Anti-HIV State but Not Apoptosis Depends on IFN Signature in CD4+ T Cells

Annette Audigé, Mirjana Urosevic, Erika Schlaepfer, Russell Walker, Doug Powell, Sabine Hallenberger, Helen Joller, Hans-Uwe Simon, Reinhard Dummer and Roberto F. Speck

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<table>
<thead>
<tr>
<th>References</th>
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</thead>
<tbody>
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Anti-HIV State but Not Apoptosis Depends on IFN Signature in CD4⁺ T Cells

Annette Audigé,† Mirjana Urosevic,‡† Erika Schlaepfer,* Russell Walker,‡ Doug Powell,‡‡ Sabine Hallenberger,§ Helen Joller,¶ Hans-Uwe Simon,‖ Reinhard Dummer,† and Roberto F. Speck*§

To gain insights into the molecular mechanisms underlying early host responses to HIV in the CD4⁺ T cell target population, we examined gene expression in CD4⁺ T cells isolated 24 h after ex vivo HIV infection of lymphocyte aggregate cultures derived from human tonsils. Gene profiling showed a distinct up-regulation of genes related to immune response and response to virus, notably of IFN-stimulated genes (ISGs), irrespective of the coreceptor tropism of the virus. This mostly IFN-α-dependent gene signature suggested the involvement of plasmacytoid dendritic cells, a principal component of the antiviral immune response. Indeed, depletion of plasmacytoid dendritic cells before HIV inoculation abrogated transcriptional up-regulation of several ISGs and resulted in increased levels of HIV replication. Treatment with a blocking anti-IFN-αR Ab yielded increased HIV replication; conversely, HIV replication was decreased in pDC-depleted cultures treated with IFN-α. Among up-regulated ISGs was also TRAIL, indicating a potential role of the IFN signature in apoptosis. However, a blocking anti-TRAIL Ab did not abrogate apoptosis of CD4⁺ T cells in CXCR4-tropic HIV-infected cultures, suggesting the involvement of pathways other than TRAIL mediated. We conclude that acute HIV infection of lymphoid tissue results in up-regulation of ISGs in CD4⁺ T cells, which induces an anti-HIV state but not apoptosis. The Journal of Immunology, 2006, 177: 6227–6237.

The immune system has only limited success in opposing infection by HIV. Although depletion of CD4⁺ T cells is the hallmark of HIV infection, the virus profoundly disturbs the entire immune system, and host factors are likely usurped by the virus to support the infection.

Early events in HIV infection critically affect the course of disease (1–5). For example, within the first few weeks after infection of macaques or rhesus monkeys with SIV or simian-HIV, respectively, viral replication (2, 3, 5) and CD4⁺ T cell depletion (4, 5) correlate with postacute viral replication and rapid disease progression. Most significantly, macaques display marked early differences in viral replication when infected with the same inoculum of SIV, implicating host factors as important determinants of viral replication (1). Furthermore, coreceptor preferences appear to influence HIV pathogenesis. CCR5-using (R5) viruses are most commonly transmitted sexually and predominate during the asymptomatic phase of HIV infection. In ~50% of HIV infections, CXCR4-using (X4) strains emerge during the chronic phase and are associated with an acceleration of the CD4⁺ T cell decline and disease progression (6).

Large-scale gene expression profiling has been used to gain insights into the genetic processes underlying the early interactions between HIV and the host. Several in vitro and animal models of acute HIV infection have been used, including macrophages (7), monocyte-derived immature dendritic cells (DCs) (8), CD4⁺ T cell lines (9–11), and PBMCs (12–14). No information, however, is available on HIV-induced changes of host gene expression in primary CD4⁺ T cells from lymphoid tissues. Knowledge of host-HIV interactions in lymphoid tissues is of crucial importance for understanding HIV pathogenesis because HIV replication takes place mainly in these tissues. Lymphoid tissue has a rich cell repertoire, including plasmacytoid DCs (pDCs). pDCs are among the initial target cells for HIV (15) and are critical for the innate immune response to viruses because they rapidly produce large amounts of IFN-α (16–18).

Therefore, we undertook a gene expression profiling study to assess the changes in CD4⁺ T cells isolated from human tonsillar cell suspensions 24 h after infection ex vivo with either a X4 or R5 HIV strain. Our data demonstrate that genes involved in immune response/response to virus, in particular IFN-stimulated genes (ISGs), represent the major group of differentially regulated genes upon HIV infection, irrespective of coreceptor selectivity. This IFN gene signature depends on IFN-α production by pDCs and enables CD4⁺ T cells to limit HIV replication. We also describe the induction of one ISG involved in apoptosis, i.e., TRAIL. Induction of TRAIL is also dependent on the presence of pDCs and IFN-α, but does not play a crucial role in apoptosis upon HIV infection.

