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HIV Induces Maturation of Monocyte-Derived Dendritic Cells and Langerhans Cells

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In HIV infection, dendritic cells (DCs) may play multiple roles, probably including initial HIV uptake in the anogenital mucosa, transport to lymph nodes, and subsequent transfer to T cells. The effects of HIV-1 on DC maturation are controversial, with several recent conflicting reports in the literature. In this study, microarray studies, confirmed by real-time PCR, demonstrated that the genes encoding DC surface maturation markers were among the most differentially expressed in monocyte-derived dendritic cells (MDDCs), derived from human blood, treated with live or aldrithriol-2-inactivated HIV-1BaL. These effects translated to enhanced cell surface expression of these proteins but differential expression of maturation markers was only partial compared with the effects of a conventional potent maturation stimulus. Such partially mature MDDCs can be converted to fully mature cells by this same potent stimulus. Furthermore, live HIV-1 stimulated greater changes in maturation marker surface expression than aldrithriol-2-inactivated HIV-1 and this enhanced stimulation by live HIV-1 was mediated via CCR5, thus suggesting both viral replication-dependent and -independent mechanisms. These partially mature MDDCs demonstrated enhanced CCR7-mediated migration and are also able to stimulate interacting T cells in a MLR, suggesting DCs harboring HIV-1 might prepare CD4 lymphocytes for transfer of HIV-1. Increased maturation marker surface expression was also demonstrated in native DCs, ex vivo Langerhans cells derived from human skin. Thus, HIV initiates maturation of DCs which could facilitate subsequent enhanced transfer to T cells. The Journal of Immunology, 2006, 177: 7103–7113.

Dendritic cells (DCs)3 are potent APCs that form a direct link between the innate and the adaptive immune systems (1–3). In their immature state, DCs are peripheral sentinels awaiting contact with microbial glycoprotein Ags which bind to C-type lectin receptors (CLR) such as DC-SIGN, mannose receptor (MR), and probably Langerin on their surface (4). These Ags are then rapidly endocytosed, digested throughout the endosomal pathway, and the resulting peptide fragments are loaded onto MHC class II molecules and transported to the cell surface for Ag presentation (2). Danger signals released from associated inflammatory foci trigger DC maturation and migration to the draining lymph nodes. Maturing DCs down-regulate the surface expression of CLR5, CCR5, and CD1a and also their endocytic capacity, and up-regulate CD40, the costimulatory molecules, CD80 and CD86, MHC class II, adhesion molecules such as CD54, CXCR4, and express the key marker maturation CD83 de novo. Upon arrival at the lymph node, mature DCs interact with and present Ag to naive or memory T cells. This process is assisted by an initial interaction between CD40L and CD28 on the T cell surface and present Ag to naive or memory T cells. This process is assisted by an initial interaction between CD40L and CD28 on the T cell surface and CD80 or CD86, respectively, on the DC surface (5).

A network of epidermal Langerhans cells (LCs) is distributed throughout anogenital skin and mucosae including the vagina and ectocervix as well as the male foreskin (6–8). Deep to this is a layer of dermal or lamina propria DCs. These cells appear to be one of the first cell types to be infected upon vaginal exposure of macaques to SIV (6). HIV may use DCs as a “Trojan horse” for transport to and possibly activation of CD4 lymphocytes in the lamina propria/submucosa and lymph nodes (9). Currently, there is intense investigation of the role of DCs in SIV/HIV entry, how these viruses traffic in DCs, and how they are transferred to T cells (10–12). Recently, we have demonstrated that after binding to surface CLR5s, HIV is either endocytosed and degraded by acid proteolytic digestion or transferred to CD4 and CCR5 followed by neutral fusion and de novo replication. The former is the major pathway. HIV can be transferred to CD4 lymphocytes via either pathway but in sequential phases, i.e., de novo replication results in late transfer (11).

Many viruses are able to infect DCs and a proportion of these have evolved mechanisms of interfering with DC function thus impairing the immune response against them. These mechanisms include induction of apoptosis, (13, 14) inhibition of maturation of DCs (15–21), or by unknown mechanisms (22–25). Conversely, some viruses enhance DC maturation after infection (26–28) and others have little affect on DC function (29–31).

Some recent reports suggest that HIV-infected DC cultures show impaired stimulation in the MLR, correlated with the level of IL-10 produced (32). Impaired secretion of IL-12 from HIV-infected DCs has also been reported (33). However, the effect of HIV-1 on DC maturation is controversial. Some studies indicate that HIV-treated or -infected DCs fail to up-regulate cell surface markers, (32, 33) and that this effect is mediated by Vpr (34) or that they are refractory to maturation stimuli (24). Conversely, other studies demonstrate abnormal or partial up-regulation of cell maturation markers and that this effect and subsequent DC
migration is triggered by signaling events induced by the binding of the virus surface glycoprotein gp120 to monocyte-derived dendritic cells (MDDCs) (35, 36).

