A dendritic cell targeted vaccine induces long-term HIV-specific immunity within the gastrointestinal tract

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Despite significant therapeutic advances for HIV-1 infected individuals, a preventative HIV-1 vaccine remains elusive. Studies focusing on early transmission events, including the observation that there is a profound loss of gastrointestinal (GI) CD4+ T cells during acute HIV-1 infection, highlight the importance of inducing HIV-specific immunity within the gut. Here we report on the generation of cellular and humoral immune responses in the intestines by a mucosally administered, dendritic cell (DC) targeted vaccine. Our results show that nasally delivered α-CD205-p24 vaccine in combination with polyICLC, induced polyfunctional immune responses within naso-pulmonary lymphoid sites that disseminated widely to systemic and mucosal (GI tract and the vaginal epithelium) sites. Qualitatively, while α-CD205-p24 prime-boost immunization generated CD4+ T-cell responses, heterologous prime-boost immunization with α-CD205-p24 and NYVAC gag-p24 generated high levels of HIV-specific CD4+ and CD8+ T cells within the GI tract. Finally, DC-targeting enhanced the amplitude and longevity of vaccine-induced immune responses in the GI tract. This is the first report of a nasally delivered, DC-targeted vaccine to generate HIV-specific immune responses in the GI tract and will potentially inform the design of preventative approaches against HIV-1 and other mucosal infections.

INTRODUCTION

Despite a marked improvement in survival of HIV-1 infected patients with combination antiretroviral therapy, HIV vaccine development remains a global priority. A key feature of HIV-1 transmission includes the preferential targeting of virus to gastrointestinal (GI) lymphocytes during acute HIV-1 (refs 1, 2) and SIV3 infections, independent of the route of viral inoculation. A recent study demonstrated a strikingly rapid seeding of viral reservoirs, including those in the GI tract, even before the appearance of systemic viremia in SIV-infected rhesus macaques.4 Therefore, it has been argued that the goal of an effective HIV vaccine should be to interrupt mucosal transmission at its earliest stages and to prevent viral production in mucosal tissues.5 Targeting antigens to dendritic cells (DC) is a strategy to enhance the effectiveness of vaccination, reviewed in ref. 6. Among the DC-associated receptors that have been targeted to boost cellular and humoral adaptive immunity are Fcγ receptors,7 MHC II molecules,8 CD40 (ref. 9), CD11b (ref. 10), CD11c11 and a number of C type lectins including CD20512, CD207,13 macrophage mannose receptor,14 CLEC9A,15 DCIR2,16 DC-SIGN,17 and dectin 1 (ref. 18). CD205 or DEC-205 targeting is perhaps best studied in the context of HIV-1 vaccine design. This involves engineering an α-CD205-p24 fusion construct that is then administered in combination with an adjuvant such as polyICLC to boost HIV-1-specific immune responses in mice,19 non human primates,20 and humans.21 In the present study, we have used an analog of polyriboinosinic-polyribocytidylid acid (Poly IC) as the adjuvant. PolyIC is a synthetic double-stranded RNA, recognized by TLR-3 and other intracellular receptors. A complex of
poly IC with poly-L-lysine and carboxymethylcellulose (poly ICLC), is five to ten times more resistant to hydrolysis by RNase than the parent poly IC. In addition, PolyICLC demonstrates a greater potency for interferon induction than its parent, PolyIC. Notably, GI mucosal immunity, highly relevant to HIV-1 vaccine development effort, has never been examined using a DC-targeted vaccine.

Our goal here was to induce and detect HIV-1-specific T- and B-cell responses in the GI tract. We focused on mucosal vaccination as it offers many attractive features including the ease of administration, potential for mass immunization, reduced cost of production, storage and delivery. In addition, mucosal vaccination is considered superior to systemic vaccination for recruiting cells to local, regional, and distant mucosal sites for non-HIV and HIV- (and SIV-) specific antigens. In studying the mechanism(s) of protection elicited by mucosal vaccines, we have previously demonstrated that intranasal vaccination licenses T cells and B cells to the GI tract through the induction of gut homing receptors α4β7 and CCR9. In the present study, we demonstrate that intranasal delivery of an α-CD205-p24 fusion antibody induces and directs HIV-specific T and B cells to the GI tract. Thus, here we define the first study of a DC-targeted vaccine to induce GI immune responses directed against HIV. The data presented herein is of relevance to the HIV-1 vaccine development effort, as well as for mucosal vaccination against other enteric and pulmonary pathogens.

