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Endogenous IFN-α Production by Plasmacytoid Dendritic Cells Exerts an Antiviral Effect on Thymic HIV-1 Infection

Kevin B. Gurney, Arnaud D. Colantonio, Bianca Blom, Hergen Spits and Christel H. Uittenbogaart

Plasmacytoid dendritic cells (pDC) are the principal producers of IFN-α in response to viral infection. Because pDC are present in the thymus, we investigated the consequences of HIV-1-induced IFN-α production by thymic pDC. We observed that thymic pDC as well as thymocytes express intracellular IFN-α upon infection with HIV-1. However, only the pDC could suppress HIV-1 replication, because depletion of pDC resulted in enhancement of HIV-1 replication in thymocytes. Thymic pDC could also produce IFN-α in response to CpG oligonucleotides, consistent with the observations of others that peripheral pDC produce IFN-α upon engagement of TLR9. Importantly, CpG considerably increased IFN-α production induced by HIV-1, and addition of CpG during HIV-1 infection enhanced expression of the IFN response protein MxA in thymocytes and strongly reduced HIV-replication. Our data indicate that thymic pDC modulate HIV-1 replication through secretion of IFN-α. The degree of inhibition depends on the level of IFN-α produced by the thymic pDC. The Journal of Immunology, 2004, 173: 7269–7276.
mAb CCR5-FITC, CXCR4-allophycocyanin, CD123-PE, and IgG1-biotin were obtained from BD Pharmingen (La Jolla, CA). Mouse anti-human biotinylated IFN-α mAb (IgG1 κ, clone 7N41) was used for intracellular staining was obtained from Endogen (Woburn, MA). Neutralizing mAb to IFN-α (IgG1 κ, clone KHC4012) was obtained from BioSource International (Camarillo, CA). mAb to MxA was a gift from Dr. O. Haller (University of Freiburg, Freiburg, Germany). CPG sequences 2243 (ggGGGAGCATGCGTgggggg) and 2216 (ggGGGACGTGCGTggggggG; CPG-A) were obtained from Oligo, Inc. (Mississippi). Amino acid sequences of actinomycin D and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich (St. Louis, MO). Actinomycin D was obtained from Roche (Indianapolis, IN); paraformaldehyde was purchased from Polysciences (Warrington, PA). Recombinant human IFN-α was obtained from BioSource International, and recombinant human IL-7 was a gift from Immunix (Seattle, WA).

**Thymocyte and thymus organ cultures**

Normal human postnatal thymus specimens were obtained from children undergoing corrective cardiac surgery. Thymocytes were prepared and cultured, as previously described, in serum-free medium consisting of IMDM (Omega Scientific, Tarzana, CA) supplemented with delipidated BSA (Sigma-Aldrich) at 1100 μg/ml, transferrin (Sigma-Aldrich) at 85 μg/ml, 2 mM glutamine, and penicillin/streptomycin at 25 U/25 μg/ml. Thymocytes were cultured at 1–2 × 10^7 cells/ml in serum-free medium as pellet cultures at 37°C in 5% CO2, in round-bottom tissue culture tubes in the presence or the absence of IL-7 (200 U/ml).

A modification of the protocol described by Galy et al. (39) was used for the postnatal thymus organ culture (40). Thymus pieces were excised from postnatal specimens and cultured on floating rafts consisting of size 4 absorbable gelatin sponges (Gelfoam; Pharmacia & Upjohn, Kalamazoo, MI) covered with 0.8-μm nitrocellulose disks. Thymus organ cultures were maintained at 37°C in 5% CO2 in 24-well plates containing serum-free medium.

**Isolation of pDC**

The pDC from thymus specimens were purified by MACS using an LS positive selection column on the VarioMAX magnet or AutoMACS according to the manufacturer’s guidelines (Miltenyi Biotec, Auburn, CA). For pDC enrichment, we used rat anti-mouse IgG1 microbeads (Miltenyi Biotec) to select pDC labeled with mouse anti-human CD123 Ab (BD Pharmingen). The purity ranged from 1–20%. For in vitro infection of highly purified pDC, the magnetically enriched pDC population was sorted on the FACSStain GelFlow FACs (BD Immunocytometry Systems) to >98% purity by CD3−, CD123+5%, CD45RA− sorting parameters.

