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Autologous Aldrithiol-2-Inactivated HIV-1 Combined with Polyinosinic-polycytidylic acid–poly-L-lysine carboxymethylcellulose as a Vaccine Platform for Therapeutic Dendritic Cell Immunotherapy

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Abstract

Therapeutic interventions for HIV-1 that successfully augment adaptive immunity to promote killing of infected cells may be a requisite component of strategies to reduce latent cellular reservoirs. Adoptive immunotherapies utilizing autologous monocyte-derived dendritic cells (DCs) that have been activated and antigen loaded *ex vivo* may serve to circumvent defects in DC function that are present during HIV infection in order to enhance adaptive immune responses. Here we detail the clinical preparation of DCs loaded with autologous Aldrithiol-2 (AT-2)-inactivated HIV that have been potently activated with the viral mimic, Polyinosinic-polycytidylic acid–poly-L-lysine carboxymethylcellulose (Poly-ICLC). HIV is first propagated from CD4+ T cells from HIV-infected donors and then rendered non-replicative by chemical inactivation with aldrithiol-2 (AT-2), purified, and quantified. Viral inactivation is confirmed through measurement of *Tat*-regulated β -galactosidase reporter gene expression following infection of TZM-bl cells. In-process testing for sterility, mycoplasma, LPS, adventitious agents, and removal of AT-2 is performed on viral preparations. Autologous DCs are generated and pulsed with autologous AT-2-inactivated virus and simultaneously stimulated with Poly-ICLC to constitute the final DC vaccine product. Phenotypic identity, maturation, and induction of HIV-specific adaptive immune

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Conflicts of Interest

Nina Bhardwaj reports a potential conflict as a co-inventor on patents related to DC function and differentiation. Andres Salazar is the CEO of Oncovir, Inc which manufactures Poly-ICLC. The remaining authors report no conflicts.

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responses are confirmed via flow cytometric analysis of DCs and cocultured autologous CD4+ and CD8+ T cells. Lot release criteria for the DC vaccine have been defined in accordance with Good Manufacturing Practice (GMP) guidelines. The demonstrated feasibility of this approach has resulted in approval by the FDA for investigational use in antiretroviral (ART) suppressed individuals. We discuss how this optimized DC formulation may enhance the quality of anti-HIV adaptive responses beyond what has been previously observed during DC immunotherapy trials for HIV infection.

Keywords

Dendritic cell; Therapeutic Vaccine; HIV-1; Poly-ICLC

Introduction

Long-term suppression of HIV-1 with antiretroviral therapy (ART) in infected individuals allows for viral persistence in cellular reservoirs, serving as a major barrier to eradication. Therapeutic vaccines for HIV-1 that successfully enhance cytotoxic T lymphocyte (CTL) function to promote killing of infected cells in combination with approaches to disrupt viral latency may result in reduction of cellular viral reservoirs that contain stably integrated, replication-competent virus [1]

As the most potent antigen presenting cells (APCs), myeloid dendritic cells (DCs) strongly stimulate the onset of CD4+ and CD8+ T cell responses, making them ideal targets for therapeutic vaccines. However, in the setting of chronic HIV infection, multiple DC functions are impaired including cytokine secretion and capacity to stimulate T cells and NK cells [2–4]. In order to bypass these defects, adoptive immunotherapies utilizing autologous monocyte-derived DCs that are activated and antigen loaded *ex vivo* have been explored [5–16]. These trials have demonstrated that DC immunization is safe and immunogenic during HIV infection, but viral control has been incomplete. Key steps in optimization of DC immunotherapies lie in maximizing the quality of DC preparations, through the provision of effective *ex vivo* maturation stimuli and HIV immunogens. Depending upon the maturation stimulus utilized, DCs upregulate co-stimulatory molecules and secrete a variable array of cytokines to modulate adaptive immune responses. In order to generate strong anti-viral immunity, selection of maturation stimuli that induce the secretion of Th1-skewing cytokines by DCs, including IL-12, is likely advantageous [17]

In terms of the HIV immunogen(s) utilized, the most successful DC immunotherapies to date have involved the use of autologous, inactivated HIV/SIV [9, 10, 16, 18]. Most notably, a recent randomized, controlled trial of autologous heat-inactivated HIV-pulsed DC resulted in a substantial decrease in viral load set point following ART interruption [10]. In contrast to conventional means of viral inactivation, including heat treatment, that result in damage to viral proteins, aldrithiol-2 (AT-2, 2,2'-dithiodipyridine) preserves the conformational and functional integrity of HIV envelope glycoproteins to allow for unaltered viral fusion, while rendering HIV non-infectious through covalent modification of key S-H- containing internal proteins [19]. Here we detail the clinical preparation of Poly-ICLC (Hiltonol, Oncovir, Inc)

activated DCs that are loaded with autologous AT-2-inactivated HIV. This DC vaccine is now approved by the FDA for investigational use as a therapeutic immunotherapy in ART suppressed individuals (BB-IND-15382). The design of this optimized DC formulation aims to enhance the quality of anti-HIV adaptive immunity beyond what has been previously observed during DC immunotherapy trials.

METHODS

The schema in Figure 1 depicts an overview of the major steps involved in generation of the final DC vaccine product. Table 2 summarizes quality control measures including specified in process testing and lot release criteria as detailed throughout the methods.

Human Subjects

HIV-1-infected, anti-retroviral therapy (ART)-naïve subjects (N=18) (plasma HIV-1 RNA ranging from 496–1,860,000 copies HIV-1 RNA/mL, median 26,225 copies/ml and CD4+ cells from 221–1074 cells/mm³, median 622 cells/mm³) and ART-treated subjects (N=5) were recruited through NYU Langone Medical Center, Aaron Diamond AIDS Research Center (ADARC) and Center for HIV/AIDS Vaccine Immunology (CHAVI 012) and underwent informed consent and leukapheresis. This study was reviewed and approved by the Institutional Review Board of New York University School of Medicine, ADARC, and CHAVI.

