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siRNA knockdown of PD-L1 and PD-L2 in monocyte-derived dendritic cells only modestly improves proliferative responses to Gag by CD8⁺ T cells from HIV-1 infected individuals

Gaëlle Breton¹, Bader Yassine-Diab^{2,3,4,5}, Lillian Cohn¹, Mohamed-Rachid Boulassel⁶, Jean-Pierre Routy^{5,6}, Rafick-Pierre Sékaly^{2,3,4,5}, and Ralph M. Steinman¹

¹ Laboratory of Cellular Physiology and Immunology, Chris Browne Center for Immunology and Immune Diseases, The Rockefeller University, New York, NY 10065, USA

² Laboratoire d'Immunologie, Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CR-CHUM) Saint-Luc, Montréal, QC H2X 1P1, Canada

³ Laboratoire d'Immunologie, Département de Microbiologie et d'Immunologie, Université de Montréal, QC H3T 1J4, Canada

⁴ INSERM U743, CR-CHUM, Université de Montréal, Montréal, QC H2X 1P1, Canada

⁵ Department of Microbiology and Immunology, McGill University, Montréal, QC H3A 2B4, Canada

⁶ Immunodeficiency Service and Division of Hematology, Royal Victoria Hospital, McGill University Health Centre, McGill University, Montréal, QC H3A 1A1, Canada

Abstract

Due to their capacity to elicit and regulate immunity, dendritic cells (DCs) are important targets to improve vaccination. Knowing that PD-1 high virus-specific T cells become functionally exhausted during chronic exposure to HIV-1, the development of a therapeutic DC-based HIV-1 vaccine might include strategies that down regulate PD-L1 and PD-L2 counter-receptors. After showing that monocyte-derived DCs rapidly up regulated PD-L1 and PD-L2 expression upon maturation with a variety of stimuli e.g. TLR ligands and cytokines, we determined that PD-L1 and PD-L2 expression could be knocked down by electroporation of a single siRNA sequence twice at the monocyte and immature stages of DC development. This knockdown approached completion, and was specific and lasting for several days. We then added the PD-L1 and PD-L2 silenced monocyte-derived DCs to PBMCs from HIV-1 infected individuals along with pools of 15-mer HIV-1 Gag p24 peptides. However, in cultures from 6 patients, there was only a modest enhancing effect of PD-L1 and PD-L2 silencing on CD8⁺ T cell proliferative responses to the DCs. These findings suggest that in monocyte-derived DCs, additional strategies than PD-L1 or PD-L2 blockade will be needed to improve the function of PD-1 high T cells.

Keywords

Dendritic Cells; PD-L1; PD-L2; siRNA; HIV-1 Gag; CD8⁺ T cell

Introduction

Development of a therapeutic vaccine for HIV-1 infection is challenging because the immune system of infected individuals is subverted due to viral persistence. During chronic exposure to the virus, HIV-1 specific CD8⁺ T cells become functionally impaired progressively, losing the capacity to proliferate and produce multiple cytokines, and facilitating viral dissemination and persistence (1-5). Some of the underlying mechanisms for this dysfunction have been elucidated and reveal an important role for the inhibitory receptor programmed death-1 (PD-1) in T cell exhaustion during chronic HIV-1 infection. Based on early findings in chronically LCMV infected mice (6), it has been shown that PD-1 is up regulated on HIV-1 specific CD8⁺ T cells in chronically infected individuals, leading to functional exhaustion of these cells. PD-1 over expression on HIV-1 specific CD8⁺ T cells also correlates with increased viral load. Interestingly blocking PD-1 signaling using antibodies restores HIV-1 specific CD8⁺ T-cell effector functions *in vitro* (7-9). During chronic SIV infection, *in vivo* PD-1 blockade also restores SIV specific CD8⁺ T cell function, reduces viral load and enhances survival of SIV infected macaques (10).

