Inactivated Simian Immunodeficiency Virus-Pulsed Autologous Fresh Blood Cells as an Immunotherapy Strategy†‡

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Practical immunotherapies for human immunodeficiency virus infection are needed. We evaluated inactivated simian immunodeficiency virus (SIV) pulsed onto fresh peripheral blood mononuclear cells in 12 pigtail macaques with chronic SIVmac251 infection for T-cell immunogenicity in a randomized cross-over design study. The immunotherapy was safe and convincingly induced high levels of SIV-specific CD4+ T-cell responses (mean, 5.9% ± 1.3% of all CD4+ T cells) and to a lesser extent SIV-specific CD8+ T-cell responses (mean, 0.7% ± 0.4%). Responses were primarily directed toward Gag and less frequently toward Env but not Pol or regulatory/accessory SIV proteins. T-cell responses against Gag were generally broad and polyfunctional, with a mean of 2.7 CD4+ T-cell epitopes mapped per animal and more than half of the SIV Gag-specific CD4+ T cells expressing three or more effector molecules. The immunogenicity was comparable to that found in previous studies of peptide-pulsed blood cells. Despite the high-level immunogenicity, no reduction in viral load was observed in the chronically viremic macaques. This contrasts with our studies of immunization with peptide-pulsed blood cells during early SIV infection in macaques. Future studies of inactivated virus-pulsed blood cell immunotherapy during early infection of patients receiving antiretroviral therapy are warranted.

The recent failure of one of the more promising human immunodeficiency virus (HIV) vaccine candidates (30) and cancellation of the highly anticipated PAVE 100 HIV vaccine trial (14) highlight the significant obstacles remaining in the successful development of a prophylactic HIV vaccine. They also underscore the desperate need for a safe, effective, and practical HIV immunotherapy. Human clinical trials evaluating dendritic cell (DC) immunotherapy are ongoing (22) and have already shown some immunogenic efficacy in the treatment of certain cancers (17, 23, 28, 32) and simian immunodeficiency virus (SIV) infection of macaques (13, 20) and in early clinical trials of HIV immunotherapy (19). DC-based immunotherapies generally rely on the induction of high levels of T-cell immunity. Unfortunately, DC vaccines require prolonged ex vivo culturing and subsequent separation of cells, thereby limiting their use as a practical and readily accessible immunotherapy for HIV-infected individuals.

By contrast, immunotherapy using peptide-pulsed peripheral blood mononuclear cells (PBMC) or whole blood is considerably simpler to administer (6, 10), rendering it more feasible for wide-scale clinical use. We have shown that overlapping peptide-pulsed autologous PBMC (OPAL) immunotherapy for SIV-infected monkeys, administered along with antiretroviral therapy (ART), is a safe and effective treatment for boosting SIV-specific T-cell responses and significantly reducing viral load following vaccination when ART is withdrawn (11). However, to adequately cover important protein targets for an outbred population with highly polymorphic major histocompatibility complex (MHC) alleles, a large number of peptides needs to be manufactured and pooled. A single immunogen still capable of stimulating broad HIV/SIV-specific T-cell immunity would be a simpler vaccine. The use of inactivated SIV as an antigen to stimulate PBMC or of whole blood in a modified OPAL immunotherapy has the potential to significantly advance clinical development of an effective HIV immunotherapy.

The zinc finger targeting compound 2,2'-dithiodipyridine (aldrithiol-2 [AT-2]) covalently modifies the cysteines of internal virion proteins, including the cysteines in the critical zinc finger motifs on nucleocapsid protein of retroviruses. AT-2 has been used to efficiently inactivate HIV/SIV virions (1, 25). Since AT-2 treatment of SIV results in the preferential covalent modification of internal viral proteins without compromising the structure or function of surface proteins, AT-2-inactivated virions are noninfectious yet retain the ability to bind and fuse with host cell membranes (25). Immunotherapy using DCs pulsed with AT-2-inactivated SIV has already proven effective at eliciting SIV-specific immune responses and controlling viral replication in SIV-infected rhesus macaques (20) and in early uncontrolled human studies (19), although the remarkable efficacy of these studies has yet to be confirmed (4). We evaluated the T-cell immunogenicity of a modified OPAL immunotherapy using inactivated SIV-pulsed autologous PBMC (IPAL) in pigtail macaques with chronic SIV infection.