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Materials and Methods
Preparation of human lymphocyte aggregate cultures
The acquisition and processing of lymphoid tissue were approved by the local ethics committee. Human tonsils from otherwise healthy adult patients were processed 1-5 h after tonsillectomy. Human lymphocyte aggregate cultures (HLACs) were prepared as described (19). Briefly, single-cell suspensions were prepared, and erythrocytes were lysed with ACK cells (Cambrex BioScience-BioWhittaker; purchased through BioConcept). Lymphoid cells were cultured at 2 x 10^6 cells in 0.2 ml/well in 96-well U-bottom plates overnight before being inoculated with HIV. Cultures were performed in triplicates. One experiment includes data from one donor; the numbers of experiments are given in the legends.

HIV infection of HLACs
HLACs were spinoculated with the X4 HIV strain NL4-3, the R5 HIV strain 49.5, or culture supernatant from mock-infected cells at 1200 f 10^6 cells in a total volume of 125 ml, washed three times with PBS, and resuspended in 200 ml of fresh medium. Virus stocks were obtained by 293T cell transfection, and HIV replication was determined by p24 ELISA as described (20).

Isolation of CD4^+ T cells from tonsillar HLACs
CD4^+ T cells were isolated 24 h postinfection (p.i.) from HIV- or mock-infected tonsillar HLACs with the CD4^+ T Cell Isolation kit II (Miltenyi Biotech). The mean purity of negatively isolated CD4^+ T cells was 93.8% (95% confidence interval [CI] = 92.6–94.9%; n = 54). CD4^+ T cells were either homogenized in lysis buffer (RNeasy Mini kit; Qiagen) containing BioConcept. Lymphoid cells were cultured at 2 x 10^6 cells in 0.2 ml/well in 96-well U-bottom plates overnight before being inoculated with HIV. Cultures were performed in triplicates. One experiment includes data from one donor; the numbers of experiments are given in the legends.

Gene expression profiling
Five sample sets (one set consisting of NL4-3, 49.5, and mock-infected CD4^+ T cells) were available for gene expression profiling. Total RNA was isolated with the RNeasy Mini kit according to the manufacturer’s recommendations (Qiagen). Total mRNA (~2 μg) was used to generate double-stranded cDNA and biotinylated cRNA by standard labeling procedures. Human targets were hybridized to Affymetrix HG-U133A oligonucleotide arrays (Affymetrix) according to the manufacturer’s directions. After hybridization, washing, and labeling with streptavidin-PE, chips were scanned with a confocal laser scanner (Agilent Technologies) at 570 nm. Pixel intensities were measured, expression signals were analyzed, and features were extracted using Affymetrix MicroArray Suite (version 5.0 software). The overall fluorescence intensity was scaled to a global intensity of 500 to allow us to compare chips. Several samples from either NL4-3, 49.5, or mock-infected CD4^+ T cells underwent the same procedure in duplicate to triplicate as reproducibility controls, showing high reproducibility and no significant variability due to the cRNA generation technique (data not shown).

Raw data were analyzed with GeneSpring (version 7.2 microarray data analysis software; Silicon Genetics). Normalization of the raw data was performed as follows. Each signal measurement was divided by the 50th percentile of all measurements in that sample, and signal for each gene was divided by the median of its measurements in all samples. If the median of the raw values was <10, each measurement for that gene was divided by 10; if the median of the raw values was >10, the measurement was excluded. Values below 0.001 were set to 0.001. NL4-3 and 49.5-infected samples were normalized against the median of “mock-infected” samples. Each measurement for each gene in those specific samples was divided by the median of that gene’s measurements in the “mock” samples. The data were median normalized by median polishing of each chip was normalized to its median and that of each gene was normalized to its median. These normalizations were repeated until the medians converged. Before median polishing, raw values below 10 were replaced with 10.

The following criteria were used to select differentially regulated genes in HIV-infected vs noninfected cultures: 1) A Student’s t test with Benjamini-Hochberg multiple-testing correction and a statistical significance level of p < 0.05; 2) a change of >1.5-fold in either direction; 3) a detection present call that coincided with the regulation in question (e.g., if a candidate gene was called up-regulated in HIV-infected cultures, at least one of the samples in the HIV-infected group had to be called present, and vice versa, if a gene was called down-regulated, at least one of the samples in the mock-infected group had to be called present). The selected differentially expressed genes were normalized to a mean of 0 and a SD of 1, log 2-transformed, and subjected to weighted, average-linkage hierarchical clustering by use of the uncentered similarity matrix. Clustering analysis was performed with GeneCluster, and the display of the rearranged data was generated with the TreeView software (21) (both programs are available through http://rana.lbl.gov/). Gene annotations were obtained through the NetAffx internet analysis system (Affymetrix) (22).

