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Preclinical evaluation of HIV-1 therapeutic ex vivo dendritic cell vaccines expressing consensus Gag antigens and conserved Gag epitopes

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Abstract

Background—Dendritic cell (DC) therapy is a promising technology for the treatment of HIV infected individuals. HIV-1 Gag- and Nef RNA-loaded DC have previously been shown to induce immune responses ex vivo following coculture with autologous lymphocytes. However, polyfunctionality and memory responses following coculture have not been evaluated. In addition, little is known regarding whether specific HIV-1 proteome components, such as highly conserved regions of the HIV-1, could enhance clinical responses following DC therapy.

Methodology and Principal Findings—To determine the breadth of the immune responses to antigen loaded DC, we analyzed polyfunctional T cell response ex vivo to Gag RNA loaded DC. Blood samples were used to generate monocyte derived DC, which were then matured and cocultured with autologous lymphocytes. We found that cytokine-matured DC loaded with Gag RNA were able to induce Gag-specific IFN- γ and IL-2 responses after a 12-day coculture. We characterized these responses by polyfunctional intracellular cytokine staining and evaluation of T cell memory phenotypes. Central memory CD8⁺ T cells were induced ex vivo after DC coculture from each of 3 patients, and the effector memory pool was increased by DC coculture from 2 patients. We also observed a decrease in the terminal effector and intermediate CD8⁺ T cell pool and an increase in the naïve/other population. There was a reduction in terminal effector and intermediate CD4⁺ T cells, and a corresponding increase in naïve/other CD4⁺ T cells. Finally, we evaluated conserved regions of Gag as a novel DC therapy immunogen and found that a

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Conserved Element (CE) p24 Gag antigen elicited IFN- γ and IL-2 responses comparable to those induced by a full-length Gag antigen.

Conclusions—We showed that RNA-loaded DC therapy induced a polyfunctional T cell response *ex vivo*, supporting the use of such DC-therapy for HIV infection. However, the central and effector memory phenotypes of T cells did not appear to be enhanced during coculture with Gag RNA-loaded DC. Furthermore, comparable antigen-specific responses were induced in HIV infected individuals using full-length Gag or only conserved elements of the Gag p24 protein. This indicates that immune responses can be focused onto the conserved elements of Gag in the absence of other Gag components.

Keywords

Dendritic Cell; Therapeutic Vaccination; HIV; RNA

Introduction

Dendritic cell therapy remains one of the more promising methods to induce an immune response against antigens in cancer patients [1-3]. Induction of robust polyfunctional T cells is essential for successful immune based therapies in HIV infection. DC are highly effective antigen-presenting cells that play a central role in pathogen-specific immune activity and the innate and adaptive immune response. There is continued interest in developing dendritic cell therapy programs for the treatment of HIV infected individuals. One approach is the use of mRNA-loaded dendritic cells [3-8]. In previous studies, mRNA has been used to encode either consensus HIV antigens able to induce responses in a variety of HIV infected patients [6], or HIV antigen sequences derived from each patient individually [5].

Prior to Phase I studies for HIV dendritic cell therapy, it is important to preclinically evaluate the response generated by mRNA-loaded DC from HIV infected patients. While most studies have tested DC function by IFN- γ ELISPOT, a more detailed analysis of T cell polyfunctionality and memory responses would be beneficial.

Another key issue for HIV DC therapy optimization is the nature of the HIV antigen. Recent studies suggest that recombinant antigens composed of only highly conserved HIV epitopes may be superior to full-length HIV antigens [9-12]. A major issue is the role of non-conserved 'decoy' epitopes encoded within the full-length antigens. These non-conserved epitopes, which are typically the dominant immune response in HIV patients, can be mutated without a fitness cost to the virus [13], leading to immune evasion. In contrast, CTL responses associated with viral control, such as those elicited in individuals carrying B*27 or B*57 HLA alleles often target conserved regions of HIV-1 [14-16].

Here we demonstrated the preclinical efficacy of a HIV DC therapy protocol using RNA-loaded DC from a cohort of HIV patients located in Miami, Florida. We also evaluated RNA encoding either conserved elements of the HIV-1 M group Gag p24 or a full-length Gag to compare induction of Gag responses. Overall these studies show that HIV patient samples can be induced to respond to dendritic cell therapy, and supports future HIV DC therapy clinical trials, as well as the use of vaccines designed to direct responses exclusively to conserved elements of viral proteins.

Materials and Methods

PBMC samples

All samples were collected in sodium Heparin unless otherwise noted. For HIV infected patient samples, patients were recruited from the University of Miami AIDS Clinical Research Unit. A total of 23 patient samples were evaluated. All patients signed informed consent in accordance with the University of Miami Institutional Review Board. Uninfected human blood samples (buffy coat) were purchased from Continental Services Group Inc., Miami, FL and were negative for HIV, HBV, and HCV infection.

