IFN-α Induces APOBEC3G, F, and A in Immature Dendritic Cells and Limits HIV-1 Spread to CD4+ T Cells

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IFN-α Induces APOBEC3G, F, and A in Immature Dendritic Cells and Limits HIV-1 Spread to CD4⁺ T Cells

Venkatramanan Mohanram,¹ Annette E. Sköld,¹,² Susanna M. Bächle, Sushil Kumar Pathak,³ and Anna-Lena Spetz

Cytokines and IFNs, such as TNF-α and IFN-α, upregulate costimulatory molecules in monocyte-derived dendritic cells (MDDCs), enabling effective Ag presentation to T cells. This activation of MDDCs is often accompanied by upregulation of apolipoprotein B mRNA–editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) (A3) family proteins that are able to restrict HIV-1 replication in MDDCs by inducing hypermutations in the viral genome. In this study, we show that TNF-α upregulates costimulatory molecules and are able to restrict HIV-1 replication in MDDCs without significant induction of A3G, A3A, or A3F. Conversely, low quantities of IFN-α failed to upregulate costimulatory molecules, did not induce IL-12p40 or migration, but significantly induced A3G, A3A, and A3F mRNA expression and restricted viral replication in MDDCs. We also showed that transmission of HIV-1 from MDDCs to autologous T cells was significantly reduced in the presence of IFN-α. Sequence analyses detected the induction of high frequency of G-to-A hypermutations in the env genes from HIV-1 BaL–infected MDDCs treated with low quantities of IFN-α. These findings show that low quantities of IFN-α can induce functional A3 family proteins and restrict HIV-1 replication in MDDCs while keeping an immature nonmigratory phenotype, supporting further investigations of modalities that enhance retroviral restriction factors. In addition, the findings highlight the role of IFN-α as a double-edged sword in HIV-1 infection, and we show that IFN-α can be powerful in reducing HIV-1 infection both in MDDCs and T cells. The Journal of Immunology, 2013, 190: 3346–3353.

Immature myeloid dendritic cells (DCs) are present in the cervico-vaginal mucosa and have been proposed to be one of the first target cells during HIV-1 transmission (1). They are characterized by low expression of costimulatory molecules and high capacity for Ag uptake. Upon encounter with Ags capable of triggering myeloid DC maturation, DCs gain a migratory potential and decrease uptake of additional Ags. Subsequently, the myeloid DCs upregulate costimulatory signals required for effective T cell priming (2, 3). Immature myeloid DCs are susceptible to HIV-1 infection, which can lead to productive infection of the cells or hijacking by the virus (4). HIV-1 can use myeloid DCs as a Trojan horse, which leads to effective dissemination of the virus from the periphery to the local lymph nodes, enabling close contact with T cells (5). Although myeloid DCs can become infected with HIV-1, they do not allow replication of the virus to a high extent, which may at least in part be explained by expression of restriction factors that can limit viral replication.
viral, antiproliferative, and clinical responses (14). The IFN receptor is coupled to a tyrosine kinase, which phosphorylates STAT proteins (15). IFN-\(\alpha\) and LPS were shown to induce MDDC maturation and upregulate A3G in MDDCs (16–18). We previously showed that TNF-\(\alpha\) plays a significant role in HIV-1\(_{\text{inad}}\) replication restriction in MDDCs that were cocultured with activated apoptotic T cells (19). The coculture with activated apoptotic T cells also led to induction of MDDC maturation as measured by up-regulation of costimulatory molecule CD80 and CD86 expression (19, 20). Induction of MDDC maturation was previously shown to be accompanied by induction of A3G and A3F expression in MDDCs (7), and maturation suppresses HIV-1 infection through multifaceted mechanisms that involve decreased viral fusion (4), a block of reverse transcription (21), and a postintegration restriction that have been proposed to exist at the transcriptional level (22). In this study, we set out to investigate whether inducers of MDDC maturation such as TNF-\(\alpha\) and IFN-\(\alpha\) result in similar induction of expression of A3G, A3F, and A3A with subsequent inhibition of HIV-1 replication. We also measured whether this treatment is accompanied by increased MDDC maturation in terms of expression of costimulatory molecules, secretion of IL-12p40, and migratory capacity, as these are central functional properties of MDDCs.

Materials and Methods

Human subjects and blood collection

 Buffy coats from healthy human blood donors were obtained from the blood bank at Karolinska University Hospital Huddinge. Ethical approval was obtained from the medical ethics committee in Stockholm.