Functional associations between differentially expressed genes were analyzed using Ingenuity Pathways Analysis (version 4.0, Ingenuity Systems, www.ingenuity.com) (23).

Real-time quantitative PCR
Total RNA was isolated with the RNeasy Mini kit as per the manufacturer’s recommendations (Qiagen). Total RNA (~1 μg) was reverse transcribed with the 1st Strand cDNA Synthesis kit for real-time PCR (Roche Diagnostics) at 42°C for 90 min. PCR amplifications were conducted with the HotStart system (LightCycler-Faststart DNA Master SYBR Green I; Roche Diagnostics) in the LightCycler thermocycler (Roche Diagnostics). Primers and standards for IFN-α and β2-M (RPA-T4) were described elsewhere (24). GAPDH and TRAIL ready-to-use primer sets were purchased (Search LC).

Depletion of pDCs from tonsillar HLACs
pDCs were depleted from tonsillar cell suspensions with the BDCA-2 Cell Depletion Kit (Miltenyi Biotech) according to the manufacturer’s recommendations. This kit contains an anti-BDCA-2 biotinylated Ab for the isolation of BDCA-2^+ pDCs. The mean purity of negatively isolated BDCA-2^+ T cells was 93.8% (95% confidence interval [CI] = 92.6–94.9%; n = 54). CD4^+ T cells were either homogenized in lysis buffer (RNeasy Mini kit; Qiagen) containing 2-ME and stored as lysates at 5°C (5 ng of p24 per 2 x 10^6 cells in a total volume of 125 ml), washed three times with PBS, and resuspended in 200 μl of fresh medium. Virus stocks were obtained by 293T cell transfection, and HIV replication was determined by p24 ELISA as described (20).

Blocking/stimulation assays
In a subset of experiments, HLACs were pretreated with one of the following compounds overnight before exposure to HIV: 1) mouse mAb against human IFN-α receptor chain 2 (anti-IFN-α-R2; PBL Biomedical Laboratories, purchased from Alexis; clone MMHAR-2; 1 μg/ml); 2) a ligand for TLR7 (852-A; a gift from 3M Pharmaceuticals; 10 μg/ml); 3) CpG2216, a ligand for TLR9 (CpG type A; Microsynth; 5 μg/ml); 4) IFN-α (pegylated IFNα2a (Pegasys); Roche, 10 ng/ml). Concentrations of these compounds were maintained throughout the experiments and replenished each time when part of the medium was replaced (i.e., 50 μl of culture supernatant every 3 days). In addition, HLACs were treated with a blocking mAb against human TRAIL (eBioscience, purchased from THP Medical Products Vertriebs; clone RIK-2; 5 μg/ml); the Ab was added immediately after spinoculation of the cultures with HIV and thereafter maintained in the culture throughout the experiments.

Immunostaining and flow cytometry
To detect p24^+ CD4^+ T cells in tonsillar HLACs inoculated with HIV, the cells were first stained with a mAb against CD4 (BD Biosciences/BD Pharmingen; clone RPA-T4) conjugated to PE, permeabilized/fixed with Cytofix/Cytoperm (BD Biosciences/BD Pharmingen), and subsequently stained for intracellular p24 with a p24-specific FITC-conjugated mAb (Beckman Coulter; KC57). To acquire all CD4^+ T cells in HIV-infected cultures, including those with CD4 down-regulated by HIV, cells were incubated with anti-CD4 mAb both before and after fixation. Mock-infected cells from autologous cultures were stained identically as cells from cultures inoculated with HIV and served as controls. Productive infection of CD4^+ T cells was assessed initially by gating on lymphocytes on forward scatter/size scatter (live cell gate) and subsequently by determining the percentage of CD4^+ T cells positive for p24. In mock-infected cultures, the percentage of CD4^+ T cells positive for p24 was 0.62% (95% CI = 0.33–0.92%; n = 8). For each experiment, the percentage of p24^+ CD4^+ T cells in mock-infected cultures was subtracted from the one in X4 and R5 HIV-infected cultures.

Depletion of pDCs from tonsillar cell suspensions before inoculation with HIV was verified by staining of both undepleted cell suspensions and the flow through of magnetic separation with BDCA-2-FITC and BDCA-4-PE mAbs (Miltenyi Biotech). Apoptosis of T cells was assessed by staining with annexin V (BD Biosciences/BD Pharmingen; labeled with FITC) and intracellular staining for active caspase 3 (BD Biosciences/BD Pharmingen; C92-695).

To detect TRAIL^+ CD4^+ T cells in tonsillar HLACs, cells were stained with an anti-TRAIL human mAb (eBioscience; RIK-2), incubated with goat anti-mouse IgG-PE (BD Biosciences/BD Pharmingen), and stained with anti-CD4-FITC mAb.