Dendritic cell preparation and cryopreservation

Whole blood from buffy coat or from HIV+ patients was centrifuged at 400×g for 10 min, and plasma was removed, centrifuged at 800×g for 20 minutes, then filtered with Centrux 0.2µm filters (Whatman) and stored in -80°C. The cell fraction was then centrifuged over ficoll at 2100 rpm for 25 min at room temperature. The monocyte layer was re-suspended in PBS, centrifuged sequentially at 1800 rpm for 10min, 1100rpm for 10min, then 1000rpm for 10 min with aspiration and resuspension between each spin. PBMC were finally resuspended in complete media: RPMI1640 (Hyclone) containing 5% human AB serum (Lonza) or autologous plasma, PenStrep (1x), and glutamine. To isolate monocytes, 8×10^7 HIV PBMC were placed in a T175 flask in 20ml of complete media, and cultured for 2 hours at 37°C in 5% CO₂. Unattached cells were removed with warm media three times by pipet. Unattached cells (defined as peripheral blood lymphocytes, PBL) were pelleted and resuspended in 80% FBS + 10% Glucose + 10% DMSO. PBL were frozen in liquid nitrogen until use. The adherent monocyte cells were cultured in 20 ml of media containing 500U/ml IL-4 (R&D systems) and 1000U/ml GM-CSF (BERLEX Inc). After 6 days of culture, flasks containing immature DC were placed at 4°C for 30 minutes to detach DC, then collected by gentle pipetting to isolate unattached and loosely attached cells. Any remaining adherent cells were discarded.

HIV antigen mRNA preparation and DC transfection

HIV-1 p24 CE construct will be described in detail in Mothe et al. (submitted). This artificial sequence contains a string of 7 highly conserved regions of p24 Gag linked together into a single coding sequence. This sequence contains >40 known HLA epitopes. HIV-1 sequences encoding codon optimized Clade B consensus Gag, Clade B consensus Nef (kindly provided by Daniel Kavanagh), and p24 CE (Mothe et al, submitted) were cloned into the pGEM4z plasmid containing a T7 promoter and a 64bp polyA tail (vector pGEM4Z/A64). The construct pGEM4Z/A64-GFP containing the GFP gene (a gift from Eli Gilboa) was used as a control in all experiments. The mMachine mMessage RNA in vitro transcription kit (Ambion) was used to generate mRNA from all constructs. Day 6 DC were washed, resuspended in OPTI-MEM medium (GIBCO) and transfected with 10µg RNA. A total of 1×10^6 immature DC in 100µl OPTI-MEM were placed in a 0.4µm Gene Pulser cuvette (Bio-Rad) for each electroporation. Electroporation was performed on a GenePulser X cell (Rio-Rad) with exponential decay at 300V and 150µF. The transfected DC were transferred to 6-well plates with 3ml complete media containing Mimic cytokine mix (5 ng/ml TNFa (R&D), 5ng/ml IL-1b (R&D), 750ng/ml IL-6 (R&D)) + PGE2 (Sigma) 1ug/ml). Cells were cultured overnight at 37°C in 5% CO₂. The supernatant of Gag RNA transfected DC was collected and tested for P24 level as a transfection efficiency indicator using the Alliance HIV-1 P24 ELISA kit (Perkin Elmer). Nef RNA transfection efficiency was tested by reverse transcription PCR, and GFP RNA transfection efficiency confirmed by flow cytometry.

Coculture of RNA transfected DC with autologous peripheral blood lymphocytes

Transfected DC were cultured at 37°C in 5% CO₂ for 18-36 hours, then placed at 4°C for 30 minutes to detach cells. DC were then stained with CD14 FITC, CD80 PE, CD86 APC, CD83 PerCP, CD11c PE cy7, CCR7 PE, HLA-DR APC cy7 (all from BD, Becton Dickinson) as maturation markers. For HIV patient-derived DC, cells were also stained with the following alternative antibody panel (all from Becton Dickinson): CD86 FITC, HLA-DR PE, CD83 APC, CCR7 PE cy7, CD40 APC in one tube and CD14 FITC, CD80 PE, and CD11c PE cy7 in a second tube. Data was acquired on an LSRII fluorometer (BD) using Diva software. For coculture, RNA transfected DC (2×10^4 /well) were mixed with 2×10^5 autologous PBL/well in round bottom 96-well plates, and cultured in 37°C in 5% CO₂ for 12 days. Either IL-2 (Chiron) at 20U/ml, or IL-7 (R&D Systems) at 5U/ml was added on day 3.

ELISPOT assays

ELISPOT plates (Millipore) were pre-coated with IFN- γ , IL-2, or granzyme B capture antibody using human ELISPOT sets (BD Biosciences). After blocking with complete media for 1 hour at room temperature, 3×10^4 to 1×10^5 PBL were added from 12 day cocultures. One of the following set of peptides were then added: 5 μ g/ml HIV consensus clade B Gag 15-mer peptides overlapping by 11 amino acids (NIH AIDS research and reference reagent program), 5 μ g/ml Nef HIV consensus clade B 15-mer peptides overlapping by 11 amino acids (NIH AIDS research and reference reagent program), 5 μ g/ml CE Gag 10-mer peptide pool overlapping by 9 amino acids, 5 μ g/ml Ova peptide (amino acids 323-339, Genscript), or 5 μ g/ml c-myc peptide (Sigma) as negative control. For positive controls, 5ng/ml PMA (Sigma) and 500ng/ml ionomycin (Sigma) was added. Plates were incubated at 37°C in 5% CO₂ overnight, washed, read and then analyzed with Immunospot CTL analyzer software (Cellular Technologies).