**HIV-1 infection of thymus organ cultures and thymocytes**

The CXCR4 tropic hybrid molecular clone HIV-1NL4-3 (NL4-3) was used for part of these studies (41). Virus stocks were prepared from 24-h harvests of supernatants from two sources: 1) CEM cells (CCRF-CEM) infected with virus derived from COS cells electroporated with plasmid pNL4-3, and 2) virus propagated in an allogeneic pool of three donor-derived peripheral blood CD4+ T cells generated in IL-2-stimulated RPMI 1640 medium with 10% FCS as described previously (42). The CCR5-tropic molecular clone HIV-1JR-CSF (JR-CSF) stocks were prepared from 24-h harvests of supernatants from IL-2-stimulated PBMC infected with the supernatant of COS cells electroporated with plasmid pYKJR-CSF (43), and virus was propagated on a CD4 allogeneic as described above. Virus Stocks were stored at −70°C and were treated with 2 μg/ml DNase (Worthington Biochemical, Lakewood, NJ) for 45 min at room temperature in the presence of 0.01 M MgCl2 before infections. All infections were standardized by determining infectious units (i.u.) in limiting dilution studies using PHA-stimulated PBMC (44).

Thymocytes were infected and cultured as previously described, but without polybrene (38). Briefly, freshly isolated thymocytes were incubated with 300–500 i.u. of NL4-3/1 × 10^6 thymocytes. Control thymocytes were mock-infected with supernatants from the same uninfected cells used to prepare the virus stocks. After infection, the cells were washed and resuspended in serum-free medium in the presence of IL-7 (200 U/ml). The medium was changed on day 1 postinfection and every 5–7 days thereafter. Virus replication was assessed by measuring p24 Ag in the supernatant by a specific p24 Ag ELISA (Coulter, Hialeah, FL). When neutralizing mAb to IFN-α was used to determine its effect on virus replication, thymocytes were cultured for 3 days after HIV-1 infection in the presence of 2 μg/ml neutralizing mAb, and culture was continued for the remainder of the experiment without mAb.

Thymus organ cultures were maintained in 24-well plates containing serum-free medium at 37°C in 5% CO2. For infection of thymus organ cultures, virus (300–500 i.u./well) or mock-infected supernatants were added to the tissues, and the cultures were incubated for 2 h, at which time the supernatants were removed. Five hundred microliters of serum-free medium was added to each well after the infection. The medium was changed on day 1 postinfection and every 2 days thereafter. Viral replication was assessed in the supernatant of individual wells by measuring p24 Ag. To test the effect of CpG, thymus pieces were cultured for 24 h before HIV-1 infection with CpG or exogenous IFN-α, which were added to the cultures after infection.

**Stimulation of IFN-α production by pDC**

IFN-α production of in vitro-infected thymus tissues was measured in supernatants of thymus organ cultures mock-infected or infected with HIV-1 NL4-3 (in quadruplicate) in the presence or the absence of CpG (6 μg/ml). Supernatants were harvested every 2 days of culture for the determination of IFN-α production and p24 Ag. IFN-α production was measured in the supernatant using the human IFN-α ELISA kit (KHC4012; BioSource). Cultures supplemented with rIFN-α were used as a positive control.

**Immunofluorescent intracellular staining and flow cytometry**

Surface immunophenotyping of purified human thymocytes with directly conjugated mAb and intracellular staining for HIV-1-Gag and IFN-α protein was performed in combination with cell surface staining as previously described (47, 48). For intracellular IFN-α staining, cells were incubated with 1 μg/ml brefeldin A (BD Pharmingen) for 5 h before intracellular staining. Briefly, cells were surface-immunophenotyped and fixed in 1% paraformaldehyde. Fixed cells were subsequently permeabilized in 0.2% FACS buffer. Fixed cells were subsequently permeabilized in 0.2% Tween 20 for 15 min at 37°C, washed with PBS containing 2% newborn calf serum and 0.1% sodium azide (FACS buffer), and blocked with human AB serum. For intracellular detection of HIV-1-Gag, cells were stained with 5 μg of fluorescent KC57 or IgG control; for intracellular detection of IFN-α protein, cells were stained with 2 μg of biotinylated mouse anti-human IFN-α or control biotinylated IgG1. For biotinylated mAb-stained conditions, cells were washed in 0.2% Tween 20 and stained with the second-step streptavidin-allophycocyanin. Finally, cells were washed with 0.2% Tween 20 and resuspended in FACS buffer before acquisition on a dual-laser FACS Calibur flow cytometer (BD Immunocytometry Systems).

