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Double-Stranded RNA Analog Poly(I:C) Inhibits Human Immunodeficiency Virus Amplification in Dendritic Cells via Type I Interferon-Mediated Activation of APOBEC3G▽†

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Human immunodeficiency virus (HIV) is taken up by and replicates in immature dendritic cells (imDCs), which can then transfer virus to T cells, amplifying the infection. Strategies known to boost DC function were tested for their ability to overcome this exploitation when added after HIV exposure. Poly(I:C), but not single-stranded RNA (ssRNA) or a standard DC maturation cocktail, elicited type I interferon (IFN) and interleukin-12 (IL-12) p70 production and the appearance of unique small (15- to 20-kDa) fragments of APOBEC3G (A3G) and impeded HIV_{Bal} replication in imDCs when added up to 60 h after virus exposure. Comparable effects were mediated by recombinant alpha/beta IFN (IFN-α/β). Neutralizing the anti-IFN-α/β receptor reversed poly(I:C)-induced inhibition of HIV replication and blocked the appearance of the small A3G proteins. The poly(I:C)-induced appearance of small A3G proteins was not accompanied by significant differences in A3G mRNA or A3G monomer expression. Small interfering RNA (siRNA) knockdown of A3G could not be used to reverse the poly(I:C)-induced protective effect, since siRNAs nonspecifically activated the DCs, inducing the appearance of the small A3G proteins and inhibiting HIV infection. Notably, the appearance of small A3G proteins coincided with the shift of high-molecular-mass inactive A3G complexes to the low-molecular-mass (LMM) active A3G complexes. The unique immune stimulation by poly(I:C) with its antiviral effects on imDCs marked by the expression of IFN-α/β and active LMM A3G renders poly(I:C) a promising novel strategy to combat early HIV infection in vivo.

Dendritic cells (DCs) are essential to initiate and regulate innate and adaptive responses to incoming pathogens. They are one of the first leukocytes that encounter human immunodeficiency virus (HIV) and elicit immune responses against HIV; however, this is insufficient to control infection. DCs subsequently can promote spread of infection by facilitating the transfer of virus to CD4 T cells (30, 50), exploiting the extensive interactions between DCs and T cells that are a part of normal physiological function (60). Immature DCs (imDCs) and mature DCs efficiently capture and internalize HIV while not becoming infected (22, 70), but imDCs are also susceptible to R5 HIV infection (9, 29, 70). Low-level infection of imDC populations can contribute significantly to the spread of HIV, often providing a reservoir of infectious virus that is beyond measurable levels of detection (70, 71). This probably provides the first site of virus replication in vivo, before virus is handed over to CD4 T cells, where robust virus replication can be detected after about 3 days (41). The constant communication between DCs and T cells and the ability of DCs to transfer captured or newly produced virus to T cells underscore the DC's key role in the onset and dissemination of HIV infection (43, 67).

imDCs predominate at the body surfaces and, following the encounter with antigen, typically migrate and undergo maturation as part of the process to elicit appropriate innate and adaptive immune responses to control incoming pathogens. DC maturation is effectively mediated via Toll-like receptors (TLRs) recognizing pathogen-associated molecular patterns or via cytokines and chemokines (3, 61). TLR signaling triggers the expression of type I interferons (IFNs) in humans (TLR3, -4, -7, -8, and -9) and in mice (TLR3, -4, -7, and -9); type I IFNs play an important role in fighting viral and nonviral pathogens (34, 40, 76). However, the antiviral activity of type I IFNs in different cell types is still poorly understood. In addition, unlike other pathogens, HIV does not fully activate imDCs (31) and appears to exploit DC biology to favor suboptimal immune activation that promotes virus dissemination (43, 67). A recent report showed that alpha IFN (IFN-α) inhibits the release of HIV particles from fibroblasts and T cells but that the HIV Vpu protein counteracts this activity in these cells (44, 45). IFN-α is also involved in the activation of APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G) (A3G) expression in macrophages, resulting in reduced susceptibility to HIV infection (47, 48), and hepatocytes (64). A3G expression is enhanced in T cells by interleukin-2 (IL-2), IL-15, and, to a lesser extent, IL-7, as well as in DCs

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after exposure to a combination of tumor necrosis factor alpha (TNF-α) and poly(I:C) (62). A3G is an innate barrier affording broad intracellular antiretroviral protection (15, 16, 58). The characterization of A3G has been carried out in T cells (17) or with recombinant A3G purified from insect cells (12), showing that the low-molecular-mass (LMM) complexes of A3G contain the antiretroviral activity. The levels of LMM A3G in imDCs reportedly contribute to the lower levels of HIV replication seen in imDC populations (49) than in activated T cells (39). More-recent reports also suggest that the amount of A3G in LMM complexes in DCs further increases during maturation (62).

HIV encounters imDCs in the periphery and utilizes them to help initiate and spread infection. We set out to determine if specific TLR stimuli could be harnessed to trigger innate pathways in virus-bearing imDCs to effectively limit imDC-driven HIV dissemination. Specifically, we compared known DC stimulators including poly(I:C) (analog of double-stranded RNA [dsRNA]); single-stranded RNA (ssRNA); and a standard maturation cocktail containing IL-1β, IL-6, TNF-α, and prostaglandin E_2, studying the impact of these treatments when applied at various times after HIV exposure in vitro. These studies provide the basis for future in vivo strategies that could use poly(I:C)-mediated stimulation to boost innate and adaptive immune function to limit the initiation of HIV infection by DCs.

MATERIALS AND METHODS

DC generation and characterization. Peripheral blood mononuclear cells were isolated from Leukopacks (New York Blood Center) using Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. $CD14^+$ cells were positively selected with the CD14 Miltenyi magnetically activated cell sorting system (Miltenyi Biotec Inc., Auburn, CA) and differentiated for 5 to 6 days in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (1,000 U/10^6 cells) (Invitrogen/Biosource, Carlsbad, CA) and IL-4 (100 U/10^6 cells) (R&D Systems Inc., Minneapolis, MN) in order to generate imDCs (22). Cells were cultured in medium (R1) consisting of RPMI 1640 (Cellgro, Springfield, NJ) supplemented with 2 mM L-glutamine (Invitrogen/Gibco, Carlsbad, CA), 10 mM HEPES (Invitrogen/Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen/Gibco), and 1% (vol/vol) heparinized human plasma (Innovative Research, Southfield, MI).

The purity and phenotype of each DC preparation were examined by flow cytometry by staining with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR combined with phycoerythrin-conjugated anti-CD25, -CD80, -CD86, and -CD206 (all from BD/Pharmingen, San Jose, CA); anti-CD83 (Immunotech-Beckman-Coulter, Marseille, France); or anti-CD209 (R&D Systems Inc.). T-cell contamination of the DCs was monitored with a phycoerythrin-conjugated anti-CD3 antibody (Ab; BD/Pharmingen). DC preparations routinely contained <2.1% T cells (averaged from 75 donors). Samples were acquired on a FACSCalibur flow cytometer (BD/Pharmingen), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Infection. HIV_{Bal} (HIV, lots P3953 and P4143) stocks and the no-virus microvesicle (MV) controls (prepared from SUPT1 cells used to generate the HIV stocks; lot P3826) were provided by the AIDS and Cancer Virus Program, SAIC-Frederick (National Cancer Institute at Frederick, MD). All stocks were sucrose density gradient purified (14) and stored at −80°C. Titers of virus were verified by titration on TZM.bl cells (18). imDCs were pulsed with HIV (6.4×10^4 to 8×10^4 50% tissue culture infective doses/10^6 DCs) or the no-virus MV control (normalized to the same amount of total protein in the virus preparation) for 1 h at 37°C in a 15-ml conical tube at a concentration of 10^6 imDCs/100 μl (maximum, 5×10^7 cells/tube). Where indicated, 10 μg of the anti-IFN-α Ab (clone MMHA-2) or the immunoglobulin G1 (IgG1) control (both from R&D Systems, Inc.) was added per 10^6 imDCs (in 100 μl) just before incubation with the virus. Cells were washed four times with ice-cold R1 and then replated in GM-CSF (1,000 U/ml) and IL-4 (100 U/ml, 0.7×10^6 to 1.5×10^6 DCs/ml). Low-level (zidovudine [AZT]-sensitive) infection of imDCs after at least 48 h of exposure to R5 HIV is readily revealed upon addition of permissive CD4 T cells (70, 71). Therefore, the various stimuli were added immediately (0 h) or 48 to 60 h later, to compare DCs that had captured virus to acutely infected imDCs.