However, in most of the studies described above, unpurified virus stocks were used and with the exception of one of these studies (35), low titer virus stocks were used. Many of these studies did not investigate the full complement of surface markers indicative of DC maturation. As part of a study on HIV-induced global gene expression in MDDCs using a high titer, highly purified HIV-1 virus stock in comparison with a chemically inactivated virus and recombinant gp120, we found up-regulation of the genes encoding maturation markers CD83 and CD80 to be two of the genes whose expression was most altered. These results were followed up by quantitative PCR and flow cytometry to investigate the cell surface expression and broaden the study to include most DC maturation markers on both MDDCs and ex vivo LCs, finally examining their functional effects in the model MDDCs.

Materials and Methods

Preparation of MDDCs

CD14⁺ monocytes derived from PBMC using CD14 magnetic beads (Miltenyi Biotec) were converted to immature MDDCs (iMDDCs) by culture in GM-CSF and IL-4 for 6 days as described previously (11, 37, 38) (high CD1a, MR, DC-SIGN, HLA-DR, moderate to low CD40, CD80, CXCR4, and negative for CD14 and CD83). At day 6, fresh medium and cytokines were added before experiments were performed. To obtain mature MDDCs, cells were cultured for 2 additional days in a maturation mixture of PGE₂ (10⁻⁶ M; Sigma-Aldrich), TNFα, IL-1β, and IL-6 (10 ng/ml; R&D Systems). Mature DCs up-regulate CD40, CD80, CXCR4, and HLA-DR, express CD83 de novo, and down-regulate CD1a, MR, and DC-SIGN.

Isolation of LCs from skin

Apronectomy and breast skin samples from healthy donors were obtained from the Royal North Shore Private Hospital (Sydney, Australia) under informed consent and Sydney West Area Health Service Ethics Committee approval. All adipose tissue was removed from the skin, which was then sectioned into 5-mm² pieces and incubated for 1 h in RPMI 1640 containing 250 µg/ml gentamicin (Invitrogen Life Technologies) at 4°C before incubation overnight in 5 mg/ml dispase II (Roche Biochemicals) and 25 µg/ml gentamicin in RPMI 1640. Epidermal layers were separated from the dermis and incubated in 5 mg/ml collagenase (Sigma-Aldrich) for 2 h at 37°C. Dissociated cells were collected, filtered, washed, and subjected to flow cytometric analysis and/or HIV infection. For measurement, surface marker expression on LC populations were gated for CD1a and langerin expression and also confirmed to express HLA-DR.

Preparation of high titer purified HIV (BaL) virus stocks

A purified high titer HIV-1 stock in the order of 5 × 10⁶ 50% tissue culture infective dose (TCID₅₀)/ml was produced as described previously, which was used to treat live HIV-infected (0.01–100 µg/ml) cells equivalent to a multiplicity of infection (MOI) 0.03–300 TCID₅₀/cell or the equivalent amount of AT-2-treated HIV (Bal) as determined by p24 gag ELISA and serial dilution Western blot and densitometry, or with recombinant monomeric gp120 at 50 ng/ml-5 µg/ml (purified from laboratory adapted Bal, strain from AIDS Research and Reference Reagent Program, National Institutes of Health, or SLCA-1 primary RS strain courtesy of Dr. J. Arthos, National Institutes of Health, Bethesda, MD (42), or mock treated.

Biotinylated gp120-binding assay

Both gp120 species were biotinylated and added to day 6 MDDCs suspended in binding buffer (RPMI 1640, 10 mM HEPES, 1% BSA (ph 7.4) at 1.1 × 10⁴ cells/ml to final concentrations ranging from 2.5 to 120 µg/ml gp120 and incubated at 4°C for 30 min as previously described (37). After washing in FACS wash (PBS, 0.1% BSA, 0.1% sodium azide), the cells were incubated 4°C for 30 min in the presence of streptavidin-PE 0.5 µg/ml (BD Pharmingen) before being analyzed by flow cytometry.

Determination of HIV-1 infectivity by real-time PCR

HIV-1 DNA in the lysate was quantified by real-time PCR for HIV-1 LTR-gag DNA in an ABI 7700 (Applied Biosystems/PerkinElmer) using primers and a molecular beacon as previously described (11, 43). Cell numbers were estimated by albumin DNA using the primers 5’-TGGT GAGAAAAACGCGAC TAA-3’ and 5’-ATGGTCGCCGGTTACCA A-3’ and the molecular beacon: 5’-FAM CGCGATGACGTACC CAAAT GCTGACAGAAAGGCCC-3’.

