J Med Primatol doi:10.1111/j.1600-0684.2008.00329.x

ORIGINAL ARTICLE

Safety, immunogenicity and efficacy of peptide-pulsed cellular immunotherapy in macaques

Robert De Rose[1], Rosemarie D. Mason[1], Liyen Loh[1], Viv Peut[1], Miranda Z. Smith[1], Caroline S. Fernandez[1], Sheilajen Alcantara[1], Thakshila Amarasena[1], Jeanette Reece[1], Nabila Seddiki[2], Anthony D. Kelleher[2], John Zaunders[2] & Stephen J. Kent[1]

1 Department of Microbiology and Immunology, University of Melbourne, Australia
2 Centre for Immunology, National Centre for HIV Epidemiology and Clinical Research, University of New South Wales, Australia

Keywords
Blood – Gag – immunotherapy – macaques – overlapping – peptide – SIV – vaccination

Correspondence
Prof S Kent, Department of Microbiology and Immunology, University of Melbourne, Australia.
Tel.: 61383449939;
fax: 61383443846;
e-mail: skent@unimelb.edu.au

Abstract

Background Simple and effective delivery methods for cellular immunotherapies are needed. We recently published on the effectiveness of using *ex vivo* pulsing of overlapping SIV Gag 15mer peptides onto fresh peripheral blood cells in 32 SIV_{mac251}-infected pigtail macaques.
Methods We now report on the safety of this approach, analysis of a novel assay for immunogenicity, the effect of an MHC allele, *Mane-A*10*, on CD8 T cell escape occurring and disease outcome.
Results The vaccine strategy was safe, with no perturbations in weight or hematological profiles in comparison to controls. The high levels of SIV-specific T cell immunogenicity of this approach was confirmed using a novel assay measuring upregulation of surface CD134 of CD4 T cells. A substantial effect of the *Mane-A*10* allele in reducing SIV viral load of pigtail macaques was observed in both vaccinees and controls; the virologic efficacy of the immunotherapy in comparison to controls was greatest in *Mane-A*10*− animals. Escape mutations at several new CD8 T cell epitopes throughout the SIV proteome were observed, primarily in animals with poorer virologic control.
Conclusions In summary, we provide further information that peptide-pulsed PBMC are a safe, immunogenic and effective immunotherapy. The observed influence of MHC alleles and immune escape allows us to design more insightful future immunotherapy studies.

Introduction

Immune control of HIV in humans and SIV in macaques can occur naturally but is rare. Substantial evidence links T cell immune responses with control of viremia [16, 17], but inducing more effective T cell immunity with practical immunotherapy techniques has been difficult. Several effective immunotherapy techniques rely on the infusion of isolated and cultured antigen-loaded dendritic cells, an approach not widely applicable [6, 22]. More practical HIV immunotherapies are required that do not necessitate extensive *ex vivo* manipulation of specialized blood cells. DNA and viral vector vaccines are also showing some promise as immunotherapeutic approaches in macaques [15, 18, 33], but these general approaches have not yet translated well into human trials, at least as effective prophylactic vaccination strategies.

We have been studying the intravenous re-infusion of fresh autologous peripheral blood mononuclear cells (PBMC) or whole blood pulsed with overlapping 15mer SIV peptides (termed 'OPAL'). This approach demonstrated remarkable immunogenicity in early macaque studies [5, 30]. We recently completed and published a large efficacy trial of peptide-pulsed PBMC in 32 SIV-mac251-infected pigtail macaques [10]. In this study, SIV-peptide pulsed PBMC induced high levels of T cell immunogenicity by intracellular cytokine staining techniques, and an approximately 10-fold reduction in viral load was observed in vaccinees in comparison to controls after withdrawal of antiretroviral therapy. Vaccinees were randomized to receive either just SIV Gag peptides (OPAL Gag group), or peptides spanning all 9 SIV proteins (OPAL All group). The OPAL All group had broader immunogenicity across multiple SIV proteins but responses to Gag were weaker in comparison to the OPAL Gag group. The virologic outcome of the OPAL All and Gag groups was almost identical [10].

Further analysis of that large study could help guide future studies and translation into human trials. First, the safety of any cellular immunotherapy will require close scrutiny in animal models prior to use in humans. Second, the immunogenicity of the peptide immunotherapy approach was studied with intracellular cytokine staining techniques; confirmation of immunogenicity using alternate methods not relying on cellular permeabilization is needed. Third, the overall virologic efficacy (approximately 1.0 log10 copies SIV RNA/ml reduction in viral load) was significant but there was a wide range of set point viral loads. Since MHC class I alleles are known to influence viral load through their restriction of CTL responses in humans and macaque species [14, 26, 28], we studied the influence of the *Mane-A*10* MHC I allele on virologic outcome. Lastly, mutational escape typically undermines CTL responses. We mapped a series of novel CTL epitopes in pigtail macaques and analysed for sequence changes within the epitopes.

Materials and methods

Animals

Juvenile pigtail macaques (*Macaca nemestrina*) free from Simian retrovirus type D were studied in protocols approved by institutional animal ethics committees and cared for in accordance with Australian National Health and Medical Research Council guidelines. At each time the animals were sedated, they were weighed and had hematology performed on peripheral blood using an ACT-diff coulter counter (Beckman-Coulter, Fullerton, CA). All pigtail macaques were typed for MHC class I alleles by reference strand mediated conformational analysis and the presence of *Mane-A*10* confirmed by sequence specific primer PCR as described [24, 27].