DC stimulation and cytokine neutralization. imDCs were left unstimulated (medium [med]) or stimulated with 0.0025 to 25 μg/ml poly(I:C) (InvivoGen, San Diego, CA; ligand of human TLR3); 4 μg/ml ssRNA40 (ssRNA; InvivoGen; 20-mer phosphothioate-protected ssRNA oligonucleotide containing a GU-rich LyoVec complexed sequence and ligand of human TLR8) (32); or the maturation cocktail containing 10 ng/ml of IL-1β, IL-6, and TNF-α and 1 μM of prostaglandin E_2 (22). DCs were also cultured with 0.01 to 10 ng/ml recombinant IFN-α (universal type I IFN) or IFN-β 1a (both from Invitrogen/PBL, Carlsbad, CA). In some experiments, the neutralizing IFN-α Ab or the respective IgG1 control was included at 10 μg/ml. To neutralize IFN-α/β signaling, we used the neutralizing IFN-α/β receptor (CD118) Ab (anti-IFNR, clone MMHAR-2) versus the IgG2A control (both from R&D Systems, Inc.). The cells were preincubated with anti-IFNR or IgG at 50 to 100 μg/ml (10 μg/10^6 DCs) for 30 min at 4°C, before additional medium (±stimulus) was added for a final concentration of 10 μg/ml in culture.

Dextran uptake. DCs (5×10^4 cells in 50 μl) were kept on ice for 30 min prior to incubation with 10 ng/ml of Alexa Fluor 488-conjugated dextran (Molecular Probes/Invitrogen, Carlsbad, CA). DCs were then incubated at 4°C (binding) or 37°C (endocytosis) for 30 min. Cells were washed four times and fixed in 125 μl Cytofix (BD Pharmingen). Endocytosed dextran was calculated by subtracting the mean fluorescence intensities (MFIs) of Alexa Fluor 488 of cells incubated at 4°C from the MFIs of cells incubated at 37°C.

Cytokine and chemokine detection. Cell-free supernatants were collected after 3 to 24 h of culture with the indicated stimuli (2×10^6 DCs in 3-ml, six-well plate or 1×10^6 DCs in 1.5-ml, 12-well plate). IFN-α and -β were measured by enzyme-linked immunosorbent assay (ELISA) (Invitrogen/PBL). The IFN-α kit detects IFN-αA and IFN-α2a. Additional cytokines and chemokines were monitored using a Beadlyte 22-Plex detection system (Upstate, Lake Placid, NY). The kit detects the following cytokines/chemokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, CCL11, CXCL8, CXCL10, IFN-γ, GM-CSF, TNF-α, CCL2, CCL3, and CCL5. The plates were read on a Luminex 100 instrument (Luminex Corporation, Austin, TX), and data were acquired and analyzed using the STarStation software (Applied Cytometry System, Inc., Sacramento, CA).

Virus concentration from infected-cell supernatants. Virus was concentrated from supernatants of imDCs that had been pulsed with HIV (above) and recultured in the presence or absence of 2.5 μg/ml poly(I:C) for 7 days with minor modifications to the published protocol (66). Supernatants were first centrifuged for 20 min at 3,000 rpm to remove debris (unconcentrated supernatant [Sup]) followed by centrifugation for 2 h at 19,400 rpm in a Beckman SW28 rotor to pellet viral particles. Pellets were gently resuspended in Hanks balanced salt solution (Cellgro) and purified by centrifugation through a 20% sucrose cushion for 2 h at 21,000 rpm in a Beckman SW55Ti rotor. Pellets were gently resuspended in 200 μl Hanks balanced salt solution (concentrated supernatant [Conc]), and samples were stored at −80°C.

Q-PCR. imDC infection by HIV was verified by measuring the levels of HIV *gag* DNA after 5 days of infection (2×10^5 to 3×10^5 DCs in 200-μl, 96-well plate) by quantitative real-time PCR (Q-PCR) using an ABIprism 7000 sequence detection system (Applied Biosystems, Foster City, CA) (8). HIV copies were normalized on cell numbers by using Q-PCR for albumin DNA (19). In brief, cells were washed twice in Tris-buffered saline (Sigma Chemicals) and resuspended at 8×10^6 cells/ml in DNA lysis buffer (10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.45% Tween 20, 0.45% Igepal-630 [all from Sigma Chemicals]) containing 1 mg/ml proteinase K (Roche Biochemicals, Indianapolis, IN). Cells were lysed for 90 min at 56°C followed by a 10-min proteinase K inactivation step at 95°C. Q-PCR was performed on 5 μl of cell lysate for 40 cycles using the ABI master mix (TaqMan Universal PCR Master Mix; Applied Biosystems). Quantification of viral copy numbers was carried out as follows. Titrated doses of the 8e5 cell line that contains one copy of HIV per cell (21) were serially diluted into a constant genomic background of SUPT1/CCR5 CL.30 cells (kindly provided by James Hoxie, University of Pennsylvania). To quantify cell numbers, titrated numbers of uninfected SUPT1/CCR5 CL.30 cells were lysed as described above and diluted in lysis buffer.

HIV p24 ELISA. HIV production by imDCs (3×10^5 DCs in 200-μl, 96-well flat-bottomed plate) was quantified by an in-house p24 ELISA (68). Plates were read using a Luminoskan Ascent (Thermo Fisher Scientific, Inc., Waltham, MA) reader and analyzed using the Ascent software for Luminoskan (Thermo Fisher Scientific, Inc.).

A3G reverse transcription-PCR. Total RNA was extracted from 2×10^6 to 10×10^6 DCs that had been cultured for 24 h with stimuli using the RNeasy

Protect minikit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized in a 20-μl reaction mixture from 2 μg of RNA using random primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen). A3G and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were separately amplified from 2 μl of RNase H (Invitrogen)-treated cDNA using Hot Start *Taq* (Invitrogen). Primers for A3G were A3GF2 (5′-GGCTCCACATAAACACGG TTTC-3′) and A3GR2 (5′-CCCACTCAGGTCTTGGCTGTGC-3′) (49). Primers for GAPDH were GAPDHhuF (5′-CCTTGGAGAAGGCTGGGG-3′) and GAPDHhuR (5′-CAAAGTTGTCATGGATGACC-3′) (47, 48). Reaction conditions for both A3G and GAPDH were an initial denaturation at 95°C for 15 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension of 72°C for 10 min.

Protein detection by Western blotting. Cells (1×10^6 DCs in 1-ml, 24-well plate) were harvested and washed once with phosphate-buffered saline (Cellgro). DCs were respuspended in DNase reaction buffer (Promega, Madison, WI) to a concentration of 2×10^7/ml. Typically, an equal volume of 2× sodium dodecyl sulfate (SDS)-containing loading buffer (1% [vol/vol] SDS, 25% [vol/vol] glycerol, 125 mM Tris, pH 6.8, 1% [vol/vol] β-mercaptoethanol, and 0.1% [vol/vol] bromophenol blue) (all from Sigma Chemicals) was added to the lysates or cell culture supernatants. In order to degrade the genomic DNA, lysates were treated with 2 to 4 U of DNase (Promega) per 10^6 cells or per 25-μl volume of supernatant (representing 10^6 cells) for 15 min at room temperature followed by 10 min at 95°C. Cells (5×10^5) or supernatant (25 μl) was subjected to SDS-12% polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Whatman Inc., Florham Park, NJ). Western blotting for A3G was performed using a polyclonal anti-A3G Ab that recognizes the C terminus of the protein (NIH AIDS Research and Reference Reagent Program) that was detected with a horseradish peroxidase-conjugated anti-rabbit Ab (Promega). Western blotting for p24 capsid protein was performed using the anti-HIV p24 monoclonal Ab produced by the 183-H12-5C hybridoma (neat hybridoma supernatant; NIH AIDS Research and Reference Reagent Program), which was detected with anti-mouse Ig-horseradish peroxidase (Promega). Bands were visualized by using the ECL kit (Pierce, Rockford, IL). The amount of protein loaded was verified by blotting for β-actin (Abcam, Cambridge, MA).