RESULTS

Intranasal immunization with α-CD205-p24 and poly ICLC induces HIV-specific CD4⁺ T-cell responses in the intestinal lamina propria

With the goal of inducing HIV-specific immune responses in the GI tract, we compared mucosal and systemic routes of vaccine delivery. C57Bl/6 mice were immunized with 5 μg of α-CD205-p24 and 50 μg of polyICLC, administered either intranasally (IN), intraperitoneally (IP), intravenously (IV), subcutaneously (SC), or via the intramuscular (IM) routes. A booster dose of the vaccine was administered 4 weeks after priming. As a control, α-CD205-empty, which did not carry any antigen, and polyICLC were administered IP (Ctrl). One-week post boost, mononuclear cells were isolated from the intestinal lamina propria and spleen (Figure 1a).

Interferon-γ (IFN-γ) producing CD4⁺ T cells were detected in the small intestinal lamina propria (SILP) following IN and IP routes of vaccine delivery. In contrast, IV, SC, and IM vaccination failed to induce detectable levels of antigen-specific CD4⁺ T cells in the SILP (Figure 1b and c). Similar findings were noted in the spleen (Figure 1d). Furthermore, IN and IP immunization induced HIV p24-specific IgA⁺ B cells in the SILP, as confirmed by ELISPOT (Figure 1e). As IN immunization is clinically feasible, it was explored further and was found to induce p24-specific IgA and IgG antibodies in the serum (Figure 1f and g).

Intranasal immunization induces IFN-γ⁺ CD4⁺ T cells locally, followed by dissemination to the effector sites of the GI tract

To examine the sites of induction of immunity following IN vaccination, we isolated mononuclear cells from the nose, mediastinal LN (Med LN) and lungs of vaccinated mice. HIV-p24-specific CD4⁺ T cells were readily detectable in lungs, Med LN, and nose suggesting a local generation of vaccine-induced immune response (Figure 2a and b). In fact, striking frequencies of HIV-specific T cells were detected in the lung, highlighting the potential efficacy of IN delivered DC-targeted vaccines against other pulmonary pathogens like pneumonic plague.

We hypothesized that GI immune responses were being induced due to the dissemination of antigen-specific cells from local sites of induction as opposed to generation of GI immune responses due to swallowed antigen. Accordingly, we examined the inductive (Peyer’s Patches-PP and mesenteric LN-MLN) and effector (SILP and colonic lamina propria (CLP)) sites of the GI tract separately. While HIV-p24-specific IFN-γ⁺ CD4⁺ T cells were detected in the SILP and CLP, the PP and MLN of immunized animals did not contain appreciable levels of antigen-specific CD4⁺ T cells (Figure 2c and d). These data demonstrated that the immune response following IN vaccination was predominantly noted in the GI effector sites as opposed to the inductive sites. Finally, to add to the GI tract, we examined the systemic compartments and female reproductive tract and found that IN immunization induces HIV-specific CD4⁺ T cells in the spleen and genital tract in addition to the GI tract (Figure 2e and f).

Combined, these data demonstrate that following IN immunization, antigen-specific T cells are induced locally within the upper and lower respiratory tracts and disseminate to systemic sites, as well as to the gastrointestinal and genital mucosa.

Classical dendritic cells mediated the induction of IFN-γ CD4⁺ T cells following intranasal vaccination

To assess the role of classical dendritic cells (cDC) in our IN immunization experiments, Zbtb46DTR mice were used. In these mice, a zinc finger transcription factor, Zbtb46, which is specific to cDCs, is conditionally deleted, thus, distinguishing cDCs from other cell types expressing CD11c.

Chimerization to WT mice was needed to avoid toxicity associated with multiple administrations of diphtheria toxin. Zbtb46DTR chimera and C57Bl/6 WT chimeric mice received 0.5 μg of DT IP on day −2 before vaccination (Figure 3a). Depletion of the cDCs was confirmed in the lungs and spleen of representative mice (data not shown). Both groups of mice were then immunized with α-CD205-p24 (5 μg) and polyICLC (50 μg) in a prime-boost regimen as described. To maintain the deletion of cDCs during the course of immunization, 0.5 μg of DT was administered IP every 5 days over the priming period and another dose of 0.5 μg of DT was administered IP on day 28 and 4 days following the booster vaccination (Figure 3a). IFN-γ⁺ T effector responses were strikingly impaired in all of
the compartments examined (lung, spleen, and GI tract) in Zbtb46DTR but not WT mice (Figure 3b and c) demonstrating that cDCs mediate the effect of IN vaccination.

α-CD205-p24 immunization enhances gastrointestinal CD4⁺ T-cell responses relative to untargeted protein immunization

Having shown that IN immunization mediated intestinal responses were DC mediated, we wanted to compare the efficacy of DC-targeted vaccination with untargeted protein vaccination. C57Bl/6 mice were immunized IN with 0.5, 5 and 15 μg of p24 protein plus polyICLC and compared with 0.5, 5, and 15 μg of IN delivered α-CD205-p24 and poly ICLC. The DC-targeted approach was more potent at inducing antigen-specific immunity within the GI tract compared with untargeted p24 immunized group (Figure 4a–c).