Intracellular staining and T cells memory marker surface staining

After 12 day coculture, at least 1×10^6 PBL or thawed patients PBL (day 0) were placed into FACS tubes with 1 ml complete media containing 1 μ l anti-CD49 and anti-CD28, 20 μ l CD107a FITC antibody plus either 5 μ g/ml Gag peptide pool, 5 μ g/ml ova peptide, or PMA/iono. Unless otherwise noted, all antibodies were from BD Biosciences. Tubes were incubated at 37°C in 5% CO₂ for 5 hours. A total of 10 μ g/ml BFA (Sigma) and 0.7 μ l monensin (eBiosciences) was added after 2 hours. Cells were washed twice with FACS buffer (1% human serum + 0.05% sodium azide in PBS), then blocked with 10% human serum in PBS for 10 minutes at RT. Cells were washed with FACS buffer twice, then stained with antibodies to CD8 APC-cy7, CD4 PerCP, CD3 Amcyan, and an exclusion mix: CD14 pacblue/CD19 pacblue/live-dead violet (Invitrogen). Tubes were incubated at room temperature in the dark for 20 minutes and then washed twice with FACS buffer. 300 μ l Cytofix/Cytoperm (BD Biosciences) was added and incubated for 15 minutes at 4°C. Cells were washed twice with PermWash buffer (BD Biosciences) then stained with IFN- γ PE-Cy7, IL-2 PE, and TNF α APC antibodies for 30 minutes at RT. Cells were washed twice with PermWash buffer then fixed with 2% buffered formalin. Data was acquired with a BD FACS Aria using Diva software.

For T cell memory marker staining, after 12 day coculture, PBL were stained with CD4 FITC, CD3 PerCP, CCR7 PE, CD45RO PE-cy7, CD8 APC, and CD27 APC-H7 antibodies, as well as Live-dead aqua (Invitrogen). Data was acquired with a BD FACS Aria machine and analyzed using FlowJo Software.

Statistics

For two-group comparison, we used unpaired Students T test. For multiple samples the Wilcoxon matched pair test was used.

Results

Ex vivo derived dendritic cells induce a de novo response to antigen

Ex vivo derived DC were stained for various markers of activation and maturation both before and after cytokine maturation. As shown in Figure 1A, both immature and mature DC cultures were CD11c⁺ and CD14⁻, indicating monocytes had fully developed into dendritic cells. Cytokine maturation with the Mimic cytokine mix induced the upregulation of chemokine receptor CCR7, activation markers CD80 and CD86, and maturation markers CD83, CD40, and HLA-DR (Figure 1A). Figure 1B showed GFP expression was induced when cells were electroporated in the presence of GFP RNA, but not following mock electroporation.

Mature DC transfected with antigen RNA would be expected to induce T cell responses after ex vivo culture with autologous lymphocytes, and it has been previously shown that lymphocytes from an antigen-naïve donor are able to induce measurable antigen-specific responses with sufficient costimulation by mature DC. To test this, DC from HIV uninfected donors were electroporated with either Gag or GFP RNA, matured, and cultured with autologous PBL for 12 days. Cells were then stimulated with overlapping 15-mer peptides to Gag. As shown in Figure 1C, a significant ($p<0.05$) increase in spot forming units (SFU) was observed only when PBL were cultured for 12 days with Gag RNA transfected DC (at a 10:1 ratio of PBL to DC), but not in the presence of DC transfected with GFP RNA, or PBL cultured in the absence of DC.

The quality of DC activation is also related to its ability to induce a recall response. To confirm that the de novo immune response we observed could be restimulated using this ex vivo model, DC were loaded with Gag RNA and matured, followed by a 9 day coculture with autologous PBL. Half the 9 day culture was tested by IFN- γ and IL-2 ELISPOT and did not induce an antigen-specific responses (data not shown). The remaining PBL culture was again mixed at a 10:1 ratio with autologous Gag RNA loaded mature DC, and restimulated in a second 9 day incubation. This second incubation induced a significant ($p<0.05$) increase in both IFN- γ and IL-2 ELISPOT responses (Figure 1D) after restimulation with Gag peptide pool compared to restimulation with control Ova peptide.

Ex vivo dendritic cells from HIV patients can enhance HIV antigen specific responses

