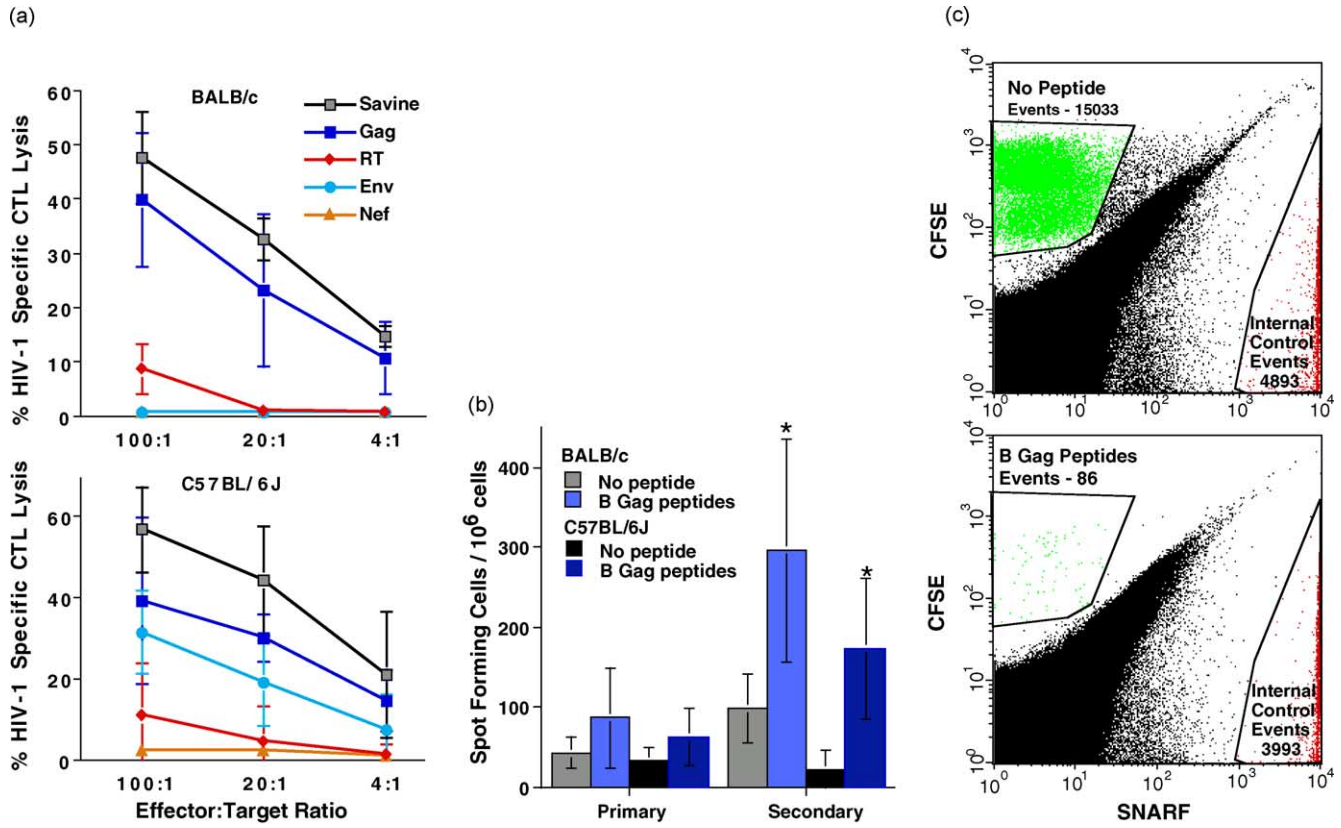


Fig. 4. T cell responses in mice immunised with the HIV-1 SAVINE by prime-boosting (DNA followed by rFPV). (a) HIV-1 specific responses in BALB/c ($n = 3$) and C57BL/6J ($n = 6$), respectively, detected in ^{51}Cr release assays using splenocytes restimulated in vitro with the rVV-HIV-1 SAVINE pool and used as effectors against P815 or MC57G target cells infected with either the rVV-HIV-1 SAVINE or rVVs expressing the native B subtype HIV-1 proteins. Lysis of uninfected or CTR VV-TK-infected target cells, was generally below 10%. Individual mice were similar as were repeat experiments. Significant responses determined by unpaired student *t*-test two-sample equal variance; VVTK vs. VVHIV-Ag targets-BALB/c VVSavine, $p = 0.00002$, VV B Gag, $p = 0.00038$; C57BL/6J mice VVSavine, $p = 0.00004$, VV B Gag, $p = 0.00031$, VV B Env, $p = 0.0062$. (b) IFN-γ ELISPOT assays showing Spot Forming Cells (SFC)/10^6 cells using either primary splenocytes or splenocytes restimulated with the rVV-HIV-1 SAVINE pool from mice ($n = 6$) immunised as above detected using a pool of overlapping B subtype Gag peptides. Significant responses determined by unpaired student *t*-test two-sample equal variance; no peptide vs. B Gag peptides-BALB/c primary, $p = 0.067$, secondary, $p = 0.0015$; C57BL/6J mice primary, $p = 0.043$, secondary, $p = 0.00056$. Individual mice were similar as were repeat experiments. (c) Significant in vivo clearance of CFSE labelled splenocytes pulsed with a pool of overlapping B subtype Gag peptides 24 h after injection into SAVINE immunised mice. Figure representative of both sets of data (total four mice, $n = 2$ for peptide labelled CSFE cells, $n = 2$ for CFSE no peptide control mice). Separate mice injected with CSFE labelled splenocytes without peptide are also a control for the assay.