Intranasal immunization with α-CD205-p24-induced antigen-specific Th1 and Th17 responses in the GI tract

Having established that IN vaccination induces antigen specific T cells in the GI effector compartments, the poly-functionality
of these cells, considered as a possible correlate of vaccine-induced protection, was assessed. Mononuclear cells were isolated from the SILP, spleen, and Med LN of vaccinated mice and re-stimulated with either p24 or p17 peptide pools and α-CD28 (1 µg ml⁻¹) for 20 h. Post restimulation the supernatant was collected and cytokine profiles determined using a Bio-Plex mouse cytokine immunoassay. The use of multiplexed enzyme-linked immunosorbent assay (ELISA) allowed us...
simultaneous detection of multiple cytokines from a relatively small (~50 μl) volume of the sample. Therefore, we chose this method over the conventional, FACS-based study of intracellular cytokines. IN immunization induced highly polyfunctional CD4\(^+\) T cells within local (Med LN), systemic (spleen), and gastrointestinal (SILP and CLP) sites (Figure 5).

Specifically, IFN-\(\gamma\), IL-2, IL-6, IL-17a, and IL-1\(\beta\)—cytokines, as well CC chemokines- MIP-1\(\alpha\) and MIP-1\(\beta\) were induced post immunization. Cell yield in the colon was low and was insufficient to provide reliable data. Thus, data from CLP were not included. Thus, IN immunization with DC-targeted \(\alpha\)-CD205-p24 vaccine produced highly polyfunctional T cells in the GI tract.

**Dendritic cell targeting induces long-term memory CD4\(^+\) T cells in the GI tract**

To test whether long-term memory could be detected within the intestinal tract, we examined recall responses in mice immunized with the DC-targeted or untargeted vaccine after 24 weeks of vaccination. Effector memory cells, i.e., T cells

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**Figure 3** Classical DCs are essential for inducing systemic and mucosal immune responses to intranasal immunization. Wild type (WT) and zbtb46\(^{\text{DTR}}\) mice were immunized IN with \(\alpha\)-CD205-p24 fusion mAb (5 μg) and poly ICLC (50 μg) in a prime-boost manner. Diphtheria toxin (DT), (0.5 μg) was administered IP, 48 h before immunization and every 5 days post vaccination. Additional doses of DT were administered on days 28 and 4 days post boost. IFN-\(\gamma\) secretion in response to HIV gag-p24 (immunizing) or p17 (control) peptide pools was evaluated 1 week post boost by intracellular cytokine staining. (a) Shows the schema of immunization. (b) FACS plots from a representative experiment, illustrating the induction of IFN-\(\gamma\) CD4\(^+\) cells in the lung, spleen, SILP, and CLP. (c) Mean data from three independent experiments (5 mice per group) is shown. Statistical comparisons between p24 levels in WT and zbtb46\(^{\text{DTR}}\) mice are shown. Error bars show mean \(\pm\) s.d. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\). CLP, colonic lamina propria; FACS, fluorescence activated cell sorting; IN, intranasally; IP, intraperitoneally; poly ICLC, poly-L-lysine and carboxymethylcellulose; SILP, small intestinal lamina propria.
producing IFN-γ were readily detected within the spleen, lung, SILP, and CLP of mice vaccinated with the DC-targeted vaccine (albeit at a lower frequency in the spleen than at mucosal sites). In contrast, untargeted p24 vaccination induced significantly lower levels of effector memory cells within the GI compartments or lungs and virtually undetectable levels in the spleen at 24 weeks (Figure 6a–c). Therefore, DC targeting induces long-term memory against p24 protein in the intestinal tissues.

Heterologous α-CD205-p24 prime, NYVAC gag/pol/nef boost immunization, generates p24-specific CD8⁺ T cells in the systemic and mucosal compartments