MDDC cultures

CD14\(^+\) monocytes were enriched from buffy coats by negative selection using RosetteSep Human Monocyte Enrichment (1 ml/10 ml blood; Stem Cell Technologies, Vancouver, BC, Canada). Monocytes were separated using Lymphoprep density gradient (Nycomed, Oslo, Norway) and were cultured for 6 d in MDDC medium containing RPMI 1640 (Life Technologies, Paisley, U.K.), supplemented with 1% HEPES [N-2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid] (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 2 mM \(l\)-glutamine (Life Technologies), 1% streptomycin (Life Technologies), 1% penicillin (Life Technologies), and 10% endotoxin-free FBS (Life Technologies) with the addition of recombiant human cytokines IL-4 (6.5 ng/ml; R&D Systems, Minneapolis, MN) and GM-CSF (250 ng/ml; PeproTech; London, U.K.) to obtain immature DCs (23). Immature MDDCs were left untreated (medium) or treated either with LPS (100 ng/ml; Sigma-Aldrich, St Louis, MO) or with TNF-\(\alpha\) at single-dose increasing concentration (0.3, 3, 30 ng/ml; R&D Systems) or with different subtypes of IFN-\(\alpha\) (pegylated [PEG] IFN-\(\alpha\)2b, IFN-\(\alpha\)2a, IFN-\(\alpha\)/IFN-\(\alpha\)/AD) at single-dose increasing concentration (10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\) U/ml; Introna; Schering-Plough, R&D Systems). MDDCs were analyzed by flow cytometer for CD80 and CD86 expression 2 d after IFN-\(\alpha\) or TNF-\(\alpha\) treatment.

Phenotypic characterization of MDDCs

MDDCs were stained with the following anti-human mAbs: CD1a (clone NA1/34; DAKO, Glostrup, Denmark), CD14 (clone TUK4; DAKO), CD3 (clone SK7; BD Biosciences), CD80 (clone L307.4; BD Biosciences), and CD86 (clone 2351/FUN-1; BD Biosciences). Cell surface expression was measured by a FACS Calibur flow cytometer (BD Biosciences). MDDCs were gated on CD1a\(^+\), CD3\(^+\), and CD14\(^+\) cells, and 5 \(\times\) 10\(^5\) cells/sample were acquired.

HIV-1 growth and preparation

The HIV-1 isolate HIV-1\(_{\text{inad}}\) (which uses CCR5 receptors) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (National Institutes of Health, Bethesda, MD). HIV-1 isolates were grown in PBMC cultures stimulated with PHA (2.5 \(\mu\)g/ml; Sigma-Aldrich) and IL-2 (75 IU/ml; Chiron, Emeryville, CA). Primary virus was isolated from HIV-infected patient blood by coculture with PHA-activated donor PBMCs. To concentrate the virus and to minimize the presence of bystander activation factors in the supernatant that could induce MDDC maturation, virus stocks were ultracentrifuged (138,000 \(\times\) g for 30 min at 4°C) (Beckman L-80 Ultra-centrifuge, rotor 70.1; Beckman Coulter, Fullerton, CA), and the virus pellets were resuspended in RPMI 1640 with 10% FBS to obtain a virus concentrate. The titers of virus stocks were 1.7 \(\times\) 10\(^{5}\) ml 50% tissue culture infectious dose for HIV-1\(_{\text{inad}}\).

HIV-1 infection of MDDCs

For infection of MDDCs, 3000–6000 50% tissue culture infectious dose/ml of HIV-1\(_{\text{inad}}\) were used (19). The percentage of infected MDDCs was determined by intracellular p24 staining after 7 d of infection, as previously described (23, 24). Cells were first stained for cell surface markers (CD1a and CD86) and then fixed in 2% formaldehyde (Sigma-Aldrich). Cells were washed in saponin solution (PBS with 2% FBS, 2% HEPES, and 0.1% saponin; Sigma-Aldrich), to allow permeabilization of the cell surface membrane, and then incubated for 1–2 h at 4°C with a p24 mAb (clone KC57; Coulter, Franklin, FL) or the corresponding isotype control Ab. p24 expression was assessed by a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star). Gates were set on CD1a\(^+\) cells, and 5 \(\times\) 10\(^4\) cells were acquired per sample. Supernatants from MDDC cultures were harvested 6, 8, and 10 d after HIV-1 infection, and RT activity was quantified using the Lenti-RT-activity kit (Cavidi Tech, Uppsala, Sweden).