Flow cytometry was performed on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).
IFN-α and soluble TRAIL (sTRAIL) ELISA

Culture supernatants from replicates were pooled for subsequent measurements and assayed for IFN-α and sTRAIL concentrations by an enzyme immunoassay (Bender MedSystems and Diaclone, respectively, purchased through BioTest), according to the manufacturer’s instructions.

Statistical analysis (other than microarrays)

Statistical analysis was performed with SPSS software package (version 12.0). Statistical significance of differences for X4/R5 HIV-infected and mock-infected and undepleted and pDC-depleted samples was determined using the paired Student t test. Samples missing respective pairs were excluded from matched (paired) statistical analysis. Values of p of <0.05 were considered statistically significant. All statistical tests were two-sided.

Results

HIV infection of tonsillar HLACs induces a distinct IFN response in CD4+ T cells

Using our filtering criteria, we found 514 differentially expressed genes in CD4+ T cells 24 h after ex vivo infection of tonsillar HLACs with HIV. IFN cluster was obtained after hierarchical clustering analysis of 514 differentially expressed genes. The similarity of gene expression profiles among experimental samples is summarized in a dendrogram above the cluster heat map, in which the pattern and the length of the branches reflect the relatedness of the samples. Red, high relative level of expression; green, low relative level of expression; black branches, mock (control) samples; blue branches, HLACs infected with X4 (NL4-3) HIV; red branches, HLACs infected with R5 (49.5) HIV. Genes are identified by their gene titles (NetAffx). Genes verified by real-time PCR are marked in bold and underlined.
Functional annotation analysis (Gene Ontology (GO) search at the NetAffx website) revealed that 17% of the genes induced by HIV were involved in the immune response, and of these, 30% were part of the response to virus (Fig. 2). Importantly, the majority of up-regulated immune response genes were ISGs, a large family of genes induced by type I IFNs and involved in mediating IFN effects (25, 26) (Figs. 1 and 2). Genes associated with apoptosis represented 6% of up-regulated genes (Fig. 2). Network analysis revealed complex functional relationships between different molecules (some of which are also ISGs) involved simultaneously.

**FIGURE 2.** Selection of up-regulated genes characterizing the response to HIV infection. Bars, The mean fold change in the mRNA level of the gene indicated 24 h after HIV infection of HLACs compared with mRNA levels in the mock-infected controls. These genes passed our filtering criteria. Genes are indicated by their gene symbol. *, Repeated gene symbols represent gene expression levels obtained by use of different probes (i.e., oligonucleotide sets) on Affymetrix arrays.

<table>
<thead>
<tr>
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gene expression intensity between the two virus strains were associated with different infection rates: at 24 h p.i., the mean percentage of infected CD4<sup>+</sup> T cells was 7.9% for X4 (95% CI = 5.4–10.8%; n = 5) and 24.1% for R5 strain (95% CI = 17.0–32.4%; n = 5), as revealed by flow cytometry. Apart from this difference in relative gene expression levels, statistical analysis revealed no significant differences in the gene expression profiles of the R5 and X4 virus. Thus, the lower gene expression intensity observed with the X4 virus was likely attributable to a lower infection rate compared with that of the R5 virus.
in antiviral immune response (e.g., IRF7, MX1, MYD88), interference with different viral functions (e.g., RSAD2, IFIH1) and apoptosis (e.g., TNFSF10, PLSCR1, MCL1, CASP1) (Fig. 3).

We decided to validate the expression levels of three ISGs by real-time PCR that represent key molecules in each of these processes (IRF7, MX1, and TNFSF10 for antiviral type I IFN response, inhibition of viral replication and apoptosis, respectively) and are functionally dependent on pDCs, i.e., their function as main type I IFN producers in response to viruses (see next paragraph). IFN regulatory factor 7 (IRF7), a transcription factor, plays a key role in type I IFN induction when TLRs are triggered by viruses (27–30). The mouse myxovirus resistance (MX) gene (human homolog, MXA; hereafter referred to as MXA) encodes a GTPase with antiviral activity selectively induced by type I IFNs (31). TNF ligand superfamily, member 10 (TNFSF10; also known as TRAIL; hereafter referred to as TRAIL), induces apoptosis in virus-infected and tumor cells (32) and can also be induced by type I IFNs (33). Indeed, consistent with microarray results, real-time PCR showed up-regulation of gene expression for IRF7, MXA, and TRAIL upon infection with HIV in nearly all of the samples analyzed (Pearson’s $r > 0.91$, $p < 0.001$ for each of these three investigated genes, array vs PCR) (Fig. 4A). Furthermore, real-time PCR performed on an additional set of samples showed up-regulated expression of the same genes in CD4$^+$ T cells from

