METHODS IN MOLECULAR MEDICINE™

DNA Vaccines

Methods and Protocols

SECOND EDITION

Edited by

W. Mark Saltzman

Department of Biomedical Engineering
Yale University, New Haven, CT

Hong Shen

Department of Chemical Engineering
University of Washington, Seattle, WA

Janet L. Brandsma

Section of Comparative Medicine,
Yale University, New Haven, CT

HUMANA PRESS TOTOWA, NEW JERSEY

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

This publication is printed on acid-free paper. ∞
ANSI Z39.48-1984 (American Standards Institute)
Permanence of Paper for Printed Library Materials.

Production Editor: Jennifer Hackworth

Cover illustration: Figure 2 from Chapter 9, "Surface-Modified Biodegradable Microspheres for DNA Vaccine Delivery," by Mark E. Keegan and W. Mark Saltzman

Cover design by Patricia F. Cleary

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel.: 973-256-1699; Fax: 973-256-8341; E-mail: orders@humanapr.com; or visit our Website: www.humanapress.com

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1
1-59745-168-1 (e-book)
ISSN 1543-1894

Library of Congress Cataloging in Publication Data
DNA vaccines : methods and protocols / edited by W. Mark Saltzman,
Hong Shen, Janet L. Brandsma. -- 2nd ed.
p. ; cm. -- (Methods in molecular medicine ; 127)
ISBN 1-58829-484-6 (alk. paper)
1. DNA vaccines--Research--Methodology. I. Saltzman, W. Mark.
II. Shen, Hong, 1972- . III. Brandsma, Janet L. IV. Series.
[DNLM: 1. Vaccines, DNA. 2. Research--methods.
W1 ME9616JM v.127 2006 / QW 805 D6295 2006]
QR189.5.D53D63 2006
615'.372--dc22

2006000446

14

Prime-Boost Strategies in DNA Vaccines

C. Jane Dale, Scott Thomson, Robert De Rose, Charani Ranasinghe, C. Jill Medveczky, Joko Pamungkas, David B. Boyle, Ian A. Ramshaw, and Stephen J. Kent

Summary

Induction of HIV-specific T-cell responses by vaccines may facilitate efficient control of HIV replication. Plasmid DNA vaccines and recombinant fowlpox virus (rFPV) vaccines are promising HIV-1 vaccine candidates, although delivering either vaccine alone may be insufficient to induce sufficient T-cell responses. A consecutive immunization strategy, known as "prime-boost," involving priming with DNA and boosting with rFPV vaccines encoding multiple common HIV antigens, is used to induce broad and high-level T-cell immunity and ameliorate AIDS in macaques. This vaccine strategy is proceeding to clinical trials. This chapter describes the use of prime-boost vaccines to induce T-cell responses against HIV-1 and protective immunity against AIDS in macaques. Methods for the construction of the vaccines, the use of animal models, and the detection of immune responses are described.

Key Words: Vaccine; DNA; recombinant fowlpox virus; prime-boost; macaque; HIV; AIDS.

1. Introduction

A major effort of vaccine development has been to induce $CD8^+$ cytolytic T-lymphocytes (CTL) responses and $CD4^+$ T-helper responses because these immune responses are associated with control of many chronic pathogens that readily evade neutralizing antibodies. Since their discovery during the early 1990s, DNA vaccines have shown great promise in inducing both cellular and humoral immunity in small animal models *(1)*. The safety and immunogenicity of injecting naked DNA over conventional vaccine strategies have meant that DNA vaccines have been applied to many diseases for which vaccines are either unavailable or ineffective. However, DNA vaccines alone have induced limited immunogenicity in nonhuman primates and human clinical trials *(2,3)*. Consecutive immunization strategies involving priming by DNA vaccination and boosting with recombinant vectors encoding common antigens have been

From: *Methods in Molecular Medicine, Vol. 127: DNA Vaccines: Methods and Protocols: Second Edition*
Edited by: W. M. Saltzman, H. Shen, J. L. Brandsma © Humana Press Inc., Totowa, NJ

shown by several research groups to generate T-cell immunity against HIV in primates *(3–11)* and, more recently, in humans *(12,13)*.

1.1. The Desired HIV Vaccine

The correlates of protection for HIV have not been defined, although there is evidence to suggest that both antibody- and cell-mediated immune responses are important *(14)*. The induction of broadly reactive neutralizing antibodies to HIV-1 is desirable, but has not been achieved with any viable vaccine to date. HIV-specific T-cell responses may facilitate control of HIV-1 infection because these responses correlate with the control of acute HIV-1 viremia *(15)* and depletion of CD8 T cells results in rises in viremia in simian immunodeficiency virus (SIV) infected macaques *(16)*. The induction of simian/human immunodeficiency virus (SHIV)-specific T-cell responses in macaques also correlates with protective immunity *(17)*.

HIV-specific T-cell immunity is, however, limited to elimination of cells already infected with HIV-1 (i.e., nonsterilizing immunity), rather than preventing infection altogether. Ongoing HIV replication also selects for T-cell escape variants *(18–21)* so HIV-specific T-cell immunity will need to exert maximal control viral replication for durable efficacy. Long-term control of HIV by T-cell-based vaccines is likely to require a high level of T-cell immunity directed toward multiple viral epitopes.

For HIV vaccines to have maximal impact on the AIDS epidemic, the vaccines should be relatively inexpensive and thermostable so that they are readily accessible to people in less developed countries. Both DNA vaccines and poxvirus vectors are relatively stable and can be lyophilized to avoid the expense associated with cold storage. However, prime-boost regimens are complicated by the necessity for multiple injections, and investments in vaccine-delivery infrastructure are likely to be required if these vaccines are found to be efficacious for expanded immunization programs.

1.2. Prime-Boost Vaccines

DNA vaccines typically induce low level immune responses in nonhuman primates and humans. For example, in our studies with pigtail macaques, interferon-gamma (IFNγ) ELISPOT responses to two to three doses of 1 mg DNA vaccines intramuscular (IM) are typically ≤50 spot forming cells/10^6 peripheral blood mononuclear cells (PBMCs) (*see* **Fig. 1**, *wk 9*) *(3–5)*. Although it is not clear, particularly in humans, whether such low-level immune responses induced by DNA may be partially protective, in general, higher levels of immunity are desired and will likely be required to support later stage clinical trial development. Priming with DNA vaccines does however induce T-cell responses with high avidity (able to recognize targets with low levels of presented antigen) *(22)*, which is a desirable characteristic of expanded levels of T-cell immunity.

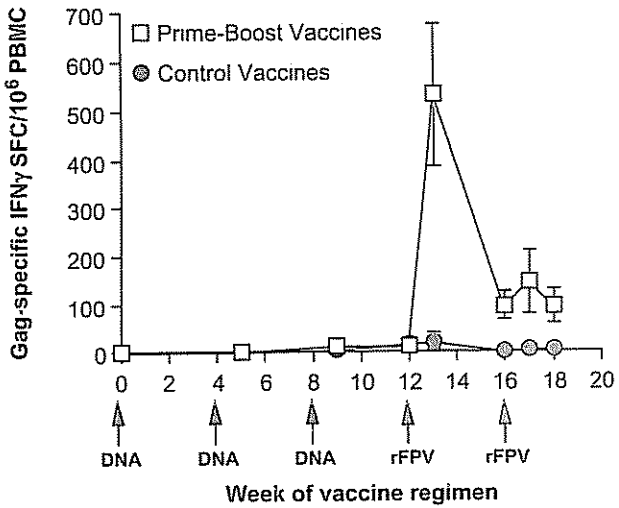

Fig. 1. Prime-boost vaccines induce antigen-specific T-cell responses. Mean (±SE of the mean) T-cell immune responses, measured by IFNγ ELISPOT over the course of the HIV-1 prime-boost vaccine regimen, are shown for immunized macaques compared with control macaques ($n = 7$). T-cell responses are low following immunization with DNA vaccines, and the responses are significantly boosted following immunization with fowlpox virus (rFPV) encoding common HIV antigens. T-cell responses are not enhanced following the second rFPV immunization.

There is a large and almost immediate surge in T-cell immune responses following recombinant fowlpox virus (rFPV) boost of macaques primed with DNA vaccines expressing homologous antigens (*see* **Fig. 1**, *wk 13*). The immediate response suggests that the rFPV vaccine is at least in part expanding preprimed T-cell responses, as this is too early for such a large primary response to vaccination. Where we have reboosted with a second dose of the same rFPV vaccine, we have not demonstrated a similar boost in immunity (*see* **Fig.1**, *wk 16*), suggesting that anti-FPV immunity may limit multiple boosting vaccinations, at least when they are used at short intervals between doses so far evaluated.

To rigorously evaluate the efficacy of HIV-1 vaccines prior to large human efficacy trials, vaccinated Asian macaque species (rhesus, cynomolgus, or pigtail macaques) can be challenged with SIV or chimeric SIV/HIV-1 viruses. Infection of Asian macaques with these viruses results in high levels of viremia, CD4 T-cell depletion, and opportunistic infections that closely mimic human AIDS.

