Attenuated *Listeria monocytogenes* Vectors Overcome Suppressive Plasma Factors During HIV Infection to Stimulate Myeloid Dendritic Cells to Promote Adaptive Immunity and Reactivation of Latent Virus

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Abstract

HIV-1 infection is characterized by myeloid dendritic cell (DC) dysfunction, which blunts the responsiveness to vaccine adjuvants. We previously showed that nonviral factors in HIV-seropositive plasma are partially responsible for mediating this immune suppression. In this study we investigated recombinant *Listeria monocytogenes* (Lm) vectors, which naturally infect and potently activate DCs from seronegative donors, as a means to overcome DC dysfunction associated with HIV infection. Monocyte-derived DCs were cocultured with plasma from HIV-infected donors (HIV-moDCs) to induce a dysregulated state and infected with an attenuated, nonreplicative vaccine strain of Lm expressing full length clade B consensus gag (KBMA Lm-gag). Lm infection stimulated cytokine secretion [interleukin (IL)-12p70, tumor necrosis factor (TNF)- α , and IL-6] and Th-1 skewing of allogeneic naive CD4 T cells by HIV-moDCs, in contrast to the suppressive effects observed by HIV plasma on moDCs on toll-like receptor ligand stimulation. Upon coculture of “killed” but metabolically active (KBMA) Lm-gag-infected moDCs from HIV-infected donors with autologous cells, expansion of polyfunctional, gag-specific CD8⁺ T cells was observed. Reactivation of latent proviruses by moDCs following Lm infection was also observed in models of HIV latency in a TNF- α -dependent manner. These findings reveal the unique ability of Lm vectors to contend with dysregulation of HIV-moDCs, while simultaneously possessing the capacity to activate latent virus. Concurrent stimulation of innate and adaptive immunity and disruption of latency may be an approach to reduce the pool of latently infected cells during HIV infection. Further study of Lm vectors as part of therapeutic vaccination and eradication strategies may advance this evolving field.

Introduction

REDUCTION OF LATENT CELLULAR RESERVOIRS during HIV-1 infection will likely require a multifaceted approach, with one component focusing on the enhancement of cytotoxic T lymphocyte (CTL) function to promote killing of infected cells.¹ As the most potent antigen-presenting cells, myeloid dendritic cells (DCs) are targets for therapeutic vaccines that aim to augment CTL function. However, the presence of DC dysfunction during HIV infection may hinder

the formation of innate and adaptive immune responses following therapeutic vaccination and limit their success.^{2–19}

We previously established that plasma factors during HIV infection suppress cytokine secretion and Th1-skewing capacity by DCs in response to Toll-like receptor (TLR) stimulation. These DC-specific suppressive effects are only partially mitigated by antiretroviral therapy (ART) and not directly mediated by HIV itself.^{18,19} Thus, novel adjuvants and/or vaccine vectors that target DCs and improve their function *in situ* during HIV infection may be necessary to

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pave the way for effective therapeutic vaccines. Another focus of curative strategies is the investigation of agents to disrupt viral latency to allow for immune recognition of these infected cells.^{1,20–22} If devised appropriately, therapeutic vaccines through transient immunostimulatory properties may simultaneously reactivate latent HIV while enhancing CTL function to promote killing of these cells.

Listeria monocytogenes (Lm) is a unique intracellular bacterium that stimulates potent innate and adaptive immunity.²³ Lm naturally infects DCs *in vivo*, which has been shown to be vital for the generation of immune responses.^{24–26} DCs from HIV-seronegative donors are strongly activated to undergo maturation and secrete cytokines following infection with Lm,²⁷ though its effect on DCs in the setting of HIV infection remains unexplored. The use of recombinant, attenuated Lm vectors in humans has been studied primarily in the context of cancer immunotherapy as a means to deliver tumor antigens to DCs and enhance antitumor immunity. Phase I trials have demonstrated enhancement of innate and adaptive antitumor immune responses with acceptable safety outcomes, with several Phase II studies now underway.^{28–30} Though there have been no human trials in HIV-infected individuals, Lm vectors encoding HIV antigens have been immunogenic in various animal models.^{31–34}