**MATERIALS AND METHODS**

**Animals.** Twelve pigtail macaques (Macaca nemestrina) with chronic SIVmac251 infection that examined in this study were from two prior SIV vaccine studies (Table 1). Three macaques participated in a replicon-based SIV vaccine trial that failed to provide any protective immunity (15) and had been infected...
with SIV\textsubscript{mac251} 32 weeks previously. Nine SIV-infected macaques were part of a previous peptide-pulsed blood cell vaccine study (11) and had been infected with SIV\textsubscript{mac251} 87 weeks previously. The 12 animals were randomized into two groups, the IPAL treatment (n = 6) and control (n = 6) groups, stratified by viral load, CD4 T-cell counts, weight, MHC class-I expression, and prior Gag-specific CD4 and CD8 T-cell responses. Prospective criteria for euthanasia for incipient AIDS, in agreement with our animal ethics committee, were weight loss (>10%), loss of appetite, persistently reduced activity, chronic diarrhea, or clinical disease consistent with infection or malignancy.

**SIV infection.** The SIV\textsubscript{mac251} strain (kindly supplied by N. Miller and R. Pal [24]) was used for infection of all animals by intravenous injection of 40–50\% tissue culture infective doses, as previously described (2, 26). Plasma SIV RNA load, CD4 T-cell counts, weight, MHC class-I expression, and prior Gag-specific CD4 and CD8 T-cell responses were evaluated by ICS as described above, with the additional antibodies anti-human interleukin-2 (IL-2), anti-human MIP-1\beta, and the degranulation marker CD107a. Polyclonal analysis of SIV Gag-specific T cells was performed 2 weeks after the third and final IPAL vaccinations were administered to the IPAL and the IPAL cross-over groups, respectively, at weeks 6 and 24, as described above. Fresh whole blood was stimulated with a pool of overlapping 15-mer peptides spanning SIV Gag, surface stained with the mono- and di-conjugated antibodies anti-CD3 fluorescein isothiocyanate (clone M-T477), anti-CD8 PerCP (clone SK1), and anti-CD107a APC Cy7, followed by intracellular staining with anti-IFN-\gamma Alexa Fluor 700 (clone B27), and anti-TNF-\alpha PE-Cy7 (clone MAB11), and anti-IL-2 APC (clone M01-17H12), and anti-MIP-1\beta PE (clone D21-13S1) (all conjugated antibodies were from BD Biosciences/Pharmingen, San Diego CA). Data analysis. Flow cytometric data were analyzed with FlowJo version 7.2.2 software (Tree Star Inc, Ashland OR). Polyclonal SIV Gag-specific CD4+ and CD8+ T-cell frequency data were output using FlowJo table editor and formatted with Pestle version 1.5.4 software (kindly provided by Mario Roederer, Vaccine Research Center, NIAID, NIH, Bethesda, MD) for subsequent analysis using simplified presentation of incredibly complex evaluations (SPICE) version 4.1.6 software (kindly provided by Mario Roederer, Vaccine Research Center, NIAID, NIH, Bethesda, MD). Background DMSO subtraction was performed for all SIV-specific responses reported.

### RESULTS

**Marked increase in SIV Gag-specific T cells following IPAL vaccination.** Pigtail macaques chronically infected with SIV\textsubscript{mac251} were used to evaluate the immunogenicity of autologous PBMC pulsed with AT-2-inactivated SIV by assessing production of IFN-\gamma and TNF-\alpha following in vitro stimulation with ICS assays. Macaques were randomized to two groups of six animals each, consisting of animals which received immunization with IPAL or control unimmunized macaques which later received IPAL immunizations in a cross-over design (IPAL cross-over group) (Table 1).