Infection and immunizations

Thirty six macaques were injected intravenously with 40 tissue culture infectious doses of SIV_{mac251} (kindly provided by R. Pal, Advanced Biosciences, Kensington, MD) as described previously [2, 26] and randomized into three groups of 12 animals (OPAL Gag, OPAL All, Controls), three weeks later. Four animals died with acute SIV infection prior to vaccinations. Animals received subcutaneous injections of dual antiretroviral therapy (ART) with tenofovir and emtricitibine (kindly donated by Gilead, Foster City, CA; both 30 mg/kg/animal) for seven weeks from week 3 [15, 18, 21, 25, 32]. Two animal groups (OPAL Gag and OPAL All) were immunized with OPAL immunotherapy using PBMC derived from 18 ml blood at weeks 4, 6, 8, 10 (i.e., under ART cover) as previously described [5, 10]. PBMC were isolated over Ficoll-paque from 18 ml of blood (anticoagulated with Na^{+}–Heparin). All isolated PBMC were suspended in 0.5 ml of normal saline to which either 125 SIV_{mac239} Gag peptides or 823 peptides spanning all SIV_{mac239} proteins (Gag, Pol, Env, Nef, Vif, Tat, Rev, Vpr, Vpx) were added at 10 μg/ml of each peptide within the pool. Peptides were 15mers overlapping by 11 amino acids at $>80\%$ purity kindly provided by the NIH AIDS reagent repository program (catalog numbers 6204, 6443, 6883, 6448-50, 6407, 8762, 6205). The peptide-pulsed PBMC were held for 1 hour in a 37°C waterbath, gently vortexed every 15 minutes and then, without washing, reinfused IV into the autologous animal. The 11 control macaques did not receive vaccine treatment.

Immunology assays

We studied a novel immunogenciity assay, termed the 'antigen-experienced cell' assay developed at the Centre for Immunology in Sydney where the antigen-specific surface expression of CD134 (OX40) and CD25 (IL-2 receptorα chain) are studied after 40 hours of *in vitro* culture (Zaunders et al., submitted for publication). Briefly, 0.25 ml of Na Heparin-anticoagulated whole blood was mixed with 0.25 ml Iscove's Modified Dulbecco's Medium (IMDM; JRH laboratories, BD, Sam Jose, CA) in 24-well plates (Becton Dickinson). Overlapping SIV peptides solubilized in DMSO (final concentration 1 μg/ml), an equivalent volume of control DMSO, or Phytohemagluttinin (PHA, 5 μg/ml, Sigma) were added and cultures were incubated, with the cap loosely attached, at 37°C for 40–44 hours in a humidified atmosphere of 5% CO_2 in air. In some experiments, cultures were performed in 24-well plates. At the end of the culture, 100 μl was stained with CD3-PerCP-Cy5.5, CD4-FITC, CD25-APC and CD134-PE (Becton-Dickinson, clones SP34-2, M-T477, 2A3 and L106, respectively) for 15 minutes at RT, treated with

Optilyse C (Beckman Coulter, Hialeah, FL) according to the manufacturer's directions, and washed once with 2 ml PBS (JRH). Cells were resuspended in 0.5 ml of 0.5% paraformaldehyde (Proscitech, Kirwan, Queensland) in PBS, and analysed on a dual-laser LSRII flow cytometer (Becton-Dickinson) using FACSDiva v4.1 software, as previously described [34]. T lymphocytes were first identified using a CD3-PerCP-Cy5.5 vs. side scatter gate, followed by gating on CD3+CD4+ T cells, which were then analysed for binding of CD25-APC and CD134-PE. Gates for CD25+ and CD134+ cells were based on comparison of negative control (no antigen) and positive control (PHA) cultures to include cells highly expressing CD25 plus positive for CD134+. A minimum of 50,000 events were analysed and compensation was checked as previously described [34].

We compared this novel 'antigen-experienced cell' immunogenicity assay with SIV-specific CD4 T-cell immune responses as analysed by expression of intracellular IFNγ as previously described [9]. Briefly, for the ICS assay 200 μl whole blood was incubated at 37°C with 1 μg/ml/peptide overlapping 15mer SIV Gag peptide pool or DMSO alone and the co-stimulatory antibodies anti-CD28 and anti-CD49d (BD Biosciences/Pharmingen San Diego CA) and Brefeldin A (10 μg/ml, Sigma) for 6 hours. Anti-CD3-PE, anti-CD4-FITC and anti-CD8-PerCP (BD, clones SP34-2, M-T477 and SK1 respectively) antibodies were added for 30 minutes. Red blood cells were lysed (FACS lysing solution, BD) and the remaining leukocytes permeabilized (FACS Permeabilizing Solution 2, BD) and incubated with anti-human IFNγ-APC antibody (BD, clone B27) prior to fixation and acquisition (LSRII, BD). The percentage of antigen-specific gated lymphocytes expressing IFNγ was assessed in $CD3^+CD4^+$ lymphocytes.

CD8 T cell epitopes were mapped by ICS on sequential blood samples as previously described [12, 23]. Briefly, positive responses to pools of peptides were mapped by studying progressively smaller peptide pools. When an individual or pair of 15mer peptides was identified as responding in some instances we purchased smaller peptides within the 15mer(s) to identify the minimal epitope.

Virology assays

Plasma SIV RNA (viral load, VL) was quantitated by real time PCR on 140 μl of plasma at the University of Melbourne (lower limit of quantitation 3.1 $\log_{10}$ copies/ml) at all time-points using a TaqMan probe as previously described [7, 9].

