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Dendritic cells infected by recombinant rabies virus vaccine vector expressing HIV-1 Gag are immunogenic even in presence of vector-specific immunity

Celestine N. Wanjalla¹, Elizabeth J. Faul¹, Emily A. Gomme¹, and Matthias J. Schnell^{1,2,*}

¹Department of Microbiology and Immunology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA

²Jefferson Vaccine Center, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA

Abstract

Dendritic cells (DC) are the most potent antigen presenting cells whose ability to interact with T cells, B cells and NK cells has led to their extensive use in vaccine design. Here, we designed a DC-based HIV-1 vaccine using an attenuated rabies virus vector expressing HIV-1 Gag (RIDC-Gag). To test this, BALB/c mice were immunized with RIDC-Gag, and the primary, secondary as well as humoral immune responses were monitored. Our results indicate that RIDC-Gag stimulated HIV-1 Gag-specific immune responses in mice. When challenged with vaccinia virus (VV) expressing HIV-1 Gag, they elicited a potent Gag-specific recall response characterized by CD8⁺ T cells expressing multiple cytokines that were capable of specifically lysing Gag-pulsed target cells. Moreover, RIDC-Gag also enhanced CD8⁺ T cell responses via a homologous prime-boost regimen. These results show that a DC-based vaccine using live RV is immunogenic and a potential candidate for a therapeutic HIV-1 vaccine.

Introduction

The 2008 UNAIDS report estimated there were approximately 33 million children and adults living with HIV, and despite administration of highly active anti-retroviral treatments (HAART), a staggering two million people died of AIDS-related illnesses in 2007 compared to 1.7 million in 2001 [1]. As such, great emphasis has been placed on the development of a vaccine against HIV. Live attenuated viral vectors used in vaccine development have had a great impact on the control of many deadly infectious diseases including yellow fever and polio [2,3]. As a group, HIV positive individuals present additional challenges as vaccine recipients as they may lack fully functional immune cells including myeloid and plasmacytoid DCs [4,5]. Thus, a vaccine that exogenously supplies functional DCs may be more efficacious [6] (for review). Due to their exceptional capacity as linkers of innate and adaptive immunity [7–9], many groups have used DC-based vaccines to immunize against infectious diseases such as HIV-1 and Hepatitis B and C [10–13].

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Correspondence should be addressed to Matthias J. Schnell, 233 S. 10th Street BLSB Room 531, Philadelphia, Pennsylvania, 19107, U.S.A., (matthias.schnell@jefferson.edu), Telephone: 215-503-4634, Fax: 215-823-9248.

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Although a good number of DC-based HIV-1 vaccines have been developed and tested, the majority were DCs pulsed with inactivated whole HIV/SIV or SIV/HIV proteins [10,14,15]. While the method has advanced DC vaccine development, none of the vaccines have proven efficacious, requiring more research and probably novel vectors to target DCs. A recombinant RABV (rRABV) expressing HIV-1 antigen is such an alternative approach.

RABV is a non-segmented, negative single-stranded RNA virus with a genome size of approximately 12 KB. The genome encodes five genes: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and L-polymerase (L) [16]. A system to obtain infectious RABV from cloned cDNA, which allows manipulation of the genome as well as expression of multiple large foreign genes, has been established [17,18]. Moreover, several approaches showed rRABV as excellent vaccine vectors against infectious diseases [19,20]. As an HIV-1 vaccine vector, studies in mice and rhesus macaques have shown that rRABV vectors expressing HIV-1/SIV proteins induce strong cellular and humoral responses [21–23]. Concerning safety, the vaccine strain of rRABV has been attenuated by several passages over cell cultures, mutations and gene deletions that render it a safer vector [24,25]. Even though wild-type RABV is a neurotropic virus that replicates in the peripheral and central nervous system, *ex vivo* experiments have now shown that the vaccine strain of RABV infects and induces maturation of human DCs highlighting the virus' potential in DC-based vaccine development [26,27]. Despite a significant number of DC-based HIV-1 vaccine studies in human clinical trials, we still do not have a vaccine that can sufficiently control HIV-1 [10,28,29]. This calls for more research to better harness the potential of DCs. Here we describe the development of a therapeutic vaccine for HIV-1 positive individuals. Using primary murine bone marrow derived DCs (BMDCs), we developed a RABV-infected DC (RIDC) vaccine expressing HIV-1 Gag (RIDC-Gag) and tested it in mice. Our results show that RIDC-Gag are functional APCs that stimulate HIV-1 Gag specific polyfunctional CD8⁺ T cells and a Th1-dominant humoral immune response. Moreover, RIDC-Gag successfully boost cellular immune responses in mice primed with live RABV expressing HIV-1 Gag (RV-Gag) or RIDC-Gag.