Cell Lines

The TZM-bl cell line (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.[20–23]) is a HeLa cell line that stably expresses CD4 and CCR5, with luciferase and β -galactosidase genes under control of the HIV-1 promoter. TZM-bl cells were sub-cultured in DMEM (90%), 10% FBS, 100 units of Penicillin and 0.1 mg/ml of Streptomycin. The Sup-T1.CCR5 cell line was obtained from Dr. Jim Hoxie [24].

Reagents and antibodies

AIM-V media and CD4+ and CD8+ Dynal beads were purchased from Invitrogen. RPMI-1640 media and X-Vivo 15 media (BioWhittaker™) were from Lonza Biologics, Inc. Pooled Human AB Serum (PHS) was from Valley Biomedical. Ficoll-Paque Premium was from GE Healthcare Sciences. Aldrithiol-2 (AT-2) and reduced L-Glutathione were from Sigma-Aldrich. Good manufacturing practice-certified (GMP-certified) Interleukin-2 (IL-2), GMP-certified Interleukin-4 (IL-4), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), GMP-certified Anti-CD3 antibody, CliniMACS CD4 and CD14 microbeads, CliniMACS LS Tubing sets, CryoMACS DMSO and CliniMACS PBS/EDTA buffer were from Miltenyi Biotec, Inc. Polyinosinic-polycytidylic acid – poly-L-lysine carboxymethylcellulose (Poly-ICLC) was from Oncovir, Inc. Fluorescent antibodies for IL-2, -IFN γ , -TNF α , -MIP-1 β , -CD3, -CD4, and -CD8 were purchased from BD biosciences. Antibodies for CD86, -CD14, -CD11c, and -HLA-DR were purchased from Biolegend. Recombinant human IL-2 and IL-7 were from R&D Systems. The following antibodies were manufactured in-house at the AIDS and Cancer Virus Program, Leidos

Biomedical Research, Inc., Frederick National Laboratory: anti-p24CA [94-0001], anti-p17MA [R193244], anti-gp120HIV [P3F5-D5-F8]. Anti-gp41TM(4E10) was obtained from Polymun [AB004].

Autologous virus production

CD4⁺ T cells from viremic donors were isolated from fresh leukaphereses via CD14-CD4⁺ selection using CD14⁺ and CD4⁺ magnetic beads and a CliniMACS Separation System. The CliniMACS device is a closed GMP system used for cell isolation. Isolated CD4⁺ T cells were activated upon culture with plate-bound anti-CD-3 antibody (5 µg/mL), and maintained in the presence of 100 IU/mL IL-2 in AIM-V media supplemented with 10% PHS, 1% HEPES buffer and gentamicin (24 µg/ml)(BioWhittaker™). Supernatants containing HIV were collected and frozen with each IL-2-supplemented media change (every 3 ± 1 days). Supernatants were monitored for virus content at each media change using HIV-1 p24 antigen capture immunoassay (AIDS and Cancer Virus Program, NCI-Frederick). Cultures that produced < 2 ng/mL at 3 consecutive time points were terminated. Frozen CD4⁺ culture supernatants were maintained at -80°C for 1-2 months until viral inactivation and purification.

Inactivation of virus

Supernatants from CD4⁺ T cell culture were thawed, pooled by donor, clarified through low speed centrifugation using a SX4750 rotor (4,000 × g × 15 mins)(Beckman Coulter), and filtered through a 0.22 µm filter. AT-2 (0.5 M) in DMSO (CryoMACS) was added to the clarified supernatant to a final concentration of 1 mM and incubated overnight for 12-18 hours at 4°C with gentle stirring. A portion of supernatant was incubated overnight under similar conditions without AT-2 for comparative use in residual infectivity assays.

Virus purification and removal of AT-2

The AT-2 inactivated virus (AT-2 HIV) from each batch of supernatant was purified and concentrated by sequential ultracentrifugation. The virus was first pelleted by centrifuging at 4°C using a SW32 rotor (100,000 × g × 60 mins) (Beckman Coulter). The resultant pellet was resuspended with 1mL of 10 mM reduced L-Glutathione to react with any residual AT-2 present and centrifuged at 4°C using a Type 50.4 Ti rotor (198,000 × g × 6 mins) (Beckman Coulter). The L-Glutathione was decanted and the pellet resuspended in 300µl PBS. The purified AT-2-inactivated virus was aliquoted and stored in liquid nitrogen.

Quantification of purified virus

The total amount of purified virus was quantified by SDS-PAGE with Coomassie staining using p24 to calibrate the gel [25].

Confirmation of purified virus identity

The purity and biochemical composition of the purified virus was evaluated by SDS-PAGE followed by immunoblotting for HIV p24, gp120, and gp41.

Confirmation of Virus Inactivation

Lack of residual infectivity of AT-2-inactivated HIV-1 was measured in a TZM-bl assay as a function of Tat-regulated β -galactosidase reporter gene expression (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.[20–23]). Infectious virus was quantified as a function of β -galactosidase activity expressed as relative spot-forming units (SFU) before and after AT-2 treatment, in donor CD4+ T cell supernatants and purified virus samples. Viral controls of infectious and AT-2 inactivated HIV-1 BaL supernatant (AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc., National Laboratory for Cancer Research) of known infectivity were included in each assay. TZM-bl plate images were captured with a Cellular Technology, Ltd. CTL-ImmunoSpot® S6 Micro Analyzer ELISPOT Reader and the number of SFUs was analyzed with ImmunoSpot® Professional Software's Immuno Capture, v6.3.

Confirmation of AT-2 removal from virus preparations

Removal of unreacted AT-2 in the purified virus preparation (lysed in 8M Guanidine-HCl) was confirmed via HPLC with a threshold sensitivity of 0.5 ng/ul of purified virus.