HIV-1 specific CTL responses were detected (Fig. 4a). Individual mice were similar as were repeat experiments. IFN-γ ELISPOT assays confirmed the induction of the T cell responses using splenocytes from mice ($n = 6$) immunised as above were pulsed with a single pool of 122 15 mer peptides overlapping by 11 amino acids spanning the Gag protein from the B subtype isolate HXB2 (Fig. 4b)[19]. Individual mice were similar as were repeat experiments. It is important to emphasize that although the HIV-1 SAVINE is not primarily a B subtype vaccine the T cell responses it generated could still recognise B subtype reagents.

Next we assessed the immunogenicity of the HIV-1 SAVINE using an in vivo CTL assay [16]. This assay was performed by injecting immunised animals with two populations of cells one population labelled with SNARF and the other labelled with CFSE and overlapping HIV-1 peptides or CFSE alone. Monitoring the clearance of peptide-labelled cells relative to the other stained cell population within the same animal (SNARF internal control) and also by comparing to the control animals (no peptides involved, external control) by FACs provides evidence of specific CTL activity in vivo. Briefly, HIV-1 SAVINE prime-boost immunized mice (DNA and rFPV SAVINE pools) (total $n = 4$) were injected with CFSE-labelled syngeneic splenocytes pulsed with the B subtype Gag overlapping 15 mer peptide pool as well as SNARF-labelled cells ($n = 2$) or the same two cell populations without peptides ($n = 2$). The number of SNARF-labelled cells in splenocytes from these animals relative to the CFSE labelled cells 24 h later was then assessed using FACs analysis. The immune mice were able to rapidly clear the Gag peptide-pulsed CFSE labelled cells but not SNARF labelled cells or CFSE cells without peptides showing that the HIV-1 SAVINE can generate HIV-1 specific cytotoxic T cell responses in vivo (Fig. 4c).