A major advantage of Lm vectors is that preexisting immunity to Lm does not cause a decrease in vaccine efficacy in animal studies.^{35–38} Concerns for potential pathogenicity have been addressed through the development of highly attenuated strains with two layers of safety. First, two virulence genes required for wild-type Lm pathogenicity, *actA* and *inlB*, have been deleted, which results in abrogation of cell-to-cell spread and infection of nonphagocytic cells, including hepatocytes, to reduce hepatotoxicity.³⁹ Second, the Lm strains are “killed” but metabolically active (KBMA), and nonreplicative due to deletion of the *uvrAB* DNA repair genes, rendering KBMA Lm vaccines exquisitely sensitive to photochemical inactivation (UV light and psoralen), which results in the formation of infrequent covalent DNA cross-links.⁴⁰ These modifications allow KBMA Lm to retain the ability to express and secrete both self and recombinant heterologous genes in the cytosol of infected antigen-presenting cells, retaining its immunogenicity.^{27,40} Furthermore, antigen expression of heterologous genes by Lm can be enhanced via specific amino acid substitutions in the transcription factor and central virulence regulator, *PrfA*, which renders it constitutively activated, along with the expression of target antigens, which are *PrfA* dependent.⁴¹

Our objective was to evaluate the potential utility of Lm vectors as a means to overcome compromised DC responsiveness observed in the context of circulating plasma factors during HIV infection to modulate the formation of adaptive immune responses. Particular focus was placed upon a potential vaccine strain: KBMA Lm expressing full-length clade B HIV consensus gag (KBMA Lm-gag) as both an antigen loading and activation platform for DCs. We found that KBMA Lm-gag infection of human monocyte-derived DCs (moDCs) in the setting of untreated HIV infection stimulates robust secretion of cytokines by DC to skew Th-1 responses from naive CD4 T cells and induce the expansion of polyfunctional, gag-specific memory CD8 T cells. Moreover, we found that moDCs infected with Lm vectors possess the capacity to reactivate latent proviruses in models of HIV

latency. These findings suggest that recombinant Lm vectors may play a role in eradication efforts by simultaneously activating the latent reservoir, while improving the ability of the innate and adaptive immune system to recognize and kill these infected cells.

Materials and Methods

Study population

The HIV-infected subjects enrolled in this cross-sectional study who donated plasma samples were recruited through New York University AIDS Clinical Trial Unit and the Center for AIDS Research. Healthy donors served as controls. Chronically infected, untreated subjects (untreated, $N=10$) were infected for ≥ 1 year (mean viral load 44,202 copies/ml, mean CD4 T cell count 342 cells/ml, mean age 44.3 years).¹⁹ The subjects enrolled in the study for induction of gag-specific memory T cell responses were recruited through the Center for HIV/AIDS Vaccine Immunology (CHAVI 012). These were HIV-infected subjects who were not receiving therapy at the time of leukapheresis. Informed consent was obtained from all study participants in accordance with the Declaration of Helsinki. This study was reviewed and approved by the Institutional Review Board of Bellevue Hospital, New York University School of Medicine, the University of North Carolina Chapel Hill, the Aaron Diamond AIDS Research Center, and CHAVI.

Listeria monocytogenes strains

All *Listeria* strains are derived from the phage-cured WT strain DP-L4056.⁴² The attenuated KBMA platform strain (Lm $\Delta actA \Delta inlB \Delta uvrAB$ *prfAG155S*) was constructed as described.⁴¹ The WT KBMA strain (Lm $\Delta uvrAB$ *prfAG155S*) was constructed using established methods. The HIV clade B consensus gag sequence was codon optimized for expression in Lm and synthesized *de novo* (DNA2.0, Menlo Park, CA). The synthetic gene was cloned downstream of the *actA* promoter and in-frame with the amino-terminus of the ActA signal sequence in a derivative of the site-specific integration vector pPL2. The gag antigen expression cassette was integrated at the *tRNA^{Arg}* locus of both attenuated and WT strain platforms.⁴² Live *Listeria* batches were prepared by growing the bacteria to early stationary phase in yeast media, washing in phosphate-buffered saline (PBS), and freezing at -80°C . KBMA batches were prepared as described previously.⁴⁰ Both live and KBMA batches were thawed and diluted to appropriate concentrations as noted.