Preparation of labeled cDNA and hybridization to microarrays

Total RNA from four independent experiments was extracted from frozen cell pellets using the RNAqueous-Midi kit (Ambion), quantified by UV spectroscopy and the integrity confirmed by gel electrophoresis. The mRNA was subsequently amplified using the messageAMP kit (Ambion) before the SuperScript Indirect cDNA Labeling Core kit (Invitrogen Life Technologies) was used to reverse transcribe the amplified RNA in the presence of aminosil and aminohexyl modified dNTPs followed by incorporation of Cy3 or Cy5 (Amersham Biosciences) fluorochromes into the cDNA. Combinations of fluorescently labeled amplified RNAs were then hybridized (in a closed loop design, see Fig. 1) to Human ResGen 8k (Australian Genome Research Facility) glass microarrays containing 8000 human cDNAs spotted in duplicate. The list of genes spotted onto each of the microarrays is available at www.agrf.org.au.

Analysis of microarray data

The hybridized microarrays were scanned using an Axon GenePix 4000B scanner and images were processed using GenePix 5.0 software. Following data extraction, all analyses were undertaken using the R (version 2.0.0) statistical computing environment and BioConductor (version 1.5). Follow-up extraction of the mean of AT-2 concentrations ranging from 2.5 to 120 µg/ml gp120 were weighted as 0.1. A weighted normalization was applied using the robust splines technique (limma package) and low quality spots and controls removed from further analysis. The MA value, or log₂ of the ratio of Cy5 to Cy3 background-subtracted intensities was used as a measure of relative expression (44). We used Bayesian linear modeling methods (limma package) for ranking genes based on their probability of being differentially expressed (45, 46). The empirical Bayes approach applies linear modeling to derive “B” values for each transcript, which were used to rank genes most likely to be differentially expressed. As illustrated below, a linear model was fitted for each time point and transcripts generating a B value > 0.50% odds of differential expression) and containing a change in expression of > 1.5-fold (arbitrarily estimate of change required for a biological effect) were defined as differentially expressed (Table I).

Real-time PCR

Real-time RT-PCR was used to determine the expression levels of genes encoding MDDC cell surface markers. Total unamplified RNA was DNase I treated (Promega), reverse transcribed using oligo d(T) and Superscript III followed by RNase H treatment (Invitrogen Life Technologies). The cDNA was then subject to quantitative PCR using defined primers and labeled probes (Invitrogen Life Technologies) and analyzed using an ABI 7700 (Applied Biosystems/PerkinElmer). The relative quantitation method (ΔΔ cycle threshold) (47) was used to evaluate the expression of selected genes with the GAPDH, β-actin, and β₂-microglobulin (β₂m)
amplicons as an internal control and the normalizer for all data. PCR assays were performed for a total of five different primers. The used primers were as follows: CD1a (forward (F)) CCACGGTTCTTCCCAACGGCC; CD1a (reverse (R)) ATCCGATGAAGGCCCTACACCTCAC; CD40 (F) CTGTGATGCTGACAGC; CD40 (R) TCGGGAAAATTGATCTCCTG; CD80(R) TCGGGAAAATTGATCTCCTG; CD80(F) ATCTGAGTCGGATGGATGAAA.

virus in a one-step growth curve and reduce domination of results. After 5 days in culture, the CFSE fluorescence was analyzed by flow cytometry. The lower chambers contained 600,000 viable MDDCs or iMDDCs that had been pretreated with live or AT-2-inactivated HIV-1BaL or the equivalent concentration of AT-2-inactivated gp120, which was 50 ng/ml, which provided a different source of gp120 and different set of potential contaminants to AT-2-inactivated HIV-1 (48, 49). The percentage of HIV-1-infected cells was determined at 6, 24, and 48 h posttreatment by real-time PCR for HIV DNA, which was only detected in those cells treated with live HIV-1_in vitro_ and ranged from 8 to 30% of cells infected after 48 h. However, previous confocal microscopy studies of MDDCs infected by a similar concentration of this HIV-1_in vitro_ showed >95% of MDDCs to be p24 Ag positive at 2 h after completion of the HIV pulse (11), suggesting that only a proportion of MDDCs initially positive for p24 Ag show evidence of infection at 48 h.

**Genes encoding DC maturation markers are some of the most highly differentially expressed cellular genes in HIV-exposed MDDCs.**

