HIV-1 and HIV-2 Differentially Mature Plasmacytoid Dendritic Cells into IFN-Producing Cells or APCs

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HIV-1 causes a progressive impairment of immune function. HIV-2 is a naturally attenuated form of HIV, and HIV-2 patients display a slow-progressing disease. The leading hypothesis for the difference in disease phenotype between HIV-1 and HIV-2 is that more efficient T cell–mediated immunity allows for immune-mediated control of HIV-2 infection, similar to that observed in the minority of HIV-1–infected long-term nonprogressors. Understanding how HIV-1 and HIV-2 differentially influence the immune function may highlight critical mechanisms determining disease outcome. We investigated the effects of exposing primary human peripheral blood cells to HIV-1 or HIV-2 in vitro. HIV-2 induced a gene expression profile distinct from HIV-1, characterized by reduced type I IFN, despite similar upregulation of IFN-stimulated genes and viral restriction factors. HIV-2 favored plasmacytoid dendritic cell (pDC) differentiation into cells with an APC phenotype rather than IFN-α–producing cells. HIV-2, but not HIV-1, inhibited IFN-α production in response to CpG-A. The balance between pDC maturation into IFN-α–producing cells or development of an APC phenotype differentiates the early response against HIV-1 and HIV-2. We propose that divergent pathways of pDC differentiation driven by HIV-1 and HIV-2 cause the observed differences in pathogenicity between the two viruses. The Journal of Immunology, 2014, 193: 000–000.

Two types of HIV have been described, HIV-1 and HIV-2. Both viruses infect and replicate in CD4-expressing cells, but they differ in evolutionary origin and disease progression rate.

HIV-1 originally derived from the SIV that infects chimpanzees. HIV-1 is the causative agent of AIDS in the current global pandemic, a progressive disease characterized by high plasma viral loads and low CD4 counts. HIV-1 causes an early defect in cellular immune responses during acute infection, epitomized by a rapid decline in CCR5+CD4+ mucosal T cells and loss of IL-2 secretion by CD4+ T cells, from which the immune system fails to recover (1). Immune alterations during HIV-1 infection are not restricted to T cell depletion. Dysregulation in dendritic cell (DC) subsets also occurs during acute HIV-1 infection and is exacerbated throughout the course of disease (2). Thus, early events in virus/host interactions are likely to critically contribute to disease progression. In particular, HIV-1 may induce a rapid dysregulation of innate immune responses, promoting the excessive and prolonged production of type I IFN (IFN-I) (3–5) and activation of the immunoregulatory enzyme IDO (3, 6). These early occurring immune alterations have been suggested to prevent the development of efficient and long-lasting antiviral adaptive T cell responses. Additionally, increasing evidence shows that early inhibition of viral activity by antiretroviral treatment preserves immune function and favors long-term control of infections (7–11).

HIV-2 originated from SIV infection of sooty mangabeys and is a naturally attenuated form of HIV. HIV-2 is less transmissible than HIV-1, and HIV-2 infection rates are progressively declining and are largely confined to West Africa (12). At the amino acid level, HIV-1 and HIV-2 share ~60% identity in the Gag and Pol proteins, and only 30–40% identity in the Env coding regions (13). HIV-2 infection is characterized by a slow rate of disease progression, with lower plasma viral loads and a slower rate of CD4+ T cell decline (14, 15). Thus, the vast majority of HIV-2 patients display a phenotype comparable to that of HIV-1–infected long-term nonprogressors. However, the expression of markers of immune activation is similar in HIV-1– and HIV-2–infected individuals with comparable levels of CD4 depletion, despite the distinct rates of CD4 decline (16). Additionally, similar reductions in circulating myeloid and plasmacytoid DC (mDC and pDC, respectively) are observed in HIV-1+ and HIV-2+ patients with comparable degrees of peripheral CD4+ T cell depletion and T cell activation (17, 18). Upon progression to AIDS, clinical manifestations in HIV-2 patients are indistinguishable from HIV-1 infection (19). Interestingly, proviral DNA levels are similar in HIV-1 and HIV-2 patients, suggesting that the slower progression of HIV-2 disease is not due to a difference in the rate of infection (20).

HIV-2 has been studied as a model of immunologically controlled HIV infection, based on evidence suggesting that HIV-2 infection is associated with an efficient cell-mediated immune response.
response. However, studies investigating immune responses against HIV-2 have shown conflicting results. When comparing asymptomatic HIV-1- and HIV-2-infected patients, some reports showed no differences in the frequencies of HIV-specific T cells or the breadth of responses (21–23). However, Duvall et al. (24) reported enhanced HIV-specific memory CD4+ T cell responses in asymptomatic HIV-2 compared with HIV-1 patients. In particular, HIV-2+ individuals preserved the ability to secrete IL-2 (25) and displayed an increased frequency of polyfunctional CD4+ and CD8+ T cells (26). A recent study comparing HIV-2 progressors and HIV-2 controllers concluded that the association between the magnitude of HIV-2 Gag-specific T cell responses and undetectable viral loads is mainly due to the CD8+ T cell response (27). Furthermore, CD8+ T cell polyfunctionality is associated with viral control, similar to HIV-1 controllers. Nonetheless, it is unclear whether an enhanced cellular immune response is the cause or consequence of low levels of circulating virus (28). Additionally, studies investigating antiviral immune response in HIV-1 and HIV-2 chronically infected patients cannot provide any information on the differences in early virus/host interactions and their contribution to shaping the phenotype of chronic disease.

In this study, we directly compared the effects of HIV-1 and HIV-2 on primary human cells from healthy uninfected humans with no previous exposure to either of the two viruses. We tested the hypothesis that acute HIV-2 exposure induces an innate immune profile that is qualitatively different from HIV-1 and associated with reduced activation of immunosuppressive mechanisms, which in turn favors the development of efficient T cell response and determines the attenuated HIV-2 disease phenotype.

