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M1 and M2a Polarization of Human Monocyte-Derived Macrophages Inhibits HIV-1 Replication by Distinct Mechanisms

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The capacity of macrophages to support productive HIV-1 infection is known to be modulated by cytokines and other extracellular stimuli. In this study, we demonstrate that cytokine-induced polarization of human monocyte-derived macrophage (MDM) into either classical (M1) or alternatively activated (M2a) MDM is associated with a reduced capacity to support productive CCR5-dependent (R5) HIV-1 infection. M1 polarization was associated with a significant down-regulation of CD4 receptors, increased secretion of CCR5-binding chemokines (CCL3, CCL4, and CCL5), and a >90% decrease in HIV-1 DNA levels 48-h postinfection, suggesting that the inhibition occurred at an early preintegration step in the viral life cycle. In contrast, M2a polarization had no effect on either HIV-1 DNA or protein expression levels, indicating that inhibition occurred at a late/postintegration level in the viral life cycle. M2a inhibition was sustained for up to 72-h postinfection, whereas M1-effects were more short-lived. Most phenotypic and functional changes were fully reversible 7 days after removal of the polarizing stimulus, and a reciprocal down-regulation of M1-related chemokines and cytokines was observed in M2a MDM and vice versa. Since reversion to a nonpolarized MDM state was associated with a renewed capacity to support HIV replication to control levels, M1/M2a polarization may represent a mechanism that allows macrophages to cycle between latent and productive HIV-1 infection. The Journal of Immunology, 2009, 182: 6237–6246.

Mononuclear phagocytes play a pivotal role in a broad range of innate and adaptive immune responses. In addition, they are important targets for HIV-1 and contribute to the dissemination and cell-to-cell spread of the virus (1–4). Unlike CD4+ T cells, mononuclear phagocytes, specifically terminally differentiated macrophages, are refractory to the cytotoxic effects of HIV-1 and, because of their longevity and ubiquitous distribution in tissues and organs, serve as viral reservoirs throughout the course of HIV-1 disease (3–5). The early establishment of such viral reservoirs during primary infection (6–8) has stimulated interest in characterizing the mechanisms underlying the persistence and replication of HIV-1 in both CD4+ T cells and monocyte/macrophages (9, 10).

Mononuclear phagocytes respond to a variety of different cytokines and microbial gene products, and their susceptibility to HIV-1 is profoundly influenced by these stimuli (11–16). IFN-α/β potently inhibits HIV-1 replication in macrophages, whereas other cytokines, including IL-6, stimulate virus replication (17–19). Depending on the stage of mononuclear phagocyte differentiation and the timing of cytokine exposure, some cytokines can either promote or inhibit HIV-1 infection. IL-4 and IL-13, for example, enhance virus replication in monocytes but inhibit replication in differentiates monocyte-derived macrophages (MDM) (20, 21). On the other hand, exposure of MDM to TNF-α before infection is inhibitory, whereas stimulation of latently infected macrophages or monocyte cell lines leads to the up-regulation of virus transcription and expression via activation of the cellular transcription factor NF-κB (16, 22, 23).

A recent concept developed primarily in mouse models is the observation that cytokine stimulation can trigger the polarization of macrophages into opposing pro- and anti-inflammatory programs. By analogy with CD4+ T cell differentiation, these polarization pathways have been designated M1 and M2 (24–26). M1 macrophages produce IL-1β, IL-12, IL-23, and TNF-α, as well as reactive oxygen and nitrogen intermediates, support Th1 responses, and mediate resistance to tumors and intracellular pathogens (24, 27–29). Conversely, M2 macrophages secrete IL-10, express scavenger and mannose receptors, contribute to Th2 responses, enhance phagocytosis, eliminate parasites, and promote tissue repair (25–27, 30–35). M2 macrophages are generally considered to be more heterogeneous than M1 cells and, to reflect these differences, have been further subdivided into M2a, M2b, and M2c cells (24). M2a (induced by exposure to IL-4 or IL-13) and M2b (induced by stimulation with immune complexes, TLG, or the IL-1 receptor antagonist (IL-1ra)) macrophages exert immunoregulatory functions and drive Th2 responses, whereas M2c cells (generated by stimulation with IL-10) play a predominant role in the suppression of immune responses and tissue remodeling (24). Recent molecular profiling of human MDM has identified >2000 transcripts that are differentially modulated during M1 vs M2a polarization, giving rise to macrophages with highly specialized...
phenotypic and functional properties (36). Although it is well documented that cytokines play a critical role in the regulation of HIV-1 replication (37), the potential effect of M1 and M2a polarization on virus infection and replication has not been specifically investigated.