α-CD205-p24 prime-boost vaccine used in the present study raised concerns regarding generation of HIV-specific CD4⁺ T cells in the GI and vaginal tracts, potentially with enhanced susceptibility to the virus.32 Therefore, we wanted to develop a platform to generate HIV-specific CD8⁺ T cells at these mucosal sites and performed heterologous prime-boost experiments (priming with α-CD205-p24 followed by a NYVAC-gag boost). We hypothesized that vaccinating with DEC-205-targeted vaccine would generate p24-specific helper T cells, which could then enhance the induction of CD8⁺ T-cell responses using a poxvirus vector such as NYVAC.33 Using a heterologous prime-boost immunization regimen, first-generation C57Bl/6 and Balb/c offspring (F1 mice) were immunized with 25 μg of α-CD40, 50 μg of polyICLC, and 5 μg of α-CD205-p24 delivered IN. For these experiments, F1 hybrid mice (first-generation C57Bl/6 and Balb/c offspring) were used as the CD8 response is directed to a defined gag 197–205 peptide presented only on H-2d but not H-2b as shown previously.34 The F1 mice were immunized with 25 μg of α-CD40, 50 μg of polyICLC, and 5 μg of α-CD205-p24 delivered IN. Previous observations have demonstrated that a combination of a TLR ligand and an agonist α-CD40 antibody elicited stronger immunity.19,35 Therefore, we immunized with a combination of α-CD40 and PolyICLC in these experiments. Four weeks later the mice were administered 10⁷ p.f.u. of NYVAC-gag/pol/nef, delivered IN (Figure 7a). In addition, IP immunization was also assessed (Supplementary Figure 1 online). 14 days post boost, IFN-γ⁺ CD8⁺ T-cell responses were observed in the lung, spleen, blood, and vaginal tract of the IN immunized mice (Figure 7b and c). Unfortunately, poor viability of intestinal mononuclear cells precluded conclusions to be drawn in two of three experiments of the IN immunized group. However, robust levels of intestinal IFN-γ⁺ CD8⁺ T cells were detected in the GI tract of the IP immunized mice. Therefore, we conclude from these experiments that while α-CD205-p24 prime-boost immunization generated predominantly CD4⁺ T-cell responses, with a heterologous α-CD205-p24 prime, NYVAC boost immunization regimen, we were able to generate gag-p24-specific CD8⁺ T cells within the systemic compartment as well as mucosal sites.

DISCUSSION

The global impact of HIV infection is staggering. Since it was recognized over 34 years ago,36 the HIV pandemic has resulted in the infection of ~60 million people worldwide, nearly half of who have died from the disease. It is widely accepted that while substantial advances have been made in the field of HIV therapeutics, an effective HIV vaccine would be the optimum solution for the ultimate control of the global AIDS pandemic.37

The present study was guided by three principles. First, given the profound effect of HIV-1 infection on GI-resident CD4⁺ T cells,13 our goal was to induce HIV-1-specific immune responses in the GI mucosa. Second, targeting DC-specific lectins and endocytic receptors has been demonstrated to enhance the potency, magnitude, and longevity of vaccine-induced systemic immune responses,12,19,38,39 therefore, we wanted to adopt a DC-targeted approach to enhance mucosal immunity. Third, as mucosal routes of vaccine delivery are superior to systemic routes in inducing mucosal immune responses,28,40–42 we wanted to immunize across a mucosal surface to induce HIV-1-specific cells in the gut. Combined, here we provide the first study of a mucosally delivered,
DC-targeted vaccine to generate HIV-1-specific immune responses in the GI tract.

Although to date no evidence exists that CD4\(^+\) T-cell activation or vaccine-induced CD4\(^+\) T cells result in heightened HIV-1 acquisition or viremia after infection,\(^{13,44}\) α-CD205-p24 prime-boost vaccine used in the present study raises concerns regarding generation of HIV-specific CD4\(^+\) T cells in the GI and vaginal tracts, potentially with enhanced susceptibility to the virus.\(^{32}\) In particular, our data need to be understood the correlates of increased susceptibility to HIV seen in this subgroup of step trial volunteers. These included the possibility that the vaccine increased the number of activated CD4\(^+\) T cells in the peripheral blood, lymphoid tissue or mucosal sites and created potential targets for HIV-1.

Subsequent analyses of peripheral blood T cells have debunked the possibility that expansion, activation or homing of Ad5-specific CD4\(^+\) T cells was responsible for increased susceptibility to HIV seen in the Step Trial.\(^{47,48}\) As mucosal sampling was not included in the Step Trial, analyses of the mucosa became available recently from a different study where rhesus macaques were immunized with rAd5-gag/pol/nef vaccine (rAd5), similar to the vaccine used in the Step Trial. This study also has revealed no increase in the phenotype, frequency or trafficking of Ad5-specific CD4\(^+\) T lymphocytes to mucosal target sites following rAd5 vaccination of rhesus monkeys with baseline Ad5 immunity.\(^{39}\) These studies provide evidence against the hypothesis that recruitment of vector-specific CD4\(^+\) T cells to mucosal sites led to increased HIV-1 acquisition in Ad5 seropositive, uncircumcised vaccinees in the step study. A number of additional findings that have emerged