HIV-1 MDDC–T cell transfer assay

Monocytes and CD4\(^+\) T cells were prepared from the same donors using RosetteSep enrichment kits (Stem Cell Technologies). The T cells were frozen at \(-80°C\) in 10% DMSO, 20% FCS, and 70% complete RPMI 1640 medium. On day 6, MDDCs were infected with HIV-1\(_{\text{inad}}\), and treated with PEG IFN-\(\alpha\)2b at single-dose increasing concentration (10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\) U/ml), whereas the T cells were thawed and cultured in complete RPMI 1640 supplemented with IL-2 (75 IU/ml; Chiron) for 2 d. Two days postinfection, the virus was thoroughly washed away from the MDDC cultures and the autologous IL-2–treated T cells were added to the MDDCs in a 2:1 ratio. Cells were cultured for 4 d in the presence or absence of PEG IFN-\(\alpha\)2b at single-dose increasing concentration (10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\) U/ml). The cells were then stained with CD1a, CD3, CD86, and p24 mAbs, and 1 \(\times\) 10\(^4\) cells/sample were acquired in a FACSCalibur.

Cytokine detection

Supernatants from HIV-1\(_{\text{inad}}\) cultures were treated with 0.5% Triton X-100 to inactivate free virus. Secretion of human IL-12p40 was then measured with an ELISA kit (Mabtech AB, Stockholm, Sweden).

Real-time PCR analysis of A3A, A3F, and A3G

RNA was extracted using the RNeasy Mini Kit (Qiagen), either from freshly isolated MDDCs or from MDDCs treated with LPS or with TNF-\(\alpha\) or with different subtypes of IFN-\(\alpha\), as described above. RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Amplification of A3A, A3F, A3G, and 18S RNA was performed using the 7900 Real-Time PCR System (Applied Biosystems) and FAM dye-labeled TaqMan MGB probes and primers (Applied Biosystems) (25, 26). We did a homology BLAST for the primer probe, which is highly specific to the above-mentioned A3 molecules. Cycle threshold values for A3 were normalized to the value for 18S in control experiments. Data are presented as fold changes in mRNA copy number in the MDDCs treated with cytokines as compared with mRNA in MDDCs cultured in medium only.

HIV-1 env sequencing and analysis

Total cell DNA was isolated from cells treated with PEG IFN-\(\alpha\)2b, 72 h postinfection with a QIAamp DNA mini kit (Qiagen). HIV-1 envelope (env) V1–V5 gene region was amplified by performing high-fidelity nested PCR using an EasyA kit (Stratagene). The primers used were as follows: round 1 (product size 1415 bp), forward 5′-TTGCAATTGAAGAAATCTTCC-3′, and reverse 5′-CTCTGGGTGTGCTACTCCTA-3′; round 2 (product size 270 bp), forward 5′-CCACGTGTTGAAGTAAACC-3′, and reverse 5′-ATGGGGACAAATGGAACTGTTGAC-3′; PCR condition as described in (27). Amplicons were purified by using a high pure PCR product purification kit (Roche), and cloned into the TA cloning vector provided in a TOPO TA cloning kit (Invitrogen) in accordance with the manufacturer’s instructions. Plasmids were isolated using Genejet plasmid mini prep kit (Fermentas), and the plasmids were sent to Eurofins MWG Operon (Operon, Germany) for sequencing. Primers M13 forward (5′-GTAAAACGAGGCCAGC-3′) and M13 reverse (5′-CAGGAAACACGTATGAC-3′) were used for sequencing. G-to-A mutations were analyzed by using the program 

http://www.jimmunol.org/ Downloaded from
Hypermut 2.0 that is freely available at http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html (28).

**Migration assay**

Transwell chambers (8 μm; BD Biosciences) were inserted into wells of 24-well plates containing 750 μl complete RPMI 1640 supplemented with CCL21 (250 ng/ml; R&D Systems) and MDDCs were added in the upper well. The cell concentration in the lower chamber was assessed after 4 h by trypan blue exclusion of nonviable cells in an Auto T4 Cellometer (Nexcelom Bioscience, Lawrence, MA).