To determine the genes differentially expressed in MDDCs in response to HIV-1 binding, entry, and replication, labeled cDNAs derived from MDDCs treated with medium alone, live HIV-1_in vitro_, AT-2 treated HIV-1_in vitro_, or recombinant gp120 for 6, 24, and 48 h were cross-hybridized in various combinations to 8 K cDNA microarrays as illustrated in Fig. 1. For cellular genes differentially expressed in response to the full virus replication cycle cDNA derived from MDDCs treated with HIV-1_in vitro_ was compared with medium. To dissect which genes from this list were differentially expressed as result of binding, entry, or later stages of replication, three additional comparisons were conducted: medium with gp120 for HIV-1 binding; AT-2-inactivated HIV-1 with gp120 for post-binding viral entry; and AT-2-inactivated HIV-1 with live HIV-1 for events in the later stages of the replication cycle. As four independent experiments each from an independent donor were conducted at each time point, a total of 48 combinations of microarrays were hybridized. Differential expression data for genes encoding proteins associated with DC maturation is presented in Table I. In MDDCs infected with live virus for 48 h, 334 genes were significantly up-regulated and the gene-encoding CD83, which is expressed de novo during maturation, was ranked first, with an 8-fold increase compared with medium-treated cells. Genes encoding two other DC maturation markers were also significantly up-regulated and the gene-encoding CD83, which is expressed de novo during maturation, was ranked first, with an 8-fold increase compared with medium-treated cells. Genes encoding two other DC maturation markers were also significantly up-regulated, CD80 showing a 2.3-fold increase (ranking 3 of 819) and CD40 showing a 1.8-fold increase (ranking 144 of 334). Furthermore, the gene encoding CXCR4, known to be up-regulated on maturing DCs, was also up-regulated 4.3-fold (ranking 11 of 659). Finally, there was a 1.9-fold decrease (ranking 71 of 334) and CD40 showing a 1.8-fold increase (ranking 144 of 334). Interestingly, AT-2-treated virions that can bind to and enter cells also significantly influenced the expression of DC maturation markers, though not to the same extent as live virus. Thus, CD83 was the most up-regulated marker showing a 3.9-fold increase (ranking 7 of 162) and CD80, CD40, and CXCR4 were also

### Table I. Linear model applied to microarray expression set

<table>
<thead>
<tr>
<th>Live HIV-1</th>
<th>AT2 HIV-1</th>
<th>gp120</th>
<th>gp120-Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp120 vs media</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Live HIV-1 vs AT2 HIV-1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Live HIV-1 vs media</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AT2 HIV-1 vs gp120</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Results**

**Treatment of MDDCs with HIV-1**

iMDDCs were either mock treated or treated with highly concentrated, purified viable R5 HIV-1_in vitro_ strain at MOI 10 (3.5 μg of p24/10^6 cells) or the equivalent concentration of AT-2-inactivated virus. The high MOI was designed to expose all the cells to the virus in a one-step growth curve and reduce domination of results by bystander effects. High purity maximizes specific effects due to exposure to HIV-1 rather than cell debris, cytokines, or other contaminants. MDDCs were also exposed to recombinant monomeric gp120_in vitro_ at 50 ng/ml, which provided a different source of gp120 and different set of potential contaminants to AT-2-inactivated HIV-1 (48, 49). The percentage of HIV-1-infected cells was determined at 6, 24, and 48 h posttreatment by real-time PCR for HIV DNA, which was only detected in those cells treated with live HIV-1_in vitro_ and ranged from 8 to 30% of cells infected after 48 h. However, previous confocal microscopy studies of MDDCs infected by a similar concentration of this HIV-1_in vitro_ showed >95% of MDDCs to be p24 Ag positive at 2 h after completion of the HIV pulse (11), suggesting that only a proportion of MDDCs initially positive for p24 Ag show evidence of infection at 48 h.

**FIGURE 1.** Diagrammatic representation of the combinations of fluororesently labeled cDNAs that were hybridized to the 8 K microarrays.
HIV INDUCES MATURATION OF MDDCs AND LCs

HIV-1 leads to partial but significant differential expression of DC maturation genes compared with a potent maturation stimulus

Real-time PCR was used to confirm the altered expression levels of the DC maturation genes and also included four genes encoding maturation markers not represented on the microarrays, CD86 and CCR7 (up-regulated by maturing DCs and involved in T cell activation and DC migration, respectively), and two CLRs DC-SIGN and MR, which are down-regulated on mature DCs (50). The effects of live and inactivated HIV-1 and gp120 were compared with a positive control “maturation mixture” containing PGE2, TNF-α, IL-1β, and IL-6, using total RNA from all four microarray experiments and from an additional fifth experiment. In view of the recent concerns regarding the use of “housekeeping genes” to normalize real-time PCR data (51, 52), we used three standard genes; GAPDH, β-actin, and β2m. There was a strong correlation between the gene expression data obtained from microarrays and that obtained from real-time PCR (Table III). Thus, treatment with gp120 showed no detectable differences in DC maturation gene expression levels (data not shown) whereas exposure to either live or inactivated HIV-1 altered the expression of all the previously studied maturation genes spotted on the microarrays. In addition, the expression of the genes encoding CCR7 and CD86 were up-regulated, although in the case of CD86 to a much lesser degree than the other up-regulated markers. The gene expression of DC-SIGN and MR was significantly down-regulated. In almost every case (24 of 27), maturation genes were differentially expressed to a greater degree in cells treated with live rather than inactivated virus. The probability of this result occurring purely by chance is <0.001 (sign test). However, in a gene-by-gene comparison the difference in gene expression levels between viable and inactivated HIV-1-treated cells was only statistically significant for CXCR4 and CD1a (p < 0.05 paired t test). In concordance with the microarray data, the magnitude of differences between live and inactivated HIV-1-treated cells was only statistically significant for CXCR4 and CD1a (p < 0.05 paired t test). In concordance with the microarray data, the magnitude of differences between live and inactivated HIV-1-treated cells was only statistically significant for CXCR4 and CD1a (p < 0.05 paired t test). In concordance with the microarray data, the magnitude of differences between live and inactivated HIV-1-treated cells was only statistically significant for CXCR4 and CD1a (p < 0.05 paired t test). In concordance with the microarray data, the magnitude of differences between live and inactivated HIV-1-treated cells was only statistically significant for CXCR4 and CD1a (p < 0.05 paired t test). In concordance with the microarray data, the magnitude of differences between live and inactivated HIV-1-treated cells was only statistically significant for CXCR4 and CD1a (p < 0.05 paired t test).