Immune dysregulation during chronic HIV infection has been shown to disrupt dendritic cell generation and HIV-specific T cell responses. DC derived from HIV patient monocytes were tested to assess whether they could enhance antigen-specific responses from the patient's autologous lymphocyte population. Immature and Mimic-matured DC derived from an HIV patient blood sample were compared by flow cytometry (Figure 2). As shown in Figure 2A, maturation induced the upregulation of CD40, CD80, CD86, and CD83 in a manner comparable to DC maturation of cells from HIV negative volunteers (compare to Figure 1A). To determine whether HIV-1 Gag antigen-specific responses could be enhanced, we performed a DC-T cell coculture with autologous PBL for 12 days, followed by Gag peptide pool stimulation and ELISPOT assay. As shown in Figure 2B, left panel, a significant increase ($p=0.016$) in IFN- γ secreting cells was observed in a set of 7 patient samples by Wilcoxon matched pair test. Similarly, IL-2 ELISPOT responses were significantly enhanced ($p=0.002$) after coculture with Gag RNA loaded DC in a set of 10 patient samples. To control for non-specific DC activation and DC-independent stimulation during the 12-

day coculture, a number of control conditions were tested in parallel. Figure 2C shows a representative ELISPOT panel while Figure 2D shows the overall responses of our patient samples under the various assay conditions. DC loaded with Gag RNA, cocultured with autologous PBL, and restimulated with Ova peptide induced only background levels of IFN- γ and IL-2 ELISPOT responses. Similarly, PBL cocultured for 12 days with DC loaded with GFP RNA and then restimulated with Gag peptide pool resulted in significantly lower levels of ELISPOT responses (Figure 2D). PBL from HIV-1 infected individuals prior to coculture were unable to induce significant Gag-specific ELISPOT responses. This likely reflects the fact that most patients had been on antiretroviral therapy (ART) for >1 year with viral load <50 copies/mL at the time of blood donation. Any memory T cells present were probably unable to generate cytokines after the brief overnight peptide stimulation employed.

Similar studies were performed with Nef RNA loaded DC. As shown in Figure 2E, only Nef loaded DC cocultured with PBL for 12 days and restimulated with Nef peptide pool were able to induce a significant number ($p<0.05$) of IFN- γ and IL-2 ELISPOTs.

Polyfunctional CD4+ and CD8+ T cells are induced by Gag RNA loaded DC

The ELISPOT responses illustrated in Figure 2 indicate a general T cell activation after DC coculture. To determine the specific role of CD4+ and CD8+ T cells in DC induced immune activation of HIV patients, multiparameter flow cytometry was performed on three patient samples after a 12-day coculture (Figure 3A). We selected the CD3+ live cell lymphocyte population and analyzed CD4+ or CD8+ T cells for their expression of IL-2, TNF- α , IFN- γ , and CD107a. Functional CD4 responses were plotted as a percentage of the total CD4+ population (Figure 3B). Prior to coculture, <1% of cells were positive for a single functional marker, with less than 0.1% positive for two cytokines or degranulation as assessed by CD107a expression. In contrast, after 12-day coculture, CD4+ T cells were able to induce a range of polyfunctional responses. Similarly, Figure 3C shows an increased percentage of CD8+ T cells induced one, two, three, or all functional markers after 12 day coculture.

To evaluate the cytotoxic T cell activity of our 12-day coculture cells, we performed Granzyme B ELISPOT assays. As shown in Figure 3D, 12-day coculture with Gag RNA loaded DC enhanced the number of Granzyme B secreting cells after restimulation with Gag peptide pool ($p<0.05$). Minimal Granzyme B responses were observed with GFP RNA loaded DC or with PBL prior to 12-day coculture (day 0 cells).

During the 12-day coculture of HIV patient samples, both naïve and memory T cells are expected to expand in response to presentation of Gag antigens together with costimulatory molecules from Gag RNA transfected, cytokine-matured DC. This expansion may shift the balance of T cell phenotypes, leading to a shift in memory phenotype ratios following costimulation. To evaluate the overall change in phenotype, three independent aviremic HIV patient samples were cocultured with autologous Gag RNA loaded DC for 12 days. Cells were then stained for markers CD3, CD4, CD8, CD27, CCR7, and CD45RO, allowing us to determine the percentage for naïve/other, central memory (T_{cm}), transitional memory (T_{tm}), effector memory (T_{em}), terminal effector (T_{eff}), and intermediate T cell (T_{int}) populations. As shown in Figure 3E, upper panel, we observed an increased proportion of central memory CD8+ T cells for all three patient samples after DC coculture. We also observed a decrease in the terminal effector and intermediate CD8+ T cell pool and an increase in the naïve/other population. For two patient samples, the effector memory pool was also increased. For the CD4+ T cell population, again there was a reduction in the terminal effector and intermediate T cell populations after 12 day coculture, and a corresponding increase in naïve/other CD4+ T cells.

Gag p24 Conserved element vaccine antigen also induces Gag-specific T cell responses after 12 day coculture

While full-length consensus B Gag antigen is useful for preclinical evaluation of DC therapy, treatment of a broad patient population will most likely require antigens that cope with the diverse set of viral sequences present in the patients. We therefore decided to evaluate a conserved element antigen [10] covering highly conserved segments of the HIV-1 M group p24 protein. The CE p24 antigen consists of 7 highly conserved peptides from Gag p24 that are typically conserved in > 98% of sequences from all HIV-1 M group viral subtypes (Mullins et al, unpublished). As shown in Figure 4A, HIV patient dendritic cells loaded with RNA encoding p24 CE were able to induce CE-specific IFN- γ responses when T cells were loaded with CE 10-mer overlapping peptides and not when loaded with Ova control peptide ($p < 0.0001$). Similar results were observed for 3 additional HIV patient samples. To characterize the ability of CE RNA to induce T cell responses to full length Gag, DC from HIV uninfected individuals were loaded with CE RNA and cocultured with T cells for a total of 18 days, including a restimulation at day 9 with CE RNA loaded DC. After coculture, T cells were stimulated with either Gag 15-mer overlapping peptides or CE 10-mer overlapping peptides. As shown in Figure 4B, equivalent ELISPOT responses were induced with full length Gag peptides and conserved element peptides. T cell responses were not induced by DC expressing GFP, indicating that human T cells specific for conserved HIV peptides could be generated in this coculture assay.