**Results**

**Induction of CD80 and CD86 expression in MDDCs after treatment with IFN-α or TNF-α**

MDDC maturation can be triggered by proinflammatory cytokines such as TNF-α and IFN-α (29). To investigate whether exogenously added TNF-α and IFN-α can trigger MDDC maturation in the present culture system, immature MDDCs were treated with TNF-α or PEG IFN-α2b at single-dose increasing concentrations (0.3, 3, 30 ng/ml and 10², 10³, 10⁴ U/ml, respectively). We used a PEG IFN, as the addition of PEG may delay clearance of the protein and is used in the clinic for treatment of hepatitis C in combination with ribavarin (30). The MDDCs were collected and stained for CD80 and CD86 for flow cytometry analysis 48 h post–TNF-α or PEG IFN-α2b treatment. LPS served as a positive control. We detected a significant increase in the expression of CD80 and CD86 in a dose-dependent manner in the cells that were treated with TNF-α (Fig. 1A, 1C). Similar data were obtained for PEG IFN-α2b displaying significant induction of CD80 and CD86 after stimulation with the highest dose of PEG IFN-α2b, but not when using the lower concentrations (10² or 10³ U/ml) (Fig. 1A, 1C). Additional phenotypic analyses showed induction of CD83 and HLA-DR using the highest concentrations of IFN-α or TNF-α. The 7-aminoactinomycin D staining did not reveal any toxic effects on the cells even at higher concentration of the cytokines (data not shown). To investigate whether the pegylation may have affected

![FIGURE 1. CD80 and CD86 expression as well as IL-12p40 secretion in human MDDCs.](http://www.jimmunol.org/)

Human in vitro differentiated monocytes cultured for 6 d in the presence of IL-4 and GM-CSF were used as the source of human immature MDDCs. Immature MDDCs were exposed to HIV-1nd, treated with cytokines for 48 h, and then analyzed for expression of CD80 and CD86 molecules by flow cytometry. Gates were set on live large CD1a⁺CD3⁻ cells. LPS, which is a potent DC activator, was used as a positive control, and MDDCs cultured in medium only were used as a negative control. Immature MDDCs were cultured in medium alone or in the presence of LPS or with TNF-α at single-dose increasing concentration (0.3, 3, 30 ng/ml) or with different subtypes of IFN-α (PEG IFN-α2b, IFN-α2a, IFN-αA/D) at single-dose increasing concentration (10², 10³, 10⁴ U/ml) in at least three independent experiments. (A) The average percentages of CD80⁺ MDDCs ± SD for 11 donors. (B) The average percentages of CD80⁺ MDDCs ± SD for 7 donors. (C) The average percentages of CD86⁺ MDDCs ± SD for 11 donors. (D) The average percentages of CD86⁺ MDDCs ± SD for 7 donors. (E) MDDCs were treated with increasing concentrations of TNF-α (0.3, 3, 30 ng/ml), PEG IFN-α2b (10², 10³, 10⁴ U/ml), or with LPS as a positive control, in the presence of HIV-1nd. The secretion of IL-12p40 was measured by ELISA in supernatants taken at 24 h of culture. The data are presented as pg/ml ± SD for 6 donors. Significant differences compared with medium control were assessed by the paired one-way ANOVA with Bonferroni’s multiple comparison test, and significance is indicated by **p < 0.01 and ***p < 0.001.
the IFN-α2b from inducing the expression of costimulatory molecules, we further treated MDDCs with non-PEG IFN-α (IFN-α2a, IFN-αA/D) at single-dose increasing concentrations (10^2, 10^3, 10^4 U/ml). We confirmed that different IFN-α subtypes (with or without PEG), at high doses, can induce expression of costimulatory molecules (Fig. 1B, 1D).

Mature MDDCs produce cytokines to influence the subsequent adaptive immune response. One cytokine important for Th1 induction is IL-12, and we therefore investigated whether IL-12p40 release into the supernatant correlated with the expression of maturation markers following HIV-1BaL infection and single-dose increasing concentrations of TNF-α or IFN-α (0.3, 3, 30 ng/ml and 10^2, 10^3, 10^4 U/ml, respectively). The release of IL-12p40 correlated with TNF-α treatment, but was not induced by PEG IFN-α2b (Fig. 1E). Significant high quantity of IL-12p40 was only induced after stimulation with the positive control LPS.