FIGURE 3. Ingenuity Pathway (IP) Analysis network that depicts functional associations between genes characterizing the response to HIV infection. The network is graphically displayed with genes/gene products as nodes (different shapes) and the biological relationships between the nodes as edges (lines). Nodes are further displayed using various shapes that represent the functional class of the gene product. The length of an edge reflects the evidence in the literature supporting that node-to-node relationship. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Genes involved in antiviral immune response (red), interference with viral function (blue) and apoptosis (violet) are shown as nodes highlighted by bold-line frames and appropriate colors. The number of genes involved in one of these processes is shown in brackets next to the description of the process. Yellow-framed nodes represent genes with overlapping functions in antiviral immune response, interference with viral function, and apoptosis. This network was generated as follows: a data set containing 514 gene identifiers and corresponding expression values (fold change with a cutoff of 1.5 in each direction) was uploaded into the application. Of the 514 up-loaded genes, 314 genes were eligible for generating networks. Each gene identifier was then mapped to its corresponding gene object in the IP Knowledge Base. Gene expression values (up-loaded fold-change values) were then used to identify genes whose expression was differentially regulated. These genes, called “focus genes,” were overlaid onto a global molecular network contained in the IP Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity and scored according to the number of focus genes (35 genes in the shown network) within the network as well as according to the strength (score) of their associations. The score is derived from a p value and indicates the likelihood of the focus genes in a network being found together due to random chance. The network shown here was the one with the highest significant score of 53, confirming $>99\%$ confidence of not being generated by random chance alone.
HIV-infected HLACs, compared with mock controls (t test for TRAIL in X4 HIV-infected HLACs, p = 0.0334) (Fig. 4B). For these additional experiments, mean percentage of infected CD4$^+$ T cells 24 h.p.i. was determined: 17.5% for X4 (95% CI = 6.8–41.2%; n = 3) and 9.8% for R5 strain (95% CI = 2.4–33.1%; n = 3). In accordance with the data from the first set of samples, a higher infectivity rate was associated with a more pronounced up-regulation of IFN-$
abla$A, MXA, and TRAIL. The difference in the infectivity between the two sets of samples (i.e., higher infection rate with R5 HIV in the first set in contrast to higher X4 HIV infection rate in the second set) is most likely due to the different infectious titers of the HIV stocks used.

**IFN response in tonsillar CD4$^+$ T cells after HIV challenge requires pDCs**

Although some ISGs are induced independently of IFN-$
abla$A, most are induced through type I IFN-triggered signaling via the IFN-$
abla$A/R/JAK/STAT pathway (34). pDCs are the main IFN-$
abla$A-producing cells in response to virus (16–18). To assess the role of pDCs in the observed ISG induction, we depleted pDCs from tonsillar cell suspensions before HIV infection and measured the expression of IFN-$
abla$A, MXA, and TRAIL gene after exposure to HIV. Depleting the pDCs nearly abolished the induction of these three ISGs in X4 HIV- and R5 HIV-infected HLACs (Fig. 5). Thus, HIV-triggered induction of ISGs, by at least the ones examined here, depends largely on the presence of pDCs in the microenvironment.

**pDCs limit HIV replication in tonsillar HLACs**

A large subgroup of ISGs codes for proteins involved in host defense, such as isoforms of MX protein and 2’-5’-oligoadenylate synthetase (OAS) that possess direct antiviral activity. We addressed the question whether pDCs, through induction of ISGs, induce an anti-HIV state in lymphoid tissue. We found that depletion of pDCs from tonsillar cell suspensions before inoculation with HIV resulted in significantly higher levels of viral replication than those in undepleted cultures (Fig. 6, A and B). This observation suggests that pDCs induce an anti-HIV state in tonsillar CD4$^+$ T cells, which is likely effected through the induction of antiviral ISGs in these cells.

**IFN-$
abla$A is essential for anti-HIV effect of pDCs**

Our data suggested that the anti-HIV effect of pDCs in tonsillar HLACs was mediated through IFN-$
abla$A produced by these cells, which then triggers the IFN response in CD4$^+$ T cells. To provide more stringent evidence for this mechanism, we first measured production of IFN-$
abla$A in the different cultures 24 h.p.i. by ELISA. Mean IFN-$
abla$A concentrations in the supernatants of X4 and R5 HIV-infected undepleted HLACs were >9-fold higher than those in mock-infected undepleted cultures (t test for R5 HIV-infected HLACs p = 0.035) (Table I). Importantly, pDC depletion before inoculation with HIV significantly reduced IFN-$
abla$A concentrations in each of the groups. Thus, the anti-HIV effect of pDCs in tonsillar HLACs seems to be primarily mediated by endogenous IFN-$
abla$A production.

