J Med Primatol 2005: 34: 282–293

JOURNAL OF MEDICAL PRIMATOLOGY

The pigtail macaque MHC class I allele *Mane-A*10* presents an immundominant SIV Gag epitope: identification, tetramer development and implications of immune escape and reversion

Smith MZ, Fernandez CS, Chung A, Dale CJ, De Rose R, Lin J, Brooks AG, Krebs KC, Watkins DI, O'Connor DH, Davenport MP, Kent SJ. The pigtail macaque MHC class I allele *Mane-A*10* presents an immundominant SIV Gag epitope: identification, tetramer development and implications of immune escape and reversion. J Med Primatol 2005; 34:282–293. © Blackwell Munksgaard, 2005

Miranda Z. Smith[1], Caroline S. Fernandez[1], Amy Chung[1], C. Jane Dale[1], Robert De Rose[1], Jie Lin[1], Andrew G. Brooks[1], Kendall C. Krebs[2], David I. Watkins[2], David H. O'Connor[2], Miles P. Davenport[3], Stephen J. Kent[1]

[1]Department of Microbiology and Immunology, University of Melbourne, Parkville, Vic., Australia, [2]Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI, USA, [3]Department of Haematology, Prince of Wales Hospital, and Centre for Vascular Research, University of NSW, Kensington, NSW, Australia

Abstract: The pigtail macaque (*Macaca nemestrina*) is a common model for the study of AIDS. The pigtail major histocompatibility complex class I allele *Mane-A*10* restricts an immunodominant simian immunodeficiency virus (SIV) Gag epitope (KP9) which rapidly mutates to escape T cell recognition following acute simian/human immunodeficiency virus infection. Two technologies for the detection of *Mane-A*10* in outbred pigtail macaques were developed: reference strand-mediated conformational analysis and sequence-specific primer polymerase chain reaction. A Mane-A*10/KP9 tetramer was then developed to quantify $CD8^+$ T lymphocytes primed by multigenic DNA vaccination, which have previously been difficult to detect using standard interferon-γ-based T cell assays. We also demonstrate mutational escape at KP9 following acute SIV infection. *Mane-A*10*$^+$ animals have lower set point SIV levels than *Mane-A*10*$^-$ animals, suggesting a significant fitness cost of escape. These studies pave the way for a more robust understanding of HIV vaccines in pigtail macaques.

Key words: $CD8^+$ T cells – major histocompatibility complex typing – tetramer – vaccines – viral escape

Accepted April 18, 2005.

Stephen J. Kent, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia. Tel.: 61 383449939; fax: 61 383443846; e-mail: skent@unimelb.edu.au

Funding: Australian National Health and Medical Research awards 299907 and 251653; National Institutes of Health award N01-A1-05395.

Introduction

The escalating global spread of HIV makes the development of an effective vaccine an incredibly urgent task. Non-human primate models of AIDS are vital to the vaccine development effort. Commonly used models are the simian immunodeficiency virus (SIV) or chimeric simian/human immunodeficiency virus (SHIV) infection of rhesus (*Macaca mulatta*), cynomolgus (*M. fascicularis*) and pigtail (*M. nemestrina*) macaques.

$CD8^+$ T cell responses significantly contribute to the control of natural HIV infection [27, 33, 36], and much attention is now focussed on understanding the underlying mechanisms of $CD8^+$ T cell control [4, 15, 32] in order to harness them for vaccine and treatment strategies. During the course of natural HIV infection of humans, and also in animal models, the $CD8^+$ T cell response is directed towards a limited number of defined viral epitopes [20, 35, 44, 45]. The characterization of these epitopes, and the discovery of the major histocompatibility complex (MHC) class I molecules that bind them, is fundamental to the detailed study necessary to understand how $CD8^+$ T cells contribute to viral control.

The characterization of the common rhesus macaque MHC class I molecule Mamu-A*01 [34], and the study of its peptide-binding motif [1], have enabled detailed studies of specific immunodominant $CD8^+$ T cell responses, particularly through the development of Mamu-A*01-peptide tetramers [2, 18, 28, 35, 42]. MHC typing of rhesus macaques is now a routine procedure, with the use of techniques such as PCR with sequence-specific primers (PCR-SSP) [26, 30] and more recently, reference strand-mediated conformational analysis (RSCA) [5], used to identify macaques expressing *Mamu-A*01* and other known MHC class I alleles. Since these advances in the rhesus macaque model, *Mamu-A*01*$^+$ animals have become much more scarce [11], thus providing significant imperative to develop alternative animal models such as the pigtail macaque.

We recently characterized an MHC class I allele in pigtail macaques, *Mane-A*10*, that restricts an immunodominant response to SIV $Gag_{164–172}$ epitope KP9 [43]. The identification of this immunodominant epitope and its restricting MHC class I allele is an important advance for the pigtail macaque model of AIDS.

Interestingly, we recently described viral escape from T cell recognition at the *Mane-A*10*-restricted KP9 epitope in SHIV-infected macaques, involving a lysine to arginine substitution at position 2 of the epitope [19]. By mapping the rapid rate of escape through serially cloning plasma SHIV RNA, we estimated that KP9-specific T cells efficiently eliminate wild-type virus. Further, when we studied infection with an SHIV strain already escaped at KP9, we saw rapid reversion to wild-type sequence, suggesting that escape at KP9 causes a significant decrease in viral fitness [19]. Prior to the present report, escape at the KP9 epitope in SIV-infected macaques (rather than SHIV) has not been reported. Furthermore, the relative utility of KP9-specific T cells acting against wild-type SIV, compared with the reduction in viral fitness as a result of escape mutations, has not been previously studied.

The identification of *Mane-A*10* in outbred populations of pigtail macaques is now an important priority for researchers using this animal model. In addition, study of KP9-specific $CD8^+$ T cells using Mane-A*10/KP9 tetramers will assist in understanding the characteristics of these T cells that contribute to viral escape and viral control. Here, we describe two alternative methods for MHC class I typing pigtail macaques – a *Mane-A*10*-specific PCR assay, and the further adaptation of RSCA – to augment the current, cumbersome methods of cloning and sequencing. In addition, we describe the design, development and validation of a Mane-A*10/KP9 tetramer, the first developed for the pigtail macaque, which will facilitate a specific and detailed analysis of KP9-specific $CD8^+$ T cells.

Materials and Methods

Reference strand-mediated conformational analysis

The previously described RSCA method using an AlfExpress DNA Analyzer (Amersham Biosciences, Piscataway, NJ, USA) [43] was adapted for the characterization of MHC class I expression patterns in *M. nemestrina* on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Briefly, a 677-bp fragment of MHC class I cDNA was amplified with 1 μl of 25 μM phosphate-labelled forward primer (5′ RSCA-P; 5′-[phos]-GCTACGTGGACGACACGC-3′), an unlabelled reverse primer (3′ RefStrandR; 5′-CAG-AAGGCACCACCACAGC-3′) and 1 U Phusion DNA polymerase (Finnzymes, Espoo, Finland) in a 50-μl reaction. An initial 30 s 98°C step was followed by 25 cycles of 98°C for 5 s, 65°C for 1 s, 72°C for 20 s, then a final extension at 72°C for 5 minutes. Following amplification, samples were digested for 30 minutes at 37°C with 10 U lambda exonuclease (Epicentre, Madison, WI, USA) to yield single-stranded amplicons. Rhesus macaque alleles *Mamu-A*15*, *Mamu-A*20* and *Mamu-B*60* were prepared as reference strands under the same amplification conditions, using 6FAM, NED or HEX-labelled forward primers (5′-[fluorescent dye]GCTACGTG-GACGACACGC-3′) and a phosphate-labelled reverse primer (5′-[phos]CAGAAGGCACC ACCACAGC-3′), then exonuclease treated as described above. Heteroduplexes of the single-stranded cDNA amplicons and reference strands were created in a reaction involving 4 minutes at 95°C, 5 minutes at 55°C and 5 minutes at 15°C. The RSCA reactions were then desalted on a Sephadex G-50 column (Amersham Biosciences), dried down in a SpeedVac (Thermo Electron, Waltham, MA, USA), and loaded onto a 4.5% polyacrylamide gel on the ABI PRISM 377 DNA Sequencer (Applied Biosystems). Samples were electrophoresed for 12 hours at 30°C, 1200 V, 60 mA current and 10 W power. Data were analysed using DAx data acquisition and analysis software (Van Mierlo Software, Eindhoven, the Netherlands).