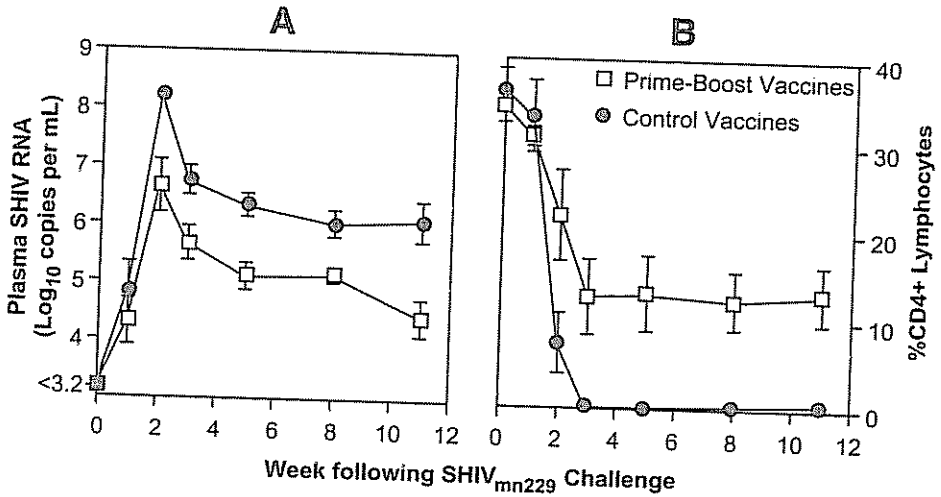

Fig. 2. Macaques immunized with prime-boost simian/human immunodeficiency virus (SHIV) vaccines display some protection against a pathogenic challenge (intrarectal) with $SHIV_{mn229}$. (**A**) Viral load: SHIV RNA copies/mL plasma, detected by quantitative real time polymerase chain reaction *(3)*. The viral load detected in immunized macaques is significantly reduced compared with control macaques at peak (wk 2; $p = 0.004$) and set-point (wk 5–11; $p = 0.002$). (**B**) CD4 T-cell decline following a highly pathogenic challenge. Prime-boost immunized macaques maintain a significant level of CD4 T cells compared with controls ($p = 0.006$; *[3]*).

In a recent study of pigtail macaques utilizing DNA and rFPV expressing shared SIVgag/pol antigens, we were able to demonstrate that, upon challenge with a highly pathogenic chimeric SIV/HIV virus ($SHIV_{mn229}$), partial protection from disease could be achieved *(3)*. There was a blunting of the peak of acute viremia 2 wk after challenge, and a reduction in chronic levels of viremia in comparison to controls (*see* **Fig. 2A**). This translated to significant retention of CD4 T cells (the primary target cell destroyed by these viruses), in the group of six DNA/rFPV vaccinated macaques in comparison with the six control animals immunized with DNA and rFPV vaccines not expressing inserted SIV/HIV-1 genes (*see* **Fig. 2B**).

Additional improvements to prime-boost vaccines can be envisaged—we have attempted to further boost immunity by encoding either IFNγ or IL-12 within the rFPV boosting vector. However, this failed to improve immunity, potentially because these cytokines may limit the persistence of the vector within cells *(4)*. Other investigators have vaccinated with DNA vaccines expressing cytokines that stimulate T-cell immunity and shown that these vaccines effectively stimulate high levels of immunity *(23–25)*. We have also recently studied heterologous prime-boost vaccination regimens utilizing two

live viral vectors (Vaccinia virus and rFPV) in mice, and detected as high (or higher) levels of T-cell immunogenicity as obtained with DNA/rFPV prime boost regimen *(26)*, suggesting this could also be a viable method to stimulate T-cell immunity.

In this chapter, we illustrate the methods used for prime-boost vaccines using HIV/AIDS and the pigtail macaque (*Macaca nemestrina*) model. The chapter will first describe the methods used to construct and evaluate both the DNA and rFPV vaccines based on our recent work *(3,4)*. Immune responses detected in the mouse confirm that the vaccine constructs express antigen in vivo. The vaccines can then be studied in outbred macaques. Regular blood sampling to assay T-cell immune responses indicates the immunogenicity of the vaccine. Methods to detect T-cell responses using the ELISPOT, intracellular cytokine staining (ICS, by FACS analysis) and lymphoproliferation (a functional assay measuring the ex vivo proliferation of T cells in response to antigens) are described. To determine whether the vaccines can protect against contact with the virus, the macaques can be challenged with HIV, SIV, or a chimeric virus SHIV, although methods to challenge the macaque and assay for viremia will not be described in this chapter (*see* **Note 1**).

2. Materials

Studies of prime-boost vaccines in macaques or mice must be carried out under the approval of the Institutional Animal Ethics Committees and within secure laboratories (physical containment level 2 and 3). Knowledge of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (*www.nap.edu/catalog/5140.html*) is also necessary for experiments using mice and macaques.

2.1. Vaccines

2.1.1. Plasmid DNA Construction, Production, and Purification

Standard molecular biology techniques and reagents are described in texts such as Sambrook et al. *(27)*.

1. Special equipment: SmartSpec 3000 Spectrophotometer (Bio-Rad; cat. no. 170-2501); Hybaid Omnigene three block polymerase chain reaction (PCR) machine; Bio-Rad agarose gel apparatus and power pack; ultraviolet (UV) light source; GelDoc system; refrigerated waterbath (Grant; cat. no LDT6G); electroporator (Bio-Rad; cat. no.165-2100); 37°C incubator with shaker.
2. PCR: oligonucleotides as shown in **Table 1** (standard, Invitrogen); Elongase Enzyme Kit (Invitrogen; cat. no. 10480-028); thin-walled PCR tubes (0.2 mL, Perkin Elmer; cat. no. N801-0540).
3. TAE agarose gel electrophoresis: 50X TAE (trisacetate ethylene-diamine tetraacetic acid) (EDTA); NuSieve GTG (Cambrex Bio Science Rockland) or Standard Ultrapure Agarose powder (Invitrogen).

Table 1
Oligonucleotide Sequences Used for Splicing and Construction of DNA Vaccine pHIS-HIV-B

Primer name	Length	Annealing temp.	Nucleotide sequence
NIH1	30mer	60°C	ggcgcggccgcgtggcgcccgaacagggac
NIH2	43mer	56°C	ggcctcgaggaattctcaaggtggtaggttaaaatcactagcc
NIH3	23mer	60°C	ctataaaactctaagagccgagc
NIH4	23mer	60°C	gatgggtcataatacactccatg
NIH5	44mer	58°C	ggctatgtgcccttctttgcccttaacagtctttctttggttcc
NIH6	44mer	60°C	ggaaccaaagaaagactgttaagggcaaagaagggcacatagcc
NIH7	47mer	64°C	ctttcatttggtgtccttcctttccgccctttttcctaggggccctg
NIH8	25mer	64°C	ctttcatttggtgtccttcctttcc
NIH9	25mer	64°C	ggaaaggaaggacaccaaatgaaag
NIH10	51mer	58°C	ctcttattaagttctctgaaatctacctttatggcaaatactggagtattg
NIH11	51mer	58°C	caatactccagtatttgccataaaggtagatttcagagaacttaataagag
NIH12	21mer	58°C	gcccacatccagtactgttac
NIH13	48mer	66°C	gtaacagtactggatgtgggcttttcagttcccttagataaagacttc
NIH14	21mer	58°C	gtaacagtactggatgtgggc
NIH15	55mer	62°C	ctatttctaagtcagatcctacatacaattgatagatgactatgtctggattttg
NIH16	55mer	68°C	caaaatccagacatagtcatctatcaattgtatgtaggatctgacttagaaatag
NIH17a	43mer	50°C	gacacaacaaatcagaagactcagttacaagcaattcatctag
NIH17b	43mer	50°C	ctagatgaattgcttgtaactgagtcttctgatttgttgtgtc
NIH26	45mer	68°C	ggaagatcttgagtgagtgattagacctggaggaggagatatgag
NIH27	23mer	66°C	ctaggtctcgagatactgctccc
NIH28	24mer	70°C	gatacttgggcaggagtggaagcc

4. DNA cloning: New England Biolabs restriction and modifying enzymes; shrimp alkaline phosphatase (Roche Diagnostics GmbH); electroporation DH10B *Escherichia coli* (Invitrogen); electroporation cuvet 0.2-cm gap (Bio-Rad); Luria broth (LB) agar plates with ampicillin (50 µg/mL) or kanamycin (25 µg/mL) added when agar is almost set.
5. Blue-White selection for pBluescript (Stratagene) based plasmids: use 4-µL filter sterilized 100 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) in water for small LB agar plates (or 20 µL for large plates) plus 50 µL 2% X-Gal in *N,N'*,dimethyl formamide for small, or 250 µL for large LB agar plates, spread onto dry plate aseptically with a disposable spreader.
6. PCR bacterial screening: each bacterial colony replica plated onto LB agar and then added directly to 10 µL PCR reaction with appropriate screening primers; reaction incubated initially for 2 min at 94°C before 30 PCR cycles; agarose gel analysis.
7. DNA plasmid amplification and purification: alkaline lysis DNA plasmid minipreps; Maxi or Mega DNA plasmid preps (Qiagen, Germany).
8. ABI Cycle Sequencing (Biomedical Resource Facility, John Curtin School of Medical Research, ANU, Canberra).