Combined surface and intracellular staining using the unconjugated mAb to MxA was performed in a different order; 0.5–1 × 10^6 cells were fixed in 1% paraformaldehyde, permeobilized with 0.2% Tween 20, and stained intracellularly with a 1/500 dilution of mAb to MxA or isotype control Ab for 30 min at 4°C. The cells were washed with 0.2% Tween 20, and goat anti-mouse FITC mAb was added at 10 μg/ml and incubated for 30 min at 4°C. Cells were washed with 0.2% Tween 20 and incubated for 20 min with 50 μl of a 1/15 dilution of normal mouse IgG (Caltag Laboratories). FITC-conjugated mAb were added for 20 min at 4°C and washed with 0.2% Tween 20. Cells were finally resuspended in FACS buffer before acquisition on a dual-laser FACS Calibur flow cytometer (BD Immunocytometry Systems). Multiparameter data acquisition and analysis were performed with CellQuest software (BD Immunocytometry Systems).

**DNA isolation and PCR**

DNA was isolated 18–24 h postinfection from thymus infection-in vitro with JR-CSF or SFN-X by phenol/chloroform extraction and ethanol precipitation as described previously (49). Semiquantitative DNA PCR for viral genomes was performed as previously described (49), with HIV PCR undergoing 35 rounds of amplification. PCR products were resolved on a 1% polyacrylamide gel and visualized on an ultraviolet transilluminator (Molecular Dynamics, Sunnyvale, CA). The phosphorus-32 content was determined by acquiring data on the STORM850 PhosphorImager and analyzing with IMAGEQUANT software (Molecular Dynamics).
Statistics

The unpaired two-tailed Student’s t test with unequal variance was used to compare differences in IFN-α expression in HIV-1-infected and mock-infected thymocytes and pDC, and p < 0.05 was considered significant.

Results

Plasmacytoid DC delay viral replication in HIV-1-infected thymocytes

Because peripheral blood pDC are decreased in number and function in HIV-infected patients, and their decrease is correlated with disease progression (50–54), pDC may play an important role in controlling HIV infection. To evaluate whether pDC in the thymus play a similar role, thymocytes were depleted of pDC by AutoMACS. The purity of the thymocytes depleted of pDC was determined by phenotyping (0% CD123+/hlgCD45RA-CD3-). Total thymocytes and CD123- thymocytes were then infected with NL4-3 or mock-infected and cultured for 2 wk in serum-free medium in the presence of IL-7 (38). Viral replication was measured in the supernatants and was found to be delayed and decreased 2- to 110-fold in total thymocytes compared with CD123-depleted thymocytes in six of six experiments (Fig. 1 and Table I). After 2 wk of culture, HIV-1 infection of the cells was monitored by immunophenotyping for intracellular expression of HIV-1-Gag proteins in combination with cell surface staining for CD3, CD4, and CD8. Our results (Table II) show that depletion of CD123+ pDC from the total thymocyte population increased productive HIV-1 infection and depletion of CD4+ cells. To determine whether the lower p24 levels might be due to IFN-α secretion by these cells, the supernatants of the HIV-1-infected and mock-infected thymocytes were assessed, but IFN-α was not detectable by ELISA (limit of detection, 12.5 pg/ml).

Neutralizing mAb to IFN-α increases viral replication in HIV-1-infected thymocytes

To determine whether the effect of pDC on viral replication in thymocytes was due to the production of IFN-α by pDC, HIV-1-infected thymocytes were cultured for 3 days in the presence of neutralizing mAb to IFN-α. Viral replication was increased 16- to 20-fold in thymocyte cultures treated with neutralizing mAb to IFN-α in two of two experiments (Fig. 1) As expected, there was little effect of neutralizing mAb to IFN-α on viral replication of CD123-depleted thymocytes (data not shown). These results indicate that IFN-α produced by pDC is probably responsible for the decrease in viral replication in thymocytes. However, the level of IFN-α produced by pDC is insufficient to prevent productive HIV-1 infection of thymocytes. Thus, we investigated whether stimuli that specifically enhance IFN-α production by pDC in the periphery can increase IFN-α production by thymic pDC to levels sufficient to interfere with viral replication in thymocytes.