Methods

Mice

BALB/c and C57BL/6 mice were obtained from the NIH and housed at Thomas Jefferson University animal facilities. Experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University.

Growth of recombinant RV

The cloning and recovery of the vaccine strain of RABV used in this paper has been previously described [30–32]. The viruses used include: SPBN (vaccine strain RABV backbone - cited as RV), SPBN-GFP (RV-GFP), BNSP-Gag and SPBN-Gag (RABV expressing HIV-1 Gag – cited RV-Gag), SPBN-NP/SIINFELK (cited as RV-OVA) and RABV lacking G protein and expressing HIV-1 Gag (Δ G-Gag) [24,33].

Generation of primary BMDCs

BMDCs were generated as published [34]. In brief, bone marrow (BM) was obtained from the tibia and femur of BALB/c mice. Following red blood cell lysis using ACK lysis buffer (Invitrogen, 0060005DG), BM cells were cultured in 24-well costar plates at a density of 1 million cells per ml of CellGro DC serum free media (CellGenix USA, Illinois, 2005) supplemented with 10ng/ml GM-CSF (Peprotech, NJ, 315-03). During the 7-day culture, two washes were performed by aspirating 600 μ l of media and adding 1ml of fresh media.

On day 7 of culture, half of the media was aspirated followed by harvesting of the semi-adherent BMDCs. Differentiation of DCs was confirmed by surface staining for CD11c by flow cytometry as described below.

Infection of BMDCs

Murine BMDCs were infected with RABV at an MOI of 10. The BMDCs were plated in either 6-well or 12-well plates at a maximum density of 10^6 cells per ml of media. RABV was added directly into the wells followed by incubation at 37°C and 5% CO₂. RIDCs used in the vaccine studies were incubated for either 2h or 24h, after which they were washed three times in PBS prior to injection into mice.

Allogeneic mixed lymphocyte reaction (MLR)

The antigen-induced T cell culture and labeling of cells with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes Inc., Eugene, OR, C34554) for proliferation studies has been previously reported. Briefly, splenocytes were obtained from homogenized spleens of C57BL/6 mice. They were incubated for 10 min at 37°C with 5 μ M CFSE. The CFSE-labeled responder cells ($1-2 \times 10^6$) were mixed with $1-2 \times 10^6$ BMDCs from BALB/c mice (uninfected, RABV infected – 24hpi) in U-bottomed 96-well plates in complete RPMI-1640. After 5 days of incubation, each of the well contents were harvested, stained with antibodies against CD8 or CD4 and fixed in 4% formaldehyde. FACS analysis was used to analyze the samples.

Apoptosis assay

The viability of RABV-infected BMDCs was monitored using 7-Amino-actinomycin D (7-AAD) as previously described [35]. In brief, BMDCs were resuspended at 1×10^6 cells/ml in complete RPMI-1640. A total of 5×10^5 cells per sample were incubated with 1 μ g/ml 7-AAD on ice for 10 minutes. These samples were then fixed with BD cytofix and immediately run on a BD FACs Calibur followed by analysis with FlowJo software (8.8.2).

Immunization and challenge protocol

BMDCs obtained from BALB/c mice were infected with RV-Gag to generate RIDC-Gag. Uninfected DCs and RIDCs infected with RABV served as negative control groups. The latter group has been designated RIDC. Mice immunized with the DC-based vaccine were primed with $4-5 \times 10^5$ cells in 50 μ l PBS via tail vein injections. Once primed, the mice were rested for 34 – 60 days, and then challenged intraperitoneally with 1×10^6 pfu of vaccinia virus expressing HIV-1 Gag (VV-Gag). The recall responses were analyzed 5 days post challenge. For the prime-boost experiments, mice were primed intramuscularly with 1×10^6 ffu of RV-Gag or 400,000 – 500,000 RIDC-Gag (intravenously). The mice were rested for at least 21 days followed by booster immunizations with 1×10^6 RIDC-Gag. Cellular responses were analyzed 7 and 11 days post boost.

IFN- γ ELISpot

A detailed protocol has previously been published [26]. In brief, ELISpot assays were done in 96-well multiscreen filtration plates (Millipore) coated with rat anti-mouse IFN- γ monoclonal antibody (BD Pharmingen, CA, 551216). Serial dilutions of splenocytes were plated in these wells and incubated with or without the MHCI-restricted immunodominant HIV-1 Gag peptide (10g/ml AMQMLKETI). Cytokine production was detected 16 – 18 hrs later by incubation with biotinylated rat anti-mouse IFN- γ mAb (Pharmingen, CA, 554410). This was followed by treatment with horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch). Spots were developed after addition of peroxidase substrate and analyzed using an ImmunoSpot C.T.L reader 5.0.

