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Rapid Communication

Delivery of immunotherapy with peptide-pulsed blood in macaques

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ABSTRACT

Simple and effective delivery methods for cellular immunotherapies are needed. We assessed *ex vivo* pulsing of overlapping SIV Gag 15mer peptides onto either whole blood or PBMC in 15 randomly assigned SIV-infected macaques. Both delivery methods were safe and immunogenic, stimulating high levels of broad and polyfunctional Gag-specific CD4 and CD8 T cells. Delivery of overlapping Gag peptides via either whole blood or PBMC is suitable for clinical evaluation.

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Introduction

Practical and effective HIV immunotherapies are needed not requiring *ex vivo* manipulation of specialized blood cells (Lu et al., 2003). Intravenous re-infusion of fresh autologous peripheral blood mononuclear cells (PBMC) or whole blood (WB) pulsed with overlapping SIV peptides (termed "OPAL") has been highly immunogenic in early pilot studies (Chea et al., 2005) and, when delivered onto PBMC, effective in reducing viral load and delaying SIV-induced AIDS in monkeys (De Rose et al., 2008). However, delivery of peptides via whole blood has not been compared for immunogenicity head-to-head with delivery of peptides via PBMC. If similarly immunogenic, pulsing WB is far preferred as a delivery method since no laboratory separation of PBMC is required, permitting a bedside technique that would be much more widely available to HIV-infected people.

Results

To compare the immunogenicity of peptide-pulsed whole blood with peptide-pulsed PBMC, we studied the 20 animals remaining from a previous immunotherapy trial (De Rose et al., 2008). In the prior study, 32 pigtail macaques were infected with SIV_{mac251} and then vaccinated with SIV Gag peptides (Gag, $n=10$), peptides spanning all

SIV proteins (All, $n=11$) or control ($n=11$) (De Rose et al., 2008). 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 (De Rose et al., 2008).

Sixty-four weeks after SIV infection, all 15 remaining, previously immunized, SIV-infected macaques were re-randomized (8:7) to receive 3 doses of 125 overlapping SIV Gag 15mer peptides (10 µg/ml) pulsed for 1 h at 37 °C onto either heparin-anticoagulated WB (9 ml) or PBMC (derived from 9 ml blood, in 0.5 ml saline) (Chea et al., 2005; De Rose et al., 2008). The animals were stratified based on prior immunizations, baseline VL, CD4 T cells, Mane-A*10 status and Gag-specific T-cell responses (Table 1). The 5 animals remaining from the control group were followed but not re-immunized.

OPAL immunization using Gag peptides pulsed onto PBMC or WB was safe with no differences between the arms in body weight or hematology profiles (not shown). The OPAL vaccinations, with both WB and PBMC, stimulated considerable Gag-specific CD4 and CD8 T-cell immunity. Similar levels of T-cell immunity were induced by the two vaccination methods (Figs. 1A, B, $P>0.2$, 2-sided *t*-tests, for all comparisons). Gag-specific CD4 T-cell responses in the PBMC group rose by a mean of 2.29% 4 weeks after the first vaccination and 1.96% 7 weeks after the first vaccination, compared to 1.94% and 0.98% respectively in the WB group. For the Gag-specific CD8 T-cell response there was a 1.89% and 1.53% mean rise in response in the PBMC group 4 and 7 weeks after the initial vaccination respectively and 1.24% and 0.78% rise in the WB group respectively. Gag-specific CD4 T-cell

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Table 1
Baseline characteristics of macaques

Group	Macaque ID	Mane-A*10 status	Prior immunizations ^a	VL week 0 ^b (log ₁₀ copies/ml)	CD4 T cells week 0 (% CD4 ⁺ T lymphocytes)
Control	6169	Negative	None	5.89	24.5
	8014	Positive	None	6.05	22.2
	9017	Positive	None	3.29	36.6
	9176	Positive	None	3.34	33.3
	9183	Positive	None	6.14	23.2
			Mean of group	4.94	28.0
		SE	0.67	2.9	
PBMC	1.3731	Positive	All	3.87	35.5
	6804	Negative	Gag	4.45	19.6
	8244	Positive	Gag	5.34	22.8
	8673	Negative	Gag	3.11	33.3
	8682	Negative	All	4.54	36.9
	9021	Positive	All	5.12	28.8
	9175	Positive	All	5.36	22.7
	9196	Negative	Gag	4.46	15.2
			Mean of group	4.53	26.9
			SE	0.27	2.8
WB	8020	Positive	Gag	6.29	23.0
	8240	Positive	All	3.19	27.0
	8251	Negative	All	5.83	20.2
	8454	Positive	Gag	3.11	27.7
	8680	Negative	All	5.75	22.4
	8873	Negative	Gag	3.21	28.9
	9020	Positive	All	3.11	38.6
			Mean of group	4.36	26.8
		SE	0.57	2.3	

^a Week 0 = first WB vs PBMC vaccination, which was week 64 after SIV infection.