PCR-SSP assay

To design *Mane-A*10*-specific primers, all known *M. nemestrina* MHC class I allele sequences were

aligned (Genbank: AY204715, AY204716, AY204723–AY204738, AY557348–AY557367). The alignment included several sequences that are likely to be further alleles but have not been found in enough clones to qualify for allele definition, in order to maximize the specificity of the primers for *Mane-A*10*. Regions unique to *Mane-A*10* were identified, and candidate primers for the amplification of 100–200 bp fragments designed (using Primer Express Version 1.0, Applied Biosystems) to (i) have a melting temperature >70°C, and (ii) exhibit sequence mismatches with the non-*Mane-A*10* alleles towards the 3′ end of the primer. Three primer sets were identified: set 1 (SSP1 forward: 5′-GGCCAACACACAGACC-TACCGAGAGAG-3′, Tm 71.4°C; SSP1 reverse: 5′-CCCTGCCGTCGTAGGCGTACTGGCTATAT-3′, Tm 74°C), set 2 (SSP2 forward: 5′-CGGGTC TCACACCTTCCAGAGGATGTAT-3′, Tm 71.3°C; SSP2 reverse: 5′-CGGTCCAGGAACGCAGG-TCCC-3′, Tm 73°C) and set 3 (SSP3 forward: 5′-GGCGCCTCCTCCGCGGATATAG-3′, Tm 72.2°C; SSP3 reverse: 5′-GGCACTCGCCCTCCACG-TAGGT-3′, Tm 71.4°C).

Primers specific for the constituently expressed CCR5 gene were used as a positive control. The CCR5 primer set amplifies a 239-bp fragment: (SSP CCR5 Forward: 5′-GGCTGTGTTTGCCT-CTCTCCCAGGA-3′, Tm 72.9°C; SSP CCR5 Reverse: 5′-CCTCACAGCCCTGTGCCTCTTC-TTCTC-3′, Tm 72.3°C).

First strand cDNA was prepared from total RNA extracted from macaques with known *Mane-A*10* status [43]. PCR amplification of 0.9 μl of cDNA or 5.3 ng of MHC clone DNA was carried out with 0.375 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) in a 15-μl reaction containing 1x PCR buffer II (Applied Biosystems), 1.5 mM $MgCl_2$ (Applied Biosystems), 0.2 mM dNTPs (Promega, Madison, WI, USA) and 0.8 μM of each sequence-specific or CCR5 forward and reverse primer. The amplification conditions for the SSP sets (SSP1, SSP2 or SSP3) were: 95°C for 5 minutes, followed by 30 cycles of 94°C for 20 s, 72°C for 20 s and 72°C for 20 s, with a final extension at 72°C for 7 minutes, using a GeneAmp 9700 Thermal Cycler (Applied Biosystems). Amplification of CCR5 cDNA was performed as above but with an annealing temperature of 60°C. A no-template reaction and an irrelevant *Mane-B*10* clone (GenBank: AY557355) served as negative controls for all primer sets. Cloned *Mane-A*10* (GenBank: AY557348) and *Mane-A*16* (GenBank: AY557354) were used as positive controls. Amplicons (5 μl) were visualized on 1.5% TBE agarose gels and sized against pGEM DNA markers (Promega).

Tetramer construction

Primers 5′A*10tet (5′-GGGCATATGGGCTCG-CACTCCATGAGG-3′) and 3′A*10tet (5′-CC-CTGGATCCGGAAGACGGCTCCCATCTC-3′) were designed to amplify the first 834 bp of the *Mane-A*10* sequence, corresponding to amino acids 1–278 of the mature MHC class I molecule (Fig. 2). The tetramer fragment was amplified from *Mane-A*10* clone DNA, using Phusion DNA polymerase (Finnzymes) with the cycling conditions: 98°C 30 s, 25 cycles of 98°C 5 s, 72°C 25 s, then 72°C for 5 minutes. The amplicon was then ligated into the pGEM-T Easy vector (Promega), transformed into *Escherichia coli*, and sequenced. The sequence-verified tetramer fragment was then subcloned into the JA5 expression vector and expressed as described by Altman et al. [3]. The Mane-A*10 polypeptide, human β_2-microglobulin and crude grade KP9 peptide (GL Biochem, Shanghai, China) were complexed to form the Mane-A*10/KP9 tetramer. The tetramer was subsequently conjugated with phycoerythrin (PE).

Tetramer validation (flow cytometry)

Initial validation of the Mane-A*10/KP9 tetramer was conducted on frozen PBMC derived from macaque 4664, which had been infected with $SHIV_{mn229}$ [12], and shown to have a large response to the KP9 epitope by intracellular cytokine staining (ICS) for interferon (IFN)-γ [14, 19, 43]. Briefly, PBMC were thawed and washed twice in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U penicillin G sodium/ml, 100 μg of streptomycin sulphate/ml, 292 μg L-glutamine/ml (PSG) (Invitrogen). Cells (1×10^6) were aliquotted into a 96-well U-bottomed plate (Nunc, Roskilde, Denmark) for staining. The total volume for each well was made up to 100 μl using FACS buffer (1x phosphate-buffered saline + 0.5% bovine serum albumin + 2 mM EDTA), and the tetramer added at a dilution of 1:200 (0.5 μl). Cells were incubated at 37°C, 5% CO_2 for 1 hour, then were counter stained with anti-CD4-FITC (BD Biosciences, PharMingen, San Diego, CA, USA, clone M-T477), anti-CD3-PerCP (BD, clone SP34) and anti-CD8-APC (BD, clone SK1), and incubated at room temperature for 40 minutes. Cells were then washed twice in FACS buffer and formaldehyde-fixed. Acquisition was performed on an LSR II Flow Cytometer (BD) and analysed using CellQuest Pro, Version 5.1.1 (BD). Following tetramer titration studies, further tetramer analysis was conducted on fresh whole blood samples, with

200 μl whole blood stained with a 1:400 dilution of tetramer for 45 minutes at room temperature in the dark. Counter-staining with anti-CD3-FITC (BD, clone SP34), anti-CD4-PerCP (BD, clone L200) and anti-CD8-APC (BD, clone SK1) was then conducted at room temperature in the dark for 45 minutes. Erythrocytes were lysed with 2 ml 1x FACS Lysing Solution (BD) for 10 minutes at room temperature incubation and washed twice in FACS buffer. Acquisition and analysis were performed as described above.