Using genome-wide expression analysis we found that HIV-2 stimulation induced a gene expression pattern distinct from HIV-1, characterized by lower expression of IFN-1 genes despite similar expression profiles of IFN-stimulated genes (ISG), pattern recognition receptors (PRR), and IFN-regulated viral restriction factors. pDC were identified as the main source of IFN-1 in the absence of high levels of IFN-1 with HIV-2 inhibited IFN-1 secretion further revealed significant differences between HIV-1 and HIV-2. HIV-2 stimulation favored the development of an APC phenotype in pDC, rather than maturation into IFN-α–producing cells, although IFN-1 levels were sufficient to enhance the expression of antiviral restriction factors and innate immune mechanisms. Importantly, stimulation with HIV-2 inhibited IFN-α production even in response to the synthetic TLR-9 ligand CpG-A. The preferential induction of an APC phenotype in the absence of high levels of IFN-α may be a critical contributor to the development of efficient T cell responses and to the lower pathogenicity observed during HIV-2 infection.

Materials and Methods

HIV-1 and HIV-2 quantification and normalization

All viruses were quantified and normalized based on RNA content, thus ensuring that the DC were exposed to the same amount of TLR ligand. Viral RNA was extracted from purified virus and reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix kit (Life Technologies, Invitrogen, Paisley, U.K.) with 50 ng/μl random hexamers, according to the following thermal profile: 25˚C for 10 min, 50˚C for 50 min, and 85˚C for 5 min. cDNA was stored at −20˚C until use. Quantitative real-time PCR was carried out using the Quantitect SYBR Green PCR kit (Qiagen, Manchester, U.K.), using prequantified plasmids containing the HIV-1 or HIV-2 amplicon sequence (Geneart, Regensburg, Germany).

For comparison, HIV-1 p24 was also quantified by ELISA (PerkinElmer Life Sciences, Waltham, MA); 13 × 10^9 RNA copies/ml HIV-1MN propagated in H9 cells corresponded to 668.7 ng/ml p24.

Isolation of cells and in vitro culture

Blood from healthy subjects was obtained from the National Health Service Blood and Transplant Service in the form of component donation leukocyte cones. PBMC were isolated by density gradient centrifugation using lymphocyte separation medium 1077 (PAA Laboratories, Somerset, U.K.) and cultured at 2 × 10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all reagents sourced from PAA Laboratories). All HIV-1 and HIV-2 isolates were added at 13, 3.9, 1.3 or 0.39 × 10^9 RNA copies/ml as described in Results. Unless otherwise specified, HIV-1 refers to HIV-1MN grown in H9 cells, and HIV-2 refers to HIV-2mac-gag grown in the HuT78 cell line. CpG-A (oligo-deoxynucleotide 2216, InvivoGen, San Diego, CA) was used at a concentration of 0.5 μM. Cells were harvested after 6 and 12 h for gene expression analysis and after 9 and 24 h for analysis of cell surface markers by flow cytometry. Cell culture supernatants were collected at up to seven different time points (3, 6, 9, 12, 18, 24, and 48 h) and immediately frozen at −80˚C for future analysis of IFN-α and IDO activity.

Plasmacytoid DC were enriched from PBMC using the negative isolation kit (pDC isolation kit II; Miltenyi Biotec, Surrey, U.K.), according to the manufacturer’s instructions. Enriched pDC were cultured at either 0.5 or 1 × 10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in the presence of 1 ng/ml IL-3. Stimulation was carried out using either 3.25 or 6.5 × 10^6 RNA copies/ml HIV-1 or HIV-2. This viral concentration was used to match the multiplicity of infection used in PBMC cultures of 2 × 10^6 cells/ml. Cells were harvested after 24 h of culture and analyzed using the flow cytometry IFN-α secretion assay as described below.

IFN-α ELISA

IFN-α analysis was performed on frozen cell culture supernatants using the a human IFN-α multi-subtype ELISA kit (PBL InterferonSource, Piscataway, NJ) according to the manufacturer’s instructions.

Kynurenine and Trp measurement

Kynurenine (Kyn) and Trp were measured in cell culture supernatants by HPLC as previously described (29). The Kyn/Trp ratio was calculated as an estimate of IDO activity.

Flow cytometry

Cells were incubated for 20 min with combinations of the following Abs: FITC-conjugated anti-human CD80 clone 2D10.4 (eBioscience, Hatfield, U.K.); PE-conjugated anti-human CD83 clone HB15e (eBioscience); PerCP-Cy5.5-conjugated anti-human CD86 clone IT2.2 (BioLegend, London, U.K.); PE-Cy7-conjugated anti-human CD123 clone 6H6 (Bio-Legend); allopurinol-conjugated anti-human CD303 (BDCA2) clone AC144 (Miltenyi Biotec); allopurinol-conjugated anti-human CD14 clone M49 (BD Biosciences, Oxford, U.K.); and Brilliant Violet 421-conjugated anti-human CD83 clone HB15e (BioLegend). Cells were acquired on a BD LSR II using FACSDiva software (BD Biosciences), and FlowJo (Tree Star, Ashland, OR) was used for data analysis.

IFN-α secretion was measured using an IFN-α secretion assay (Miltenyi Biotec) according to the manufacturer’s instructions. Briefly, after stimulation in the presence or absence of either HIV-1 or HIV-2, cells were labeled with IFN-α catch Ab (Miltenyi Biotec). To allow for the cells to secrete IFN-α, cells were diluted in culture media to a concentration of 1 × 10^6 cells/ml. Tubes were then placed at 37˚C with 5% CO2 for 20 min and rotated every 5 min to resuspend settled cells. Cells were subsequently labeled with IFN-α detection Ab conjugated to PE plus a combination of fluorescently conjugated mAbs (listed above) for 20 min. Following this, cells were incubated with Fixable Viability Dye eFluor 506 (eBioscience) at 4°C to exclude dead cells from analysis. Cell were acquired on a BD LSR II using FACSDiva software (BD Biosciences), and FlowJo (Tree Star) and SPICE (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA) were used for data analysis.

