**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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26 **ABSTRACT**

27 Therapeutic interventions for HIV-1 that successfully augment adaptive immunity to promote killing of infected
28 cells may be a requisite component of strategies to reduce latent cellular reservoirs. Adoptive immunotherapies
29 utilizing autologous monocyte-derived dendritic cells (DCs) that have been activated and antigen loaded *ex vivo*
30 may serve to circumvent defects in DC function that are present during HIV infection in order to enhance
31 adaptive immune responses. Here we detail the clinical preparation of DCs loaded with autologous Aldrithiol-2
32 (AT-2)-inactivated HIV that have been potently activated with the viral mimic, Polyinosinic-polycytidylic acid–
33 poly-L-lysine carboxymethylcellulose (Poly-ICLC). HIV is first propagated from CD4+ T cells from HIV-infected
34 donors and then rendered non-replicative by chemical inactivation with aldrithiol-2 (AT-2), purified, and
35 quantified. Viral inactivation is confirmed through measurement of *Tat*-regulated β -galactosidase reporter gene
36 expression following infection of TZM-bl cells. In-process testing for sterility, mycoplasma, LPS, adventitious
37 agents, and removal of AT-2 is performed on viral preparations. Autologous DCs are generated and pulsed with
38 autologous AT-2-inactivated virus and simultaneously stimulated with Poly-ICLC to constitute the final DC
39 vaccine product. Phenotypic identity, maturation, and induction of HIV-specific adaptive immune responses are
40 confirmed via flow cytometric analysis of DCs and cocultured autologous CD4+ and CD8+ T cells. Lot release
41 criteria for the DC vaccine have been defined in accordance with Good Manufacturing Practice (GMP) guidelines.
42 The demonstrated feasibility of this approach has resulted in approval by the FDA for investigational use in
43 antiretroviral (ART) suppressed individuals. We discuss how this optimized DC formulation may enhance the
44 quality of anti-HIV adaptive responses beyond what has been previously observed during DC immunotherapy
45 trials for HIV infection.

46 **Keywords:** Dendritic cell, Therapeutic Vaccine, HIV-1, Poly-ICLC

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51 **Introduction**

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

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

69 In terms of the HIV immunogen(s) utilized, the most successful DC immunotherapies to date have
70 involved the use of autologous, inactivated HIV/SIV [9, 10, 16, 18]. Most notably, a recent randomized,
71 controlled trial of autologous heat-inactivated HIV-pulsed DC resulted in a substantial decrease in viral load set
72 point following ART interruption[10]. In contrast to conventional means of viral inactivation, including heat
73 treatment, that result in damage to viral proteins, aldrithiol-2 (AT-2, 2,2'-dithiodipyridine) preserves the

74 conformational and functional integrity of HIV envelope glycoproteins to allow for unaltered viral fusion, while
75 rendering HIV non-infectious through covalent modification of key S-H- containing internal proteins[19]. Here
76 we detail the clinical preparation of Poly-ICLC (Hiltonol, Oncovir, Inc) activated DCs that are loaded with
77 autologous AT-2-inactivated HIV. This DC vaccine is now approved by the FDA for investigational use as a
78 therapeutic immunotherapy in ART suppressed individuals (BB-IND-15382). The design of this optimized DC
79 formulation aims to enhance the quality of anti-HIV adaptive immunity beyond what has been previously
80 observed during DC immunotherapy trials.

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82 **METHODS**

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

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

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

97 **Reagents and antibodies.** AIM-V media and CD4+ and CD8+ Dynal beads were purchased from Invitrogen.
98 RPMI-1640 media and X-Vivo 15 media (BioWhittaker™) were from Lonza Biologics, Inc. Pooled Human AB
99 Serum (PHS) was from Valley Biomedical. Ficoll-Paque Premium was from GE Healthcare Sciences. Aldrithiol-2
100 (AT-2) and reduced L-Glutathione were from Sigma-Aldrich. Good manufacturing practice-certified (GMP-
101 certified) Interleukin-2 (IL-2), GMP-certified Interleukin-4 (IL-4), Granulocyte-Macrophage Colony Stimulating
102 Factor (GM-CSF), GMP-certified Anti-CD3 antibody, CliniMACS CD4 and CD14 microbeads, CliniMACS LS Tubing
103 sets, CryoMACS DMSO and CliniMACS PBS/EDTA buffer were from Miltenyi Biotec, Inc. Polyinosinic-polycytidylic
104 acid – poly-L-lysine carboxymethylcellulose (Poly-ICLC) was from Oncovir, Inc. Fluorescent antibodies for IL-2, -
105 IFN γ , -TNF α , -MIP-1 β , -CD3, -CD4, and -CD8 were purchased from BD biosciences. Antibodies for CD86, -CD14, -
106 CD11c, and -HLA-DR were purchased from Biolegend. Recombinant human IL-2 and IL-7 were from R&D
107 Systems. The following antibodies were manufactured in-house at the AIDS and Cancer Virus Program, Leidos
108 Biomedical Research, Inc., Frederick National Laboratory: anti-p24CA [94-0001], anti-p17MA [R193244], anti-
109 gp120HIV [P3F5-D5-F8]. Anti-gp41TM(4E10) was obtained from Polymun [AB004].