Sequencing across SIV-specific epitopes was performed as previously described. Briefly, plasma cDNA PCR amplification of SIV sequences was performed using specific primer pairs to conserved regions of the SIV genome. PCR conditions used Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) at 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, annealing temp for 20 sec and 72°C for 20 sec. A final phase of 72°C for 7 minutes completed the cycle. Adding Taq polymerase (Promega) to amplicons at 72°C for 10 minutes facilitated 'A' tailing. PCR products were cleaned using a Qiagen PCR spin kit. Amplicons were ligated into pGEM-T easy vector (Promega, Madison, WI) and transformed into *E. coli* JM109 (Promega) competent cells. Individual clones were sequenced by BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA) and sequence analysis done on Sequencher 4.1 (Gene Codes Corp, Ann Arbor, MI).

Results

Safety of OPAL immunotherapy

We recently reported the main immunologic and virologic outcomes of a large immunotherapy trial with SIV peptide pulsed PBMC [10]. The 32 pigtail macaques were infected with SIV_{mac251} and then vaccinated with SIV-Gag peptides (Gag, n = 10), peptides spanning all SIV proteins (All, n = 11) pulsed onto fresh PBMC derived from 18 ml of blood or left as unvaccinated controls (n = 11) [10]. The immunotherapy resulted in high levels of SIV-specific CD4 and CD8 T cells, a 10-fold reduction in SIV viral load in both the Gag and All groups and delayed progression to AIDS-related mortality [10].

This immunotherapy approach is now heading towards human trials. An issue to be addressed in both preclinical and human trials is the safety of this approach. We therefore analyzed the weights of the animals and hematolgic profiles of hemoglobin, white cell counts and platelets prior to and throughout the vaccination period (Fig. 1). The animals remained healthy and there were no differences in mean weights of hematologic profiles between the two vaccine groups and controls throughout follow up. The animals were juvenile and gained weight normally throughout follow up (Fig. 1A). Mean hemoglobin levels dipped slightly in the first few weeks after SIV infection, but were similar in controls and vaccinees (Fig. 1B). Similarly, platelets and total white cell counts dipped uniformly in all the three groups at week 2 of SIV infection (prior to ART or vaccinations, Figs 1C and D) but remained fairly steady thereafter; again there were no differences between vaccinees and controls. Thus, although acute SIV infection

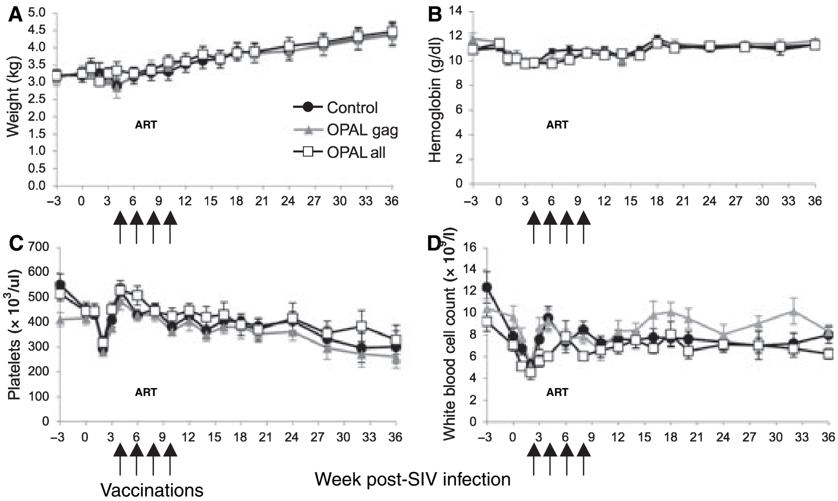

Fig. 1. Safety of OPAL immunotherapy. Pigtail macaques were infected with SIV_{mac251} (week 0) and randomly assigned to controls (n = 11), OPAL Gag immunized animals (n = 10) or OPAL All immunized animals (n = 11) and serially monitored for weight (A), hemoglobin (B), platelets (C) and white cell count (D). All animals received dual ART weeks 3–10 and vaccinations occurred at weeks 4, 6, 8 and 10 after SIV_{mac251} infection.

has an effect on hematology parameters, this was not compounded by the OPAL vaccinations.

CD4 T cell immunogenicity of OPAL immunotherapy

Our previous reports on OPAL immunotherapy have primarily analysed immunogenicity by intracellular expression of various cytokines after short incubation times *ex vivo*. Although a robust measure of T cell immunity, the assay is reasonably labor intensive, requiring a fixation and permeabilization step. Furthermore, background responses in unstimulated control wells can occasionally be problematic. We recently developed a novel flow cytometry based assay to measure antigen-specific CD4 T cells. Here, expression of surface CD134 (OX40, a costimulatory molecule) and CD25 (the IL-2 receptor α chain) are measured after 40 hours of culture. The assay is much simpler, does not rely on T cell expression of a particular cytokine, and typically has low background levels of activation (Zaunders et al., submitted for publication).

At 4 weeks after the set of OPAL vaccinations, we measured CD4 T cell immunogenicity by this novel antigen-experienced cell (AEC) assay to multiple SIV antigens on fresh blood samples from all 32 animals. CD134+25+ cells were low in control, unstimulated wells and were generally robust in mitogen-stimulated cultures. Representative examples of flow cytometry plots of gated CD4 T cells are shown in Fig. 2A. SIV-specific CD4 T cell responses were modest in control SIV-infected animals. CD4 T cell responses to SIV Gag antigens were generally strongest in animals administered only Gag peptides (OPAL Gag group) but broader in animals administered peptides spanning all nine SIV proteins (OPAL All group).

The observed pattern of immunogenicity was broadly similar to that observed with the ICS assay previously published [10]. We therefore directly compared results from the antigen-experienced cell assay to the IFNγ ICS assay performed on the same day (Fig. 2B). There was a significant correlation between results from the two assays, suggesting both assays reliably detect antigen-specific CD4+ T cells.