Restriction of HIV-1BaL replication in MDDCs upon treatment either with TNF-α or IFN-α

We previously showed that TNF-α plays a significant role in restriction of HIV-1BaL replication in MDDCs that are cocultured with activated apoptotic T cells (19). In addition, α-IFNs are known to play a major role in antiviral activity (13, 14, 31). To further investigate the role for TNF-α and IFN-α in inhibition of viral replication, HIV-1–infected MDDCs were treated either with TNF-α at single-dose increasing concentration (0.3, 3, 30 ng/ml) or with three different subtypes of IFN-α (PEG IFN-α2b, IFN-α2a, IFN-αA/D) (10^2, 10^3, 10^4 U/ml), respectively. The cells were collected and quantified for expression of the HIV-1 structural protein p24 by flow cytometry 6 d after TNF-α and IFN-α treatments (Fig. 2A, 2B). The release of viral particles into the supernatant of infected cells did not increase after this time point (Fig. 2C). We have previously shown that the intracellular detection of p24 requires de novo synthesis of viral proteins and fails to detect capture of HIV-1 virions (23). There was large variability between donors with regard to HIV-1 infection efficiency, ranging from 8.9 to 59.4% virions (23). There was large variability between donors with regard to HIV-1 infection efficiency, ranging from 8.9 to 59.4% virions (23). There was large variability between donors with regard to HIV-1 infection efficiency, ranging from 8.9 to 59.4% virions (23). There was large variability between donors with regard to HIV-1 infection efficiency, ranging from 8.9 to 59.4% virions (23).

FIGURE 2. Frequency of HIV-infected MDDCs. Immature MDDCs were exposed to HIV-1BaL, in the presence of LPS or with TNF-α at single-dose increasing concentration (0.3, 3, 30 ng/ml) or with different subtypes of IFN-α (PEG IFN-α2b, IFN-α2a, IFN-αA/D) at single-dose increasing concentration (10^2, 10^3, 10^4 U/ml). The percentage of infected MDDCs was assessed by flow cytometry analysis of intracellular p24 staining after 6–7 d in at least three independent experiments. (A) The average percentage of p24iferous MDDCs ± SD after 6–7 d after treatment with cytokines for 10 donors. (B) The average percentage of p24iferous MDDCs ± SD after 6–7 d after treatment with non-PEG IFN-α for 6 donors. Significant differences compared with HIV-1BaL–infected control were assessed by the paired one-way ANOVA with Bonferroni’s multiple comparison test, and significance is indicated by ***p < 0.001. (C) MDDCs were infected with HIV-1BaL. After 2 d, the cells were thoroughly washed and further cultured for 8 d. Supernatants from 4 donors were taken at 6, 8, and 10 d postinfection, and the reverse-transcriptase activity was measured with a Lenti RT activity kit.

Reduced HIV-1 transfer from MDDCs to autologous T cells in the presence of PEG IFN-α2b, but not TNF-α

We next investigated the viral transfer from HIV-1–infected MDDCs to cocultured autologous IL-2–activated T cells. MDDCs were infected with HIV-1BaL, and treated with increasing concentrations of PEG IFN-α2b (10^2, 10^3, 10^4 U/ml) or the highest concentration of TNF-α for 2 d. Thereafter, the cultures were extensively washed, to prevent further infection from unbound virus. IL-2–activated autologous T cells were added to the MDDCs in a ratio of 2:1 and either left untreated (Fig. 3A) or continuously treated with PEG IFN-α2b (Fig. 3B) for 4 d. The p24 expression was then investigated in both T cells and MDDCs. MDDCs pre-treated with the highest dose of PEG IFN-α2b (10^4 U/ml) displayed significantly reduced viral transfer to T cells even without addition of new PEG IFN-α2b (Fig. 3A). An even more impressive reduction of viral transfer was measured if the PEG IFN-α2b (10^2, 10^3, 10^4 U/ml) treatment was continued also with the addition of T cells. A significant reduction of the viral transfer was measured for both the intermediate and the higher concentration of PEG IFN-α2b (10^3 and 10^4 U/ml) (Fig. 3B). Similar results were obtained for the MDDC infection in the coculture (data not shown). However, pretreating the MDDCs with the TNF-α concentration that protected the MDDCs from HIV-1BaL infection (30 ng/ml) (Fig. 2A) did not significantly alter the viral transfer to autologous T cells as compared with nontreated control (Fig. 3). Hence, TNF-α protects MDDCs from high HIV-1BaL infection by the induction of maturation, but this is not protecting interacting T cells from the infection. However, PEG IFN-α2b protects MDDCs from high HIV-1BaL infection without inducing a mature phenotype, and also protects interacting T cells, even in the absence of exogenously added PEG IFN-α2b. Therefore, we continued to compare the induction of host-restriction factors in MDDCs treated with either PEG IFN-α2b or TNF-α at single-dose increasing concentrations (10^2, 10^3, 10^4 U/ml and 0.3, 3, 30 ng/ml, respectively).