In this study, we investigated the effects of M1 (TNF-α plus IFN-γ) and M2a (IL-4) polarization on the capacity of MDM to support productive CCR5-dependent (R5) HIV-1 infection. The study focuses primarily on M1 and M2a cells because these phenotypes mediate Th1 and Th2 responses, and it has been proposed that a switch from a Th1 to a Th2 immune response may play a role in the pathogenesis of HIV-1/AIDS (38, 39). We found that both polarization pathways led to a decreased capacity to support R5 HIV-1 replication in association with specific alterations in the expression of cell surface receptors, chemokines, and cytokines involved in HIV-1 entry, replication, and dissemination. However, the quality, magnitude, and duration of these inhibitory responses and the mechanisms underlying these responses were clearly different in M1 vs M2a MDM.

Materials and Methods

Reagents

Human recombinant cytokines were purchased from R&D Systems and used at concentrations of 2 ng/ml (TNF-α) and 20 ng/ml (IFN-γ and IL-4), respectively. All cytokines were declared by the manufacturer to contain <0.1 ng of LPS per µg of protein. Brefeldin A and BSA were purchased from Sigma-Aldrich. Ficol-Hypaque was purchased from Amersham Biosciences. DMEM, PBS, FBS, normal human serum (NHS), penicillin, streptomycin, and glutamine were obtained from Cambrex.

Isolation of human monocytes and differentiation into MDM

PBMC were isolated from the buffy coats of healthy HIV-1-seronegative blood donors by Ficol-Hypaque density gradient centrifugation. The cells were then washed, resuspended in DMEM containing pen/strep (1%), heat-inactivated FBS (10%), and heat-inactivated NHS (5%) (complete medium), and seeded into 75-cm² flasks (Falcon; BD Biosciences) at 2 × 10⁵ cells/ml. Nonadherent cells, mostly T lymphocytes, were removed by gentle pipette aspiration after 2 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂. An equal volume of fresh complete medium was then added to each flask. After 24 h of culture, adherent cells were washed twice with PBS, detached from the flask by scraping with a rubber policeman, and counted after trypan blue dye staining. The cells (≥85% monocytes as determined by flow cytometric analysis after staining with anti-CD14 mAb) were seeded at 48-well plate density (Falcon) at the concentration of 2.5 × 10⁵ cells/well and were cultured for 7–8 additional days at 37°C in 5% CO₂ to promote their full differentiation into MDM (12). These MDM (≥95% CD14⁺) were then stimulated for 18 h with either TNF-α plus IFN-γ or IL-4 to obtain M1 or M2a phenotypes, respectively (24). Unpolarized control MDM were maintained in culture for 18 h in complete medium alone. At the end of the 18-h period, MDM were washed with cytotoxic-free culture medium, resuspended in complete medium, and further analyzed in parallel cultures.