Furthermore, we examined the effects of increasing or reducing the levels of IFN-$
abla$A, respectively, with an anti-IFN-$
abla$A-R blocking Ab or with compounds stimulating IFN-$
abla$A release by pDCs through triggering of TLR7 or TLR9, which are pathogen recognition receptors preferentially expressed by pDCs (35). Indeed, treatment with anti-IFN-$
abla$A-R blocking Ab significantly increased replication of both X4 and R5 HIV, and treatment with TLR7 and TLR9 ligands significantly decreased HIV replication (Fig. 6C). We further investigated whether rIFN-$
abla$A could compensate for IFN-$
abla$A endogenously produced by pDCs in pDC-depleted cultures. Indeed, exogenous IFN-$
abla$A caused a significant reduction of HIV replication in X4 and R5 HIV-infected HLACs (Fig. 6D).
Role of IFN response in apoptosis of CD4+ T cells in HIV-infected HLACs

IFNs are also involved in apoptosis, and this effect is mediated by a subgroup of ISGs with apoptotic function, including TRAIL (36). In line with the mRNA data obtained by microarray analysis and real-time PCR, TRAIL cell surface expression on CD4+ T cells was up-regulated in undepleted HIV-infected cultures, regardless of coreceptor preference, compared with pDC-depleted cultures infected with HIV (Fig. 7A) and mock-infected cultures (data not shown) as assessed by flow cytometry. We also assessed sTRAIL (32) levels in the cultures 24 h p.i. by ELISA. Concentrations of sTRAIL in the supernatants of X4- and R5 HIV-infected undepleted HLACs were significantly higher than those in mock-infected undepleted cultures; sTRAIL levels were reduced by pDC depletion before infection with X4 or R5 HIV and in mock controls (Table II). Thus, levels of soluble TRAIL were increased in response to HIV infection, and this response appeared to be mediated largely by pDCs, in particular during R5 HIV infection (reflected in a profound decrease in sTRAIL levels upon pDC depletion as compared with X4-infected samples).

To examine whether the pDC-mediated IFN response in tonsillar CD4+ T cells triggers apoptosis of CD4+ T cells, we examined the effects of pDC depletion on CD4+ T cell apoptosis on day 7 p.i., by staining with annexin V and for active caspase 3. Infec-

Discussion

Initial host-pathogen interactions are pivotal for the extent of HIV replication and the host’s capacity to mount an efficient immune response. CD4+ T cells are the main target cells of HIV and therefore knowledge about host-pathogen interactions in these cells is of prime importance. Previous studies examining early modulation of gene expression by HIV in this cell population have exclusively used CD4+ T cell lines (9–11). They found differentially expressed genes involved in TCR-mediated signaling, subcellular trafficking, transcriptional regulation, and various cellular metabolic pathways (9); mitochondrial function, DNA repair, and apoptosis (10); cell division, transcription, translation, and splicing (11).

Findings obtained in CD4+ T cell lines need to be corroborated by models that mimic more closely the in vivo situation. Thus, in this study, we examined host gene expression in CD4+ T cells isolated 24 h p.i. from suspension cultures of human tonsillar cells infected ex vivo with HIV. We concentrated on CD4+ T cells from lymphoid tissues because HIV replicates mainly in these tissues. Strikingly, the gene signature we observed was substantially different from that previously obtained in CD4+ T cell lines (9–11). They found differentially modulated genes were those involved in immune response and response to virus, with ISGs representing the major subgroup.

The gene profiles observed by us were similar between X4 and R5 HIV, suggesting that cellular gene expression in lymphoid CD4+ T cells early in HIV infection is largely independent of preference for a coreceptor. This suggests that viral and/or host factors later in infection or the selective cellular tropism of the HIV strains (e.g., R5-tropic strains and macrophages) are likely to underlie the different pathogenicities of R5 and X4 HIV strains. Our findings are in contrast to a recent study by Cicala et al. (14), who reported distinct gene expression profiles in PBMCs induced by R5 and X4 envelopes. This discrepancy is likely explainable by the different experimental settings: first, while Cicala et al. (14) used PBMCs with no separation of cellular subsets, we used CD4+ T cells isolated from tonsillar cell suspensions. In the former experimental setup, the HIV-induced gene profiles cannot be attributed to a specific cell subset. Second, Cicala et al. (14) used recombinant HIV envelope proteins, whereas we used infectious virus. It has to be considered that in addition to the HIV envelope protein gp120, other HIV proteins, including Nef (37, 38) and Tat (8, 39, 40), alter the expression of cellular genes. Moreover, by adding rHIV envelope, subsets of cells such as macrophages and
myeloid DCs may be the cause of the distinct gene profiles. Most importantly, recognition of viral infection by innate immune cells occurs through binding of genomic DNA and RNA or dsRNA produced in virally infected cells through different pattern recognition receptors (41). Thus, the use of infectious virus resembles more closely the in vivo situation.