Influence of Mane-A*10 on outcome of OPAL immunotherapy

OPAL immunotherapy with either Gag peptides or peptides spanning all nine SIV proteins resulted in a durable 10-fold reduction in VL [10]. In this study, we randomly stratified animals across the three groups for the presence of *Mane-A*10*, since we had previously shown this allele restricts a highly dominant Gag-specific CTL response and unvaccinated pigtail macaques possessing this allele have lower VLs than animals without this allele [12, 26, 27]. The *Mane-A*10* allele was present in 13 (36%) of animals starting the trial, consistent with our previous observations on the prevalence of this allele in unselected pigtail macaques [24]. The 13 *Mane-A*10*+ animals were randomly allocated to four in the control group, four in the OPAL Gag group and five in the OPAL All group.

To assess the overall effect of *Mane-A*10*, on the outcome across all 32 animals studied out to 36 weeks, we compared VLs and total peripheral CD4 T cell counts in the 13 *Mane-A*10*+ animals to the 19 *Mane-A*10*− animals (Figs 3A and B). Viral load was significantly higher in the *Mane-A*10*− animals; mean peak VL was 0.7 log10 copies/ml higher in *Mane-A*10*+ group compared to *Mane-A*10*− group, and set point VL was

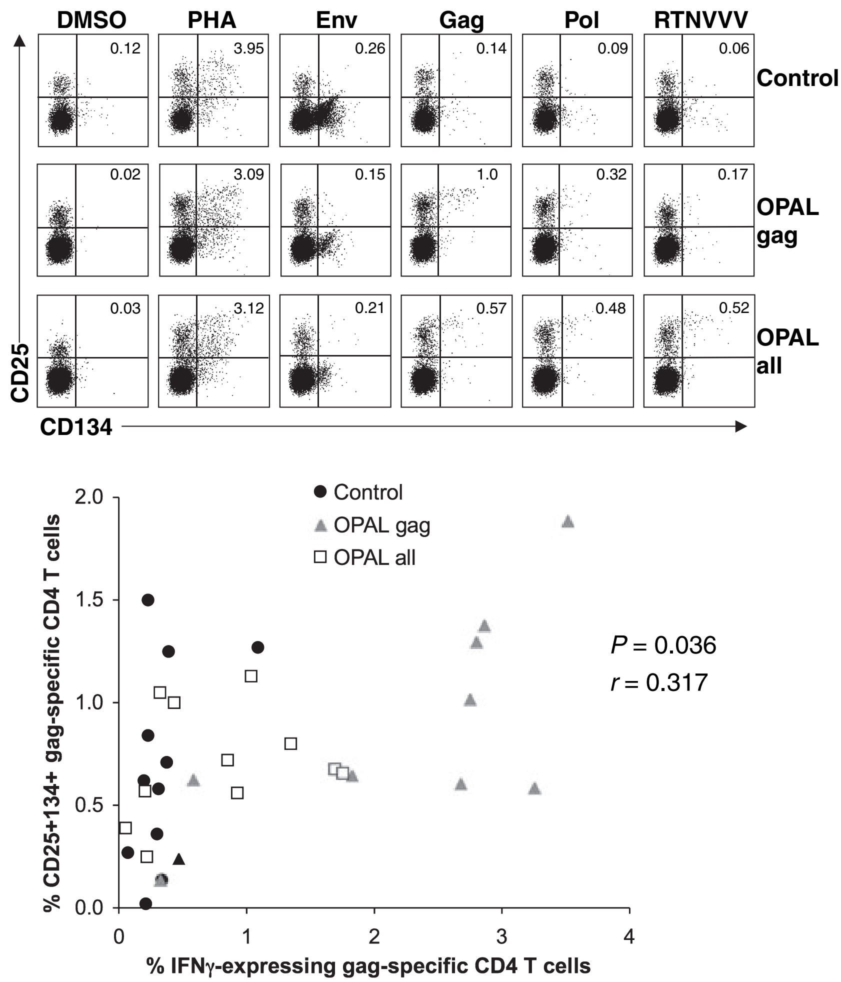

Fig. 2. Immunogenicty of OPAL immunotherapy. We evaluated a novel assay studying the upregulation of surface CD134 and CD25 on CD4+ T cells after 40 hours *in vitro* stimulation of whole blood obtained at week 14 with peptide pools spanning SIV proteins. (A) Flow cytometry plots show representative control, OPAL Gag, and OPAL All immunized animals. Stimulation of blood with overlapping peptides spanning Env, Gag, Pol or a combined pool of the six regulatory/accessory SIV proteins (Rev, Tat, Nef, Vif, Vpx and Vpr, named RTNVVV). The percent of CD134+25+ cells from within the CD4+3+ lymphocyte gate is shown in the upper right quadrant. (B) Correlation of Gag-specific CD4 T cell responses by IFNγ expression using intracellular cytokine staining and by expression of CD25 and CD134 by the antigen experienced cell assay. The different symbols show animals that received control, OPAL Gag or OPAL All vaccinations, illustrating the stronger Gag-specific responses induced in the OPAL Gag group, despite the same dose of Gag peptides used in the immunizations.

consistently 0.6–0.9 $\log_{10}$ copies/ml higher in *Mane-A*10*+ animals throughout the 26 weeks of follow up off ART. Concomitantly, there was a significantly faster decline in peripheral CD4 T cells, which did not fall at all in the *Mane-A*10*+ animals (1% above baseline in this group) but declined 19.3% from baseline in the *Mane-A*10*− animals by 36 weeks after SIV infection.