SIV infection and analysis of viral escape

Eight naïve pigtail macaques were infected intravenously with SIV_{mac251} as previously described [43]. Intracellular IFN-γ secretion was assessed by flow cytometry using 200 μl whole blood incubated with overlapping SIV_{mac239} Gag 15-mer peptides (kindly supplied by the National Institutes of Health AIDS Research and Reference Reagent Program) at 1 μg/ml/peptide, using protocols described elsewhere [19, 43]. SHIV RNA was extracted from plasma at each timepoint, and cDNA generated as previously described [19]. The Gag region containing the KP9 epitope was then amplified using SIV Gag-specific primers 4 and 5 and Phusion DNA polymerase (Finnzymes) using conditions as previously outlined [19]. The amplicons were then cloned into the pGEM-T Easy vector (Promega), transformed into competent JM109 *E. coli* (Promega), and individual clones sequenced using the BigDye Terminator v3.1 chemistry (Applied Biosystems).

Results

*Mane-A*10* typing of pigtail macaques by RSCA

The RSCA is a sensitive method to characterize multiple MHC class I alleles. The technique involves heteroduplexing known labelled rhesus macaque reference alleles with unknown pigtail macaque cDNA. We have previously described the use of RSCA to MHC class I genotype pigtail macaques through the use of rhesus macaque fluorescent reference strands and an AlfExpress DNA Analyser [43]. While this technique is sensitive and useful for detecting multiple unknown MHC class I alleles in pigtail macaques, it is limited by the fact that only one reference strand can be used in each lane, and that the size standard has to be run in a separate lane from the samples. Using three separate reference strands per animal, and leaving lanes for the external size standards, throughput is limited to 12 animals per gel. This technique has recently been adapted to the ABI PRISM 377 DNA Sequencer, which enables four separate fluorescent traces to be analysed per lane (K. Krebs, Z. Jin, R. Rudersdorf, A. Hughes, D.H. O'Connor, submitted for publication). This significantly increases the throughput potential of the RSCA technique, as samples need to be run with multiple fluorescent reference strands in order to eliminate false positives. With 96 lanes available, up to 96 animals can be analysed in a single gel.

We analysed amplified cDNA from a total of 79 pigtail macaques by RSCA. Of these, 13 were definitively positive for *Mane-A*10*, with peaks corresponding to the *Mane-A*10* clones with all three rhesus macaque MHC class I reference strands tested. Traces from two of these *Mane-A*10*$^+$ animals are shown in Fig. 1 in comparison with a cloned *Mane-A*10* allele. Both animals were recently vaccinated against SIV Gag and confirmed to present the KP9 epitope (data not shown). In addition, both have been shown to be *Mane-A*10*$^+$ by PCR-SSP (Fig. 3 and data not shown).

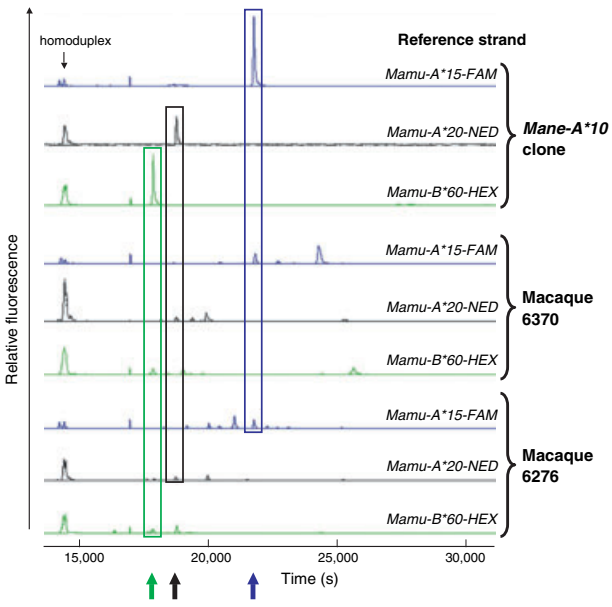

Fig. 1. Identification of *Mane-A*10* expression by reference strand-mediated conformational analysis. Raw trace data from a *Mane-A*10* clone and cDNA from macaques 6370 and 6276 are displayed. Each sample has been heteroduplexed to three separate reference strands, as indicated on the right-hand side of each trace. *Mane-A*10* mobility with the *Mamu-A*15*-FAM reference strand is indicated with the blue box, *Mamu-A*20*-NED indicated in black, and the *Mamu-B*60*-HEX in green. Arrows below the traces indicate the presence of a peak in each respective trace that corresponds to the *Mane-A*10* clone.

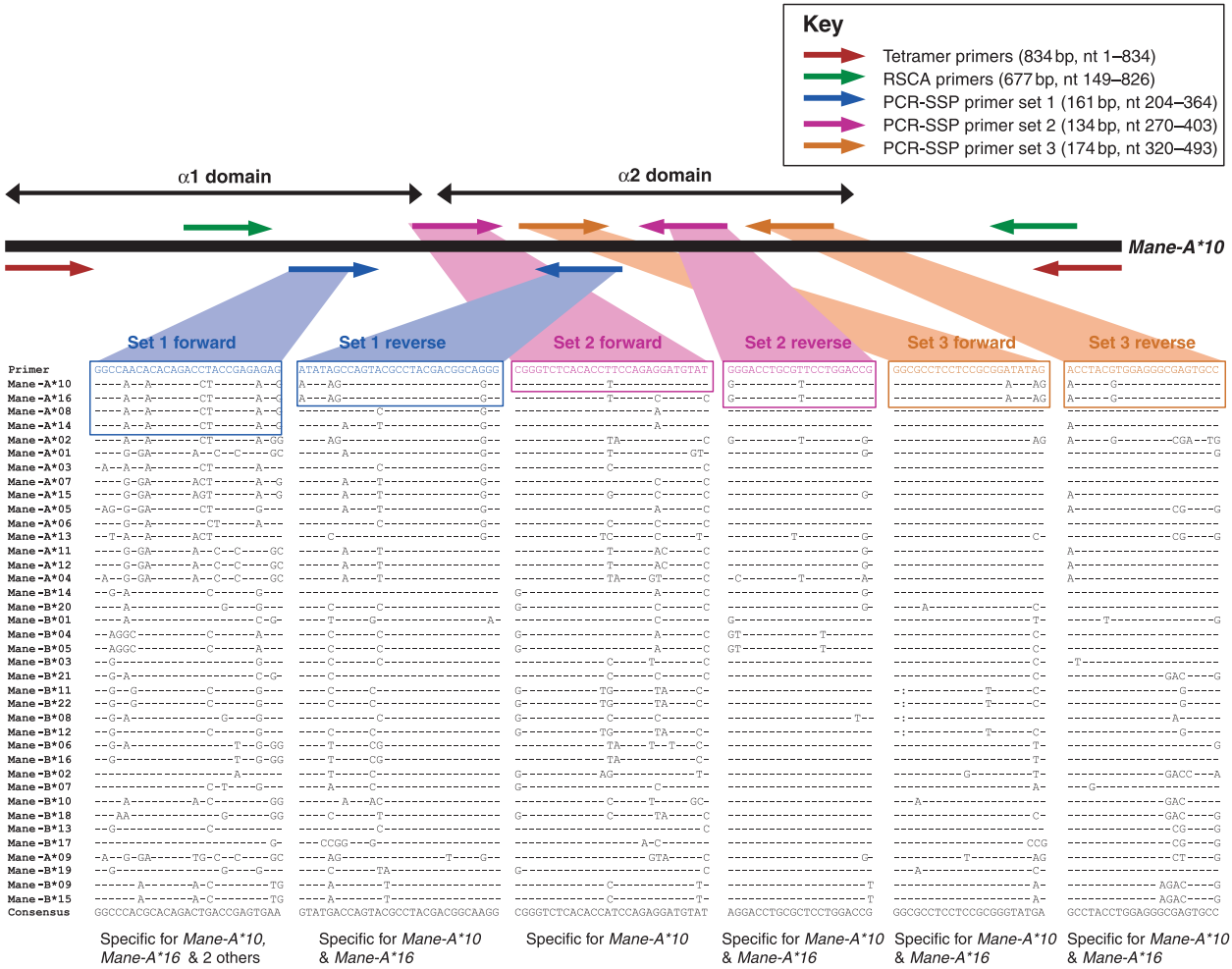

Fig. 2. Design of sequence-specific primers for *Mane-A*10* PCR assay. The positions of all the primers described in this paper are shown across a schematic of the *Mane-A*10* sequence. In addition, the sequences of all known *Macaca nemestrina* MHC class I alleles across the primer binding regions are shown to demonstrate the specificity of the PCR-SSP primers. Dashes indicate homology with the consensus sequence at the bottom of the list. The number of alleles that each primer is expected to amplify is indicated below its respective sequence. The key at the top indicates the colour scheme, the size of the fragment that each primer set amplifies and the exact nucleotide locations of the primer sites.