Flow cytometry

Protein expression of DCs and T cells was analyzed using flow cytometry as published [26]. The cells were incubated in 100µl FACS buffer (2% Bovine Serum Albumin in PBS) containing 2µl anti-CD16/32 (BD Biosciences, CA, 553142) on ice for 15 – 60 minutes as per the manufacturer's protocol. For tetramer staining, the T cells were treated with unconjugated streptavidin in 100µl FACS buffer for 1h on ice. After two washes with FACS buffer, cells were incubated for 30 minutes at room temperature with antibodies against cell surface markers. These samples were then washed two times and fixed with BD-cytofix buffer (BD Biosciences, CA, 554655) for 20 minutes on ice. This was followed by a 30-minute (room temperature) incubation of the DCs with anti-RABV N diluted in BD-cytoperm buffer (BD Biosciences, CA, 554723). The stained cells were washed and resuspended in FACS buffer prior to FACS analysis. FACS analysis was performed on a BD FACS Calibur machine available at the Kimmel Cancer Center Flow Cytometry Facility (Thomas Jefferson University). For DC experiments, 25,000 – 50,000 events were collected during the run. 200,000 events were collected for analysis of T cells from immunized mice. All the data was analyzed using FlowJo software (8.8.2).

Immunofluorescence

The immunofluorescence assays were done as previously described [36]. In brief, primary BMDCs were infected as described above with RV-GFP at an MOI of 10. In order to monitor infection by GFP fluorescence, infected cells were harvested two days after the infection and analyzed by fluorescence microscopy.

Intracellular cytokine stain (ICS)

For T cell analysis, 1×10^6 splenocytes/ml were cultured in 96-well plates. For each mouse analyzed, two groups were set up; one pulsed with 10ug/ml AMQMKLETI peptide and the other without peptide. Both wells were treated with GolgiPlug™ (BD Biosciences, CA, 555029) followed by 6 hr incubation. Surface stains against CD8 were done prior to fixation, after which the cells were washed, followed by staining against internal proteins. FACS analysis was done as above. Antibodies: Single cell suspensions obtained from the spleen were stained for CD8a, CD44, CD62L, IFN, IL2, TNF-, IL6, IL10 (BD Biosciences, CA).

Rabies Virus G protein enzyme-linked immunosorbent assay (ELISA)

The RABV-G ELISA assays were done as previously published [26].

Calcein-AM release assay—Calcein-release assays measuring cell cytotoxicity were conducted as previously published [26].

Statistics

Statistical analyses in this paper were done using Graph Pad Prism version 4.0, GraphPad software, San Diego California USA, www.graphpad.com. Where more than two experimental groups were analyzed, one-way ANOVA test with Bonferroni post-test was performed. Unpaired T-test analysis was performed when two groups of data were compared; two-tailed P value, confidence interval of 95%.

Results

Characterization of primary mouse derived BMDCs infected with RABV (RIDCs)

RABV has been shown to infect primary human DCs and a murine DC-derived cell line (JAWSII) [26,27,36]; however, the immunogenicity of RIDCs *in vivo* has not been

determined. To establish a mouse model for the DC-based HIV-1 vaccines, we first assessed whether RABV could infect primary mouse derived BMDCs. Following seven days of differentiation, we confirmed the phenotype of these BMDCs as CD11c positive cells (Fig. 1a). Subsequently, we determined whether RABV could successfully infect and replicate in primary BMDCs. For a DC-based vaccine approach, it is important that the virus expresses the foreign antigens against which an immune response is targeted. To this end, a recombinant RABV expressing GFP (RV-GFP) was used to infect the cells. GFP is a useful reporter gene because its expression is easy to monitor by fluorescence microscopy. RIDCs were analyzed at 48 hpi for expression of GFP and RABV nucleoprotein (RABV-N) (Figs. 1b–c). We found that RIDCs express RABV-N and GFP as early as 24hpi (data not shown). Based on GFP expression in RABV infected BMDCs, we concluded that foreign proteins can successfully be expressed in BMDCs by recombinant vaccine strain-based RABV.