DC preparation and stimulation

MoDCs were generated from buffy coats (New York Blood Center) from HIV-seronegative individuals as previously described.⁴³ Using a system we have previously described and validated on both moDCs and circulating myeloid DCs,¹⁹ moDCs were harvested on day 4, washed, and resuspended in RPMI supplemented with 10% plasma from each control and untreated HIV-infected donor for 24 h ($N=8-9$ per plasma group per experiment) prior to infection/stimulation.¹⁹ For each individual experiment, moDCs generated from a single uninfected donor were exposed to the entire cohort of plasma from HIV-infected subjects in addition to control plasma donors prior to infection/stimulation.

On day 5, moDCs were infected with Lm vectors (MOI 10–50) as previously described,²⁷ or stimulated with poly ICLC (Oncovir) at 5 $\mu\text{g}/\text{ml}/10^6$ overnight (15–18 h). The MOIs used to infect DCs (10–50) were based on titrations of attenuated Lm vectors revealing reproducible activation of DCs while minimizing cell toxicity (data not shown).²⁷ Secretion of interleukin (IL)-12p70, tumor necrosis factor (TNF)- α , IL-6, and IL-1 β was measured in moDC supernatants using the Human Inflammatory Cytokine Cytometric Bead Array (BD Pharmingen).

Naive CD4 T cell coculture

Allogeneic naive CD4 T cells were purified by magnetic cell sorting (Naive CD4 T cell Isolation Kit II, Miltenyi Biotec) from uninfected donor peripheral blood mononuclear cells (PBMCs) (New York Blood Center) and cocultured with moDCs (ratio of 1:5 DC/T cell) that had been cultured with plasma from control and HIV-infected subjects and infected/stimulated with Lm vectors or poly ICLC as above.¹⁹ After 6 days, culture supernatants were assayed for interferon (IFN)- γ using Human Cytokine Cytometric Bead Arrays (BD Pharmingen).

Expansion of gag-specific CD4 and CD8 memory T cell responses

Memory CD4 and CD8 T cells were enriched from untreated HIV-infected donor PBMCs following magnetic bead sorting (Negative Selection for CD56, CDRA, CD14, CD56, and CD19, Miltenyi Biotec). T cells were then cocultured with autologous moDCs following infection with Lm vectors at a ratio of 1:5 (DC/T cell) or with pools of overlapping 15-mer HIV-1 gag-peptides (1.5 $\mu\text{g}/\text{ml}$) (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) for 10 days in IMDM (GIBCO) supplemented with 1 mM HEPES (Life Technologies), 2 mM L-glutamine (Sigma), streptomycin (100 $\mu\text{g}/\text{ml}$)/penicillin (100 IU/ml) (Sigma), and 5% PHS in the presence of rhIL-2 (10 IU/ml) and IL-7 (5 ng/ml) (R&D Systems).

To evaluate for the presence of gag-specific T cell responses at baseline and following coculture with DCs or gag-peptides, T cells were stimulated with pools of gag-peptides (5 $\mu\text{g}/\text{ml}$) versus control peptides (Neural MOG, proimmune) for 6 h in the presence of brefeldin A (10 $\mu\text{g}/\text{ml}$) and Monensin (1 $\times$) (Biolegend), and intracellular cytokine production was assessed following fixation, permeabilization, and fluorescent staining for IFN- γ (BD Pharmingen), TNF- α (ebiosciences), and CD107a (LAMP-1) (Biolegend). Antigen specificity was defined by the percentage of cells secreting cytokine if it exceeded background (cytokine-secreting cells in the presence of control peptide stimulation) by more than 2-fold and consisted of more than 0.5% of responding cells following subtraction of background for at least one cytokine. Data were acquired with a FACScalibur cytometer (BD Biosciences) and analyzed using FlowJo software.

Latency models

ACH-2 cells (HIV-1 latent T cell clones, NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Dr. Thomas Folks^{44,45}) were cultured in the presence of TNF- α (10 ng/ml), PMA (1 μM), SAHA (vor-

inostat) (Sigma), or moDCs $\pm$ Lm vectors. After 48 h, infectious HIV in the supernatant was quantified in a β -galactosidase reporter assay in TZM-bl cells (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc.^{46–49}). Infectious virus was quantified by either measurement of spot-forming units (SFU) captured with a Cellular Technology, Ltd. (C.T.L.) CTL-ImmunoSpot S6 Micro Analyzer ELISpot Reader and analyzed with ImmunoSpot Professional Software's ImmunoSpot Analysis Pack or via colorimetric quantification (OD) using a β -Galactosidase Assay Kit (Thermo Scientific). In select experiments, moDCs and ACH-2 cells were separated in culture wells using Transwell plates (0.4- μm polycarbonate membrane, Costar).

