

p38 Mitogen-Activated Protein Kinase/Signal Transducer and Activator of Transcription-3 Pathway Signaling Regulates Expression of Inhibitory Molecules in T Cells Activated by HIV-1-Exposed Dendritic Cells

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Human immunodeficiency virus type 1 (HIV-1) infection enhances the expression of inhibitory molecules on T cells, leading to T-cell impairment. The signaling pathways underlying the regulation of inhibitory molecules and subsequent onset of T-cell impairment remain elusive. We showed that both autologous and allogeneic T cells exposed to HIV-pulsed dendritic cells (DCs) upregulated cytotoxic T-lymphocyte antigen (CTLA-4), tumor-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), lymphocyte-activation gene-3 (LAG3), T-cell immunoglobulin mucin-3 (TIM-3), CD160 and certain suppression-associated transcription factors, such as B-lymphocyte induced maturation protein-1 (*BLIMP-1*), deltex homolog 1 protein (*DTX1*) and forkhead box P3 (*FOXP3*), leading to T-cell suppression. This induction was regulated by p38 mitogen-activated protein kinase/signal transducer and activator of transcription-3 (P38MAPK/STAT3) pathways, because their blockade significantly abrogated expression of all the inhibitory molecules studied and a subsequent recovery in T-cell proliferation. Neither interleukin-6 (IL-6) nor IL-10 nor growth factors known to activate STAT3 signaling events were responsible for STAT3 activation. Involvement of the P38MAPK/STAT3 pathways was evident because these proteins had a higher level of phosphorylation in the HIV-1-primed cells. Furthermore, blockade of viral CD4 binding and fusion significantly reduced the negative effects DCs imposed on primed T cells. In conclusion, HIV-1 interaction with DCs modulated their functionality, causing them to trigger the activation of the P38MAPK/STAT3 pathway in T cells, which was responsible for the upregulation of inhibitory molecules.

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INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) has adopted myriad ways to evade host immune responses and hijacks the immune system to establish persistent infection. The virus utilizes numerous receptors to interact with immune cells such as T cells, macrophages

and dendritic cells (DC), an approach that facilitates viral survival within the host. HIV-1 use DCs at the sites of initial infection as a Trojan horse for transport to regional lymph nodes, where HIV-1 is transmitted to T cells (1).

Stimulation via positive costimulatory molecules, for example CD28, at the im-

munological synapse leads to efficient T-cell receptor (TCR) engagement that augments initial activation and provides additional signals for cell division (2). These events favor T-cell survival and induction of effector functions, such as cytokine secretion and cytotoxicity, whereas negative costimulatory signals, for example, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1: CD279), inhibit TCR-mediated responses, impair T-cell division and functional maturation, and induce T-cell tolerance (2,3). An array of these molecules and factors, for example, CTLA-4 (4), PD-1 (3), lymphocyte activation gene-3 (LAG3) (5), T-cell immunoglobulin mucin-3 (TIM-3) (6), CD160, tumor necrosis factor (TNF)-related

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apoptosis-inducing ligand (TRAIL) (7) and fork-head box P3 transcription factor (FOXP3) (8,9), are overexpressed on T cells in HIV-1-infected individuals and in HIV-1 *in vitro* assays and are thought to aid HIV-1 immune evasion. Exposure of immature DCs to HIV-1 gp120 induced increased production of interleukin-10 (IL-10), impaired responsiveness to maturation stimuli and impaired ability to stimulate T-cell growth, which was due to the interaction of gp120 mannoses with DC-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) and subsequent signaling (10). Increased expression of B-lymphocyte-induced maturation protein-1 (BLIMP-1), FOXP3 and deltex homolog 1 protein (DTX1) has been linked to immune impairment in certain chronic diseases (11–13). BLIMP-1 expression has been associated with T-cell exhaustion in chronic viral infections (13,14). Furthermore, nuclear factor of activated T cells (NFAT) has been shown to regulate expressions of CTLA-4 (15), PD-1 (16) and FOXP3 (17). Recent studies showed an increase in FOXP3 and regulatory T-cell (Treg) levels in HIV-1-infected individuals (12,18,19).

HIV-1, via a series of unidentified mechanisms, induces high levels of PD-1, CTLA-4, TRAIL, TIM-3, CD160 and LAG3, both *in vitro* and *in vivo* (4–6,14). T-cell impairment in HIV-1-infected individuals has been largely attributed to increased PD-1 and CTLA-4 levels (3–5) and further shown to be contact dependent because blockade of these molecules significantly restored T-cell activity (3–5). Inhibitory molecules regulate cell functions through diverse mechanisms, for example, engagement of CTLA-4 or PD-1 to their respective ligands, CD28- or PD-L1/2-induced signaling cascades, leading to impaired TCR-mediated IL-2 production and T-cell proliferation (20,21). Recent HIV-1 studies suggest a role of TIM-3 in mediating T-cell impairment acting via ligation with galactin-9 and/or phosphatidylserine ligands, triggering T-cell dysfunction and eventual cell death (6). Likewise, LAG3 binds with a

higher affinity to major histocompatibility complex class II (MHC II) molecules relative to CD4 and facilitates immunosuppression, especially when expressed by Tregs (22). We recently showed that HIV-1-pulsed DCs upregulated the expression of a broad array of inhibitory receptors in primed T cells (14).

Increased understanding of the diverse molecules involved in regulating the immune system has been achieved in the last few years, whereas the underlying mechanisms and signaling pathways regulating their expression remain ambiguous and are of paramount importance to investigate. We showed increased activation (phosphorylation) of signal transducer and activator of transcription-3 (STAT3) in T cells activated by HIV-1-exposed DCs. STAT3 was shown to be involved in the increased expression of PD-1, CTLA-4, TRAIL, LAG3, TIM-3 and CD160, because their inhibition significantly abolished the expression of inhibitory molecules and restored T-cell proliferation. The transcriptional repressors *BLIMP-1*, *DTX1* and *FOXP3* were also regulated by STAT3. Of note, viral access to the DC cytosol seemed to be involved in the induction of expression of inhibitory molecules on activated T cells and subsequent T-cell impairment. We did not find increased levels of IL-10, IL-6, transforming growth factor- β (TGF- β), epidermal growth factor (EGF), granulocyte colony-stimulating factor (G-CSF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), the known activators of STAT3 signaling (23–27), but rather increased activation of P38 mitogen-activated protein kinase (P38MAPK) in T cells primed by HIV-1-exposed DCs. Moreover, inhibition of P38MAPK significantly abolished the expression of inhibitory molecules and restored T-cell proliferation. Therefore, HIV-1-exposed DCs trigger P38MAPK/STAT3 signaling in T cells, resulting in enhanced expression of inhibitory molecules and transcriptional repressors, which lead to T-cell impairment.

MATERIALS AND METHODS

Culture Medium, Cytokine and Reagents

RPMI1640 was supplemented with 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Fisher Scientific, Leicestershire, UK), 20 μ g/mL gentamicin (Fisher Scientific), 2 mmol/L L-glutamine (Sigma Aldrich, St. Louis, MO, USA) and 1% plasma (1% plasma culture medium) or 5% heat-inactivated pooled human serum (5% PHS medium) (Fisher Scientific). Recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) (Immunex, Seattle, WA, USA) (100 IU/mL) and recombinant human IL-4 (rhIL-4) (R&D Systems, Minneapolis, MN, USA) (300 U/mL) were used for the *in vitro* differentiation of monocyte-derived DCs (MDDCs). STAT3 inhibitors (Cat. No. 573095 Calbiochem[®]) (EMD4Biosciences, La Jolla, CA, USA, and WP1066-Sigma, Sigma-Aldrich) as well as STAT5 inhibitor (Cat No. 573108 Calbiochem) (EMD4Biosciences) were used to block the respective pathways. P38MAPK inhibitor SB220025 and NFAT inhibitor VIVIT (N7032) were purchased from Sigma-Aldrich. C34 peptide was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, and b12 monoclonal antibody (mAb) was a generous gift from D Burton, Scripps Research Institute, La Jolla, CA, USA. HIV-1_{Bal} gp120 protein was purchased from Immune Technology Corp., New York, NY, USA.

Propagation of Human DCs

Buffy coats from anonymous HIV-seronegative healthy donors were purchased from the Transfusion Medicine Center, Karolinska Institute, Huddinge Hospital, Stockholm, Sweden. Peripheral blood mononuclear cells (PBMCs) were separated by density-gradient centrifugation by use of Ficoll-Paque[™] (Amersham Pharmacia Biotech, Piscataway, NJ, USA). CD14⁺ progenitor cells were selected from PBMCs by adherence to tissue culture plates for 1 h at 37°C. Nonad-

herent cells were removed by washing the plates with RPMI1640. Adherent DC progenitors were cultured in 1% plasma, rhIL-4, and rhGM-CSF. The cytokines were replenished every second day and immature DCs were collected after 5–6 d of culture. DC maturation was induced by 24-h exposure to 30 ng/mL polyinosinic acid:polycytidylic acid (poly I:C) (Sigma Aldrich).