One advantage of DC-based vaccines is the additive capacity of the DCs to process and present the antigen to the immune system. Both live and apoptotic RIDCs could contribute to the immune responses elicited [37]. To characterize the composition of the RIDCs used in the vaccine, uninfected BMDCs and RIDCs infected with RV or RV-Gag were stained with 7-AAD followed by FACS analysis. Stress induced NA cells were used as a positive control. We found that RABV induces apoptosis in DCs independent of the MOI and foreign insert. The apoptotic cells are denoted as R2 (Figure 1D–F). Similar results were obtained in an independent experiment with 21.6% apoptotic RIDCs via Tunnel-BrdU assay compared to 0.82% in uninfected cell culture, indicating virus-induced cell death (data not shown). Taken together, we established that RABV infects murine BMDCs with expression of foreign antigens. Moreover, both apoptotic and viable RIDCs could contribute to the immunogenicity of the vaccine.

RIDCs are functional APCs

Having determined that RABV infects BMDCs, we next wanted to analyze their maturation phenotype. This is a key quality control checkpoint in the development of DC-based immunotherapy, because DCs are capable of inducing tolerance based on their activation status [38]. To determine whether RIDCs have a mature phenotype, BMDCs were infected with RV and analyzed at 48 hpi. LPS treated cells served as a positive control. RIDCs had higher levels of CD80 and CD86 expression compared to uninfected DCs, cells incubated with UV-inactivated RV, or cells infected with vesicular stomatitis virus (VSV) (Figs. 2a). This is similar to previously published data where recombinant VSV infected BMDCs had low levels of CD86 and CD80 expression due to the deleterious effects of the matrix protein on host gene expression [39]. The results above indicated the successful activation and maturation of RIDCs. In general, the maturation phenotype of RIDC-Gag used in the vaccination regimen were similar to RIDCs (Data not shown).

Next, we tested the capacity of such RIDCs to act as APCs by monitoring the processing and presentation of foreign antigens. BMDCs were infected with RV or RV-OVA_{257–264} and stained 48hpi with anti-MHCI Kb-SIINFEKL. BMDCs pulsed with SIINFEKL peptide (10µg/ml) were included as a positive control (Fig. 2b). In addition to processing of antigens, we tested the function of RIDCs as APCs gauged by their presentation of the antigen to T cells in an allogeneic mixed lymphocyte reaction (MLR). As shown in (Fig. 2c), RIDCs could stimulate the proliferation of CD8+ T cells as determined by CFSE dilution whose average geometric mean fluorescent intensity (MFI) was 30.17 whereas CD8+ T cells incubated with uninfected DCs had an MFI of 50.02. Proliferation of CD4+ T cells incubated with RIDCs had an MFI of 84.79 compared to uninfected DCs with an MFI of 125.16. Collectively, these results demonstrate that RIDCs mature after infection and are able to present foreign antigens on their MHC secondary to the infection. Furthermore,

RABV does not interfere with the capacity of DCs to induce proliferation of both CD4+ and CD8+ T cells.

Mice immunized with RIDC-Gag exhibit a Gag-specific immune response

To determine whether RIDC-Gag induce HIV-1 Gag specific T-cell responses, BALB/c mice were injected intravenously with $4-5 \times 10^5$ RIDC-Gag in PBS. Uninfected DCs and RIDCs were included as negative controls, whereas mice immunized i.m. with RV-Gag (1×10^6 ffu) were included as a positive control. Primary immune responses were analyzed by flow cytometry 7 and 11 days post prime as shown (Fig. 3a). Splenocytes from immunized and control mice were analyzed by FACS and IFN- γ ELISpot. The results indicated that there was no significant difference in the number of activated CD8+ T cells (CD44^{hi} and CD62L^{lo}) for all four groups (Fig. 3b). However, AMQMLKETI tetramer specific CD8+ T cells were detected for both RIDC-Gag and RV-Gag immunized mice 7 days post prime. No differences between the four groups were detected on day 11 (Fig. 3c). Furthermore, splenocytes from mice immunized with RIDC-Gag and RV-Gag expressed IFN- γ in the presence of AMQMLKETI peptide (Fig. 3d). These results illustrate that an RIDC-based HIV-1 vaccine is capable of inducing an antigen-specific primary immune response.

Mice immunized with RIDC-Gag induce memory

The main goal of a vaccine is to establish antigen-specific memory, which can be tested by challenge with the vaccine-directed pathogen. In the case of HIV-1 vaccines, challenge experiments cannot be performed due to the lack of a productive infection of HIV-1 in mice. Therefore, we analyzed the presence of a HIV-1 Gag-specific memory response 60 days post prime by challenging the mice with recombinant VV expressing HIV-1 Gag (VV-Gag). We first analyzed the percentage of activated T cells five days after challenge. Similar levels of activated CD8+ T cells (CD44^{hi} and CD62L^{lo}) were detected in all groups, most likely because they received the same amount of VV-Gag (Fig. 4a). However, mice that had been primed with RIDC-Gag exhibited a significantly higher number of AMQMLKETI tetramer specific CD8+ T cells compared to uninfected DC and RABV vector infected controls (Fig. 4b). To analyze the quality of the immune response, we analyzed the functionality of the CD8+ T cells from the immunized mice focusing on the expression of multiple cytokines by intracellular cytokine staining (Fig. 4d). CD8+ T cells from RIDC-Gag immunized mice that were stimulated with AMQMLKETI peptide expressed significantly higher amounts of IFN- γ , TNF- α , IL2, IL6 and IL10 compared to control mice (Fig. 4c-d). Of note, all the CD8+ T cells that expressed TNF- α also expressed IFN- γ (Fig. 4d). These results show that RIDC-Gag immunization induces HIV-1 Gag specific CD8+ memory T cells in the immunized host that are functional as shown by expression of multiple cytokines.