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**Figure 5** Intranasal immunization with α-CD205-p24 induced predominantly antigen-specific Th1 and Th17 responses in the GI tract. C57Bl/6 mice were immunized IN with α-CD205-p24 fusion mAb (5 μg) and poly ICLC (50 μg) in a prime-boost regimen. One-week post boost, mononuclear cells were isolated from the SILP, spleen and Med LN and re-stimulated with either p24 or p17 peptide pools and α-CD28 (1 μg ml\(^{-1}\)) for 20 h. Multiplexed ELISA was used to quantify the levels of secreted IFN-γ, IL-17, IL-6, IL-1β, IL-2, MIP-1α and MIP-1β. Mean data from two individual experiments is shown (5 mice per experiment). Statistical comparisons to p17 control are shown. Error bars show mean ± s.d. *P<0.05, **P<0.01, ***P<0.001. ELISA, enzyme-linked immunosorbent assay; GI, gastrointestinal; IFN, interferon; IL, interleukin; IN, intranasally; poly ICLC, poly-L-lysine and carboxymethylcellulose; SILP, small intestinal lamina propria.
from the analyses of step trial data that may explain the lack of vaccine efficacy. These include reduced vaccine-specific immunity in Ad5 seropositive individuals, narrow epitope recognition by CD8 T cells, and the induction of intestinal microbiota polyreactive HIV non-neutralizing gp-41 reactive antibody repertoire response to HIV Env DNA- rAd5 vaccine. Another key feature that has been highlighted by the step trial is the fact that HIV vaccine efficacy studies need to incorporate evaluation of mucosal immune responses in their study design. This is an aspect that our study attempts to address.

In contrast to the Step Trial, The RV 144 efficacy trial conducted in Thailand provided encouraging evidence that an HIV vaccine could provide modest level of protection against HIV acquisition—31.2% at 42 months of follow up. Immune correlates of protection demonstrated that IgG antibodies to V1V2 loops in the HIV envelope correlated inversely with the rate of HIV-1 infection. Notably, again HIV-specific CD4 T cells were not associated with infection risk in these analyses. In contrast, studies done on a subgroup of RV 144 vaccine recipients confirmed the presence of V2 specific, polyfunctional effector CD4 T cells.

Further proof that HIV-specific CD4 T cells are not associated with increased risk of disease acquisition comes from the pre-exposure prophylaxis initiative trial. In this study, T-cell responses to HIV-1 gag, protease, integrase, reverse transcriptase, vif, and nef antigens were examined in exposed-sero negative subjects. IFN-γ CD4+ and -CD8+ responses against vif and integrase were associated with reduced HIV-1 infection risk.

Together, based on these data, we infer that although there has been a theoretical risk, the abundance of scientific evidence refutes the claim that HIV-specific CD4+ T cells enhance susceptibility to HIV-1 infection. In fact, we would argue that as the vast majority of neutralizing antibodies and cytotoxic T cells are critically dependent on CD4+ T-cell help, inducing specific, potent, broad, and long-lived CD4+ T-cell response will be essential in the HIV-1 vaccine effort. This was the rationale behind the heterologous prime-boost experiments (priming with α-CD205-p24 followed by a NYVAC-gag boost) in our study. We hypothesized that vaccinating with DEC-205 targeted vaccine would generate robust levels of p24-specific helper T cells, which could then enhance the induction of CD8+ T-cell responses using a poxvirus vector such as NYVAC. A recent phase 1b clinical trial with recombinant adenovirus 5–vectored prime, followed by NYVAC boost showed enhanced vaccine immunogenicity and further validated our rationale. In confirmation of our hypothesis, we detected significant numbers (~10–20% of all CD8+ T cells) of p24-specific CD8+ T cells in both the GI tract and the vaginal mucosa. This is the first demonstration of DEC-205 prime, NYVAC boost vaccination and given the robust generation of CD8+ T cells at mucosal surfaces.

The migration of cells to the gut in response to sensitization in the lung (and vice versa) led to the proposal of a "common mucosal immunological system" by Bienenstock et al. in 1978. In support of this hypothesis, we have recently demonstrated that intranasal vaccination can target lung DCs to induce gut homing receptors α4β7 and CCR9 and recruit vaccine primed, antigen specific, T cells to the GI tract. Further, administration of an adjuvant such as polyICLC appeared to boost the expression of α4β7-on T cells following IN vaccination. In the present study we show that an IN
delivered α-CD205-p24 vaccine induces gag-p24-specific cells in the inductive sites of the upper (nose-associated lymphoid tissue) and lower (Med LN) respiratory tract but not the GI tract (PP and MLN). Interestingly, in addition to effector respiratory sites (lung), gag-p24-specific T cells could also be detected in the effector sites of GI (SILP and CLP) and genital (vaginal mucosa) tracts. Although we did not test for the expression of α4β7 (targeting to the gut) or α4β1 (targeting to the vaginal mucosa), our data echo recent findings by Stary et al. who show that nasal vaccination against Chlamydia trachomatis targets CD4+ T cells to the vaginal mucosa and offers protection against genital chlamydia infection. It follows therefore, that IN vaccine-induced immune responses, generated locally within the upper and lower respiratory tracts are disseminated widely to other mucosal sites. Thus, the present study provides further functional evidence of IN vaccine-induced DC-mediated mucosal cross talk.