Propagation and Purification of HIV-1_{BaL}

HIV-1_{BaL}/SUPT1-CCR5 CL.30 (Lot #P4143) was produced with chronically infected cultures of the ACVP/BCP cell line (No. 204), originally derived by infecting SUPT1-CCR5 CL.30 cells (graciously provided by J Hoxie, University of Pennsylvania) with an infectious stock of HIV-1_{BaL} (NIH AIDS Research and Reference Reagent Program, Cat. No. 416, Lot No. 59155). Virions were purified by continuous flow centrifugation with a Beckman CF32Ti rotor at ~90,000g at a flow rate of 6 L/h followed by banding for 30 min after sample loading. Sucrose density-gradient fractions were collected, virus-containing fractions were pooled and diluted to <20% sucrose and virus was pelleted at ~100,000g for 1 h. The virus pellet was resuspended at a concentration of 1000 fold and concentrated relative to the cell culture filtrate, and aliquots were frozen in liquid N₂ vapor. HIV-1_{BaL} was used at a concentration of 175–750 ng p24 equivalent. Neutralization of viral CD4 binding was achieved by pretreating HIV-1 with 20 µg/mL HIV-1 gp120 neutralizing b12 mAb for ~60 min at 37°C before being added to the DCs. HIV-1 fusion was inhibited by adding 0.5 µg/mL (100 µmol) C34 during HIV pulsing of DCs.

Pulsing of Mature DCs with HIV-1

Mature DCs were exposed to HIV-1 (175–750 ng/mL) overnight and washed repeatedly with RPMI1640, and the viability of DCs was determined by annexin V staining analyzed by flow cytometry and 0.4% Trypan blue exclusion by microscopy.

Allogeneic DC-T-cell Proliferation

Allogeneic naïve (T_N) or memory (T_M) bulk T cells were negatively selected by depleting monocytes (CD14), B cells (CD19), natural killer cells (NK cells) (CD56), and T_M (CD45RO) or T_N cells (CD45RA) (Miltenyi Biotec, Auburn, CA, USA) from PBMCs by use of magnetic beads. Mature DCs, mock or HIV-1 pulsed, were cocultured with 10 µmol/L carboxyfluorescein succinimidyl ester (CFSE) (Fisher Scientific)-labeled T_N or T_M bulk T cells at a ratio of 1:10 (10⁴ DCs:10⁵ T cells) in flat-bottomed 96-well plates [BD Falcon, Franklin Lakes, NJ, USA] in 5% PHS. We also examined the effects of direct addition of HIV-1 to mock DC T cells; HIV-1 preexposed DC-T-cell cocultures or naïve T cells at the onset of coculture and incubated for 7 d. The cocultures were restimulated on d 7 with the corresponding groups of DCs. Inhibitors for P38MAPK (200 nmol/L), STAT3 (200 µmol/L), STAT5 (500 µmol/L) or NFAT-c (30 µg/mL) were added to DC-T-cell cocultures at the onset of cocultures. Anti-human IL-10 and IL-6 neutralizing or isotype control mAbs (R&D Systems, Minneapolis, MN, USA) were added to DC-T-cell cocultures on d 0 and 7. T-cell proliferation was assessed on d 8 by flow cytometry (FACSCalibur™, BD Immunocytometry Systems, San Jose, CA, USA) and/or 4 µCi of ³H-thymidine (Amersham Pharmacia Biotech). In addition, mRNA, and protein expression levels were measured with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and flow cytometry, respectively.

Autologous Staphylococcal Enterotoxin B DC-T-Cell Proliferation

Mock or HIV-1-exposed mature DCs were pulsed with 1 ng/mL staphylococcal enterotoxin B (SEB) (Sigma Aldrich) for 3 h and washed in RPMI1640. DCs were cocultured with bulk autologous T_N cells. Because of rapid activation, the cocultures were assessed for T-cell proliferation and gene and protein expressions on d 4 and/or d 5 as described for the allogeneic system.

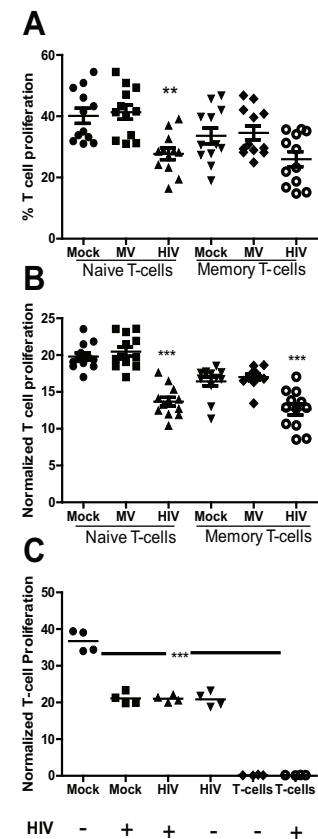


Figure 1. HIV-1 impairs the ability of DCs to activate both T_N- and T_M-cell responses. Mature DCs were pulsed with mock, microvesicles (MV), or HIV-1_{BaL} (HIV) for ~24 h, washed and cocultured with CFSE-labeled T_N or T_M cells. HIV-1 (375 ng p24 equivalent of HIV-1) was also added directly to mock DC-T cells, HIV-1 preexposed DC-T-cell cocultures or naïve T cells at the onset of culture. Cocultures were restimulated on d 7 with the same DC conditions as d 0. T-cell proliferation was measured on d 8 by CFSE dilution using flow cytometry and ³H-thymidine incorporation using a β counter. (A) Percentage T cell proliferation for T_N and T_M cocultured with DCs preexposed to HIV-1 (n = 12); (B) Normalized T-cell proliferation for T_N and T_M cocultured with DCs preexposed to HIV-1 (n = 12); and (C) T-cell proliferation in naïve T cells, mock DC-T-cell cocultures, and HIV-1 pulsed DC-T-cell cocultures with or without supplementary HIV-1 (n = 4). The experiments were normalized (n = 12) and statistical analysis was performed by one-way ANOVA (Tukey *post hoc* test). **P* < 0.05, ***P* < 0.005 and ****P* < 0.001 were considered statistically significant.