^b Immunized animals previously received either Gag or All SIV peptides pulsed onto PBMC at weeks 4, 6, 8, 10, 36, 39, and 42 after SIV infection.

responses rose >0.5% in 6 of the 8 PBMC vaccinated animals compared to 3 of the 7 WB-immunized animals. Gag-specific CD8 responses rose >0.5% in 4 of 8 animals in the PBMC group and 4 of 7 in the WB group. There was no rise in CD4 or CD8 T-cell responses in the Control group.

Since the animals had been previously immunized with OPAL vaccinations, we were able to compare the effect of this set of immunizations with those administered previously. The rises in Gag-specific CD4 and CD8 T-cell responses were similar to that observed with the prior Gag-based immunotherapy with PBMC, even in animals previously receiving peptides spanning all SIV proteins, which initially blunted the Gag-specific T-cell responses (Fig 1C, D).

The breadth of the Gag-specific T-cell immunity was studied by analyzing responses to 5 subpools of 25 Gag 15mer peptides at week 0, 4 and 7 and determining whether a rise of ≥0.1% of CD4 or CD8 T cells after vaccination occurred. For Gag-specific CD4 T-cell responses, a mean of 2.14 (range 0–4) of the 5 pools were stimulated in WB-immunized animals compared to 2.37 (0–5) pools in the PBMC immunized group. For Gag-specific CD8 T-cell responses, a mean of 1.71 pools were stimulated in the WB group (0–5) compared to 2.00 in the PBMC group (0–5). There were no significant rises in SIV-specific CD4 or CD8 T-cell responses in the 5 control animals.

Increasing evidence suggests that T cells which express multiple effector molecules (“polyfunctional” T cells) are most effective in controlling virus replication (Betts et al., 2006). We therefore analyzed the SIV Gag-specific T cells for expression of TNFα, MIP1β, IL-2 and the degranulation marker CD107a. Multiple effector molecules were expressed by Gag-specific T lymphocytes, although the expression of IL-2 was low, consistent with the long duration of infection and active viral replication prior to this study. There were no significant differences in the expression of the various molecules between the 2 delivery methods (Fig. 2).

This study immunized animals with partial control of viremia due to prior immunizations (De Rose et al., 2008). No further reductions in mean plasma viremia or changes in CD4 T cells occurred during the course of this re-boosting protocol (Fig. 3A). This likely reflects the

long duration of SIV infection prior to this study, and lack of antiretroviral cover during the immunization process (De Rose et al., 2008). Using a last-observation-carried-forward analysis to account for missing data (De Rose et al., 2008), a significant difference ($p=0.027$, 2 sided t -test of time weighted area-under-the-curve VL weeks 12–73) in viremia was maintained between controls and vaccinated animals during this protocol (Fig 3B).

We previously observed that the immunogenicity of peptide-pulsed blood cells in macaques was inversely proportional to VL (Chea et al., 2005). In a post-hoc analysis, the non-significantly lower immunogenicity of the WB group was explored by examining the animals that did not mount Gag-specific CD4 or CD8 T-cell responses to the immunotherapy. Although the PBMC and WB groups were matched for mean (\pm SE) VL (4.53 ± 0.27 vs 4.36 ± 0.57 log₁₀ copies/ml respectively, Table 1) at the start of this study, the WB group had a larger upper range of pre-treatment VLs. The 3 animals in the WB-immunized group that failed to generate a significant T-cell response had the highest baseline VLs of all 15 immunized animals (5.75–6.29 log₁₀ copies/ml, Table 1) and had experienced some CD4 T-cell depletion (all to <15% of total lymphocytes) by the end of the study. The other 4 WB vaccinated animals (baseline viral loads all ≤ 3.2 log₁₀ copies/ml) all had significant rises in Gag-specific CD4 or CD8 T cells. Our results suggest that initial clinical trials should occur in subjects with well-controlled viremia, for example during antiretroviral therapy.