Preparation of Monocyte-derived Dendritic Cells and Final DC Vaccine Product

PBMCs from leukaphereses were frozen using a controlled rate freezer and later thawed to generate monocyte derived dendritic cells (DCs). Monocytes were enriched from frozen PBMCs by plastic adherence and cultured in RPMI 1640 media supplemented with 5% PHS, 1% HEPES, and Gentamicin (24ug/ml) in the presence of 100 IU/mL GM-CSF (Leukine) and 400 IU/mL IL-4 for 6 days. On day 6, immature DCs (iDCs) were pulsed overnight with autologous, purified AT-2 HIV at a concentration of 100–300 ng/mL of p24 equivalents [26–29]. These DCs were matured with 5 μ g/mL Poly-ICLC to constitute the final DC vaccine product. The final DC vaccine product was frozen in PHS/10% DMSO.

Detection of Bacteria, Mycobacteria, and Fungus

BacT/Alert culture bottles (aerobic, anaerobic, and mycobacteria) were inoculated with culture supernatant and incubated for 2 weeks to detect bacterial and fungal contamination, and 8 weeks to detect mycobacterial at the beginning and end of CD4+ T cell culture, on day 5 of DC preparation, and on the final DC vaccine product. Throughout CD4+ T cell culture, supernatants were added to a TSA (Trypticase Soy Agar) 5% Sheep's Blood/MacConkey Agar Biplate weekly and incubated for 7 days to detect the presence of bacteria.

Detection of Mycoplasma

Culture supernatants were sent to Clongen Laboratories LLC to evaluate for the presence of mycoplasma at the beginning and end of CD4+ T cell culture, on day 5 of DC preparation, and on the final DC vaccine product. Cultures are maintained for 14 days and DNA fluorochrome staining performed after 5 days.

Detection of Endotoxin

The level of Gram negative bacterial endotoxin was measured in culture supernatants using a Kinetic Chromogenic LAL Kit (Associates of Cape Cod, Inc., range 0.005 EU/ml – 50.0

EU/ml) per manufacturer's protocol, at the beginning and end of CD4+ T cell culture, on day 5 of DC preparation, and on the final DC vaccine product.

Detection of Adventitious Viruses

The presence of adventitious virus was evaluated at the end of each CD4+ T cell culture via in vitro virus testing (Clongen Laboratories LLC). Three indicator cells lines (MRC-5, Vero, and SC-1) were inoculated with culture supernatants and observed for 14 days for development of cytopathic effects (CPE) per Clongen's protocol.

Viability, Identity, and Maturation of the DC vaccine

The identity of DCs after pulsing with AT-2 HIV and maturation with Poly-ICLC was evaluated by flow cytometry. Frozen DC were thawed and stained with antibodies against CD3, HLA-DR, CD11c, CD14, CD86, and CD40. The viability of each DC vaccine product was determined by Live/Dead staining with Guava Viacount Reagent (Millipore, Billerica, MA).

Additional experiments utilizing DC derived from ART- suppressed HIV-infected individuals were performed under non-GMP conditions to compare maturation with Poly-ICLC to the widely used standard cytokine cocktail Monocyte Conditioned Medium (MCM) MIMIC (1 ug/mL PGE2, 5 ng/mL TNF α and IL-1 β , 150 ng/mL IL-6). Up regulation of CD86 and CD40, and secretion of cytokines, including the pro-Th1 cytokine, IL-12, was measured after pulsing DCs with AT-2 HIV and maturation with Poly-ICLC (5 ug/mL) or MCM MIMIC. Cytokines were measured by Human Inflammatory Cytokine Bead Array (BD Biosciences).

Immunogenicity of the DC Vaccine

Immunogenicity was evaluated as a function of activation of autologous HIV-specific CD4+ and CD8+ T cell responses. DCs were derived as described and matured with Poly-ICLC +/- pulsed with AT-2 HIV (100 ng/mL-300 ng/mL) on d6 of growth and co-cultured with magnetic bead sorted autologous CD4+ and CD8+ T cells at a ratio of 1:5 (DC:T cell). Cultures were maintained for 10 days in RPMI-1640 supplemented with 1mM HEPES, 2mM L-glutamine and 10% PHS in the presence of rhIL-2 (10 IU/mL) and rhIL-7 (5ng/mL). To evaluate for the expansion of HIV-specific T cell responses following co-culture, T cells were restimulated with autologous DC +/- pulsed with autologous AT-2 HIV at a ratio of 1:5 (DC:T cell) for 6 hours followed by fixation, permeabilization, and fluorescent staining for IFN γ , TNF α , MIP1 β , and CD107a. Data were acquired with an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

RESULTS

HIV Production, Purification and Quantification

Donor CD4+ T cells cultured under GMP conditions (N=4) remained viable and contained 2 ng/mL p24 by ELISA for 36-55 days (Fig. 2A). Following purification via ultracentrifugation, quantification of the AT-2 inactivated virus batches yielded an average of 200,071 ng p24CA [range 27,904 – 489,224 ng] (Fig. 2B). To further establish feasibility

of virus production, CD4+ T cells from 14 additional untreated HIV-infected donors were cultured, and virus containing supernatants were inactivated and purified under non-GMP conditions with similar culture duration and viral yield (Supplemental Figure 1). No correlation was found between viral yield and donor plasma HIV-1 RNA or CD4+ T cell count (not shown).

Immunoblots confirmed the presence of whole virions in the purified virus preparations in all donors (N=4) (Fig. 2C).

Confirmation of viral inactivation and removal of AT-2 from virus preparation

Virus inactivation was confirmed in CD4+ T cell culture supernatants before and after AT-2 treatment (Fig. 3A) and also following purification and concentration of viral supernatants (data not shown) for each donor via TZM-bl assays. Following AT-2 treatment, there was no detectable infectivity present in all culture supernatants and purified viruses tested. The removal of residual AT-2 was confirmed in all virus preparations by HPLC (N=4) (Fig. 3B).