A number of immunomodulatory agents including toxin based adjuvants, cytokines, CpG DNA, α-Gal-cer, chitosan, and non-replicative delivery systems, reviewed in ref 57 have been tested as mucosal adjuvants. We chose microbial mimetic and TLR-3 ligand, poly ICLC as an adjuvant based on its enhanced potency compared with other adjuvants when co-administered with protein antigens. Furthermore, poly-ICLC has an established safety record in cancer patients and, in a recent study by our lab, polyICLC, administered subcutaneously to a group of human volunteers demonstrated excellent safety. This combined with the data presented here supports the use of polyICLC as a mucosal adjuvant for further studies.

Our study establishes that intranasal vaccine-induced immunity is mediated by zbtb46-dependent classical DCs. In addition, two other features of DC targeting with intranasal vaccines stand out here. First, DC-targeting boosts the amplitude of vaccine-induced mucosal immunity. Second, DC-targeting results in a striking increase in the longevity of mucosal immune responses. We speculate that enhanced longevity of DEC-205-targeted immune response could be due to a stronger priming effect. Alternatively, as DEC-205 is expressed in the intestinal epithelium, we hypothesize that epithelial DEC-205 signals could maintain the longevity of DEC-205 DC-primed mucosal immune responses. We are in the process of testing this hypothesis and if proven, this could make DEC-205 targeting particularly relevant for mucosal vaccines.

Several characteristics of T cells such as phenotype, function, antigen specificity, and MHC restriction have been investigated as potential correlates of immune protection after vaccination. Recent data, however, suggests that the quality of the immune response, measured through increased levels of antigen-specific polyfunctional T cells capable of producing multiple, relevant cytokines, is a better correlate of sustained protective immunity. The use of multiplexed ELISA allowed us simultaneous detection of multiple cytokines from a relatively small (~50 μL) volume of the sample. Therefore, we chose this method over the conventional, FACS-based study of...
intracellular cytokines. Our study demonstrates that IN vaccination with α-CD205-p24 vaccine generated polyfunctional T cells, both systemic and mucosal, capable of producing T_{H}1, T_{H}2, and T_{H}17 cytokines as well as β-chemokines. Although vaccine-induced IFN-γ and IL-2 production is perhaps best studied, there is evidence that β chemokines such as MIP-1 α and MIP-1 β are associated with a better clinical status in HIV-1 infected patients.64 In addition, IL-17 producing CD4+ T cells have been associated with protective efficacy of vaccines against tuberculosis65 and rotavirus infections,66 whereas reduced intestinal IL-17a production is associated with loss of mucosal integrity and with SIV disease progression in rhesus macaques.67,68 The induction of IL-17 producing cells by our α-CD205-p24 vaccine is particularly interesting in this context and worthy of further examination.

Humoral immunity can completely suppress viremia in humanized mice,69,70 SIV-infected rhesus macaques71 and, in combination with viral inducers, decrease rebound from latent reservoirs of HIV-1 in humanized mice.72 In addition, as discussed above, the RV 144 Thai trial43 has demonstrated that vaccine recipients who produced V2-loop antibodies were 31% less likely to get HIV-infected.73 Therefore, although the focus of the present study was induction of T cell immunity, we also investigated vaccine-induced humoral response in the GI mucosa. Notably, antigen targeting to DCs has previously been shown to elicit long-lived T-cell help for antibody responses.74 Our study found that the IN administered, DC-targeted vaccine-induced antigen-specific IgA secreting B cells in the intestinal lamina propria. We are planning a more detailed investigation on GI humoral immunity elicited by DC-targeted vaccines.

We would like to mention an important caveat here- how these findings will translate to protection against human infection are unclear at this point and additional studies of in-vivo protection are needed. However, to our knowledge, there are no challenge models to test the efficacy of vaccine-induced, HIV-specific immune responses within the murine GI tract. The vaccinia-gag model, previously used in the lab19 is less relevant for delivery across the GI mucosa. As more studies examine the induction of HIV-specific immune responses in the GI tract, the creation of a GI-specific challenge model will become increasingly relevant.

In summary, our study is the first demonstration of a DC cell targeted, mucosally delivered vaccine to induce HIV-1-specific immunity in the GI tract. The data presented herein offers insights into future research in identifying preventative strategies not only against HIV-1 infection but also against other enteric and pulmonary pathogens.