PCR with *Mane-A*10* SSP

A PCR-based assay for the detection of the *Mane-A*10* allele will be a valuable tool for the bulk screening of macaques, being quicker and more efficient than the current methods of cloning and sequencing, and less resource intensive than RSCA. We designed three primer sets with specificity for regions across the *Mane-A*10* sequence encoding the polymorphic α1 and α2 domains (Fig. 2). The primers were designed to have high melting temperatures (>70°C) to increase specificity, mismatches with non-*Mane-A*10* alleles towards the 3′ end, and to amplify short (100–200 bp) fragments. All primers, except the set 2 forward primer, are specific for both *Mane-A*10* and the closely related allele, *Mane-A*16*. The *Mane-A*16* sequence differs from *Mane-A*10* by only two nucleotides within the regions studied [43]. Both mismatches between *Mane-A*10* and *Mane-A*16* are contained within the region covered by the set 2 forward primer, the only primer not specific for *Mane-A*16* (Fig. 2). The set 1 forward primer is the least specific, detecting *Mane-A*10*, *Mane-A*16*, *Mane-A*08* and *Mane-A*14*, though the reverse primer is specific only for *Mane-A*10* and *Mane-A*16* (Fig. 2).

All three primer sets were able to detect *Mane-A*10* in four animals shown to be *Mane-A*10*$^{+}$ by RSCA (Fig. 3). None of the three primer sets detected any product in four *Mane-A*10*$^{-}$ animals. Interestingly, there was no detectable difference between the three primer sets when used on cDNA from *Mane-A*10*$^{-}$ animals, suggesting that the

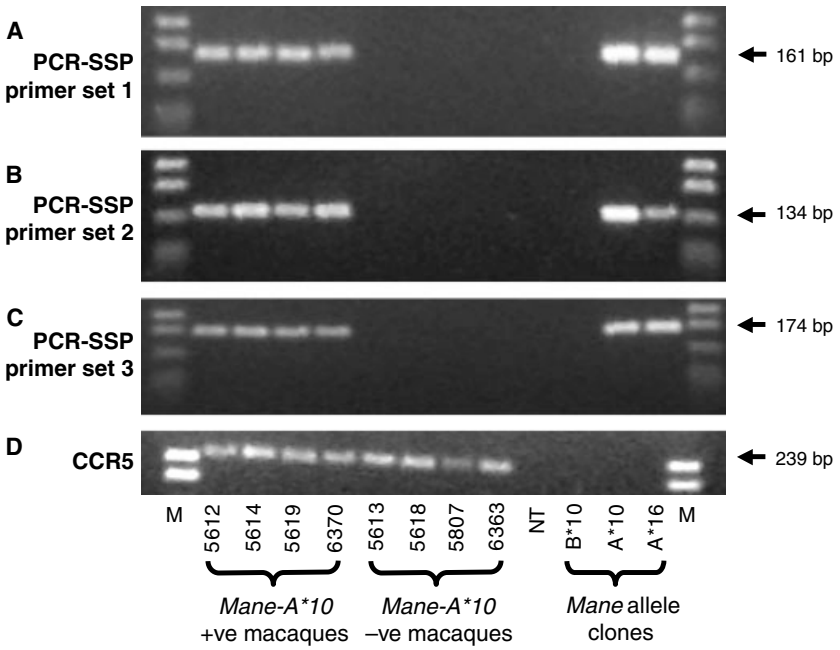

Fig. 3. Validation of specificity of sequence-specific primers. cDNA from four *Mane-A*10*$^{+}$ and four *Mane-A*10*$^{-}$ macaques was amplified using each of the three PCR-SSP primer sets. As negative controls, a no-template sample (NT) plus a clone of allele *Mane-B*10* were also amplified, with *Mane-A*10* and *Mane-A*16* clones acting as positive controls. pGEM markers are shown flanking the samples on either side. Amplicons from PCR-SSP primer set 1 (161 bp), primer set 2 (131 bp) and primer set 3 (173 bp) are shown in panels **A**–**C** respectively. Panel **D** shows amplification of a 239-bp fragment of CCR5, demonstrating that the cDNA from all eight cDNA samples was intact. The *Mane* allele clones do not contain the CCR5 sequence.

relative specificity of the three sets for *Mane-A*10* is equivalent. When tested on cloned MHC alleles, none of the three primer sets detected *Mane-B*10*, and all three sets detected both the *Mane-A*10* and *Mane-A*16* clones. Unexpectedly, even primer set 2, which had 3′ mismatches for *Mane-A*16* in the reverse primer, was able to detect the *Mane-A*16* clone DNA, though the band appears to be weaker than that of the *Mane-A*10* clone.

Mane-A*10/KP9 tetramer analysis of PBMC and whole blood

The development of a Mane-A*10 tetramer, complexed with the immunodominant SIV Gag epitope, KP9, facilitates the analysis of KP9-specific CD8^{+} T cells in vaccinated and SIV-infected pigtail macaques. The Mane-A*10 tetramer was constructed using standard *E. coli* expression systems and conjugated with PE. To validate the tetramer, we first analysed a frozen PBMC sample from an animal, 4664, from a timepoint where a large response to KP9 had been detected by ICS for IFN-γ [14, 19, 43]. A large tetramer-positive population was detected in this sample, accounting for 8.88% of all CD8^{+} T lymphocytes (Fig. 4A), a similar magnitude to that detected by ICS on fresh blood (9.8%) [19, 43].

Most tetramer analysis described in the literature is conducted on PBMC [9, 31, 37, 42], but the preparation of PBMC requires considerable cell handling and results in significant cell loss. We therefore adapted whole blood protocols we used for intracellular IFN-γ staining [14] for tetramer analysis. These protocols are simpler and facilitate the analysis of rare populations such as tetramer-positive populations on small volumes of blood as there is no significant cell loss.