In Process Testing and Lot Release Criteria

In process and lot release criteria testing was performed on select samples depending on availability of specimens manufactured under GMP conditions as detailed in Table 1. All CD4+ T cell culture supernatants evaluated weekly for gross contamination with bacteria tested negative for each culture (N=4; ranging from 4–6 time points per culture). Supernatants did not contain bacteria, fungus, mycobacteria, or Mycoplasma at the start or end of culture (N=4). Supernatants did not contain adventitious virus at the end of culture (N=4). All samples evaluated for endotoxin levels met the criteria for in process testing and lot release of <50 EU/mL.

Viability, Identity and Maturation of DC Vaccine

The DC vaccine was stored in the vapor phase of liquid nitrogen for 5 months and evaluated for viability, phenotype, and sterility after thawing. Lot release criteria were met for viability (>70%) and identity (>50% CD11c+, <30% CD11c+/CD14+, >50% CD11c+CD86+). Viability was >90% after thawing and DCs were >75% CD11c+, <5% CD11c+/CD14+, and >70% CD11c+CD86+ (N=2) (Fig 4A). Given the limited number of GMP samples, these criteria along with additional parameters were also evaluated under non-GMP conditions to further confirm identity and maturation (N=5). Poly-ICLC resulted in significant up regulation of CD86 and CD40 compared with unstimulated DCs (both nonpulsed and AT-2 HIV pulsed conditions) (Fig. 4B). When compared with the standard clinical DC maturation stimulus, MCM MIMIC, DCs matured with Poly-ICLC produced robust levels of the pro-Th1 cytokine, IL-12 (Fig. 4C). Additionally, Poly-ICLC induced strong secretion of TNF α , IL-1 β , and IL-6 from moDCs (both nonpulsed and AT-2 HIV pulsed conditions) (Supplemental Figure 2).

Immunogenicity of DC Vaccine

The DC vaccine stimulated polyfunctional autologous HIV specific CD4+ and CD8+ T cells in all donors tested as evidenced by secretion of IFN γ , TNF α , MIP1 β , and/or CD107a (N=3) (Fig. 5). For all donors, cytokine levels secreted by CD4+ T cells following expansion and

restimulation with AT-2 HIV-pulsed (either 100ng/ml or 300ng/ml) DCs were >3X higher in at least 2 cytokines and >2X higher in at least 3 cytokines when compared with control (background) conditions (T cells expanded and restimulated with nonpulsed Poly-ICLC matured DCs). Overall, CD8+ T cell responses were generally lower in magnitude compared with CD4+ T cell responses, but remained >3X higher in at least 1 cytokine and >2X higher in at least 2 cytokines, in all donors compared with background. There was no consistent dose response when comparing DCs that were pulsed with 100ng versus 300ng of AT-2 HIV. This pattern of induction of CD4+ >>CD8+ T cells has been observed in previous *in vitro* studies of AT-2 HIV pulsed DCs and also in a clinical trial in untreated individuals [16, 27]. AT-2 HIV has been found to stimulate HIV-specific CD8+ T cells via the classical endogenous pathway following CD4+ binding and entry [28].

Discussion

We detail GMP methods for the generation and subsequent AT-2 inactivation, purification, quantification and quality control of sufficient quantities of autologous HIV from donor CD4+ T cells to allow for its use as an immunogen in DC immunotherapies. These methods can be readily reproduced in GMP laboratories without the need for highly specialized equipment. Upon pulsing autologous DCs with purified autologous AT-2 HIV and maturation with Poly-ICLC, we demonstrate the *in vitro* stimulation of autologous, polyfunctional HIV-specific adaptive immune responses. This is consistent with previous *in vitro* and *in vivo* studies by our group and others, revealing that AT-2 HIV/SIV pulsed, mature DCs effectively prime and expand polyfunctional HIV/SIV-specific CD4+ and CD8+ T cell responses, resulting in enhanced viral control during untreated HIV/SIV-infection [16],[18],[27, 28, 30, 31]. In contrast with autologous heat-inactivated HIV [10], AT-2 HIV DC preparations have not been studied in ART suppressed individuals, in part because they have not been previously approved for investigational use in North America or Europe. The use of AT-2 HIV may enhance the immunogenicity of DC therapeutic vaccines beyond what has been seen with heat-inactivated HIV, given it minimizes conformational damage to viral proteins. One potential limitation of the use of autologous virus-containing vaccines is that cells from aviremic individuals are less optimal for generating large quantities of virus. This requires that the initial leukapheresis for virus generation be performed prior to the initiation of ART or, alternatively, following a closely monitored structured treatment interruption in select patient populations. Notably, in the aforementioned *in vivo* study using DCs pulsed with heat-inactivated HIV, autologous HIV was generated using cells from ART-suppressed patients without treatment interruption [10]. Therefore, significantly lower amounts of immunogen may be sufficient for generating an effective vaccine, and early phase studies that evaluate dose titrations of inactivated virus will be useful.

Furthermore, we describe a means to augment the quality of DC immunotherapies for HIV-infected individuals through the *ex vivo* activation of DCs with Poly-ICLC, a synthetic dsRNA that is recognized by TLR3 in endosomes and cytoplasmic sensors, including MDA5 and DHX/DDX RNA helicases [32–34]. Consistent with prior studies in seronegative donors [35, 36], we demonstrate that Poly-ICLC matures DCs from HIV-infected donors, as evidenced by upregulation of costimulatory molecules and secretion of

cytokines. Notably, Poly-ICLC stimulated robust secretion of the pro-Th1 cytokine, IL-12, by DCs from donors on suppressive ART, compared with standard cytokine cocktails that have been previously employed in clinical trials, which has likely limited their anti-viral potency [9, 10, 16]. Powerful anti-viral effects were observed by Poly-ICLC and AT-2-inactivated SIV in a therapeutic tonsillar vaccine study in non-human primates [37]. Here viral loads following treatment interruption were 1–2 logs lower in vaccinated animals compared with unvaccinated controls or when Cytosine-phosphate-guanine class C (TLR 9 agonist) was used as an adjuvant. Our finding of increased IL-12 secretion by DCs from ART-treated HIV donors aligns with our previous work revealing that Poly-ICLC significantly rescues DC cytokine secretion in the presence of plasma from ART-suppressed individuals, when compared with plasma from untreated HIV donors, though some defect in cytokine production still remains, compared with plasma from uninfected donors [4]. Given the suppressive role that HIV donor plasma has been shown to have on DCs, an important factor in our culture system is the use of clinical grade plasma from seronegative donors, during the derivation and activation of DCs, rather than autologous plasma which is commonly utilized and may diminish DC cytokine secretion upon ex vivo stimulation [3, 4].