A3G siRNA knockdown. A3G-specific small interfering RNA (siRNA) (si-A3G) was a mixture of three siRNAs targeting A3G (17, 49): siA3G-1 (5′-GCAUCGUGACCAGGAGUAU, dTdT-3′), siA3G-2 (5′-GCAACCAGGCUC CACAUAA, dTdT-3′), and siA3G-3 (5′-CCGCAUCUAUGAUGAUCAA, dTdT-3′). The control siRNA (si-Ctrl) was a nonspecific siRNA derived from siA3G-1 by a two-nucleotide inversion (5′-GCAUCGUGCACAGGAGUAU, dTdT-3′) (17). All siRNAs were synthesized by Dharmacon RNA Technologies (Chicago, IL). For proof-of-concept experiments, RNA interference was performed in 293 cells stably expressing A3G (293-A3G; NIH AIDS Research and Reference Reagent Program: 293-APOBEC3G-HA cell line). 293-A3G cells were transfected (2×10^5 cells/well, 24-well plate) on day 0 with 30 nM si-A3G (10 nM [each] A3G-specific siRNA) or 30 nM si-Ctrl, using 1 μl Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. At 48 h posttransfection, cells were harvested, and A3G knockdown was verified by Western blotting.

Monocytes were cultured for 4 days in GM-CSF/IL-4 (above) before being transfected (1×10^6 cells/well, 24-well plate) on day 4 and again on day 5 with 210 nM si-A3G (70 nM [each] A3G-specific siRNA) or 210 nM si-Ctrl, using 1.5 μl Lipofectamine RNAiMAX according to the manufacturer's instructions. Successful transfection was confirmed using the Block-iT Alexa Fluor red fluorescent oligonucleotide (Invitrogen). On day 6 (48 h after the first transfection) cells were harvested, pulsed with HIV, and recultured for 24 h (above). Knockdown was monitored 24 h later by Western blotting and densitometry analysis of A3G bands, and infection was assessed after 5 days by Q-PCR.

Velocity sedimentation of proteins. Native complexes of A3G in imDCs and 293-A3G cells were size fractioned by velocity sedimentation through sucrose as described previously (65). Cells were collected in phosphate-buffered saline and resuspended in ice-cold buffer (0.626% NP-40, 10 mM Tris acetate, pH 7.4, 50 mM potassium acetate, 100 mM NaCl, 10 mM EDTA prepared in 1× EDTA-free protease inhibitor cocktail) at a concentration of 6×10^7 cells/ml. Cell lysates were prepared by manual disruption through a 22-gauge needle and then clarified by low-speed centrifugation at $162 \times g$ for 10 min. For 293-A3G cells, lysates were then incubated for 10 min at 37°C in the presence or absence of 250 μg/ml RNase A (Novagen, EMD Biosciences, Gibbstown, NJ). Lysates were centrifuged again at $18{,}000 \times g$ for 30 s, and supernatants from this spin were loaded on discontinuous sucrose gradients containing 50%, 30%, 20%, 15%, and 10% sucrose and centrifuged at 41,500 rpm ($163{,}000 \times g$) for 45 min at 4°C in a Beckman Sw55 Ti rotor. Fractions were collected from the top of the gradient, and 40 μl of each was diluted in 3× SDS loading buffer and subjected to SDS-polyacrylamide gel electrophoresis and Western blotting for A3G. Larger (40-μl) sample volumes were examined to ensure that the smaller amounts of A3G protein in the lower fractions were detected.

Statistical analyses. Statistical analyses were performed using the nonparametric Wilcoxon matched pair test. Data were analyzed using SPSS software (Chicago, IL). Differences were considered significant when P was <0.05.

RESULTS

Poly(I:C) shuts down DC-driven HIV replication. As peripheral immunologic sentinels, imDCs are sensitive to a variety of pathogenic ligands. Typically pathogenic stimuli engage imDCs and trigger signaling that culminates in various degrees of DC maturation. During this process, the sentinel role of DCs is downregulated and, in its place, the capacity to engage and stimulate naïve and memory T cells is enhanced. Thus, our initial aim was to determine how stimulation via such ligands would impact HIV replication in imDCs. Two TLR stimuli, ssRNA and poly(I:C), which represent ssRNA viruses and dsRNA genomes or intermediates during the replication cycle of many pathogens, respectively, were tested against a mix of proinflammatory cytokines ("cocktail") known to terminally differentiate imDCs into mature DCs (22). The stimuli were added to HIV-pulsed imDCs, and their ability to replicate virus was measured by ELISA and Q-PCR.

Only poly(I:C) fully prevented HIV replication in the imDCs when added immediately after virus pulsing (Fig. 1A and B) [$P < 0.0001$, medium versus poly(I:C)]. Low, though significantly reduced, levels of infection persisted in the presence of ssRNA ($P < 0.03$, medium versus ssRNA), while cocktail stimulation had a more limited impact on infection ($P > 0.05$, medium versus cocktail). Although infection was reproducibly higher in the ssRNA-treated cultures, this was not significantly different from the negligible infections detected in poly(I:C)-treated cells ($P > 0.05$). In contrast, infection levels in the cocktail-stimulated cultures were significantly greater than those detected in poly(I:C) ($P < 0.03$) but not those seen in ssRNA ($P > 0.05$)-treated cells. Addition of poly(I:C) at later time points after virus exposure continued to significantly reduce HIV replication (data not shown and Fig. 4B) ($P < 0.03$ for 48 h, $P < 0.05$ for 60 h). Inhibition of infection was not a consequence of cell death in the presence of poly(I:C), and actually DC viability was slightly better in poly(I:C)-stimulated DCs (Fig. 1C) ($P > 0.05$). Closer examination confirmed that the supernatants and cells from the poly(I:C)-treated cultures contained no infectious virus and negligible amounts of p24 protein (Fig. 1D and E).

The differences in the abilities of the diverse stimuli to inhibit HIV replication could not be attributed to a particular lack of responsiveness of the DCs to the stimuli. Under the conditions tested, poly(I:C) effectively matured the DCs, upregulating CD25, CD80, CD83, and CD86 and downregulating CD206 and CD209 (Fig. 2), much like cocktail-matured DCs (see Fig. S1 in the supplemental material). ssRNA stimulation, which inhibited DC infection more effectively than did cocktail maturation but less than did poly(I:C), nevertheless upregulated the costimulatory molecule expression to a lesser extent (see Fig. S1 in the supplemental material). HIV capture by (0 h) or infection of (48 h) DCs did not affect the ability of the stimuli to modify these markers (Fig. 2; see also Fig. S1B in the supplemental material) (compare with the MV controls, which