Initial experiments were carried out on macaques receiving DNA only or DNA/fowlpoxvirus vaccinations against SIV Gag [14]. We have previously shown that although these DNA vaccines prime for boosting with viral vectors such as fowlpoxvirus, the level of immunity after DNA vaccination alone is difficult to detect using standard IFN-γ ICS or ELISpot (enzyme-linked immunosorbent spot-forming cell assay) techniques [14, 13, 16, 25]. Tetramer analysis was used to investigate the level of KP9-specific CD8^{+} T lymphocytes in *Mane-A*10*$^{+}$ vaccinees after DNA-only or DNA/fowlpoxvirus vaccination. The background level of tetramer-positive cells in an unvaccinated *Mane-A*10*$^{+}$ animal was negligible, demonstrated by staining 200 μl of whole blood from animal 6167 (Fig. 4B, left panel). This animal was

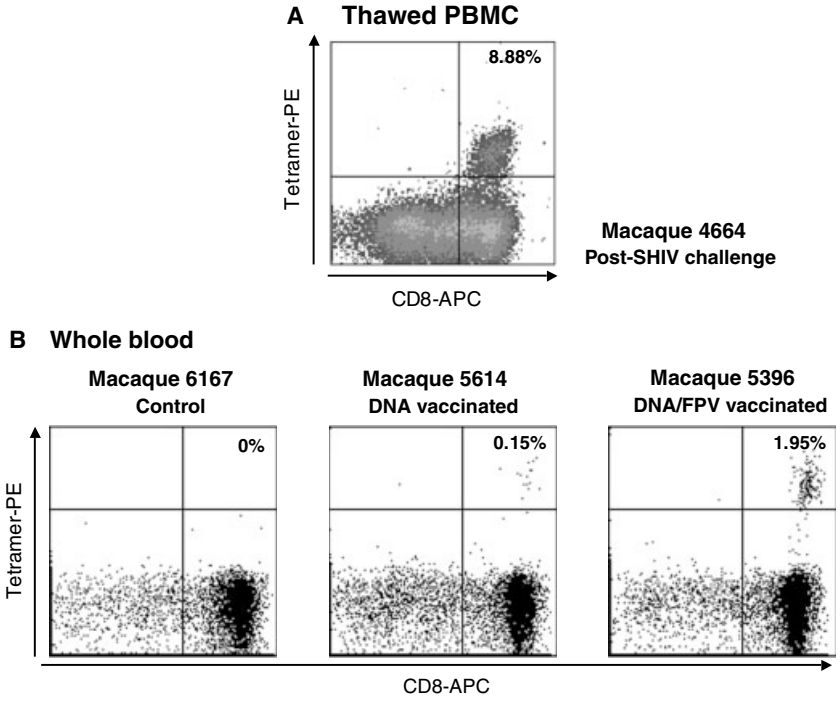

Fig. 4. Detection of KP9-specific $CD8^+$ T lymphocytes by Mane-A*10 tetramer analysis. (A) Tetramer staining on thawed PBMC from DNA/fowlpoxvirus-vaccinated macaque 4664, 3 weeks following $SHIV_{mn229}$ challenge. The tetramer-positive population, in the upper right quadrant, is expressed as a percentage of $CD3^+CD8^+$ T cells. (B) Tetramer staining on fresh whole blood from *Mane-A*10*$^+$ macaques 1.5 weeks following vaccination. The left panel is blood from control unvaccinated macaque 6167. The middle panel is blood from DNA-only vaccinated macaque 5614, which shows a small detectable tetramer-positive population. The right-hand panel is blood from DNA/fowlpoxvirus-vaccinated macaque 5396, which displays a larger tetramer-positive population.

vaccinated with an empty plasmid construct, and has clearly not elicited a KP9-specific response. Animal 5614 had been DNA-vaccinated against SIV Gag three times at 4-week intervals and was analysed with the Mane-A*10/KP9 tetramer 1.5 weeks after the last vaccine. Tetramer staining shows a small but clearly discernable tetramer-positive population at this time, accounting for 0.15% of $CD8^+$ T lymphocytes (Fig. 4B, middle panel). Another *Mane-A*10*$^+$ animal, macaque 5396, was vaccinated at 4-week intervals: twice with SIV Gag-expressing DNA, then boosted once with a fowlpoxvirus expressing SIV Gag. Blood from this animal was also analysed 1.5 weeks after the last vaccine, and displayed a larger and more distinct tetramer-positive population (1.95% of $CD8^+$ T lymphocytes; Fig. 4B, right panel) compared with that detected in the DNA-only vaccinated animal.

Immune escape at KP9 following SIV_{mac251} infection: importance of viral load

We have previously reported that a response to KP9 correlates with a lower viral load during acute SIV infection [43], and separately, that immune escape occurs at the KP9 epitope in SHIV-infected animals, resulting in a significant fitness cost to the virus [19]. The mechanism mediating SIV control in *Mane-A*10*$^+$ animals is not clear. Do *Mane-A*10*$^+$ animals produce a $CD8^+$ T cell response that effectively dampens viral replication, or does the fitness cost to the virus caused by the escape mutation result in deficient replication? To further probe the relationship between KP9-specific $CD8^+$ T cell responses and control of SIV replication, we followed eight pigtail macaques infected with SIV_{mac251}, including five *Mane-A*10*$^-$ animals and three *Mane-A*10*$^+$ animals (Table 1). These macaques were typed for *Mane-A*10* primarily by RSCA as described above, and following SIV_{mac251} infection, we studied their $CD8^+$ T cell responses to SIV Gag (by intracellular staining for IFN-γ) and to the KP9 epitope (by *Mane-A*10* tetramer, as above). The proportion of viral clones bearing wild-type or mutant sequence at the KP9 epitope was determined by cloning and sequencing of serial viral RNA samples derived from plasma.

The viral loads of the *Mane-A*10*$^+$ animals were significantly lower than those of the *Mane-A*10*$^-$ animals ($P < 0.05$ [43]), and correlate with a higher retention of $CD4^+$ T cells, even at later timepoints (e.g. week 21 post-infection) (Table 1). All three

Table 1. Immunological profiles of SIV_{mac251}-infected pigtail macaques studied for viral escape

Animal	*Mane-A* 10*[1]	Peak $CD8^+$ T cell response		Viral load (log_{10} copies/ml)		$CD4^+$ T cells (%)			KP9 sequence (proportion of clones)																					
									Week 8											Week 17/21											
		Gag[2]	KP9[3]	Week 2	Week 8	Week 0	Week 8	Week 17/21	K	K	F	G	A	E	V	V	P		K	K	F	G	A	E	V	V	P		
5053	–	0.07	0.02	7.69	6.6	40.47	32.9	39.36	.	.	.	.	.	.	.	.	.	(10/10)	.	.	.	.	.	.	.	.	.	(10/10)
5169	–	0.1	0.01	6.8	6.09	32.26	19.16	8.51	Not studied										.	.	.	.	.	.	.	.	.	(10/10)	
5176	–	0.04	0.01	8.41	7.25	37.06	20.53	15.97[4]	.	.	.	.	.	.	.	.	.	(10/10)	.	.	.	.	.	.	.	.	.	(10/10)[4]	
5607	–	0.06	0.01	7.08	6.09	21.74	16.33	11.8	.	.	.	.	.	.	.	.	.	(9/9)	.	.	.	.	.	.	.	.	.	(10/10)	
6138	–	0.03	0.01	7.92	7.47	26.37	12.1	5.54[4]	Not studied										.	.	.	.	.	.	.	.	.	(10/10)[4]	
									.	R	.	.	.	.	.	.	.	(4/10)	.	R	.	.	.	.	.	.	.	(8/10)	
5284	+	2.63	7.39	7.89	5.57	29.88	27.21	27.04	.	.	.	.	.	.	.	.	S	(6/10)	.	.	.	.	.	.	.	.	S	(2/10)	
									.	R	.	.	.	.	.	.	.	(3/10)	.	R	.	.	.	.	.	.	.	(8/10)	
5715	+	1.11	1.02	7.3	5.31	34.94	28.89	21.97	.	.	.	.	.	.	.	.	.	(7/10)	.	.	.	.	.	.	.	.	.	(2/10)	
																			.	R	.	.	.	.	.	.	.	(8/10)	
5424	+	0.58	1.73	6.79	3.7	25.63	23.19	28.63	.	.	.	.	.	.	.	.	.	(9/9)	.	.	.	.	.	.	.	.	.	(2/10)	

[1]Status determined by RSCA (5607 by RSCA and cloning and sequencing; 5169 by RSCA and PCR-SSP).
[2]Measured by intracellular cytokine staining for IFN-γ following stimulation with overlapping Gag peptides.
[3]Measured by tetramer staining on thawed PBMC from week 7 post-infection (week 5 for 5715).
[4]Animals 5176 and 6138 were killed at week 17, so final $CD4^+$ T cell counts and KP9 sequences are from this timepoint.