Immunization with IPAL was safe, with no apparent differences between the body weights, hematology profiles, or clinical observations of IPAL vaccines and those of control animals (data not shown). SIV Gag-specific T cells were substantially boosted following IPAL vaccination, with mean Gag-specific CD4+ T cells reaching 5.9% ± 1.3% of all CD4+ T cells and mean Gag-specific CD8+ T cells reaching 0.7% ± 0.4% of all CD8+ T cells (Fig. 1A). Five out of six IPAL-vaccinated animals had greater than threefold increases in SIV Gag-specific CD4+ T cells by 2 weeks after the third and final IPAL vaccinations, and two of these animals had concomitantly large increases in SIV Gag-specific CD8+ T cells (Fig. 1B, left panels). There was no increase in either the CD4+ or the CD8+ T-cell response to SIV Gag in any of the six control animals during the same time frame (Fig. 1B, right panels). The primary end point, a time-weighted area-under-the-curve analysis for weeks 0 to 18, demonstrated significant differences between the frequency of SIV Gag-specific CD4+ T cells of the
IPAL group and that of the control group (Mann-Whitney test, $P = 0.04$; Fig. 1C).

**IPAL vaccination also boosts SIV Env- but not Pol- or RT NVVV-specific T cells.** Although Gag is the most abundant protein within HIV/SIV, virions also contain smaller amounts of Env, Pol, and some of the regulatory/accessory proteins. We determined the frequency of T cells specific for all SIV proteins following IPAL vaccination by measuring responses to
Env, Pol, and regulatory/accessory protein peptide pools by ICS. SIV Env-specific CD4\(^+\)/H11001 T-cell responses were clearly increased in only two out of six IPAL animals (Fig. 2A, left panel). IPAL vaccination did not boost SIV Env-specific CD8\(^+\)/H11001 T-cell responses (data not shown). We also evaluated CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T-cell responses to SIV Pol and a combined accessory/regulatory protein pool containing Rev, Tat, Nef, Vif, Vpr, and Vpx (RTNVVV) at baseline (week 0) and at week 6 and found no increase in these responses in either the IPAL group or the control group (Fig. 2B), consistent with the smaller levels of these proteins within virions.

**IPAL cross-over vaccination confirms observations in the IPAL group.** To rigorously confirm T-cell immunogenicity of the IPAL vaccinations, we performed a cross-over vaccination of the control animals, with IPAL-vaccinated animals serving as cross-over controls. A washout period of 14 weeks following the final IPAL vaccination was selected to allow T-cell responses to return toward baseline. Animal 5831 was euthanized at week 16, based on prospectively set criteria; thus, five out of six control animals were available for the IPAL cross-over arm of the study. At weeks 18, 20, and 22, the control animals received IPAL vaccinations, while animals previously vaccinated with IPAL served as controls. We continued to monitor T-cell responses to SIV Gag, Env, Pol, and RTNVVV in all animals for an additional 16 weeks (until week 34). As with the previous IPAL animals, there was a marked increase in SIV Gag-specific CD4\(^+\)/H11001 T cells in four of the five IPAL cross-over animals (Fig. 1B, right panels). SIV Gag-specific CD8\(^+\)/H11001 T-cell responses were also boosted in three of five animals. The SIV Gag-specific CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T-cell responses observed in the IPAL cross-over animals at week 24 reflected a 4- to 29-fold increase over baseline levels at week 18. One animal also displayed a 13-fold increase in SIV Env-specific CD4\(^+\)/H11001 T cells after cross-over vaccinations (Fig. 2A, right panel). The IPAL cross-over vaccinations did not boost SIV Pol- or RTNVVV-specific CD4\(^+\)/H11001 or CD8\(^+\)/H11001 T-cell responses (not shown).