Gag specific immune response is not due to viral spread from infected BMDCs to other cells

One of the key features of DCs is their ability to directly prime T cells and interact with other cells such as NK and B cells [7,8]. Both *ex vivo* and *in vivo* analysis of the RIDCs was post infection with live, replication-competent RV-Gag. In order to address whether the immune response might have been due to secondary infection by RV-Gag released from RIDCs, viral titrations of supernatants collected from RIDC-Gag were performed and showed no detectable viral production [40]. Failure to detect any virus in the supernatants implies that DCs do not support high levels of viral release, if any. However, small amounts of BNSP-Gag could spread to secondary tissue and amplify. Therefore, a more stringent experiment was performed using primary BMDCs infected with rRV-Gag lacking the RABV-G gene, denoted Δ G-Gag. Due to lack of the G protein, Δ G-Gag can only undergo one round of replication in target cells [24]. Both RIDC-Gag and RIDC- Δ G-Gag showed comparable levels of infection. Analysis of the CD86 upregulation of the two groups

showed that RIDCs (RV-N +) had a more mature phenotype than uninfected cells in the same culture or compared to the negative control of uninfected cells. (Fig. 5a) of note, both RIDC-Gag and RIDC-ΔG-Gag contain apoptotic cells based on a 7-AAD apoptosis assay. BMDCs infected with either RV-Gag or ΔG-Gag were harvested 36hpi and the percentage of early apoptotic cells compared. Preliminary comparisons of these two groups showed 34.2% apoptotic cells RIDC-Gag compared to 19.3% apoptotic RIDC-ΔG-Gag (Data not shown). Although vaccine strain RABV-G has been shown to induce apoptosis in mammalian cells [41], RABV undergoes limited replication BMDCs [40]. Hence RABV-G protein together with other anti-viral mechanisms may contribute to PCD of mature DCs downstream of activation by pattern recognition receptors.

Splenocytes from mice immunized with either RIDC-Gag or RIDC-ΔG-Gag and challenged 30days later with VV-Gag had similar levels of activated CD8⁺ T cells (CD44^{hi} and CD62L^{lo}) and AMQMLKETI tetramer specific CD8⁺ T cells 5 days after VV-Gag challenge (Fig. 5b–c). Furthermore, groups immunized with RIDC-Gag or RIDC-ΔG-Gag showed similar levels of IFN-γ and TNF-α production (Fig. 5d–e). Therefore, we determined that the HIV-1 Gag-specific immune response elicited by the RIDC-Gag is dependent on antigen presentation by RIDC-Gag. This also indicates that replication-deficient RABV-based vectors are safe and potent vaccine candidates for immunization utilizing DCs.

RIDC induced CD8 T cells are cytotoxic

CD8⁺ T cells have been purported to be important in controlling HIV-1 replication and preventing AIDS after infection. So far, we have established that the RIDC vaccine induces a significant number of tetramer specific cells that produce a variety of cytokines. In order to determine their ability to kill infected cells, we performed a functional cytotoxicity assay. All groups were challenged with VV-Gag 30days post prime. Compared to uninfected DCs, RIDC-Gag and RIDC-ΔG-Gag both elicited CD8⁺ T cells that were able to lyse AMQMLKETI pulsed cells in a dose dependent manner (Fig. 6). The number of lysed Gag pulsed P815 target cells had a positive correlation with the percentage of tetramer cells in the samples obtained from both RIDC-Gag and RIDC-ΔG-Gag groups (0.8157 and 0.9629 respectively) (data not shown). Whereas cells pulsed with the irrelevant Env peptide had a negative correlation with the percentage of tetramer cells (data not shown). Together, this data demonstrates that the CD8⁺ T cells elicited by the RIDC-Gag vaccine are functional.

RIDC-Gag enhance cellular responses in a homologous prime-boost regimen

There are several advantages to a prime-boost vaccine regimen in the induction of protective immune responses. One of the major challenges in vaccine development is that homologous prime-boost approaches are often not effective in the induction of cellular immune responses. This is also the case for RABV-based vaccine vectors [23].