PEG IFN-α2b, but not TNF-α, upregulates mRNA expression of A3G, A3F, and A3A

IFN-α is known to upregulate A3 molecules that possess cytidine deaminase activity in various cell types (32, 33). A3 molecules are
potent in inducing mutations of a broad spectrum of retroviruses, including HIV-1 (34–37). A3A was recently reported to be expressed in MDDCs and inhibited early phases of HIV-1 infection (8). In this study, we investigated whether A3G, A3F, and A3A were upregulated in MDDCs exposed to HIV-1 and treated with TNF-α and PEG IFN-α2b at single-dose increasing concentration (0.3, 3, 30 ng/ml) or with TNF-α at 30 ng/ml for 2 d. The cells were then extensively washed and thereafter cocultured with autologous T cells that had been treated with IL-2 (75 IU/ml) for 2 d. The PEG IFN-α2b was either washed away (A) or replenished in the same concentration as during the HIV-1 infection (B). After additional 4 d, the cultures were assessed for p24 expression and phenotypical markers in at least three independent experiments. The average percentage of CD3+ p24+ T cells ± SD for 10 donors is displayed. Significant differences compared with HIV-1_BaL–infected control were assessed by the paired one-way ANOVA with Bonferroni’s multiple comparison test, and significance is indicated by *p < 0.05 and **p < 0.01.

PEG IFN-α2b is sufficient to induce up to a 100-fold (range fold increase 9.5–285) increase of A3A as well as significant induction of A3G and A3F. PEG IFN-α2b induces G-to-A hypermutation in MDDCs infected with HIV-1_BaL.

To investigate whether there were signs of A3 family protein activity, we screened for G-to-A hypermutation in the HIV-1 env genes that were amplified from the DNA obtained from the infected MDDCs treated with PEG IFN-α2b (10^2 U/ml). The env region was chosen for analyses because several studies have confirmed that A3 exhibits a pronounced 5’ to 3’ editing gradient, resulting in a particular susceptibility to A3 cytidine deaminase activity (38, 39). Sixty different clones were sequenced, and the total numbers of bp investigated were 51,000. We observed a high frequency of G-to-A hypermutations in the DNA that were obtained from the cells treated with the relatively low concentration...
of PEG IFN-α2b (Table I). There was a similar distribution within the V3 env region as compared with other variable parts of env, in total, 14 of the 60 clones harbored significant mutation rates (Fisher exact \( p \) value <0.05). The total numbers of G-to-A mutations were 2,475, and of these 741 were in GG context, whereas 1,191 were in GA context. The average number of G-to-A mutations per 100 bp was 4.85. Our data suggest that even a relatively low concentration of PEG IFN-α2b, which is not able to induce full MDDC maturation, is yet able to inhibit viral replication by upregulating A3 molecules, which in turn induce G-to-A hypermutation in the viral genome, causing the viruses to become replication incompetent.

**MDDC migration is not induced by PEG IFN-α2b treatment**

Because PEG IFN-α2b did not induce strong maturation of MDDCs, but upregulated sufficient amounts of A3 molecules to reduce HIV-1 infection, we investigated whether a migratory phenotype was induced by the cytokine. MDDCs were treated with single-dose increasing concentrations (10^2, 10^3, 10^4 U/ml) of PEG IFN-α2b, either overnight or directly before addition to the upper well in a Transwell setting (Fig. 5). To stimulate migration of activated cells, the medium below the Transwell contained the chemokine CCL21, and the MDDCs were allowed to migrate for 4 h before the number of migrated cells was counted. Untreated cells were used as a background control, and LPS-treated cells were used as a positive control for migration. For some donors, CCL21 was instead added in the top chamber as a control, but the number of migrating cells did not differ from untreated cells (data not shown). In accordance with the relative lack of costimulatory molecules, MDDCs treated with PEG IFN-α2b did not migrate toward the CCL21 gradient (Fig. 5). The LPS-treated cells, however, migrated efficiently after pretreatment, but not when stimulated just prior to the migration assay.

**Discussion**

In this study, we measured the induction of A3G, A3F, and A3A antiviral restriction factors and activation/maturation of MDDCs after HIV-1 infection and treatment with TNF-α or IFN-α. Our study shows significant inhibition of HIV-1 replication and induction of A3G, A3F, and A3A in MDDCs after incubation with relatively low concentration of PEG IFN-α2b (10^2 U/ml). The lower PEG IFN-α2b concentrations of 10^2 U/ml and 10^3 U/ml did not induce MDDC maturation, as we did not detect significant induction of the costimulatory molecules CD80 and CD86, or secretion of IL-12p40. The presence of 10^2 U/ml PEG IFN-α2b was, however, sufficient to protect autologous IL-2–activated T cells from high HIV-1 infection in a MDDC–T cell transmission setting. None of the investigated PEG IFN-α2b concentrations induced a migratory phenotype in MDDCs. However, higher concentrations of PEG IFN-α2b induced expression of costimulatory molecules and a dose-dependent induction of APOBEC3 family proteins. Notably, the relatively low concentration of 10^2 U/ml was sufficient to induce significant G-to-A mutations (4.85 per 100 bp) in env sequences obtained from the MDDCs.