J-lat cells encoding GFP and full length HIV-1 (clone 10.6)⁵⁰ were cultured in the presence of supernatants obtained from Lm-infected moDCs for 24 h and compared to additional activators including PMA/ionomycin, TNF- α , and SAHA (vorinostat) (Sigma). Reactivation of HIV-1 was measured via flow cytometry as a function of cellular GFP expression. Infliximab (Remicade), a chimeric monoclonal antibody against TNF- α , was added to ACH-2 and J-lat cultures in select experiments.

Statistical analysis

Separate analyses were performed for each experiment individually. Nonparametric specific pairwise comparisons between independent groups were performed (Mann–Whitney nonparametric *U* test) for the secreted cytokine (Figs. 1 and 2). For results in Figs. 3–5 analyses of variance was performed for an overall comparison among independent groups if ≥ 3 groups were present, and Student's *t* tests were then used for specific pairwise comparisons. In all cases, *p*-values < 0.05 using two-sided tests were considered significant. For rtPCR arrays, statistical analysis was performed via software provided by the manufacturer (www.sabiosciences.com/pcrarraydataanalysis.php) to determine *p*-values for individual fold-changes (see Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid), and linear regression was performed using Graph Pad Prism 4.

Results

Lm infection stimulates robust cytokine secretion by HIV plasma-suppressed DC

Monocyte-derived DCs were cocultured with plasma from HIV-infected donors (HIV-moDCs) to induce a dysregulated state and subsequently infected with KBMA Lm or stimulated with TLR ligand (poly ICLC). Suppression of IL-12p70 secretion by HIV-moDCs was observed upon poly ICLC stimulation but not following infection with KBMA Lm in all moDC donors tested (Fig. 1A). The overall median IL-12p70 secretion (represented as % of mean control plasma moDC secretion upon infection/stimulation) for KBMA Lm-infected moDCs was $106.9 \pm 33.9\%$ versus $43.1 \pm 18.4\%$ for poly ICLC moDCs. The *p*-values (performed on each individual moDC donor experiment) were < 0.001 for all donors (Fig. 1A). Levels of secreted IL-12p70 did not differ significantly among control plasma and HIV plasma moDCs following infection with KBMA Lm (Supplementary Fig. S1A).

FIG. 1. *Listeria monocytogenes* (Lm) infection stimulates robust cytokine secretion by HIV plasma-suppressed dendritic cells (DCs). Measurement of cytokine secretion by monocyte-derived DCs (moDCs) from uninfected donors was performed following exposure to plasma from control ($N=9$) and untreated HIV-infected subjects ($N=9$) and subsequent infection with “killed” but metabolically active (KBMA) Lm-gag (MOI 10) or stimulation with the TLR3 agonist poly ICLC ($5 \mu\text{g/ml}$). Each experiment was performed on three independent moDC donors (denoted as DC donor 1–3 below the x -axis). (A) Interleukin (IL)-12p70, (B) tumor necrosis factor (TNF)- α , and (C) IL-6 secretion by moDCs following exposure to HIV plasma and stimulation by poly ICLC or infection with KBMA Lm-gag. Each dot represents an individual HIV plasma donor to the mean of control plasma moDC secretion following infection/stimulation for each condition. The asterisks indicate p -values: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Similarly, inhibition of TNF- α and IL-6 secretion by HIV plasma observed upon TLR stimulation was significantly less following infection with KBMA Lm (Fig. 1B and C) in all moDC donors. The overall median TNF- α secretion (represented as % of mean control plasma moDC secretion upon infection/stimulation) for KBMA Lm moDCs was $92.6 \pm 65.4\%$ versus $38.0 \pm 21.2\%$ for poly ICLC moDCs. The median IL-6 secretion for KBMA Lm moDCs was $100.4 \pm 66.8\%$ versus $36.5 \pm 19.8\%$ for poly ICLC moDCs. The p -values for both TNF- α and IL-6 (performed on each individual moDC donor experiment) were < 0.05 for all donors (Fig. 1B and C). Of note, TNF- α and IL-6 secretion by KBMA Lm was decreased in HIV-moDCs in one of the three moDC donors tested when compared with controls (Supplementary Fig. S1B and C). IL-1 β secretion was observed in KBMA Lm-infected moDCs in both control and HIV groups with a trend similar to TNF- α and IL-6 (Supplementary Fig. S1D).