Table III. Real-time PCR derived differential expression data for genes encoding DC maturation markers

<table>
<thead>
<tr>
<th>Gene Name/Accession No.</th>
<th>Live Virus</th>
<th>AT-2 Virus</th>
<th>Maturation Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD86 (AA973397)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD83 (AA111969)</td>
<td>3.84 ± 1.33</td>
<td>2.97 ± 0.55</td>
<td>2.13 ± 0.20</td>
</tr>
<tr>
<td>CD80 (AA983817)</td>
<td>4.56 ± 1.77</td>
<td>1.73 ± 0.44</td>
<td>16.71 ± 8.91</td>
</tr>
<tr>
<td>CD81 (AA983818)</td>
<td>1.52 ± 0.16</td>
<td>1.48 ± 0.18</td>
<td>1.49 ± 0.14</td>
</tr>
<tr>
<td>CXCR4* (T62636)</td>
<td>5.12 ± 1.49</td>
<td>1.49 ± 0.14</td>
<td>13.30 ± 1.97</td>
</tr>
<tr>
<td>CXCR7 (AI672677)</td>
<td>4.37 ± 1.68</td>
<td>2.48 ± 0.71</td>
<td>140.62 ± 70.47</td>
</tr>
<tr>
<td>CD1A* (AI240210)</td>
<td>−1.52 ± 0.14</td>
<td>−1.22 ± 0.04</td>
<td>−10.70 ± 5.35</td>
</tr>
<tr>
<td>MR (AA953297)</td>
<td>−1.62 ± 0.20</td>
<td>−1.43 ± 0.19</td>
<td>−12.32 ± 3.94</td>
</tr>
<tr>
<td>CD209 (NM_021155)</td>
<td>−1.40 ± 1.30</td>
<td>−1.44 ± 0.23</td>
<td>−4.35 ± 1.43</td>
</tr>
</tbody>
</table>

a cDNA derived from RNA extracted from MDDCs cultured with either a maturation mixture, live and AT-2-inactivated HIV-1 (cDNA derived from RNA extracted from MDDCs cultured with either a maturation mixture, live and AT-2-inactivated HIV-1 (MOI 10, 3.5 μg of p24 per 10⁷ cells), gp120 (50 ng/ml), or with media only was quantified by real-time PCR using primers directed towards DC maturation marker genes. The differential expression data are presented for the 48-h time point after being normalized using three “housekeeping genes” (GAPDH, β-actin, and β2m) as a mean with the SE from five independent experiments. Those genes indicated by an asterisk (*) show a statistically significant difference in their differential expression between cells treated with live and inactivated HIV-1 (p < 0.05, paired t test) when normalized to at least one housekeeping gene.
all three were reliable normalizers. As fully maturing DCs significantly change their morphology and MHC class I expression, it is not surprising that the genes encoding β-actin and β2m were also altered in their expression levels.

Differential expression of genes encoding DC maturation markers results in a parallel change in surface expression

Changes in surface expression of the DC maturation markers after exposure to either maturation mixture, gp120_LV_b, gp120_SLCA-1, live HIV-1_BAL, AT-2-inactivated HIV-1_BAL, or mock for 48 h were studied by flow cytometry using fluorophore-conjugated mAbs to CD1a, CD40, CD80, CD83, CD86, CXCR4, DC-SIGN, and MR (Fig. 2A). No significant changes were detected in cells treated with either source of purified gp120 ranging from 50 ng/ml to 5 μg/ml (data not shown), despite the fact that both biotinylated gp120s in parallel with biotinylated mannan, a natural CLR ligand, demonstrated specific binding to MDDCs at this range of concentrations (data not shown). As previously shown, gp120_SLCA-1 was able to bind soluble CD4 as demonstrated by immunoprecipitation (42). In response to live or inactivated HIV-1 differential surface expression of the maturation markers correlated with changes at the mRNA level, with the exception of CD86 which showed the least change in mRNA levels (Table III) but greatest in surface expression. As with the gene expression studies, HIV-1 induced partial yet significant changes in gene expression of cell surface markers when compared with a potent maturation stimulus. Furthermore, the live virus-induced significantly greater alterations in the surface expression of maturation markers than the inactivated virus (Fig. 2 and see Fig. 6). In addition only a subset of HIV-1-treated cells showed increased CD83 and CD86 expression compared with cells treated with a maturation mixture.