Cell-free HIV-1 virions can be relatively poor inducers of type I IFN production compared with HIV-1–infected cells, as suggested in a recent study showing that HIV-1–infected cells induced 10–100 times more IFN-α (mean 600 U/ml) than cell-free HIV-1 virions in vitro (40). It remains to be elucidated whether a local IFN-α release at the site of transmission, without concomitant proinflammatory responses, can induce sufficient restriction factors to protect from infection. In a recent study, significant induction of A3G and A3F as well as reduction (\(-0.921 [\pm 0.858] \log_{10}\) copies/ml) of viral load was demonstrated in HIV-1/hepatitis C–coinfected patients undergoing treatment with PEG IFN-α and ribavirin (30). The plasma concentrations measured in patients after s.c. injection of PEG IFN-α are in the same order (mean week 1, 1710 pg/ml or week 4, 2380 pg/ml) as the lower doses used in the current study (10^2 U/ml equals 1430 pg/ml PEG IFN-α2b). However, it remains to be elucidated whether PEG IFN-α can effectively penetrate into tissue compartments to modulate HIV-1 restriction factors in DCs and macrophage reservoirs. Although IFN-α has antiviral activity and can induce factors that limit viral replication (such as APOBEC molecules, TRIM-5 α, tetherin, and SAMHD1)

### Table I. G-to-A mutation frequencies in HIV-1 env sequences obtained from HIVBaL–infected MDDCs treated with PEG IFN-α2b

<table>
<thead>
<tr>
<th>Item Analyzed</th>
<th>MDDC PEG IFN-α2b–Treated 10^2 U/ml (env V1, V2, V4, V5)</th>
<th>MDDC PEG IFN-α2b–Treated 10^2 U/ml (env V3 Region)</th>
<th>MDDC PEG IFN-α2b–Treated 10^2 U/ml (Total env)</th>
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</thead>
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<tr>
<td>Total no. of clones*</td>
<td>60</td>
<td>60</td>
<td>60</td>
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<tr>
<td>Total no. of bp</td>
<td>33,240</td>
<td>17,760</td>
<td>51,000</td>
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<tr>
<td>Total no. of clones Fisher exact ( p ) value &lt;0.05</td>
<td>9</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Total no. of G-to-A mutations</td>
<td>1,615</td>
<td>860</td>
<td>2,475</td>
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<td>No. of G-to-A mutations (GG context)</td>
<td>496</td>
<td>245</td>
<td>741</td>
</tr>
<tr>
<td>No. of G-to-A mutations (GA context)</td>
<td>831</td>
<td>360</td>
<td>1,191</td>
</tr>
<tr>
<td>Average no. of G-to-A mutations/100 bp</td>
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<td>4.84</td>
<td>4.85</td>
</tr>
</tbody>
</table>

*Representative clones from three donors were sequenced.