Poly ICLC-stimulated moDCs did not consistently secrete appreciable levels of IL-1 β in either control or HIV groups. However, in the one donor in whom IL-1 β secretion was observed, levels were reduced following exposure to HIV plasma as previously described (data not shown).¹⁹ When moDCs were left unstimulated following plasma exposure, minimal cytokine secretion was observed with no differences between HIV plasma and control plasma groups (data not shown). KBMA strains of Lm induced levels of cytokine secretion (IL-12p70, TNF- α , and IL-6) similar to both WT and attenuated ($\Delta actA \Delta inlB$) strains of Lm despite attenuation and/or inactivation with psoralen and UV light (Supplementary Fig. S2).

In the experiment displayed in Supplementary Fig. S2, the IL-12 secreted by control DCs in response to poly ICLC was greater than in response to infection with Lm vectors. In contrast, the secretion of TNF- α was greater in response to

Lm vectors. This general trend is often observed *in vitro* at the utilized doses of poly ICLC and Lm, though significant donor-to-donor variability exists (not shown). Furthermore, there is a dose dependency in regard to magnitude of secreted cytokines by DCs in response to poly ICLC and Lm vectors. Determination of equivalent doses of poly ICLC versus Lm vectors is not feasible, nor is the physiologic relevance of the utilized doses known or easily estimated.

Consistent with the observed cytokine secretion, transcriptional responses by DCs in the setting of HIV infection are comparable to controls following infection with Lm vectors as measured using the Human Toll-Like Receptor (TLR) Signaling Pathway RT² Profiler PCR Array (SABiosciences) (Supplementary Fig. S3). Fold changes (HIV plasma moDCs/control plasma moDCs) for 84 genes central to DC activation, including transcription factors, cytokines, and chemokines, following infection with KBMA Lm-gag (MOI 10) versus poly ICLC ($5 \mu\text{g/ml}$) are listed with p -values (Supplementary Table S1). Though on an individual level only a few of these differences demonstrated significance (Supplementary Table S1), when taken together, fold changes (HIV plasma moDCs/control plasma moDCs) were significantly higher following KBMA Lm-gag infection compared with poly ICLC stimulation ($p < 0.0001$) (Supplementary Fig. S3A). Furthermore, the majority of fold-change values clustered around 1 for KBMA Lm-gag [Supplementary Fig. S3A (top)].

To better quantify the relationship of transcriptional responses between HIV and control groups, linear regression and comparison of slopes were performed, revealing that HIV plasma moDCs more closely resembled control plasma exposed moDCs following KBMA Lm-gag infection compared with poly ICLC [slope = 0.77, $R^2 = 0.81$ (KBMA Lm) versus slope = 0.17, $R^2 = 0.27$ (poly ICLC), $p < 0.0001$] (Supplementary Fig. S3B).

HIV plasma-suppressed DCs retain Th-1 skewing capacity following infection with Lm vectors

When plasma-exposed moDCs were cocultured with allogeneic naive CD4⁺ T cells, suppression of IFN- γ secretion observed upon TLR stimulation (poly ICLC) by HIV plasma was observed to a significantly lesser degree following infection with KBMA Lm in all moDC donors tested (Fig. 2). The overall median IFN- γ secretion (represented as % of mean control plasma moDC/T cell IFN- γ secretion for each respective condition) for the KBMA Lm group was $98.0 \pm 32.1\%$ versus $39.0 \pm 18.8\%$ for poly ICLC. The p -values (performed on each individual moDC donor experiment) were <0.05 for all donors (Fig. 2B). Levels of secreted IFN- γ did not differ significantly among control plasma and HIV-moDCs following infection with KBMA Lm in two of three moDC donors (Supplementary Fig. S1E).