Flow cytometric analysis of cell surface determinants

Adherent MDM were washed with cold EDTA/PBS (2 mM) and detached from the plastic plates by incubation with cold EDTA/PBS (2 mM) for 30 min at 4°C and scraping with a rubber policeman. Nonspecific binding of Ab was eliminated by preincubating the cells in medium containing 10% FBS and 5% NHS for 15 min at 4°C. The cells were then incubated with anti-CD4 (PE) and anti-CD14 (PE) (BD Biosciences) mAbs for 30 min at 4°C, washed with cold PBS, and fixed with 2% paraformaldehyde. CCR5 expression was assessed using biotinylated human CCL4 (Fluorokine; R&D Systems). Flow cytometry was performed using a CYAN ADP instrument (DakoCytomation), and the results were analyzed with the FlowJo software version 8.4.3 (Tree Star). Results are reported as the percentage of positive cells and as mean fluorescence intensity (MFI), the latter being used to describe the level of expression on a population of positive cells.

To investigate the persistence of MDM polarization, M1, M2a, and control MDM were thoroughly washed and cultivated for 7 additional days in the absence of further stimulation. These cells were then monitored for cell surface Ag expression and cytokine/chemokine secretion after 0, 3, and 7 days postpolarization.

Cytokine and chemokine secretion and expression

Cytokines were quantified in culture supernatants of MDM 18 h after their polarization (day 0) as well as on days 3 and 7 postpolarization. The levels of CXCL8, CCL3, CCL22, and CCL24 released into the supernatants of MDM cultures were quantified by commercial ELISA kits (R&D Systems), whereas the concentrations of IL-1β, IL-1ra, IL-6, IL-10, IL-12, CCL4, CCL5, CCL2, and CXCL10 were determined in customized Bio-Plex plates (Bio-Rad) following the manufacturer’s instructions.

Intracellular staining for CCL3 was determined after 18 h of MDM polarization in medium containing brefeldin A (1 µg/ml). Control, M1-, M2a-, and lipid A-stimulated MDM (1 µg/ml) were detached from the plastic surface by scraping and fixed in 2% paraformaldehyde. The cells were then permeabilized with a Saponin buffer (0.1% saponin and 0.5% BSA in PBS) and stained with allophycocyanin-conjugated anti-CCL3 mAb for 30 min at 4°C. The cells were then washed twice with cold PBS and analyzed with a CYAN ADP apparatus (DakoCytomation).

R5 HIV-1 infection of MDM

Control, M1, and M2a MDM were infected with the macrophage-tropic, laboratory-adapted R5 strain HIV-1agl at the multiplicity of infection of 0.1. In certain experiments, MDM were also infected with HIV-1agl, 3 and 7 days after polarization, as further specified. Multiple aliquots of culture supernatants were collected every 3–4 days over a 3-week period and stored at −20°C to assess virus production. At the end of each infection experiment, supernatants were thawed and analyzed for their viral content by measuring the levels of virion-associated reverse transcriptase (RT) activity present in the supernatant, as described previously (12).

Quantification of HIV-1 DNA by real-time PCR

Unpolarized control, M1, and M2a MDM were infected with DNase/RNase-free HIV-1agl at the multiplicity of infection of 0.1) and were then cultivated in complete medium for 48 h, a period estimated to be required for completion of a single round of HIV replication in macrophages (40). The infected MDM (10⁶ cells/ml) were then washed, resuspended in lysis buffer containing polyoxyethylene 10, lauryl ether (0.1%), and proteinase K (0.1 mg/ml) from Sigma-Aldrich, and digested at 65°C for 2 h; proteinase K was then heat inactivated at 95°C for 15 min (41). An amount of lysate corresponding to 2.5 × 10⁵ cells was amplified by real-time quantitative PCR reactions using primers and a probe that recognize the HIV-1 gag gene (42, 43); forward primer, 5′-GATACAGCGAAGCATGCAAAT-3′; reverse primer, 5′-ATCT GCCGCTTGCAATTAGG-3′; and probe, 5′-FAM-CAATCGAGAAAGCTGCAATGATGATA-GAG-3′ (TAAR MDM). This primer/probe combination detects all forms of viral DNA synthesized after second-strand transfer mediated by RT. The number of HIV-1 DNA copies were normalized to those of human GAPDH by an external standard curve showing a linear response between 10¹ and 10¹⁴ copies (40). The primers and probe for GAPDH were forward primer, 5′-ACCACAGTCCTGACATCCT-3′; reverse primer, 5′-GGCCATGCAACAGTCAGG-3′; and probe, 5′-FAM-CCACAGAAGCTGCTAGGTGGCAG-GAG-3′ (TAAR MDM). Thermal cycling conditions for real-time PCR with SYBR Green quantification were 50°C for 2 min and 95°C for 15 min followed by 40 cycles at 95°C for 1 s and 65°C for 1 min (43).