9. Storage of clones: 40% glycerol bacterial culture storage at –70°C.

2.1.2. Recombinant rFPV Construction

The materials used for the construction of rFPV expressing multiple HIV-1 antigens have been described in detail elsewhere *(28)*.

2.2. Use of Small Animal Models to Test Vaccine Constructs

1. Special equipment: Class II Biohazard Hood; Pipetboy motorized pipetor; air displacement pipets and associated tips; centrifuge and adaptors to hold 15-mL centrifuge tubes.
2. 1-mL syringes with 27-gage needles attached.
3. Sterile stainless steel sieves or disposable cell strainers (Falcon, cat. no. 352360).
4. Sterile cotton swabs.
5. Sterile 15-mL tubes (e.g., Greiner Bio-One, cat. no. 188271).
6. Complete media: RPMI 1640 (Gibco, cat. no. 21870-076) supplemented with fetal calf serum (FCS) (CSL Australia), 100 m*M* sodium pyruvate (Trace Scientific, Clayton Australia), 200 m*M* L-glutamine (Invitrogen), 14.3 mol/L 2-mercaptoethanol (Sigma), 1 *M* HEPES buffer (Trace Scientific), 5000 IU/mL penicillin and 5 mg/mL streptomycin (Trace Scientific).
7. Tris red blood cell lysis buffer. To make 1 L: add 900 mL, 0.16 *M* NH_4Cl to 100 mL, 0.17 *M* Tris, pH 7.65. Filter-sterilize and store at 4°C. Check pH after filtering as filtering may increase the pH.
8. Petri dishes (Falcon).
9. Sterile 10- and 2-mL pipets (Falcon, cat. nos. 357551 and 357525).

2.3. Intramuscular Delivery of Vaccines to Macaques

1. Sedative (ketamine 10 mg/kg IM).
2. Needles: 21- and 27-gage.
3. Syringes: 1 and 3 mL.

2.4. Blood Collection From Macaques and PBMC Preparation

1. Special equipment: Class II Biohazard Hood, Coulter Counter (Ac.T diff; Beckman Coulter), centrifuge and adaptors to hold 15-mL centrifuge tubes (Allegra X-12R Centrifuge; Beckman Coulter).
2. Sodium heparin (anti-coagulant): Vacuette blood collection tubes (Interpath, cat. no. 455051).
3. Luer 1.5-in. 21-gage needle (Interpath, cat. no. 450076) and barrel.
4. Centrifuge tubes: 1.5-mL screw cap (Interpath, cat. nos. SS2230-00 and SS2001-00), 15 mL (Interpath, cat. no. TPP91015).
5. Transfer pipets (Lomb Scientific, cat. no. 222-205).
6. Ficoll-Paque Plus (Amersham Biosciences, cat. no. 17-1440-03).
7. RPMI: RPMI-1640 media (Gibco, cat. no. 21870-076), supplemented with penicillin, streptomycin, and L-glutamine (Invitrogen, cat. no. 10378-016).

2.5. Detection of Cellular Immune Responses

2.5.1. ELISPOT

1. Special equipment: pipets, including a single channel stepper (Eppendorf Multipette plus with combitips); Class II Biohazard Hood; 37°C 5% CO_2 humidified incubator; centrifuge and adaptors to hold tissue culture plates (Allegra X-12R centrifuge; Beckman Coulter); automated ELISPOTcounter (Autoimmun Diagnostika, AID, GmbH, Strassberg, Germany).
2. HIV-1 antigens used for in vitro stimulation: HIV-1 Gag, Pol, and Env peptide pools (prepared from peptides sets acquired from the NIH AIDS Research and Reference Reagent Program) (*see* **Note 2** for preparation); whole inactivated HIV and control antigen (whole inactivated HIV-1 has proved to be a useful antigen for quantifying T-cell responses in vitro as it is taken up and processed for both class I and II presentation efficiently *(4,28,29)*; available from Rossio et al. *(28)* (NCI-FCRDC).
3. Control stimulation: dimethylsufoxide (DMSO) as a negative control for stimulation (all peptides were dissolved in DMSO); superantigen *Staphylococcus* enterotoxin B (SEB) at 10 ng/mL as a positive control.
4. Monkey IFNγ cytokine ELISPOT kit (U-CyTech, Netherlands), includes: IFNγ capture monoclonal antibody (MAb), MD-1, biotinylated IFNγ detector antibody, pAB, gold-conjugated goat anti-biotin secondary antibody, activators I and II, bovine serum albumin (BSA), and 96-well micro-titer plates.
5. 48-Well tissue culture plates (Edward Kellar, Nunclon).
6. RPMI: RPMI-1640 media supplemented with penicillin, streptomycin, and L-glutamine.
7. FCS (CSL, Australia).
8. RF-5 media: RPMI-1640 as above, supplemented with 5% FCS.
9. Phosphate-buffered saline (PBS): refer to laboratory manual for preparation; e.g., Sambrook et al. *(27)*.
10. PBS-Tween-20: PBS containing 0.05% (v/v) Tween-20.
11. BSA: 1% stock dissolved in PBS.

2.5.2. Intracellular Cytokine Staining (ICS)

1. Special equipment: centrifuge with adaptors to hold various tube sizes, 37°C 5% CO_2 humidified incubator, FACScalibur flow cytometer.
2. 96-Well U-bottom tissue culture trays (Medos, cat. no. 163320).
3. HIV-1 antigens used for in vitro stimulation: peptide sets available from the NIH AIDS Research and Reference Reagent Program (HIV-1 Gag, Pol, Env) as previously mentioned for ELISPOT assay.
4. Control stimulation: DMSO as a negative control for stimulation (as all peptides were dissolved in DMSO); *Staphylococcus* enterotoxin B (at 10 µg/mL as a positive control.
5. Costimulatory antibodies: CD28 (Clone L293, BD Biosciences, cat. no. 340975) and CD49d (Clone L25.3, BD Biosciences; cat. no. 340976). Dilute the purchased stock 1:10 in sterile PBS to make a working stock of 100 µg/mL. Store at 4°C.
6. Brefeldin-A (Sigma, cat. no. B7651) (*see* **Note 3**).
7. Fluorochrome-conjugated MAbs to detect macaque cell surface markers: anti-human CD4-FITC conjugated antibody, anti-human CD3-PE, anti-human CD8-PerCP, anti-human IFNγ-APC (*see* **Note 4**). We also use anti-human CD8-APC as a FACS compensation control for APC. Store antibodies at 4°C in dark.
8. PBS.
9. FACS Lysing Solution (BD Biosciences, cat. no. 349202) and FACS permeabiliszing solution (BD Biosciences, cat. no. 340973). These are supplied as 10X stock reagents.
10. FACS tubes: polystyrene round-bottom 5 mL (BD Biosciences, cat. no. 352008) and caps (BD Biosciences, cat. no. 352032). It is important to use the correct type of tube for your FACS equipment and caps.
11. Formaldehyde (Polysciences Inc., cat. no. 04018). Prepare 5% working stock solution in PBS.

2.5.3. Lymphoproliferation

1. Special equipment: multichannel pipet, 37°C 5% CO_2 humidified incubator, cell harvester (Inotech AG), Top Count NXT microplate scintillation and luminescence counter (Packard).
2. RPMI: RPMI-1640 media supplemented with penicillin, streptomycin, and L-glutamine.
3. Sera: heat-inactivated (56°C) autologous sera (*see* **Note 5**).
4. HIV-1 antigens used for in vitro stimulation: HIV-1 P55 (or P27) Gag and control antigen, whole inactivated HIV and control antigen *(28)* (NCI-FCRDC), and Pokeweed mitogen (as a positive control).
5. Methyl-^{3}H tritiated thymidine (Perkin Elmer, cat. no. NET-221X).
6. Tissue culture plates: 96 well (6-well plates and reagent reservoirs are useful during assay preparation).
7. Glass fiber filters (Packard, cat. no. 6005422).

2.6. Humoral Immunity

1. Commercially available EIA kit to detect HIV-1 antibodies (Murex HIV-1.2.0, Abbott Murex).
2. Western blot against HIV-1.

3. Methods

3.1. Vaccines

3.1.1. DNA Vaccine Construction and Purification

The DNA vaccine construct contained as many of the potential antigens of the virus as possible, without compromising the safety of the vaccine. The HIV-1 genome was modified and inserted into the plasmid DNA vaccine vec-

tor pHIS-64 (H. Davis, Coley Pharmaceuticals) containing kanamycin-selection, a cytomegalovirus (CMV) promoter, the bovine growth hormone polyA signal, and 14 primate-optimized CpG immunostimulatory sequences *(4)* (Purcell et al., manuscript in preparation). DNA vaccine pHIS-HIV-B contained approx 65% of the B subtype pNL(AD8) provirus, with sequences expressing modified Gag, modified RT (reverse transcriptase), protease, Rev, Tat, Vpu, truncated Nef (the first 31 amino acids), and truncated Env (the first 275 amino acids only). HIV-1 genes for Integrase, LTRs, Vif, Vpr, whose function posed a theoretical risk, were deleted.

3.1.1.1. Construction of pHIS-HIV-B

Standard molecular biology techniques were used to construct the DNA vaccines. Refer to texts such as Sambrook et al. *(26)* and product information available with the kits and enzymes.

