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 SIVmac251-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 vaccines in comparison to controls after withdrawal of antiretroviral therapy. Vaccines 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 *Mae-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 analyzed 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 *Mae-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, Kensing-ton, 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 emtricitabine (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) from Simian retrovirus type D were studied in protocols. 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 immunogenicity 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 receptor) 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 Phytohemagglutinin (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% CO2 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 any 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 log10 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 pigtailed macaques were infected with SIVmac251 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 hematologic 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...
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 \( \alpha \) 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 measure antigen-specific CD4 T cells. Here, expression of surface CD134 (OX40, a costimulatory molecule) and CD25 (the IL-2 receptor \( \alpha \) 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).

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
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 indistinguish-
able from Mane-A*10+ animals in the OPAL All

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 SIV164–172 Gag 9 amino
acid epitope presented by Mane-A*10 in pigtail maca-
ques, 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.
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 log₁₀ 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
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.

Table 1 Immune escape following OPAL immunotherapy

<table>
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<tr>
<th>Epitope</th>
<th>Source/animal</th>
<th>Group</th>
<th>Week</th>
<th>Viral sequence$^2$</th>
<th>Likely escape</th>
<th>Controlled Virus at w20</th>
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</tr>
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<tr>
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<td>(3.11)</td>
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<tr>
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<td>Y/a</td>
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<tr>
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</table>

Proportion of responses with likely escape

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 repository) present within the peptide pool. The SIVmac251 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.

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