Plasmacytoid DC in thymus secrete IFN-α in response to CpG

Because peripheral blood pDC express TLR9 that recognizes unmethylated bacterial DNA sequences through a CpG motif (55), we examined whether thymic pDC respond to CpG sequences. Total thymocytes, pDC enriched thymocytes (CD123+), and CD123-depleted thymocytes were cultured with the TLR9 ligand CpG2216 or negative control CpG2243 sequences. IFN-α production was determined in the culture supernatants over time. In five of seven experiments, IFN-α production (0–890 pg/ml) was detected in supernatants of thymocytes enriched for pDC in response to CpG2216, but not in medium or negative control CpG2243. Thymic pDC from two of seven donors did not produce IFN-α after stimulation with CpG; thus, the amounts of IFN-α produced by thymic pDC in response to CpG2216 were lower than those produced by adult PBMC (data not shown), reminiscent of lower IFN-α production by PBMC from newborns (56) and by cells from tonsils (10). In addition, there was donor variability in the levels of IFN-α secreted by thymic pDC in response to CpG, as has been described for peripheral pDC stimulated with HSV (57). CD123-depleted thymocytes did not produce IFN-α in six of six experiments (Fig. 2). These results show that thymic CD123+ pDC, but not thymocytes, secrete IFN-α upon stimulation by CpG oligonucleotides.

Comparison between IFN-α secretion induced by HIV-1 and CpG

Because HIV-1 replication clearly occurs in thymus tissue in vivo and in vitro, the levels of IFN-α induced by HIV-1 infection might modulate, but do not prevent, viral replication. IFN-α titration experiments with HIV-1-infected thymus organ cultures revealed reproducible viral suppression at concentrations >50 pg/ml (data not shown). To evaluate the dose effects of endogenous IFN-α production, thymus organ cultures were treated with CpG2216, infected with NL4-3, or both. Levels of IFN-α induced by HIV-1 infection were comparable to those in CpG2216-treated organ cultures (4–20 pg/ml). However, the combination of both stimuli (i.e., HIV infection and CpG treatment) induced 4-fold greater IFN-α production over either stimulus alone (8–100 pg; Fig. 3A). The high levels of IFN-α produced by pDC in HIV-1-infected thymus organ cultures in response to CpG corresponded to a reduction in viral replication.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Days Post infection</th>
<th>Total Thymocytes p24 (ng/ml)</th>
<th>CD123+ Thymocytes p24 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>483</td>
<td>731</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>311</td>
<td>1222</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>223</td>
<td>415</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>292</td>
<td>514</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>17</td>
<td>265</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>4</td>
<td>442</td>
</tr>
</tbody>
</table>

*Total thymocytes and CD123-depleted thymocytes (0% CD123+/hlgCD45RA-CD3-) were infected with HIV-1 (NL4-3) or mock supernatant and cultured at 2 × 10⁷ cells/ml for 1–2 wk in serum-free medium with IL-7 (200 U/ml). Cultures were fed fresh medium every 5–7 days. Levels of p24 production in the supernatants of whole thymocytes and CD123-depleted thymocytes, measured at the end of the experiment, are indicated.

FIGURE 1. The presence of pDC delays viral replication in HIV-1-infected thymocytes. Total thymocytes or thymocytes depleted of CD123+ cells by AutoMACS were infected with NL4-3 and cultured at 2 × 10⁷ cells/ml in serum-free medium in the presence of IL-7 (200 U/ml). Neutralizing mAb (2 μg/ml) to IFN-α was added to some cultures for 3 days after HIV-1 infection. The cultures were continued for the remainder of the experiment without mAb. Supernatants from the thymocytes were tested for the presence of HIV-1 p24 by ELISA.

Table I. The lack of pDC increases HIV-1 replication in thymocyte suspension cultures

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replication comparable to that after the addition of 500 U/ml (300 pg/ml) exogenous rIFN-α (Fig. 3B). Thus, CpG can boost endogenous IFN-α production levels, decreasing viral replication in the thymus. The data also demonstrate that, like CpG, HIV-1 infection alone fails to induce the full capacity of pDC for IFN-α production.