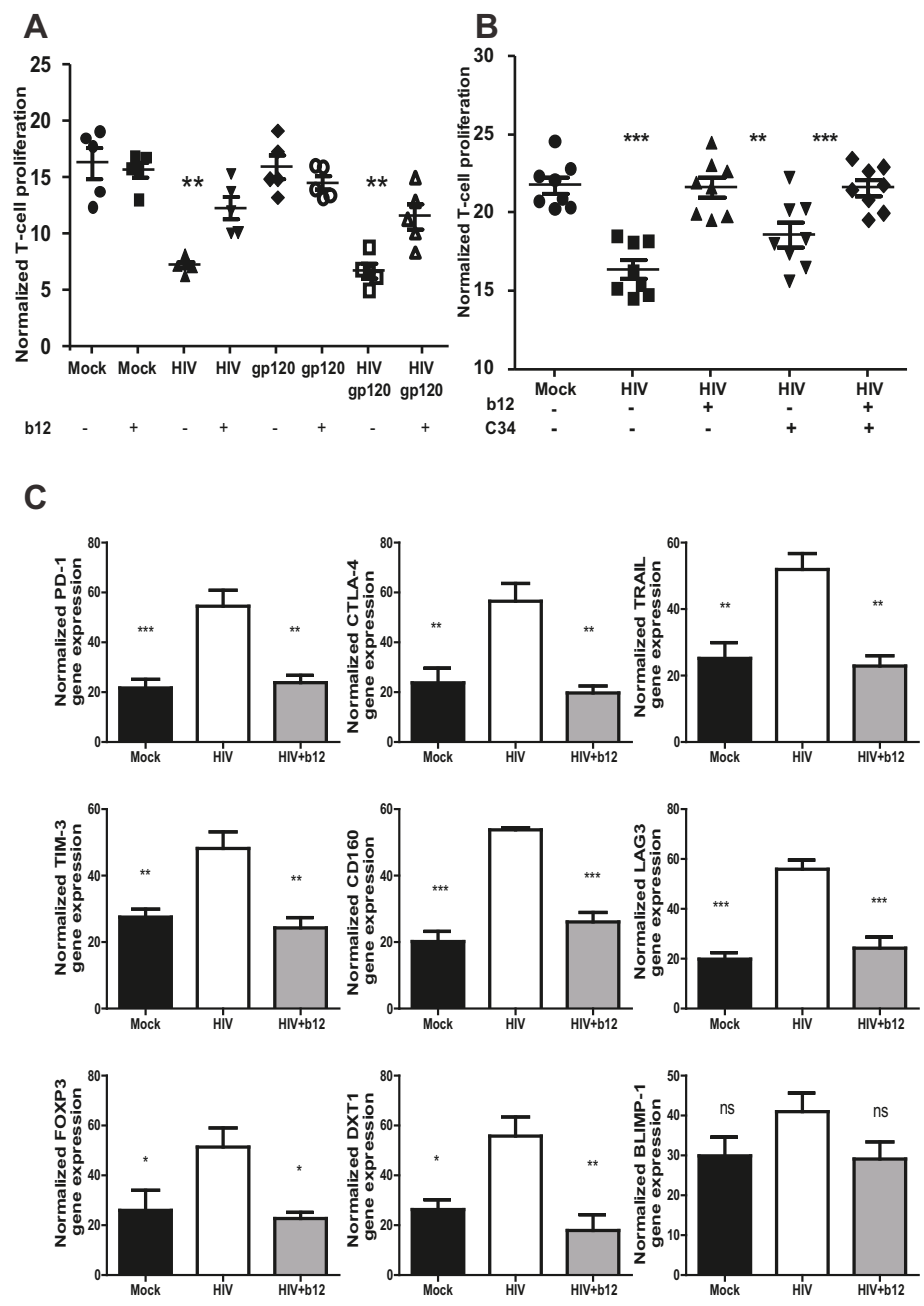


Figure 2. HIV-1 access to DC cytosol is a prerequisite for HIV-1 impairment of the ability of DCs to prime T_N cell responses. HIV-1_{Bal} or HIV-1gp120_{Bal} protein was pretreated with anti-gp120 mAb (b12) for ~60 min at 37°C. The matured DCs were treated with mock, mock-b12, HIV-1, HIV-1-b12, HIV-1gp120, HIV-1gp120-b12, HIV-1-HIV-1gp120, and HIV-1-b12-HIV-1gp120-b12. DCs were also pulsed with fusion inhibitor (C34), or in combination with b12 antibody. The DCs were pulsed for ~24 h, washed and cocultured with CFSE-labeled T_N cells. DC-T-cell cocultures were restimulated on d 7 and harvested on d 8. (A, B) T-cell proliferation was measured for CFSE dilution by flow cytometry and 3H -thymidine incorporation by β counter. (C) The gene profiles of *CTLA-4*, *PD-1*, *TRAIL*, *TIM-3*, *CD160*, *LAG3*, *BLIMP-1*, *FOXP3* and *DTX1* were analyzed by qRT-PCR (n = 5) in an assay with or without b12 mAb. The experiments (A, n = 5, and B, C, n = 8) were normalized and statistical analysis was performed by one-way ANOVA (Tukey *post hoc* test). ***P* < 0.005, ****P* < 0.001.

Immunostaining

Direct conjugated mAbs against CD3-fluorescein isothiocyanate (FITC), CD8⁺ phycoerythrin (PE), CD8⁺ allophycocyanin (APC), CD4 PE, CD4 APC, PD-1-FITC, TRAIL-PE, CTLA-4-PE, CD45RO-PECY5, CD45RA-PECY5, FOXP3-FITC TIM-3-APC, CD160-APC, LAG3-PE, B- and T-lymphocyte attenuator (BTLA)-PE, anti-STAT3-Alexa Fluor 488 and anti-P38 MAPK-PE (BD Pharmingen) were used for T-cell phenotyping before or after the DC-T-cell coculture. Cell acquisition was done on a FACSCalibur and analyzed with FlowJo software (TreeStar Inc., Ashland, OR, USA).

STAT3 and P38MAPK Phospho Flow Cytometry Assays

Allogeneic DC-T-cell coculture assays were prepared as mentioned above. After restimulation on d 7, cocultures were harvested on d 8 for intracellular staining for phosphorylated STAT3 and P38MAPK. Briefly, the mock or HIV-1 DC-mediated expanded T cells were stained for surface expression of CD3 and inhibitory molecules (PD-1, TRAIL, CTLA-4, LAG3, TIM-3 and CD160). Cells were later fixed in 4% paraformaldehyde (PFA) for 10 min, permeabilized in 0.2% saponin, and incubated with anti-STAT3 and anti-P38MAPK for 30 min. Samples were acquired on a FACSCalibur and data analyzed with FlowJo software.

Cytokine Assays

The levels of cytokines and growth factors present in the supernatants of DC-T-cell assays were measured on d 8 using a Bio-Plex™ Cytokine Luminex assay (Bio-Rad, Hercules, CA, USA). Mean fluorescent intensity (MFI) levels for IL-6, IL-10, EGF, G-CSF, PDGF and VEGF were compared between mock and HIV-1-primed DC-T-cell cocultures.

Quantitative Real-Time PCR

The expression profiles for genes of interest were investigated by qRT-PCR (SYBR® Green: Applied Biosystems, Stockholm, Sweden) as described previously (17). β -Actin served as a house-

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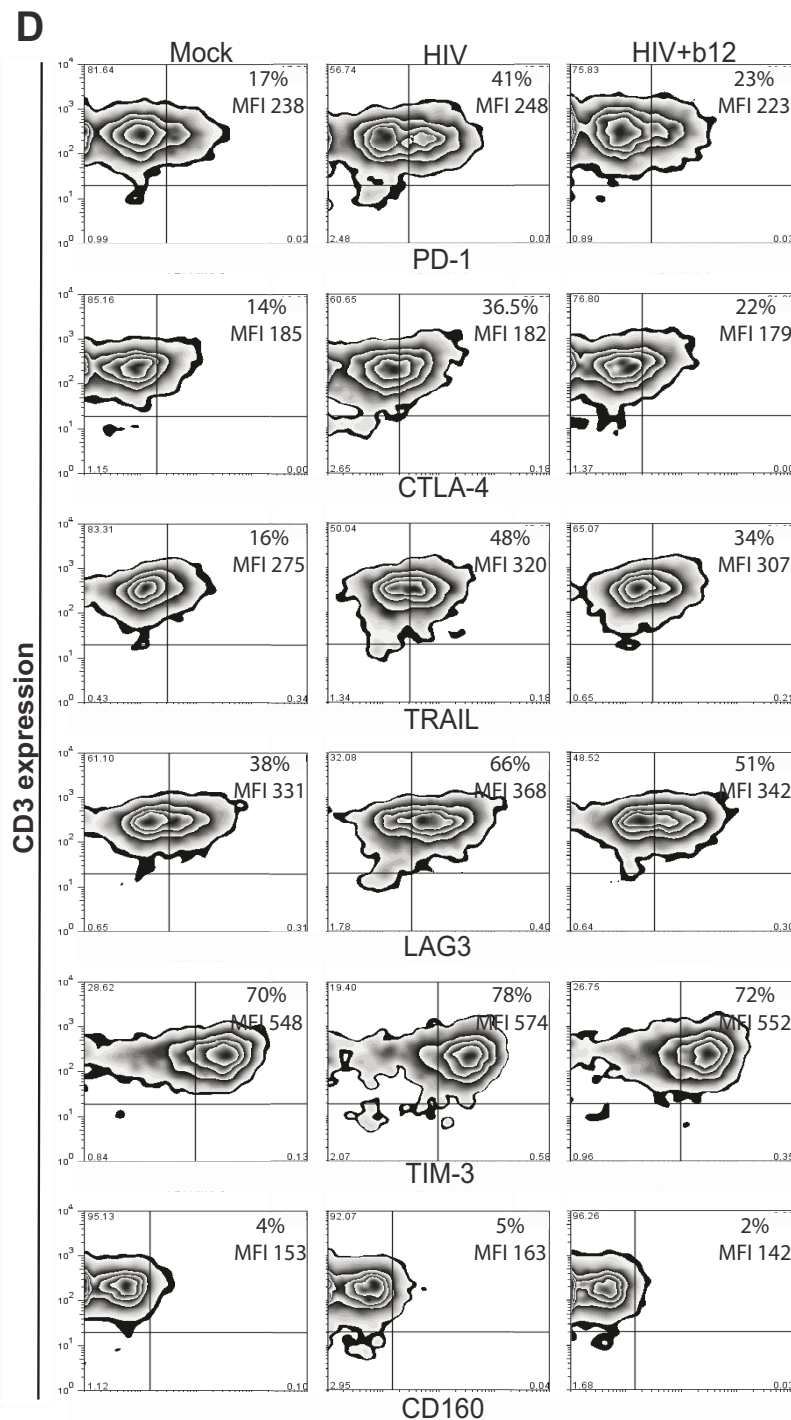


Figure 2. Continued. (D) Protein expression profiles of CTLA-4, PD-1, TRAIL, TIM-3, CD160 and LAG3 were measured by flow cytometry and shown by one representative of 4 experiments ($n = 4$).

keeping gene to define the relative gene expression levels by use of the $2^{-\Delta\Delta Ct}$ formula, where Ct refers to the threshold value.

Statistical Analyses

Because of variability between different donors used in the experiments, that is, levels of basic T-cell proliferation and

expression of inhibitory/suppressor molecules, the data were transformed/normalized. The value from one group was divided by the sum of all the values from all groups in the data set. This process was performed individually for each experiment. Statistical analysis of the normalized values was performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA (Tukey *post hoc* test) and unpaired *t* tests were used for revealing statistical significance, whereby *P* values < 0.05 were considered significant and indicated by asterisks ($*P < 0.05$, $**P < 0.005$, $***P < 0.001$).