Discussion

Immunotherapy with overlapping Gag peptides delivered briefly onto either fresh WB or PBMC *ex vivo* for 1 h stimulated high levels of CD4 and CD8 T-cell immunity in SIV-infected animals. Gag-specific responses of over 10% of all CD4 or CD8 T cells were induced in some animals. There was no significant difference in the immunogenicity of OPAL immunotherapy delivered by either WB or PBMC.

The T cells induced by the OPAL immunotherapy in both the PBMC and WB groups had similar breadth (approximately 2 of 5 pools of Gag peptides recognised). There was no significant positive or negative relationship between the breadth and strength of T-cell immunity in this study (not shown) as observed following our original vaccinations (De Rose et al., 2008). Such a relationship may have been obscured either by the lower numbers of animals or the effect of prior immunizations. There was similar functionality of the SIV-specific T cells as assessed by expression of up to 5 effector molecules. Minimal T-cell responses were detectable in the control group of 5 animals.

This study began just over 1 year after the animals first came off antiretroviral treatment (FTC and tenofovir). A significant difference in virologic control was maintained between treated and control groups during this extended period, although no further reductions in virus levels occurred during this study. There are limitations in interpreting this data, however, since 12 animals were euthanized prior to this study. However, this data does suggest that the virologic effect of OPAL immunotherapy can be durable.

The T-cell immunogenicity of Gag peptides pulsed onto either WB or PBMC is comparable or superior to more complex dendritic cell-based immunotherapies (Lu et al., 2003) and exceeds those generally obtainable with DNA of viral vector approaches (Hel et al., 2000; Lisiewicz et al., 2005; von Gegerfelt et al., 2007). Overall, our data support moving WB and PBMC delivery approaches of peptide immunotherapy vigorously into clinical trials.