3.1.1.2. Standard Procedures Used

1. PCR (Elongase mix kit, Invitrogen).
2. PCR SOEing (Elongase mix kit, Invitrogen).
3. TAE agarose gel electrophoresis (NuSieve GTG, Cambrex Bio Science Rockland or Standard Ultrapure, Invitrogen).
4. Restriction endonuclease (New England Biolabs) digestion.
5. Phenol/chloroform purification of digested plasmid DNA.
6. Phosphatasing (shrimp alkaline phosphatase, Roche Diagnostics GmbH).
7. DNA plasmid ligation
8. Electroporation (Bio-Rad Electroporator, Electroporation ready DH10B *E. coli*, Invitrogen).
9. Blue-white selection (initial pBluescript based plasmids only).
10. PCR bacterial screening subsequent plasmids.
11. Alkaline lysis plasmid DNA minipreps.
12. Maxi or mega plasmid DNA purification kit (Qiagen).
13. ABI cycle sequencing (Biomedical Resource Facility, John Curtin School of Medical Research, ANU, Canberra).
14. 40% glycerol bacterial culture stock; store at –70°C.

3.1.1.3. Procedures to Modify B Clade HIV-1 Genome

DNA template fragments were obtained from the NIH AIDS Research and Reference Reagent Program. Specifically, two plasmids containing approximately the 5' half of HIV-1 isolate pNL4-3 and a 3' half of HIV-1 pNL88 (pNL4-3 with AD8 envelop fusion at nucleotides 6343-8047). A PCR splicing strategy was developed in which safety modifications noted above were made

in the 5' half (*see* **Fig. 3A**) and 3' half (*see* **Fig. 3B**). These modification were done in parallel, then the inserts were joined together later in the target pHIS-64 plasmid DNA vector. This last part also included the insertion of a synthetic intron following the CMV promoter and upstream of the modified HIV-1 sequences to improve expression.

3.1.1.4. Modifications to 5' HIV-1 Sequences in pNL43

Primers (**Table 1**) were dissolved in water, the A_{260}nm absorbance was measured and the concentration adjusted to 20 µ*M*. Initially, bases 635–4319 of pNL43 were PCR amplified using primers NIH1 and NIH2 (**Table 1**) to add a *Not*I site at the 5'-end and a stop codon, *Eco*RI, and *Xho*I sites at the 3'-end. This was done using the Invitrogen Elongase Enzyme Kit and 100 ng of the template plasmid in a 50-µL reaction in a Hybaid Omnigene PCR machine for 25 cycles. The resulting fragment was then purified by 0.8% TAE agarose gel electrophoresis. The gel slice with the DNA fragment was centrifuged through Whatman 3MM paper to release the DNA fragment. The DNA was cloned into pBluescript KS (Stratagene) using *Not*I and *Xho*I restriction enzymes and standard plasmid ligation techniques to create pBSFront. Three plasmid clones were fully sequenced to ensure sequence integrity. While this sequencing was being done, PCR splicing starting with the pNL43 template, was used to generate a DNA fragment with all the desired 5'-half deletions. This fragment could then be inserted into the selected pBSFront clone.

3.1.1.5. Splicing Strategy

The splicing strategy was carried out in a stepwise fashion, using multiple rounds of PCR, PCR splicing by Overlap Extension (SOEing), and TAE agarose gel electrophoresis (*see* **Fig. 3A**). The 50-µL PCR reactions used Elongase and 25 cycles (95°C hot start) using the Hybaid PCR machine at the required annealing temperatures (lowest annealing temperature for each primer pair) and extension times (approx 45 s/kb). The resulting spliced fragment was cloned into the selected pBSFront clone using *Hin*dIII and *Age*I at sites 1712 and 3485 bp, respectively (*Hin*dIII removed from pBluescript KS-MCS during construction of pBSFront) and standard cloning techniques to generate pBSFrontΔ. Six clones were then sequenced (often many more clones are required when using this technique owing to the PCR errors introduced by the collective number of PCR cycles). An alternative to the sequential splicing approach followed by cloning is to clone each fragment at each step to reduce the mutation rate, but this can slow construction down. A further point muta-

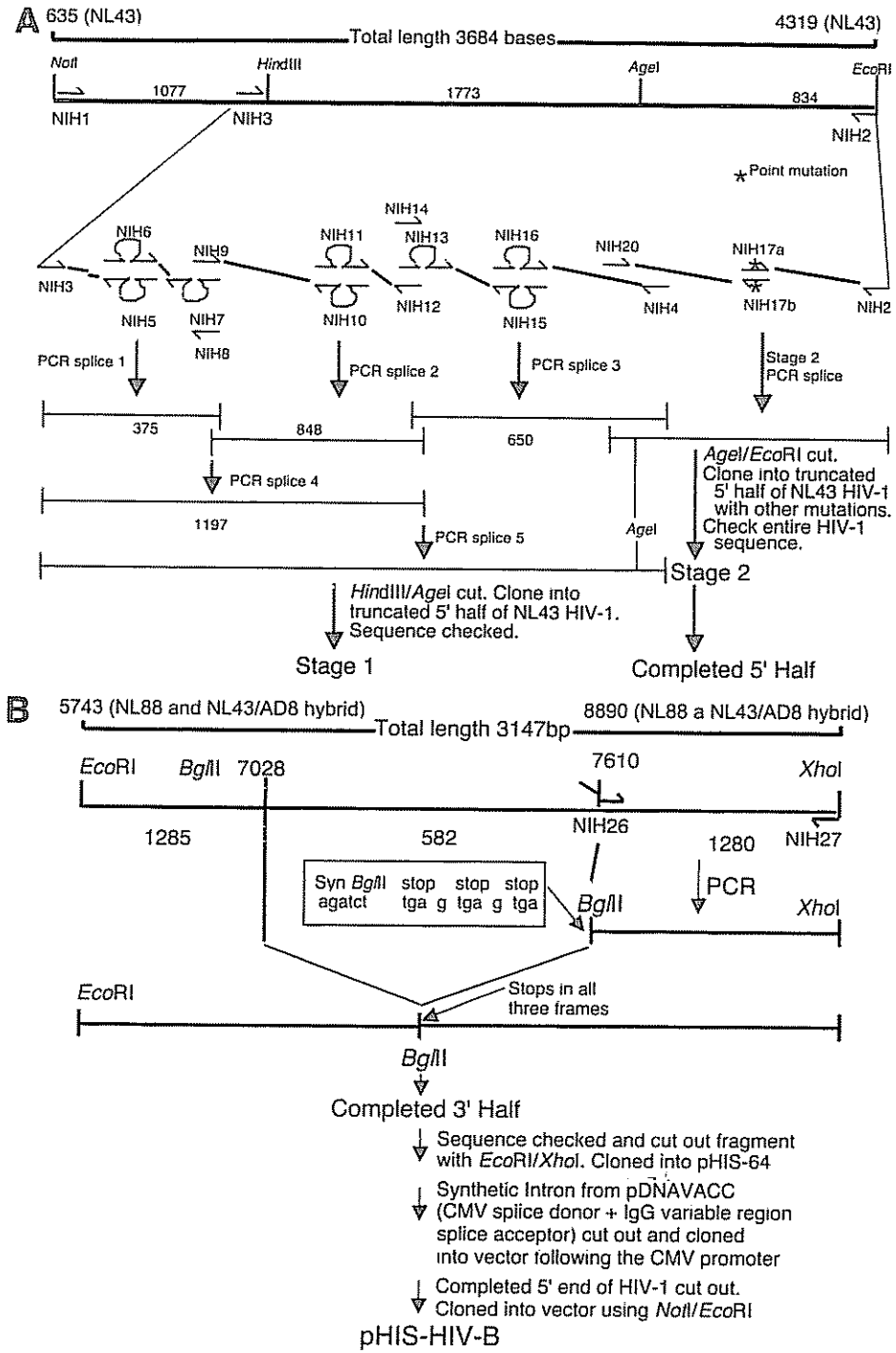

Fig. 3.

tion in the RNAse H domain of the RT protein (Gln 478-Glu478) was introduced into pBSFrontΔ by PCR amplification using primers NIH20/NIH17b and NIH17a/NIH2 followed by SOEing these two fragments together. The fragment containing the RNAse mutation was cloned into a correct pBSFrontΔ using *AgeI*/*Eco*RI and standard cloning techniques. A clone with a correct sequence pBSFrontΔ2 was then used to generate the DNA vaccine plasmid outlined next.

3.1.1.6. Modifications to 3' HIV-1 Sequences in pNL88 (pNL4-3 With AD8 Envelop Fusion 6343-8047)

A 3' HIV-1 fragment with a truncated *env* gene was generated by first PCR amplifying fragment 7610–8889 using primers NIH26/NIH27 to reintroduce a *Bgl*II site at 7610 (absent in the NL88 provirus isolate) using PCR, electrophoresis, and SOEing as previously described. This was also used to insert stop codons in all three frames 3' of the *Bgl*II site (*see* **Fig. 3B**). The spliced fragment was then cloned back into the pNL88 plasmid using *Bgl*II/*Xho*I and standard cloning techniques to join it to the sequences 5742–7028 at the same time generating a large deletion in the HIV-1 *Env* gene. Six clones of pBSBackΔ were sequenced to identify a clone without mutations.