The median number of events recorded for pDC was 414 (interquartile range [IQR], 283–607).

Materials and Methods

HIV-1 and HIV-2 quantification and normalization

All viruses were quantified and normalized based on RNA content, thus ensuring that the DC were exposed to the same amount of TLR ligand. Viral RNA was extracted from purified virus and reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix kit (Life Technologies, Invitrogen, Paisley, U.K.) with 50 ng/μl random hexamers, according to the following thermal profile: 25˚C for 10 min, 50˚C for 50 min, and 85˚C for 5 min. cDNA was stored at −20˚C until use. Quantitative real-time PCR was carried out using the Quantitect SYBR Green PCR kit (Qiagen, Manchester, U.K.), using the following primers (Invitrogen) at 0.5 μM: HIV-1 gag forward, 5′-GGC TTT CAG CCC AGA AGT AAC ACC C-3′, reverse, 5′-TGG CAT GGC TGG TTG ATG TCC CC-3′; HIV-2 gag forward, 5′-TGT GGG CGA CCA TCA AGC AGC-3′, reverse, 5′-CCG GTG CTA AGG GCC GTG CTA-3′. Reaction plates were incubated as follows: 95˚C for 15 min, followed by 30 cycles of 94˚C for 15 s, 60˚C for 20 s, and 72˚C for 30 s. For quantification purposes, a standard curve was run on every plate. Standards were generated from prequantified plasmids containing the HIV-1 or HIV-2 amplicon sequence (Geneart, Regensburg, Germany).
The Journal of Immunology

subsequently concentrated using the RNasy MiniElute cleanup kit (Qiagen) according to the manufacturer’s instructions. The quality and concentration of extracted RNA was determined by measuring absorbance at 260-, 230-, and 280-nm wavelengths (NanoDrop 1000; Thermo Fisher Scientific, Wilmington, DE), and the quality was checked using an Agilent Bioanalyzer, with only samples having an RNA integrity number of 7.5 being used. An Ambion WT expression kit was used to prepare the cDNA, which was then labeled using the Affymetrix GeneChip WT terminal labeling kit (Affymetrix, High Wycombe, U.K.). Samples were run on the human gene 1.0ST version 2 arrays (Affymetrix), which contain probe sets for >40,000 transcripts. Partek Genomics 6.6 (Partek, St. Louis, MO) was used for data analysis. Individual CEL files were corrected using RNA background correction. Normalization was performed using quantiles, and median polish was used for probe set summarization. Results were expressed as log2. Data sets from different time points were treated separately. Gene microarray data are available on the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE58994 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58994).

Statistical analysis

Statistical analyses for the gene array data were performed with Partek genomics using a two-way ANOVA, cross comparing HIV-1, HIV-2, and CpG-A to media alone, with donor as a random variable. Only genes that had a 2-fold change in expression relative to media and were considered statistically significant (Benjamini and Hochberg corrected p value < 0.05) were selected for further analysis. The Partek gene ontology enrichment function was used to identify groups of genes based on their biological function. Gene enrichment was performed on three separate lists of genes: those differentially regulated by 1) both HIV-1 and HIV-2, 2) HIV-1 and not by HIV-2, and 3) HIV-2 and not by HIV-1. Spearman rank tests were performed for correlation analysis using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Statistical analyses for all other data were performed using SPSS version 20 (IBM, Armonk, NY). Different culture conditions were compared using a non-parametric Friedman test. Pairwise comparisons were subjected to Dunn’s correction. A p value < 0.05 was considered significant.

Results

HIV-1 and HIV-2 differentially regulate type I IFN genes but induce similar innate antiviral responses

HIV-1MN and HIV-2NIH-Z were normalized based on viral RNA (13 × 10⁶ RNA copies/ml, final concentration, measured by real-time PCR) and used as stimuli for cultures of PBMC from healthy uninfected donors. Viral nucleic acids are pathogen-associated molecular patterns that potently stimulate innate immunity via PRR. Thus, normalization based on nucleic acid content guarantees that the cells were exposed to the same amount of pathogen-associated molecular patterns from the two viruses.

Whole-gene array was performed on PBMC from three uninfected donors cultured with HIV-1MN, HIV-2NIH-Z (referred to henceforth as HIV-1 and HIV-2), CpG-A oligodeoxynucleotide, or cell culture media alone for 6 or 12 h. Differential regulation of specific subsets of genes by HIV-2 compared with HIV-1 or CpG-A was observed in all donors and highlighted by principal components analysis (Supplemental Fig. 1A), ANOVA-based analysis of differentially expressed genes (Supplemental Fig. 1B), and unbiased hierarchical clustering of genes shortlisted by ontology enrichment (Fig. 1A).

Classification of genes into subgroups according to AmiGO analysis (version 1.8, GO database release 2013-11-09) and available literature indicated that the most substantial differences were observed in IFN-I gene expression, which was higher in PBMC stimulated with HIV-1 and CpG-A compared with HIV-2 (Fig. 1B). This pattern was observed across all three donors at both 6 and 12 h. Interestingly, all stimuli induced similar increases in the expression of genes involved in IFN signaling at 6 (IRF7, IRF8, and STAT2) and 12 h (IRF7, IRF8, STAT2, STAT1, and IRF5) (Fig. 1B), as well as ISG (Supplemental Fig. 1C). Furthermore, a similar upregulation of viral restriction factors (Supplemental Fig. 1D) and apoptosis-related genes (data not shown) was observed with HIV-1, HIV-2, and CpG-A compared with media alone at both time points. Expression of 11 PRR-associated genes (including TLR3, TLR7, and NOD1) was equally increased in response to all three stimuli at 6 h (data not shown). Similar upregulation of 13 PRR-associated genes (including TLR3, TLR7, TLR8, and NOD1) was observed after 12 h, whereas the expression of CLEC4A, CLEC5A, and NLRC4 was reduced in HIV-1– and CpG-A–stimulated cells, but only marginally in HIV-2–stimulated cells compared with media alone after 12 h (data not shown).