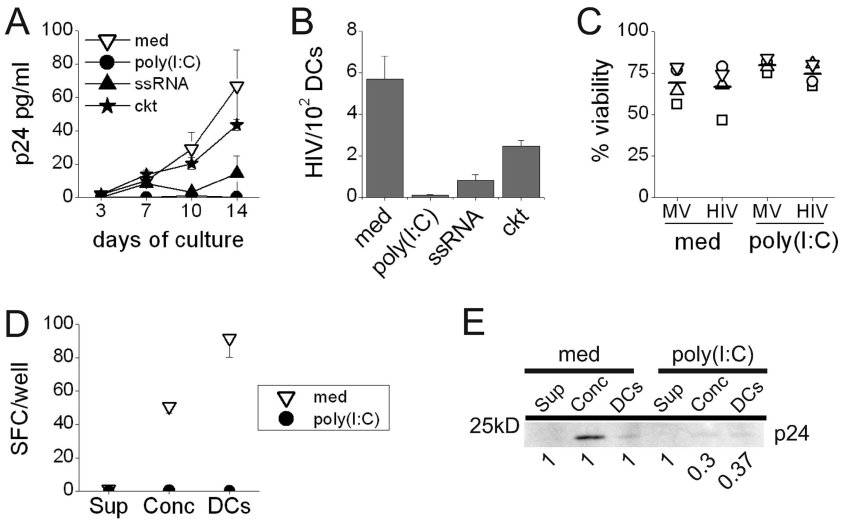

FIG. 1. Poly(I:C) stimulation inhibits HIV amplification in imDCs. (A) HIV p24 gag release by HIV-pulsed DCs cultured with 25 μg/ml poly(I:C) (●), ssRNA (▲), cocktail (ckt, ⋆), or medium (med, ▽). Mean (±SEM) p24 values are shown from three experiments [$P < 0.09$, $P > 0.05$, and $P > 0.05$ for medium versus poly(I:C)-, ssRNA-, or ckt-treated cells at day 14, respectively]. (B) Copies of HIV *gag* DNA as the mean (±SEM) number per 100 DCs are shown from 26 experiments comparing medium- and poly(I:C)-cultured cells, including five experiments where ssRNA and cocktail were tested in parallel. (C) DCs were pulsed with HIV or the no-virus MV control before being recultured in the presence or absence of 25 μg/ml poly(I:C). DC viability is shown as the percentage of viable cells (trypan blue exclusion) harvested after 14 days relative to the number of input cells from four independent experiments (the horizontal bar shows the mean). Each symbol represents a different donor. (D and E) Virus-loaded imDCs were cultured for 7 days in the presence or absence of 2.5 μg/ml poly(I:C), before the cells and supernatants were collected. The supernatants were assayed directly (Sup) or after concentration on a 20% sucrose cushion (Conc). (D) The presence of infectious virus was measured by culturing the supernatants (1:10 dilution in culture medium) or 10^5 DCs with TZM.bl cells. The numbers of spot-forming cells (SFC) per well are shown (mean ± SEM, two independent experiments). (E) Western blotting for HIV p24 gag protein was performed on the supernatants and DCs described for panel D. A representative blot from duplicate experiments is provided. The differences in the band intensities are indicated by the numbers under each lane, relative to the respective medium-treated control (set as 1 for each condition; note that no bands were evident in either of the unconcentrated supernatants).

represent nonviral components accruing during viral preparation from the producer cell). Virus capture or the low-level infection present after 48 h in the absence of other maturation stimuli did not lead to DC maturation. Endocytic activity was also evaluated as a functional measure of DC maturation after exposure of virus-loaded DCs to the different stimuli. Endocytic function was reduced by all stimuli (least effectively by ssRNA) as expected, but it was not influenced by the presence of HIV (Fig. 2; see also Fig. S1B in the supplemental material).

Poly(I:C) induces unique innate responses in DCs. Cytokines and chemokines are important in cell-cell communication for effective immunity, as well as in triggering cells to clear incoming pathogens. The triggering of imDCs by various pathogenic ligands may result in cytokine/chemokine secretion and be the key in molding a terminally differentiated DC. Thus, we compared the cytokines and chemokines produced in response to the tested stimuli to seek additional insight into how poly(I:C) maturation was more effective at limiting HIV replication (Fig. 3; see also Fig. S2 in the supplemental material). The rapid release of IL-1α, IL-10, TNF-α, IFN-γ, CCL2, CCL3, CCL5, and CXCL10 induced by poly(I:C) (versus ssRNA) is shown for HIV-pulsed cells in Fig. 3A. Cytokine/chemokine levels increased after 24 h of poly(I:C) stimulation, with the ssRNA- and cocktail-stimulated cells now producing some of these factors (often at lower levels) (Fig. 3; see also Fig. S2 in the supplemental material). Comparable responses were seen after poly(I:C) stimulation of 48-h-infected imDCs (see Fig. S2 in the supplemental material). While the presence

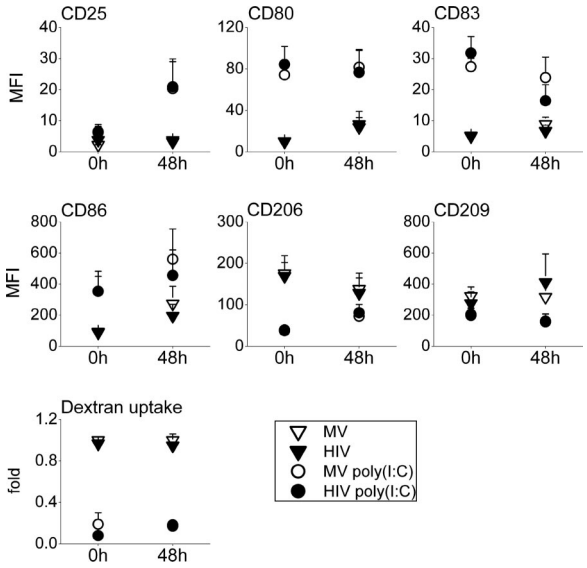

FIG. 2. HIV has minimal impact on DC membrane phenotype and endocytic function. DCs exposed to HIV (filled symbols) or the MV no-virus control (open symbols) were stimulated with 25 μg/ml poly(I:C) (circles) immediately (0 h) or 48 h after virus (versus MV) exposure (compared to nonstimulated controls [triangles]). Cells were harvested 48 h later and analyzed by flow cytometry. The MFIs (means ± SEMs) of the indicated markers expressed by the HLA-DR^+ DCs are summarized for nine donors (0 h) and five donors (48 h). Aliquots of cells were assessed for their ability to take up FITC-dextran. The MFI of FITC-dextran uptake by MV-loaded nonstimulated DCs was set as 1, and the reductions in FITC-dextran uptake by the variously treated cells are shown (means ± SEMs) for three donors (0 h) and four donors (48 h).

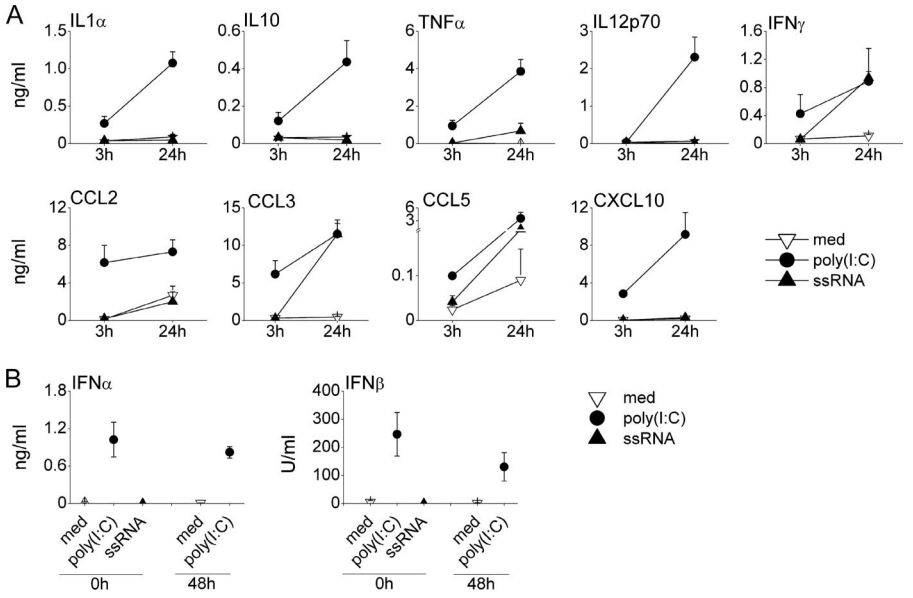

FIG. 3. Poly(I:C) induces rapid and unique cytokine and chemokine responses. (A) Time course of cytokine and chemokine secretion by HIV-exposed DCs immediately treated with 25 μg/ml poly(I:C) (●), ssRNA (▲), or medium (med, ▽). If chemokine levels were above standard range, a default value corresponding to the concentration of the highest standard was used; this may result in a relative underestimation of actual levels. Shown are the means (±SEMs) of factors released by the cells from four donors (3 h) and 15 donors (24 h). (B) IFN-α and -β production by HIV-loaded (0 h) or -infected (48 h) DCs after 24 h of stimulation as described for panel A. No IFN-α or -β production was detected after 3 h of culture (not shown). The mean (±SEM) values in ng/ml from six donors are shown.

of HIV had minimal impact on the majority of the cytokine and chemokine responses (versus the MV controls [see Fig. S2 in the supplemental material]), poly(I:C) induced more TNF-α ($P < 0.02$) and IFN-γ ($P < 0.003$) and ssRNA induced more IFN-γ ($P < 0.05$) release in HIV-pulsed DCs after 24 h (see Fig. S2 in the supplemental material [asterisks]). Notably, only poly(I:C) stimulated virus-loaded and virus-infected DCs to secrete considerable amounts of IFN-α and IFN-β (Fig. 3B), and HIV did not alter these responses (see Fig. S2 in the supplemental material).