Increased surface expression is dependent on the concentration of HIV-1

The effect of varying concentrations of inactivated and viable virus on the altered cell surface expression of DC maturation markers was investigated. DCs were treated with a range of live HIV-1 concentrations (MOI 0.03, 0.3, 3, 30, or 300 as determined by TCID50 in PM1 cells) and the equivalent dose of AT-2 inactivated HIV. Cell surface marker expression increased with increased MOI of either live or AT-2 inactivated virus particles (especially at MOI ≥3, Fig. 3).

DCs exposed to HIV-1 can still undergo full maturation

Using a low viral inoculum, reports on the ability to further upregulate the surface maturation markers on HIV-1-treated MDDCs by a potent maturation stimulus are contradictory (32, 33). Therefore, we repeated these studies using our purified high titer virus stock. DCs were either mock treated or exposed to live HIV-1 or AT-2-inactivated HIV-1 (at MOI 10), in the presence or absence of a cytokine maturation mix and the level of cell surface expression of maturation markers determined 48 h posttreatment. In addition, a cytokine maturation mix was added to MDDCs 48 h after HIV-1/mock treatment and marker levels determined another 24 h later. In both experimental designs, HIV-1-treated DCs are fully able to mature (Fig. 4). Thus, treatment with high titer purified HIV-1_BAL does not inhibit further DC maturation after exposure to a more potent stimulus.
HIV-1-treated MDDCs can stimulate T cell proliferation in a MLR

To determine whether HIV-1-treated, partially mature DCs could stimulate T cells in a MLR, CFSE-labeled PBMCs were mixed with MDDCs pretreated with varying doses of live or AT-2-inactivated HIV-1 virions or a cytokine maturation mixture. After 5 days, the proportion of T cells that had proliferated was determined by measuring CFSE dilution by flow cytometry. Live/inactivated HIV-1 induction of partial maturation of DCs was associated with an increased ability to induce T cell proliferation (Fig. 5A). Consistent with the effects on maturation markers, live HIV-1 was more efficient at T cell stimulation than the AT-2-inactivated virus and the effect was concentration dependent.

HIV-1-treated MDDCs show enhanced migration toward CCL21

To investigate whether HIV-1 treatment of iMDDCs resulted in enhanced CCR7-mediated chemotaxis, iMDDCs were either pretreated with viable or AT-2-inactivated HIV-1 or a cytokine maturation mixture or untreated and compared in their ability to migrate toward the CCR7 agonist CCL21 or medium alone, using Transwell chambers. In concordance with the data presented thus far, CCL21 induced a 15-, 8-, 5-, and 3-fold increase in migration of maturation mixture, viable HIV-1, AT-2-inactivated HIV-1 or medium only treated MDDCs, respectively (Fig. 5B).

Inhibition of maturation effects induced by live but not inactivated HIV by a CCR5 Ab

The consistently greater degree of MDDC maturation induced by live virus compared with inactivated virus suggests that HIV-1-associated DC maturation occurs by at least two mechanisms, especially as the AT-2-inactivated virus was derived from the same stock as the live virus and adjusted to the same concentration of viral particles. Such mechanisms might occur during endocytosis and subsequent trafficking or in the alternative minor pathway, binding/fusion via CD4/CCR5 and additional steps in the replication cycle. To distinguish mechanisms associated with these two pathways, we compared the effects of both live and inactivated HIV-1 on DC maturation in the presence or absence of an Ab directed against CCR5 (clone 2D7) that will block virus entry and therefore replication, but that will have no effect on Ag uptake via
CLR-mediated endocytosis. Treatment of MDDCs with anti-CCR5 had no significant effect on the ability of AT-2-inactivated HIV-1 to induce DC maturation but it significantly reduced the effect on maturation caused by live HIV-1 by at least 20–40%, for CD40, CD80, CD83, and CD86 (p<0.05) (Fig. 6B) and reduced the infection of MDDCs by 50% (Fig. 6A). This shows that a proportion of the enhanced effects on maturation by live HIV-1 are mediated via CCR5 but live HIV-1 also induces non-CCR5-mediated effects on maturation, whereas the effects of inactivated HIV-1 are mostly non-CCR5 mediated.

HIV-1 also leads to partial maturation of LCs

Finally, to determine whether the effect of HIV-1 on MDDC maturation could also be demonstrated on native ex vivo DCs, we exposed LCs freshly isolated from human skin to HIV-1 at high MOI of 10. The LCs were examined for maturation markers at 24 h
monomeric HIV-1BaL gp120 as well as high concentrations of model. In these experiments, the effects of recombinant soluble recombinant gp120 provide different sources of potential contaminants and strengthens any similarities obtained with the two different reagents.