PD-1 and its ligands play a significant role in immune regulation (for review, see 11-13). PD-1, whose expression is up regulated on activated T cells, has been shown to deliver an inhibitory signal when triggered by its counter-receptors and to block TCR-induced T cell proliferation and cytokine production (14-17). PD-1 has two known ligands, PD-L1 and PD-L2. PD-L2 is induced on macrophages and dendritic cells (DCs), whereas PD-L1 is constitutively expressed on monocytes, macrophages and some DCs, and is further up regulated upon activation with e.g. TLR ligands and cytokines. PD-L1 is also expressed on a wide range of nonhematopoietic cells, allowing PD-L1 to potentially negatively regulate PD-1 in peripheral tissues.

An association between PD-L1 expression and HIV-1 disease progression was first reported by Trabattoni *et al.* who showed that PD-L1 expression levels are augmented in mainly CD19⁺ and CD14⁺ cells of HIV-1 infected individuals and that a direct correlation is observed between PD-L1 expression and HIV-1 plasma viremia (18). PD-L1 is also significantly up regulated on peripheral DCs in HIV-1 infected typical progressors and AIDS patients, but is maintained at relatively low levels in LTNPs (19). To date, no data involving PD-L2 in HIV-1 infection have been reported.

Due to their capacity to elicit and regulate immune responses, DCs are being studied as adjuvants for vaccination (20,21). A potential benefit for therapeutic vaccination with DCs in HIV-1 infection is suggested by a number of studies (22-25). The most extensive study, by Lu *et al.*, showed activation of HIV-1 specific immunity by *ex-vivo* cytokine matured monocyte-derived DCs, which correlated with a prolonged reduction in viral load in 8 out of 18 of the treated subjects (22). However, the boosting of antiviral T cell responses after vaccination did not occur in some individuals and failed to completely eradicate the virus. The potential of therapeutic HIV-1 vaccines using DCs might be enhanced by strategies aimed at down regulating negative pathways on DCs, e.g. the PD-1/PD-1 ligands pathway. In chronically LCMV infected mice in which virus-specific CD8⁺ T cells up regulate PD-1 and become functionally exhausted, the combination of PD-1 blocking and therapeutic vaccination boosted CD8⁺ T cell immunity and enabled viral control (26).

Therefore, we proposed to silence PD-L1 and PD-L2 expression in cytokine matured monocyte-derived DCs using siRNA. Silencing statistically improves HIV-1 Gag specific CD8⁺ T cell stimulation by DCs, but there was only a modest enhancing effect.

Methods

Human Subjects

The Royal Victoria Hospital and the CR-CHUM hospital review boards approved the study and six HIV-1 infected individuals were recruited and signed informed consent. Buffy coats obtained from New York Blood Center were used as a source of mononuclear cells from healthy seronegative donors.

Preparation of PBMCs

PBMCs were isolated from heparinized blood by density gradient centrifugation using Ficoll-Hypaque. A fraction of those PBMCs was freshly used to prepare DCs whereas the rest of it was cryopreserved in liquid nitrogen until coculture assay time.

Generation of DCs

CD14⁺ cells were freshly separated from PBMCs using CD14 microbeads and LS columns (Milenyi) following the manufacturer's protocol. iDCs were generated by culturing CD14⁺ cells in RPMI 2% human serum (GemCell). GM-CSF (20 ng/ml; Berlex) and IL-4 (20 ng/ml; R&D Systems) were added to the culture on days 0, 2 and 4. On day 5, iDCs were matured for 24, 48, 72 or 96 hours by adding to the culture either inflammatory cytokines - IL-1 β (10 ng/ml; R&D Systems), IL-6 (1000 U/ml; R&D Systems), TNF- α (10 ng/ml; R&D Systems) and Prostaglandin E₂ (1 μ g/ml; Sigma) - LPS (100 ng/ml; Sigma), Poly I:C (25 μ g/ml; InvivoGen), ssRNA40 (7.5 μ g/ml; InvivoGen), Imiquimod (5 μ g/ml; InvivoGen), Zymosan (5 μ g/ml; InvivoGen), Flagellin (5 μ g/ml; InvivoGen), IFN- α 2b (750 U/ml, Schering-Plough) or IFN- γ (100 ng/ml; R&D Systems). DCs maturation phenotype was monitored by flow cytometry using specific antibodies as described in the Antibodies and Flow Cytometry section.