Discussion

Previous studies have generated HIV specific immune responses from HIV patient monocyte derived DC [4-6], with analyses that mostly consisted of DC maturation and antigen presentation as measured by ELISPOT IFN- γ cytokine secretion assays. The present study confirms that HIV patient monocyte derived DC loaded with HIV Gag or Nef RNA can induce T cell responses. Initially, we confirmed that mature DC could be generated from HIV uninfected monocytes (Figure 1A). These DC were also successfully transfected using electroporation (Figure 1B), and induced Gag-specific immune responses in autologous T cells after DC-T cell coculture (Figure 1C). Similar to PBMC from HIV uninfected individuals, HIV+ patient monocyte derived DC were able to induce upregulation of CD40, CD80, CD83, and CD86 following Mimic cytokine cocktail-induced maturation (compare Figure 2A to Figure 1A).

In the HIV+ patient cohort, we observed a statistically significant increase in both IFN- γ and IL-2 Gag-specific responses after DC-T cell coculture (Figure 2B and 2C). Despite chronic HIV infection, most patients were unable to mount more than background ELISPOT responses prior to coculture (Figure 2E and 2F). This likely reflects the low levels of antigen present after long term ART, as well as immune dysregulation in this patient population [17-21]. In addition, the overnight culture ELISPOT assay would only detect effector T cells, but not memory T cells present in the PBMC. Typically, memory T cell responses are quantified after a 7-10 day culture of PBMC in the presence of antigen [22], a similar process to our DC-T cell coculture protocol. Previous studies have also documented low levels of HIV antigen specific effector T cells present following ART, as well as dysfunction of HIV-specific T cells in patients chronically infected with HIV [21,23-25]. In addition, recent evidence suggests that monocyte-derived DC from HIV+ patients (either on ART or treatment naïve) are impaired in their ability to induce T cell responses in a Mixed Leukocyte Reaction [26]. However, in our study most patients were able to generate high levels of IFN- γ and IL-2 ELISPOT responses following the 12-day coculture (Figure 2B and 2C). Increased numbers of IL-2 secreting cells indicate that these cocultures generated a robust antigen-specific memory T cell response as well as effector T cell response. This suggests that patients treated with Gag-loaded autologous DC will be able to mount an IL-2

dependent immune response after DC immunotherapy. IL-2 dysregulation is an important aspect of HIV immune dysfunction [27,28], and our results suggest that DC therapy with Gag RNA antigen may be able to enhance IL-2 secretion by patient T cells in vivo similar to what was observed ex vivo, generating a central memory phenotype in the resulting Gag-specific T cell pool. Results with Nef antigen (Figure 2G and 2H) confirmed that the induction of antigen-specific and IL-2 secretion occurs with Nef as well as Gag HIV antigens. It is also interesting to note that we observed a significant increase in IFN- γ secretion but not IL-2 secretion prior to coculture (Figure 2G, Day0/Nef). This is consistent with suppression of IL-2 secretion in the context of chronic HIV infection, even under ART, while IFN- γ secretion persists in the context of chronic immune activation and inflammation.

Polyfunctional flow cytometry has previously been used to document correlates of protection for SIV vaccines, with cells positive for >2 cytokines present in non-human primates that generate enhanced T cell responses during infection. Studies by Letvin, Nabel and others [29-31] have highlighted that vaccinated primates with either a high proportion of polyfunctional T cells or a larger proportion of memory T cells have reduced SIV viral load and survive longer after challenge. In our cohort, the number and proportion of polyfunctional CD4+ and CD8+ T cells increased following coculture, consistent with an improvement in the polyfunctionality of the T cell population. The increase in the level of CD4+ T cells co-expressing two cytokines or CD107a from <0.1% to >1% was especially promising, as was the increase in CD4+ T cells co-expressing three or more cytokines or CD107a from <0.01% to >0.1%. These data suggest that CD4+ as well as CD8+ T cells are generated during coculture and may mount a potent anti-HIV response. Surprisingly, when patient samples were assayed for memory phenotype (Fig. 3D and 3E), we did not observe a corresponding increase in memory cells as would be expected from the increase in polyfunctional T cells (Fig. 3B and 3C) and the lower proportion of T_{eff} cells following coculture. This may reflect the differences in T cell populations generated ex vivo compared to those induced by in vivo vaccine responses. However, we did observe an increase in the number of T_{cm} CD8+ T cells (Fig. 3E) that did not reach statistical significance. One potential explanation for the lack of memory T cell generation is the increase in the number of naïve/other T cells during coculture. The increase in this cell population may be a consequence of this ex vivo culture method, and not reflect the cell populations expected in vivo where we would expect a loss of the naïve antigen-specific cell population following antigen presentation. We also observed an increase in the number of double negative CD3+CD4-CD8- T cells after 12 days of coculture (data not shown). Typically, double negative T cells are deleted in vivo, but these cells can be retained in an ex vivo assay due to an absence of CD95-mediated apoptosis [32]. This double negative population may explain the low relative proportion of memory cells despite a robust polyfunctional response.