**METHODS**

**Mice.** C57Bl/6 mice (B6) and Balb/c mice were purchased from Taconic Labs or bred at the Rockefeller University. F1 hybrid mice (cross between BALB/c females and C57Bl/6 males; heterozygous at all loci), were bred at in-house. Zbtb46DTR mice were kindly provided by Dr M. Nussenszweig (Rockefeller University, New York). All mice were maintained in specific pathogen–free conditions and used at 6–8 weeks unless otherwise specified. Protocols were approved by the Rockefeller University Animal Care and Use Committee.

**Construction and production of fusion mAbs.** DNA for HIV gag-p24 (aa 133–363 derived from HIV isolate BH10) was cloned in frame into the COOH terminus of the heavy chains of α-mouse-DEC-205 as described previously.15 The fusion α-CD205-p24 mAb was produced by transient transfection (cationic phosphate) in 293 T cells in serum-free DMEM supplemented with Nutridoma SP (Roche Applied Science, Indianapolis, IN). The mAbs were purified on protein G columns (GE Healthcare Bio-Sciences, Pittsburgh, PA) and characterized by polyacrylamide gel electrophoresis/PAGE and western blotting using α-mouse IgG1-HRP (Southern Biotech, Birmingham, AL) or HRP-α-gag-p24 (ImmunoDiagnostics, Espoo, Finland). mAb binding was verified on CHO cells stably transfected with the respective receptor by FACS using phycoerythrin-conjugated goat α-mouse IgG (Jackson Immunoresearch, West Grove, PA). Unconjugated α-DEC-205 mAb expressed by stably transfected CHO cells was similarly purified. Recombinant NYVAC-encoding HIV BX009gp120-H1B gag/Pol/Nef is a replication incompetent vector, kindly provided by Dr Giuseppe Pantaleo (Centre Hospitalier Universitaire Vaudois (CHUV) Lausanne, Switzerland) and prepared as previously described.75

**Soluble gag-p24 protein.** Soluble FLAG gag-p24 protein76 was expressed by CHO cells and purified from culture supernatant using anti-FLAG M1 Affinity gel (Sigma-Aldrich, St Louis, MO) following the manufacturer’s instructions. All proteins had <0.125 endotoxin units per μg using a Limulus Amebocyte Lysate assay, QCL-1000 (Bio Whittaker, Walkersville, MD).

**HIV gag peptides.** Overlapping (staggered by 4 aa) gag p17 and p24 15-mer peptides were obtained from the NIH AIDS Reference Reagent Program (catalog no. 8117). Peptides, including the H-2Kb binding peptide p24 197–205 (ref. 34), were also synthesized by the Proteomics Resource Center at The Rockefeller University. As previously described,19 the 30- and 60-member gag p17 and p24 libraries were divided into 3 and 5 pools of 9 to 12 peptides, respectively. The respective gag p17 peptide pools span from aa 1–51 (pool 1), aa 41–91 (pool 2), and aa 81–135 (pool 3) of the HIV gag p17 protein. The respective gag-p24 peptide pools span from aa 125–138 (pool 1), aa 173–231 (pool 2), aa 221–279 (pool 3), aa 269–327 (pool 4), and aa 317–363 (pool 5) of the HIV gag-p24 protein. Response to individual pools has been mapped previously in our lab.18 Also, previous work from the lab has demonstrated that CD8+ T-cell responses elicited by pooled p24 peptides were comparable to the 197–205 nonamer peptides.15 Hence, in the present study, we combined all three p17 peptide pools and all five p24 peptide pools together while testing for recall responses.

**Immunization.** Mice were injected by various routes including IN, IP, IV, IM or SC (in the hind footpads) with the indicated doses of fusion α-CD205-p24 mAb, unconjugated α-CD205-empty mAb, or HIV gag-p24 protein along with stimuli for DC maturation—polyICLC (Oncovir, Washington, DC) 50 μg. and when indicated—25 μg of agonist α-CD40 mAb.35 Unless otherwise specified, vaccines were delivered in a prime-boost fashion with the booster given 4 weeks after priming. Mice were killed 7 weeks after the booster dose. For determining long-term immune responses, mice were killed 25 weeks post boost.