Western blot analysis of cell-associated HIV-1 proteins

MDM were scraped from the wells with a rubber policeman and lysed with buffer C (20 mM HEPES, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM MgCl₂, and 0.5% glycerol; 100 µl per million cells). Cellular proteins were denatured in an equal volume of 2× sample buffer (0.125 M Tris-HCl (pH 6, 8), 4% SDS, 10% 2ME, and 20% glycerol) for 5 min at 100°C, separated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences) by electroblotting. Membranes were blocked in 5% nonfat milk, 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.2% Tween 20 for 30 min at room temperature and further incubated for 1 h with the serum (1/1000 dilution) from an HIV-1-individual containing high titers of anti-HIV-1 Abs. Ab binding was visualized by using HRP-conjugated anti-human Abs (Amersham Biosciences). The signal was detected using ECL reagents (Amersham Biosciences) according to the manufacturer’s instructions.

Statistical analysis

Results are reported as mean values ± SD. To minimize interdonor variability, values were normalized relative to unstimulated control cells. One-way ANOVA and the Tukey posttest were used for multivariate analysis.
To further compensate for interdonor variability, all assays were performed on triplicate samples with MDM derived from four to six independent donors, as further specified.

Results

**M1 and M2a polarization shapes MDM morphology**

Before polarization, control MDM showed approximately equal numbers of spindle-shaped fibroblastoid and large flat-round/fried egg-shaped cells (50.5 ± 6.2% and 49.5 ± 6.1%, n = 5), respectively (Fig. 1). Exposure to M1-inducing cytokines for 18 h led to a significant increase in the percentage of fibroblastoid-like cells (69.3 ± 6.2%, n = 5, p = 0.027 vs controls) and in the length of the cellular projections extending from the cell poles. Conversely, exposure to IL-4 led to an increase in the percentage of flat-round cells (73.7 ± 1.4%, n = 5, p = 0.016 vs controls; Fig. 1). When compared with the flat-round cells in control cultures, M2a cells exhibited a more condensed morphology with short cellular projections accumulating at the poles of the cell. Neither M1 nor M2a activation affected MDM survival as assessed by trypan blue dye exclusion. Thus, we can conclude that the polarization protocol adopted in our study affected the majority of cells in the MDM population, at least in terms of morphological features.

**Both M1 and M2a polarization of human MDM inhibits R5 HIV-1 replication**

To assess the relative capacities of M1 and M2a MDM to support productive infection by R5 HIV-1, MDM cultures were established from 15 independent HIV-1-seronegative donors and were either
left unpolarized (controls) or were polarized with M1 (TNF-α plus IFN-γ) or M2a (IL-4)-inducing cytokines for 18 h before infection with R5 HIV-1_BaL. Virus replication in control MDM increased progressively reaching peak levels 14–18 days postinfection, followed by a drop to low levels of persistent replication (Fig. 2A). In contrast, HIV-1 replication was strongly inhibited in both M1- and M2a-polarized MDM with the former showing a significantly stronger inhibition in comparison to the latter (Fig. 2A).

The levels of viral suppression in M1 MDM ranged from 51 to 75% in 20% of independent cultures to 76–100% in the remaining 80% of cultures, whereas the levels of suppression in M2a MDM ranged from <25% in 7% of donors to 26–50, 51–75, and >76% in 7, 33, and 53% of independent MDM cultures, respectively (Fig. 2B).