We then addressed the issue of the effect of the *Mane-A*10* allele on VLs within each vaccine group. The comparisons are now with smaller groups and the error bars larger, however some distinct effects were observed (Fig. 3C). In the controls (circles in Fig. 3C), the mean set point VL was as expected much higher in the *Mane-A*10*− animals compared to the *Mane-A*10*+ animals (difference of 1.3 $\log_{10}$ copies/ml at week 36). The same trend (lower VLs in the *Mane-A*10*+ animals) was observed in the OPAL All group (difference of 1.0 $\log_{10}$ copies/ml at week 36) but, interestingly, not the OPAL Gag group. Remarkably, the VL in the *Mane-A*10*+ controls was indistinguishable from *Mane-A*10*+ animals in the OPAL All group and actually slightly *lower* than the *Mane-A*10*+ animals in the OPAL Gag group. Thus, the net effect of including a fairly representative proportion of *Mane-A*10*+ macaques was to substantially *reduce* the power of the study to detect differences between the controls and vaccines since the *Mane-A*10*+ controls do well without vaccination.

Mutations within CTL epitopes

An Achilles heel of vaccination strategies based on induced CD8 T cell immunity is the ability of the virus to mutate to escape CTL control. We previously showed that the dominant $SIV_{164–172}$ Gag 9 amino acid epitope presented by *Mane-A*10* in pigtail macaques, termed KP9, frequently undergoes mutational escape [12]. The mutated KP9 epitope reverts back to wild-type when passaged into naïve pigtail macaques [13]. Our group has begun to explore Gag epitopes outside KP9 [19] but until this study had not mapped epitopes in other SIV proteins.

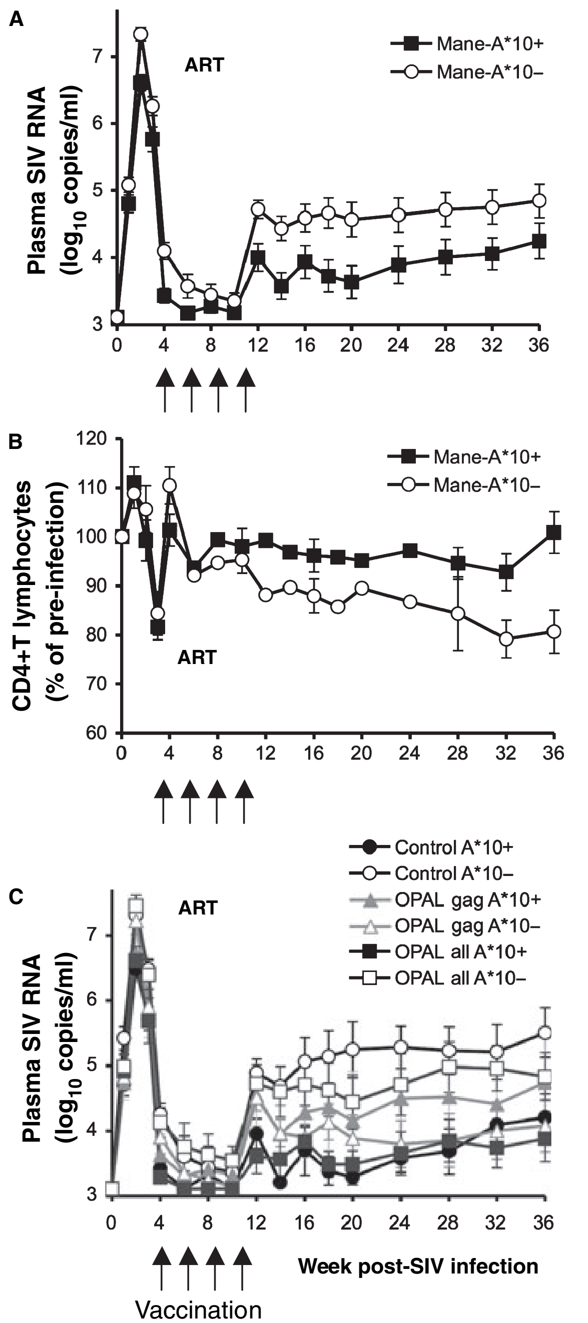

Fig. 3. Effect of the *Mane-A*10* allele on outcome. (A) Viral load (mean ± SE) in the 13 *Mane-A*10+* pigtail macaques compared to the 19 *Mane-A*10–* macaques. (B) CD4 T cell proportions (mean ± SE) in the *Mane-A*10+* macaques compared to the *Mane-A*10–* macaques, expressed as a percent of baseline pre-infection levels. (C) Viral Load (mean ± SE) in the *Mane-A*10+* (closed symbols) and *Mane-A*10–* macaques (open symbols) divided by vaccine group (controls in circles, OPAL Gag in triangles, and OPAL All in squares).

We mapped a series of CTL epitopes in the SIV Tat, Pol, and Nef proteins by using progressively smaller pools of overlapping 15mer peptides. We identified 14 novel epitopes, including two in Tat shared by multiple animals (Table 1).

We then sequenced plasma virus at around week 20 of infection (10 weeks after withdrawal of ART) to determine whether there were any mutations away from wild-type sequence. We first attempted to sequence across KP9 in all 13 *Mane-A*10+* animals. In four animals no useful sequence information was obtained; all of these animals had undetectable levels of plasma viremia in our VL assay (Table 1). In the other nine animals, eight had strong evidence of immune escape, with seven having the canonical K165R mutation and one the P172S mutation, both previously shown to be immune escape mutations [12, 29]. The one animal that did not have immune escape mutations identified was the only other *Mane-A*10+* animal to control VL to the limits of detection (<3.1 $\log_{10}$ copies/ml, shaded in Table 1).