A subset of genes associated with the activation and regulation of the adaptive immune response were downregulated after 6 h in response to all stimuli, but their expression remained higher in HIV-2–stimulated cells compared with either HIV-1 or CpG-A (Supplemental Fig. 1E). The expression of genes associated with Ag processing and presentation was equally increased in response to all stimuli after 12 h (Supplemental Fig. 1E). Similar expression profiles were observed for cytokine and chemokine genes in HIV-1–, HIV-2–, and CpG-A–stimulated samples, with the exception of reduced expression of a selected number of chemokine genes after 12 h stimulation with HIV-1, an effect that was less pronounced in response to HIV-2 exposure (data not shown).

Genome-wide expression data collectively demonstrate that acute responses to HIV-1 and HIV-2 exposure differ primarily in the signature and intensity of acute responses to HIV-1 than HIV-2 in all conditions tested. Specifically, HIV-1 induced significantly greater IFN-α secretion compared with media alone at all time points between 9 and 48 h. In contrast, HIV-2 induced significantly higher IFN-α production compared with media alone after 24 h. Increased sample size at 9 and 24 h also revealed that HIV-1 was significantly more potent than HIV-2 in stimulating IFN-α production. Similar results were found across all concentrations tested. When IFN-α production at 24 h was analyzed, we observed that stimulation with both viruses achieved the maximum response within the range of concentrations used (Supplemental Fig. 2A), and we confirmed that the difference between HIV-1 and HIV-2 is not resolved by simply increasing HIV-2 concentrations.

We also tested the ability of isolates other than HIV-1MN and HIV-2NIH-Z to induce IFN-α secretion. Although differences were observed between viral strains, overall, HIV-1MN, HIV-1INF, and HIV-1ADA induced the secretion of higher levels of IFN-α compared with HIV-2NIH-Z and HIV-2SY (Fig. 2E). Similar concentrations of IFN-α could detect following stimulation with replication-competent CXCR4-tropic HIV-1IN and reverse transcription–deficient aldriothiol-2 (At-2)–treated HIV-1MN (Fig. 2E). Furthermore, IFN-α levels remained unchanged when PBMC were stimulated with either HIV-1MN or HIV-2NIH-Z in the presence of the reverse transcriptase inhibitor zidovudine (3’-azido-3’-deoxythymidine) (Supplemental Fig. 2B), indicating that viral replication did not contribute to enhanced IFN-α responses. Similar IFN-α production was also observed using CCR5-tropic
At-2 HIV-1Ada and HIV-1MN grown in either H9 cells or CE-Mx174 cells (Fig. 2E), suggesting that co-receptor usage and the cell line used for virus propagation also did not influence IFN-α secretion in response to HIV-1.

HIV-1 also proved to be a more potent inducer of the activity of the immunosuppressive enzyme IDO, measured as the ratio between the concentrations of Trp and its catabolite Kyn in culture supernatants. No measurable Trp catabolism was observed after 9 h of stimulation with either HIV-1 or HIV-2 (data not shown). After 24 h HIV-1, but not HIV-2, significantly stimulated IDO activity compared with unstimulated cells when used at both 13 and 1.3 \times 10^9\text{copies/ml} (Fig. 2F).

Additionally, using 13 \times 10^9\text{RNA copies/ml} virus, HIV-1 induced a significantly higher Kyn/Trp ratio, indicating increased IDO activity, compared with HIV-2–stimulated PBMC (Fig. 2F).

FIGURE 1. Gene expression profile of PBMC stimulated with HIV-1, HIV-2, or CpG-A. (A) Heat map of genes showing significant differences in expression (at least 2-fold change and p < 0.05 as per ANOVA) and belonging to enriched gene ontology categories in PBMC from three independent donors stimulated with HIV-1 (blue), HIV-2 (green), CpG-A (yellow), or media alone (orange) for 6 (left heat map) and 12 h (right heat map). Each column represents the response from a single donor in each condition; color and grayscale bars on top of each heat map indicate culture conditions and donor code (A, B, or C), according to the legend on the right; columns were ordered by unbiased hierarchical clustering. (B) Heat maps of IFN-I, IFN-II, IFN signaling genes in PBMC stimulated with HIV-1 (blue), HIV-2 (green), CpG-A (yellow), or media alone (orange) for 6 (top panels) and 12 h (bottom panels). Each heat map represents responses in one individual donor. In both (A) and (B), gene expression levels are indicated by color transitions from blue (lowest expression) to red (highest expression) according to the legend displayed below each graph.
We investigated whether the differential effect of HIV-1 and HIV-2 on PBMC stimulation extended to the production of other inflammatory cytokines. Production of IL-8, TNF-α, and IL-1β was not significantly changed in response to either HIV-1 or HIV-2 (Supplemental Fig. 2C). Increased IL-6 production was observed in response to both viruses at 24 h, and it significantly exceeded control in the case of HIV-2 stimulation (Supplemental Fig. 2C).