We hypothesized that RIDC-Gag can directly present antigens to T-cells; therefore, they should be functional in a homologous prime-boost regimen and not be affected by a preexisting humoral response against RABV G. For this approach, we primed mice with either RV-Gag or RIDC-Gag. Twenty-one days after priming, the mice were boosted with either RV-Gag or RIDC-Gag. At 7 days post boost, there was no significant difference in the number of AMQMLKETI tetramer specific CD8⁺ T cells between the groups (data not shown). At 11 days post boost, RV-Gag immunized mice that had obtained the RIDC-Gag boost had higher numbers of AMQMLKETI tetramer specific cells, $P = 0.0619$ (Fig. 7b). Homologous RIDC-Gag/RIDC-Gag also resulted in significantly higher numbers of Gag specific CD8⁺ T cells, $P = 0.0431$, when compared to the RV-Gag/RV-Gag group. Similarly, IFN-γ ELISpot revealed higher levels of peptide specific expression of IFN-γ in

RV-Gag/RIDC-Gag and RIDC-Gag/RIDC-Gag groups (Fig. 7c). These results indicate that DC infected with RABV vaccine vectors are still efficient vaccines even in the presence of anti-RABV specific host immune responses.

RIDC-Gag mounts a RABV vector specific humoral immune response

RABV vaccine strain-based vectors used in vaccine studies have been shown to induce strong humoral immune responses in both mice and monkeys [23,31,32,42–46]. We wanted to analyze if RIDCs can also elicit a humoral immune response in the immunized host. To do this, BALB/c mice were primed with either RV-Gag or RIDC-Gag. Similar to live RABV, mice immunized with RIDC-Gag mounted antibodies against the G protein (Fig. 8a). It has been previously shown that a DC-based vaccine against HIV-1 elicited a Th2 skewed response [47], whereas for vaccines against HIV-1 infections both Th1 and Th2 responses are desirable. RABV-based vaccine vectors have been shown to induce Th1 skewed responses [48]. Therefore, serum from both groups of mice 14 days post prime were analyzed for anti-RABV-G IgG1 and IgG2a. Both RV-Gag and RIDC-Gag induced a Th1 dominant response marked by higher levels of IgG2a compared to IgG1, as indicated by a ratio greater than 1 (Fig. 8b). Taken together, this data shows that the RIDC vaccine can induce a strong Th1-biased humoral immune response.

Discussion

In order for DC-based vaccines to stimulate an immune response, it is important that they attain a mature state and are functional. RABV is a neurotropic virus that has been shown to infect different cells *in vitro* including DCs [27,36]. Our studies show that murine BMDCs support RABV transcription and expression and presentation of foreign antigens. Although RABV is generally a non-cytolytic virus, the RABV vaccine strain has been shown to induce programmed cell death (PCD) in different cell types including T cells and neuroblastoma cells [41]. Similarly, RIDCs also undergo PCD secondary to RABV infection resulting in a heterogenous DC vaccine that includes both live and apoptotic cells that could contribute to the immune response. Unlike wildtype VSV, VV, measles, and herpes simplex virus, vaccine strain RABV infection leads to the maturation of DCs [39,49–51]. Similar to previous studies with human DCs, UV-inactivated RABV failed to mature BMDCs illustrating that RABV replication is indispensable in the maturation of murine DCs [27]. Of note, while RABV undergoes replication in BMDCs resulting in the expression of viral and foreign proteins, the infection is non-productive with no detectable viral progeny in the supernatants. Interestingly, although RABV-P has been shown to interact with STAT1, which would interfere with interferon induction, this phenomenon does not appear to significantly affect the ability of BMDCs to obtain a mature phenotype [40,52].

For an HIV-1 vaccine, the induction of polyfunctional CD8⁺ T cells and antibodies are currently accepted as endpoints for potential HIV-1 vaccines. RABV as a vaccine vector has been shown to induce both humoral and cellular immune responses [21,24,31,42]. Our results show that exogenous RIDCs infected with vaccine strain rRABV are functional as APCs. They can present foreign antigen on their MHC and induce the proliferation of both CD4⁺ and CD8⁺ T cells. *In vivo*, these RIDCs stimulate both cellular and Th1 skewed humoral immune responses. The total IgG response against RABV-G protein was higher in the RIDC-Gag mice compared to RV-Gag possibly due downstream effects of interaction between RIDCs and B/T cells or due to the route of inoculation used to immunize the mice – RIDC-Gag (i.v.) versus RV-Gag (i.m.). Moreover, the immune response is not due to secondary infection by viral particles from RIDCs, because BMDCs infected with a single-cycle RABV induce a similar immune response. We can speculate based on the *ex-vivo* experiments, that RIDCs can directly present to T cells leading to their proliferation. Furthermore, the heterogenous pool of live and apoptotic RIDCs could contribute to the