To assess the other novel CTL epitopes for potential immune escape, we also sequenced across each of these epitopes in animals responding at the epitope (Table 1). In the 12 epitopes recognized by the total of 30 animals, we identified mutations within the epitopes on 14 occasions. In four occasions where we did not detect mutations despite the presence of readable sequence, the VL was undetectable (shaded in Table 1). Overall, we detected potential or proven immune escape mutations in 14 of all 23 (61%) CTL responses identified, and in 14 of 19 (74%) of CTL responses where there was detectable viremia.

Discussion

This report complements and expands our recent findings on a peptide-pulsed blood cell immunotherapy in pigtail macaques [10]. We also recently showed that peptide-pulsing of unfractionated whole blood is similarly immunogenic compared to peptide-pulsing PBMC and offers a simpler delivery technique [11]. We now present in more detail the safety of the vaccine strategy and confirm the high level of CD4 T cell immunogenicity of this approach with a novel antigen experienced cell assay. We illustrate the considerable effect of the *Mane-A*10* allele on the virologic and immunologic outcomes of this study and show how immune escape likely undermines some of the benefit of this immunotherapy. This immunotherapy technique is proceeding towards human trials using overlapping HIV Gag peptides.

The safety of the approach was remarkable given that controls did not have the same volume of blood taken or re-infusion procedures. We reasoned before starting the study that it would be a more rigorous test of safety of the entire procedure to not take blood or

J Med Primatol **37** (Suppl 2) (2008) 69–78 © 2008 The Authors
Journal compilation © 2008 Blackwell Munksgaard

Table 1 Immune escape following OPAL immunotherapy

Epitope	Source/animal	Group	Week	Viral sequence[2]	Likely escape	Controlled Virus at w20
KP9[1] (Gag)	SIV_{mac251}		0	KKFGAEVVP		
	8014	Control	20	.R.......	Yes	N (3.61)
	9176	Control	20	....	No[4]	Y (3.11)
	9183	Control	20	.R.......	Yes	N (3.36)
	9017	Control	20	No amp[3]	n/a	Y (3.11)
	8020	Gag	20	.R.......	Yes	N (3.39)
	8241	Gag	20	........S	Yes	N (6.35)
	8244	Gag	20	.R.......	Yes	N (3.80)
	8454	Gag	20	No amp[3]	n/a	Y (3.11)
	1.3731	All	20	.R.......	Yes	N (3.97)
	9021	All	20	.R.......	Yes	N (4.00)
	9175	All	20	.R.......	Yes	N (3.23)
	8240	All	20	No amp[3]	n/a	Y (3.11)
	9020	All	20	No amp[3]	n/a	Y (3.11)
Tat 5429	9017,8020, 8454,9021		2	TPKKAKANTSSASNK		
	9017	Control	24	No amp[3]	n/a	Y (3.11)
	8020	Gag	24	....	No	N (3.39)
	8454	Gag	24	......T........	Yes	Y (3.11)
	9021	All	18	No amp[3]	n/a	N (4.00)
Tat 5435-6	9176,8240, 9021		2	AKKETVEKAVA		
	9176	Control	20	....	No	Y (3.11)
	8240	All	18	.E.........	Yes	Y (3.11)
	9021	All	18	..E......M.	Yes	N (4.00)
Pol 5734-5	2.3308	All	2	QGGDRGFAAPQ		
			24	...N...V...	Yes	N (5.44)
Nef 8594-5	6169	Control	2	NQGQYMNTPWR		
			24	..E........	Yes	Y (3.11)
Nef 8631-2	9017	Control	2	EVLAWKFDPTL		
			24	....	No	Y (3.11)
Nef 8582	9176	Control	2	RSKPAGDLRQRLLRA		
				....	No	Y (3.11)
Nef 8639	9176	Control	2	SGLSEEEVRRRLTAR		
			20	....	No	Y (3.11)
Nef 8606	8012	Gag	2	RVPLRTMSYKLAIDM		
			18	....	No	N (5.12)
Nef 8614	1.3731	All	2	RRHRILDMYLEKEEG		
			24	....	No	N (3.97)
Nef 8611	8251	All	2	EKGGLEGIYYSARRH		
			18	.....G.........	Yes	N (4.72)
Nef 8621	8680	All	2	IRYPKTFGWLWKLVP		
			20	....	No	N (3.32)
Proportion of responses with likely escape				All epitopes	14/23 (61%)	
				Epitopes where VL detectable	14/18 (78%)	

[1]Escape at KP9 was studied in all 13 *Mane-A*10*+ pigtail macaques. (.) denotes wild-type; [2]Amino acids denote CD8+ T-cell epitopes. Tat, Pol and Nef epitopes have been mapped to individual or paired overlapping 15mers peptides (e.g., 5429, using the designation of the NIH AIDS reagent reposi tory) present within the peptide pool. The SIV_{mac251} challenge stock or viral sequences from week 2 after infection (before escape is likely) are used as the reference wild-type sequence for each epitope; [3]No amplification of viral PCR product; [4]Shaded responses indicate no escape where VL is controlled

mock re-infuse the controls, since some adverse effects could have resulted from excess blood sampling (18 ml at each occasion for the ~4 kg macaques) or complications of incubating the PBMC and the reinfusion process. Despite being conducted shortly after acute SIV infection, the vaccination procedures were safe. This should engender considerable confidence moving into clinical trials.

The novel antigen-experienced cell assay demonstrated the robust CD4 T cell immunogenicity of the vaccinations and the assays correlated well with standard ICS assay. The antigen-experienced cell assay is much simpler than the ICS assay since the permeabilization steps are not required. Since live cells can theoretically be gated on, further sorting of cells for advanced immunology studies are possible in future studies, a significant advantage over ICS based studies.