**FIG. 2.** IPAL vaccination also boosts SIV Env- but not Pol- or RTNVVV-specific T cells. (A) Kinetics of SIV Env-specific dual IFN-γ- and TNF-α-producing CD4\(^+\)/H11001 T cells following IPAL vaccination (indicated by arrows) in individual animals (listed across the top). (B) Frequencies of dual IFN-γ- and TNF-α-producing SIV Pol- and RTNVVV-specific CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T cells pre- and post-IPAL vaccination. Each bar represents the mean of all animals in the group ± standard error of the mean.
Broad SIV Gag-specific T-cell responses following IPAL vaccination. Due to the considerable boosting of SIV Gag-specific T cells following IPAL vaccination, we further studied these responses to determine the breadth of SIV Gag responses.

Analysis of CD4+ and CD8+ T-cell responses to five pools of 25 SIV Gag peptides revealed that there was variation in both the breadth and magnitude of SIV Gag-specific CD4+ T-cell responses in immunized animals (Fig. 3). Eight out of nine Gag
responders had CD4 \(^+\) T-cell responses to more than one SIV Gag peptide pool which ranged from 0.5% to more than 12% of CD4 \(^+\) T cells, with a mean of 2.7 CD4 \(^+\) T-cell epitopes recognized per animal. Responses greater than 0.5% of CD4 \(^+\) T cells were subsequently mapped to individual 15-mer SIV Gag peptides. Fourteen SIV Gag CD4 \(^+\) T-cell epitopes (responses of \(>\)0.5%) were identified, of which 13 were novel epitopes and three of these were common to two or more animals. The CD4 \(^+\) T-cell response to SIV Gag peptide 21 (TVCVWIICHHAWWKVK) was shared by animals 6804 and 8454, and responses to peptides 25 to 26 (KQIVQRHLVVE) were identified in animals 1.3731 and 6158. Three animals had CD4 \(^+\) T-cell responses directed against SIV Gag peptide 50 (QAAMQIIRDIINEEA). Of the five SIV Gag CD8 \(^+\) T-cell responders (Fig. 1B), two animals (5612 and 8454) had CD8 \(^+\) T cells specific for more than one SIV Gag peptide pool. The sizeable SIV Gag-specific CD8 \(^+\) T-cell response in animal 5612 was mapped to the immunodominant Mane-A\(*10\)-restricted Gag KP9 epitope (26). We followed the frequency of Mane-A\(*10\)-specific CD8 \(^+\) T-cell response in SIV Gag KP9 tetramer \(^*\) T cells in fresh whole blood from animal 5612. IPAL vaccinations are indicated by arrows.

**IPAL vaccination of pigtail macaques with chronic SIV infection does not lower viral load.** Although our primary goal was to examine the immunogenicity of this novel immunotherapeutic approach in nonhuman primates, we also followed the plasma viral loads and CD4 \(^+\) T-cell counts of all animals. Despite the high levels of T-cell immunogenicity observed, there were no overall differences between the mean viral load measurements or CD4 \(^+\) T-cell count depletion of the vaccinated group and that of the control groups throughout the study (Fig. 6). Indeed, there were several animals that had a transient increase in viral load immediately after IPAL immunotherapy (Fig. S1 in the supplemental material), consistent with activation of a population of CD4 T cells by the immunotherapy and an increase in virus replication.

**Comparison of IPAL and OPAL immunotherapeutic approaches.** Since many of the animals used in the IPAL study had been study subjects in previous OPAL studies (10, 11), in post hoc analyses, we were able to directly compare the magnitude of SIV Gag-specific CD4 \(^+\) and CD8 \(^+\) responses following IPAL vaccination with those observed following prior OPAL vaccinations. Immunization with IPAL induced CD4 \(^+\) T-cell responses to SIV Gag to a magnitude that was similar to that observed with OPAL immunization using Gag peptides and was greater than that observed with OPAL using peptides spanning all SIV proteins (11) (Fig. 7A). The SIV Gag CD8 \(^+\) T-cell responses with IPAL immunizations, however, were smaller than that observed with OPAL Gag peptide immunotherapy and similar to that observed with OPAL immunotherapy using peptides from all nine SIV proteins (Fig. 7A, OPAL All).