*Mane-A*10*$^+$ animals displayed vigorous responses to the KP9 epitope when measured by ICS (data not shown), with even greater responses detected using the Mane-A*10/KP9 tetramer (Table 1). The *Mane-A*10*$^-$ animals showed negligible Gag responses, and no KP9-specific responses, emphasizing the immunodominance of the KP9 epitope within Gag.

The study of viral sequence at the KP9 epitope shows that the wild-type KP9 sequence is maintained in all of the *Mane-A*10*$^-$ animals at both early (week 8) and late (week 17/21) timepoints following SIV_{mac251} infection (Table 1). In two of three *Mane-A*10*$^+$ animals, viral escape was detected at week 8 following infection, and by week 21, all three *Mane-A*10*$^+$ animals displayed mutant sequence at the KP9 epitope. The predominant escape mutant detected was the previously described K165R mutation [19]. Macaque 5424, the *Mane-A*10*$^+$ animal that did not display any viral escape at week 8, also had the lowest viral load of all the animals.

Discussion

Pigtail macaques have emerged as an important alternate non-human primate model for the study of HIV vaccines. The MHC class I allele, *Mane-A*10*, is common in outbred pigtail macaques and restricts an immunodominant response to the SIV Gag epitope, KP9. Importantly, the presence of *Mane-A*10*, and a response to the KP9 epitope, confers a significantly lower viral load during acute SIV_{mac251} infection [43]. Furthermore, rapid immune escape has been shown to occur during primary infection at the KP9 epitope following both SHIV infection [19] and SIV infection, emphasizing the efficacy of KP9-specific $CD8^+$ T cells. This data now provides a valuable framework for the study of immunodominance, $CD8^+$ T cell dynamics and phenotypes, and viral escape. *Mane-A*10*$^-$ macaques infected with virus containing mutant sequence at the KP9 epitope rapidly revert to wild-type sequence [19]. This reversion to wild-type sequence implies that the escape mutation comes at a fitness cost to the virus, as it is only maintained in the presence of a KP9-specific immune response. This paper further describes methods for the detection of *Mane-A*10* and its associated cellular immune responses in outbred pigtail macaques, including a novel sequence-specific PCR assay, and the development and validation of a Mane-A*10/KP9 tetramer, first developed for the pigtail macaque model of AIDS. We also explored the relationship between SIV viral load and the occurrence of immune escape at the KP9 epitope.

The RSCA represents a powerful and accurate technique for MHC class I typing pigtail macaques. The RSCA method using the ABI 377 DNA Sequencer has already been used to identify shared MHC class I alleles from cynomolgus macaques (K. Krebs, Z. Jin, R. Rudersdorf, A. Hughes, D.H. O'Connor, submitted for publication) and to select pigtail macaques for vaccine trial groups, with equal numbers of *Mane-A*10*$^+$ animals assigned to

each group. The current RSCA method is still, however, limited by the lack of an appropriate size standard and differences in gel-to-gel sample mobilities, leading to some ambiguities that have had to be resolved through cloning and sequencing. The further development of appropriate size standards is a high priority and will be necessary to ensure that accurate mobility tracking and minimization of lane-to-lane variability can be achieved.

In contrast to RSCA, PCR-SSP assay is a comparatively straightforward and simple method for the detection of *Mane-A*10*. The PCR-SSP assay will be very useful as a method for bulk screening of macaques for *Mane-A*10*, as it is much less resource intensive than RSCA, making it suitable for use in macaque breeding colonies both in developed and in less developed countries. The PCR-SSP assay requires further validation to ensure its accuracy, which will involve studying much larger numbers of pigtail macaques with known genotypes, plus cross-referencing with techniques such as RSCA and MHC class I cloning and sequencing. All three of the PCR-SSP primer sets described here detect *Mane-A*10* as well as the very closely related allele, *Mane-A*16*. This is the case even for primer set 2, which has a forward primer with a 3′ mismatch for *Mane-A*16*. *Mane-A*16* was originally characterized in a macaque which responded to KP9, but did not have the *Mane-A*10* allele, suggesting that *Mane-A*16* may also present the KP9 epitope [43]. The Mane-A*16 protein has since been shown to re-fold around the KP9 epitope during tetramer production, which provides further evidence that *Mane-A*16* can elicit a KP9-specific response (data not shown). It is therefore of interest to know whether animals carry the *Mane-A*16* allele, although the impact of the more rare *Mane-A*16* allele on the outcome of SIV infection is not yet known. The specificity of the PCR-SSP assay for *Mane-A*10* could potentially be increased through the adaptation of the assay to real-time PCR, involving the incorporation of a fluorescent probe that binds to a third *Mane-A*10*-specific sequence within the amplified fragment. Taken together, the PCR-SSP and RSCA techniques for MHC class I typing provide important advances for the pigtail macaque model of human AIDS.

The development of a functional Mane-A*10/KP9 tetramer is another significant development for the use of pigtail macaques as an animal model for AIDS research. Highly sensitive, tetramer analysis can detect and quantify very small numbers of antigen-specific $CD8^+$ T cells [3, 31]. Apart from enabling more sensitive and accurate quantification, tetramer analysis also facilitates the phenotyping of antigen-specific $CD8^+$ T cells without requiring *in vitro* stimulation. Tetramer analysis will also be very useful for the measurement of mucosal immune responses, which are typically difficult to measure in pigtail macaques with standard IFN-γ-based T cell assays due to high levels of background activation [24]. Furthermore, tetramer-positive $CD8^+$ T cell populations can be sorted by flow cytometry for further analysis, including phenotyping, study of gene expression patterns by microarray and the induction of cellular regulatory and transcriptional networks [8, 17, 23]. We recently showed that DNA vaccination alone primes for protection against SHIV challenge almost as effectively as a DNA/fowlpoxvirus regimen, despite negligible immunogenicity post-vaccination as assessed by standard assays such as ELISpot and ICS [14]. The Mane-A*10/KP9 tetramer now provides a powerful tool for isolating the very small antigen-specific populations elicited by DNA vaccination in order to phenotype them and dissect what characteristics may contribute to their efficacy following viral challenge.

Viral escape occurs during acute and chronic phases of HIV and SIV infection [6, 7, 10, 22, 38, 40, 41], presenting a formidable challenge to the development of an effective HIV vaccine. While mutation at dominant $CD8^+$ T cell epitopes can undermine immune control of viral replication, it is becoming apparent that escape mutations can carry significant fitness costs to the virus, as indicated by the reversion to wild-type sequence that occurs in the absence of immune pressure [19, 21, 29, 39]. We show here that viral escape occurs at the KP9 epitope in SIV_{mac251}-infected *Mane-A*10*$^+$ macaques. Interestingly, escape at KP9 occurred at different rates in the animals studied, and in all cases did not occur completely. In two of three animals (macaques 5284 and 5715), escape was detected by week 8 post-infection and in the third (macaque 5424), escape was not detected until later. In all three animals, the K165R escape variant accounted for only 80% of viral clones sequenced at week 21, with the less common P172S mutation persisting in 20% of clones from animal 5284. These results contrast with the more rapid and complete escape observed at the KP9 epitope in animals challenged with $SHIV_{SF162P3}$ [19]. Macaque 5424 had a lower viral load than the other two *Mane-A*10*$^+$ macaques, suggesting that the $CD8^+$ T cell response may have constrained viral replication sufficiently to prevent the emergence of the escape variant until later in infection. KP9-specific T cells constrain viral replication by

acting on cells infected with wild-type virus, resulting in variable rates of escape at the KP9 epitope. Combined with the rapid reversion of KP9 escape variants seen in *Mane-A*10*$^{-}$ animals [19], this provides a defined scenario in which to dissect the influence of T cell immunity on viral load, and on the rate and extent of viral escape in pigtail macaques.