The effect of *Mane-A*10* on the outcome of this immunotherapy study was remarkable and helps guide future pigtail macaque studies. We confirmed the significant benefit in virologic control of SIV afforded by *Mane-A*10*. Presumably this corresponds to *Mane-A*10* restricting the dominant Gag CTL epitope KP9. Although this epitope can escape, and indeed escape at KP9 was very common in this study, escape comes at a significant fitness cost, illustrated by the reversion of KP9 escape mutations upon passage to naive macaques [12, 19]. Although we adequately stratified macaques in this study for *Mane-A*10*, the remarkable control of viremia in *Mane-A*10+* controls meant that the overall virologic efficacy of the approach was diluted, since the benefit of vaccination was primarily observed only in approximately two-thirds of the animals that were *Mane-A*10−*. The marginally higher VLs in *Mane-A*10+* animals in the OPAL Gag group compared to the *Mane-A*10−* animals in the same group was curious. We have recently speculated that for some CTL epitopes where the benefit of CTL efficacy against virus infected cells is late, a possible effect of vaccination could be to 'speed up' escape (i.e., encourage it to occur earlier) which might be counterproductive on viral control [8, 20]. Similar observations have been made on the effect of *HLA-B*27* on control of HIV in humans, where there is a late beneficial effect of *HLA-B*27*-restricted CTL responses [14], but, at least in an anecdote reported, bringing the response to bear earlier by vaccination had a detrimental effect on HIV infection [3].

Mutations within CTL epitopes are encouraged by active virus replication, much as the effect of partial control of viremia by antiretroviral drugs promotes drug resistance. We show that the majority of CTL mutations occur in animals with detectable viremia and that CTL mutations are less common in animals without detectable viremia. An implication of this work is that CTL responses need to be either effective enough as individual responses, or combined with other immune responses, to control viremia otherwise they will likely be ultimately undermined. Although a 'fitness cost' usually comes with CTL escape, in some flexible proteins this can be quite modest [23, 31], or compensated at other parts of the relevant protein [4]. Functionally highly constrained proteins such as parts of Gag probably incur the greatest fitness cost of escape and may make the best CTL vaccine or immunotherapy targets [1]. This may underlie the overall equivalent effectiveness of the OPAL Gag strategy, where stronger Gag responses were generated, in comparison to the OPAL All where broader overall responses, but weaker Gag responses, were observed [10].

In summary, we extend our findings on OPAL immunotherapy in macaques and add to the compelling set of safety, immunogenicity and effectiveness data towards moving this relatively simple immunotherapy approach into clinical trials.

Conflicts of interest

Dr Kent spun out a start-up biotech company, OPAL Therapeutics to advance this technology into clinical trials. He and the University of Melbourne hold shares in this company.

References

1 Altfeld M, Allen TM: Hitting HIV where it hurts: an alternative approach to HIV vaccine design. *Trends Immunol* 2006; **27**:504–10.

2 Batten CJ, Rose RD, Wilson KM, Agy MB, Chea S, Stratov I, Montefiori DC, Kent SJ: Comparative evaluation of simian, simian-human, and human immunodeficiency virus infections in the Pigtail Macaque (Macaca nemestrina) model. *AIDS Res Hum Retroviruses* 2006; **22**:580–8.

3 Betts MR, Exley B, Price DA, Bansal A, Camacho ZT, Teaberry V, West SM, Ambrozak DR, Tomaras G, Roederer M, Kilby JM, Tartaglia J, Belshe R, Gao F, Douek DC, Weinhold KJ, Koup RA, Goepfert P, Ferrari G: Characterization of functional and phenotypic changes in anti-Gag vaccine-induced T cell responses and their role in protection after HIV-1 infection. *Proc Natl Acad Sci USA* 2005; **102**:4512–17.

4 Brockman MA, Schneidewind A, Lahaie M, Schmidt A, Miura T, Desouza I, Ryvkin F, Derdeyn CA, Allen S, Hunter E, Mulenga J, Goepfert PA, Walker BD, Allen TM: Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. *J Virol* 2007; **81**:12608–18.

5 Chea S, Dale CJ, De Rose R, Ramshaw IA, Kent SJ: Enhanced cellular immunity in macaques following a novel peptide immunotherapy. *J Virol* 2005; **79**:3748–57.

6 Connolly NC, Whiteside TL, Wilson C, Kondragunta V, Rinaldo CR, Riddler SA: Therapeutic Immunization

with HIV-1 Peptide-Loaded Dendritic Cells is Safe and Immunogenic in HIV-1-Infected Individuals. *Clin Vaccine Immunol* 2007.

7 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 virus-like-particle vaccines: immunogenicity and protective efficacy in macaques. *Virology* 2002; **301**:176–87.

8 Davenport M, Loh L, Petravic J, Kent SJ Rates of HIV immune escape and reversion: implications for vaccination. *Trends Microbiol* 2008; in press.

9 De Rose R, Batten CJ, Smith MZ, Fernandez CS, Peut V, Thomson S, Ramshaw IA, Coupar BE, Boyle DB, Venturi V, Davenport MP, Kent SJ: Comparative efficacy of subtype AE simian-human immunodeficiency virus priming and boosting vaccines in Pigtail Macaques. *J Virol* 2007; **81**:292–300.

10 De Rose R, Fernandez CS, Smith MZ, Batten CJ, Alcantara S, Peut V, Rollman E, Loh L, Mason RD, Wilson CM, Law MG, Handley AJ, Kent SJ: Control of viremia following immunotherapy of SIV-infected macaques with peptide pulsed blood. *Plos Pathogens* 2008; **4**:e12.