3.4. Impact of delivery strategy and route in mice

The intramuscular route is a preferred delivery route for human vaccines. To examine this using the HIV-1 SAVINE we immunized mice ($n = 6$) as above but delivered the rFPV-HIV-SAVINE pool intramuscularly instead of intravenously or intraperitoneally. For comparison a further group of mice were immunized with a whole gene based vaccine using similar delivery vectors and immunization route [20], Surprisingly, no significant T cell responses were generated with the SAVINE in any mouse unlike the significant Gag responses generated with the whole gene approach (data not shown). This result suggests that the intramuscular route for the rFPV booster is suboptimal compared to other routes for the delivery of the HIV SAVINE. It also showed that there is a difference between the immunological potential of whole gene vaccine approach and the synthetic unstructured SAVINE strategy when delivered by this route.

Recently we have developed an improved prime boost approach where both the prime and boost utilizes poxvirus vectors. The substantial differences between VV and FPV delivery vectors, means that immune responses induced by one vector does not preclude the use of the other as a boost. The improved prime-boost approach was used to determine if HIV SAVINE could induce significant immune responses when delivered by the intramuscular route only. Groups of BALB/c mice ($n = 5$) were immunized with either the rFPV or rVV-HIV-SAVINE pool and then boosted 2 weeks later with the rVV and rFPV-HIV-SAVINE pools, respectively. Fig. 5 shows the results of an IFN-gamma ELISPOT assay using different overlapping peptide pools to multiple HIV proteins (NIH AIDS Reference and Reagent Program). Interestingly, clear significant differences were detected depending on the poxvirus order, with the rVV-prime/rFPV boost clearly inducing a significant Pol response. This Pol response has been consistently much higher than any previous response to Pol detected using our other vaccine candidates (data not shown). Individual mice were similar as were repeat experiments. Also interesting was the apparent change in the immunodominance pattern in the BALB/c mice from the Gag response induced by the prime DNA i.m./ boost rFPV i.p. (Fig. 4a), to the Pol response induced only by the prime rVV i.m./boost rFPV i.m. immunisation regime (Fig. 5).