3.1.1.7. Construction of the DNA Vaccine

The modified 3' HIV-1 fragment in pBSBackΔ was excised with *Eco*RI/*Xho*I and cloned into the same sites in pHIS-64 (Coley Ptd Ltd) using standard techniques to generate pHIS-HIV3'. A synthetic intron in the plasmid pDNAVACC *(31)* was excised by cutting with *Nco*I(in CMV promoter)/*Not*I and cloning into the same sites in pHIS-HIV3'. The HIV-1 sequences in pBSFrontΔ2 were excised using *Not*I/*Eco*RI and cloned into pHIS-HIV3' using the same sites to generate the plasmid used for DNA immunization in the following protocols, namely pHIS-HIV-B. The insert was fully sequenced in both directions using ABI Sequencing. The DNA vaccine, pHIS-HIV-B, was manufactured under GMP conditions (Qiagen).

3.1.2. Recombinant Fowlpox Virus Vaccine Construction

Construction of rFPV-HIV-B will be described briefly as it is extensively covered elsewhere *(5,28,32)*. The FPV M3 strain, a tissue culture-passaged

Fig. 3. Construction of DNA vaccine pHIS-HIV-B. (**A**) Diagram showing the splicing and polymerase chain reaction steps involved in constructing the 5' half of the DNA vaccine. (**B**) Diagram showing the steps involved to complete the 3' half. Both the 5' and 3' inserts were then inserted into the target vector to complete the DNA vaccine pHIS-HIV-B.

strain derived from the mild vaccine strain (Fort Dodge Pty Ltd, Sydney, Australia), was used to construct the rFPV-HIV-B. Chicken embryo skin (CES) cells were used for growth and titration of FPVs *(33)*. A rFPV expressing the mutated Gag/Pol from p-HIS-HIV-B was constructed using the insertion vector pAF09 and the parent fowlpox, FPV-M3, using techniques previously described *(28)*. The *gag/pol* sequences were inserted under the control of the FPV P.E/L promoter such that the *gag/pol* is expressed from the initiation of the P.E/L promoter.

Inserted gene sequences were scanned for T_5NT motifs, which are early poxvirus terminator sequences. Any instances were removed by PCR mutagenesis. The selection and generation of the rFPV based on dominant selection using plasmid pKG10 have been previously described *(27)*.

3.2. Small Animal Experiments to Test Vaccines

3.2.1. Prime-Boost Immunization of Mice

BALB/c (h-2d) mice aged 6–10 wk were divided between control and treatment groups ($n = 5$). Mice in the treatment group were primed by injecting plasmid pHIS-HIV-B (50 µg in 100 µL PBS), into the left (50 µL IM) and right (50 µL IM) quadriceps muscle bundles on week 0 and 2, and boosted with the rFPV-Gag/Pol (5×10^6 pfu in 100 µL) vaccine on week 6. Mice in the control group were injected in the same way with plasmid DNA (pHIS) and FPV-M3 not containing any HIV-1 gene inserts.

3.2.2. Sample Collection and Preparation of Lymphocytes

Mice were sacrificed using CO_2, 2–4 wk post-immunization with rFPV-Gag/Pol to measure immune responses. Blood was collected from the tail vein, pre- and post-immunization to measure antibody responses. Plasma was separated by centrifugation and stored at –20°C until assayed by enzyme-linked immunosorbent assay (ELISA) against HIV-1 P24 Gag. Spleen and lymph nodes were removed and single cell suspensions were prepared. The splenocytes were treated with lysis buffer to remove red cells and the nodes were not subjected to this treatment. Cells were counted and T-cell responses were evaluated by ELISPOT and ICS.

1. Preparation of media. Prepare sterile, complete RPMI media on the day prior to harvesting mouse tissue as follows: to one 500-mL bottle of RPMI-1640, add 50 mL of heat-inactivated fetal calf serum, 10 mL sodium pyruvate, 5 mL l-glutamine, 5 mL 2-mercaptoethanol, and 12.5 mL HEPES buffer. Add 5 mL of penicillin/streptomycin immediately prior to use. FCS is heat inactivated by thawing at 37°C and then placing in a 56°C water bath for 1 h. The 2-mercaptoethanol working stock solution is prepared by adding 40 µL of the 14.3 mol/L stock to 100 mL of sterile distilled water (2-mercaptoethanol should only be opened in the fume hood). Store complete RPMI at 4°C.
2. Prepare Tris red blood cell lysis buffer.
3. Label two 15-mL tubes for the treatment and control groups and for each tissue to be removed. Add 5 mL of complete RPMI aseptically to one of the tubes for each of the tissues.
4. Clean and autoclave enough scissors, forceps, and sieves (or use sterile, disposable cell strainers). A fresh set of scissors/forceps per group and a sterile sieve for each set of tissues are required.
5. Sacrifice each mouse just prior to tissue collection using CO_2. Swab the abdomen of the mouse with 70% ethanol. Pinch up the skin on the abdomen and make a small cut in the skin using nonsterile scissors. Holding each side of the cut with your fingers, pull back the skin. Using sterile scissors and forceps, cut open the body cavity to expose the organs. Locate the required tissues (e.g., spleen, caudal/lumbar lymph nodes or mesenteric lymph nodes) and remove using curved forceps. Place each tissue into the labeled 15-mL tube containing 5 mL complete RPMI and place on ice.
6. Dispose of mouse carcases by incineration.
7. Use a class II biosafety cabinet for cell preparation and setting up immune assays.
8. Tip tissue and medium from the 15-mL tube onto a sterile stainless steel sieve (or disposable cell strainer). Gently push the tissue through the sieve with the rubber plunger of a 3-mL syringe to form a cell suspension. The cell suspension is transferred to a sterile 50-mL tube using a transfer pipet.
9. Pellet the cells by centrifugation at 140*g* at 4°C for 5 min.
10. Discard the supernatant into a 2-L beaker containing a small amount of bleach.
11. Wash cells by resuspending the cell pellet in 5 mL of complete RPMI by gently tapping the side of the tube. Do not vortex the sample.
12. Centrifuge at 140*g*, at 4°C for 5 min.
13. For spleen cells, resuspend pellet in 10 mL of Tris red blood cell lysis buffer and leave on ice for 5 min. Add 30 mL of complete RPMI to dilute lysis buffer before centrifugation at 140*g* at 4°C for 5 min.
14. Spleen and lymph node cell preparations should be washed twice by resuspending in complete RPMI and centrifugation, as previously mentioned.
15. Resuspend the pellet in 5–10 mL of complete RPMI for spleens and 1–2 mL for lymph nodes. Perform a viable leukocyte count. Place cells on ice until required.
16. Set up T-cell assays (ELISPOT, ICS) using similar protocols as described next.

3.3. Delivery of Vaccines Into Macaques.

Macaques are randomized into vaccine groups: control and treatment. Randomization is based on weight and sex of the animal. The number of macaques per group required in order to power the study to detect significant differences

between the control and the treatment groups will vary depending on the primary endpoint of the study.

Seven macaques were randomly allocated to the treatment and control groups to study the HIV-1 clade B prime-boost vaccines. Macaques were sedated by intramuscular injection of Ketamine (0.1 mL/kg body weight). Blood samples were collected regularly, and routine physical examinations and weight checks were performed each time the animals were sedated to ensure macaques were healthy and gaining weight. Vaccines formulated in 1–2 mL were injected intramuscularly into the quadriceps muscle bundles using 26-gage needles. A typical prime-boost vaccine regimen is shown in **Table 2**.

3.4. Blood Collection From Macaques and PBMC Preparation

Baseline blood samples are collected from the femoral vein of the macaque prior to injecting vaccines, and regularly following each immunization to detect immune responses as indicated in **Table 2**. Blood is collected into tubes (typically Vacuettes) containing the anti-coagulant sodium-heparin, compatible with the in vitro stimulation assays to detect cellular immune responses.

3.4.1. PBMC Preparation

1. Collect blood into sodium-heparin Vacuette tubes.
2. Centrifuge samples at room temperature at 900*g*, for 7 min.
3. Remove plasma to labeled 1.5-mL screw cap centrifuge tubes.
4. Store plasma in aliquots at –70°C for use in assays to detect antibodies.
5. Replace plasma by adding RPMI to the packed cells (i.e., add 5–6 mL RPMI to dilute remaining blood in Vacuette tube). Mix well using a transfer pipet. The total volume of the blood and RPMI mix should be approx 10 mL.
6. Layer blood/RPMI mix over 5 mL 95% Ficoll-Paque Plus (*see* **Note 6**) in a 15-mL centrifuge tube. Centrifuge at 1000*g* at room temperature for 25 min. Ensure centrifugation brakes are turned off to avoid any disturbance at the PBMC interface.
7. Transfer the band of mononuclear cells (white cell layer in the middle of the gradient) to 10 mL RPMI. Mix and centrifuge at 500*g* at 4°C for 7 min.
8. Remove supernatant by tipping off RPMI.
9. Wash PBMC a second time using a transfer pipet filled with chilled (4°C) RPMI to resuspend cell pellet. Top up tube to 14 mL with RPMI. Use of chilled RPMI will help prevent the cells from adhering to the transfer pipets and centrifuge tubes.
10. Pellet PBMC as before: centrifuge at 500*g* at 4°C for 7 min. Discard supernatant.
11. Resuspend cell pellet in 1 mL RPMI and count cells using a Coulter Counter or haemocytometer. Keeping PBMC on ice will help prevent the cells from adhering to the walls of the centrifuge tube.