11 De Rose R, Fernandez CS, Loh L, Peut V, Mason RD, Alcantara S, Reece J, Kent SJ: Delivery of immunotherapy with peptide-pulsed blood in macaques. *Virology* 2008; **187**:204–10.

12 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 simian-human immunodeficiency virus cytotoxic T-lymphocyte epitope exacts a dramatic fitness cost. *J Virol* 2005; **79**:5721–31.

13 Fernandez CS, Smith MZ, Batten CJ, De Rose R, Reece JC, Rollman E, Venturi V, Davenport MP, Kent SJ: Vaccine-induced T cells control reversion of AIDS virus immune escape mutants. *J Virol* 2007; **81**:4137–44.

14 Gao X, Bashirova A, Iversen AK, Phair J, Goedert JJ, Buchbinder S, Hoots K, Vlahov D, Altfeld M, O'Brien SJ, Carrington M: AIDS restriction HLA allotypes target distinct intervals of HIV-1 pathogenesis. *Nat Med* 2005; **11**:1290–2.

15 Hel Z, Venzon D, Poudyal M, Tsai WP, Giuliani L, Woodward R, Chougnet C, Shearer G, Altman JD, Watkins D, Bischofberger N, Abimiku A, Markham P, Tartaglia J, Franchini G: Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. *Nat Med* 2000; **6**:1140–6.

16 Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, Ho DD: Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 1999; **189**:991–8.

17 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* 1994; **68**:4650–5.

18 Lisziewicz J, Trocio J, Xu J, Whitman L, Ryder A, Bakare N, Lewis MG, Wagner W, Pistorio A, Arya S, Lori F: Control of viral rebound through therapeutic immunization with DermaVir. *Aids* 2005; **19**:35–43.

19 Loh L, Batten CJ, Petravic J, Davenport MP, Kent SJ: In vivo fitness costs of different Gag CD8 T cell escape mutant simian-human immunodeficiency viruses in macaques. *J Virol* 2007; **81**:5418–22.

20 Loh L, Petravic J, Batten CJ, Davenport MP, Kent SJ: Vaccination and timing influence SIV immune escape viral dynamics in vivo. *PLoS Pathog* 2008; **4**:e12.

21 Lori F, Lewis MG, Xu J, Varga G, Zinn DE Jr, Crabbs C, Wagner W, Greenhouse J, Silvera P, Yalley-Ogunro J, Tinelli C, Lisziewicz J: Control of SIV rebound through structured treatment interruptions during early infection. *Science* 2000; **290**:1591–3.

22 Lu W, Wu X, Lu Y, Guo W, Andrieu JM: Therapeutic dendritic-cell vaccine for simian AIDS. *Nat Med* 2003; **9**:27–32.

23 Peut V, Kent SJ: Utility of human immunodeficiency virus type 1 envelope as a T-cell immunogen. *J Virol* 2007; **81**:13125–34.

24 Pratt BF, O'Connor DH, Lafont BA, Mankowski JL, Fernandez CS, Triastuti R, Brooks AG, Kent SJ, Smith MZ: MHC class I allele frequencies in pigtail macaques of diverse origin. *Immunogenetics* 2006; **58**:995–1001.

25 Shen A, Zink MC, Mankowski JL, Chadwick K, Margolick JB, Carruth LM, Li M, Clements JE, Siliciano RF: Resting CD4+ T lymphocytes but not thymocytes provide a latent viral reservoir in a simian immunodeficiency virus-Macaca nemestrina model of human immunodeficiency virus type 1-infected patients on highly active antiretroviral therapy. *J Virol* 2003; **77**:4938–49.

26 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* 2005; **79**:684–95.

27 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**:282–93.

28 Smith MZ, Kent SJ: Genetic influences on HIV infection: implications for vaccine development. *Sexual Health* 2005; **2**:53–62.

29 Smith MZ, Asher TE, Venturi V, Davenport MP, Douek DC, Price DA, Kent SJ: Limited maintenance of vaccine-induced simian immunodeficiency virus-specific CD8 T-cell receptor clonotypes after virus challenge. *J Virol* 2008; **82**:7357–68.

30 Stratov I, Dale CJ, Chea S, McCluskey J, Kent SJ: Induction of T-cell immunity to antiretroviral drug-resistant human immunodeficiency virus type 1. *J Virol* 2005; **79**:7728–37.

31 Troyer RM, Collins KR, Abraha A, Fraundorf E, Moore DM, Krizan RW, Toossi Z, Colebunders RL, Jensen MA, Mullins JI, Vanham G, Arts EJ: Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. *J Virol* 2005; **79**:9006–18.

32 Villinger F, Brice GT, Mayne AE, Bostik P, Mori K, June CH, Ansari AA: Adoptive transfer of simian immunodeficiency virus (SIV) naive autologous CD4(+) cells to macaques chronically infected with SIV is sufficient to induce long-term nonprogressor status. *Blood* 2002; **99**:590–9.

33 von Gegerfelt AS, Rosati M, Alicea C, Valentin A, Roth P, Bear J, Franchini G, Albert PS, Bischofberger N, Boyer JD, Weiner DB, Markham P, Israel ZR, Eldridge JH, Pavlakis GN, Felber BK: Long-lasting decrease in viremia in macaques chronically infected with simian immunodeficiency virus SIVmac251 after therapeutic DNA immunization. *J Virol* 2007; **81**:1972–9.

34 Zaunders JJ, Dyer WB, Wang B, Munier ML, Miranda-Saksena M, Newton R, Moore J, Mackay CR, Cooper DA, Saksena NK, Kelleher AD: Identification of circulating antigen-specific CD4+ T lymphocytes with a CCR5+, cytotoxic phenotype in an HIV-1 long-term nonprogressor and in CMV infection. *Blood* 2004; **103**:2238–47.