Polyfunctional gag-specific T cell responses are expanded by KBMA Lm-gag-infected DC

Expansion of gag-specific CD8 and CD4 T cell responses from each untreated HIV-infected donor tested was observed by autologous KBMA Lm-gag-infected moDCs (Fig. 3 and Supplementary Fig. S4). The CD8 T cell responses expanded by KBMA Lm-gag moDCs were of greater magnitude than gag-peptide-expanded cells as measured by secretion of IFN- γ and CD107a, and a trend toward TNF- α ($p=0.051$) (Fig. 3A and B). Though increases in cytokine levels were observed following expansion with both KBMA Lm (-) and KBMA Lm-gag moDCs (Fig. 3A), subtraction of background (control peptide stimulated) from gag-peptide-stimulated wells revealed that only KBMA Lm-gag vectors exhibited gag-specific responses (Fig. 3B). These CD8 T cell responses were polyfunctional with 93.26% secreting two or more cytokines and 47.46% secreting three cytokines (Fig. 3C). Polyfunctional CD4 T cell responses were similarly ex-

panded by KBMA Lm-gag moDCs, though these were not significantly greater in magnitude as compared with gag-peptide-expanded cells (Supplementary Fig. S4).

Reactivation of latent HIV by DCs infected with Lm vectors

Upon coculture with ACH-2 cells, moDCs infected with Lm vectors [wild-type Lm, attenuated Lm, and KBMA Lm (MOI 10)] each resulted in significant reactivation of infectious HIV compared with uninfected DCs ($p<0.05$), similar to positive controls TNF- α and PMA (Fig. 4A) and greater than SAHA (vorinostat) (Fig. 4C). When moDCs and ACH-2 cells were separated in culture using Transwell permeable membranes, levels of infectious HIV were overall unaltered indicating that reactivation was not dependent on cell-to-cell contact, but rather was mediated by cytokines and/or other soluble factors (Fig. 4B). HIV reactivation by Lm-infected moDC supernatants was almost entirely abrogated in the presence of infliximab, indicating a dependence on TNF- α in this model (Fig. 4B).

In the J-lat 10.6 model (expressing full-length HIV GFP), supernatants from Lm-infected moDCs resulted in reactivation of latent provirus as evidenced by high levels of GFP expression (Fig. 5). These levels again were comparable to known activators such as TNF- α and PMA/ionomycin, but greater than SAHA. GFP expression induced by Lm-infected moDC supernatants was again abrogated in the presence of infliximab, indicating that reactivation is largely mediated by TNF- α in this model (Fig. 5B).

Discussion

We report that Lm vectors are able to overcome suppressive plasma factors during chronic untreated HIV infection to potentially activate DCs as evidenced by secretion of cytokines and expression of transcriptional responses similar to

FIG. 2. HIV plasma-suppressed DCs retain Th-1 skewing capacity following infection with Lm vectors. MoDCs from uninfected donors were exposed to plasma from control ($N=9$) and untreated HIV-infected subjects ($N=8-9$) infected with KBMA Lm-gag (MOI 10), or stimulated with poly ICLC ($5 \mu\text{g/ml}$), and then cocultured with allogeneic naive CD4 T cells for 6 days at which point supernatants were assayed for interferon (IFN)- γ . (A) IFN- γ secretion is displayed from a representative experiment. Control conditions performed with unstimulated moDCs following plasma exposure are denoted by (-). Each dot represents an individual control or HIV plasma donor. (B) Values from three separate moDC donors are displayed as percent (%) control following normalization of IFN- γ secretion from each HIV-infected plasma donor to the mean of control plasma moDC/T cell IFN- γ secretion for each respective condition. The asterisks indicate p -values: *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

FIG. 3. Expansion of polyfunctional gag-specific CD8 T cells by KBMA Lm-gag DCs from untreated HIV-infected donors. **(A)** Autologous memory CD8 T cells from untreated HIV-infected donors were expanded via coculture with overlapping gag-peptides versus autologous moDCs infected with KBMA Lm (-) or KBMA Lm-gag for 10 days. Expansion of gag-specific CD8 T cells was measured by intracellular cytokine staining for IFN- γ , TNF- α , and CD107a following restimulation with gag-peptides. These responses were compared to baseline (unexpanded) gag-specific responses. Data are displayed from a representative donor. **(B)** To account for variability in background cytokine levels per condition (cytokine-secreting cells in the presence of control peptide restimulation), gag-specific responses from each donor are represented as the percentage of cells secreting cytokine following subtraction of background (* $p \leq 0.05$). **(C)** The polyfunctionality of the CD8 T cell responses expanded by KBMA Lm-gag-infected moDCs was analyzed as the ability to produce one (IFN- γ alone), two, or all three cytokines in response to restimulation with gag-peptides.