Poly(I:C)-induced inhibition of HIV replication in DCs is mediated by type I IFNs. To determine if the type I IFN responses elicited by poly(I:C) contributed to the inhibition of infection, recombinant IFNs were added to virus-loaded DCs. Recombinant IFN-α and -β also inhibited HIV replication in the imDCs in a dose-dependent manner, comparable to the inhibition seen in poly(I:C)-treated cultures (Fig. 4A). As little as 0.01 ng/ml of the type I IFNs ($P < 0.003$ [IFN-α] and $P < 0.02$ [IFN-β]) or 0.25 μg/ml poly(I:C) ($P < 0.02$) was needed to significantly block HIV replication. Poly(I:C) and the type I IFNs were able to limit replication even when added after infection was established [Fig. 4B, $P < 0.004$ for 25 μg/ml poly(I:C), 1 ng/ml IFN-β, and 10 ng/ml IFN-α; 48 h]. The inhibition was less pronounced when the stimuli were added 60 h after infection, although the poly(I:C)-induced inhibition was still significant [$P < 0.05$ for poly(I:C) versus $P > 0.05$ for 1 ng/ml IFN-β and 10 ng/ml IFN-α]. Recombinant type I IFNs upregulated CD25, CD80, CD83, and CD86 expression, much as was observed with poly(I:C) treatment (Fig. 4C). Moreover, IFN-α and -β stimulated patterns of cytokine and chemokine release similar to those for poly(I:C) (always above the levels of medium-cultured cells), although they were typically of considerably lower magnitudes (Fig. 4D).

In an attempt to directly demonstrate the involvement of poly(I:C)-induced type I IFNs in the suppression of virus infection in the DCs, we used neutralizing Abs to impede type I IFN signaling of the DCs. A neutralizing Ab against IFN-α could not be used to dissect this biology; it inhibited HIV replication in imDCs (and TZM.bl cells) through an unrelated mechanism (see Fig. S3 in the supplemental material). Ab recognition of the immunosuppressive domain in gp41 of HIV (IS-D, Env amino acids 583 to 599) that has homologies to the receptor binding sites of IFN-α and -β (13, 75) might explain this interference. As an alternative, we tested the neutralizing anti-IFNR Ab. Addition of the anti-IFNR Ab to the virus-carrying DCs reduced HIV replication in some donors (8.15 ± 0.022 HIV copies/100 DCs in IgG-treated cells versus 4.6 ± 0.01 HIV copies/100 DCs in anti-IFNR-treated cells, mean ± standard error of the mean [SEM] from 15 donors), but this was not statistically significant ($P > 0.05$). This was likely due to (at most) partial DC activation via the IFNR (slight upregulation of CD80 and CD86 but no induction of cytokine/chemokine production [see Fig. S4 in the supplemental material]). Despite this, the anti-IFNR Ab was able to reverse the inhibitory effects of poly(I:C) and recombinant type I IFN on HIV infection (Fig. 4E). Although the levels of infection were variable and the dose of poly(I:C) at which the Ab reversed the block varied between donors, the anti-IFNR Ab reproducibly reversed the inhibition induced by poly(I:C) [Fig. 4F; $P < 0.05$ for 0.25 and 2.5 μg/ml versus $P > 0.05$ for 25 μg/ml poly(I:C), although the latter approached significance, $P < 0.07$].

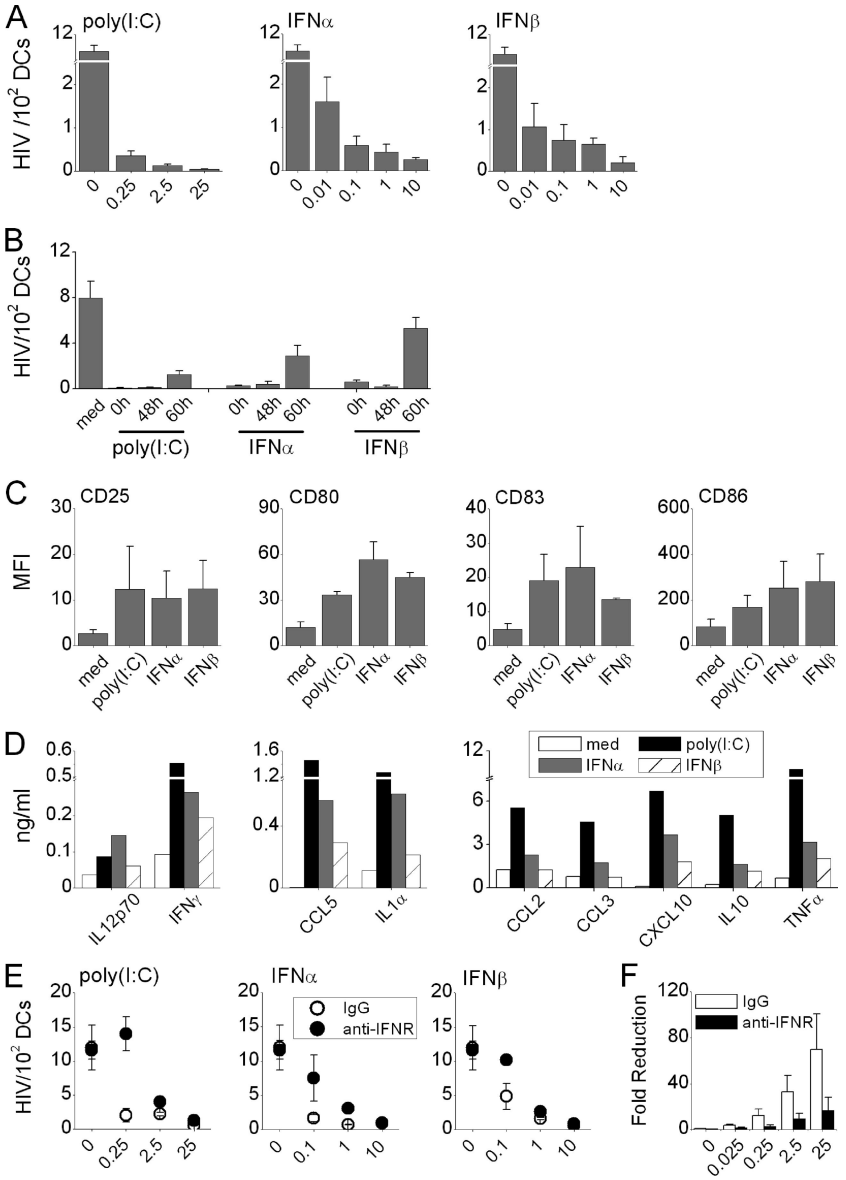

FIG. 4. Type I IFNs mimic poly(I:C), stimulating DCs and limiting HIV replication in DCs. (A) HIV-pulsed imDCs were incubated with poly(I:C) (0.25 to 25 μg/ml), IFN-α (0.01 to 10 ng/ml), or IFN-β (0.01 to 10 ng/ml), or not incubated (0), for 5 days before infection was measured by Q-PCR. The mean (±SEM) numbers of HIV copies per 100 DCs from at least six donors are shown. (B) Poly(I:C) (25 μg/ml), IFN-α (10 ng/ml), IFN-β (1 ng/ml), or medium was added to the cultures immediately (0 h), 48 h, or 60 h after virus exposure. HIV infection was measured 5 days after virus loading of the DCs. Data are shown from at least three donors (means ± SEMs). (C) The DC phenotype was monitored in HIV-pulsed DCs by fluorescence-activated cell sorting analyses after 48 h of culture with 25 μg/ml poly(I:C), 10 ng/ml IFN-α, or 10 ng/ml IFN-β (versus medium). The MFIs (means ± SEMs) of the indicated markers expressed by HLA-DR$^+$ DCs are summarized for four or five donors. Similar responses were seen after 24 h (data not shown). (D) Cytokine and chemokine production after 24 h was monitored in the cultures from panel C. Shown are data from one representative donor (out of four). (E) HIV-pulsed DCs were preincubated with 10 μg/ml anti-IFNR Ab or the control IgG before being cultured with the indicated doses of the respective stimuli [poly(I:C), μg/ml; IFNS, ng/ml]. Infection was assessed after 5 days by Q-PCR for HIV gag. Mean (±SEM) numbers of HIV copies/100 DCs are shown from a representative experiment [which is typical of five donors for poly(I:C) and three donors for the IFNs]. (F) The reductions of infection by various doses of poly(I:C) (relative to the medium-cultured cells, set as 1) in the presence or absence of the anti-IFNR Ab were calculated. Mean (±SEM; five donors) data are summarized.