Taken together, these modifications may improve the success of DC immunotherapies beyond what has been observed in previous trials when administered to ART suppressed individuals. Unfortunately, due to practical limitations and high cost of DC immunotherapies, these strategies are unlikely to be feasible on a large-scale for a wide population of infected individuals or as a measure to prevent the acquisition of HIV infection in at-risk individuals. However, given that DCs have been shown to be dysfunctional *in situ* during HIV infection, successful DC immunotherapy would provide an important proof of concept, that augmentation of DC function is vital for generating more effective HIV-specific adaptive immune responses to ultimately lower barriers to functional cure and/or eradication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Autologous AT-2 inactivated HIV can be derived from donor CD4+ T cells for use as a DC vaccine immunogen.

This immunogen can be generated in GMP laboratories without highly specialized equipment.

AT-2 HIV-pulsed DCs matured with Poly-ICLC secrete IL-12 and induce HIV-specific immunity.

This formulation is now approved for investigational use in ART-suppressed individuals.

This formulation may enhance anti-viral immunity beyond previous therapeutic DC vaccine trials.

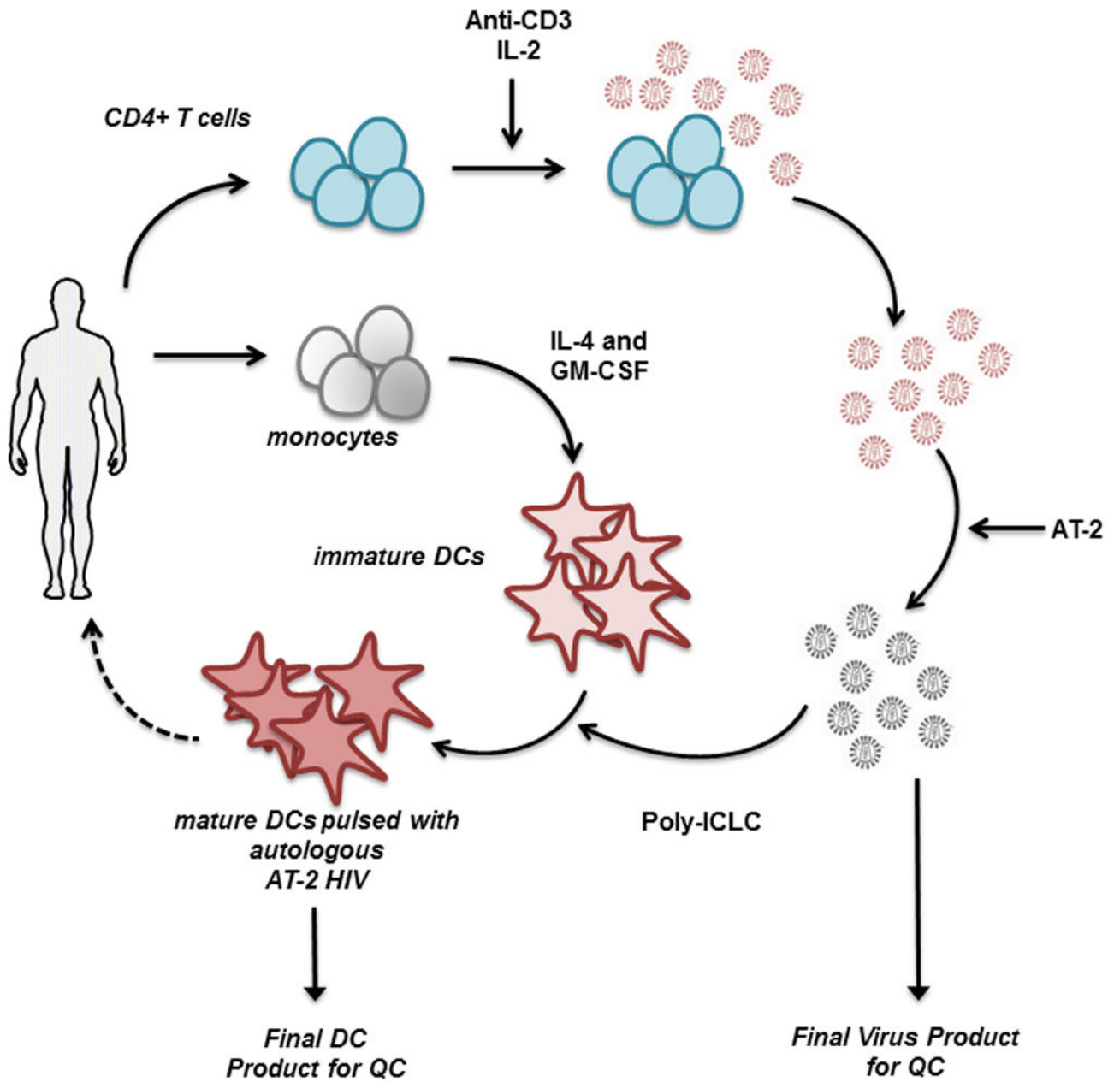


FIGURE 1. Autologous DC Vaccine Production Schema

Following leukapheresis of viremic HIV-infected donors, virus is propagated from anti-CD3-activated CD4+ T cells in the presence of IL-2. Viral supernatants are inactivated with AT-2 and then purified. This autologous, inactivated HIV is then pulsed onto autologous monocyte-derived DCs in combination with Poly-ICLC. These mature, antigen-loaded moDCs may be injected back into the patient as a form of HIV immunotherapy.