controls. Notably, this is in sharp contrast to traditional DC stimuli, including TLR ligands and CD40L, where cytokine secretion, in particular IL-12, is impaired,^{5,6,8,12,15,16,18,19} potentially hindering adaptive responses to pathogens and vaccine adjuvants during chronic infection. This defect in IL-12 secretion by DCs during HIV infection is most prominent during untreated infection, but persists during suppressive ART therapy.¹⁹ Our finding of preservation of IL-12 secretion during chronic infection is especially noteworthy due to the critical role it plays in the formation of Th-1 type responses to generate antiviral CTLs, in addition to enhancing natural killer (NK) cell cytotoxicity.^{51–53}

Consistent with this finding of maintained IL-12 secretion, we also observe largely preserved Th1 skewing of CD4 T cells by HIV-moDC infected with Lm vectors. Remarkably, an impaired Th-1 skewing capacity has been previously described not only for TLR-stimulated DCs, but also for CD40L-activated DCs.⁶ The findings of preserved cytokine

secretion following Lm infection of HIV-moDCs were supported by transcriptional responses that were more similar to control moDCs following Lm infection when compared with TLR stimulation of moDCs. Taken together, these findings suggest that Lm vectors possess adjuvant properties that may enhance vaccine-induced immune responses during chronic HIV infection. Alternatively, the design of novel adjuvants that mimic signaling initiated by Lm may be of use in the future. In addition to signaling via TLRs, activation by Lm occurs via a multitude of membrane-bound and cytoplasmic pattern recognition receptors (PRRs) including nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and the cytosolic DNA sensor STING.^{54–61}

It is possible the activation of these additional pathways either singly or in combination allows Lm to maintain its stimulatory capacity despite the suppressive milieu that circulates during HIV infection. It remains unclear which of these pathway(s) is key to overcoming DC suppressive

FIG. 4. Reactivation of latent HIV by DCs infected with Lm vectors in an ACH-2 model. **(A)** The latently infected T cell line (ACH-2) was cultured in the presence of TNF- α (10 ng/ml), PMA (1 μ M), or moDC $\pm$ Lm vectors [wild-type (WT) Lm, attenuated (Att) Lm, and KBMA Lm (all MOI 10)]. After 48 h, infectious IV in the supernatant was quantified in a β -galactosidase reporter assay in TZM-bl cells [expressed as spot forming units (SFU)]. This was carried out in five independent moDC donors and data were pooled (*top panel*) (* $p \leq 0.05$). The *bottom panel* displays TZM-bl images from a representative donor. **(B)** In certain experiments, moDCs and ACH-2 were separated in culture using Transwell plates to determine the impact of cell-to-cell contact versus the sole effect of soluble mediators. This was carried out in four independent moDC donors and data were pooled (*top panel*). The *bottom panel* displays TZM-bl images from a representative donor. **(C)** Supernatants from moDC $\pm$ Lm infection were cocultured with ACH-2 cells and infectious virus was quantified using a colorimetric β -galactosidase assay (OD) and compared to TNF- α (5 ng/ml) and SAHA (Vorinostat) (300 nM). In certain conditions, J-lat cells were pretreated with 100 μ g/ml infliximab, a monoclonal antibody against TNF- α , immediately prior to being applied to the J-lat cells.

factors, and thus studies to better delineate the respective role each plays in this process are warranted given the relevance to therapeutic vaccine strategies for HIV.

We additionally characterized the formation of antigen-specific responses by DCs infected with KBMA Lm-gag vectors, reporting that they effectively expand polyfunctional, gag-specific CD8⁺ T cells that express multiple cytokines and markers for cytotoxicity. Similar phenotypes have been implicated in superior viral control during HIV infection and are found more frequently in long-term nonprogressors.^{62,63} The generation of such responses by therapeutic vaccination may serve as an important component of eradication efforts. Purging of the latent reservoir will ultimately rely on the death of infected cells following virus reactivation; however, it has been demonstrated that induction of viral replication in the absence of cellular

activation, as is accomplished with histone deacetylase (HDAC) inhibitors, is not sufficiently cytopathic to induce cell death.¹

Furthermore, endogenous CTLs from HIV-infected individuals on suppressive ART were shown to be ineffectual in killing infected cells following reactivation. Rather, high-quality CTLs, such as those from either elite suppressors or from ART-treated individuals following *in vitro* stimulation, were more effective in killing infected cells following reactivation.¹ These findings strongly support a central role for the enhancement of HIV-specific adaptive responses by effective therapeutic vaccines as a part of eradication strategies. Lm vectors appear to possess the requisite qualities for successful therapeutic vaccine platforms for HIV given their ability to concurrently deliver antigen and strongly activate DCs to stimulate adaptive immunity.