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

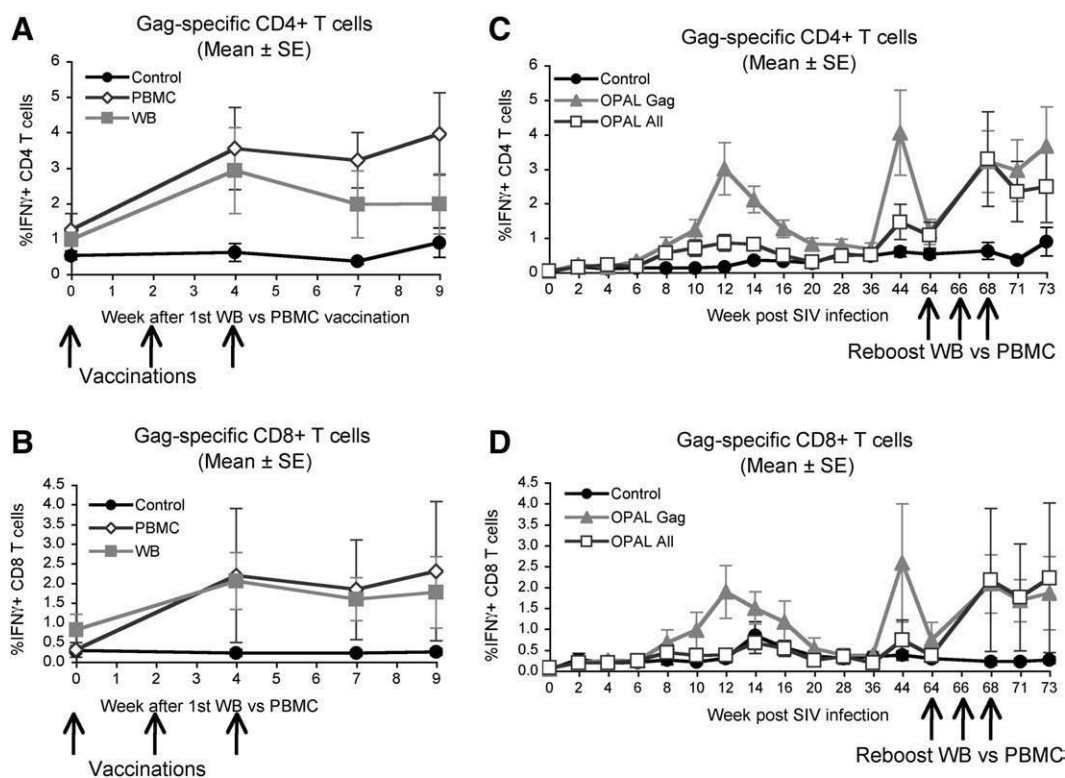


Fig. 1. OPAL immunotherapy with whole blood or PBMC. Fifteen SIV-infected pigtail macaques received overlapping SIV Gag 15mer peptides pulsed onto either PBMC or whole blood 3 times over 4 weeks and were compared to 5 controls. A, B. Percent Gag-specific CD4⁺ and CD8⁺ T lymphocytes expressing IFN γ over time after WB or PBMC-based vaccinations. Mean (\pm SE) responses are shown. Vaccinations are shown with arrows. C, D. Gag-specific CD4⁺ and CD8⁺ T cells expressing IFN γ over time based on initial vaccination status (Gag or All SIV peptides).

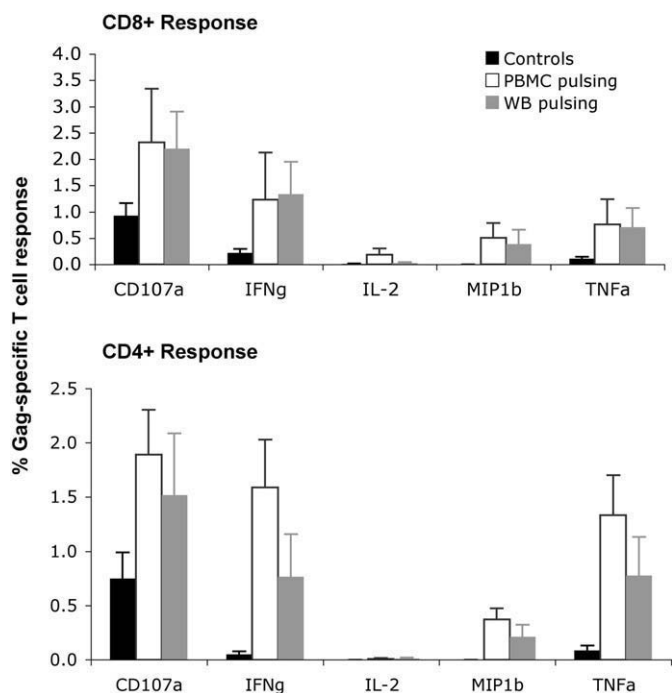


Fig. 2. Expression of multiple effector molecules by Gag-specific CD8 and CD4 T lymphocytes. Fresh blood obtained 3 weeks after the last vaccination from all animals was stimulated with overlapping Gag 15mer peptides and expression of IFN γ , TNF α , MIP1 β , and CD107a studied on gated CD8 β ⁺ and CD8 β ⁻ lymphocytes by flow cytometry. Mean levels of total Gag-specific expression of all effector molecules in each group are shown.

National Health and Medical Research Council guidelines. 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 (Pratt et al., 2006; Smith et al., 2005b). 36 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 (Batten et al., 2006; Smith et al., 2005a) and randomized into 3 groups of 12 animals (OPAL-Gag, OPAL-All, Controls) 3 weeks later. Animals received subcutaneous injections of dual antiretroviral therapy with tenofovir and emtricitibine (kindly donated by Gilead, Foster City, CA; both 30 mg/kg/animal) for 7 weeks from week 3 (Hel et al., 2000; Lisiewicz et al., 2005; Lori et al., 2000; Shen et al., 2003; Villinger et al., 2002).

Immunizations

In the earlier study, 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, 36, 39 and 42 as previously described (Chea et al., 2005; De Rose et al., 2008). In the current study, we re-randomized the remaining 15 previously vaccinated animals to receive Gag peptides pulsed onto PBMC derived from 9 ml of whole blood or onto 9 ml of fresh whole blood. The 15 monkeys were stratified based on VL, CD4 T cells, Gag-specific CD4 and CD8 T-cell immunity, *Mane-A*10* status (Smith et al., 2005a) and prior OPAL immunotherapy (Gag or All) to match the groups as evenly as possible (Table 1). Briefly, PBMC were isolated over Ficoll-paque from 9 ml of blood (anticoagulated with Na⁺-heparin). All isolated PBMC were suspended in 0.5 ml of normal saline. The 125 SIV_{mac239} Gag were added to the 9 ml blood or 0.5 ml of PBMC at 10 μ g/ml of each peptide within the pool. Peptides were 15mers overlapping by 11 amino acids