**Isolation of mononuclear cells from various organs and cell preparation.** Mice were killed and the mononuclear cells were isolated from the following organs:

**Intestinal mononuclear cell isolation.** As previously described,77 small and large intestines were removed in toto and placed in cold HBSS media containing 5% FCS. The intestines were carefully cleaned from the mesentery and flushed of fecal content. Peyer’s patches were removed and analyzed separately where indicated. Intestines were...
opened longitudinally and then cut into 1 cm pieces. The intestinal tissue was incubated with 1.3 mM EDTA (Cellgro, Manassas, VA) in HBSS at 37 °C for 20 min. The EDTA incubation step was repeated a second time. The supernatants containing intestinal epithelial cell with some superficial villi cells, referred to as the “intestinal epithelial cell fraction”, were not used in the present study. To isolate the lamina propria lymphocytes, the remaining intestinal tissue was minced and transferred to conical tubes. The minced pieces were resuspended in 20 ml of complete RPMI containing 0.125 mg ml⁻¹ of collagenase (Sigma, St Louis, MO) and shaken at 200 r.p.m. for 50 min at 37 °C. The tissue suspension was collected and passed through a 70-μm cell strainer and the cells were pelleted by centrifugation at 1,600 r.p.m. The cells were then resuspended and layered onto a 20/80% Percoll (GE Biochemicals, Pittsburgh, PA) gradient, centrifuged and collected, washed and resuspended in complete RPMI media. These purified cells constituted the SI LP or colonic lamina propria (CLP) lymphocyte population.

**Lung mononuclear cell isolation.** The pulmonary circulation was removed in toto. Organs were thoroughly minced using iridectomy scissors and incubated for 60 min in digestion medium containing 0. Collagenase (5 mg/ml; Sigma) in a humidified incubator at 37 °C and 5% CO₂. In the last 5 min, 10 mM EDTA was added. Tissue fragments were disrupted mechanically by pipetting and passed through a 70-μm cell strainer and mononuclear cells were washed twice in RPMI. The cells were then layered onto a 20/80% Percoll gradient, centrifuged and collected, washed and resuspended in complete RPMI media. Percoll or colonic lamina propria (CLP) lymphocyte population.

**Spleen and LN cell isolation.** The spleen and LN were harvested in RPMI media with 5% FCS and digested at 37 °C in the presence of 400 units per ml of collagenase D (Sigma-Aldrich) or 25 min. In the last 5 min, 10 mM EDTA was added. Spleen but not LN samples went through subsequent ACK lysis (Gibco, Waltham, MA), were washed twice and counted.

**Vaginal mononuclear cell isolation.** The vaginal tract was excised, cut into fine pieces and incubated in collagenase (Sigma) at 0.5 mg ml⁻¹ at 37 °C for 1 h. Tissue was then homogenized, filtered, and washed. The resulting cell suspension was layered on a 20/80% Percoll gradient and the interface was collected to obtain an enriched mononuclear cell population.

**Peripheral blood mononuclear cell isolation.** Blood was obtained from the experimental mice by cardiac puncture in heparinized syringes. Mononuclear cells were obtained by layering on Histopaque (Sigma-Aldrich). Mononuclear cells were obtained by incubation with pooled p17 or p24 peptide pools and ρ17/CD28 (1 μg ml⁻¹) for 20 h. The supernatant was collected and analyzed using a Bio-Plex Pro Mouse Cytokine 23-plex assay (Biorad, Hercules, CA). Briefly, the Bio-Plex assay is an immunoassay, based on magnetic beads, consisting of a sandwich ELISA. Capture antibodies directed against the desired biomarkers were covalently coupled to beads. Coupled beads (50 μl) were added to the supernatant (50 μl) and incubated for 1 h at room temperature. After a series of washes to remove unbound protein, a biotinylated detection antibody (25 μl) was added and incubated at room temperature for 30 min. The final detection complex was formed with the addition of a streptavidin-phycocyanin conjugate. The samples were re-suspended in assay buffer at a volume of 125 μl. Phycocyanin served as a fluorescent reporter. The fluorescence was measured in a Luminex-based reader (Biorad). The data were analyzed using Bio-Plex data Pro software.

**ELISPOT.** Plates were coated with 5 μg ml⁻¹ gag-p24 protein overnight. Following washing, plates were blocked with RPMI media containing 10% FBS (Gibco), 1% non-essential amino acids (Gibco), 1% anti-anti cocktail (Gibco), and 0.1% 2-mercaptoethanol (Gibco). Cells were plated in a concentration of 1–2 × 10⁶ cells per well (G1) in triplicate and cultured for 24 h at 37 °C, washed, and isotype-specific antibody was detected using HRP-conjugated goat anti-mouse IgG secondary (Southern Biotech). Spots were developed using BD ELISPOT AEC substrate set (551951) according to manufacturer’s protocol. Spots were quantified using a CTL-ImmunoSpot Analyzer and Software (Shaker Heights, OH).
Statistical analysis. Data reported in the figures represent the average of at least three independent experiments. Error bars represent the s.d. Statistical analysis using the unpaired t-test between three groups was done using Prism software (La Jolla, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE
The authors declared no conflict of interest.

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