A number of approaches have been taken in clinical studies to improve the antigen used in HIV DC therapy. Generating viral antigen mRNA from the patients' own viral clones provides the most precise method of matching vaccine and donor viral sequences. However, this does not change the capacity of the virus population to quickly mutate in response to DC therapy, thereby reverting to pre-vaccination viral load levels. As an alternative approach that attempts to mitigate the benefit of viral escape, we explored the use of a prototype HIV-1 Gag p24 Conserved Element (CE) vaccine encoding of 7 segments of highly conserved p24 peptides (Figure 4). We observed that ex vivo coculture using CE-loaded DC induced antigen-specific immune responses in both HIV infected patients (Fig. 4A) and HIV uninfected donors cells (Fig. 4B). The uninfected donor generated a de novo immune response to CE using an 18-day culture method. Others have previously shown that HIV specific responses can be generated ex vivo from antigen-naïve donors using similar methods [33]. While these experiments cannot evaluate the in vivo efficacy of this approach,

recent studies using mice, HLA-transgenic mice and rhesus models suggest that the same CE antigen is able to induce immune responses equivalent to those induced by full-length Gag protein (G. Pavlakis, unpublished; D. Weiner, personal communications). This would support the use of CE for dendritic cell therapy in order to generate a robust immune response only to those HIV epitopes most likely to be essential for viral control.

Overall, this preclinical evaluation provides data to support the evaluation of Gag and other HIV-derived antigens such as CE for DC immunotherapy of HIV infected individuals. Based on these preclinical data, a DC therapy trial is being planned at the University of Miami to evaluate a CE DC immunotherapy vaccine for the treatment of HIV infected individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Kim S, Lee JB, Lee GK, Chang J. Vaccination with recombinant adenoviruses and dendritic cells expressing prostate-specific antigens is effective in eliciting CTL and suppresses tumor growth in the experimental prostate cancer. *Prostate* 2009;69:938–948. [PubMed: 19267351]
2. Higano CS, Small EJ, Schellhammer P, Yasothan U, Gubernick S, et al. Sipuleucel-T. *Nat Rev Drug Discov* 9:513–514. [PubMed: 20592741]
3. Gilboa E, Vieweg J. Cancer immunotherapy with mRNA-transfected dendritic cells. *Immunol Rev* 2004;199:251–263. [PubMed: 15233739]
4. Kavanagh DG, Kaufmann DE, Sunderji S, Frahm N, Le Gall S, et al. Expansion of HIV-specific CD4+ and CD8+ T cells by dendritic cells transfected with mRNA encoding cytoplasm- or lysosome-targeted Nef. *Blood* 2006;107:1963–1969. [PubMed: 16249391]
5. Routy JP, Boulassel MR, Yassine-Diab B, Nicolette C, Healey D, et al. Immunologic activity and safety of autologous HIV RNA-electroporated dendritic cells in HIV-1 infected patients receiving antiretroviral therapy. *Clin Immunol* 134:140–147. [PubMed: 19889582]
6. Van Gulck ER, Vanham G, Heyndrickx L, Coppens S, Vereecken K, et al. Efficient in vitro expansion of human immunodeficiency virus (HIV)-specific T-cell responses by gag mRNA-electroporated dendritic cells from treated and untreated HIV type 1-infected individuals. *J Virol* 2008;82:3561–3573. [PubMed: 18234800]
7. Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. *Proc Natl Acad Sci U S A* 107:13824–13829. [PubMed: 20631300]
8. Rinaldo CR. Dendritic cell-based human immunodeficiency virus vaccine. *J Intern Med* 2009;265:138–158. [PubMed: 19093966]
9. Nickle DC, Rolland M, Jensen MA, Pond SL, Deng W, et al. Coping with viral diversity in HIV vaccine design. *PLoS Comput Biol* 2007;3:e75. [PubMed: 17465674]
10. Rolland M, Nickle DC, Mullins JJ. HIV-1 group M conserved elements vaccine. *PLoS Pathog* 2007;3:e157. [PubMed: 18052528]
11. Liu Y, McNevin J, Rolland M, Zhao H, Deng W, et al. Conserved HIV-1 epitopes continuously elicit subdominant cytotoxic T-lymphocyte responses. *J Infect Dis* 2009;200:1825–1833. [PubMed: 19909083]