**IFN-α expression in HIV-1-infected thymus tissue in the SCID-hu mouse**

Although our findings strongly suggested that IFN-α production by pDC affects HIV-1 viral replication, it cannot be excluded that IFN-α produced by thymocytes contributes to this effect. IFN-α expression in both pDC and thymocytes was therefore evaluated in the SCID-hu mouse model after HIV-1 infection.

Using immunohistochemical staining, we found that IFN-α-positive cells were present in thymic implants infected with NL4-3 (X4) and JR-CSF (R5), but some positive cells were also detectable in mock-infected and noninfected implants (data not shown). To confirm that IFN-α is expressed by pDC in vivo, we combined cell surface with intracellular immunophenotyping of the thy/liv implants (Fig. 4A). There was a large variation in the percentages of IFN-α-positive pDC in the HIV-infected and mock-infected implants. The frequencies of pDC that were positive for IFN-α by intracellular staining in one implant series were as follows: X4 HIV-1-infected implants, 18 ± 0%; mock-X4, 17 ± 11%; R5 HIV-1-infected implants, 26 ± 16%; and mock-R5, 21 ± 1% (Fig. 4A). Thus, our data show that pDC in HIV-1-infected implants express IFN-α, but positive cells can also be detected in mock-infected implants. There was no difference in the percentages of IFN-α-positive cells between mock-infected and noninfected implants in mice from another implant series (data not shown).

Although pDC are known to be the main producers of IFN-α, we observed the expression of IFN-α not only in pDC from SCID-hu mice, but also in CD3+ and CD3- thymocytes (Fig. 4B). The percentages of total IFN-α-positive thymocytes varied from 0.7–3.3% (mean, 1.61 ± 1.5) in X4 HIV-1-infected implants, 0.14–12% (mean, 6.3 ± 5.3) in R5 HIV-1-infected, and 0.4–3.5% (mean, 1.9 ± 1.2) in mock-infected implants. There were some virus-infected implants with a dramatic increase in the percentage

**Table II. pDC delay productive HIV-1 infection and depletion of CD4+ cells in thymocyte suspension cultures**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Cells</th>
<th>Condition</th>
<th>% CD4+CD8+</th>
<th>Total CD4+</th>
<th>% KC57+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total thymocytes</td>
<td>NL4–3</td>
<td>6</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CD123-depleted</td>
<td>NL4–3</td>
<td>2</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HI-NL4–3</td>
<td>22</td>
<td>74</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mock</td>
<td>17</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Total thymocytes</td>
<td>NL4–3</td>
<td>7</td>
<td>39</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>CD123-depleted</td>
<td>NL4–3</td>
<td>1</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HI NL4–3</td>
<td>18</td>
<td>77</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mock</td>
<td>9</td>
<td>62</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*a Total thymocytes and CD123-depleted thymocytes (0% CD123+highCD45RA−CD3−) were infected with live, heat-inactivated (HI) HIV-1 (NL4–3) or mock supernatant and cultured at 2 × 106 cells/ml for 2 wk in serum-free medium with IL-7 (200 U/ml). Virus expression was determined by intracellular staining for HIV-1-Gag proteins using the KC57 Ab in combination with cell surface staining for CD4, CD8, and CD3.

**FIGURE 2.** Thymic pDC secrete IFN-α in response to CpG. CD123+ thymocytes were enriched by positive selection on AutoMACS. Total, CD123-enriched, and CD123-depleted thymocytes were cultured in parallel in seven experiments. Thymocytes were cultured in serum-free medium for 18 h in the presence or the absence of CpG2216 (6 μg/ml) or the control sequence CpG2243 (6 μg/ml). Results obtained with CpG2243 were similar to those with medium. IFN-α was measured by ELISA in culture supernatants collected at 24 h. CD123-enriched cells contained 6–53% CD123+highCD45RA−CD3− cells, and CD123-depleted thymocytes did not contain any CD123+ cells (0% CD123+highCD45RA−CD3−).