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

120 **Inactivation of virus.** Supernatants from CD4+ T cell culture were thawed, pooled by donor, clarified through
121 low speed centrifugation using a SX4750 rotor (4,000 x g x 15 mins)(Beckman Coulter), and filtered through a
122 0.22 µm filter. AT-2 (0.5 M) in DMSO (CryoMACS) was added to the clarified supernatant to a final
123 concentration of 1 mM and incubated overnight for 12-18 hours at 4°C with gentle stirring. A portion of
124 supernatant was incubated overnight under similar conditions without AT-2 for comparative use in residual
125 infectivity assays.

126 **Virus purification and removal of AT-2.** The AT-2 inactivated virus (AT-2 HIV) from each batch of supernatant
127 was purified and concentrated by sequential ultracentrifugation. The virus was first pelleted by centrifuging at
128 4°C using a SW32 rotor (100,000 x g x 60 mins) (Beckman Coulter). The resultant pellet was resuspended with
129 1mL of 10 mM reduced L-Glutathione to react with any residual AT-2 present and centrifuged at 4°C using a
130 Type 50.4 Ti rotor (198,000 x g x 6 mins)(Beckman Coulter). The L-Glutathione was decanted and the pellet
131 resuspended in 300µl PBS. The purified AT-2-inactivated virus was aliquoted and stored in liquid nitrogen.

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

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

136 **Confirmation of Virus Inactivation.** Lack of residual infectivity of AT-2-inactivated HIV-1 was measured in a
137 TZM-bl assay as a function of Tat-regulated β-galactosidase reporter gene expression (NIH AIDS Research and
138 Reference Reagent Program, Division of AIDS, NIAID, NIH, Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme
139 Inc.[20-23]). Infectious virus was quantified as a function of β-galactosidase activity expressed as relative spot-
140 forming units (SFU) before and after AT-2 treatment, in donor CD4+ T cell supernatants and purified virus
141 samples. Viral controls of infectious and AT-2 inactivated HIV-1 BaL supernatant (AIDS and Cancer Virus
142 Program, Leidos Biomedical Research, Inc., National Laboratory for Cancer Research) of known infectivity were
143 included in each assay. TZM-bl plate images were captured with a Cellular Technology, Ltd. CTL-ImmunoSpot®

144 S6 Micro Analyzer ELISPOT Reader and the number of SFUs was analyzed with ImmunoSpot® Professional
145 Software's Immuno Capture, v6.3.

146 **Confirmation of AT-2 removal from virus preparations.** Removal of unreacted AT-2 in the purified virus
147 preparation (lysed in 8M Guanidine-HCl) was confirmed via HPLC with a threshold sensitivity of 0.5 ng/ul of
148 purified virus.

149 **Preparation of Monocyte-derived Dendritic Cells and Final DC Vaccine Product.** PBMCs from leukaphereses
150 were frozen using a controlled rate freezer and later thawed to generate monocyte derived dendritic cells (DCs).
151 Monocytes were enriched from frozen PBMCs by plastic adherence and cultured in RPMI 1640 media
152 supplemented with 5% PHS, 1% HEPES, and Gentamicin (24ug/ml) in the presence of 100 IU/mL GM-CSF
153 (Leukine) and 400 IU/mL IL-4 for 6 days. On day 6, immature DCs (iDCs) were pulsed overnight with autologous,
154 purified AT-2 HIV at a concentration of 100-300 ng/mL of p24 equivalents[26-29]. These DCs were matured with
155 5µg/mL Poly-ICLC to constitute the final DC vaccine product. The final DC vaccine product was frozen in
156 PHS/10% DMSO.

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

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

167 **Detection of Endotoxin.** The level of Gram negative bacterial endotoxin was measured in culture supernatants

168 using a Kinetic Chromogenic LAL Kit (Associates of Cape Cod, Inc., range 0.005 EU/ml - 50.0 EU/ml) per
169 manufacturer's protocol, at the beginning and end of CD4+ T cell culture, on day 5 of DC preparation, and on the
170 final DC vaccine product.