immunogenicity of the vaccine with the help of endogenous DCs [37],[53]. Although a significant number of DC-based HIV-1 vaccines have been investigated [10,14,15], there is a paucity of vaccine research using viral vectors to deliver HIV antigens to DCs *ex vivo*. Yet the advantage of a live vector is that it may undergo several rounds of replication making the antigens available for presentation over a longer period of time. Furthermore, *ex-vivo* delivery of antigens to DCs allows streamlining of the quality and function of the DC vaccine administered[6]. To our knowledge, there are few other viruses such as Canarypox that have been used to transduce DCs *ex vivo* with HIV-antigens and tested in human clinical trials [54]. Based on current DC-vaccine research, it is evident that more work needs to be done to optimize DC-based vaccines. Our studies present RABV as a vector that serves very well to transduce immunogenic BMDCs that readily induce a robust humoral and cellular immune response. Furthermore, RABV has several advantages including the ability to express multiple foreign genes [18]. Our group has extensively studied and documented the immunogenicity of RABV co-expressing HIV-1 proteins and different cytokines (IL-2, IL-4, IFN- β) [26,43]. Future studies will now focus on optimizing the RIDC vaccine and testing it in humanized SCID mice infected with HIV prior to using them in monkeys based on the outcomes.

The importance of prime-boost regimens in HIV vaccines has been greatly emphasized. A recent study using DC-targeted lentivector expressing HIV-1 Gag showed that the virus was able to efficiently boost Gag-specific cellular responses in mice that had previously been immunized by the same virus [55]. Similar to this, we were able to show that RIDC-based HIV-1 Gag vaccine was able to boost cellular immune responses in mice primed with live RABV or RIDCs. Further studies will focus on improving the boost responses by using RABV co-expressing cytokines that can enhance CD8+ T cell proliferation.

In conclusion, we have shown that RABV can infect murine DCs *ex vivo*. These RIDCs are viable and functional as APCs. When tested in immune competent mice, RIDC-Gag induce robust humoral and cellular immune responses. Moreover, they boost cellular responses in mice primed with live RV-Gag and RIDC-Gag. We present a DC-vaccine model that can be further manipulated to co-express HIV antigens with pro-inflammatory cytokines and chemokines to improve the immune responses elicited.

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Figure 1. Characterization of RIDCs

Bone marrow from BALB/c mice was differentiated in the presence of 10ng/ml GM-CSF for a period of 7 days (See materials and methods). Primary BMDCs were confirmed by expression of CD11c (a). BMDCs were infected with SPBN-GFP at an MOI of 10. After 48h, GFP positive cells were identified by immunofluorescence (b). To identify viral protein expression, cells were stained with a FITC-tagged antibody against RV-N and analyzed by flow cytometry (c). The viability of the RV, RV-Gag infected and uninfected BMDCs from 5 independent mice was determined by 7-AAD staining versus FSC – early apoptotic cell populations were identified as R2, whereas R3 denotes live cells(d). The average apoptotic

BMDCs from n=5 independent mice is denoted by the bar graphs at 24 (**e**) and 36hpi (**f**). Apoptotic NA cells were included as a positive control.

Figure 2. RIDCs are functional APCs

BMDCs were infected with RV at an MOI of 10. The maturation phenotype of RIDCs was determined 48hpi by FACS analysis of CD80 and CD86 expression, with numerical inserts of the Geometric mean fluorescence (GMF) (a). Presentation of antigen by RIDCs was analyzed after infection of BMDCs from C57BL/6 mice with either RV-OVA or RV at MOI-10. 48hpi the cells were harvested and stained with an antibody against the MHC I Kb SIINFEKL complex. Cells pulsed with OVA (SIINFEKL) peptide were used as a positive control.(b). BALB/c derived RIDC-Gag were incubated with CFSE pulsed splenocytes from C57BL/6 mice in an allogeneic MLR for 5 days as described in materials and methods.

CFSE dilution in CD4 and CD8 populations were determined by GMF intensities and compared to splenocytes incubated with uninfected DCs (c).