**FIGURE 3.** IFN-α production in HIV-1-infected thymus organ cultures is enhanced by CpG, and viral replication is decreased by CpG. Thymus organ cultures were treated with CpG (6 μg/ml) and infected with HIV-1 (NL4-3) or were cultured in the presence of CpG without infection. A, Supernatants from the thymus organ cultures were tested for the presence of IFN-α by ELISA on days 8–11 of culture. B, Supernatants from the thymus organ cultures were tested for the presence of HIV-1 p24 by ELISA on days 4–11 of culture. Exogenous IFN-α (500 U/ml) was used as a control.
of IFN-α expression in the thymocytes over mock-infected implants, but the difference did not reach statistical significance (p > 0.08).

MxA expression in thymocytes after exposure to CpG or HIV-1
Prolonged production of IFN-α is dependent on positive feedback via the IFN-α receptor and induction of secondary response genes such as the Mx genes (23). We and others (21) found that CD118, the β-chain of the IFN-α receptor, is expressed on thymocytes at multiple stages of development (data not shown), indicating the potential for thymocytes to respond to IFN-α produced in the thymus. We therefore used the IFN response protein MxA as a marker for thymocyte responsiveness to IFN-α in the thymic organ culture system. Thymus organ cultures were either NL4-3-infected or mock-infected and were cultured in the presence or the absence of CpG2216. Both mature CD3⁺ and immature CD3⁻ thymocytes responded to the IFN-α produced upon HIV-1 infection by increasing expression of MxA protein (Fig. 5A). Thymocytes obtained from CpG-treated, HIV-1-infected organ cultures expressed higher levels of MxA protein than thymocytes from HIV-1-infected cultures not treated with CpG. Furthermore, thymocytes from CpG-treated, HIV-1-infected organ cultures expressed MxA levels similar to those in mock-infected cultures treated with exogenous IFN-α (Fig. 5B).

Plasmacytoid DC are essential for MxA expression in thymocytes
Because HIV-1 can replicate in thymocytes despite the intracellular expression of IFN-α, the role of pDC in MxA expression in thymocytes was examined. Total thymocytes, but not CD123-depleted thymocytes, expressed MxA proteins after infection with NL4-3 (Fig. 5C). These results may explain the increased viral replication observed in thymocytes depleted of pDC, because secondary response genes are necessary for the antiviral effects of IFN-α (23). Thus, only in thymocytes containing pDC is IFN-α secretion sufficient to induce the secondary response protein MxA and suppress HIV-1 replication.

Plasmacytoid DC are productively infected by HIV-1 in SCID-hu mice
Given the involvement of pDC in producing IFN-α in response to HIV-1 infection of the thymus, we investigated whether pDC cells are infectable in vivo. To this end, we examined CD4, CXCR4, and CCR5 expression on pDC and, as reported by Keir et al. (21), observed that pDC expressed CD4 and the two major HIV-1 coreceptors. CXCR4 expression on pDC was 64 ± 18 (n = 7). The expression of CCR5 on pDC varied considerably (50 ± 36%; n = 18), but CCR5 expression was considerably higher on pDC than on the total thymocyte population (0.2%–1%)(49). To further assess productive infection of CD123⁺high DC by HIV-1 in vivo, we used the SCID-hu mouse system. Intracellular HIV-1-Gag expression in pDC was identified in seven of the 13 implants only at late time points when the overall frequency of virus replication in the total thymus population exceeded 5%, and many of the CD4⁺ cells were depleted. At this time point, the frequency of virus-expressing cells in pDC ranged from 7–40%, as determined by intracellular flow cytometric analysis for HIV-1-Gag protein; compared

FIGURE 4. Plasmacytoid DC and thymocytes from thy/liv implants of SCID-hu mice express IFN-α. Thymocytes obtained from thy/liv implants in SCID-hu mice infected with X4 HIV-1 (NL4-3; three mice) and R5 HIV-1 (JR-CSF; four mice) or mock-infected (four mice) were phenotyped simultaneously for cell surface markers and intracellular expression of IFN-α at 4 wk postinfection. The cells were surface-stained with Abs CD3-TC, CD123-PE, and intracellular IFN-α-biotin-streptavidin-allophycocyanin. Appropriate isotype control Abs were used in parallel. To ensure the specificity of the IFN-α staining, the curves were set on cells stained in parallel with the intracellular IgG1 isotype control Ab in combination with the surface-immunophenotyping Abs. A, IFN-α expression in pDC was determined by gating on CD3⁺CD123⁺high cells. B, IFN-α expression vs CD3 is shown in total thymocytes.