RESULTS

Activation by HIV-1 Exposed DCs Impairs both T_N - and T_M -Cell Responses

T_M cells are known to be more sensitive to antigenic stimuli than T_N cells and to mount a more rapid and broader pathogen-specific response following a recurrent activation by DCs. Our aim was to compare the responses mounted between T_N and T_M cells upon activation by HIV-1-exposed DCs. We recently showed that HIV-1 affected the ability of DCs to activate T_N cells (5), and hence we sought to establish if HIV-1 also affected the ability of DCs to activate T_M cells. In brief, T_N and T_M cells from the same donor were negatively selected and cocultured with mock or HIV-1-exposed DCs at a ratio of 10:1. The level of T-cell proliferation was measured by CFSE dilution using flow cytometry and/or 3H -thymidine incorporation. We showed that HIV-1 impaired the ability of DCs to activate both T_N and T_M cells by $\sim 40\%$ ($P < 0.001$) (Figure 1A, B). We did not observe enhanced T-cell death (annexin V staining) in HIV-1 assays compared with the mocks (data not shown [5,14]). Of note, the proliferation was higher for T_N cells than T_M cells. We also observed increased expression of inhibitory molecules on HIV-1-activated T_M cells (data not shown) similar to T_N cells (5,14). We went further to elucidate if DCs needed

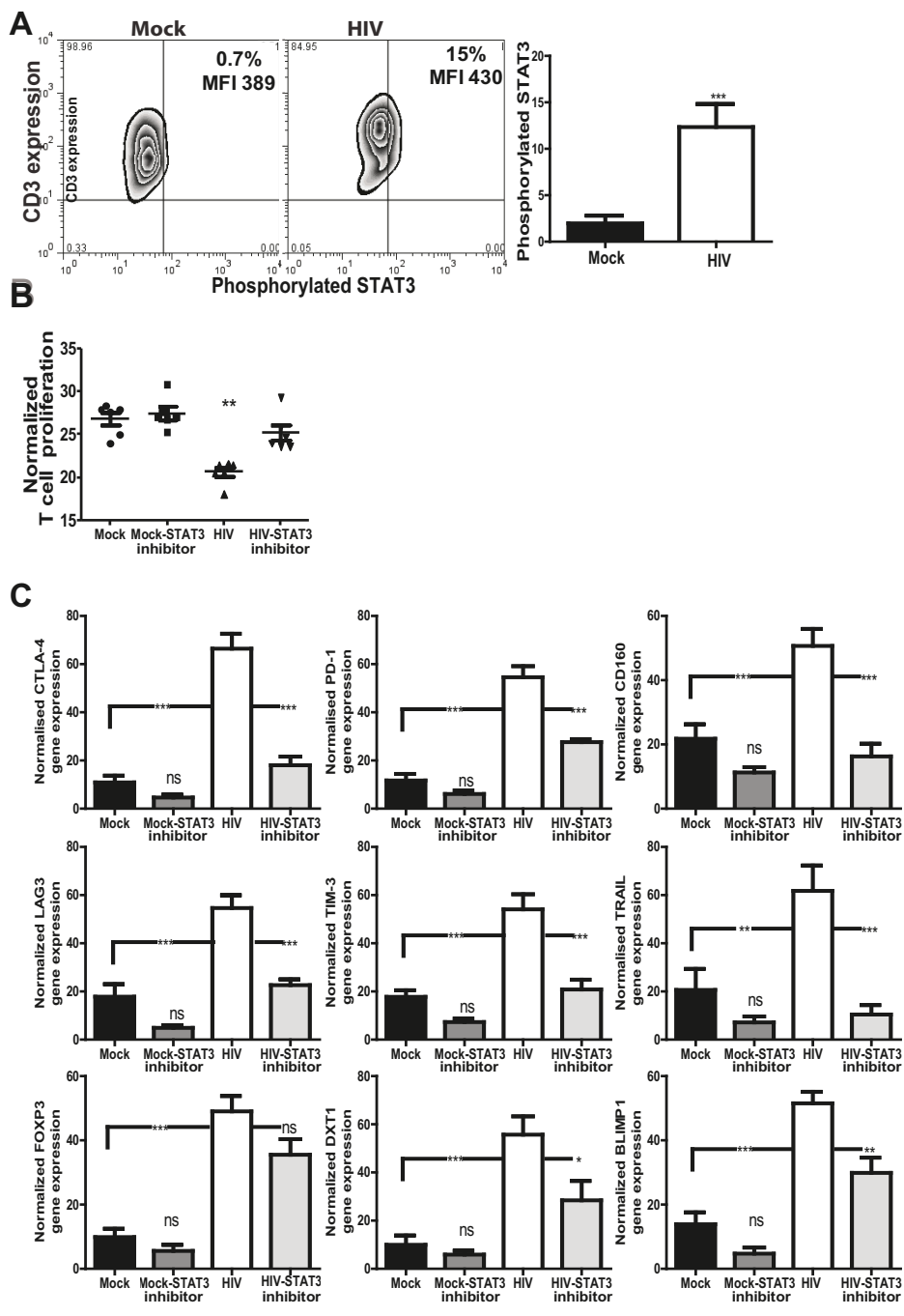


Figure 3. HIV-1 induces STAT3 activation and impairs T-cell functionality. Matured DCs were pulsed with mock or HIV-1_{BaL} (HIV), cultured for ~24 h, washed and cocultured with T_N cells for 8 d. (A) Intracellular staining for phosphorylated STAT3 was performed after 8 d to compare between HIV-1 primed and mock DC primed T cells. MERK-573095 (200 μmol/L) or Sigma WP1066 (2 μmol/L) STAT3 inhibitors were added to the wells at the onset of cocultures. (B) T-cell proliferation was measured by CFSE dilution by flow cytometry and ³H-thymidine incorporation (n = 6). (C) The expression levels of *CTLA-4*, *PD-1*, *TRAIL*, *TIM-3*, *CD160*, *LAG3*, *FOXP3*, *BLIMP-1* and *DTX1* genes were assessed by qRT-PCR (n = 6). The experiments in (A–C) were normalized and statistical analysis was performed by one-way ANOVA (Tukey *post hoc* test). **P* < 0.05, ***P* < 0.005, ****P* < 0.001.

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to be preexposed to HIV-1 before the coculture to induce T-cell impairment or if the presence of HIV-1 during the DC–T-cell coculture was sufficient. Presence of HIV-1 in the DC–T-cell coculture was a prerequisite for the impairment of T-cell proliferation (Figure 1C) or expression of inhibitory molecules (data not shown). Of note, direct exposure of HIV-1 to naïve T cells neither affected T-cell proliferation (Figure 1C) nor induced expression of inhibitory molecules (data not shown).

Neutralization of HIV-1 Reversed the Impaired T-Cell Proliferation and Elevated Expression of Inhibitory Molecules

HIV-1 induces T-cell exhaustion and/or expansion of inhibitory T cells, a mechanism the virus evokes to evade exuberant host immune responses (3–5,20). These cells are characterized by increased surface expression of negative costimulatory molecules resulting in contact-dependent inhibitory T cells (5,14). We investigated whether HIV-1 binding to CD4 or viral fusion was required to impair the ability of DCs to trigger T_N-cell responses. To address this question, we incubated HIV-1 and/or HIV-1_{BaL} gp120 protein with b12, a neutralizing anti-gp120 mAb, which prevents binding to the CD4 receptor (28). We also incubated HIV-1 with C34, an anti-gp41-specific C-peptide fusion inhibitor, or a combination of b12 and C34. C34 reportedly blocks both HIV-1 infection of Langerhans cells (LCs) and LC-mediated viral transfer to T cells (29). We found that T_N-cell proliferation was restored when b12 (*P* < 0.001) was used to neutralize HIV-1 (Figure 2A,B). The binding and uptake of gp120 by DCs had no impairing effects on the ability of DCs to activate T-cell proliferation, and there was no effect when gp120 was neutralized with b12 mAb (Figure 2A). The combination of b12 and C34 showed a similar rescue (*P* < 0.001) as b12, whereas C34 alone was less efficient (*P* < 0.005) (Figure 2B). In addition, the gene expression of inhibitory molecules *PD-1*,