In summary, this paper describes the MHC class I typing of pigtail macaques, an increasingly common animal model for HIV infection, the development of the first MHC class I tetramer for use in this model and an investigation of the effect of immune escape on SIV viral loads. Together, these developments represent a considerable advance for the use of pigtail macaques in HIV research, providing the opportunity to undertake detailed study of CD8^{+} T cells directed towards the immunodominant KP9 epitope. MHC class I typing for *Mane-A*10* will also enable the selection of animals for future vaccine trials, facilitating a more robust comparison of vaccine regimens and better understanding of CD8^{+} T cell immunity to SIV and SHIV.

References

1. Allen TM, Sidney J, Del Guercio MF, Glickman RL, Lensmeyer GL, Wiebe DA, Demars R, Pauza CD, Johnson RP, Sette A, Watkins DI: Characterization of the peptide binding motif of a rhesus MHC class I molecule (Mamu-A*01) that binds an immunodominant CTL epitope from simian immunodeficiency virus. J Immunol 160:6062–6071, 1998.
2. Allen TM, Mothe BR, Sidney J, Jing P, Dzuris JL, Liebl ME, Vogel TU, O'Connor DH, Wang X, Wussow MC, Thomson JA, Altman JD, Watkins DI, Sette A: CD8(+) lymphocytes from simian immunodeficiency virus-infected rhesus macaques recognize 14 different epitopes bound by the major histocompatibility complex class I molecule mamu-A*01: implications for vaccine design and testing. J Virol 75:738–749, 2001.
3. Altman JD, Moss PA, Goulder PJ, Barouch DH, Mcheyzer-Williams MG, Bell JI, Mcmichael AJ, Davis MM: Phenotypic analysis of antigen-specific T lymphocytes. Science 274:94–96, 1996.
4. Amara RR, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staprans SI, Montefiori DC, Xu Y, Herndon JG, Wyatt LS, Candido MA, Kozyr NL, Earl PL, Smith JM, MA HL, Grimm BD, Hulsey ML, Miller J, Mcclure HM, McNicholl JM, Moss B, Robinson HL: Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 292:69–74, 2001.
5. Arguello JR, Little AM, Bohan E, Goldman JM, Marsh SG, Madrigal JA: High resolution HLA class I typing by reference strand mediated conformation analysis (RSCA). Tissue Antigens 52:57–66, 1998.
6. Barouch DH, Kunstman J, Kuroda MJ, Schmitz JE, Santra S, Peyerl FW, Krivulka GR, Beaudry K, Lifton MA, Gorgone DA, Montefiori DC, Lewis MG, Wolinsky SM, Letvin NL: Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 415:335–339, 2002.
7. Barouch DH, Kunstman J, Glowczwskie J, Kunstman KJ, Egan MA, Peyerl FW, Santra S, Kuroda MJ, Schmitz JE, Beaudry K, Krivulka GR, Lifton MA, Gorgone DA, Wolinsky SM, Letvin NL: Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys. J Virol 77:7367–7375, 2003.
8. Baskin CR, Garcia-Sastre A, Tumpey TM, Bielefeldt-Ohmann H, Carter VS, Nistal-Villan E, Katze MG: Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). J Virol 78:10420–10432, 2004.
9. Bertley FM, Kozlowski PA, Wang SW, Chappelle J, Patel J, Sonuyi O, Mazzara G, Montefiori D, Carville A, Mansfield KG, Aldovini A: 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, 2004.
10. Borrow P, Lewiki H, Wei X, Horwitz MS, Peffer N, Meyers H, Nelson JA, Gairin JE, Hahn BH, Oldstone MBA, Shaw GM: Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 3:205–211, 1997.
11. Cohen J: Aids research. Vaccine studies stymied by shortage of animals. Science 287:959–960, 2000.
12. Dale CJ, Liu XS, De Rose R, Purcell DF, Anderson J, Xu Y, Leggatt GR, Frazer IH, Kent SJ: Chimeric human papilloma virus-simian/human immunodeficiency virus-like-particle vaccines: immunogenicity and protective efficacy in macaques. Virology 301:176–187, 2002.
13. Dale CJ, De Rose R, Wilson K, Croom H, Thomson SA, Coupar BE, Ramsay AJ, Purcell DF, Ffrench R, Law M, Emery S, Cooper DA, Ramshaw IA, Boyle DB, Kent SJ: Evaluation in macaques of HIV-1 DNA vaccines containing primate CpG motifs and Fowlpoxvirus vaccines co-expressing IFNg or IL-12. Vaccine 23:188–197, 2004.
14. Dale CJ, De Rose R, Stratov I, Chea S, Montefiori D, Thomson SA, Ramshaw IA, Coupar BE, Boyle DB, Law M, Kent SJ: Efficacy of DNA and fowlpox virus priming/boosting vaccines for simian/human immunodeficiency virus. J Virol 78:13819–13828, 2004.
15. Davenport MP, Ribeiro RM, Perelson AS: Kinetics of virus-specific CD8+ T cells and the control of human immunodeficiency virus infection. J Virol 78:10096–10103, 2004.
16. De Rose R, Chea S, Dale CJ, Reece JC, Fernandez CS, Wilson CM, Thomson SA, Ramshaw IA, Coupar BE, Boyle DB, Sullivan MT, Kent SJ: Subtype AE HIV-1 DNA and recombinant Fowlpoxvirus vaccines encoding 5 shared HIV-1 genes: safety and T cell immunogenicity in macaques. Vaccine 23:1949–1956, 2005.
17. Dunbar PR, Ogg GS, Chen J, Rust N, Van Der Bruggen P, Cerundolo V: Direct isolation, phenotyping and cloning of low-frequency antigen-specific cytotoxic T lymphocytes from peripheral blood. Curr Biol 8:413–416, 1998.
18. Egan MA, Kuroda MJ, Voss G, Schmitz JE, Charini WA, Lord CI, Forman MA, Letvin NL: Use of major histocompatibility complex class I/peptide/beta2M tetramers to quantitate CD8(+) cytotoxic T lymphocytes specific for dominant and nondominant viral epitopes in