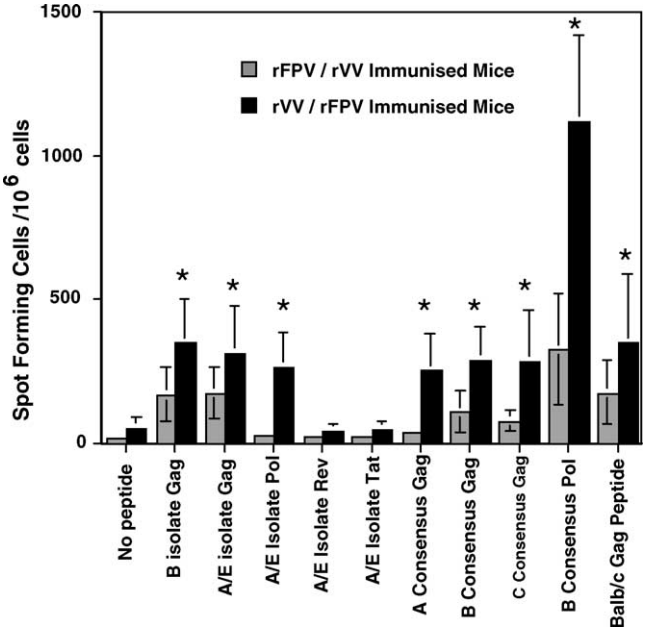


Fig. 5. T cell responses in groups of BALB/c mice ($n = 5$) immunised by the improved prime/boost approach utilising the rFPV and rVV HIV SAVINE pools. T cell responses measured as spot forming cells (SFC)/10^6 cells using primary splenocytes exposed to a standard pool of overlapping B subtype Gag peptides, Gag peptide pool to an A/E isolate (p93TH293)[8], Pol A/E isolate pool, A/E Rev peptide pool, A/E Tat peptide pool, NIH consensus ovelapping peptide sets for Gag A subtype, B subtype, C subtype and B subtype Pol (NIH Reagent program) and a single known Gag BALB/c CTL epitope peptide. Individual mice were similar as were repeat experiments. Significant differences (marked (*)) as determined by unpaired student *t*-test ($n = 5$) two-sample equal variance; FPV/VV vs. VV/FPV immunisation-B consensus Pol, $p = 0.00057$, A/E isolate Pol, $p = 0.0013$, A Gag consensus, $p = 0.0032$, B Gag consensus, $p = 0.0081$, C Gag consensus, $p = 0.016$, BALB/c Gag peptide, $p = 0.018$, B Gag isolate, $p = 0.022$, A/E Gag isolate, $p = 0.024$; FPV/VV B Pol consensus vs. FPV/VV B Gag peptide, not significant $p = 0.088$, VV/FPV B Pol consensus vs. B Gag peptide, significant (unmarked) $p = 0.0011$.

4. Discussion

The SAVINE vaccine strategy is clearly immunogenic, being able to restimulate human HIV-1 specific responses in vitro and induce HIV-1 specific immunity in vivo in mice. The main advantage of the SAVINE strategy is that large amounts of potentially immunogenic information can be delivered and expressed safely without having to thoroughly characterise each and every protein antigen. This approach also eliminates the need to identify and characterize large numbers of T cell epitopes as required for example by the polyepitope approach [21,22]. Furthermore, potentially important T cell epitopes presented by rare HLAs can be easily included in the vaccine. Because the HIV-1 SAVINE incorporates virtually all the protein sequences in HIV-1 it may be more likely to remain protective against subtype variation and new subtypes in the future. The structure and function(s) of the HIV-1 protein sequences were destroyed by the fragmentation and scrambling process. This enhances vaccine safety and also may improve T cell epitope presentation by producing misfolded proteins that are rapidly proteolytically processed. A potential disadvantage of the strategy is that it reduces the possibility of generating potentially advantageous neutralizing antibody responses, which may be important in some situations. The design flexibility of the SAVINE strategy, however, means that it could be combined in the future with other genetic-based strategies that might be able to generate neuralizing antibody responses.

One of the aims of using the SAVINE approach to develop an HIV-1 vaccine was to improve coverage by using two key design features, firstly encoding as much of the virus as possi-

ble and secondly by using a consensus sequence. We clearly show here that the SAVINE was able to restimulate functional cytotoxic and cytokine producing cells from the blood of HIV-1 infected individuals. These restimulated responses were broad and directed against multiple HIV proteins in different HLA backgrounds. We also showed that the HIV-1 SAVINE was able to sensitize human target cells for lysis by cytotoxic T cells. These data indicate that a range of natural HIV-1 T cell epitopes are correctly processed and presented by the SAVINE vaccine.

Generating broadly reactive T cell responses in vivo with a vaccine, however, can be more difficult than restimulating naturally generated responses in vitro. This was demonstrated here by the relatively narrow breadth of T cell responses induced in mice in vivo following immunization with the HIV-1 SAVINE. Furthermore, we have found clear differences in the immune responses induced by our whole gene HIV-1 vaccine compared to the HIV-1 SAVINE using identical delivery approaches. For example, in BALB/c mice our whole gene vaccine candidate always induced an immunodominant T cell response to the known BALB/c Gag CTL epitope [23] whereas the SAVINE vaccine candidate delivered using identical delivery strategies and routes induced the immunodominant Gag response (DNA i.m./rFPV i.p., Fig. 4a), an immunodominant Pol response (rVV i.m./rFPV i.m., Fig. 5) or no response (DNA i.m./rFPV i.m. data not shown). This was a consistent finding in individual mice in the different experiments. Interestingly, the SAVINE induced BALB/c Pol response is likely to recognise a previously characterized Pol epitope identified using a codon optimised Pol gene only and delivered by DNA or a DNA/VV prime boost [24]. The reason why the Pol response is generally subdominant to the Gag response when both genes are present in whole gene vaccines may be due to the low expression of Pol caused by the dependence on the Gag/Pol frameshift and Rev dependent expression [24]. Also vaccines expressing whole Gag often produce virus-like particles [25]. The capacity to form such particles could be especially important for some vaccination routes and when utilizing poxvirus delivery vectors that are known to cause immature dendritic cell death following virus entry [26,27]. Similarly, the ability to cross-prime CTL responses under these circumstances may be important [28]. Together with the data, these issues suggest that the characteristics of the expression vector(s) and choice of delivery route may be more critical for synthetic unstructured antigens, such as the HIV SAVINE than for whole gene vaccines that retain the ability to produce virus-like particles.