Table 2
A Typical HIV-1 Prime-Boost Immunization Regimen in Macaques

Wk	-2	0	4	5	8	9	12	13	14	16	17	18	20	24
Physical examination and weight check	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Collect blood sample	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
DNA vaccine (IM)		✓	✓		✓									
Dose: 1 mg in 1 mL														
rFPV vaccine (IM)							✓			✓				
Dose: 5×10^7 pfu in 1 mL														
Assay for cellular immunity	✓	✓		✓		✓	✓	✓		✓	✓	✓		
Assay for humoral immunity		✓					✓	✓		✓	✓	✓		

3.5. Cellular Immunity

Cellular immune responses stimulated by the vaccine regimen are detected from individual macaques using a series of assays. Macaques are outbred animals, and, hence, PBMC samples cannot be pooled for assays. Multiple assays using various antigens help confirm results obtained from each assay.

3.5.1. ELISPOT

The ELISPOT assay is a highly sensitive assay used to determine frequencies of functional antigen-specific activated or memory T cells ex vivo. Both CD4 and CD8 T cells are measured by this assay, which has become the "gold standard" with which to measure T-cell immunity in HIV vaccine trials (*34*,*35*). For our HIV vaccine studies in macaques, we routinely use the monkey IFNγ ELISPOT kit (U-CyTech Bv, Utrecht, Netherlands) as a marker for cellular immune responses. We perform the assay using two culture steps to improve the signal:noise ratio (spot intensity:background). That is, the cells (PBMC) are incubated with antigen (or controls) for 18 h in a tissue culture plate. The nonadherent cells are then transferred to the ELISPOT plate, precoated with an anti-IFNγ MAb, and restimulated with antigen (or controls) for 5 h. The cells are removed, and IFNγ secretion is detected using an anti-IFNγ polyclonal antibody, a gold-labeled anti-biotin antibody, and an "activator" that deposits silver to form spots. All reagents (except PBS) and protocols are supplied in the kit.

3.5.1.1. Coat ELISPOT Plates With Capture Antibody

1. Prepare 96-well plate (supplied with kit) with capture antibody, MD-1 (1:100) in PBS.
2. Add 50 µL to each well of the ELISPOT plate.
3. Vortex the plate briefly on the microtiter plate shaker (or tap the sides) in order to

spread the capture antibody solution over the entire base of the well. Check that the entire surface of each well is covered by antibody.
4. Wrap plate in foil and incubate at 4°C overnight.
5. Remove capture MAb from the wells (flick out contents).
6. Wash wells five times with 200 µL sterile-filtered PBST.
7. Block wells with 200 µL of 1% BSA/PBS. Incubate 1 h or longer at 37°C.
8. Remove the blocking agent (pat dry) immediately prior to the addition of cells (no washes).

3.5.1.2. Prepare Macaque PBMC and Set Up ELISPOT Assay on the Day of Blood Sample Collection.

1. For stimulation of PBMC, prepare each antigen as a 2X concentration (antigens can be prepared earlier in the day and stored on ice until required). Peptide pools (HIV Gag, Pol, Env, and others): prepare 2 µg/mL in RF-5, to give a final concentration of 1 µg/mL in the assay. Whole inactivated HIV-1: prepare 10 µg/mL in RF-5, to give a final concentration of 5 µg/mL. SEB (super antigen, positive control): prepare 20 ng/mL in RF-5, to give a final concentration of 10 ng/mL. Prepare equivalent amounts of negative control antigens.
2. Add 150 µL of each antigen preparation to wells of a 48-well tissue culture plate just prior to the addition of PBMC.
3. Prepare PBMC for each macaque: 4×10^5 PBMC is required for stimulation of each macaque. Make up PBMC in RF-5. Add 150 L PBMC/RF-5 to wells of a 48-well tissue culture plate containing antigen or controls.
4. Incubate plate at 37°C in a humidified incubator (5% CO_2) for 18 h.

3.5.1.3. Prepare the PBMC for Restimulation in ELISPOT Plates Containing Capture Antibody

1. Using a stepper-pipet, add 1 mL of prewarmed (37°C) RF-5 to each well of the 48-well tissue culture plate
2. Pellet cells at 200*g* for 10 min at room temperature (use low brake).
3. Carefully discard 1 mL supernatant.
4. Add 1 mL prewarmed RF-5 to wash cells a second time, as previously mentioned.
5. Pellet cells at 200*g* for 10 min at room temperature (use low brake). Discard 1 mL supernatant.
6. Each well should now contain approx 200 µL RF-5, containing 4×10^5 PBMC.
7. Restore the initial concentration of stimulating antigen: keep antigen volume to a minimum (e.g., use 5 µL from a 40X concentrated stock), dilute in RF5 and add to washed PBMC in the 48-well plate.
8. Use a pipet set at 100 µL to mix the cells with the antigen in the 48-well plate, and transfer 100 µL cell suspension from each well equally into duplicate wells of the prepared 96-well ELISpot plate. Each well now contains 100 µL with 2×10^5 PBMC.

9. Incubate the plates for 5 h at 37°C, 5% CO_2 humidified incubator. *Note:* in order to achieve sharp spot formation, avoid excessive vibration the plate during incubation.
10. Remove plate to 4°C following 5 h incubation. Leave overnight, if desired.
11. Discard cell suspension from the wells and lyse remaining cells by adding 200 µL ice-cold deionized water to each well.
12. Incubate plate on wet ice for 10 min.
13. Wash wells 10 times with PBST and pat dry.

3.5.1.4. Detection of Spot Formation,

1. Prepare biotinylated detector antibody solution, pAB (1:100) in 1% BSA/PBS. Add 100 µL to each well. Incubate 1 h at 37°C or overnight at 4°C.
2. Discard biotinylated detector antibody solution from wells.
3. Wash wells five times with PBST and pat dry.
4. Prepare gold-conjugated anti-biotin antibody (GABA, 1:50) in 1% BSA/PBS. Add 50 µL to each well. Incubate 1.5 h at 37°C or overnight at 4°C.
5. Discard antibody solution from wells, and wash wells five times with PBST. After the final wash, ensure that all wash buffer has been removed before adding the substrates (Act I and Act II) because excess wash buffer will interfere with the development of spots.
6. Sites of cytokine deposition are revealed with the addition of the activator mix (U-Cytech). Mix equal volumes of substrates Act I and Act II. Add 30 µL to each well. *Note:* Thaw the activators in a cold-water bath and keep them on ice until they are required. Mix them together just before they are needed, otherwise they will not work optimally. The mitogen/super antigen controls will develop a lot faster than the antigen- and background-stimulated wells, so add the activators to these wells last.
7. Incubate at room temperature for 30 min in the dark.
8. Commence observation of spot formation after 25 min.
9. When the spots are intense and the background is just beginning to develop (fine dots become visible in clear areas), stop the reaction by rinsing several times with tap water. Pat-dry plate on paper towel.
10. Allow plates to air-dry before using AID ELISPOT counter to acquire data according to manufacturer's instructions.
11. Data are expressed as spot forming cells/10^6 PBMC (i.e., multiply the mean number of spots in duplicate wells by 5) after subtraction of nonspecific spots in the negative-control wells.

3.5.2. Intracellular Cytokine Staining

The intracellular cytokine staining (ICS) assay is a sensitive assay for the detection of cytokines expressed in response to activation of cells with antigen stimulation. The advantage of ICS is that the cells expressing the cytokine can be phenotyped using cell markers, thus providing further information about the relative proportions of T-cell immune responses detected (e.g., CD4 or CD8 T-

cell responses). ICS and flow cytometry can be used to measure T-cell immune responses to defined antigens using whole blood or PBMC. The assay we describe is based on the method by Maecker et al. (*36,37*). Whole blood is incubated with HIV-1 antigens and antibodies to costimulatory molecules CD28 and CD49d. The addition of a secretion inhibitor (Brefeldin A) allows cytokines to accumulate within the activated cells for a defined length of time. Following activation of cells, the cells are first stained for surface cell markers and then fixed and permeabilized before staining for cytokines (such as IFNγ). Cells are enumerated and phenotyped by flow cytometry. Pools of overlapping 15mer peptides, spanning the entire predicted protein sequence, provide an excellent source of antigen with which to stimulate CD8 and CD4 T-cell responses in vitro. Reducing the number of peptides within the pool helps define immunodominant epitopes within an antigen.