Such rigor is important in investigating the effects of HIV-1 on MDDCs where two different processes are occurring at different kinetics. First, most virus (probably >98%) is taken up via endocytosis after binding to DC-SIGN and mannose receptor (37) and, in the absence of T cells, degraded almost completely within 12 h (11). A lesser proportion of the virus bound to CLR (probably <10%) is transferred to CD4/CCR5, probably at the cell surface, and enters the DC cytoplasm via viral fusion with the cell membrane, followed by de novo replication. HIV-1 DNA levels then increase over 48 h as previously shown (11). Thus, inactivated HIV-1 or gp120 may influence the host cell gene expression by signaling through the CLRs on the cell surface, via TLRs in the endosome, or after transfer from CLRs to CD4 and CCR5 on the surface. In addition, live HIV-1 may influence gene expression via these mechanisms or at later stages of the replication cycle.

Somewhat surprisingly, CD83 was found to be the gene whose expression was most changed by live virus in comparison with medium (8-fold increase). Other maturation markers, including CXCR4, CD80, and CD40, were also found to be significantly increased (4.3- to 1.8-fold changes) and the magnitude of these changes was significantly correlated with the extent of infection. Inactivated virus also led to significant changes in most of these maturation markers but to a lesser extent and no correlation with the infectability of the donor cells (as assessed in the parallel treatments using live virus). In contrast, monomeric gp120 induced no significant changes. Most of the changes in MDDCs were significant at 24 h but more marked at 48 h. These findings were confirmed and extended by real-time PCR where down-regulation of the CLRs, MR, and DC-SIGN, up-regulation of CCR7 and a minor up-regulation of CD86, were also observed. Similar findings were observed with flow cytometry and a strong correlation between transcriptional and posttranscriptional changes was observed except for the marked increase in CD86 which was posttranscriptional only. With both live and inactivated virus, the effects were found to be highly concentration dependent with the most marked effects on CD83 and CD86 being demonstrated above an MOI ≥3. Purity of the virus preparations is also highly likely to be important as contaminating cytokines may have variable effects on DC maturation. Cytokines would be excluded by the highly purified, high titer preparations used in these experiments and no residual TNF-α was detected in any virus stocks, though they may still have contained some contaminating microvesicles. However, the differences induced by very similar concentrations of AT-2-inactivated and live virus and the partial but significant inhibition of the live virus effects by an anti-CCR5 Ab demonstrated that the virus is clearly mediating the effect.

The reported effects of HIV-1 or gp120 on maturation of MDDCs are contradictory and confusing (32, 33, 35, 36). Our study of the model MDDCs places such changes in the context of the effects of HIV-1 on global gene expression. The published reports used widely differing conditions which probably explain the widely differing results. In particular, three of the four studies used quite low titers of HIV-1 (32, 35, 36) and the failure of two reports (32, 33) to demonstrate effects on maturation markers may be due to these low titers and low purities, as strongly suggested by our results on the concentration dependence of the HIV-1 inoculum. None examined the full complement of maturation markers and only surface expression of proteins was examined, not RNA. The induction of partial maturation of blood myeloid DCs (especially CD86 expression) by live HIV-1 (54) showed marked similarities to our own results using high titer viruses on MDDCs. We were unable to duplicate a report of high titer R5 strain gp120 induction of MDDC maturation (35) despite using soluble recombinant gp120 from both laboratory adapted (BaL) and primary
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(SLCA-1) R5 strains at similar or higher concentrations (as immature MDDCs express CCR5 rather than CXCR4, and are preferentially infected by R5 strains, the use of R5 rather than X4 strain gp120 is appropriate). Both gp120 species bound normally to MDDCs even after biotinylation demonstrating that their conformation was preserved. The failure of gp120 to induce stimulation of any of the maturation markers, compared with the effects of inactivated or live virus in our study suggest that either oligomeric gp120 in its native conformation is required for the stimuli or, more likely, that endosomal uptake or entry of inactivated virus and/or replication of live virus are the key stimulatory steps. The marked concentration dependence of live HIV-induced maturation may be partly explained by the predominance of CLR-mediated endosomal uptake and degradation over entry by fusion with the plasma membrane. Relatively high MOI (>3) may be required to achieve infection of a significant proportion of the cell sheet, sufficient to induce the observed changes in host gene expression. This agrees with the observed correlation between the extent of infection of DCs from different donors (despite equivalent exposure to virus) and the magnitude of maturation marker differential gene expression in response to live HIV-1. Furthermore, these high concentrations of inactivated virus may be required to overcome the high proportion (probably >90%) of bound virus which undergoes CLR-mediated endosomal uptake and degradation. This may explain some of the differences in findings between the published reports with MDDCs. The loss of virus through endolysosomal degradation and the fact that HIV-1-infected activated T cells are present in semen and may have burst sizes of over 1000 virions per cell (55) justify the concentration of virus used in this study. The local HIV-1 concentrations in vivo could well deliver such MOI to epithelial LCs across "microabraded" noncornified mucosal epithelium. Interestingly, if the concentration dependence of live HIV-induced maturation is indeed due to the fact that most virus is lost through CLR-mediated uptake and degradation, the absence of DC-SIGN and MR expression on myeloid DCs may allow viruses to exclusively induce maturation of these cells via CD44/CCR5 (54) and a direct comparison with MDDCs would therefore be interesting.