Poly(I:C) induces unique expression of small A3G proteins via type I IFNs. Type I IFNs were recently shown to increase the expression of A3G in macrophages coinciding with their diminished susceptibility to HIV infection (47, 48). To determine if the poly(I:C)-induced type I IFNs increased A3G expression in DCs, A3G mRNA levels were measured after stimulation with poly(I:C) and compared to those in cells stimulated with ssRNA or cocktail (Fig. 5A and B). A3G mRNA was elevated most strongly in poly(I:C)-treated cells, although this was not statistically significant ($P > 0.1$) from untreated cells (Fig. 5B).

Although there were no significant changes in A3G mRNA

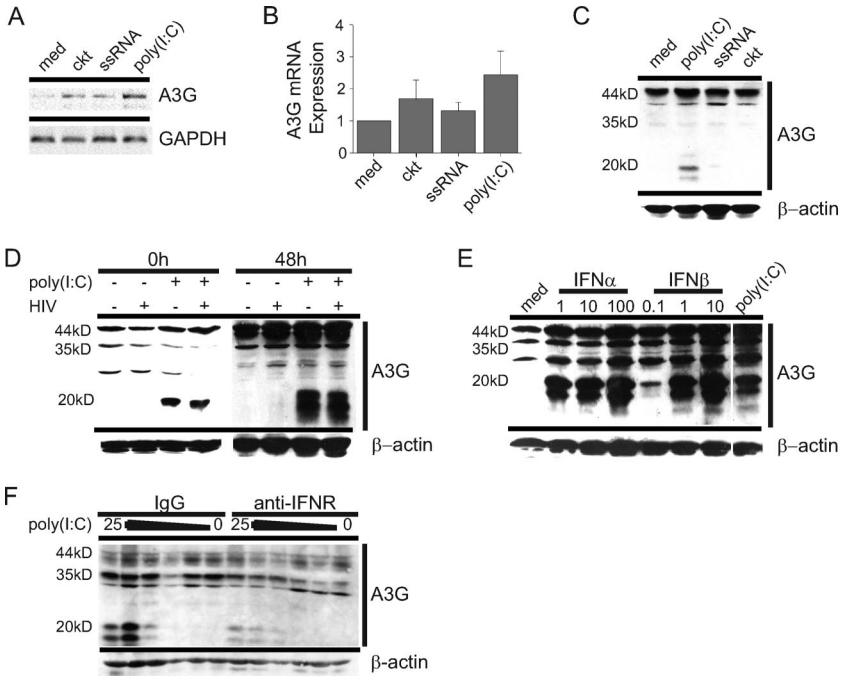

FIG. 5. Poly(I:C) and type I IFNs induce the appearance of small A3G proteins in imDCs. (A and B) imDCs were cultured for 24 h in the presence of 2.5 μg/ml poly(I:C), ssRNA, or cocktail (ckt) (versus medium only [med]), before the levels of A3G mRNA were measured by reverse transcription-PCR. (A) A representative gel showing the levels of A3G mRNA (versus GAPDH) is provided. (B) The A3G band intensity of each sample was normalized to the respective GAPDH control, and the values for the stimulus-treated samples were then compared to the medium-treated cell control (set as 1) from the same donor to determine the relative A3G mRNA expression. The mean (±SEM) values for three or four donors are provided. (C) HIV-loaded DCs were cultured with 25 μg/ml poly(I:C), ssRNA, cocktail (ckt), or medium (med) for 24 h. Cells were lysed, and the total cell lysates were run on an SDS gel before being transferred to a nitrocellulose membrane. Western blotting for A3G was performed, and the membranes were reprobed for β-actin to ensure that comparable numbers of cells were loaded for each lane. A representative blot of three is shown. (D) Poly(I:C) (25 μg/ml) was added immediately (0 h) or 48 h after HIV (+) versus MV (−) loading of imDCs, and the cells were cultured for 12 h before being processed for Western blotting. A representative result from four independent experiments is shown. (E) HIV-pulsed DCs were stimulated for 24 h with 25 μg/ml poly(I:C), 1 to 100 ng/ml IFN-α, and 0.1 to 10 ng/ml IFN-β (versus medium only). Western blotting was performed, and a blot representative of at least three experiments is provided. (F) DCs were incubated for 24 h with 10-fold dilutions of poly(I:C) (from 25 to 0.0025 μg/ml, versus 0 μg/ml) in the presence of the anti-IFNR Ab or the IgG control. A representative A3G Western blot of two replicate experiments is shown. The A3G proteins (~44-kDa monomer band, 30- to 35-kDa bands, and the small 15- to 17-kDa bands) and β-actin are shown in each Western blot (C to F).

after poly(I:C) stimulation, we examined A3G protein expression by Western blotting since earlier reports indicated increased A3G expression in DCs after poly(I:C)/TNF-α (62) or lipopolysaccharide (LPS) (49) activation. Expression of the ~44-kDa A3G monomer appeared unchanged after exposure to any of the stimuli tested (Fig. 5C). Interestingly, exposure to poly(I:C), but not ssRNA or cocktail, induced the appearance of 15- to 20-kDa A3G protein bands (one to three bands in this size range) (Fig. 5C). As little as 12 h post-poly(I:C) stimulation was sufficient for the appearance of the small A3G proteins (see Fig. S5A in the supplemental material). The small A3G bands were similarly detected in virus-loaded (0-h) or -infected (48-h) DCs cultured with poly(I:C) (Fig. 5D). Poly(I:C) did not induce the appearance of these bands in CD4 T cells (data not shown). Both type I IFNs also induced the appearance of the 15- to 20-kDa A3G proteins in imDCs (Fig. 5E), and as expected, the appearance of the small A3G proteins occurred rapidly (within 6 h) upon direct exposure to the IFNs (see Fig. S5A in the supplemental material). The anti-IFNR Ab blocked the appearance of the small A3G bands induced by poly(I:C) and type I IFNs (Fig. 5F; see also Fig. S5B in the supplemental material), especially at lower doses of poly(I:C). In the presence of 25 μg/ml of poly(I:C), anti-IFNR Ab induced at least partial reductions in the intensity of the small A3G bands in half of the donors tested (8 of 16 donors; Fig. S5B in the supplemental material shows a nonresponsive donor, and Fig. 5F shows a responsive donor), paralleling the less complete effect of the anti-IFNR Ab at reversing the inhibition of infection at the higher poly(I:C) doses. Appearance of the small A3G bands reproducibly corresponded to poly(I:C)- or IFN-induced inhibition of HIV replication in the DCs.