Based on the results obtained by microarray analysis, we addressed the question of whether HIV induction of ISGs in CD4+ T cells might depend on the microenvironment, in particular the presence of pDCs, which are the most efficient producers of IFNα. Some ISGs are induced in virally infected cells independently of IFN; most ISGs are up-regulated through IFN-triggered signaling via the IFN-αR/JAK/STAT pathway (34). Indeed, depletion of pDCs from tonsillar cell suspensions before inoculation with HIV abrogated induction of the three ISGs analyzed (i.e., IRF7, MXA, and TRAIL) in CD4+ T cells. Lower levels of IFN-α in the supernatants of pDC-depleted cultures than in those of undepleted cultures suggest that the observed IFN gene signature is mediated by pDC-produced IFN-α.

The dependence of ISG induction in CD4+ T cells by HIV on the presence of pDCs observed in our study easily explains why these gene profiles were not found in previous microarray studies using CD4+ T cell lines (9–11). We have already emphasized the critical role of the experimental system in studying the host response to HIV in a previous study: while the transcription factor NFIB-2 was highly up-regulated in HIV-infected CD4+ T cell lines (10), mRNA levels of this gene were not affected in lymphoid tissue infected ex vivo with HIV (20).
Some ISGs, such as MXA and OAS, have antiviral activity (42). Our data suggest that the pDC-mediated IFN response in CD4+ T cells also conferred an inhibitory effect against HIV. First, depletion of pDCs from tonsillar cell suspensions before HIV challenge resulted in increased levels of HIV replication. Second, HIV replication was increased in undepleted HLACs treated with a blocking anti-IFN-α Ab and decreased in pDC-depleted cultures treated with rIFN-α. Third, both TLR7 and TLR9 ligands strongly reduced replication of X4 and R5 HIV. TLR7 and TLR9 are pattern recognition receptors, which are expressed predominantly in pDCs and B cells (43), and their triggering results in increased secretion of IFN-α (18, 35). Thus, HIV triggers an antiviral program in CD4+ T cells, which depends on signals provided by pDCs, in particular IFN-α. However, this antiviral response cannot fully control HIV replication. Our data are consistent with previous reports, demonstrating that the innate immunity does sense and respond to HIV, but is inefficient in constraining the virus (13, 44, 45). Molecular mechanisms permitting HIV to escape from the antiviral IFN response likely contribute to this failure of the innate immune system. For instance, HIV-1 Tat protein associates with dsRNA-dependent protein kinase and inhibits its activity (46).

Approximately 6% of genes up-regulated in CD4+ T cells were related to apoptosis, including TRAIL, an ISG and member of the TNF family, that induces apoptosis in tumor and virus-infected cells (32). Several groups have emphasized the role of TRAIL in HIV-induced apoptosis of infected and uninfected CD4+ T cells (47–49). In contrast, induction of ISGs, such as TRAIL, did not appear to promote apoptosis of CD4+ T cells in our study. Neither depletion of pDCs nor treatment with a blocking anti-TRAIL Ab prevented CD4+ T cell apoptosis in X4 HIV-infected cultures. It is of note that in the studies mentioned above, anti-TRAIL Abs did not completely prevent apoptosis either, suggesting concurrent involvement of other death mechanisms. The Fas/FasL pathway alone did not appear to be responsible for apoptosis, but a combined action of mitochondrial intrinsic pathway, TRAIL, FasL, TWEAK, and TNF-α has been proposed to trigger cell death (47–51). TRAIL death receptor 5 (DR5) is up-regulated in PBMCs from HIV-infected patients and plays a role in HIV-induced CD4+ T cell apoptosis (49). In our study, DR5 mRNA expression was not significantly changed by HIV as assessed by microarray analysis and real-time PCR (data not shown). Our characterization of gene profiles provides the basis for examining other possible mechanisms mediated by other molecules, such as phospholipid scramblase (PLSCR1), another up-regulated ISG related to apoptosis (25, 36), or IFIH1 (IFN-induced with helicase C domain 1, also known as MDA5) (52–54). Taken together, the HIV-induced IFN response in CD4+ T cells has only a minor, if any, proapoptotic effect.

Despite the fact that the infection rate with the R5 HIV was somewhat higher than the one in the X4 HIV-infected cultures in the first set of samples, induction of apoptosis was considerably higher in X4 HIV- than in R5 HIV-infected cultures (data not shown). Moreover, depletion of pDCs from HLACs infected with X4 HIV further increased the percentage of annexin V+ cells with no significant effect on R5 HIV- and mock-infected cultures. The coreceptor for X4 HIV, CXCR4, is expressed on nearly all CD4+ T cells in contrast to CCR5, the coreceptor for R5 HIV, which is expressed on ~15–30% of these T cells (reviewed in Ref. 55).