Comparison of the effects of the virus on MDDCs with maximal maturation stimuli (a mixture of TNF-α, IL-1, IL-6, and PGE2) showed that virus-induced maturation was only partial. Is this virus-induced maturation aberrant or just partial? Why does the virus induce CD83 and CD80 mRNA and proteins differently to the maturation mixture? Furthermore, some of the previous studies mentioned above demonstrate that HIV-1-infected DCs either could not be induced to maximal maturation (higher expression of maturation markers) (32, 33, 35, 36) and/or were functionally deficient (32, 35) and/or produced low levels of Th1 cytokines (32, 33). However, our studies with relatively high titer pure virus showed the ability of conventional maturation stimuli to enhance the (partial) maturation and migration of HIV-1-infected DCs and demonstrated that effects of HIV-1 are not mediated through an aberrant "dead end" pathway. Furthermore, this partial maturation was functionally important as these DCs had an increased ability to stimulate T cell proliferation in a MLR and this was also enhanced with live virus. This data complements and supports the results of two other studies: one showed that such HIV-1 treatment of MDDCs induced migration (36) and the other showed that HIV-induced partial maturation of blood myeloid DCs which could still be induced to complete maturation by the TLR7/8 agonist R846 (54).

Thus, these results resolve the conflict in the literature about the effect of HIV-1 on maturation markers in MDDCs and demonstrate the reason that the conflict is probably due to the use of different titters and purities of virus inocula as well as individual variation in the susceptibility of MDDCs to infection. Our studies also indicate that these changes are some of the most important transcriptional changes in DCs induced by HIV-1 and show that the changes occur mostly at a transcriptional level, with the exception of CD86 where they are mostly posttranscriptional. Furthermore, the experiments on concentration dependence, comparison between live and inactivated virus and gp120 and inhibition by anti-CCR5 mAb strongly suggest that these effects are partly due to steps in the viral replication cycle beyond binding and entry.

The next step was to determine whether the effects of HIV infection on the model MDDCs were also observed in native dendritic cells, especially those exposed to HIV at the site of viral entry, immature epidermal LCs. To obtain these cells in the immature state, they were dissociated from epidermal explants using collagenase. Whether purified or not, these LCs slowly up-regulated maturation markers spontaneously over 48 h of observation, as previously reported in a study that used trypsin dissociation, which may cleave surface molecules (56). Nevertheless, such maturation was observed in both laboratories whether collagenase or trypsin digestion of cell sheets was used and whether unpurified, partially affinity purified, or density gradient purified LCs were used. Thus, it seems likely that LC maturation proceeds when these cells are exposed to dissection or deprived of contact with surrounding keratinocytes. Therefore, our experiments with HIV were conducted as early as possible, at 24 h postinfection, to determine whether HIV may accelerate this process. There was a marked and significant increase in all maturation markers including CD83. As with MDDCs, basal CD86 expression was much higher than the rest but still significantly up-regulated. This acceleration of maturation by HIV in both MDDCs and LCs is likely to be important in transfer of HIV from such infected cells to CD4 lymphocytes resident in the underlying submucosa or in the lymph nodes after migration. Thus, immature LCs which express high levels of CLRs, including langerin, and are highly endocytic, in the upper layers of the epidermis or genital tract mucosa are well-prepared to transfer maximal HIV binding and uptake. Initiation or acceleration of maturation through up-regulation of maturation markers by HIV infection augmented by HIV induced migration as suggested by the complementary results of Willflingseder et al. (36) (now confirmed here) and/or danger signals from surrounding keratinocytes subjected to trauma or coinfection may all facilitate this process. Mature DCs have been shown to more efficiently transfer virus and immature MDDCs up to contact to T cells probably through stronger and more stable attachments at "viral synapses" (57, 58). Furthermore, such HIV-1-induced maturation of myeloid or interstitial DCs may contribute to the characteristic T cell activation in HIV-1 infection and to transfer of HIV-1 to HIV-specific T cells.

The exact mechanisms of virus induction of these maturation stimuli need to be further dissected. Our results showing greater effects by live virus and partial inhibition by anti-CCR5 and those of Willflingseder et al. (36) suggest there may be two mechanisms, one triggered by HIV-1 Ag endocytosis and conventional MAPK 38 pathways as for TNF-α, and the other induced by virus replication, probably beyond viral entry. Recent findings of up-regulation of DC maturation markers after gag RNA transfection (59) are consistent with the latter, suggesting an effect via TLRs. In addition, the effects that HIV-1 exerts on these DCs need to be confirmed in epithelial DCs in vivo. Nevertheless, the microarray results suggest these HIV-1-induced effects on maturation are important adaptations likely to assist the virus in its dissemination.
HIV INDUCES MATURATION OF MDDCs AND LCs

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Disclosures

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