3.5.2.1. ICS Method

1. Collect 1–2 mL blood into sodium-heparin Vacuette tubes.
2. Label a 96-well tissue-culture tray with animal identification numbers and each antigen. Include five wells for fluorochrome/FACS compensation use.
3. Aliquot 200 µL blood per well.
4. Prepare antigens and add to each well: peptides at 1 µg/mL, DMSO (negative control, use same volume as used for the peptides) and SEB at 10 µg/mL (positive control). It is a good practice to prepare each antigen in sterile PBS for the total number of animals to be studied and aliquot 10 µL/well.
5. Add costimulatory antibodies: prepare costimulatory antibodies to CD28 and CD49d in sterile PBS and add at a final concentration of 1 µg/mL in 200 µL blood per sample tested (i.e., from a 1 mg/mL stock: 0.2 µL of CD28, 0.2 µL of CD49 in 9.6 µL sterile PBS to aliquot 10 µL/well).
6. Using a multichannel pipet (set > 100 µL), mix antigen and antibody with blood samples.
7. FACS compensation wells: aliquot 200 µL blood (from one animal) for each of the fluorochromes used in the assay (i.e., no stain, FITC, PE, PerCp, and APC). It is not necessary to add costimulatory antibodies or antigen to the compensation wells.
8. Incubate plate for 2 h at 37°C in 5% CO_2.
9. Thaw Brefeldin A and add 10 µg/mL to each well in a 10 µL aliquot in sterile PBS. Mix thoroughly using a multichannel pipet (*see* **Subheading 3.4.2.1., step 6**).
10. Incubate plate for an additional 5 h at 37°C in 5% CO_2.
11. Remove plate from incubator and place at 4°C overnight. Incubation at 4°C postactivation allows the assay to be done over 2 d and does not appear to affect the assay.

3.5.2.2. Staining of Cell Surface Markers

1. Compensation controls:

   a. No stain: antibodies are not added.
   b. FITC: add 5 µL CD4 FITC.
   c. PE: add 5 µL CD3-PE.
   d. PerCp: add 8 µL CD8-PerCp.
   e. APC: add 8 µL CD8-APC.
2. Prepare a mix of antibodies to cell surface markers. We routinely use an antibody combination of 5 µL CD4-FITC, 5 µL CD3-PE, and 8 µL CD8-PerCp/sample. Aliquot 18 µL of the antibody mix to each well (excluding the compensation control wells). Mix well using a multichannel pipet (*see* **Subheading 3.4.2.1., step 6**).
3. Incubate antibodies with blood at room temperature for 30 min. Cover plate with foil to exclude light.
4. Label a FACS tube for each sample.
5. Lyse red blood cells: dilute stock FACS lysis buffer 1:10 in distilled H_2O. Add 2 mL FACS lysis buffer to each of the labeled FACS tubes (equivalent to 10 vol of lysis buffer).
6. Using a pipet, remove blood from the 96-well culture tray and add to FACS tubes containing lysis buffer. Mix well. Incubate at room temperature for 10 min in the dark.
7. Add 2 mL PBS. Cap tubes tightly and invert to mix. Centrifuge samples at 600*g*, for 7 min, at room temperature.
8. Decant supernatant. The lysis buffer can cause the cells to become buoyant, therefore, it is important to decant carefully.
9. Permeabilize cells for intracellular cytokine staining: dilute stock of Permeabilization buffer 1:10 in distilled H_2O. Add 0.5 mL/tube. Vortex gently and incubate at room temperature for 10 min. Exclude light.
10. Add 3.5 mL PBS to each tube. Cap tubes tightly and invert to mix. Centrifuge samples at 600*g*, for 7 min at room temperature.
11. Decant supernatant as before.

3.5.2.3. Stain for IFNγ

1. The amount of IFN-APC antibody to use per sample requires optimization (0.7 L IFN-APC per sample gives consistent results in our assay). Prepare a mix: no. samples (0.7 µL IFN-APC + 9.3 µL PBS). Add 10 µL to each sample and vortex gently. Incubate at room temperature for 40–60 min, vortexing every 20 min.
2. Add 4 mL PBS to tubes. Cap tubes tightly and invert to mix. Centrifuge samples at 600*g* for 7 min at room temperature.
3. Decant supernatant as before.
4. Fix cells: add 50 µL 5% formaldehyde to each tube. Vortex briefly to mix.
5. Analyze samples within 24 h of staining.
6. Perform FACS analysis according to protocols supplied with equipment. Use compensation controls to ensure good separation between each of the fluorochromes detected.

3.5.3. Lymphoproliferation

Lymphoproliferative responses can be assessed by standard ^{3}H-thymidine incorporation assays to detect T cells undergoing ex vivo cell division in response to antigen stimulation. This 7-d assay generally measures CD4 T-cell proliferation. PBMC isolated from individual animals are stimulated in vitro with relevant antigens and their controls. Activated PBMC divide following in vitro antigen stimulation and indicate a response to the vaccine. Cell proliferation is measured against background stimulation by adding radio-labeled (methyl-^{3}H) thymidine, which will be incorporated into the DNA of the dividing cells. The cells are harvested onto glass fiber filters and thymidine uptake is measured by counting. Results are represented as a stimulation index: mean thymidine uptake of triplicate wells stimulated with antigen over the mean response to the control antigens.

3.5.3.1. Lymphoproliferation Method

1. Label a 96-well tissue plate. Include information about the animal identification and antigen to be assessed. Set up each test in triplicate.
2. Prepare the antigens for stimulation at twice the desired concentration in RPMI-1640 media: whole inactivated HIV at 20 µg/mL, control antigen at 20 µg/mL, HIV-1 P55 (or P27) Gag and control at 20 µg/mL, pokeweed mitogen at 2 µg/mL (antigens should be titrated before use to check for optimum concentration).
3. Using a multichannel pipet, add 100 µL antigen/RPMI to respective wells.
4. Prepare a suspension of PBMC in RPMI-1640 media and heat inactivated autologous sera (*see* **Note 5**). Calculate number of PBMC required for 2×10^5 PBMC/well. Add PBMC and 10% (final concentration is 5%) heat-inactivated autologous serum to the RPMI and aliquot 100 µL/well.
5. Incubate PBMC with antigen at 37°C, 5% CO_2 in a humidified incubator for 7 d.
6. Pulse wells with ^{3}H-tritiated thymidine 18 h prior to harvest. Prepare a mix of ^{3}H thymidine and RPMI for 1 Ci 3H thymidine in 20 µL RPMI per well. Aliquot using a multichannel pipet. Incubate plate at 37°C, at 5% CO_2 in a humidified incubator for 18 h.
7. To lyse macaque PBMC and virus, add 0.05% NP40 detergent per well. Prepare a mix of NP40 and RPMI and aliquot 20 µL/well with a multichannel pipet.
8. Harvest cells onto glass fiber filters using the cell harvester as per operator instructions.
9. Dry filter and count using a β counter.
10. Analyze data: use a spreadsheet (e.g., Microsoft Excel) to take the mean of the triplicate wells. The stimulation index is calculated as the mean proliferative response to the antigen over the mean response to control antigens.

3.6. Humoral Immunity

3.6.1. EIA and Western Blot

There are research facilities that can provide a service for the detection of antibodies to HIV-1 and HIV-2. We are grateful to Dr. Kim Wilson and colleagues at the National Serological Reference Laboratory (NRL, St Vincent's Hospital, Melbourne Australia) for performing all antibody tests and for their help with our studies. Antibodies to HIV-1 were detected using a competitive enzyme immunoassay (EIA; Wellcozyme HIV Recombinant, UK), and to specific HIV-1 antigens by Western blot using 200µg standardized HIV1 viral lysate as described *(38)*.

4. Notes

1. In this chapter we describe the use of vaccines against HIV-1 B clade as a model for prime-boost vaccines. Macaques in this study were not challenged as we (and others) have shown previously that HIV-1 infects macaques poorly *(4,5)*. There has been much discussion in the literature about the appropriate choice of virus with which to challenge macaques and the route of inoculation *(38)*. The amplification and in vitro titration of a challenge stock of HIV-1_{LAI} or $SHIV_{mn229}$ has been described *(4,37)*.
2. Preparation of pools of peptides for use in immunological assays. Use of 15mer overlapping peptides (e.g., 11 amino acid overlap) to include all linear epitopes of HIV proteins has provided a helpful resource for in vitro detection of immune responses *(35)*. Individual peptides are supplied lyophilized and dissolved in 100% DMSO, molecular biology grade at a high concentration before combining into a single pool. To each vial containing 1 mg peptide add 10 µL 100% DMSO. Vortex the vial to mix the peptide and DMSO. Observe whether the peptide has dissolved (i.e., that there is no evidence of precipitate in the vial). Use a centrifuge to collect the vial contents. Use a pipet to transfer dissolved peptide to a prelabeled screw-cap, 1.5-mL microfuge tube. Keep the tube containing the pooled peptides on ice. Most peptides will dissolve in 10 µL of DMSO. If peptides do not dissolve, increase the volume to 20 µL DMSO. Peptides that fail to dissolve can be incubated in a 37°C water bath for 2 h. Continue to add DMSO and heat the peptides (max. vol. 50 µL DMSO) until peptide has dissolved. In rare cases it may be necessary to heat the vial to 55°C. After transferring the dissolved peptide to the pool, centrifuge the individual vials to collect any residual peptide. The second centrifugation step often recovers an additional 2 µL peptide. Carefully record the volume used to dissolve each of the peptides to calculate the final peptide pool concentration. DMSO is toxic to cells at concentrations greater than 0.5% of the culture. Store peptide pools at –70°C in small aliquots. Avoid repeated freeze/thawing of aliquots.
3. Preparation of Brefeldin-A stock solution: dissolve 5 mg in 1 mL DMSO. Store at or below –20°C in small aliquots (20 µL). Discard the aliquot once it has been

thawed for use during an ICS experiment; i.e., minimize freeze/thaw cycles to maintain activity of Brefeldin-A.