### Table I. Production of IFN-α in tonsillar HLACs infected ex vivo with HIV

<table>
<thead>
<tr>
<th></th>
<th>Undepleted</th>
<th>pDC Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFN-α (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL4-3</td>
<td>102</td>
<td>5.49</td>
</tr>
<tr>
<td>95% CI</td>
<td>(11.0–193)</td>
<td>(1.73–9.24)</td>
</tr>
<tr>
<td>49.5</td>
<td>161±c,d</td>
<td>4.74</td>
</tr>
<tr>
<td>95% CI</td>
<td>(26.0–297)</td>
<td>(1.49–8.00)</td>
</tr>
<tr>
<td>Mock</td>
<td>11.0</td>
<td>5.79</td>
</tr>
<tr>
<td>95% CI</td>
<td>(6.00–16.0)</td>
<td>(2.03–9.54)</td>
</tr>
</tbody>
</table>

a Data are given as mean (95% CI); n = 7.

b Value of p = 0.046 compared with pDC-depleted NL4-3.

c Value of p = 0.035 compared with undepleted Mock.
d Value of p = 0.032 compared with pDC-depleted 49.5.
e Value of p = 0.037 compared with pDC-depleted Mock.

**FIGURE 7.** Role of ISGs for HIV-induced apoptosis of CD4+ T cells. Untreated and pDC-depleted HLACs were spinoculated with HIV NL4-3, 49.5, or mock-infected, and cells were analyzed for apoptosis by immunostaining and flow cytometry. A, The percentage of TRAIL+ CD4+ T cells was assessed 24 h.p.i. B, The percentage of annexin V+ (left panel) and active caspase 3+ (right panel) CD4+ T cells in X4 HIV-infected cultures was assessed 7 days p.i. C, HLACs were left untreated or treated with a blocking mAb against human TRAIL immediately after spinoculation of the cultures with X4 HIV and thereafter maintained in the culture; apoptosis of CD4+ T cells was assessed 7 days p.i.
This accounts, at least in part, for a profound deleterious effect of X4 HIV on CD4 T cells due to its particular capacity to induce apoptosis through interaction of the HIV envelope glycoprotein gp41/gp120 (Env) and/or soluble gp120 to CXCR4 (50, 51)–an effect also observed in our study. The type I IFN response mediated by pDCs seems to only partially diminish the depletion of CD4 T cells induced by X4 HIV, reflecting the use of other death pathways by the virus.

High-throughput gene expression profiling has also been performed on other cell types, such as monocyte-derived macrophages (7), monocyte-derived immature DCs (8), and peripheral blood from simian-HIV-infected macaques (13). In these systems, up-regulation of ISGs was also found. However, the study by Woelk et al. (7), the authors could not detect induction of IFN-αβ genes; they speculate that their induction was likely missed due to a narrow time frame when this induction takes place. Likewise, in monocyte-derived immature DCs (8), IFN induction could not be detected at the mRNA and protein level; here, the authors conclude that HIV protein Tat rather than IFN was directly responsible for the up-regulation of several ISGs, such as IRF7, MXA, STAT1, IFIs, and TRAIL. However, they did not take into account pattern recognition receptors and IFN-producing cells (first description term for pDCs). In our study, we could not detect induction of IFN-αβ genes, even though numerous ISGs were up-regulated at 24 h after the infection. This phenomenon very likely reflects the narrow time window of IFN-α mRNA transcription that starts as early as 4 h after viral stimulation and reaches its peaks at 12 h (56) followed by the protein production and subsequent induction of various ISGs.

A number of differentially expressed genes other than IRF7, MXA, and TRAIL, are also worth mentioning. One of the most highly up-regulated ISGs was (mouse) RSAD2, the human homolog of which is CIG5. Its gene product, viperin, has inhibitory activity against hepatitis C virus and human CMV (57, 58). CIG5 is one of the ISGs induced in monocyte-derived macrophages after exposure to HIV (7). Among up-regulated genes related to immune response/responses to virus independent of IFN-α was MyD88, a key factor in virus-triggered TLR signaling (59), reflecting the induction of various innate immune mechanisms in response to HIV. This group also contained a gene encoding for HIV-induced TAR RNA-binding protein 1, which is involved in HIV Tat binding to TAR RNA and the regulation of HIV replication (60, 61).

In conclusion, gene expression profiling revealed a distinct IFN signature in CD4 T cells after ex vivo acute infection of lymphoid tissue with HIV. This IFN response is mediated by pDCs through IFN-α production and enables these cells to mount an anti-HIV state, which is however insufficient to fully control the virus. It will be of interest to dissect the contribution of individual ISGs to the overall anti-HIV effect. Assessment of their relevance will be useful in the design of novel therapeutic interventions and prevention strategies.

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Disclosures
The authors have no financial conflict of interest.

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