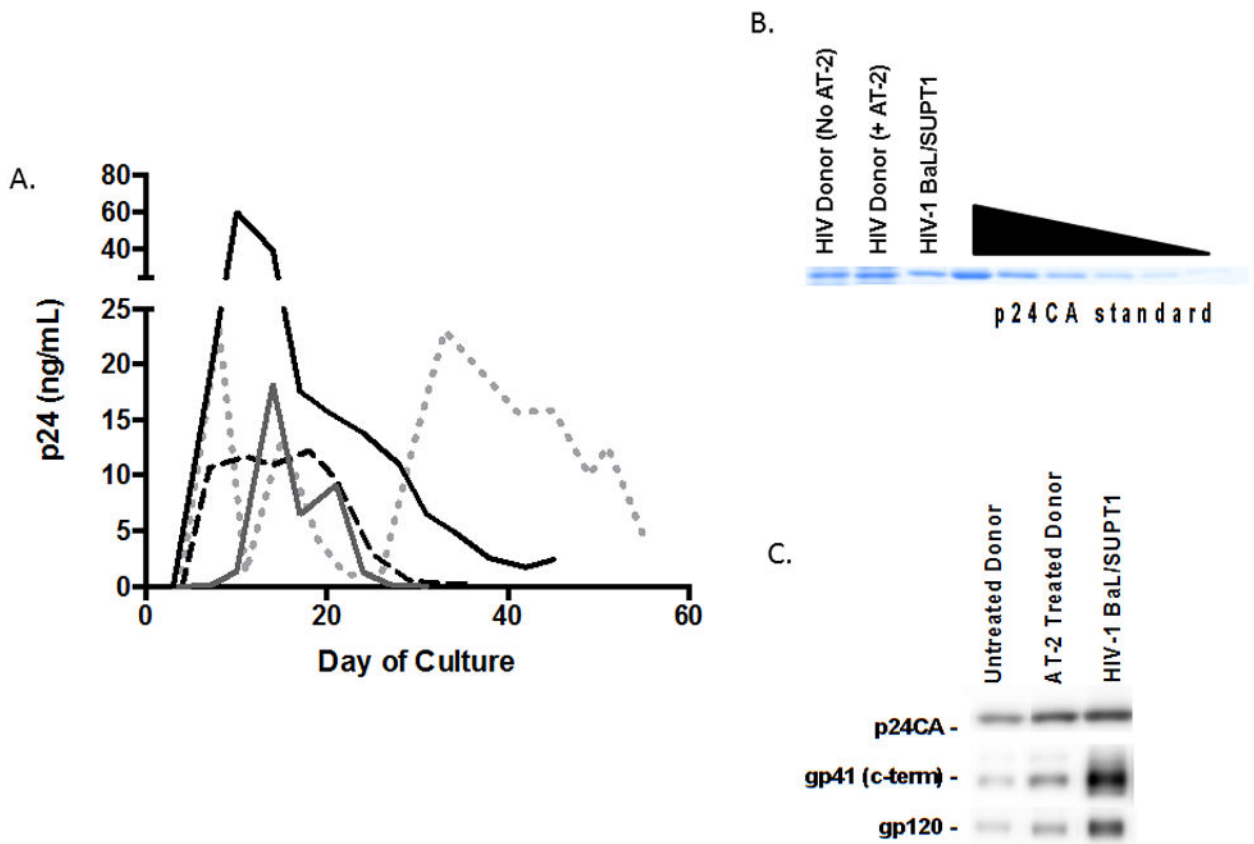


FIGURE 2. HIV Production, Purification and Quantification

(A) p24 content of CD4+ T cell culture supernatants from HIV-infected donors (N=4) under GMP conditions was quantified by ELISA. To further establish feasibility, 14 additional donors were cultured under non-GMP conditions demonstrating similar concentration of p24 (Supplemental Figure 1).

(B) Coomassie staining of purified, inactivated virus from a representative HIV-infected donor before and after treatment with AT-2. p24CA standard ranges from 500 to 15.1625 ng. This was performed on all purified virus samples cultured under GMP conditions (N=4), with an average yield of 200,071 ng p24CA [range 27,904 –489,224 ng]. Viruses from 14 additional untreated HIV-infected donors were cultured, inactivated, and purified under non-GMP conditions with similar yield of p24CA (data not shown).

(C) Immunoblots for the viral proteins p24CA, gp41TM (c-terminus), and gp120 were performed on purified, inactivated virus preparations (N=4, figure displays representative donor). This was performed to confirm the presence of whole virions rather than free p24CA in the virus preparations.