FIG. 5. Reactivation of latent HIV by DCs infected with Lm vectors in the J-lat 10.6 model. **(A)** Supernatants from moDC $\pm$ Lm infection were cocultured for 24 h with the J-lat model of latency encoding gfp and full length HIV-1. Reactivation of latent provirus as evidenced by GFP expression was compared with PMA (10 nM)/ionomycin (1 μ M) and SAHA (Vorinostat) (300 nM). **(B)** Supernatants from moDC $\pm$ Lm infection were cocultured with J-lat cells and GFP expression was compared to TNF- α (5 ng/ml). In certain conditions, J-lat cells were pretreated with 100 μ g/ml infliximab, a monoclonal antibody against TNF- α , immediately prior to being applied to the J-lat cells.

We present evidence in models of HIV latency that DCs infected with Lm vectors are able to promote reactivation via bystander effects on infected cells. Importantly, this was not dependent on DC:T cell contact, but was achieved in equal magnitude by soluble mediators secreted by Lm-infected DCs indicating that the effects of systemically administered Lm vectors may have the potential to broadly reach latently infected cells. Reactivation of HIV in this context is dissimilar to current approaches that directly target latency promoting mechanisms by HIV without inducing cellular activation, including HDAC inhibitors.^{1,20–22}

In contrast, attenuated Lm vectors lead to secretion of proinflammatory cytokines by DCs, several of which have been shown to trigger cellular activation pathways (e.g., NF- κ B) and promote HIV reactivation.^{64–70} Here, we show that TNF- α secreted by DCs that have been infected and activated with Lm is largely responsible for HIV reactivation in the context of the models utilized. Thus, the effects observed on HIV latency are not likely to be Lm specific, but may occur with any stimuli/vector that efficiently induces secretion of TNF- α by HIV-DCs. Though our findings represent data from models of HIV infection, the role TNF- α plays in the reactivation of latent HIV has been extensively described in multiple primary models of HIV latency and, more importantly, in resting CD4⁺ T cells from HIV-infected individuals using viral outgrowth assays.^{69,71,72}

The level of reactivation has varied in primary models ranging from no effect in models described by Greene and Planelles^{72–74} to modest effects in models described by Lewin, Siliciano, and Spina.^{69,71–73} Notably, aside from cell lines, the most robust reactivation by TNF- α has been observed via viral outgrowth assays in resting CD4 T cells isolated from HIV-infected donors following leukapheresis (approximately 50% of maximal stimulation).⁷²

The reasons for the variable effects observed by TNF- α on HIV latency in various models remain unclear, though it has been postulated that there may be a dependence on TNFR expression on infected cell targets.⁷² In general, recent clinical approaches to disrupt latency have avoided strategies that result in cellular activation due to concerns for toxicities from undesired or inappropriate immune activation. However, if inflammation is transient following vaccination, as occurs with attenuated Lm vectors,^{28,40} and is simultaneously directed at enhancing anti-HIV adaptive immunity, it may represent a safe and effective singular approach to reduce the latent reservoir. Our findings suggest that future therapeutic vaccine trials utilizing systemic adjuvants should not only evaluate for immunogenicity endpoints, but also assess the latent reservoir, including measures such as cell-associated HIV-1 RNA and DNA and viral outgrowth assays.

Though it remains to be seen whether therapeutic vaccination alone versus vaccination in combination with targeted antilateness drugs will be a successful means to tackle latent viral reservoirs, agents that are able to combat the suppressive milieu during HIV infection to stimulate appropriate innate responses, such as Lm vectors, hold promise to advance this evolving field.

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Author Disclosure Statement

D.B., P.L., and T.D. are paid employees of Aduro BioTech Inc., hold stock in the company, and are inventors on several patents related to the Lm vaccines described. N.B. is the co-inventor on patents related to DC biology and differentiation.

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