12. Rousseau CM, Lockhart DW, Listgarten J, Maley SN, Kadie C, et al. Rare HLA drive additional HIV evolution compared to more frequent alleles. *AIDS Res Hum Retroviruses* 2009;25:297–303. [PubMed: 19327049]
13. Rolland M, Carlson JM, Manocheewa S, Swain JV, Lanxon-Cookson E, et al. Amino-Acid Co-Variation in HIV-1 Gag Subtype C: HLA-Mediated Selection Pressure and Compensatory Dynamics. *PLoS One* 5.
14. Leslie AJ, Pfafferoth KJ, Chetty P, Draenert R, Addo MM, et al. HIV evolution: CTL escape mutation and reversion after transmission. *Nat Med* 2004;10:282–289. [PubMed: 14770175]
15. Altfeld M, Addo MM, Rosenberg ES, Hecht FM, Lee PK, et al. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *Aids* 2003;17:2581–2591. [PubMed: 14685052]
16. Hendel H, Caillat-Zucman S, Lebuane H, Carrington M, O'Brien S, et al. New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. *J Immunol* 1999;162:6942–6946. [PubMed: 10352317]
17. Sabado RL, O'Brien M, Subedi A, Qin L, Hu N, et al. Evidence of dysregulation of dendritic cells in primary HIV infection. *Blood*.
18. Chougnet C. Role of CD40 ligand dysregulation in HIV-associated dysfunction of antigen-presenting cells. *J Leukoc Biol* 2003;74:702–709. [PubMed: 12960257]
19. Boasso A, Shearer GM. Chronic innate immune activation as a cause of HIV-1 immunopathogenesis. *Clin Immunol* 2008;126:235–242. [PubMed: 17916442]
20. Kaufmann DE, Kavanagh DG, Pereyra F, Zaunders JJ, Mackey EW, et al. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol* 2007;8:1246–1254. [PubMed: 17906628]
21. Paiardini M, Frank I, Pandrea I, Apetrei C, Silvestri G. Mucosal immune dysfunction in AIDS pathogenesis. *AIDS Rev* 2008;10:36–46. [PubMed: 18385779]
22. Calarota SA, Folli A, Maserati R, Baldanti F, Paolucci S, et al. HIV-1-specific T cell precursors with high proliferative capacity correlate with low viremia and high CD4 counts in untreated individuals. *J Immunol* 2008;180:5907–5915. [PubMed: 18424710]
23. Rehr M, Cahenzli J, Haas A, Price DA, Gostick E, et al. Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy. *J Virol* 2008;82:3391–3404. [PubMed: 18199637]
24. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, et al. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 2006;12:1198–1202. [PubMed: 16917489]
25. Zhang D, Shankar P, Xu Z, Harnisch B, Chen G, et al. Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. *Blood* 2003;101:226–235. [PubMed: 12393740]
26. Buisson S, Benlahrech A, Gazzard B, Gotch F, Kelleher P, et al. Monocyte-derived dendritic cells from HIV type 1-infected individuals show reduced ability to stimulate T cells and have altered production of interleukin (IL)-12 and IL-10. *J Infect Dis* 2009;199:1862–1871. [PubMed: 19419334]
27. Erikstrup C, Kronborg G, Lohse N, Rye Ostrowski S, Gerstoft J, et al. T-Cell Dysfunction in HIV-1-Infected Patients With Impaired Recovery of CD4 Cells Despite Suppression of Viral Replication. *J Acquir Immune Defic Syndr*. 2009
28. Fuse S, Tsai CY, Molloy MJ, Allie SR, Zhang W, et al. Recall responses by helpless memory CD8+ T cells are restricted by the up-regulation of PD-1. *J Immunol* 2009;182:4244–4254. [PubMed: 19299723]
29. Letvin NL, Mascola JR, Sun Y, Gorgone DA, Buzby AP, et al. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* 2006;312:1530–1533. [PubMed: 16763152]
30. Mattapallil JJ, Douek DC, Buckler-White A, Montefiori D, Letvin NL, et al. Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge. *J Exp Med* 2006;203:1533–1541. [PubMed: 16735692]

31. Precopio ML, Betts MR, Parrino J, Price DA, Gostick E, et al. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 2007;204:1405–1416. [PubMed: 17535971]
32. Mehal WZ, Crispe IN. TCR ligation on CD8+ T cells creates double-negative cells in vivo. *J Immunol* 1998;161:1686–1693. [PubMed: 9712032]
33. Subramanya S, Armant M, Salkowitz JR, Nyakeriga AM, Haridas V, et al. Enhanced Induction of HIV-specific Cytotoxic T Lymphocytes by Dendritic Cell-targeted Delivery of SOCS-1 siRNA. *Mol Ther*.

FIGURE 1. RNA-loaded cytokine-matured DC induce de novo antigen-specific immune responses

(A) DC from an HIV uninfected donor were cultured in the presence of a mixture of IL-6, IL-1 β , TNF α , and PGE $_2$ (Mimic). After 24 hours, cells were stained for markers of maturation and activation. Grey filled – isotype control, thin line – immature DC, thick line – Mimic matured DC. (B) Transfection of DC from an HIV uninfected donor with RNA encoding GFP. Immature DC were electroporated in the presence of 10 μ g/ml in vitro transcribed GFP RNA. GFP expression was compared between mock electroporated DC and GFP RNA electroporated DC. (C) De Novo generation of Gag-specific immune responses. DC from an HIV negative donor were electroporated with RNA encoding Gag or GFP and matured with Mimic cytokines. DC were then cultured for 12 days with autologous PBL. After restimulation with a pool of 15-mer Gag peptides, T cell responses were determined as the number of ELISPOTS after 12-day coculture with DC electroporated with Gag RNA compared to irrelevant RNA (GFP) or irrelevant peptide (Ova). (D) Restimulation enhances de novo antigen responses. DC from a HIV negative donor were electroporated with Gag RNA and cocultured for 9 days with autologous PBL. The 9-day stimulated PBL were then restimulated for an additional 9 days with a second addition of autologous DC electroporated with Gag RNA, followed by ELISPOT in the presence of Gag 15-mer peptide pool or Ova control peptide. ELISPOT responses were compared to responses of PBL prior to coculture.