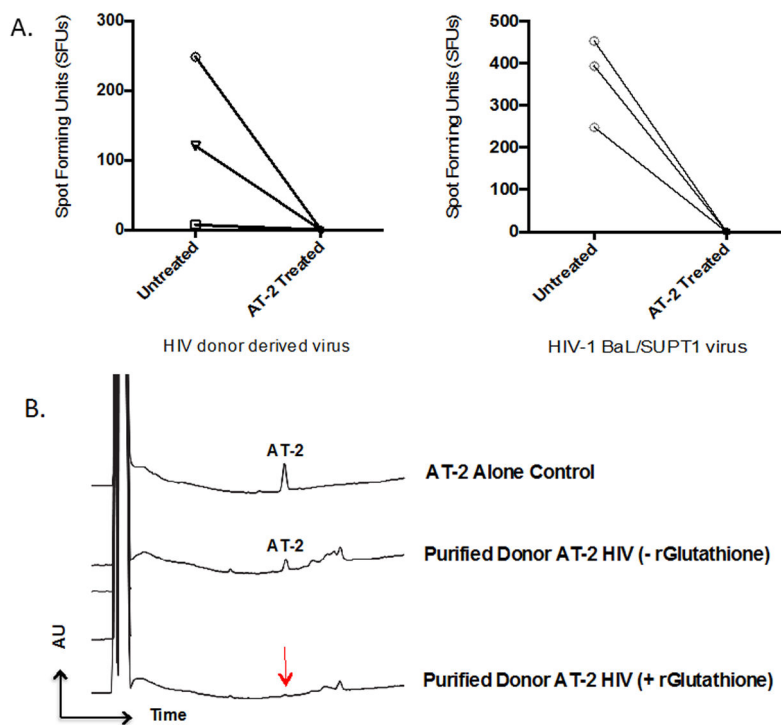


FIGURE 3. Confirmation of viral inactivation and removal of AT-2 from viral preparation
 (A) Evaluation for residual infectivity was performed via TZM-bl reporter assays. Infectious virus (quantified as spot forming units (SFU)) is shown before and after AT-2 treatment in donor CD4+ T cell supernatants (N=3) (left) and HIV-1 BaL/SUPT1 supernatants (right). Viral inactivation was also confirmed following purification and concentration of viral supernatants for each donor with no detectable infectivity following AT-2 treatment (data not shown).
 (B) HPLC analysis of 0.5M AT-2 solution (AT-2 alone control) and purified AT-2-inactivated HIV from a representative donor with and without the addition of 10 mmol r-Glutathione. HPLC was performed at a flow rate of 300 mL/min on 3 m, 2 × 100 mm Luna C18(2), (Phenomenex), using aqueous acetonitrile/trifluoroacetic/acid solvents on a Shimadzu HPLC system. Peaks were detected by UV absorption at 206 and 280 nm.

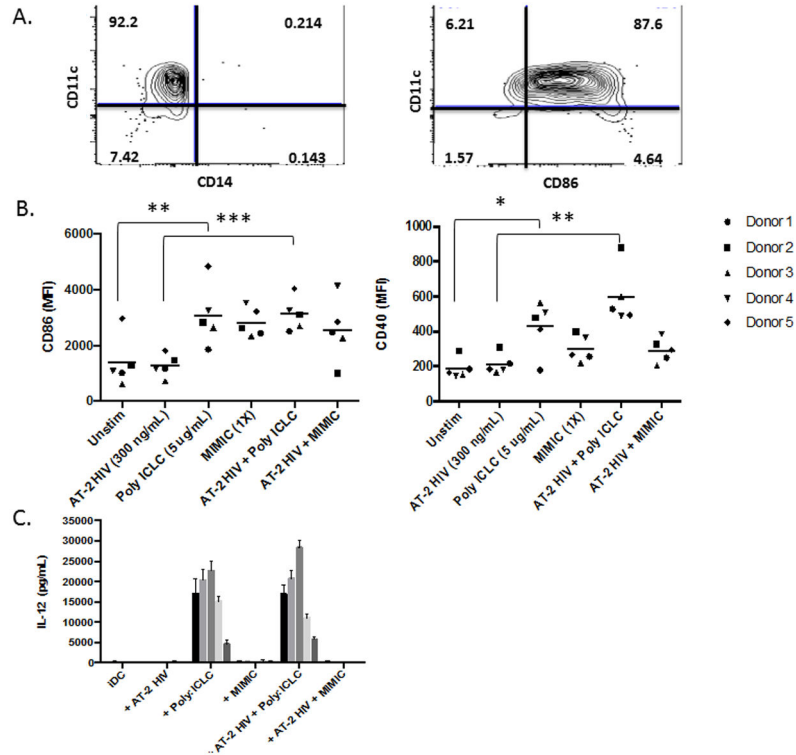


FIGURE 4. Identity, Maturation, and IL-12 Secretion

(A) To ascertain fulfillment of identity and maturation lot release criteria, thawed final DC vaccine products were stained for CD11c, CD14, and CD86. Representative donor is displayed. (B) DCs from ART-suppressed donors (N=5) were pulsed with patient-propagated AT-2 HIV and stimulated with Poly-ICLC or the standard cytokine cocktail, MCM MIMIC. Poly-ICLC resulted in upregulation of CD86 and CD40 compared with unstimulated DCs (both nonpulsed and AT-2 pulsed conditions). (C) IL-12 secretion by DCs from these same donors and conditions was evaluated. In contrast to unstimulated and MCM MIMIC DCs, Poly ICLC induced robust secretion of IL-12 by DCs.