**HIV-1 and HIV-2 induce comparable levels of pDC activation**

Previous data indicate that pDC are the main producers of IFN-α and IDO among HIV-stimulated PBMC in vitro (6, 30, 31). Thus, we investigated the induction of costimulatory molecules, CD80 and CD86, as well as the activation marker CD83 on pDC following PBMC stimulation with HIV-1 or HIV-2 (gating strategy shown in Supplemental Fig. 3A–D). CD80 expression was negligible after 9 h under all culture conditions (Fig. 3A). When used at $13 \times 10^9$ RNA copies/ml, HIV-1 induced a statistically significant upregulation of CD80 expression on pDC after 24 h compared with unstimulated PBMC (Fig. 3A). HIV-2 stimulation induced CD86 expression on a higher frequency of pDC compared with media alone after 9 h, whereas HIV-1–induced CD86 expression reached statistical significance after 24 h (Fig. 3B). CD83 expression was minimal in cultures with media alone after 9 and 24 h (Fig. 3C). Both HIV-1 and HIV-2 increased the frequency of CD83-expressing pDC compared with unstimulated PBMC at both 9 and 24 h. Analysis of CD80, CD86, and CD83 mean fluorescence intensity (MFI) showed profiles similar to those observed for the frequencies of marker-expressing cells, and confirmed that the enhanced induction of IFN-α by HIV-1 compared with HIV-2 is not mirrored by differences in phenotypic pDC activation (Fig. 3). Similar profiles, characterized by milder changes in the expression of all three markers, were observed when lower concentrations ($1.3 \times 10^9$ RNA copies/ml) of HIV-1 and HIV-2 were used (Fig. 3).

Because IFN-I promotes the activation and maturation of other APC, we also tested the regulation of CD80 and CD86 on mDC and monocytes, the two other main APC subtypes present in PBMC (gating strategy shown in Supplemental Fig. 3A–D). CD80 expression by mDC was negligible in all conditions tested, whereas the frequency of CD86+ mDC approached 100% in all conditions (Supplemental Fig. 4A, 4B). CD86 MFI was mildly increased in mDC following PBMC stimulation with HIV-1 or HIV-2, but no substantial differences were observed between the two viruses (Supplemental Fig. 4B). Minor changes were observed in the frequencies of CD80+ monocytes in response to both viruses, which tested significant only in response to HIV-1, whereas CD80 MFI as well as CD86 MFI and the frequency of CD86+ monocytes were enhanced by both viruses at comparable levels (Supplemental Fig. 4C, 4D).

**FIGURE 2.** IFN-α secretion in response to HIV-1 and HIV-2 stimulation. (A–D) Concentrations of IFN-α detected by ELISA in PBMC culture supernatants after stimulation for 6–48 h with varying concentrations of virus, as indicated in the top left of each graph. Responses to HIV-1 are denoted by open boxes, HIV-2 by diagonal pattern filled boxes, and responses to media alone are shown by solid boxes. Horizontal lines within boxes represent median values, boxes show IQR, and lines show extent to 90th and 10th percentiles. (E) IFN-α detected by ELISA in PBMC culture supernatants after stimulation for 24 h with HIV-1MN/H9, HIV-1MN/CEMx174, HIV-1BB/85/H9. At-2–treated HIV-1MN/H9, At-2 HIV-1MN/CEMx174, At-2 HIV-1Ada/SupT1-CCR5/HIV-2NIH-Z/Hut78, or HIV-2 ST/CEMx174. Solid circles indicate responses against replication-competent HIV-1 isolates, solid squares represent At-2–treated isolates, and open circles represent HIV-2 isolates. Horizontal lines represent median values. (F) Kyn/Trp ratios in PBMC culture supernatants after stimulation for 24 h with HIV-1 (solid circles) or HIV-2 (open circles), at $13 \times 10^9$ RNA copies/ml (left panel) and $1.3 \times 10^9$ RNA copies/ml (right panel), or media alone (triangles). Horizontal lines indicate median values, and vertical lines show the IQR. In all graphs, responses to HIV-1, HIV-2, and media alone within individual time points and virus concentrations were compared using a Friedman test with a Dunn’s posttest for multiple analyses. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Reduced IFN-α production in response to HIV-2 is associated with the development of an APC phenotype by pDC

Functionally distinct mature pDC subsets develop after stimulation with different synthetic ligands, either promoting the development of IFN-I-producing cells or inducing the expression of costimulatory molecules (32, 33). Therefore, we investigated the expression of CD86 and CD83 on pDC in conjunction with active secretion of IFN-α following stimulation with either HIV-1 or HIV-2 (gating strategy shown in Supplemental Fig. 3E, 3F).

We observed a strong positive correlation between the frequency of IFN-α-secreting pDC and IFN-α levels in supernatants in different culture conditions at both 9 and 24 h (Fig. 4A, 4B), confirming that pDC are the main cell type responsible for IFN-α production in this experimental setting.

The frequency of triple-positive pDC (CD83+, CD86+, IFN-α+) was low in all conditions at both 9 and 24 h (Fig. 4C and 4D), confirming previous observations that pDC maturation into IFN-I-producing cells or APC are mutually exclusive. Additionally, both IFN-α secretion and CD86 expression by pDC were associated with increased CD83 expression, suggesting that upregulation of the activation marker CD83 occurs independent of the pDC differentiation pathway.

HIV-1 stimulation induced a significantly higher frequency of IFN-α+/CD86-/CD83+ pDC compared with unstimulated cells (Fig. 4C, 4D). Conversely, HIV-2 induced a significantly greater proportion of IFN-α-/CD86+/CD83+ pDC compared with unstimulated cells.

These data suggest that HIV-1 and HIV-2 stimulate pDC differentiation along different pathways, namely IFN-I production and APC maturation, respectively.

A similar pattern, in which HIV-2 induced a higher frequency of CD86+ pDC, whereas HIV-1 promoted more potent IFN-α secretion, was observed after 24 h culture of enriched pDC (isolated by negative selection with magnetic beads and cultured in the...
presence of the survival factor IL-3; purity >90%), albeit the frequency of activated pDC appeared to be lower than that observed with unseparated PBMC (Supplemental Fig. 3G). These data suggest that the presence of cells other than pDC may not be necessary to drive differential maturation, but may be required to achieve optimal pDC stimulation.