Figure 3. Mice immunized with RIDC-based HIV-1 vaccine exhibit a Gag-specific primary immune response

BALB/c mice were immunized with $4 - 5 \times 10^5$ DCs (uninfected, RIDCs or RIDC-Gag) or 1×10^6 ffu of live RV-Gag. Primary immune responses were monitored seven and eleven days post immunization. Spleens were harvested, stained and analyzed as shown in the representative charts (a). CD8 T cells were analyzed for the expression CD44^{hi}, CD62L^{lo} and AMQMLKETI HIV-1 Gag tetramer (a-c). IFN- γ expression after AMQMLKETI HIV-1 Gag peptide stimulation was detected in an ELISpot assay 7 days post immunization and represented as the mean with the standard error of the mean (SEM) (n=4: Uninfected, RV-Gag) and (n=3 RIDC and RIDC-Gag). (d). The data presented here is representative of two independent experiments. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post-test. $P < 0.05$ * and $P < 0.001$ ***.

Figure 4. Mice immunized with RIDC-Gag mount a recall response

Previously immunized BALB/c mice were rested for 60 days followed by a challenge with a dose of 1×10^6 pfu of VV-Gag. The resulting cellular immune responses were analyzed five days post challenge. Splenocytes were obtained from the three immunization groups (Uninfected DC, RIDC and RIDC-Gag) and stained for the expression of CD8, AMQMLKETI HIV-1 Gag tetramer, CD44^{hi}, and CD62L^{lo} (a–b). Functional expression of IFN-γ determined via ELISpot (c) and ICS (d). Functional expression of TNF-α, IL2, IL6, and IL10 by CD8 T cells was determined ICS (d). The data presented here is representative of at least three independent experiments. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post test. $P < 0.05$ *, $P < 0.01$ ** and $P < 0.001$ ***.

Figure 5. Gag specific immune response is not due to secondary infection by RV-Gag
 RIDC-Gag and -ΔG-Gag infected at MOI-10 were stained with antibodies against RV-N and CD86 and analyzed by flow (a). Mice were primed with either RIDC-Gag or RIDC-ΔG-Gag. Thirty days post prime, the mice were challenged with a dose of 1×10^6 pfu of VV-Gag. Activated and AMQMLKETI specific CD8 T cell induction was determined by FACS analysis (b–c). Functional expression of inflammatory cytokines by antigen pulsed splenocytes was determined by IFN-γ ELISpot (d), presented as the mean with the SEM (n=2: Uninfected, n=3: RIDC-Gag and n=5: RIDC. ΔG-Gag), as well as ICS (IFN-γ, TNF-α, IL6) (e). The data presented is representative of two repeat experiments. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post-test.

Figure 6. RIDC induced CD8 T cells are cytotoxic

P815 target cells were pulsed overnight with either AMQMKLETI peptide or Env (89.6 R10) peptide. The target cells were then harvested and stained with Calcein as described in materials and methods. P815 target cells were incubated in different E:T ratios (100:1, 33:1, 11:1, 3.7:1) with splenocytes obtained from mice first immunized with uninfected DCs, RIDC-Gag or RIDC.ΔG.Gag and then challenged with 1×10^6 pfu of VV-Gag. After the 3hr incubation, supernatants were collected and the amount of fluorescence read to determine the percentage of lysed cells (see materials and methods).

Figure 7. RIDC-Gag enhance cellular responses in a homologous prime-boost regimen

BALB/c mice were immunized with 1×10^6 ffu RV-Gag or 5×10^5 RIDC-Gag and rested for at least 21 days. The mice received booster shots of either RV-Gag or RIDC-Gag and the cellular immune responses analyzed. The cells were gated as shown (a). On day 11 post boost, AMQMLKETI tetramer specific CD8 T cells (b) and IFN- γ producing cells in the presence of AMQMLKETI peptide were quantified in an ELISpot assay (c) and presented as the mean (n=2: Naive, n=3: RV-Gag/RV-Gag, n=5: RV-Gag/RIDC-Gag and n=5:RIDC-Gag/RIDC.Gag). Statistical analyses were performed using two-tailed unpaired t-tests with welch's correction where necessary.

Figure 8. Mice immunized with RIDCs mount a RV vector specific humoral immune response BALB/c mice were immunized with either 1×10^5 ffu live RV expressing HIV-1 Gag (i.m.) or 5×10^5 RIDC-Gag (i.v.). Eye bleeds of eight mice per group were done 7, 14 and 21 days post prime. The OD (490nm) readings of total anti RV-G IgG levels in the immunized mice were measured. The results from 1:450, 1:1350, 1:4050 dilutions were charted to monitor the development of the humoral immune response (a). The ratio of anti RV-G IgG2a to IgG1 antibodies induced 14 days post immunization was measured (b). Statistical analyses were performed using two-tailed unpaired t-tests, $P < 0.05$ *, $P < 0.01$ ** and $P < 0.001$ ***. The mean and SEM are presented (n=8 per group).