Administration of OPAL peptide immunotherapy during early SIV infection along with ART resulted in a durable 10-fold decrease in SIV load in comparison to that of controls. However, in the absence of ART, the SIV load of macaques with chronic SIV infection often transiently increased immediately after OPAL (peptide) vaccinations without concomitant ART (10). We therefore compared pre- and postvaccination viral loads in OPAL-treated macaques, either with or without ART, to those in macaques receiving IPAL immunotherapy (Fig. 7B). The pattern of nonsustained increase in viral load immediately following IPAL vaccination was similar to that previously seen in OPAL-vaccinated animals during chronic SIV infection without ART (10). This finding contrasts with the decrease in viremia in comparison to controls follow-
We evaluated T-cell immunogenicity of IPAL vaccination in pigtail macaques during chronic SIV infection. IPAL vaccination was a well-tolerated and simple immunotherapy to administer. Our results clearly demonstrate that IPAL vaccination significantly boosts SIV Gag-specific T cells with broad specificity and multiple functions. Most IPAL-vaccinated animals had robust SIV Gag-specific CD4+ T-cell responses, and a subset of these animals had concomitant SIV Gag-specific CD8+ T-cell responses. Our results are consistent with previous reports that documented induction of both CD4+ and CD8+ T-cell responses using AT-2-inactivated SIV-pulsed DC immunotherapy (13, 20); however, the use of fresh PBMC is a considerably simpler and safer delivery technique than gener-

**FIG. 5.** Polyfunctional SIV Gag-specific T-cell responses following IPAL vaccination. (A) A gating strategy used to identify polyfunctional SIV Gag-specific CD4+ T cells. The gating strategy for CD4+ and CD8+ lymphocytes is shown in the upper panels. The lower panels show the gating strategy for CD107a, IFN-γ, IL-2, MIP-1β, and TNF-α. (B) Functional profile of average SIV Gag-specific CD4+ and CD8+ T-cell responses from Gag responders from combined IPAL and IPAL cross-over animals. Black bars represent relative frequencies (percentages) of SIV Gag-specific CD4+ and CD8+ T cells expressing a particular combination of cytokines, with black dots indicating positive CD107a, IFN-γ, IL-2, MIP-1β, and/or TNF-α expression. Responses shown were derived by subtracting background DMSO control values. (C) Pie charts represent mean numbers of effector molecules expressed by SIV Gag-specific CD4+ and CD8+ T-cell responses grouped by numbers of functions with colors matched to the panel in B.
ating DC populations. We recently showed that peptide-based therapy using whole blood produced immunogenic results that were similar to that of therapy utilizing PBMC, further simplifying delivery of this broad immunotherapy technique (10).

While there was a clear increase in SIV-specific CD8⁺ T cells in a subset of animals, IPAL-induced CD4⁺ T-cell responses were more prevalent and of higher frequency than CD8⁺ T-cell responses. The mode of vaccination and/or the presence of immune dysfunction in chronically infected animals may account for fewer SIV-specific CD8⁺ T cells detected by ICS in response to peptide stimulation. IPAL vaccination may lead to preferential uptake by immature DCs, resulting in enhanced presentation to CD4⁺ T cells via the MHC class-II pathway. Another possibility is that deletion or functional exhaustion of CD8⁺ T cells in animals with chronic SIV-infection may have resulted in lower frequencies of SIV-specific CD8⁺ T cells following IPAL vaccination. Virus-specific CD8⁺ T-cell deletion and functional exhaustion have been reported in various chronic infections including those of HIV/SIV (8, 18, 29, 33). Functional exhaustion is characterized by progressive functional impairment of virus-specific T cells, driven by persistent antigen stimulation, and usually involves expression of the programmed death-1 (PD-1) receptor, which can be epitope specific (31). Thus, functional exhaustion and