StealthTM siRNA duplexes

All StealthTM siRNA duplexes were obtained from Invitrogen and dissolved to a final concentration of 100 μ M according to manufacturer's instructions. RNAi target sequence for PD-L1 (GenBank Accession No. NM_014143) is 5'-GATATTTGCTGTCTTTATA-3' and for PD-L2 (GenBank Accession No. NM_025239) is 5'-AGCAGAGGTGTGTGGAAT-3'. Scrambled siRNA are ordered for each target sequence. Sequences of the two synthesized oligonucleotides for PD-L1 are 5'-GAUGAGGAUAAUUGCUGUCUUUAUA-3' (sense) and 5'-UAUAAAGACAGCAAAUAUCCUCAUC-3' (antisense). Sequences of the two synthesized oligonucleotides for PD-L2 are 5'-AAAAGCAGAGGUGUGGAAAUAUC-3' (sense) and 5'-GAAAUUCCACACACCUCUGCUUUU-3' (antisense). These target sequences were submitted to a BLAST search to ensure that only the PD-L1 or PD-L2 genes were targeted. Duplexes were stocked in aliquots at -80°C.

Electroporation with siRNA

Cells were treated with targeting or scrambled siRNA either once (at day 5) or twice (at day 0 and day 5). For electroporation, cells were resuspended in Opti-MEM without phenol red (Invitrogen) at a concentration of 4×10^7 cells/ml. 4×10^6 cells were electroporated with 1 nmol of siRNA duplexes in a 4-mm cuvette and in a total volume of 200 μ l of Opti-MEM. Cells were pulsed using the ECM830 Electro Square PoratorTM (BTX Harvard Apparatus). The pulse conditions were a unique square wave pulse of 500 V and 0.5 milliseconds. Immediately after electroporation, cells are transferred in complete medium [RPMI 2% human serum (GemCell)] supplemented with GM-CSF (20 ng/ml; Berlex) and IL-4 (20 ng/ml; R&D Systems). For the electroporation at day 5, cells were matured for 24, 48, 72 or 96 hours using inflammatory cytokines: IL-1 β (10 ng/ml; R&D Systems), IL-6 (1000 U/ml; R&D Systems), TNF- α (10 ng/ml; R&D Systems) and Prostaglandin E₂ (1 μ g/ml; Sigma). Knockdown efficacy was

monitored by flow cytometry using specific antibodies as described in the Antibodies and Flow Cytometry section.

Peptide libraries

The Gag library [HIV-1 Consensus B Gag (15-mer) peptides – complete set (123 peptides)] was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The control Ova library (15-mer peptides – 107 peptides) was designed and synthesized by the Proteomics Resource Center, The Rockefeller University. Each individual vial of peptide was reconstituted in DMSO to a final concentration of 20 mg/ml. We next prepared a mix of all Gag or Ova peptides, which contains each peptide at a 50 µg/ml concentration in sterile water. These stock mixes were aliquoted and stored at –80°C for long-term storage. In some conditions, the Gag library was split into two pools containing each peptide at a 50 µg/ml concentration.

Coculture Assay

Frozen PBMCs were thawed and stained with CFSE as described in the CFSE Labeling section. Pro-inflammatory cytokines matured DCs, treated or untreated with siRNA, were collected 24 hours after adding the maturation stimuli and resuspended in RPMI 10% human serum. CFSE-labeled PBMCs, plated at a final concentration of 2×10^6 PBMCs/ml in 5 ml Polypropylene Round-Bottom tubes, were cocultured with DCs at a 1:100 ratio. Gag or Ova peptide mixes were added to the culture to a final concentration of 250 ng/ml for each peptide. Proliferation of the CFSE-labeled cells was assessed by flow cytometry after 6 or 7 days of culture. In some conditions, we added blocking antibody specific to PD-L1 and PD-L2 (eBiosciences) to cell cultures at a final concentration of 10 µg/ml.

CFSE Labeling

5-(and-6)-CarboxyFluorescein diacetate Succinimidyl Ester (CFSE; Molecular Probes) is resuspended in DMSO at a concentration of 5 mM and stored at –80°C. Each batch of CFSE was titrated beforehand on PBMCs to determine optimal concentration for labeling (0.5 µM in our study). PBMCs were washed twice in PBS and resuspended in PBS at a 2×10^7 cells/ml concentration. A 1 µM working solution of CFSE was prepared from the stock by dilution in PBS. For labeling, one volume of working solution of CFSE was added to one volume of PBMCs. The cells were incubated in the dark with gentle mixing at room temperature for 8 minutes. The reaction was quenched by addition of an equal volume of human serum for 2 minutes. Cells were washed twice with PBS and resuspended in RPMI 10% human serum.