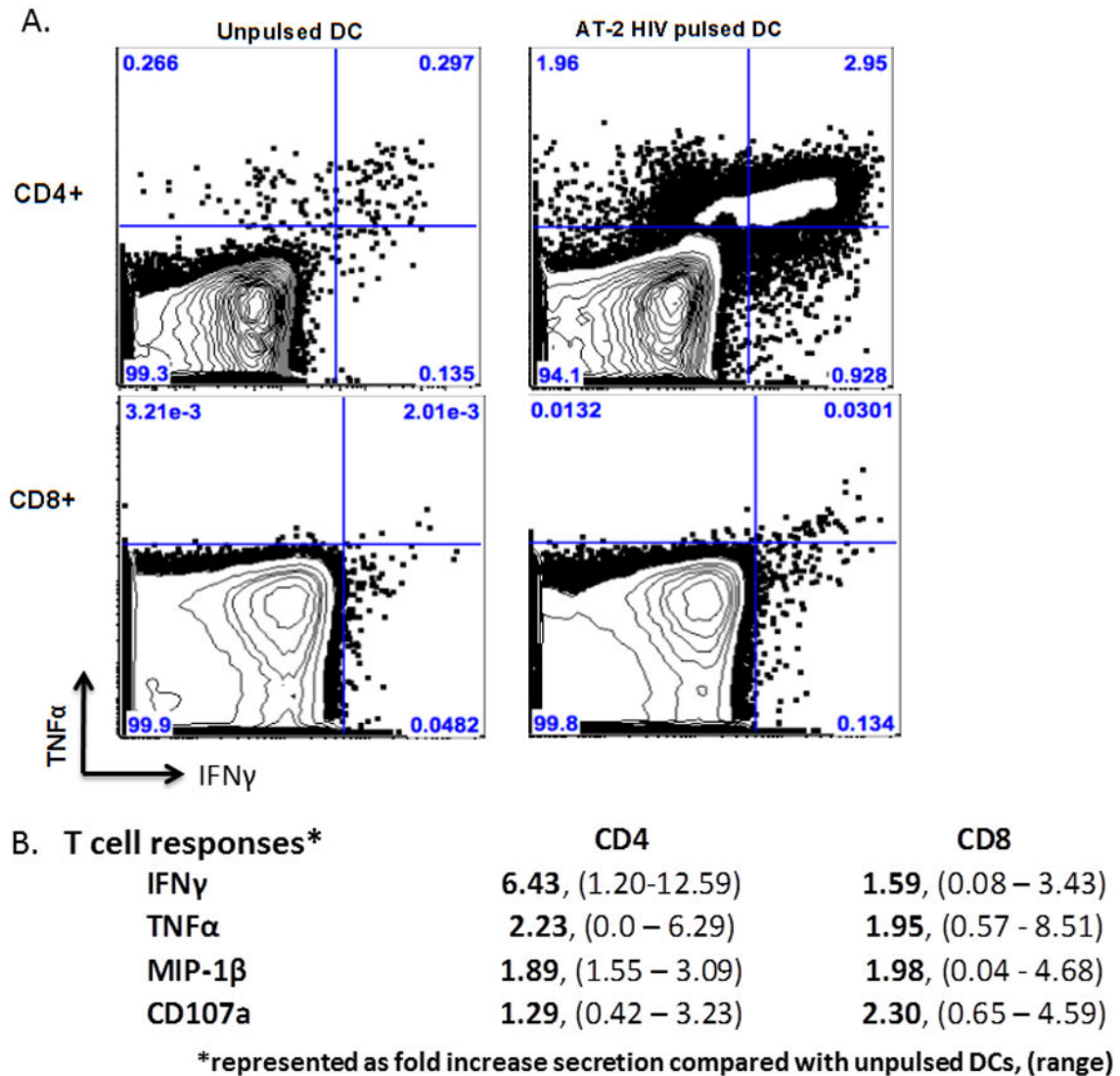


FIGURE 5. Immunogenicity of DC Vaccine

To evaluate potency/immunogenicity, the autologous DC vaccine (Poly-ICLC matured DCs pulsed with AT-2 HIV (100ng/ml and/or 300ng/ml) or nonpulsed autologous DCs) were co-cultured with autologous CD4+ (top) and CD8+ (bottom) T cells at a ratio of 1:5 (DC:T cell). Following 10 days of co-culture, expansion of HIV-specific T cell responses was measured via flow cytometry as a function of intracellular cytokine secretion in response to restimulation with corresponding autologous Poly-ICLC matured DCs \pm AT-2 HIV. A) Results are displayed from representative donors for IFN γ and TNF α . Top panel shows expansion of CD4+ T cells upon coculture with autologous DCs pulsed with AT-2 HIV (100ng/ml). Bottom panel shows expansion of CD8+ T cells upon coculture with autologous DCs AT-2 HIV (300ng/ml). B) Summary of intracellular cytokines secreted by CD4+ and CD8+ T cells following expansion with autologous DCs pulsed with AT-2 HIV. Data are represented as fold change in cytokine secretion of T cells expanded and restimulated with

autologous AT-HIV pulsed DCs compared with control (nonpulsed) conditions (**mean**, (range)).

TABLE 1

In Process Testing and Lot Release Criteria.

Virus Production Tests (In process tests)	Method	Criteria
Virus Quantification	HIV p24 ELISA	Culture terminated when p24 > 2 ng/mL at 3 consecutive time points
Sterility – Bacteria, Mycobacteria, and Fungi	Detection of aerobic and anaerobic bacteria and fungi by gram stain (7 days) and direct culture using BacTAlert (2 weeks for bacteria and fungi and 8 weeks for mycobacteria)	No growth
Mycoplasma	Direct Culture on mycoplasma selective agar (14 days) and DNA Fluorochrome Staining Assay with Vero Indicator Cell Line (5 days)	Negative
Endotoxin	Kinetic Chromogenic LAL Assay	< 50 EU/mL
Adventitious Virus Testing	In vitro culture using 3 cell lines (14 days)	Negative
Purity – Residual AT-2	HPLC for AT-2	< 0.5 ng of AT-2 detected in analyte
Identify – Viral Proteins	Western blot to confirm presence of full virion	Presence of HIV p24CA, gp41TM, and gp120
Virus Quantification	Quantification of p24CA by Coomassie staining and densitometry analysis	Density of p24CA compared to p24CA standard
Infectious Titre	Residual infectivity by TZM-bl reporter assay	SFU _s of media control (1.48 × 10 ⁻⁴ to 1.65 × 10 ⁻⁵ ng p24 HIV)
DC vaccine Tests (Lot Release Criteria)	Method	Criteria
Cell Viability	Viability staining after storage in LN ₂ vapor phase	> 70% viable
Identity	Flow Cytometry - % CD11c+	> 50%
	Flow Cytometry - % CD11c+CD14+	> 30%
	Flow Cytometry - % CD11c+CD86+	> 50%
Sterility – Bacteria, Mycobacteria, and Fungi	Direct Culture for aerobic and anaerobic bacteria, mycobacteria and fungi (2 weeks)	No growth
Mycoplasma	Direct Culture on mycoplasma selective agar (14 days) and DNA Fluorochrome Staining Assay with Vero Indicator Cell Line (5 days)	Negative
Endotoxin	Kinetic Chromogenic LAL Assay	< 50 EU/mL