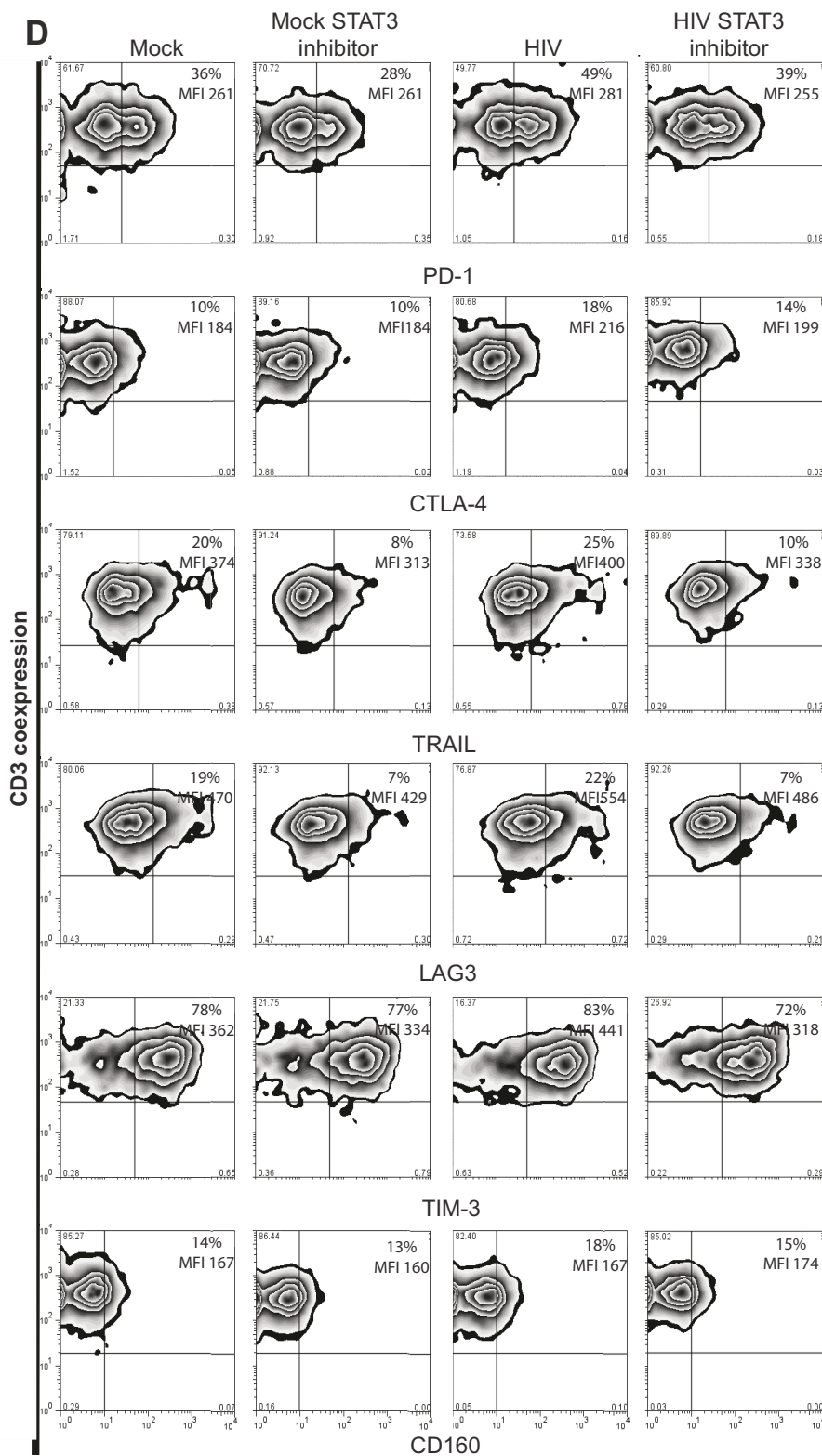


Figure 3. Continued. (D) Protein profiles of CTLA-4, PD-1, TRAIL, TIM-3, CD160 and LAG3 were measured by flow cytometry ($n = 3$, one representative experiment shown).

CTLA-4, TRAIL, TIM-3, CD160, LAG3 and the transcriptional repressors *BLIMP-1*, *DTX1* and *FOXP3* was assessed by qRT-PCR (Figure 2C), and protein levels by flow cytometry (Figure 2D). When viral binding to CD4 and fusion was blocked by b12, the array of inhibitory molecules and transcriptional repressors triggered in the presence of HIV-1 normalized both at the gene and protein levels (Figure 2C,D). It is noteworthy that the mRNA levels do not always reflect protein levels, which could potentially be the explanation for the CD160 and TIM-3 protein profiles. These findings indicated that viral fusion but not initial binding to CD4 on DCs was involved in the impairment of T cells.

Signaling through STAT3 is Critical for HIV-1 Induction of T Cells

The STAT3 signaling pathway regulates multiple cellular events activated by cytokines such as IL-6 (30), IL-10 (31) and growth factors like EGF (32), G-CSF (33), PDGF (32) and VEGF (34). STAT3 is the convergence point for commonly activated oncogenic pathways and plays a critical role in carcinogenesis, downregulation of T_H1 responses, promotion of expression of immunosuppressive genes and generation of Tregs, all favoring tumorigenesis (23,25,27,35). Similarly, during chronic HIV-1 infection, HIV-1 exploits the immune regulatory pathways to avoid a functional response (36,37). However, to the best of our knowledge the involvement of STAT3 in HIV-1-induced immune suppression/evasion has never been studied. Using FACS analysis, we detected increased STAT3 phosphorylation in T cells activated by HIV-1-exposed DCs (8–23%) ($P < 0.001$) compared with mock cultures (1–5%) (Figure 3A). Blockade of the STAT3 pathway with the specific inhibitor MERK-573095 significantly rescued T-cell proliferation ($P < 0.005$) (Figure 3B) and decreased the gene expression of *PD-1* ($P < 0.005$), *TRAIL*, *CTLA-4*, *TIM-3*, *LAG3*, *CD160* ($P < 0.001$), *BLIMP-1*, *FOXP3* ($P < 0.05$) and *DTX1* ($P < 0.005$) (Figure 3C). Moreover, surface expres-

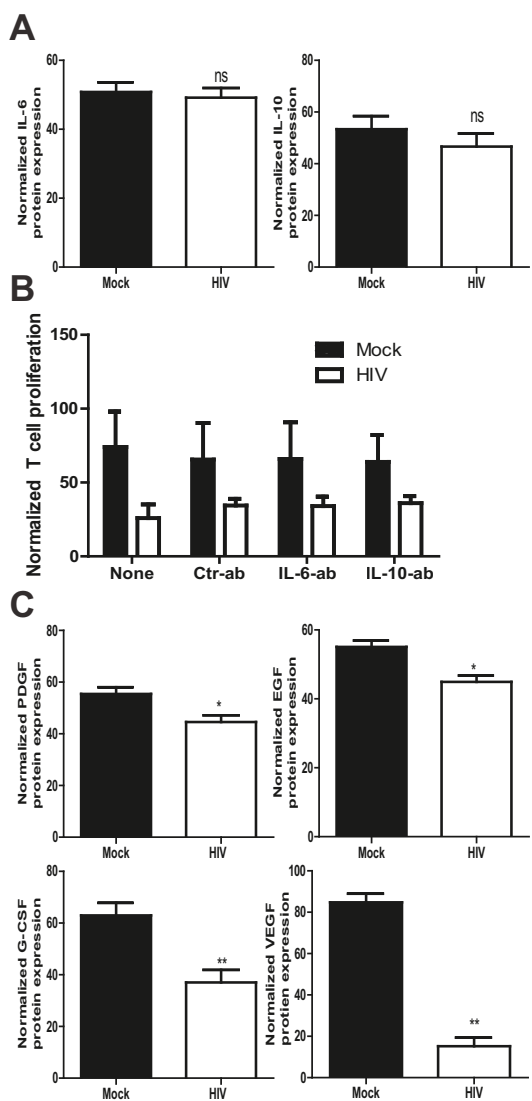


Figure 4. IL-6 or IL-10 neutralization did not reverse HIV-1-induced T-cell impairment. Mature DCs were pulsed with mock or HIV-1_{BoL} cultured for ~24 h, washed and cocultured with T_N cells for 8 d and restimulated on d 7. Neutralizing anti-IL-6, anti-IL-10, or isotype control mAbs (10 μ g/mL) were added and replenished on d 4 and 7 following restimulation. Cocultures were harvested on d 8 to measure T-cell proliferation, gene expression and cytokine secretion. IL-6, IL-10, PDGF, G-CSF, EGF, and VEGF levels were measured in culture supernatants by a Bio-Plex Cytokine Luminex assay. (A) IL-6 and IL-10 cytokine levels in the coculture supernatants. (B) T-cell proliferation was measured for CFSE dilution by flow cytometry in IL-6 and IL-10 neutralized assays ($n = 6$). (C) PDGF, G-CSF, EGF and VEGF levels in the coculture supernatants. The experiments were normalized and statistical analysis was performed by one-way ANOVA (Tukey *post hoc* test). * $P < 0.05$, ** $P < 0.005$.

sion of the inhibitory molecules reflected their gene profiles (Figure 3D). We also found decreased (not significantly) expression of inhibitory molecules in the mock primed cells, which emphasizes the possibility that STAT3 could be a nat-

ural regulator of inhibitory molecules. To the best of our knowledge this is the first evidence that STAT3 tightly regulates the expression of inhibitory molecules on T cells in the presence of HIV-1 *in vitro* (Figure 3A–D). Of note, similar responses

were seen for both STAT3 inhibitors used in our experiments. Because STAT5 is intertwined with STAT3 functions, we also investigated the effect of STAT5 on T-cell inhibition. However, in contrast to STAT3 inhibition, blockade of STAT5 led to further impairment of T-cell activity (data not shown). This result was in line with previous findings showing that blockade of STAT5 signaling curtails IL-2, IL-15 and IL-7 signaling, a feedback loop identified to exert a negative effect on the T-cell proliferation (38).

Neutralization of IL-6 or IL-10 Did Not Restore HIV-1-Induced T-Cell Impairment

Because IL-6 and IL-10 are known activators of the STAT3 pathway (31,39), we investigated IL-6 and IL-10 protein levels in our cocultures and the effect neutralization of these cytokines had on the activation of STAT3. There were no significant differences in IL-6 and IL-10 levels between the mock and HIV-1-exposed DC–T-cell cocultures (Figure 4A). Neutralization of IL-6 and IL-10 had no effects on T-cell proliferation (Figure 4B) or mRNA levels of inhibitory molecules (data not shown). Subsequently, we examined the potential involvement of other STAT3-activating factors like EGF, PDGF, G-CSF and VEGF. The presence of HIV-1 in the DC–T-cell assays did not increase the expression of these factors (Figure 4C), which therefore exempted further investigation of these factors. These findings indicate the contribution of other STAT3 signaling activators, which may involve activity from Src or P38MAPK kinases (34,40).