Antibodies and Flow Cytometry

Flow cytometry was used to monitor maturation of DCs as assessed by the up regulation of CD83 (clone HB15e), CD80 (clone 2D10), CD86 (clone IT2.2) and HLA-DR (clone L243) expression. The cell surface expression of PD-L1 and PD-L2 in siRNA treated and untreated DCs are assessed using PD-L1 (clone MIH1) and PD-L2 (clone MIH18) antibodies. Cells were also labeled with appropriate isotype control antibodies in each experiment. CD8⁺ T cell proliferation was assessed by CFSE dilution gating on CD3⁺ (clone SK7) CD8⁺ (clone SK1) double positive cells. Titrated surface antibodies were added to either 10^5 DCs or 2×10^6 PBMCs in 50 µl of PBS for 20 minutes at 4°C. Washed cells were fixed in 2% formaldehyde and stored at 4°C until flow cytometry analysis. Flow cytometry was performed using a LSR II flow cytometer (BD Biosciences). Data were analyzed using Diva 4.1 software (BD Biosciences) or FlowJo (Tree Star Inc).

Statistical analysis

Correlations were performed by Wilcoxon match pairs test using Prism 3.0 software (GraphPad Software).

Results

PD-L1 and PD-L2 expression is highly up regulated in monocyte-derived DCs upon maturation

When immature DCs (iDCs) are stimulated to mature, there is not only up regulation of co stimulatory molecules like CD80 and CD86, but also other co inhibitory B7 family members with the potential to negatively regulate the T cell response e.g., PD-L1 and PD-L2 that ligate PD-1 on T cells (11-13). iDCs can receive maturation signals through pro-inflammatory cytokines such as IL-1 β , IL-6, type-I interferon that are secreted during the innate response; also DCs directly sense microbial components via TLRs and other pattern recognition receptors (27). After this recognition, the DCs differentiate extensively, a process called maturation.

As we proposed to knockdown PD-L1 and PD-L2 expression in monocyte-derived DCs, we first determined to what extent PD-L1 and PD-L2 were up regulated on these cells after maturation with a variety of different stimuli, e.g. pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α and PGE₂), TLR ligands (LPS, Poly I:C, ssRNA, Imiquimod, Zymosan or Flagellin), IFN- α 2b or IFN- γ . Therefore, iDCs from healthy donors were incubated for 24, 48, 72 or 96 hour with each of these different stimuli and then analyzed by FACS for PD-L1 and PD-L2 expression (Figure 1). Monocytes and iDCs expressed little PD-L2 whereas they did express some PD-L1. PD-L1 and PD-L2 expression were both up regulated upon maturation and stayed high over a 96 hour period. The expression of PD-L1 and PD-L2 varied with the stimulus encountered by the DCs. Pro-inflammatory cytokines, LPS (TLR-4 agonist), Poly I:C (TLR-3 agonist), Zymosan (TLR-2 agonist) and Flagellin (TLR-5 agonist) induced the highest expression of both PD-L1 and PD-L2 molecules. In contrast, ssRNA (TLR-7 agonist), Imiquimod (TLR-7/8 agonist) and IFN- α 2b did not induce an up regulation of PD-L1 and PD-L2 expression on monocyte-derived DCs as these cells expressed levels similar to those observed in iDCs. Finally, IFN- γ increased both PD-L1 and PD-L2 expression, but this up regulation was transient. In the functional studies that follow, we used monocyte-derived DCs matured in the presence of pro-inflammatory cytokines, which expressed high levels of PD-L1 and PD-L2 (arrows, Figure 1).