HIV-2–induced pDC maturation into phenotypic APC prevents IFN-α production in response to the synthetic TLR-9 ligand CpG-A

We investigated whether HIV-2 stimulation affected the ability of pDC to produce IFN-α in response to the synthetic TLR-9 ligand CpG-A. PBMC were stimulated with $13 \times 10^9$ RNA copies/ml of either HIV-1 or HIV-2 in the presence or absence of CpG-A. Supernatants were collected after 24 h and IFN-α concentration was determined by ELISA. As expected, CpG-A alone was a potent stimulus for IFN-α production, which remained unaltered in the presence of HIV-1 (Fig. 5A, 5B). However, when HIV-2 and CpG-A were added together there was a significant reduction in secreted IFN-α compared with CpG-A alone, highlighting the dominant effect of HIV-2 over the IFN-I–inducing stimulus. A similar inhibition was observed when HIV-2 was added to PBMC 3 h before (Fig. 5C) or up to 2 h after (Fig. 5D) CpG-A stimulation. Conversely, the inhibitory effect of HIV-2 on CpG-A–induced IFN-α production was dramatically reduced when the virus was added 3 h after CpG-A (Fig. 5D).

Discussion

HIV-2 shares many of the biological features of HIV-1, but it is less pathogenic, causing a slow progressing disease characterized by low plasma viral load and reduced levels of immune activation (15, 27, 34–36). One leading hypothesis to explain the different
disease outcome is that more efficient T cell responses are induced and maintained during HIV-2 infection, and they allow prolonged immune-mediated control of viral replication (12). Thus, the course of disease in HIV-2–infected patients is similar to that observed in HIV-1 long-term nonprogressors, in that adaptive immunity controls viral activity. The reasons why HIV-2 elicits a more efficient antiviral adaptive response compared with HIV-1 are still obscure, but immunologic and inflammatory events that occur early during viral exposure are likely to influence the quality and potency of the adaptive response.

We examined the effects of acute exposure of uninfected PBMC to HIV-1 and HIV-2. We found that HIV-2 stimulation induced a gene expression profile distinct from HIV-1, characterized by reduced expression of IFN-1 genes, which was due to preferential induction of an APC phenotype in pDC rather than maturation into IFN-producing cells. Most importantly, HIV-2–induced pDC maturation into phenotypically defined APC was dominant over IFN-1 production even when cells were stimulated with CpG-A. Although additional studies investigating the APC function of HIV-1– and HIV-2–stimulated pDC are required, our data suggest that the balance between pDC maturation into IFN-producing cells or APC is a critical factor differentiating the early response against the two viruses. Based on these data, we hypothesize that the pathway of pDC differentiation during early viral exposure is an important determinant of the outcome of HIV infection.

Differential pDC maturation in response to HIV-1 and HIV-2 was also observed in enriched pDC cultures, but the levels of pDC activation appeared lower compared with those observed with unseparated PBMC. These data suggest that the presence of other cells may be required to facilitate full pDC activation, either by secretion of other immunomodulatory factors or by facilitating viral interaction with pDC. One caveat is that cultures of enriched pDC require the addition of exogenous IL-3 as a survival factor, which has been shown to modify pDC maturation by promoting the development of APC that stimulate Th2 CD4 T cell differentiation (37). Thus, in vitro culture of enriched pDC supplemented with IL-3 may not represent a biologically relevant experimental setting, and results obtained in this system should be treated with caution.

Interestingly, Cavaleiro et al. (17) reported that similar reductions of pDC numbers are observed in HIV-1– and HIV-2–infected patients with similar levels of CD4 depletion, despite significant differences in plasma viral load. The decrease in circulating pDC may be secondary to comparable degrees of pDC activation and relocation to lymphoid tissues during HIV-1 and HIV-2 infection. This is consistent with our data indicating that both viruses activate pDC to a similar degree, as measured by CD83 expression, but differentially modulate the function of activated pDC.

We have previously shown that removing the ability of HIV-1 to stimulate IFN-1 secretion and IDO, although preserving the capacity to induce high expression of costimulatory molecules on pDC, resulted in a greater ability to stimulate secondary HIV-1–specific memory T cells (5). This is consistent with the findings by O’Brien et al. (32) showing that CpG-B–stimulated pDC, which activated costimulatory molecule expression over IFN-1 secretion, elicited more efficient T cell proliferation than did pDC stimulated with CpG-A or HIV-1. Importantly, the activation of mDC and monocytes, measured as upregulation of costimulatory molecules, was comparable between HIV-1 and HIV-2, suggesting that efficient stimulation of other APC occurs even in conditions of low IFN-1 production upon exposure to HIV-2.

Patients infected with HIV-2 show enhanced HIV-specific T cell responses compared with HIV-1+ patients. A study comparing HIV-2+ patients with undetectable and high viral loads showed that the ability to control viral replication correlated with HIV-2 Gag-specific CD8+ T cell responses (27). Consistent with this view, we observed that the levels of IDO activity induced by HIV-2 were significantly lower compared with HIV-1. IDO is an immunoregulatory enzyme that suppresses T cell proliferation and drives the differentiation of regulatory T cells (38, 39). Increased IDO activity has been reported in HIV-1–infected patients and is associated with disease progression (6, 40–42), likely contributing...
to viral persistence by suppressing antiviral T cell responses. The gene array data we report in the present study are consistent with the hypothesis that the adaptive immune response is more efficiently induced by HIV-2 than by HIV-1. Thus, genes involved in B cell signaling (CD79B, GAPT), T cell activation (CD27, LAT) and T cell migration (ITGB7, AMICA1) were expressed at higher levels in PBMC stimulated with HIV-2 compared with HIV-1.