P38MAPK Pathway Is Associated with Suppressor T-Cell Induction by HIV-1

P38MAPK acts in response to external stress stimuli, for example, cytokines, ultraviolet radiation, heat and osmotic shocks (41), and plays cardinal roles in cell differentiation and apoptosis (42). Elevated P38MAPK activation has also been reported in cancers and shown to suppress immune responses directed against tumors in a manner similar to

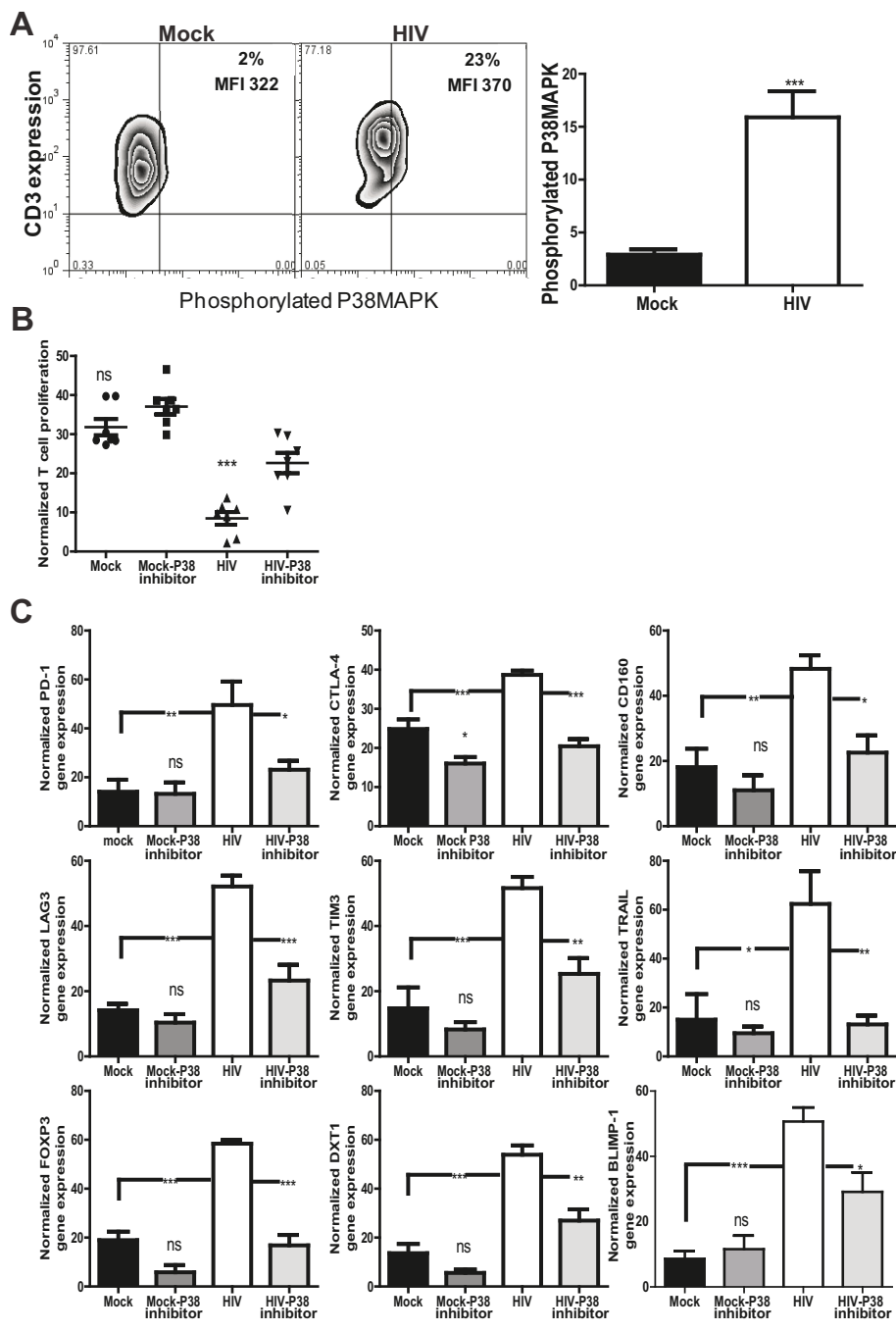


Figure 5. HIV-1 induces inhibitory molecules and T-cell impairment through P38MAPK pathway. Mature DCs were pulsed with mock or HIV-1_{Bol} cultured for ~24 h, washed and cocultured with T_N cells for 8 d. (A) Intracellular staining for phosphorylated P38MAPK was performed after 8 d of culture to compare between HIV pulsed and mock DC primed T cells. P38MAPK inhibitor (500 nmol/L) (Sigma-SB220025) was added to the wells at the onset of coculture. The cocultures were restimulated on d 7 and (B) T-cell proliferation was measured for CFSE dilution by flow cytometry (n = 6). (C) The gene levels of *CTLA-4*, *PD-1*, *TRAIL*, *TIM-3*, *CD160*, *LAG3*, *FOXP3*, *BLIMP-1* and *DTX1* were assessed by qRT-PCR (n = 4). The experiments (A–C) were normalized, and statistical analysis was performed by one-way ANOVA (Tukey *post hoc* test). **P* < 0.05, ***P* < 0.005, ****P* < 0.001.

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STAT3 (43). An increasing number of studies show that P38MAPK regulates divergent pathways and phosphorylates downstream targets inducing a broad spectrum of biological and cellular effects, including STAT3 activation (40,41,44). STAT3 could therefore be a potential target for P38MAPK (41) in our *in vitro* system. On the other hand, HIV-1 utilizes P38MAPK and ERK pathways for the propagation of new virions and the depletion of CD4⁺ T cells (45). In addition, the HIV-1 Nef protein has also been shown to induce the expression of PD-1 and FasL via the P38MAPK pathway in CD4⁺ T cells (46). We investigated the role of P38MAPK in our *in vitro* system and their potential involvement in STAT3 signaling. Interestingly, P38MAPK was phosphorylated to a higher extent in HIV-1 DC–T-cell cocultures (10–25%) (*P* < 0.001) compared with mock cultures (1–6%) (Figure 5A). Blockade of P38MAPK with inhibitor resulted in increased T-cell proliferation (*P* < 0.001) (Figure 5B), and decreased the gene (Figure 5C) and protein (Figure 5D) expressions of inhibitory molecules.

HIV-1 Impairs the Ability of DCs to Activate Autologous T-Cell Responses against Staphylococcus Enterotoxin B

We used staphylococcus enterotoxin B (SEB)-exposed DCs to induce autologous T-cell activation to confirm our findings from the allogeneic system. We performed the assay as described for the allogeneic system and established that the presence of HIV-1 impaired the ability of DCs to activate autologous T_N-cell responses (*P* < 0.001) against SEB (Figure 6A). Furthermore, T cells primed by HIV-1-exposed DCs had enhanced expression of *PD-1*, *CTLA-4*, and *TRAIL* (*P* < 0.005); *LAG3* and *TIM-3* (*P* < 0.001); *CD160* (not significant); *BLIMP-1* (*P* < 0.001); *FOXP3* (*P* < 0.005); and *DTX1* (*P* < 0.05) genes (Figure 6B). Surface protein expressions of PD-1, CTLA-4, TRAIL, LAG3, TIM-3 and CD160 also correlated with corresponding gene expressions (Figure 6C). As was shown in our previous study in allogeneic cocultures (14),