Identification of PD-L1 and PD-L2 siRNA sequences that show efficient, specific and long lasting knockdown of targeted proteins

We first tested different siRNA sequences (3 for PD-L2 and 4 for PD-L1) for each of the targeted protein (data not shown). Initially, iDCs were electroporated with 10 nmol of siRNA and maturation was induced immediately after electroporation with IL-1 β , IL-6, TNF- α and PGE₂. We selected one siRNA duplex that allowed complete knockdown of PD-L2 at 48 hour (Figure 2A). For PD-L1, we were not able to obtain a complete knockdown of the protein at 48 hour using a single siRNA sequence (Figure 2A) or a pool of 4 siRNA sequences (data not shown). Of note, the efficiency of siRNA mediated gene knockdown was highly dependent on the expression level/turnover of the targeted protein and PD-L1 was highly expressed on DCs activated by pro-inflammatory cytokines. Therefore, we studied a second protocol to silence PD-L1. Cells were electroporated twice: once at day 0 (at the monocyte stage) and a second time at day 5 (at the iDCs stage). Electroporating DCs twice with siRNA significantly improved the efficiency of PD-L1 knockdown (Figure 2B).

To make sure that our identified sequences did not have any off-target effects, we looked at other surface markers expressed by mature DCs such as CD83, CD80, CD86 and HLA-DR.

The expression of these markers at 48 hour was not influenced by the electroporation of PD-L1 siRNA (Figure 2C) and PD-L2 siRNA (data not shown). Finally, we assessed PD-L1 expression in silenced monocyte-derived DCs over time to determine if this knockdown was long-lasting (Figure 2C). PD-L1 knockdown was observed at least 96 hour after electroporation with specific siRNA. In conclusion, PD-L1 and PD-L2 expression could be knocked down by electroporation of a single siRNA sequence twice at the monocyte and immature DC stages of development. This knockdown approached completion, and was specific and long lasting.

Modest enhancing effect of PD-L1 and PD-L2 silencing on Gag specific CD8⁺ T cell proliferative responses to monocyte-derived DCs in HIV-1 infected individuals

PD-L1 or PD-L2 silenced, cytokine matured, monocyte-derived DCs were tested for their ability to expand Gag specific CD8⁺ T cell memory responses *in vitro* in comparison with scrambled siRNA treated monocyte-derived DCs. We cultured DCs with PBMCs from the same HIV-1 infected donor at a DC:PBMC ratio of 1:100 for 6-7 days. Using flow cytometry, we evaluated by CFSE dilution the percentage of CD8⁺ T cells that were able to proliferate after stimulation with Gag or Ova peptide libraries. In addition, PD-L1 or PD-L2 blocking antibodies were added to scrambled siRNA treated DCs at day 0 of coculture as control for blocking the PD-1 pathway. Each condition was done in triplicate.

In preliminary experiments, we compared single knockdowns of PD-L1 and PD-L2 to double knockdowns, but the combination knockdown did not significantly improve the single knockdown. Because of the demands of working with blood from HIV-1 infected individuals, i.e., to have enough cells to set up triplicate cultures of each variable and to compare knockdowns of PD-L1 and PD-L2 with control scrambled siRNA, we decided to focus on single knockdowns. In addition, blockade of single molecules especially PD-L1 is being moved by others into the clinic to overcome the “exhaustion” of PD-1 high expressing lymphocytes.

In Figure 3A, we show a representative example of the proliferative responses from one HIV-1 infected subject. Both PD-L1 and PD-L2 silenced DCs were able to modestly expand the percentages of Gag specific CD8⁺ T cells relative to scrambled siRNA treated DCs. We observed a 15.8% and 14.1% augmentation for PD-L1 and PD-L2 silenced DCs respectively. PD-L1 silenced DCs induced a lower response than DCs blocked with PD-L1 blocking antibodies (48.1% for siRNA and 57.5% for blocking antibody). In contrast, PD-L2 blocking antibody did not seem to have an effect on the Gag specific CD8⁺ T cell proliferative responses to monocyte-derived DCs (34.3% of CD8⁺ CFSE^{low} cells with PD-L2 blocking antibody versus 32.3% with no blocking antibody). Of note, the PD-L1 blocking antibody has been extensively used in studies and showed efficacy in blocking the PD-1 pathway (7-9). However, no study has described blocking with the PD-L2 antibody we tested and it did not improve the CD8⁺ T cell response. We used, as a non-relevant peptide, an Ova library. No enhancement of non-specific CD8⁺ T cell proliferative responses was measured in any of the PD-L1 and PD-L2 silencing conditions with Ova peptides, which support the fact that our knockdown specifically enhanced Gag responses.