pDC produce 1000-fold more IFN-I than any other cell type in the blood, and they are key mediators of the innate immune response by both limiting viral replication and enhancing the adaptive arm of the immune response (43). Dysregulation of the IFN-I system has been suggested by us and others to contribute to different aspects of HIV-1 immunopathogenesis, including induction of CD4 T cell apoptosis, suppression of CD8 T cell responses, generalized lymphadenopathy, and promotion of an activated phenotype in T cells (3, 44–47). However, the contribution of different cell types to the sustained and prolonged production of IFN-I may change during the transition between acute and chronic infection. Recent evidence suggests that pDC may be the main source of IFN-I during acute infection, and they may be replaced by mDC and macrophages during the chronic phase (48). Macrophages and mDC, different from pDC, do not produce IFN-I following stimulation of endosomal TLR, but secrete IFN-I following stimulation of cytoplasmic PRR triggered during the early steps of the viral life cycle (49). Additionally, Harman et al. (50) demonstrated that productive infection of monocyte-derived DC by HIV-1 inhibits IFN-I secretion via IRF1 induction, and that this effect is characteristics of cells expressing newly synthesized viral proteins. Our data showing that similar levels of IFN-α are induced by HIV-1 and HIV-2 independent of the replicative capacity of the viruses argue against the possibility that a similar mechanism may occur in pDC. Thus, pDC may be critical in influencing the innate immune response and the development of adaptive immunity following viral exposure during primary HIV-1 infection, but may play a secondary role in IFN-I-mediated pathogenesis during the chronic phase.

Microarray analysis showed different gene expression profiles in PBMC exposed to HIV-1 and HIV-2, respectively. In particular, we found that HIV-2 induced lower expression of IFN-I genes compared with both HIV-1 and CpG-A, and expression of some IFN-I genes following HIV-2 stimulation was more similar to unstimulated than HIV-1-stimulated cells. Measurement of IFN-α concentrations in cell culture supernatants in vitro confirmed these results at a translational level across a wider range of virus concentrations and throughout a 48-h kinetic. The differences in IFN-α secretion extended to other HIV-1 and HIV-2 isolates, irrespective of HIV-1 co-receptor usage and the cell line used for viral propagation, and they were not a result of different replication rates. We normalized virus concentrations based on RNA, ensuring that the difference in IFN-α production is not due to differences in the amount of TLR ligand. Additionally, even when PBMC were stimulated with HIV-2 at concentrations up to 10-fold higher than that used for HIV-1, the difference in IFN-α production was preserved. These data demonstrate that the discrepancy between early immune responses against HIV-1 and HIV-2 are due to qualitative differences in virus/host interactions rather than caused by different amounts of viral stimuli.

Despite the striking difference in IFN-α stimulation, HIV-2 induced a robust innate immune response, similar to HIV-1. In particular, the expression profiles of PRR-associated and IFN-regulated viral restriction factor genes were similar in response to the two viruses. The upregulation of IFN-stimulated viral restriction factors (APOBEC3A, TRIM family members, SAMHD1, Tetherin/BST2) is of particular biological interest, because it suggests that molecular mechanisms of viral inhibition may be equally induced by HIV-1 and HIV-2 during the early stages of viral exposure.

The production of other cytokines, such as TNF-α, IL-1β, IL-8, and IL-6, was similar between HIV-1 and HIV-2, suggesting that the difference in inflammatory responses against the two viruses was limited to IFN-α. The strong correlation between the frequency of IFN-α-secreting pDC and the concentration of IFN-α detected in cell culture supernatants underscores the role of pDC in driving IFN-α responses during acute viral exposure.

CD83 is rapidly upregulated by pDC upon activation via TLR. We observed that although secretion of IFN-α was always associated with CD83 expression, there was a high frequency of activated pDC that expressed only CD83 after viral stimulation. Three non–mutually exclusive explanations may be considered for this phenomenon: 1) only a fraction of IFN-α–secreting cells can be detected within the 20 min window when cells are incubated with the detection Ab in our assay; 2) CD83 single-positive pDC may represent activated cells that are not yet committed to become either IFN-producing or APC; or 3) CD83+IFN-α–CD86+ cells may represent a population of activated pDC exerting functions other than IFN-α secretion, for example production of other inflammatory cytokines.

Our data indicate that HIV-2 promotes pDC differentiation along a different pathway compared with HIV-1, favoring costimulatory molecule expression over IFN-I. Very few CD86+IFN-α–pDC were detected, suggesting either that 1) the subpopulation of CD83+CD86+ cells secreted IFN-α only during the early phases of exposure, before the assessment made at 9 and 24 h; or 2) IFN-1 production and APC maturation are mutually exclusive. The latter hypothesis is supported by studies comparing the effect of CpG-A and CpG-B stimulation on pDC, in which alternative differentiation is observed (32, 33). A growing body of evidence suggests that different intracellular trafficking of TLR agonists within pDC results in alternative maturation pathways and different activated phenotypes. Similar to CpG-A, HIV-1 has been shown to traffic to the early endosome where it stimulates persistent IFN-α secretion via IRF7 induction, whereby CpG-B traffics to the lysosome resulting in preferential activation of the NF-κB pathway and upregulation of co-stimulatory molecules, yet weakly stimulating IFN-I secretion (32). In our experiments, we normalized HIV-1 and HIV-2 based on RNA copies/ml, thus ensuring that the same amount of TLR agonist was added to the cultures. Thus, the differences in pDC activation may be a consequence of different uptake, intracellular trafficking, or accessibility of viral RNA for TLR stimulation due to different dynamics of uncoating between the two viruses.