FIGURE 5. MxA proteins are expressed in response to HIV-1 infection and CpG in thymocytes. A, Thymocytes were obtained from mock-infected and NL4-3-infected thymus organ cultures 12 days postinfection and phenotyped simultaneously for cell surface markers and intracellular expression of MxA protein. The cells were surface-stained with Abs to CD3-allophycocyanin, CD4-PE, and CD8-TC and stained intracellularly with MxA-goat anti-mouse FITC. Isotype control Abs were used in parallel. MxA vs CD3 expression is shown. B, Histograms of MxA expression in HIV-1-infected or mock-infected organ cultures with or without CpG2216 or IFN-α (500 U/ml). C, Histograms of MxA expression in HIV-1 (NL4-3)-infected total thymocytes and thymocytes depleted of pDC (CD123-depleted). This experiment is representative of three independent experiments.
with CD4+ thymocytes, pDC were relatively spared from HIV-1-induced depletion (Fig. 6A).

In contrast to NL4-3-infected SCID-hu mice, only two of 18 JR-CSF-infected mice expressed detectable virus in pDC 8 wk postinfection when virus induced severe depletion of CD4+ cells (Fig. 6A). Yet, in another R5-infected SCID-hu transplant series, HIV-1 replication proceeded for as much as 9 wk postinfection without inducing noticeable CD4 T cell loss, and accordingly, no HIV-1-Gag protein expression was detected in pDC despite expression in thymocytes (data not shown).

With such a rare occurrence of R5 HIV-1 productively infecting pDC in vivo, we wanted to further verify the susceptibility of pDC to R5 HIV-1 infection. The pDC were sorted from postnatal thymus tissue and pulsed with either R5 HIV-1 strain NFN-SX or JR-CSF in vitro for 24 h. DNA was isolated and subjected to semiquantitative PCR for proviral sequences. Sequences for both initial and late RT products, R/U5 and LTR-gag sequences, respectively, were detected in the pDC population, confirming the susceptibility of these cells to viral infection (Fig. 6B). Thus, pDC are productively infected by X4 HIV-1 and R5 HIV-1 in the thymus, but only at late stages, when viral replication progresses sufficiently to cause depletion of the abundant CD4+ thymocytes.

Discussion

We have shown for the first time that pDC play an essential role in controlling the levels of viral replication in the thymus. However, the antiviral state induced by pDC upon HIV infection is suboptimal and can be enhanced by additional stimuli, such as CpG oligonucleotides. Virus replication was 2–110 times greater in the CD123-depleted thymocyte population compared with the total population, indicating that the few pDC in the total population are responsible for suppressing virus replication. Plasmacytoid DC make up ~0.2% of lymphoid cells in the thymus, and CD123 depletion efficiently decreased this population to undetectable levels. With high levels of CD123 expression primarily on pDC, removal of CD123+ cells did not change the overall proportion of CD4+, CD8+, and CD4+CD8+ thymocytes with respect to the total population (data not shown). Because the CD123+ population is not biased in thymocyte subset composition, the increased replication kinetics in the CD123+ population are due to the loss of pDC. Several reports describe a decrease in the frequency and function of peripheral blood pDC in HIV-1-infected patients (50, 52–54, 58). A decrease in pDC has been correlated to an increase in HIV-1 RNA virus load and opportunistic infections, suggesting that a loss of these cells may contribute to disease progression in HIV-1-infected patients (54, 59).

The presence of neutralizing mAb to IFN-α in the cultures had similar effects on HIV replication as the removal of pDC, indicating that IFN-α produced by pDC is probably responsible for the decrease in p24 production by total thymocytes. Because antiviral effects of IFN-α are dependent on the induction of secondary response proteins (23), we examined MxA expression in HIV-1-infected thymus organ cultures. The pDC were essential for the induction of MxA expression in HIV-1-infected thymocytes, because thymocytes depleted of pDC expressed intracellular IFN-α, but not MxA. We and others (21) found that all thymocyte subsets express IFN-α receptor; thus, the absence of secreted IFN-α in CD123-depleted thymocyte cultures seems responsible for the lack of MxA expression in thymocytes. Because MxA proteins have antiviral activity, this can explain the increase in HIV-1 replication in cultures in which pDC were depleted. In SIV-infected rhesus macaques, higher SIV RNA levels in lymphoid tissues, including the thymus, correlated with increased IFN-α and Mx mRNA levels (60). IFN-α mRNA levels in the thymus were similar to those in other lymphoid tissues, but viral RNA was lower, suggesting that increased IFN-α mRNA levels may provide some protection against viral replication in the thymus (60).