In Figure 3B, we show a summary of the proliferative responses to Gag and Ova obtained in 6 different HIV-1 infected subjects with each condition in triplicate. PD-L1 silenced DCs induced a statistically significant increase in the percentage of CD8⁺ CFSE^{low} T cells in comparison to scrambled siRNA treated DCs ($p=0.004$). PD-L2 silenced DCs also induced a statistically significant greater expansion of CD8⁺ CFSE^{low} T cells ($p=0.008$). However, while statistically significant effects were observed, the magnitude of the changes was modest. Finally, PD-L1 blocking antibodies allowed a statistically significant expansion of Gag specific CD8⁺ T cells ($p=0.02$) whereas PD-L2 blocking antibody had no effect ($p=0.50$). In conclusion, our data indicate a modest enhancing effect of PD-L1 and PD-L2 silencing in monocyte-derived DCs on Gag specific CD8⁺ T cell proliferative responses.

Discussion

Many studies have reported that HIV-1 specific CD8⁺ T cells up regulate the inhibitory receptor PD-1 during chronic HIV-1 infection, which contributes to their dysfunction (7-9). *In vitro* blockade of the PD-1 pathway restores the function of these exhausted CD8⁺ T cells, improving their capacity to proliferate, to produce cytokines, and to survive. These data suggest that the engagement of PD-1 by its counter-receptor is an important inhibitory pathway during chronic HIV-1 infection and that strategies aiming at blocking it *in vivo* may be crucial for the development of effective therapeutic treatment.

Up to now, studies on blocking the PD-1 pathway in chronic HIV-1 infection have used blocking antibodies and bulk PBMCs. We have developed a method using siRNA that specifically knocks down PD-L1 and PD-L2 expression in antigen presenting cells, in this case monocyte-derived DCs. During the DC maturation process, PD-L1 and PD-L2 molecules, together with surface molecules such as CD83, HLA-DR, CD80 and CD86, were strongly up regulated on these DCs. Of note, the expression levels of these markers varied with the maturation stimuli. In our study, we chose to work with pro-inflammatory cytokines matured monocyte-derived DCs, which have shown potential to be used in DC-based HIV-1 immunotherapy (22-25) and therefore may be a relevant model to study the consequences of blocking PD-L1 and PD-L2 *in vivo*. Since iDCs expressed little PD-L2, one electroporation with a single siRNA duplex was sufficient to achieve a complete knockdown of PD-L2 in cytokine matured monocyte-derived DCs. In contrast, as iDCs expressed very high levels of PD-L1, we found that a two-step method was needed to approach a complete PD-L1 knockdown in monocyte-derived DCs. This protocol consisted of electroporating cells at the monocyte stage as well as the iDC stage. The knockdown obtained for both PD-L1 and PD-L2 was specific to the targeted protein and lasted at least 96 hour. We next assessed whether those PD-L1 and PD-L2 silenced monocyte-derived DCs had a superior potential to expand Gag specific CD8⁺ T cells in HIV-1 infected individuals *in vitro*. PD-L1 and PD-L2 deficient DCs did statistically improve CD8⁺ T cell proliferative responses to Gag, but the magnitude of the enhancement was modest.

Some limitations of our *in vitro* coculture system may partly explain the relative modest improvement in CD8⁺ T cell responses following siRNA knockdown or blocking antibodies. For technical reasons, we added the siRNA-treated DCs to PBMCs rather than pure T cells, so the remaining APCs in the PBMCs might have contributed to APC function in the cultures. Also many other co inhibitory receptors are involved in T cell responses and have been shown to be up regulated in chronic infections, i.e. CTLA-4, LAG-3, TIM-3 (28-30). These pathways might also need to be knocked down to further improve T cell responses.