4. Several antibodies raised against human cell surface markers cross-react with nonhuman primate species, including *M. nemestrina*. The BD Biosciences catalog lists known species with which their antibodies cross-react. Antibodies will vary from batch to batch and the volume to be used should be optimized for use in whole blood. Set up a simple experiment titrating volumes of each antibody from 1 µL to the BD Biosciences suggested volume of 20 µL. The volumes we have given in the protocol give consistent results in our hands.
5. Use of FCS in proliferation assays using *M. nemestrina* PBMC results in high background proliferation. Hence, it is necessary to collect autologous sera from macaques prior to commencing the trial. Sera samples are stored in 0.5 to 1-mL aliquots at –20°C. Aliquots are heat inactivated at 56°C for 30 min. Sera should be cooled to room temperature before use in the proliferation assay.
6. Use of 95% Ficoll-Paque Plus. We have found that 95% Ficoll-Paque Plus (Amersham-Pharmacia) gives the optimum separation of *M. nemestrina* PBMC. Ficoll-Paque Plus is diluted to 95% by adding sterile (autoclaved), deionized-distilled water.

References

1. Ulmer, J. B., Donnelly, J. J., Parker, S. E., et al. (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259,** 1745–1749.
2. Liu, M. A. (2003) DNA vaccines: a review. *J. Intern Med.* **253,** 402–410.
3. Dale, C. J., De Rose, R., Stratov, I., et al. (2004) Efficacy of DNA and fowlpoxvirus priming/boosting vaccines for simian-human immunodeficiency virus. *J. Virol.* **78,** 13,819–13,828.
4. Dale, C. J., De Rose, R., Wilson, K. M., et al. (2004) Evaluation in macaques of HIV-1 DNA vaccines containing primate CpG motifs and fowlpoxvirus vaccines co-expressing IFNγ or IL-12. *Vaccine* **23,** 188–197.
5. Kent, S. J., Zhao, A., Best, S. J., Chandler, J. D., Boyle, D. B., and Ramshaw, I. A. (1998) 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.* **72,** 10,180–10,188.
6. Leong, K. H., Ramsay, A. J., Morin, M. J., Robinson, H. L., Boyle, D. B., and Ramshaw, I. A. (1995) Generation of enhanced immune responses by consecutive immunisation with DNA and recombinant fowlpox viruses. *Vaccines* **95,** 327–331.
7. Robinson, H. L., Montefiori, D. C., Johnson, R. P., et al. (1999) Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant poxvirus booster immunizations. *Nat. Med.* **5,** 526–534.
8. Amara, R. R., Villinger, F., Altman, J. D., et al. (2001) Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292,** 69–74.
9. Allen, T. M., Vogel, T. U., Fuller, D. H., et al. (2000) Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *J. Immunol.* **164,** 4968–4978.
10. Wee, E. G., Patel, S., McMichael, A. J., and Hanke, T. (2002) A DNA/MVA-based candidate human immunodeficiency virus vaccine for Kenya induces multi-specific T cell responses in rhesus macaques. *J. Gen. Virol.* **83,** 75–80.
11. Shiver, J. W., Fu, T. M., Chen, L., et al. (2002) Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415,** 331–335.
12. McConkey, S. J., Reece, W. H., Moorthy, V. S., et al. (2003) Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat. Med.* **9,** 729–735.
13. Mwau, M., Cebere, I., Sutton, J., et al. (2004) A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. *J. Gen. Virol.* **85,** 911–919.
14. Esparza, J. and Osmanov, S. (2003) HIV vaccines: a global perspective. *Curr. Mol. Med.* **3,** 183–193.
15. Koup, R. A., Safrit, J. T., Cao, Y., et al. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68,** 4650–4655.
16. Schmitz, J. E., Kuroda, M. J., Santra, S., et al. (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283,** 857–860.
17. Barouch, D. H., Santra, S., Schmitz, J. E., et al. (2000) Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290,** 486–492.
18. Goulder, P. J., Brander, C., Tang, Y., et al. (2001) Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature* **412,** 334–338.
19. O'Connor, D. H., Allen, T. M., Vogel, T. U., et al. (2002) Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. *Nat. Med.* **8,** 493–499.
20. O'Connor, D. H., Mothe, B. R., Weinfurter, J. T., et al. (2003) Major histocompatibility complex class I alleles associated with slow simian immunodeficiency virus disease progression bind epitopes recognized by dominant acute-phase cytotoxic-T-lymphocyte responses. *J. Virol.* **77,** 9029–9040.
21. Oxenius, A., Price, D. A., Trkola, A., et al. (2004) Loss of viral control in early HIV-1 infection is temporally associated with sequential escape from CD8+ T cell responses and decrease in HIV-1-specific CD4+ and CD8+ T cell frequencies. *J. Infect. Dis.* **190,** 713–721.
22. Estcourt, M. J., Ramsay, A. J., Brooks, A., Thomson, S. A., Medveckzy, C. J., and

Ramshaw, I. A. (2002) Prime-boost immunization generates a high frequency, high-avidity CD8(+) cytotoxic T lymphocyte population. *Int. Immunol.* **14,** 31–37.
23. Kim, J. J., Yang, J. S., Montaner, L., Lee, D. J., Chalian, A. A., and Weiner, D. B. (2000) Coimmunization with IFN-gamma or IL-2, but not IL-13 or IL-4 cDNA can enhance Th1-type DNA vaccine-induced immune responses in vivo. *J. Interferon Cytokine Res.* **20,** 311–319.
24. Boyer, J. D., Chattergoon, M., Muthumani, K., et al. (2002) Next generation DNA vaccines for HIV-1. *J. Liposome Res.* **12,** 137–142.
25. Bertley, F. M., Kozlowski, P. A., Wang, S. W., et al. (2004) Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. *J. Immunol.* **172,** 3745–3757.
26. Ranasinghe, C., Medveczky, C. J., Woltring, D. et al. (2006) Evaluation of fowlpox-vaccinia virus prime-boost vaccine strategies for high-level mucosal and systemic immunity against HIV-1 *Vaccine* in press.
27. Sambrook, J. E., Fritsch, F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
28. Boyle, D. B., Anderson, M., Amos, R., Voysey, R., and Coupar, B. E. H. (2004) Construction of recombinant fowlpox viruses carrying multiple vaccine antigens and immunomodulatory molecules. *BioTechniques.* **37,** 104–111.
29. Rossio, J. L., Esser, M. T., Suryanarayana, K., et al. (1998) Inactivation of HIV-1 infectivity with preservation of conformational and functional integrity of virion surface proteins. *J. Virol.* **72,** 7992–8001.
30. Kent, S. J., Dale, C. J., Preiss, S., Mills, J., Campagna, D., and Purcell, D. F. J. (2001) Vaccination with attenuated simian immunodeficiency virus by DNA inoculation. *J. Virol.* **75,** 11,930–11,934.
31. Thomson, S. A., Sherritt, M. A., Medveczky, J., et al. (1998) Delivery of multiple CD8 cytotoxic T cell epitopes by DNA vaccination. *J. Immunol.* **160,** 1717–1723.
32. Coupar, B. E., Teo, T., and Boyle, D. B. (1990) Restriction endonuclease mapping of the fowlpox virus genome. *Virology* **179,** 159–167.
33. Silim, A., El Azhary, M. A., and Roy, R. S. (1982) A simple technique for preparation of chicken-embryo-skin cell cultures. *Avian Dis.* **26,** 182–185.
34. Russell, N. D., Hudgens, M. G., Ha, R., Havenar-Daughton, C., and McElrath, M. J. (2003) Moving to human immunodeficiency virus type 1 vaccine efficacy trials: defining T cell responses as potential correlates of immunity. *J. Infect. Dis.* **187,** 226–242.
35. Hudgens, M. G., Self, S. G., Chiu, Y. L., Russell, N. D., Horton, H., and McElrath, M. J. (2004) Statistical considerations for the design and analysis of the ELISpot assay in HIV-1 vaccine trials. *J. Immunol. Methods* **288,** 19–34.
36. Maecker, H. T., Dunn, H. S., Suni, M. A., et al. (2001) Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J. Immunol Methods* **255,** 27–40.
37. Dale, C. J., Liu, X. S., De Rose, R., et al. (2002) Chimeric human papilloma virus-simian/human immunodeficiency virus virus-like-particle vaccines: immunogenicity and protective efficacy in macaques. *Virology* **301,** 176–187.

38. Kent, S. J., Woodward, A., and Zhao, A. (1997) Human immunodeficiency virus type 1 (HIV-1)-specific T cell responses correlate with control of acute HIV-1 infection in macaques. *J. Infect. Dis.* **176,** 1188–1197.
39. Nishimura, Y., Igarashi, T., Donau, O. K., et al. (2004) Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses. *Proc. Natl. Acad. Sci. USA* **101,** 12,324–12,329.