We have shown the HIV-1 SAVINE can restimulate human HIV-1 specific responses in vitro and induce HIV specific immunity in vivo in mice. When combined with its enhanced safety advantages this strategy may prove with further development to be an extremely useful antigen design approach. The SAVINE strategy may be applicable to other pathogens where strong T cell immunity and destruction of protein functions may be important, e.g. Hepatitis C virus. It may also prove useful as a cancer vaccine strategy to enable the use of sequences from cancer-associated proteins. The SAVINE strategy could eventually prove to be a significant advance in the development of safer and more effective vaccines.

Acknowledgments

Grant support: this work was supported by ANU, JCSMR and the National Centre for HIV Virology Research (Australia). The authors would like to acknowledge the following: (1) National Institutes of Health, AIDS Research and Reference Reagent Program. (2) Human Retroviruses and AIDS 1997: a Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Korber B., Hahn B., Foley B., Mellors J.W., Leitner T., Myers G., McCutchan F., Kuiken C.L. (Eds.). Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM. (3) Clients and Staff at the Albion St Clinic, Surrey Hills, Sydney, Australia. (4) Professor Gordon Ada for ongoing support.

References

[1] Girard MP, Mastro TD, Koff W. Human immunodeficiency virus. Ch. 44. In: Plotkin SA, Orenstein WA, editors. Vaccine. 4th ed. Elsevier Publications; 2003. p. 1219–58.
[2] McMichael A, Hanke T. The quest for an AIDS vaccine: is the CD8+ T-cell approach feasible? Nat Rev Immunol 2002;2:283–91.
[3] Gaschen B, Taylor J, Yusim K, Foley B, Lang D, Novitsky V, et al. Diversity considerations in HIV-1 vaccine selection. Science 2002;296:2354–60.
[4] Nabel G, Makgoba W, Esparza J. HIV-1 diversity and vaccine development. Science 2002;296:2335.
[5] Yusim K, Kesmir C, Gaschen B, Addo MM, Altfeld M, Brunak S, et al. Clustering patterns of cytotoxic T-lyphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. J Virol 2002;76:8757–68.
[6] Ferrari G, Neal W, Jones A, Olender N, Ottinger J, Ha R, et al. CD8 CTL responses in vaccines: emerging patterns of HLA restriction and epitope recognition. Immunol Lett 2001;79:37–47.
[7] Korber B, Hahn B, Foley B, Mellors JW, Leitner T, Myers G, et al. Human retroviruses and AIDS A compilation and analysis of nucleic acid and amino acid sequences. Theoretical biology and biophysics group. Los Alamos 1997.
[8] Gao F, Robertson DL, Morrison SG, Hui H, Craig S, Fultz PN, et al. The heterosexual HIV-1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. J Virol 1996;70:7013–29.
[9] Salminen MO, Johansson B, Sonnerborg A, Ayehunie S, Gotte D, Leinikki P, et al. Full length sequence of an Ethiopian human immunodeficiency virus type 1 (HIV-1) isolate of genetic subtype C. AIDS Res Hum Retro 1996;12:1329–39.
[10] Del Val M, Schlicht H, Ruppert T, Reddehase MJ, Koszinowski UH. Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. Cell 1991;66:1145–53.
[11] Sandhu GS, Aleff RA, Kline BC. Dual asymmetric PCR: one-step construction of synthetic genes. Biotechniques 1992;12:14–6.
[12] Thomson SA, Sherritt M, Medveczky J, Elliott S, Moss DJ, Fernando GJP, et al. Delivery of multiple CD8+ cytotoxic T cell epitopes by DNA vaccination. J Immunol 1998;160:1717–23.