**Polarization-induced inhibition of HIV-1 replication is transient and of shorter duration in M1 vs M2a MDM**

Next, we examined the duration of the inhibitory effect on HIV-1 replication following a single 18-h exposure to M1- and M2a-polarizing cytokines. Control and polarized MDM were thoroughly washed and infected with HIV-1_BaL either immediately (day 0) or 3 or 7 days after polarization (Fig. 3). Exposure to HIV-1 immediately after polarization led to a rapid decrease in HIV-1 replication in both M1 and M2a MDM relative to control cells with the nadir of inhibition in M2a MDM occurring on day 10 (Fig. 3, upper left panel). A more prolonged suppression of virus replication lasting for up to 25 days was observed in M1 MDM (Fig. 3, upper left panel).

When infection was delayed until 3 days after polarization, M2a MDM maintained their inhibitory phenotype. In contrast, M1 MDM had already lost their inhibitory phenotype and supported HIV replication at the same level as control cells (Fig. 3, upper right panel). When M1 and M2a MDM were infected 7 days after polarization, no inhibitory effects on HIV-1 replication were observed in comparison to control cells (Fig. 3, lower panel). Thus, the inhibitory effects of MDM polarization on HIV-1 replication are transient, particularly in M1 MDM, and do not render these cells permanently refractive to HIV-1 infection and replication.

**M1 and M2a polarization differentially modulates the expression of CD4, CCR5, and CXCR4**

To investigate the effects of M1 and M2a polarization on MDM susceptibility to HIV-1 infection, we monitored changes in the expression of CD4, CCR5, and CXCR4, the primary receptor, and the major coreceptors for HIV-1 entry into target cells, respectively (44, 45). M1, and to a lesser extent M2a, polarization resulted in a significant reduction in the percentage of MDM expressing CD4, a finding that may account, at least in part, for their decreased capacity to support productive infection (Table I).

### Table I. Fold changes of HIV receptor and coreceptor expression in M1 and M2a MDM vs control MDM

<table>
<thead>
<tr>
<th>Receptor</th>
<th>N°</th>
<th>Parameter</th>
<th>Average Expression (control) (%)</th>
<th>M1 (fold vs control)</th>
<th>M2a (fold vs control)</th>
<th>M1 vs control</th>
<th>M2a vs control</th>
<th>M1 vs M2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>5</td>
<td>Positive cells</td>
<td>41 ± 3.3</td>
<td>0.14 ± 0.08</td>
<td>0.32 ± 0.08</td>
<td>**</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>MFI</td>
<td>5</td>
<td>Positive cells</td>
<td>12 ± 2.3</td>
<td>0.92 ± 0.07</td>
<td>0.91 ± 0.05</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>5</td>
<td>Positive cells</td>
<td>17 ± 7.7</td>
<td>0.28 ± 0.23</td>
<td>0.50 ± 0.09</td>
<td>*</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>CCR5</td>
<td>5</td>
<td>Positive cells</td>
<td>16 ± 2.8</td>
<td>1.13 ± 0.29</td>
<td>1.14 ± 0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFI</td>
<td>5</td>
<td>Positive cells</td>
<td>21 ± 1.8</td>
<td>1.27 ± 0.06</td>
<td>1.87 ± 0.72</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFI</td>
<td>44 ± 3.1</td>
<td>0.98 ± 0.01</td>
<td>0.91 ± 0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean fold change in the number of positive cells and MFI in M1 and M2a MDM vs. control MDM. MFI and expression of surface receptors/coreceptors was analyzed by cytofluorimetry. *, p < 0.05; **, p < 0.001, as assessed by one-way ANOVA and Tukey posttest. N° denotes the number of MDM donors studied.*
contrast, expression of CCR5, the major coreceptor for macrophage-tropic strains of HIV-1, remained unchanged and, in some donors, was even up-regulated on the surface of M2a MDM vs M1 and controls. CXCR4, an entry coreceptor for X4-dependent viruses (which do not usually cause productive infection in MDM) (46, 47), was also down-regulated in polarized MDM vs controls (Table I).