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

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

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

185 **Immunogenicity of the DC Vaccine.** Immunogenicity was evaluated as a function of activation of autologous
186 HIV-specific CD4+ and CD8+ T cell responses. DCs were derived as described and matured with Poly-ICLC +/-
187 pulsed with AT-2 HIV (100 ng/mL-300 ng/mL) on d6 of growth and co-cultured with magnetic bead sorted
188 autologous CD4+ and CD8+ T cells at a ratio of 1:5 (DC:T cell). Cultures were maintained for 10 days in RPMI-
189 1640 supplemented with 1mM HEPES, 2mM L-glutamine and 10% PHS in the presence of rhIL-2 (10 IU/mL) and
190 rhIL-7 (5ng/mL). To evaluate for the expansion of HIV-specific T cell responses following co-culture, T cells were
191 restimulated with autologous DC +/- pulsed with autologous AT-2 HIV at a ratio of 1:5 (DC:T cell) for 6 hours

192 followed by fixation, permeabilization, and fluorescent staining for IFN γ , TNF α , MIP1 β , and CD107a. Data were
193 acquired with an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

194 **RESULTS**

195 **HIV Production, Purification and Quantification**

196 Donor CD4+ T cells cultured under GMP conditions (N=4) remained viable and contained ≥ 2 ng/mL p24 by ELISA
197 for 36-55 days (**Fig. 2A**). Following purification via ultracentrifugation, quantification of the AT-2 inactivated
198 virus batches yielded an average of 200,071 ng p24CA [range 27,904 – 489,224 ng] (**Fig. 2B**). To further establish
199 feasibility of virus production, CD4+ T cells from 14 additional untreated HIV-infected donors were cultured, and
200 virus containing supernatants were inactivated and purified under non-GMP conditions with similar culture
201 duration and viral yield (**Supplemental Figure 1**). No correlation was found between viral yield and donor plasma
202 HIV-1 RNA or CD4+ T cell count (not shown).

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

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

206 Virus inactivation was confirmed in CD4+ T cell culture supernatants before and after AT-2 treatment (**Fig. 3A**)
207 and also following purification and concentration of viral supernatants (data not shown) for each donor via TZM-
208 bl assays. Following AT-2 treatment, there was no detectable infectivity present in all culture supernatants and
209 purified viruses tested. The removal of residual AT-2 was confirmed in all virus preparations by HPLC (N=4) (**Fig.**
210 **3B**).

211 **In Process Testing and Lot Release Criteria**

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

216 Supernatants did not contain adventitious virus at the end of culture (N=4). All samples evaluated for endotoxin levels
217 met the criteria for in process testing and lot release of <50 EU/mL.

218 **Viability, Identity and Maturation of DC Vaccine.**

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

229 **Immunogenicity of DC Vaccine**

230 The DC vaccine stimulated polyfunctional autologous HIV specific CD4+ and CD8+ T cells in all donors tested as
231 evidenced by secretion of IFN γ , TNF α , MIP1 β , and/or CD107a (N=3) (**Fig. 5**). For all donors, cytokine levels
232 secreted by CD4+ T cells following expansion and restimulation with AT-2 HIV-pulsed (either 100ng/ml or
233 300ng/ml) DCs were >3X higher in at least 2 cytokines and >2X higher in at least 3 cytokines when compared
234 with control (background) conditions (T cells expanded and restimulated with nonpulsed Poly-ICLC matured
235 DCs). Overall, CD8+ T cell responses were generally lower in magnitude compared with CD4+ T cell responses,
236 but remained >3X higher in at least 1 cytokine and >2X higher in at least 2 cytokines, in all donors compared
237 with background. There was no consistent dose response when comparing DCs that were pulsed with 100ng
238 versus 300ng of AT-2 HIV. This pattern of induction of CD4+>>CD8+ T cells has been observed in previous in
239 vitro studies of AT-2 HIV pulsed DCs and also in a clinical trial in untreated individuals[16, 27]. AT-2 HIV has been

240 found to stimulate HIV-specific CD8+ T cells via the classical endogenous pathway following CD4+ binding and
241 entry[28].