We detected IFN-α-positive cells in HIV-1-infected thyliv implants in the SCID-hu mouse. Although thymus tissue obtained from HIV-1-infected implants produced IFN-α, we questioned why the secreted IFN-α did not prevent viral infection. Our results suggest that the levels of IFN-α induced by HIV-1 infection are
insufficient to abort viral replication. However, we found that endogenously produced IFN-α was enhanced by distinct CpG motifs in oligodeoxynucleotides that stimulate pDC through TLR9 (55, 61). Only pDC, not thymocytes, produced IFN-α production in response to CpG2216, a sequence found to induce high levels of IFN-α production by peripheral blood pDC (15, 16). IFN-α secreted in response to CpG was most likely responsible for the delay in viral replication of X4 HIV-1, because CCR5 ligands that can be produced by pDC (62) do not interfere with HIV-1 infection with an X4 virus.

In our SCID-hu mouse experiments we detected intracellular IFN-α expression not only in pDC, but also in thymocytes. Indeed, mammalian cells are capable of increasing IFN-α production above the basal level and generating a type I IFN response to a large number of stimuli (reviewed in Ref. 63), including cell-to-cell interactions (13). Intracellular expression of IFN-α in CD123−PBMC of HIV-1-infected and healthy individuals after stimulation with HSV was shown by Feldman et al. (52). Thus, our findings that thymocytes express IFN-α in HIV-1-infected implants are not surprising, but are novel. However, the presence of intracellular IFN-α expression did not correlate with (detectable levels of) secreted IFN-α and was independent of the presence of pDC in HIV-1-infected cultures. It is possible that thymocytes expressing IFN-α intracellularly are at least temporarily protected against HIV-1 infection; however, this idea is not supported by our observation that there were thymocytes and pDC that expressed both HIV-1-Gag proteins and IFN-α (data not shown).

IFN-α-positive cells were found in HIV-1-infected implants as well as in mock-infected implants (Fig. 5) and noninfected implants (data not shown), consistent with data from the literature showing IFN-α-expressing cells in normal fetal and postnatal tissue (34). Thus, in contrast to Keir et al. (21), we cannot conclude from these data that IFN-α-positive cells are only present in the thy/lym/implant after HIV-1 infection. Our results emphasize the pitfall of assuming that intracellular IFN-α equates with secretion.

HIV-1 infection stimulates an IFN-α response in the thymus, yet levels of IFN-α that would prove beneficial in aborting HIV replication are not reached. The failure of HIV-1 to induce protective levels of IFN-α by pDC could be due to direct or indirect effects of HIV-1. Both systemically (64) and in the thymus, IL-10 levels are increased by HIV-1 infection (65), and IL-10 decreases IFN-α production and frequency of natural IFN-producing cells in response to viral stimulation (66). Alternatively, HIV-1 could disrupt pDC by direct infection. As reported by Keir et al. (21), we found that thymic pDC can be infected in vitro with HIV-1. In vitro infection with HIV-1 of peripheral blood pDC has been reported (19, 20). In addition, Donaghy et al. (67) detected proviral sequences and a decrease in function in peripheral blood pDC from HIV-1-infected individuals. We now show in vivo infection of thymic pDC isolated from thy/lym implants. However, productive infection is only detected after HIV-1 induces severe loss of CD4+ thymocytes at late time points postinfection. Plasmacytoid DC in the thymus may not get the CD40 stimulatory signal necessary for virus production until late in the course of HIV infection (19). Direct HIV-1 infection of pDC probably contributes to the loss of these cells, because in the midst of HIV-1 depletion of thymocytes, the frequency of pDC is similar to that in mock-infected implants. In conclusion, we found that pDC produce IFN-α in response to HIV-1, limiting HIV-1 replication in the thymus. However, the IFN-α response generated is not sufficient to control virus replication and is not maximal. Thus, efforts to specifically enhance or preserve pDC function would contribute to better innate immunological control of virus replication in infected individuals.

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