Removal of the polarizing cytokines led to a near complete reversal of M1 and M2a cell surface phenotypes by day 7. In particular, cell surface CD4 expression returned to control levels in M1 MDM 3 days after the removal of the polarizing stimuli, whereas this occurred more slowly in M2a MDM (Table II).

Table II. Kinetics of CD4 expression following MDM polarization

<table>
<thead>
<tr>
<th>Days after polarization</th>
<th>N*</th>
<th>Polarization</th>
<th>% CD4+ MDM</th>
<th>M1 vs control</th>
<th>M2a vs control</th>
<th>M1 vs M2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>Control</td>
<td>41 ± 3.3</td>
<td>**</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1</td>
<td>6 ± 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2a</td>
<td>13 ± 2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>Control</td>
<td>35 ± 6.7</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1</td>
<td>31 ± 9.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2a</td>
<td>13 ± 5.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>Control</td>
<td>33 ± 4.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1</td>
<td>31 ± 6.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2a</td>
<td>26 ± 2.9</td>
<td></td>
<td></td>
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</tbody>
</table>

* Mean CD4 expression on control, M1, and M2a MDM. **p ≤ 0.05; ***p ≤ 0.001, as assessed by one-way ANOVA and Tukey posttest. N* denotes the number of individual MDM donors tested.

*M1 and M2a polarization differentially modulates the secretion of chemokines and cytokines relevant to HIV-1 replication

Next, we investigated the effects of M1 and M2a polarization on the CCR5-binding chemokines CCL3, CCL4, and CCL5 that are potent inhibitors of R5 HIV-1 entry in CD4+ T lymphocytes and MDM (16, 48). Secretion of CCL3 and CCL4 was detected after 18 h of cytokine stimulation in all MDM cultures and was significantly up-regulated in M1 vs M2a MDM (25-fold in M1 vs ~2-fold in M2a-MDM for CCL3; 14-fold in M1 vs ~2-fold in M2a for CCL4) (Fig. 4A and Table III). CCL5, which was undetectable in both control and M2a MDM, was significantly up-regulated in M1 MDM (range, 61–186 pg/ml) in three of six donors (Table III). We further determined the intracellular levels of CCL3 in both unpolarized and polarized MDM. A clear increase in the percentage of CCL3+ cells was observed in M1 vs control MDM (from 1–3 to 12–21%, with a mean increase of 7 ± 2.31-fold vs Nil, n = 5, p = 0.023), although no changes were detected in the MFI of CCL3+ M1 vs control MDM (Fig. 4B). Stimulation with lipid A, a highly potent M1 stimulus (24), resulted in a greater (20-fold) increase in both the number and MFI of MDM expressing intracellular CCL3 (Fig. 3B), indicating that the M1 polarizing cytokines used in this study induced CCL3 production only in a subset of MDM.

In addition to increased secretion of CCR5-binding chemokines, M1, and to a lesser extent M2a, polarization led to a significant up-regulation of CXCL10 (215-fold in M1 and 25-fold in M2a MDM vs controls), a chemokine previously linked to the up-regulation of HIV-1 replication in vitro (49) (Table III). CCL2, a chemokine that is elevated in the cerebrospinal fluid of individuals with HIV-1 and CMV encephalitis (50, 51), was unaffected by M1 polarization but was consistently down-regulated (0.5-fold) in M2a MDM (Table II). This chemokine has been shown to enhance HIV-1 replication in activated PBMC (52). CXCL8, a chemokine that is also increased during HIV-1 infection (53) and that has been shown to enhance HIV-1 replication in vitro (54, 55), was significantly up-regulated in M1-MDM. This chemokine was partially down-regulated in four of five independent M2a MDM compared with control cultures (Table III). Finally, CCL2, a chemokine constitutively expressed by unpolarized MDM (11, 56) and originally described as an inhibitor of HIV-1 replication in both CD4+ T cells and MDM (12, 57), was significantly up-regulated in M2a MDM vs M1 and control MDM (Table III).