simian-human immunodeficiency virus-infected rhesus monkeys. J Virol 73:5466–5472, 1999.
19. Fernandez CS, Stratov I, De Rose R, Walsh K, Dale CJ, Smith MZ, Agy MB, Hu SL, Krebs K, Watkins DI, O'Connor DH, Davenport MP, Kent SJ: Rapid viral escape at an immunodominant SHIV CTL epitope exacts a dramatic fitness cost. J Virol 79:5721–5731, 2005.
20. Frahm N, Korber BT, Adams CM, Szinger JJ, Draenert R, Addo MM, Feeney ME, Yusim K, Sango K, Brown NV, Sengupta D, Piechocka-Trocha A, Simonis T, Marincola FM, Wurcel AG, Stone DR, Russell CJ, Adolf P, Cohen D, Roach T, Stjohn A, Khatri A, Davis K, Mullins J, Goulder PJ, Walker BD, Brander C: Consistent cytotoxic-T-lymphocyte targeting of immunodominant regions in human immunodeficiency virus across multiple ethnicities. J Virol 78:2187–2200, 2004.
21. Friedrich TC, Mcdermott AB, Reynolds MR, Piaskowski S, Fuenger S, De Souza IP, Rudersdorf R, Cullen C, Yant LJ, Vojnov L, Stephany J, Martin S, O'Connor DH, Wilson N, Watkins DI: Consequences of cytotoxic T-lymphocyte escape: common escape mutations in simian immunodeficiency virus are poorly recognized in naive hosts. J Virol 78:10064–10073, 2004.
22. Goulder PJ, Phillips RE, Colbert RA, Mcadam SN, Ogg GS, Nowak MA, Giangrande P, Luzzi G, Morgan B, Edwards A, Mcmichael AJ, Rowland-Jones SL: Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med 3:212–217, 1997.
23. Kaech SM, Hemby S, Kersh E, Ahmed R: Molecular and functional profiling of memory CD8 T cell differentiation. Cell 111:837–851, 2002.
24. Kent SJ, Dale CJ, Ranasinghe C, Stratov I, De Rose R, Chea S, Montefiori DC, Thomson S, Ramshaw IA, Coupar BEH, Boyle DB, Law M, Wilson KM, Ramsay AJ: Mucosally-administered Human-Simian Immunodeficiency Virus DNA and fowlpoxvirus-based recombinant vaccines reduce acute phase viral replication in macaques following vaginal challenge with CCR5-tropic $SHIV_{SF162P3}$. Vaccine 2005; in press.
25. 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 72:10180–10188, 1998.
26. Knapp LA, Lehmann E, Piekarczyk MS, Urvater JA, Watkins DI: A high frequency of Mamu-A*01 in the rhesus macaque detected by polymerase chain reaction with sequence-specific primers and direct sequencing. Tissue Antigens 50:657–661, 1997.
27. Koup RA, Safrit JT, Cao Y, Andrews CA, Mcleod G, Borkowsky W, Farthing C, Ho DD: 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, 1994.
28. Kuroda MJ, Schmitz JE, Barouch DH, Craiu A, Allen TM, Sette A, Watkins DI, Forman MA, Letvin NL: Analysis of Gag-specific cytotoxic T lymphocytes in simian immunodeficiency virus-infected rhesus monkeys by cell staining with a tetrameric major histocompatibility complex class I-peptide complex. J Exp Med 187:1373–1381, 1998.
29. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M, Tang Y, Holmes EC, Allen T, Prado JG, Altfeld M, Brander C, Dixon C, Ramduth D, Jeena P, Thomas SA, St John A, Roach TA, Kupfer B, Luzzi G, Edwards A, Taylor G, Lyall H, Tudor-Williams G, Novelli V, Martinez-Picado J, Kiepiela P, Walker BD, Goulder PJ: HIV evolution: CTL escape mutation and reversion after transmission. Nat Med 10:282–289, 2004.
30. Lobashevsky AL, Thomas JM: Six mamu-A locus alleles defined by a polymerase chain reaction sequence specific primer method. Hum Immunol 61:1013–1020, 2000.
31. Loffredo JT, Sidney J, Wojewoda C, Dodds E, Reynolds MR, Napoe G, Mothe BR, O'Connor DH, Wilson NA, Watkins DI, Sette A: Identification of seventeen new simian immunodeficiency virus-derived CD8+ T cell epitopes restricted by the high frequency molecule, Mamu-A*02, and potential escape from CTL recognition. J Immunol 173:5064–5076, 2004.
32. McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G, Vuola JM, Blanchard TJ, Gothard P, Watkins K, Hannan CM, Everaere S, Brown K, Kester KE, Cummings J, Williams J, Heppner DG, Pathan A, Flanagan K, Arulanantham N, Roberts MT, Roy M, Smith GL, Schneider J, Peto T, Sinden RE, Gilbert SC, Hill AV: Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. Nat Med 9:729–735, 2003.
33. McMichael AJ, Rowland-Jones SL: Cellular immune responses to HIV. Nature 410:980–987, 2001.
34. Miller MD, Yamamoto H, Hughes AL, Watkins DI, Letvin NL: Definition of an epitope and MHC class I molecule recognized by gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. J Immunol 147:320–329, 1991.
35. Mothé BR, Horton H, Carter DK, Allen TM, Liebl ME, Skinner P, Vogel TU, Fuenger S, Vielhuber K, Rehrauer W, Wilson N, Franchini G, Altman JD, Haase A, Picker LJ, Allison DB, Watkins DI: Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. J Virol 76:875–884, 2002.
36. Musey L, Hughes J, Schacker T, Shea T, Corey L, Mcelrath MJ: Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. N Engl J Med 337:1267–1274, 1997.
37. Nacsa J, Radaelli A, Edghill-Smith Y, Venzon D, Tsai WP, Morghen CDEG, Panicali D, Tartaglia J, Franchini G: Avipox-based simian immunodeficiency virus (SIV) vaccines elicit a high frequency of SIV-specific CD4+ and CD8+ T-cell responses in vaccinia-experienced SIVmac251-infected macaques. Vaccine 22:597–606, 2004.
38. O'Connor DH, Allen TM, Vogel TU, Jing P, Desouza IP, Dodds E, Dunphy EJ, Melsaether C, Mothe B, Yamamoto H, Horton H, Wilson N, Hughes AL, Watkins DI: Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat Med 8:493–499, 2002.
39. Peyerl FW, Bazick HS, Newberg MH, Barouch DH, Sodroski J, Letvin NL: Fitness costs limit viral escape from cytotoxic T lymphocytes at a structurally constrained epitope. J Virol 78:13901–13910, 2004.
40. Price DA, Goulder PJ, Klenerman P, Sewell AK, Easterbrook PJ, Troop M, Bangham CR, Phillips RE:

Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc Natl Acad Sci U S A 94:1890–1895, 1997.

41. Price DA, West SM, Betts MR, Ruff LE, Brenchley JM, Ambrozak DR, Edghill-Smith Y, Kuroda MJ, Bogdan D, Kunstman K, Letvin NL, Franchini G, Wolinsky SM, Koup RA, Douek DC: T cell receptor recognition motifs govern immune escape patterns in acute SIV infection. Immunity 21:793–803, 2004.
42. Sharpe SA, Cope A, Dowall S, Berry N, Ham C, Heeney JL, Hopkins D, Easterbrook L, Dennis M, Almond N, Cranage M: Macaques infected long-term with attenuated simian immunodeficiency virus (SIVmac) remain resistant to wild-type challenge, despite declining cytotoxic T lymphocyte responses to an immunodominant epitope. J Gen Virol 85:2591–2602, 2004.
43. Smith MZ, Dale CJ, De Rose R, Stratov I, Fernandez CS, Brooks AG, Weinfurter JT, Krebs K, Riek C, Watkins DI, O'Connor DH, Kent SJ: Analysis of pigtail macaque major histocompatibility complex class I molecules presenting immunodominant simian immunodeficiency virus epitopes. J Virol 79:684–695, 2005.
44. Yamamoto H, Miller MD, Tsubota H, Watkins DI, Mazzara GP, Stallard V, Panicali DL, Aldovini A, Young RA, Letvin NL: Studies of cloned simian immunodeficiency virus-specific T lymphocytes gag-specific cytotoxic T lymphocytes exhibit a restricted epitope specificity. J Immunol 144:3385–3391, 1990.
45. Yu XG, Addo MM, Rosenberg ES, Rodriguez WR, Lee PK, Fitzpatrick CA, Johnston MN, Strick D, Goulder PJ, Walker BD, Altfeld M: Consistent patterns in the development and immunodominance of human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T-cell responses following acute HIV-1 infection. J Virol 76:8690–8701, 2002.