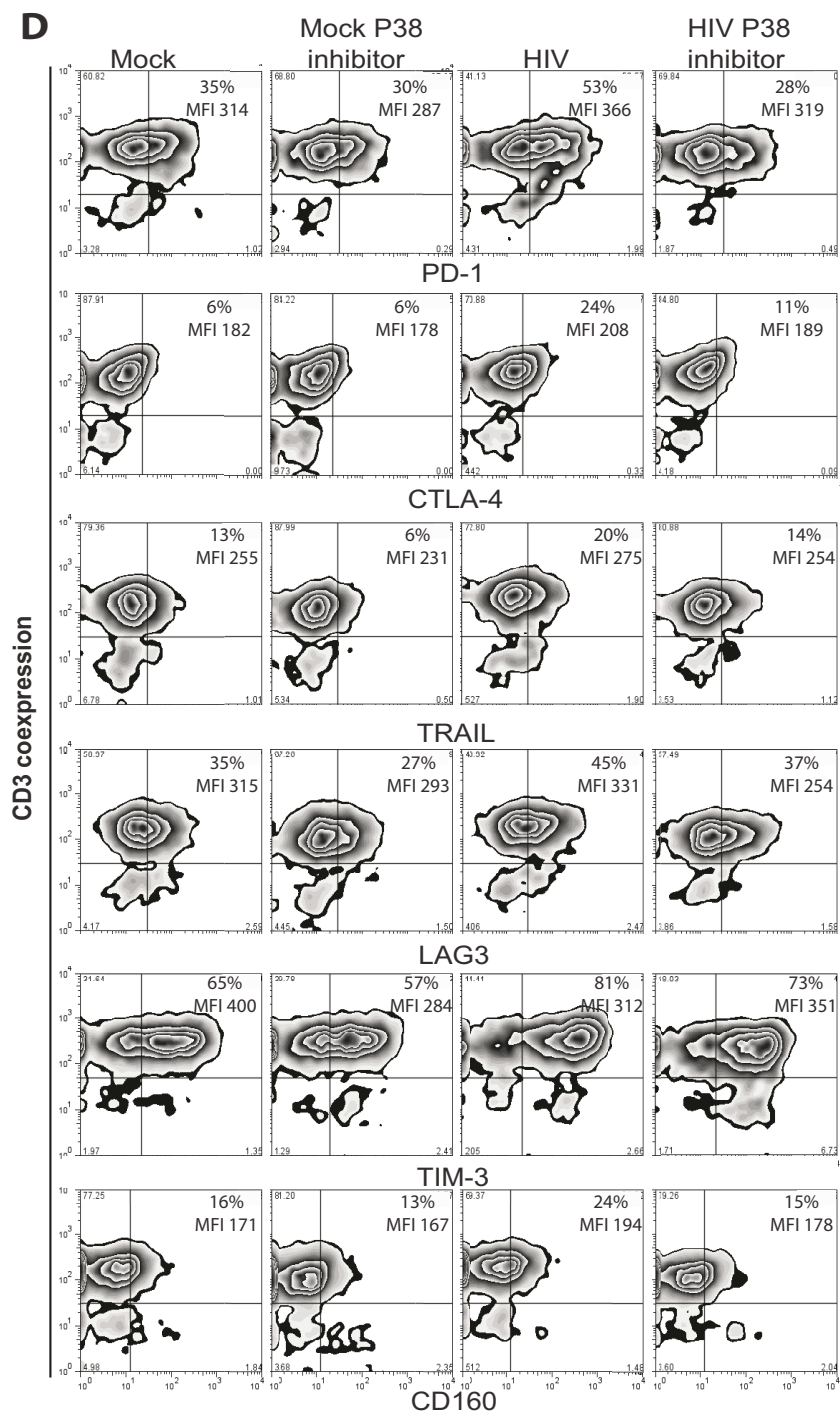


Figure 5. Continued. (D) The protein profiles of CTLA-4, PD-1, TRAIL, TIM-3, CD160 and LAG3 were measured by flow cytometry (n = 4: one representative experiment shown).

PD-1 coexpression with CTLA-4, TRAIL, LAG3, TIM-3 or CD160 was also substantial in our present autologous system, with all being increased in T cells primed by HIV-1-exposed DCs (Figure 6D). Fur-

thermore, blockade of STAT3 ($P < 0.005$) and P38MAPK ($P < 0.001$) significantly restored T-cell proliferation (Figure 6E, F), respectively. These findings suggest that our established allogeneic cocultures re-

flected the events occurring in an autologous system. However, SEB has been shown to induce massive T-cell activation *in vivo* (20%) in a manner that circumvents normal mechanisms of T-cell activation (47), which prompts us to believe that the allogeneic system might be a better *in vitro* T-cell priming model than SEB.

DISCUSSION

A great deal of attention has been focused on elucidating the potential mechanisms underlying HIV-1-associated immune abrogation. Either directly or indirectly, chronic HIV-1 infection rapidly impairs the host immune system. Of the various factors possibly associated with mediating immune impairment in HIV-1 infection, the role played by suppressive factors expressed on T cells has received considerable attention in recent years. Increased expression of inhibitory/suppressor molecules such as PD-1, CTLA-4, TRAIL, CD160, TIM-3 and LAG3 has been documented in HIV-1-infected individuals (4,6,7,20,48). Furthermore, T_N cells activated in the presence of HIV-1 *in vitro* expressed elevated levels of inhibitory molecules (14), and blockade of PD-1 and CTLA-4 dramatically improved T-cell functions (3–5,20). Herein, we have established the same event in T_M cells, although these cells appeared less responsive compared with T_N cells. However, the potential molecular mechanisms underlying the upregulation of inhibitory molecules both *in vivo* and *in vitro* remain elusive. We found that HIV-1-mediated upregulation of inhibitory molecules in T cells under *in vitro* conditions was attributable to STAT3 signaling events. After excluding the involvement of IL-6, IL-10 and growth factors in STAT3 activation, we suggest a possible mechanism through P38MAPK. The interruption of signaling via P38MAPK/STAT3 significantly suppressed the expression of inhibitory molecules and transcriptional repressors *BLIMP-1*, *DTX1* and *FOXP3*. We confirmed these allogeneic findings in au-

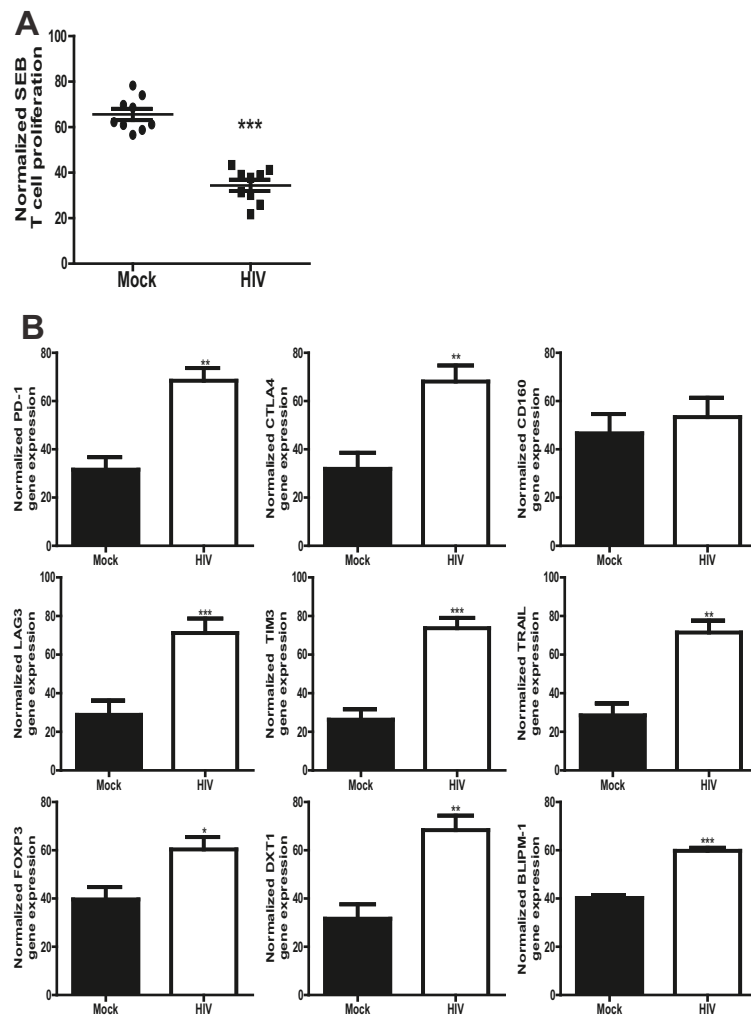


Figure 6. HIV-1 impaired the ability of DCs to activate autologous T-cell responses against SEB. Mature DCs were pulsed with mock or HIV-1_{BaL} cultured for ~24 h and thereafter, incubated with mock or 1 ng/mL SEB for 3 h. The cells were washed and cocultured with T_N cells with and without STAT3 or P38MAPK inhibitors. The cocultures were restimulated on d 7 with the same DC conditions and harvested on d 8. (A) T-cell proliferation was measured by ³H-thymidine incorporation (n = 9). (B) The gene levels of *CTLA-4*, *PD-1*, *TRAIL*, *TIM-3*, *CD160*, *LAG3*, *BLIMP-1*, *FOXP3* and *DTX1* were assessed by qRT-PCR (n = 4). The experiments were normalized, and statistical analysis was performed by one-way ANOVA (Tukey *post hoc* test). **P* < 0.05, ***P* < 0.005, ****P* < 0.001.

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tologous DC–T-cell cocultures activated with SEB.

Our previous study demonstrated that HIV-1 exposure to DCs affected their ability to prime T-cell responses in a contact-dependent manner, giving rise to suppressor T cells (5). Furthermore, in line with the immunomodulatory role of STAT3 in cancer (23,25–27,32), we showed that STAT3 also modulated the

immune responses against HIV-1 by increasing the expression of negative regulatory molecules that suppressed T-cell proliferation. It is noteworthy that the reduced expression in mock primed T cells suggests the possibility that STAT3 regulates expression of inhibitory molecules even under normal physiological conditions. Viral access to the cytosol was required for DC-mediated activation of

suppressor T cells and expansion because blockade of viral fusion significantly improved T-cell proliferation and decreased the expression of inhibitory molecules. This phenomenon could be direct viral access to the DCs cytosol but may also be attributed to decreased viral levels in the T-cell cytosol due to diminished viral transfer from DCs to T cells. A study by Van Montfort *et al.* revealed that the HIV-1 transfer from mature DCs to CD4⁺ T cells was blocked when b12 was used to neutralize the virus (49), whereas Ganesh *et al.* found that transfer of b12-neutralized HIV-1 could occur at later time point after an initial lag phase (50).