242 Discussion

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

263 Furthermore, we describe a means to augment the quality of DC immunotherapies for HIV-infected
264 individuals through the *ex vivo* activation of DCs with Poly-ICLC, a synthetic dsRNA that is recognized by TLR3 in
265 endosomes and cytoplasmic sensors, including MDA5 and DHX/DDX RNA helicases[32-34]. Consistent with prior

266 studies in seronegative donors[35, 36], we demonstrate that Poly-ICLC matures DCs from HIV-infected donors,
267 as evidenced by upregulation of costimulatory molecules and secretion of cytokines. Notably, Poly-ICLC
268 stimulated robust secretion of the pro-Th1 cytokine, IL-12, by DCs from donors on suppressive ART, compared
269 with standard cytokine cocktails that have been previously employed in clinical trials, which has likely limited
270 their anti-viral potency[9, 10, 16]. Powerful anti-viral effects were observed by Poly-ICLC and AT-2-inactivated
271 SIV in a therapeutic tonsillar vaccine study in non-human primates[37]. Here viral loads following treatment
272 interruption were 1-2 logs lower in vaccinated animals compared with unvaccinated controls or when Cytosine-
273 phosphate-guanine class C (TLR 9 agonist) was used as an adjuvant. Our finding of increased IL-12 secretion by
274 DCs from ART-treated HIV donors aligns with our previous work revealing that Poly-ICLC significantly rescues DC
275 cytokine secretion in the presence of plasma from ART-suppressed individuals, when compared with plasma
276 from untreated HIV donors, though some defect in cytokine production still remains, compared with plasma
277 from uninfected donors[4]. Given the suppressive role that HIV donor plasma has been shown to have on DCs,
278 an important factor in our culture system is the use of clinical grade plasma from seronegative donors, during
279 the derivation and activation of DCs, rather than autologous plasma which is commonly utilized and may
280 diminish DC cytokine secretion upon ex vivo stimulation[3, 4].

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

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Acknowledgements/Funding

This work has been supported by National Institutes of Health [K08 AI84578 to E.A.M., R37 AI044628 to N.B., and U01 A1067854]; National Institutes of Health, National Cancer Institute under contract HHSN261200800001E; Bill and Melinda Gates Foundation [Collaboration for AIDS Vaccine Discovery Grant ID: 38645]; Center for AIDS Research [P01AI057127]; and the New York University Langone Medical Center Grunebaum AIDS Research Fund and Saul Farber Scholarship.

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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.

TABLE 1: In Process Testing and Lot Release Criteria.

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).

431 (B) HPLC analysis of 0.5M AT-2 solution (AT-2 alone control) and purified AT-2-inactivated HIV from a
432 representative donor with and without the addition of 10 mmol r-Glutathione. HPLC was performed at a flow
433 rate of 300 mL/min on 3 m, 2 x 100 mm Luna C18(2), (Phenomenex), using aqueous
434 acetonitrile/trifluoroacetic/acid solvents on a Shimadzu HPLC system. Peaks were detected by UV absorption at
435 206 and 280 nm.

436

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

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

445

446 **FIGURE 5: Immunogenicity of DC Vaccine.**

447 To evaluate potency/immunogenicity, the autologous DC vaccine (Poly-ICLC matured DCs pulsed with AT-2 HIV
448 (100ng/ml and/or 300ng/ml) or nonpulsed autologous DCs were co-cultured with autologous CD4+ (top) and
449 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
450 responses was measured via flow cytometry as a function of intracellular cytokine secretion in response to
451 restimulation with corresponding autologous Poly-ICLC matured DCs $\pm$ AT-2 HIV. A) Results are displayed from
452 representative donors for IFN γ and TNF α . Top panel shows expansion of CD4+ T cells upon coculture with
453 autologous DCs pulsed with AT-2 HIV (100ng/ml). Bottom panel shows expansion of CD8+ T cells upon coculture
454 with autologous DCs AT-2 HIV (300ng/ml). B) Summary of intracellular cytokines secreted by CD4+ and CD8+ T

455 cells following expansion with autologous DCs pulsed with AT-2 HIV. Data are represented as fold change in
456 cytokine secretion of T cells expanded and restimulated with autologous AT-HIV pulsed DCs compared with
457 control (nonpulsed) conditions (**mean**, (range)).

458

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
Identity – 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	$\leq$ SFUs of media control ($\leq 1.48 \times 10^{-4}$ 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+ Flow Cytometry - % CD11c+CD14+ Flow Cytometry - % CD11c+CD86+	$> 50\%$ $< 30\%$ $> 50\%$
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

Figure 1

Figure 2

Figure 3

Figure 4

B. T cell responses*

	CD4	CD8
IFNγ	6.43, (1.20-12.59)	1.59, (0.08 – 3.43)
TNFα	2.23, (0.0 – 6.29)	1.95, (0.57 - 8.51)
MIP-1β	1.89, (1.55 – 3.09)	1.98, (0.04 - 4.68)
CD107a	1.29, (0.42 – 3.23)	2.30, (0.65 – 4.59)

Figure 5 *represented as fold increase secretion compared with unpulsed DCs, (range)