In contrast to chemokines, polarization-induced changes in cytokine production were quantitatively modest. In particular, neither IL-1β nor IL-1ra production was significantly modulated in M1 or M2a MDM vs control cells. IL-6, a cytokine that, like IL-1, has
been reported to up-regulate HIV-1 expression in MDM and chronically infected promonocytic cells (18), showed a relatively weak 2-fold increase in M1 vs control MDM. Finally, IL-10, a cytokine that has been accredited with both enhancing and suppressive effects on virus replication in monocytes, primary MDM, and promonocytic cell lines (58–61), was significantly up-regulated in M2a MDM vs control and M1 MDM (Table III).

“Contra-regulatory” effects of M1 and M2a polarization

Most secreted molecules, including CCL3, IL-6, and IL-10 returned to control levels 3 to 7 days after polarization (Fig. 5). In contrast, the secretion of CXCL10 remained significantly up-regulated for at least 7 days in M1 vs control and M2a MDM (p = 0.004; Fig. 5). Conversely, secretion of the M2a-associated chemokine CCL22 was further up-regulated after removal of IL-4 and remained above control levels for at least 7 days (p = 0.032; Fig. 5). In addition, we have also observed an impaired secretion of chemokines and cytokines typically secreted by the opposite polarization program with peak secretion levels occurring ~3 days post-polarization. In particular, secretion of M1-related CCL3, CXCL10, and IL-6 was significantly decreased in M2a MDM. Conversely, the release of the M2a-associated CCL22, IL-10, and

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**Table III. Cytokine and chemokine secretion in polarized and unpolarized MDM**

<table>
<thead>
<tr>
<th>Chemokine/Cytokine</th>
<th>N°</th>
<th>Range (control) (pg/ml)</th>
<th>Mean± SD</th>
<th>M1 (fold vs control)</th>
<th>M2a (fold vs control)</th>
<th>M1 vs control</th>
<th>M2a vs control</th>
<th>M1 vs M2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>4</td>
<td>2519–8506</td>
<td>5006 ± 2783</td>
<td>1.4 ± 0.6</td>
<td>0.5 ± 0.3</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>6</td>
<td>26–274</td>
<td>82.5 ± 96</td>
<td>25 ± 8.3</td>
<td>2.2 ± 1.1</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>6</td>
<td>154–4532</td>
<td>222 ± 58</td>
<td>14 ± 5.4</td>
<td>1.6 ± 0.8</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>6</td>
<td>n.d.</td>
<td>69 ± 85.8</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
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<tr>
<td>CCL22</td>
<td>4</td>
<td>165–886</td>
<td>398 ± 330</td>
<td>1 ± 0.4</td>
<td>3.9 ± 0.3</td>
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<tr>
<td>CXCL10</td>
<td>4</td>
<td>185–6186</td>
<td>1734 ± 2526</td>
<td>215 ± 157</td>
<td>2.5 ± 3.3</td>
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<tr>
<td>CXCL8</td>
<td>5</td>
<td>118–3607</td>
<td>1267 ± 1156</td>
<td>2.6 ± 1.4</td>
<td>0.45 ± 0.4</td>
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<td>IL-1β</td>
<td>4</td>
<td>0.17–0.28</td>
<td>0.21 ± 0.04</td>
<td>1.1 ± 0.5</td>
<td>1.1 ± 0.3</td>
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<td>IL-1ra</td>
<td>6</td>
<td>115–3301</td>
<td>1572 ± 1458</td>
<td>1.0 ± 0.3</td>
<td>0.9 ± 0.3</td>
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<td>IL-6</td>
<td>4</td>
<td>0.38–18</td>
<td>6.9 ± 7.6</td>
<td>2.3 ± 1.1</td>
<td>1.0 ± 0.3</td>
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<tr>
<td>IL-10</td>
<td>6</td>
<td>0.99–18</td>
<td>6.2 ± 6.9</td>
<td>1.7 ± 0.7</td>
<td>5.2 ± 3.5</td>
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* Mean fold change in chemokine/cytokine production (±SD) in M1 and M2a MDM relative to control MDM. °Control, unpolarized MDM. *, p ≤ 0.05, as assessed by one-way ANOVA and Tukey posttest. N° denotes the number of independent MDM cultures studied. n.d., not detectable.
FIGURE 6. Inhibition of HIV DNA and protein synthesis in M1 but not in M2a MDM. A, Control, M1 and M2a MDM were infected with HIV-1BaL and harvested after 48 h. Levels of HIV-1 gag DNA ranged from 52 to 4,463 copies per 1,000,000 cells in infected MDM, whereas uninfected MDM routinely expressed <1 copy viral RNA per 1,000,000 cells. These results represent the mean (±SD) of eight individual infections of independent MDM cell cultures. Statistical differences were assessed using the multivariate one-way ANOVA and Tukey’s posttest. B, Accumulation of HIV-1 proteins was determined in control, M1, and M2a MDM 7 days after infection using the serum from an HIV+ individual containing high titters of anti-HIV Abs. A sharp decrease of viral protein synthesis was observed in M1 but not in M2a MDM; these results are representative of three independent infections with cells of different donors.