![FIGURE 5. Migration of MDDCs toward CCL21. MDDCs were treated with PEG IFN-α2b at single-dose increasing concentration (10^2, 10^3, 10^4 U/ml) either overnight (o.n.) or directly before addition to Transwells. LPS-treated cells were used as positive control and medium treated as a negative control. The lower wells contained CCL21 as a migratory gradient. The number of migrated cells in the lower chamber was counted 4 h after addition of cells. Data represent the mean ± SD from eight different donors analyzed in at least three independent experiments. Significant differences compared with medium control were assessed by the nonparametric Wilcoxon matched-pairs signed rank test and are indicated as **\( p < 0.01 \).](http://www.jimmunol.org/)
(9, 43–46), it is becoming clear that HIV-1 infection is characterized by acute and chronic immune activation, which partly may be due to dysfunctional cytokine and IFN-α production (47). In natural hosts of SIV (such as African Green Monkeys and Sooty Mangabeys), the infection is often pathogenic despite the presence of high viral load (48). Experiments showed that type I IFN production was only transient after viral inoculation in natural hosts that do not develop disease (49–51). In contrast, in macaques in which SIV infection is pathogenic, there was a local accumulation of plasmacytoid DCs in the mucosal tissue shortly after primary infection, producing high quantities of IFN-α that can fuel the infection. It is possible that similar events take place during HIV-1 transmission and acute infection, which may be associated with high production of IFN-α and proinflammatory cytokines (47). High production of IFN-α and proinflammatory cytokines such as TNF-α will induce myeloid DC maturation and trigger migration of myeloid DCs into lymph nodes facilitating dissemination of the infection (1). The finding reported in this study suggests that a window of low IFN-α concentration can inhibit viral replication without inducing MDDC maturation and migration. Furthermore, the intermediate concentration of PEG IFN-α2b significantly prevented transmission of HIV-1 from infected MDDCs to interacting IL-2–activated autologous T cells. TNF-α, in contrast, could only limit replication in MDDCs in a relatively high concentration (30 ng/ml) that also induced MDDC maturation, and this treatment did not protect interacting T cells from HIV-1 infection. None of the concentrations of TNF-α (0.3–30 ng/ml) that we used in the current study showed induction of APOBEC3 family proteins. The antiviral effect exerted by TNF-α in MDDCs is in sharp contrast with its proviral activity in other cell types, such as monocyte-derived macrophages (52). Studies aiming to elucidate the mechanism of how TNF-α is acting to limit replication in MDDCs may shed light on these cellular differences.

Earlier reports showed that A3A is highly expressed in monocytes (53–55), and a recent report demonstrated expression of A3A in MDDCs (8). Our study confirms expression of A3A in MDDCs (range fold increase 9.5–285) and shows a high mutation frequency after treatment with 10⁴ U/ml PEG IFN-α2b. The induction of A3A, A3G, and A3F occurred in a dose-dependent manner, but the relative induction of A3A was higher compared with A3G and A3F in MDDCs. Of note is that A3A is the only member of the APOBEC3 family that is preferentially expressed in myeloid cells compared with peripheral blood lymphoid cells (8), A3G induces G-to-A mutations preferentially in a GG context, A3F prefer a GA context, whereas A3A can induce mutations in GG as well as in GA contexts (6). In the current study, we sequenced 60 clones and in total 51,000 bp, 2,475 G-to-A mutations were revealed. The G-to-A mutations occurred in both GG (n = 741) and GA (n = 1191) contexts, and the current study did not determine the relative contribution of each APOBEC family member. We did, however, detect significant inhibition of HIV-1 replication in the DCs, and it is conceivable that the total proportion of proviral sequences mutated by A3 proteins is underestimated, because extremely hypermutated sequences may fail to replicate and amplify by PCR.

Although the treatment with low quantity of PEG IFN-α2b in MDDCs led to lethal hypermutations and inhibited viral replication, as shown in the present study, there is the theoretical possibility that APOBEC-mediated hypermutations may contribute to viral adaptation and even be involved in the acquisition of immune escape mutations early in HIV-1 infection (56). However, a recent study opposes this hypothesis and suggests that A3G-induced hypermutations of the HIV-1 genome are rather an “all or nothing” phenomenon that is lethal to the virus (57). Moreover, it was shown that patients with low viral set point had significantly higher levels of A3G mRNA both pre- and postinfection compared with patients that have high viral set point (56). Furthermore, HIV-1–exposed seronegative individuals were reported to have higher A3G expression levels than healthy controls (58), and PBMCs of HIV-1–exposed seronegative individuals have higher A3G expression and decreased susceptibility to infection of HIV-1 in vitro (59). Hence, it remains to be further explored which levels of APOBEC family proteins are required for effective block of HIV-1 acquisition in vivo and means to induce their expression without providing fuel for HIV-1 infection. It can be noted that highly exposed seronegative women were reported to have upregulated IFN-α expression in the cervix as quantified by immunohistochemistry (60). In addition, immunomodulatory or immunization strategies inducing A3G expression were reported to correlate with reduced viral load or protection against infection in macaques (26, 61, 62). In this study, we showed that relatively modest quantity of IFN-α can significantly induce expression of A3G, A3F, and, in particular, A3A, without inducing signals that induce MDDC maturation and migration in vitro. We also showed that transmission of HIV-1 from MDDCs to autologous T cells is significantly reduced in the presence of IFN-α. This study supports future development of interventions that can enhance APOBEC expression and suggests that this can be achieved in myeloid DCs without inducing maturation and migration.

Disclosures
The authors have no financial conflicts of interest.

References