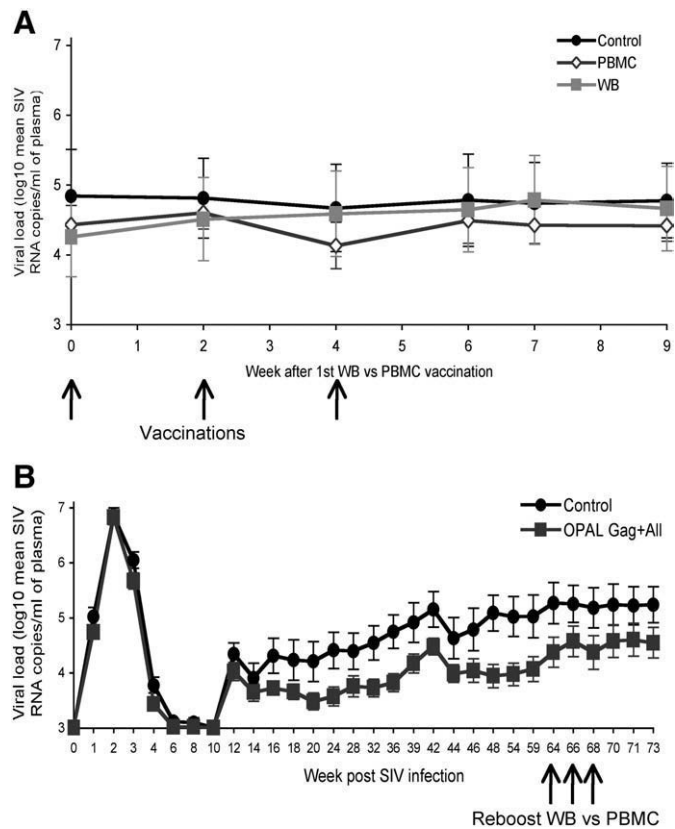


Fig. 3. Viral Loads. A. Mean (\pm SE) viral load from week 64, the time of the first WB or PBMC boost is shown across the vaccine groups. B. SIV viral load over time. Animals that controlled SIV viral load on the initial ART therapy are shown. A last-observation-carried-forward analysis was used for animals euthanized with incipient AIDS.

at >80% purity kindly provided by the NIH AIDS reagent repository program. To pool the peptides, each 1 mg vial of lyophilised 15mer peptide was solubilized in 10–50 μ l of pure DMSO and added together. The concentration of the SIV Gag peptide pool was 629 μ g/ml/peptide. The peptide-pulsed PBMC were held for 1 h in a 37 °C waterbath, gently vortexed every 15 min and then, without washing, re-infused IV into the autologous animal. The 5 control macaques did not receive vaccine treatment.

Immunology assays

SIV-specific CD4 and CD8 T-cell immune responses were analyzed by expression of intracellular IFN γ as previously described (De Rose et al., 2007). Briefly, 200 μ l whole blood was incubated at 37 °C with 1 μ g/ml/peptide overlapping 15mer SIV Gag peptide pool (described above) or DMSO alone and the co-stimulatory antibodies anti-CD28 and anti-CD49d (BD Biosciences/Pharmingen San Diego CA) and Brefeldin A (10 μ g/ml, Sigma) for 6 h. Anti-CD3-PE, anti-CD4-FITC and anti-CD8-PerCP (BD, clones SP34, M-T477 and SK1 respectively) antibodies were added for 30 min. Red blood cells were lysed (FACS lysing solution, BD) and the remaining leukocytes were permeabilized (FACS Permeabilizing Solution 2, BD) and incubated with anti-human IFN γ -APC antibody (BD, clone B27) prior to fixation and acquisition (LSRII, BD). The percentage of antigen-specific gated lymphocytes expressing IFN γ was assessed in both CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocyte subsets. To assess the expression of multiple effector molecules, we performed a simultaneous analysis of IFN γ , TNF α , MIP1 β , IL-2 and CD107a expressions on CD8 β ⁺ and CD8 β ⁻ lymphocytes at week 7 after the initial vaccination as previously described (Betts et al., 2005; Betts et al., 2006).

Virology assays

Plasma SIV RNA was quantitated by real time PCR on 140 μ l 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 (Dale et al., 2002; De Rose et al., 2007).

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