To further explore the potential involvement of A3G in the poly(I:C)-mediated inhibition of infection, we attempted to knock down A3G expression using siRNA. A3G knockdown by siRNA was first verified in 293-A3G cells (Fig. 6A). Following a recently published protocol (49), DCs were transfected with A3G-specific or control siRNA (versus the nontransfected control), and the impact on A3G expression and HIV replication was measured. Unfortunately, A3G-specific and control siRNA activated the DCs (consistent increase of CD80 and CD86 expression; data not shown) and induced the expression of the small A3G bands (Fig. 6B and C) (compared to the nontransfected control). A3G expression was reduced in the

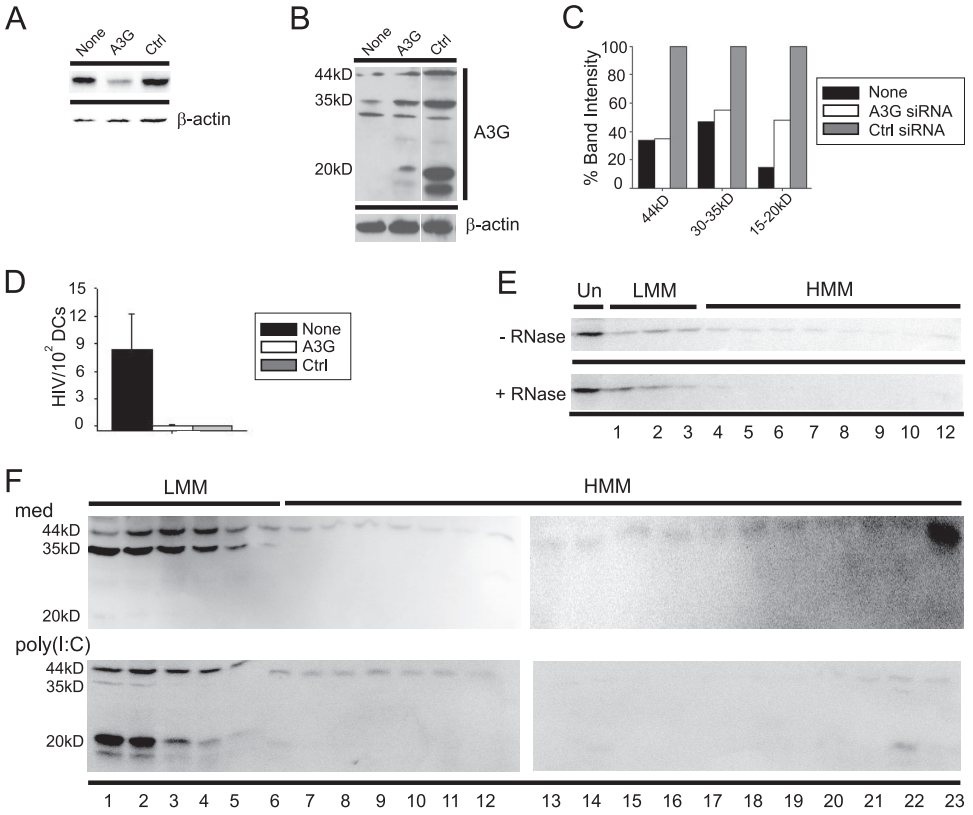

FIG. 6. Poly(I:C) increases the expression of A3G LMM complexes. (A) 293-A3G cells were transfected with control (Ctrl) versus A3G siRNA, and 24 h later the knockdown of A3G protein was monitored by Western blotting. Nontransfected cells were included as an additional control (None). A representative blot (of two) is provided. (B and C) imDCs were transfected twice, 24 h apart, with A3G versus Ctrl siRNA or not (nontransfected control) and then cultured for 24 h more. (B) An A3G Western blot assay was performed on the cells, and a typical result (from three identical donors) is shown. (C) The percent expression of A3G was determined by comparing the band intensities of the A3G ~44-kDa monomer and 30- to 35-kDa and 15- to 20-kDa bands in the A3G siRNA-transfected and nontransfected controls to the Ctrl siRNA-transfected sample (set as 100%). (D) imDCs transfected with A3G-specific versus Ctrl siRNA (or not [None]) were pulsed with HIV_{Bal} and then cultured for 5 days. HIV infection was measured by Q-PCR, and the numbers of HIV copies per 100 cells are shown (mean ± SEM, seven donors). (E) 293-A3G cells were lysed, and the extracts were treated with RNase A (+) or not (−) prior to being run over a velocity sedimentation gradient. Each 400-μl fraction (1 to 12, top to bottom of the gradient) was then analyzed by A3G Western blotting. Unfractionated samples were included as additional controls (Un). One of two similar blots is shown. (F) Extracts of imDCs cultured in medium (med) or 25 μg/ml of poly(I:C) were separated by velocity sedimentation, and 23 200-μl fractions were collected (1 to 23, top to bottom of the gradient). The presence of A3G proteins in each fraction was monitored by Western blotting. Shown is a representative blot of four to six donors [using pooled cells from three donors in the example shown; representative of six donors for poly(I:C)-stimulated cells and four donors for medium-treated cells]. Faint bands denoting the A3G monomers were detected in the lower 12 fractions of medium- but not poly(I:C)-treated cells.

A3G siRNA-transfected cells compared to that in cells transfected with control siRNA, although some A3G expression persisted due to the nonspecific effect of siRNA on DCs. Just as we saw with poly(I:C)- or IFN-treated cells, the presence of the small A3G bands in the transfected cells correlated with inhibition of infection (Fig. 6D). Supporting the previous report using vesicular stomatitis virus (VSV) glycoprotein-pseudotyped HIV (49), slightly higher levels of infection were observed in the A3G-specific siRNA-transfected imDCs in two of the seven donors tested (0.27 versus 0 DNA copies/100 DCs for A3G-specific versus control siRNA), paralleling the partial reduction in the A3G levels by the A3G-specific siRNA in the face of the nonspecific DC activation by the siRNAs. However, this was significantly less than the virus replication detected in the nontransfected controls. Thus, it was impossible to use siRNA to reverse the antiviral effects of poly(I:C). Notably, the induction of the small A3G bands and inhibition of imDC infection by siRNAs supported the innate dsRNA-triggered mechanism described herein.

Because siRNA knockdown could not be used to demonstrate A3G involvement in the poly(I:C)-mediated inhibition of HIV replication in DCs, we examined whether poly(I:C) increased the amount of A3G in the active LMM complex in DCs by velocity sedimentation (65). Velocity sedimentation allows the identification of A3G proteins in the LMM versus high-molecular-mass (HMM) complexes, and this was first confirmed using 293-A3G cells treated or not with RNase A (Fig. 6E). Using this approach, the previously reported dominance of LMM A3G complexes in imDCs (49) was documented, with smaller amounts of A3G monomer being detected in the HMM complex-containing fractions of medium-treated imDCs (Fig. 6F). Treatment with poly(I:C) shifted the expression of A3G to the LMM complex form, with the A3G monomer primarily being detected within the top fractions (fractions

1 to 12 instead of throughout fractions 1 to 23 [Fig. 6F]), much like the RNase A-treated 293-A3G cells. The poly(I:C)-induced small A3G bands were detected in the first six fractions.

DISCUSSION

imDCs are a critical player in the onset of HIV infection across the body surfaces (43, 67). imDCs likely provide one of the first sites of HIV replication in vivo before virus is handed over to CD4 T cells, where robust virus amplification occurs (41, 70, 71). Identifying ways to trigger innate responses to prevent imDC-driven HIV amplification represents a potential strategy to limit HIV infection and possibly overcome HIV-induced immune dysfunction. To investigate the potential of TLR ligands to limit DC-driven HIV spread, we studied the effect of the TLR7/8 and TLR3 stimuli ssRNA40 and poly(I:C), which are known to elicit antiviral responses (56, 57). The TLR4 ligand LPS, although activating DCs, was not considered due to the limitations in ultimately extending this to the in vivo settings. Unlike most previous studies demonstrating minimal infection of already matured DCs (5, 9, 29, 70), we specifically evaluated the impact of these stimuli on virus replication when the stimuli were applied to imDCs after virus exposure. This would determine if we could activate innate imDC responses that could be effective in limiting HIV replication while boosting immune functions during the earliest stages of infection.

Only poly(I:C) reproducibly inhibited HIV replication in imDCs when added up to 48 h after virus exposure and still significantly reduced infection when added 60 h after exposure. The more potent activity of poly(I:C) than of recombinant type I IFNs suggests that type I IFN-independent mechanisms might also be involved (38), particularly at the later time point. These findings are consistent with results from the work of Sanghavi and Reinhart, who showed that TLR3 ligation through poly(I:C) impeded virus replication in lymphoid tissues isolated from simian immunodeficiency virus-infected macaques and in human cell lines (56). In contrast, ssRNA only partially limited HIV replication in imDCs, and this appeared to be independent of type I IFNs. TLR7/8 stimulation and its subsequent effect on HIV replication depend on the cell type and on the replication cycle of HIV (57). Specifically, treatment of human peripheral blood mononuclear cells with ssRNA40 prior to HIV infection significantly limited virus replication, while latently infected monocytoid-like cell lines showed enhanced HIV replication in response to TLR7/8 stimulation using imidazoquinoline R-848. Moreover, single-stranded siRNA derivatives, unlike the double-stranded siRNAs, were unable to support DC activation (37). This is in agreement with our observations of more-effective activation of innate DC responses by dsRNA to limit HIV spread. Although potently activating the DCs, cocktail maturation had the least impact on virus replication in the DCs. We recently reported a similar persistence of virus replication in DCs maturing in response to *Candida albicans*, which ultimately facilitated enhanced spread upon T-cell contact (72). These data underscore how different stimuli uniquely impact the fate of HIV in DCs and how critical it is to completely shut down HIV replication in imDCs.