Figure 2. Ex vivo DC from HIV infected patients can enhance Gag-specific and Nef-specific immune responses

(A) Ex vivo derived DC from an HIV infected individual were matured in the presence of mimic. After 24 hours cells were stained for markers of activation and maturation. (B) HIV patient T cells before and after 12-day coculture with DC loaded with Gag RNA were compared by IFN- γ (left) or IL-2 (right) ELISPOT. A total of 12 patient samples were evaluated. T cell spots per million cells on Day 0 (prior to coculture) were compared to responses on Day 12 of DC coculture by Wilcoxon matched pair test. (C) Sample ELISPOT response. For all patient samples, controls were tested in addition to responses after coculture with matured, Gag loaded DC and stimulation with Gag 15-mer peptide pool. Controls included culture of T cells with Ova peptide, or coculture of HIV patient PBL with DC transfected with GFP RNA, followed by stimulation with Gag peptide pool. A positive control stimulated with PMA/ionomycin was also assayed for each patient (D) Comparison of sample and control ELISPOT responses. Mean responses for all patients tested under both sample and control conditions are shown for both IFN- γ (left) and IL-2 (right) ELISPOT. (E) Nef-specific responses induced by Nef loaded DC. HIV patient derived DC were loaded with Nef RNA, matured, and cocultured with autologous PBL. A total of 4 patients were evaluated. Cocultured PBL were stimulated with either Nef 15-mer peptide pool or Ova

control peptide. Control DC were electroporated with GFP RNA, cocultured with HIV patient PBL, and stimulated with Nef peptide pool. PBL were also assayed on Day 0 for initial Nef-specific immune responses. ELISPOT responses to IFN- γ (left) and IL-2 (right) are shown. E: Nef response

Figure 3. Functional and phenotypic responses of PBL to Gag RNA-loaded ex vivo DC
(A) Gating strategy for functional analysis of PBL cocultured for 12 days with Gag RNA-loaded DC. After selection of CD3+CD4+ and CD3+CD8+ T cell populations, cells were analyzed for IL-2, TNFα, IFN-γ, and CD107A expression. PBL stimulated with PMA/

ionomycin is shown. **(B, C)** Functional responses of CD4⁺ T cells **(B)** or CD8⁺ T cells **(C)** before and after 12-day coculture with Gag RNA-loaded cytokine-matured DC. The percentage of cells positive for each marker (alone or in combination) are shown. **(D)** Granzyme B ELISPOT responses from HIV infected patient sample. Number of PBL secreting Granzyme B are shown either before or after 12 day coculture with Gag loaded DC (or GFP loaded as a control) followed by Gag 15-mer peptide pool stimulation. Control cultures received Ova peptide for restimulation. **(E)** Memory phenotype responses after 12 day coculture. Cells were cocultured as above, followed by phenotyping by staining with CD3, CD4, CD8, CD27, CCR7, and CD45RO. “Other” includes naïve T cells and undefined cells. Tcm: CD45RO+CD27+CCR7+, Tem: CD45RO+CD27-CCR7-, Ttm: CD45RO+CD27+CCR7-, Tinter: CD45RO-CD27+CCR7-, Teff: CD45RO-CD27-CCR7-, Tnaive/Other: CD45RO-CCR7+. B: CD4⁺ T cell Responses to coculture with Gag transfected DC. C: CD8⁺ T cell Responses to coculture with Gag transfected DC. D: Granzyme B. E: Memory responses

Figure 4. Ex vivo DC loaded with a Conserved Element Gag p24 RNA enhance T cell responses in HIV infected and uninfected individuals

(A) HIV+ patient T cell responses before and after coculture with p24 CE RNA loaded DC. CE RNA induced a significant increase in IFN- γ T cell responses after 12-day coculture. No response to a CE 10-mer peptide pool was observed prior to coculture (Day 0), suggesting that no preexisting responses to CE conserved epitopes were present prior to DC therapy. Similar results were observed for 3 other HIV+ patient samples, suggesting this observation is reproducible across different HLA restrictions. (B) HIV negative donor immune responses to DC loaded with p24 CE RNA. p24 CE RNA induced a significant increase in ELISPOT IFN- γ responses after restimulation with either a CE 10-mer peptide pool or full-length Gag 15-mer peptide pool. Cells were cultured for a total of 18 days, with RNA-loaded DC mixed with autologous lymphocytes on Day 0 and restimulation with DC on Day 9. DC loaded with irrelevant GFP RNA did not induce above-background responses to these peptides.