HIV-2 infection is not only less pathogenic than HIV-1, but it may also be beneficial in the context of coinfection. HIV-1–infected patients with pre-existing HIV-2 infection display a slower disease progression phenotype, characterized by higher CD4+ T cell counts (51). A study by Jaehn et al. (33) reported that pDC simultaneously exposed to CpG-A and CpG-B respond with an activated phenotype more similar to that induced by CpG-B alone. Consistent with this report, we found that when PBMC were stimulated with HIV-2 and CpG-A together, IFN-α secretion was significantly reduced compared with CpG-A alone. Thus, it is possible that in conditions of pre-existing HIV-2 infection, HIV-1 fails to induce an inflammatory phenotype dominated by IFN-α, alleviating the immune suppression and resulting in slower disease progression. Interestingly, HIV-2 exerted an inhibitory effect on IFN-α secretion only when added within 2 h of CpG-A-stimulation, suggesting a time-limited window of opportunity to
condition the system and suppress IFN-α responses. Further studies are required to identify which steps of virus/cell interaction are necessary for HIV-2 to prevent pDC maturation in vivo, and to determine whether similar events occur in vivo in HIV-2/HIV-1 coinfected patients.

Our data demonstrate that HIV types that cause different disease phenotypes in vivo can be characterized by their effect on pDC maturation in vitro. The conditioning of pDC during the acute stages of infection, depending on whether the response is dominated by IFN-α or an APC phenotype, may be a critical determinant of disease outcome. We propose that the pDC differentiation profile during the early phases of infection is critical in shaping the immune response against HIV and contributes to the differences in pathogenicity between HIV-1 and HIV-2.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1. Gene expression profile of PBMC stimulated with HIV-1, HIV-2 or CpG-A. (A) shows principal component analysis (PCA) of complete gene expression profiles of PBMC stimulated for 6 and 12 hours with HIV-1 (blue), HIV-2 (green), CpG-A (yellow) or media alone (orange); two perspective angles of 3D visualization are shown for each time point. (C) represents Venn diagrams indicating the overlap of the number of genes differentially regulated by HIV-1, HIV-2 and CpG-A compared to media, as determined by ANOVA after 6 and 12 hours. (C), (D) and (E) heat maps of IFN-stimulated genes (C), viral restriction factor genes (D) and adaptive immunity genes (E) in PBMC stimulated with HIV-1 (blue), HIV-2 (green), CpG-A (yellow) or media alone (orange) for 6 and 12 hours. In heat maps, each column represents responses from an individual donor; gene expression levels are indicated by colour transitions from blue (lowest expression) to red (highest expression) according to the legend displayed below the graphs.
Supplemental Figure 2. Cytokine secretion in response to HIV-1 and HIV-2. (A) shows the secreted levels of IFN-α after 24 hours only across all four concentrations of virus used. Solid circles and solid line indicate median responses to HIV-1, the grey shaded area extends to the IQR for HIV-1 responses. Open circles and dashed line indicate median responses to HIV-2, the pattern filled shaded area extends to the IQR for HIV-2 responses. (B) shows IFN-α responses in PBMC stimulated for 24 hours with HIV-1MN/H9 or HIV-2NIH-Z/Hut78 in presence or absence of different concentration of the reverse transcriptase inhibitor AZT. Solid symbols indicate responses to HIV-1 and open symbols indicate responses to HIV-2; each symbol represents one individual donor. (C) shows concentrations of IL-8, IL-1β, TNF-α and IL-6 measured by ELISA in supernatants from PBMC cultured for 24 hours with HIV-1 (solid circles), HIV-2 (open circles) or control media alone (triangles). Horizontal bars represent median values and vertical lines extend to the IQR; responses to HIV-1, HIV-2 and media alone were compared using a Friedman test with a Dunn’s post test for multiple analyses. *p≤0.05, **p≤0.01.
Supplemental Figure 3. Gating strategies and example plots for flow cytometry experiments. (A), (B), (C) and (D) show gating strategies and example plots for experiments shown in Figure 3, Figure 4 and Supplemental Figure 4. (A) Flow cytometry dot plots showing live cell gating, duplets exclusion and identification of monocytes (CD14+), pDC (CD14- BDCA2+ CD123+) and mDC (Lineage- HLA-DR+ CD11c+) , (B), (C) and (D) show flow cytometry histograms for CD80, CD86 and CD83 staining in pDC (B), as well as CD80 and CD86 staining in monocytes (C) and mDC (D) in PBMC stimulated for 24 hours with 13 x10^9 viral RNA copies/ml of either HIV-1 or HIV-2, or with cell culture media alone. Shaded histograms indicate isotype control staining. One example of N=8 individual experiment is shown. (E) and (F) show gating strategies and example plots for IFN-α secretion experiments shown in Figure 4. (E) Flow cytometry dot plots showing live cell gating, exclusion of dead cells by staining with FVD, duplets exclusion and identification of pDC as CD14- BDCA2+ CD123+ cells. (F) Flow cytometry dot plots for IFN-α secretion versus CD86 in negatively isolated pDC cultured for 24 hours with either HIV-1 or HIV-2, or with cell culture media alone. One example of N=6 individual experiment in shown. (G) Flow cytometry dot plots for IFN-α secretion versus CD86 in negatively isolated pDC cultured for 24 hours with either HIV-1 or HIV-2, or with cell culture media alone. One example of N=3 individual experiment in shown.
Supplemental Figure 4. Expression of co-stimulatory molecules on mDC and monocytes after HIV-1 and HIV-2 stimulation.

Expression of CD80 (A and C) and CD86 (B and D) on mDC (A and B) and monocytes (C and D) were analysed by flow cytometry after 24 hours stimulation with \(13 \times 10^9\) or \(1.3 \times 10^9\) viral RNA copies/ml of either HIV-1 or HIV-2, or with cell culture media alone (0 x 10^9 viral RNA copies/ml). Left panels show the frequencies of cells expressing the marker of interest, and right panels show the MFI for each marker. Each dot represents one individual donor. Horizontal bars represent median values and vertical lines extend to the IQR. Responses to HIV-1, HIV-2 and media alone within individual time points and virus concentrations were compared using a Friedman test with a Dunn’s post test for multiple analyses. *p≤0.05, **p≤0.01, ***p≤0.001.