The mechanisms whereby pathogens, or HIV-1 in particular, transform DCs to tolerogenic cells remain ambiguous. One could speculate that the presence of nucleic acids in the DC cytosol have immunomodulatory effects, as shown for CD4⁺ T cells (51).

The suppression of immune responses in HIV-1-infected individuals is similar to that in cancer patients, who show generalized immune impairment both in immune cell numbers and function. Both cancer and HIV-1 infection exhibit increased expression of immune suppressive factors like IL-10, CTLA-4, PD-L1, B7-H4, indoleamine 2, 3 dioxygenase (IDO), PD-1, SOCS1 and COX2 (23,27). Studies have established that STAT3 signaling is induced primarily by IL-6 or IL-10 (39,52). In addition to activation by IL-6 and IL-10, STAT3 can be activated by growth factors, for example, EGF, PDGF, insulin-like growth factor (IGF), and G-CSF (32–34), and MAPKs (40,41). We did not observe any significant increase in the levels of IL-6 and IL-10 in our HIV-1-exposed DC–T-cell cocultures, and neutralization of these factors failed to restore T-cell proliferation. In addition, the growth factors were unlikely to have been involved in STAT3 activation because their expression was significantly decreased in HIV-1 cocultures. Interestingly, we detected increased phosphorylation of P38MAPK in T cells primed by HIV-1-exposed DCs, which is also

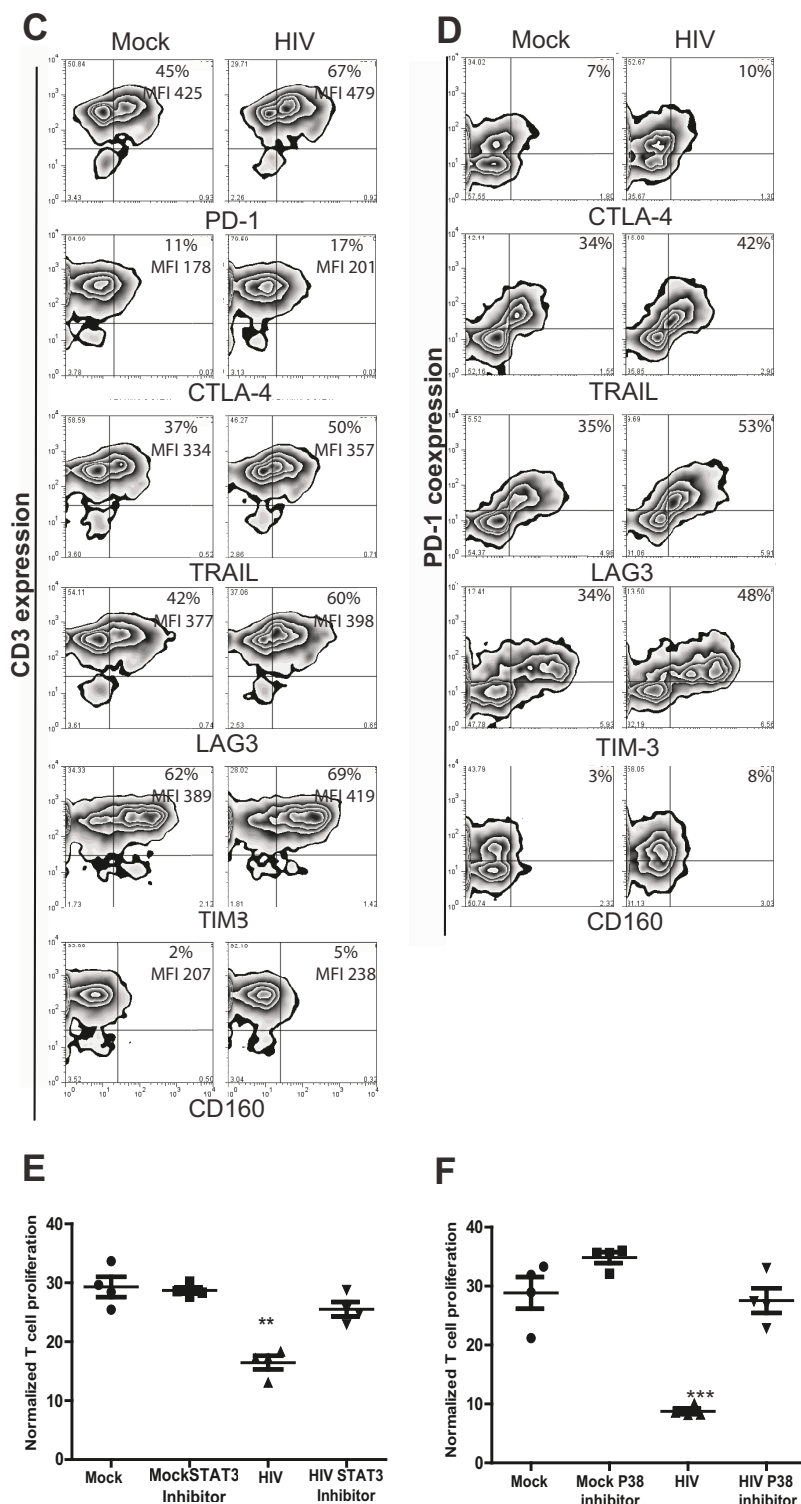


Figure 6. Continued. (C) The protein expression profile on T cells for CTLA-4, PD-1, TRAIL, TIM-3, CD160, BTLA and LAG3 and (D) PD-1 coexpression with CTLA-4, TRAIL, LAG3, TIM-3 and CD160 on T cells were measured by flow cytometry (n = 4: one representative experiment shown). (E, F) Normalized T-cell proliferation after STAT3 (E) and P38 (F) blockade.

known to regulate STAT3 activation (40,41). Better still, the blockade of P38MAPK significantly reduced HIV-1-mediated expression of inhibitory molecules and subsequently improved T-cell proliferation. We are for the first time suggesting a possible route via the P38MAPK/STAT3 pathway for the regulation of inhibitory molecules expressed on T cells primed by HIV-1-exposed DCs. The P38MAPK pathway can be stress activated, and HIV-1, being an error-prone virus capable of inducing stress on immune cells (41), may propagate the herein described responses through exposure to the DCs. The precise events presiding over P38MAPK/STAT3-induced suppressor T-cell activation by DCs could be the effects exerted by viral-derived nucleic acids in the cytosol, which have previously been established in CD4⁺ T cells to have immunomodulatory effects (51), although this possibility warrants further investigation. It is also important to note that the possibility of HIV-1 transfer from DCs to T cells is not fully exempted and could play a role in the induction of stress in T cells, another possibility that requires further investigation.

Intriguingly, the transcription repressors *BLIMP-1*, *FOXP3*, and *DTX1* were also regulated by the P38MAPK/STAT3 signaling pathway. Clinical studies showed that lack of FOXP3 leads to hyperimmune activation and onset of autoimmunity in healthy subjects (53), whereas BLIMP-1 is known to repress normal CD8⁺ T_M-cell differentiation and promote high expression of inhibitory molecules during chronic viral infections (13,54). Furthermore, *DTX1* is a transcriptional target of NFAT-c and negatively regulates T-cell functions (11). HIV-1 induced increased expression of *DTX1* mRNA in the T cells primed by HIV-1-exposed DCs, which correlated with increased *NFAT* mRNA (data not shown). Inhibition of NFAT decreased *DTX1* and *PD-1* mRNA and protein expressions, but surprisingly increased the expression of *CTLA-4*, *TRAIL*, *LAG3*, *CD160* and *TIM-3*, which could explain

the failure of the NFAT blockade to restore T-cell proliferation (data not shown). This result showed that the inhibitory molecules are under the control of diverse pathways, that is, *PD-1* is up-regulated by both P38MAPK/STAT3 and NFAT pathways, whereas *CTLA-4*, *TRAIL*, *LAG3*, *CD160* and *TIM-3* are regulated by P38MAPK/STAT3. We recapitulated these mechanisms in autologous assays in which SEB was used to initiate T-cell proliferation, findings that strengthen the possible occurrence of these mechanisms *in vivo*.

CONCLUSION

In conclusion, HIV-1-exposed DCs prime T_N-cell responses with impaired functionality owing to increased expression of inhibitory molecules and transcriptional repressors in T cells. This process was regulated by the STAT3 pathway, because blockade of this signaling cascade rescued T-cell proliferation. STAT3 activation occurred via P38MAPK intersection. This was evident from the phosphorylation of P38MAPK and STAT3 in T cells primed in the presence of HIV-1. Moreover, HIV-1 access to DC cytosol and possibly T-cell cytosol was involved in the induction of suppressive T cells. The impaired T cells in HIV-1-infected individuals express multiple inhibitory molecules and might need a combination of inhibitors and/or antibodies to abolish the immune impairment. Therefore, it is essential to find a common pathway responsible for the expression of inhibitory molecules, which potentially can be intersected to restore immune functions. Herein, we have shown that targeting the P38MAPK/STAT3 signaling pathway should be seriously considered in the design of immunotherapeutic agents against HIV-1 and other chronic infections that impair the immune system via the generation of suppressive T cells.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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