[13] Coupar BEH, Andrew ME, Boyle DB. A general method for construction of recombinant vaccinia viruses expressing multiple foreign genes. Gene 1988;68:1–10.
[14] Heine HG, Boyle DB. Infectious bursal disease virus structural protein VP2 expressed by a fowlpox virus recombinant confers protection against disease in chickens. Arch Virol 1993;131:277–92.
[15] Kent SJ, Zhao A, Best SJ, Chandler JD, Boyle DB, Ramshaw IA. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. J Virol 1998;72:10180–8.
[16] Estecourt MJ, Ramsay AJ, Brooks A, Thomson SA, Medveckzy CJ, Ramshaw IA. Prime-boost immunization generates a high frequency, high-avidity CD8(+) cytotoxic T lymphocyte population. Int Immunol 2002;14:31–7.
[17] Oehen S, Brduscha-Riem K. Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. J Immunol 1998;161:5338–42;
(a) Gao F, Li Y, Decker JM, Peyerl FW, Bibollet-Ruche F, Rodenburg CM, et al. Codon usage optimization of HIV type 1 subtype C gag, pol, env, and nef genes: in vitro expression and immune responses in DNA-vaccinated mice. AIDS Res Hum Retroviruses 2003;19(9):817–23.
[18] Cohen J. Vaccines: monkey puzzles. Science 2002;296:2325–8.
[19] AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.
[20] The Australian Thai HIV Vaccine Consortium. Subtype AE HIV-1 DNA and recombinant Fowlpoxvirus vaccines encoding 5 shared HIV-1 genes: safety and T cell immunogenicity in macaques. Vaccine 2005;23:1949–56.
[21] Thomson SA, Elliott SL, Sherritt MA, Sproat KW, Coupar BEH, Scalzo AA, et al. Recombinant polyepitope vaccines for the delivery of multiple CD8+ cytotoxic T cell epitopes. J Immunol 1996;157:822–6.
[22] Hanke T, McMichael AJ, Mwau M, Wee EG, Ceberej I, Patel S, et al. Development of a DNA-MVA/HIVA vaccine for Kenya. Vaccine 2002;20:1995–8.
[23] Selby MJ, Doe B, Walker CM. Virus-specific cytotoxic T-lymphocyte activity elicited by co-immunisation with human immunodeficiency virus type 1 genes regulated by the bacteriophage T7 promoter and T7 RNA polymerase protein. J Virol 1997;71:7827–31.
[24] Casimiro DR, Tang A, Perry HC, Long RS, Chen M, Heidecker GJ, et al. Vaccine-induced immune responses in rodents and nonhuman primates by use of a humanized human immunodeficiency virus type 1 *pol* gene. J Virol 2002;76(1):185–94.
[25] Young KR, Smith JM, Ross TM. Characterization of a DNA vaccine expressing a human immunodeficiency virus-like particle. Virology 2004;327(2):262–72.
[26] Engelmayer J, Larsson M, Subklewe M, Chahroudi A, Cox WI, Steinman RM, et al. Vaccinia virus inhibits the maturation of human dendritic cells: a noval mechanism of immune evasion. J Immunol 1999;163(12):6762–8.
[27] Akahata W, Yang ZY, Nabel GJ. Comparative immunogenicity of human immunodeficiency virus particles and corresponding polypeptides in a DNA vaccine. J Virol 2005;79(1):626–31.
[28] Fonteneau JF, Kavanagh DG, Lirvall M, Sanders C, Cover TL, Bhardwaj N, et al. Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells. Blood 2003;102(13):4448–55.