Poly(I:C), but not ssRNA or cocktail, induced rapid cytokine and chemokine responses. Though HIV had minimal impact on poly(I:C)-, ssRNA-, or cocktail-induced DC activation after 24 h, HIV-pulsed (not infected) DCs produced significantly more IFN-γ in response to poly(I:C) or ssRNA and even secreted small amounts of IFN-γ when cultured in medium alone (compared to MV-treated cells). This suggests that the DCs recognized and responded to the viral particles (but not the integrated virus), highlighting the limited direct activation of DCs by HIV, which would continue to enable virus replication in the absence of an additional stimulus (31). It is unlikely that the IFN-γ secretion was due to contaminating T cells since there was $<0.9\%$ T cells in these cultures. IFN-γ production by DCs has been detected in response to intracellular pathogens and certain cytokines (24, 25, 27). The early (IFN-γ and TNF-α) and later, more-potent (IL-12 p70, IFN-γ, and TNF-α) responses induced by poly(I:C) might contribute to the more effective DC maturation (7, 36, 46, 62), resulting in efficient inhibition of infection. A recent report highlighted that poly(I:C) was able to boost mucosal HIV-specific CD4 T-cell responses in mice when antigen was targeted to DCs by Abs specific to CD205 (69). Thus, poly(I:C)-stimulated imDCs would ultimately favor the activation of stronger HIV-specific T-cell responses to further help control HIV spread (26).

Unique to poly(I:C) and type I IFN stimulation was the appearance of small A3G proteins and inhibition of HIV replication in imDCs. IFN-α-induced expression of CD317 (tetherin) has been shown to correlate with decreased HIV infection by sequestering viruses within endocytic compartments and at the cell surface (45). In this study, CD317 expression (surface and intracellular) by DCs was unchanged after stimulation with poly(I:C), ssRNA, or cocktail (for 24 or 48 h; three donors, data not shown), indicating that an IFN-α-induced CD317 mechanism of virus retention by the DCs was not responsible for the antiviral effects of poly(I:C). Poly(I:C)- and type I IFN-induced inhibition of infection and the appearance of the small A3G proteins were reversed by neutralizing the IFNR. The low-level triggering by the anti-IFNR Ab seen in some donors coincided with partial DC activation and some restriction of HIV replication, underscoring the involvement of the type I IFN-mediated signaling in limiting HIV growth.

The antiviral function of type I IFNs is at least partially due to the induction of proteins with specific antiviral activity (54, 59). While type I IFNs do not have an effect on A3G expression in the H9 T-cell line (51) or in CD14-depleted peripheral blood mononuclear cells (62), IFN-α is a potent inducer of A3G in hepatocytes (64) and macrophages (47, 48, 62), leading to a reduction of HIV replication (47, 48). The antiviral activity of A3G requires the shift from HMM to LMM forms of A3G (12, 17). imDCs were found to predominantly express A3G in LMM form, rendering them less permissive to infection than other cell types (49). DC activation with poly(I:C)/TNF-α (62) or LPS (49) was shown to further increase the levels of LMM A3G. We showed that, coincidentally with the appearance of novel small A3G proteins in the LMM fractions, poly(I:C) alone also induced the shift from the HMM to LMM form of A3G, even though the levels of A3G mRNA or monomer expression were unchanged. These small A3G proteins were not induced by other stimuli that we tested, ruling out the possibility that the 15- to 20-kDa fragments are simply degradation products of proteins from maturing DCs per se. Additional controls confirmed that the 15- to 20-kDa proteins were

detected only with the polyclonal anti-A3G Ab and not with control Abs against RNase A and Trim5α (completely negative; data not shown), Abs against RNase L (single ~85-kDa band; not shown), or Abs against β-actin (single ~43-kDa band, shown as loading controls). This demonstrates that the 15- to 20-kDa proteins were specific A3G epitopes, and the appearance of these bands was not due to nonspecific binding of the anti-A3G Ab. This was further supported by the reduction in these proteins following A3G siRNA knockdown. These small A3G proteins are likely cleaved A3G monomer and represent a novel marker of the HMM-to-LMM shift. Interestingly, cleavage of A3G has been reported and correlated with loss of antiviral activity (1).

Knockdown of A3G expression by siRNA could not be utilized to further dissect the involvement of A3G in this biology since siRNA itself activated the DCs, inducing the appearance of small A3G proteins and inhibiting HIV replication. This contrasts with a report that A3G siRNA knockdown makes imDCs more susceptible to HIV infection (49). It is difficult to appreciate if there was a nonspecific siRNA effect in their hands, as nontransfected controls were not shown. In our hands, slightly higher replication in A3G-specific siRNA-treated cells was seen only in two of seven donors, compared to control siRNA. Moreover, Pion et al. utilized VSV glycoprotein-pseudotyped HIV, which results in considerably higher infection frequencies that likely revealed low-level infection that might have been missed herein using HIV_{Bal}. It is also possible that nonspecific siRNA-mediated activation of DCs has more impact on HIV envelope-mediated entry than on the endocytic entry by VSV-pseudotyped viruses. Our data do, however, support an earlier observation that transfection of imDCs with siRNAs resulted in DC activation while the ssRNA derivatives of those siRNAs did not (37). Together, these observations support the idea that dsRNA-induced responses in imDCs prevent HIV replication.

Clinical-grade TLR3 ligands are being considered as immunostimulatory and antiviral agents for in vivo applications (6, 10, 52, 53). Early studies demonstrated promise for poly(I:C) treatment in reducing the viral burden in HIV-infected individuals (10, 52). Moreover, the antiviral effects of poly(I:C) were found to reduce the amounts of AZT required to limit HIV replication in vitro (42), and poly(I:C) was active against drug-resistant strains of HIV (20). With the advent of highly active antiretroviral therapy (HAART) in more recent years, additional antiviral strategies such as this may prove even more effective in helping to control infection while boosting immune function. A recent report described how combined HAART and IFN-α treatment of acute HIV infection elicited stronger anti-HIV Ab and innate responses and better virus control upon ceasing HAART (2). Above all, our data suggest that type I IFN-mediated inhibition by poly(I:C) would be particularly effective during the earliest stages of HIV infection to prevent infection of imDCs. If poly(I:C) was applied too late, imDC infection would be reduced, but the persistence of low-level viral replication might continue to provide an infectious source of virus to spread to neighboring T cells (23, 70–72, 74). Moreover, the activation of DCs without completely shutting down virus replication would further enhance DC-mediated transmission of HIV to T cells (55, 70, 72, 74). Poly(I:C)-mediated activation through type I IFNs may be especially advantageous following mucosal (rather than systemic) application of poly(I:C) (35, 73). Thus, poly(I:C) might have most potential as a topical strategy that could be applied immediately within 24 h after exposure to curb local spread. In addition, the ability of TLR3 ligation to limit herpes simplex virus infection (4, 28, 33, 63, 76) would also help to control the spread of HIV (11).

These data demonstrate that poly(I:C) triggers unique innate mechanisms within imDCs that limit HIV replication, even after infection has been initiated. This is largely mediated through type I IFNs, although additional IFN-independent mechanisms may also contribute. Interestingly, this coincided with the presence of small A3G proteins that reflected the increased LMM A3G expression. Thus, the appearance of these small A3G proteins represents a novel marker of poly(I:C)-mediated DC activation and correlates with DC resistance to HIV infection. The combined immunostimulatory and antiviral effects of poly(I:C) on imDCs suggests that this represents a promising strategy that could be employed in vivo to limit early mucosal DC-driven HIV dissemination, while augmenting the innate and adaptive immune activities needed to control HIV.

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None of the authors have any financial conflicts of interest.

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