One way to determine the biological significance of the *in vitro* enhancement would be to evaluate the *in vivo* impact of PD-L1 and PD-L2 knockdown in DCs. Various laboratories including ours have utilized different strategies to exploit DCs in immunotherapy. The first method is based on the *ex-vivo* preparation of DCs presenting HIV-1 antigens, which are then injected to the patient (22-25). We could consider manipulating those DCs *ex-vivo* to silenced PD-L1 and PD-L2 expression prior to injection into the HIV-1 infected donor. The second approach is based on the *in vivo* targeting of HIV-1 antigens to maturing DCs via an antibody that bind receptors such as the DEC-205 endocytosis receptor (31). Combining the *in vivo* targeting of the antigen with the *in vivo* delivery of siRNA as described by Lieberman's group might also provide a strategy to treat HIV-1 infection (32).

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Abbreviations

APCs	Antigen Presenting Cells
CFSE	5-(and-6)-CarboxyFluorescein diacetate Succinimidyl Ester
DCs	Dendritic Cells
mDCs	mature Dendritic Cells
iDCs	immature Dendritic Cells
PBMCs	Peripheral Blood Mononuclear Cells
siRNA	small interfering RNA
ssRNA	single strand RNA
TLRs	Toll-Like Receptors
PD-1	Programmed Death-1
LTNPs	Long-Term Non-Progressors

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Figure 1.

Expression of PD-L1 (upper panel) and PD-L2 (lower panel) on monocyte-derived DCs is up regulated upon maturation with cytokines or agonists for TLR. iDCs were generated from healthy donors by culturing CD14⁺ cells in RPMI 2% human serum in which GM-CSF and IL-4 were added on day 0, 2 and 4. On day 5, iDCs were matured for 24, 48, 72 and 96 hour with either pro-inflammatory cytokines (arrows), TLR ligands (LPS, Poly I:C, ssRNA, Imiquimod, Zymosan, Flagellin), IFN- α 2b or IFN- γ . The cell surface expression of PD-L1 and PD-L2 was assessed by FACS (gating on living cells). PD-L1 (clone MIH1) and PD-L2 (clone MIH18) antibodies were used for staining. Isotype control was done with iDCs at 72 hour. The data are representative of three independent experiments on three different donors.

Figure 2.

PD-L1 and PD-L2 siRNA knockdown in monocyte-derived DCs is efficient, specific and long lasting. **A.** Identification of PD-L1 and PD-L2 siRNA duplexes. 4 million iDCs from a healthy donor were electroporated with 1 nmol of PD-L1 or PD-L2 siRNA and maturation was induced right after electroporation with IL-1 β , IL-6, TNF- α and PGE₂. FACS analysis of the surface expression of the protein was performed at 48 hour. **B.** Electroporation of cells twice improved significantly PD-L1 knockdown. Cells of a healthy donor were electroporated either at 5 days of monocyte culture in GM-CSF and IL-4 (1X), or twice in monocyte at day 0 and immature DC at day 5 (2X) with 1 nmol of PD-L1 siRNA or scrambled siRNA. FACS analysis of the surface expression of the protein was performed at 48 hour. **C.** PD-L1 knockdown is specific. Surface expression of CD83, CD80, CD86 and HLA-DR on PD-L1 silenced DCs were analyzed by flow cytometry 48 hour after electroporation. **D.** PD-L1 knockdown is long lasting. As in C, but PD-L1 surface expression was assessed 24, 48, 72 and 96 hour after electroporation

and induction of maturation. The data in A, B, C and D are representative of three different donors.

Figure 3.

PD-L1 and PD-L2 siRNA silenced DCs show a modest increase in their capacity to expand Gag specific CD8⁺ T cells *in vitro*. **A.** Example of CD8⁺ T cell proliferative responses after Gag and Ova (control) stimulation of PBMCs with either scrambled siRNA treated DCs (–), PD-L1 silenced DCs, scrambled siRNA treated DCs in presence of PD-L1 blocking antibody, PD-L2 silenced DCs and scrambled siRNA treated DCs in presence of PD-L2 blocking antibody (DC:PBMC ratio is 1:100). CFSE dilution of peptide specific T cells was evaluated after 6-7 days of stimulation using flow cytometry. One of 6 patients is shown. **B.** Summary data of CD8⁺ T cell proliferative responses to Gag and Ova in six different patients. Each condition is performed in triplicate. Statistical comparisons were made using Wilcoxon match pairs test.