![FIG. 6. No significant differences were seen in the viral load of IPAL versus control groups following IPAL vaccinations. Dynamics of plasma SIV RNA levels and CD3⁺CD4⁺ T-cell counts of individual animals (identified by numbers listed across the top) in IPAL (A) and IPAL cross-over (B) groups during chronic SIV infection following IPAL vaccinations (indicated by arrows). (C) Group plasma SIV RNA levels (means ± standard errors of the means). (D) Group CD4⁺ T-cell counts (means ± standard errors of the means).](image-url)
deletion could account for the low or absent SIV-specific CD8⁺ T-cell frequencies relative to that of CD4⁺ T cells following IPAL vaccination in some animals, despite remarkably multifunctional SIV-specific CD8⁺ T-cell responses in other animals. Prior immune escape at dominant CD8⁺ T-cell epitopes may also diminish the ability to boost immune responses. Indeed, plasma SIV RNA sequence across the KP9 Gag164-172 epitope contained typical escape motifs (K165R and P172S) prior to vaccination in four of four KP9-responding animals studied (not shown). Future studies of the effects of PD-1 expression on the induced SIV-specific CD8⁺ T cells and immune escape at CD8⁺ T-cell epitopes may help guide more appropriate timing of immunotherapy studies.

SIV Env-specific T cells were also boosted in IPAL-vaccinated animals, although the number of Env responders was fewer than Gag responders. This difference is not surprising, since it is estimated that there are ~1,500 Gag molecules (5) compared to ~14 Env trimers per native virion (34). Responses to Pol and the regulatory/accessory proteins were absent, again consistent with smaller amounts of these molecules in native virions. The lack of breadth across multiple viral proteins contrasts with our work with OPAL immunotherapy using peptides spanning all nine SIV proteins (11). However, the use of peptides spanning all nine SIV proteins comes at the cost of diminishing responses to the Gag protein, presumably by competition for presentation, and there was no advantage in virologic control using peptides spanning all nine SIV proteins in comparison to just Gag peptides (11). This is consistent with reports of the effectiveness of Gag-specific T cells and the relative ineffectiveness of responses to other proteins in large HIV-infected cohorts, in macaques and in vitro (16, 21). In this context, the Gag-specific T-cell immunogenicity of the IPAL vaccinations is encouraging.

Immunization with IPAL induced Gag-specific CD4⁺ T-cell responses which were as good as or better than those observed with OPAL (11). Several epitopes throughout SIV Gag were targeted, indicating that IPAL immunotherapy is as effective as OPAL for inducing broad T-cell responses. Furthermore, both the CD4⁺ and the CD8⁺ SIV Gag-specific T-cell responses detected following IPAL vaccination consisted of predominantly polyfunctional T cells capable of producing multiple cytokines. The lack of IL-2 secretion was not surprising since this is the first function lost during chronic viral infection (31), including HIV-infection (3). A limitation of this study is that we analyzed 12 animals surviving from a total of 48 animals that had been enrolled in previous vaccine and immunotherapy trials. Although we studied all available animals, a selection bias toward animals more capable of responding to the immunotherapy may have occurred. Nonetheless, our results provide a strong impetus for future prospective studies with unselected subjects.

Although IPAL immunotherapy did not decrease viral load in this study, this was not unexpected given that animals were chronically infected, some for as long as 2 years. These results were similar to those with OPAL immunotherapy, which, although effective for early SIV infection with ART, does not decrease chronic SIV viremia without the ART cover. Nonetheless, the increase in SIV Gag-specific CD4⁺ and CD8⁺ T cells with multiple functions clearly establishes the immunogenicity of IPAL immunotherapy during chronic SIV infection. Taken together, these results suggest that IPAL immunotherapy, if administered during acute SIV-infection under the cover of ART, could provide both increased SIV-specific T-cell immunogenicity and improved outcomes. Our IPAL immunotherapy using inactivated virus-pulsed fresh blood cells is a promising immunotherapy technique.
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S. Kent elounded a start-up company, OPAL. Therapeutics, to pursue peptide-based immunotherapies into clinical trials. He and the University of Melbourne hold an interest in this company.

REFERENCES