IL-1ra was reduced in M1-MDM (Fig. 5). These patterns of cytokine and chemokine secretion suggest that the functional consequences of M1/M2a polarization may be further amplified by a delayed down-modulation of soluble factors released by MDM that have been polarized along the opposite program.

M1, but not M2a, polarization inhibits early events of R5 HIV-1 infection of MDM
To further examine the mechanisms underlying the inhibitory patterns associated with macrophage polarization, we quantified the levels of viral DNA that accumulated during the first 48 h of infection in M1 and M2a or control MDM. This time point was chosen because it is indicative of a single round of virus replication in MDM (40). M1 polarization consistently led to a marked decrease in the accumulation of HIV-1 DNA (range, 54–97%). In contrast, no statistical differences were observed in terms of HIV-1 DNA accumulation in M2a vs control MDM (Fig. 6A). These results, taken together with the differences in the inhibitory kinetics in M1 and M2a MDM, indicate a clear-cut difference in the HIV inhibitory mechanisms induced by M1 vs M2a polarization.

To further characterize the steps in the viral life cycle that are inhibited by M1 and M2a polarization, we investigated the accumulation of cell-associated viral proteins during the first 7 days of infection using Western blotting. Consistent with the decreased accumulation of viral DNA, a marked decrease of HIV-1 protein synthesis was observed in M1 MDM (Fig. 6B). In contrast, M2a polarization had no effect on the synthesis or accumulation of viral proteins, despite the marked reduction in the levels of cell-free HIV-1 released into the culture supernatant, as determined by the RT activity (Figs. 2 and 3). In this regard, it should be emphasized that the RT enzyme is almost exclusively associated with HIV-1 virions and is not shed by infected cells like the p24 Gag Ag (62) (Fig. 6B). This observation strongly suggests that the polarization of MDM into M2a cells induces a restriction in one or more late steps of the viral life cycle.

Discussion
In this study, we demonstrate that the polarization of MDM into M1 and M2a macrophages leads to a significant restriction in the capacity of these cells to support productive R5 HIV-1 infection. M1 polarization was associated with a more profound suppression of HIV-1 replication that was rapidly lost when cells were infected 3 days after removal of the polarizing cytokines (TNF-α plus IFN-γ). M1-dependent inhibition was also associated with a sharp decrease in HIV-1 DNA synthesis at 48 h and a decrease in the accumulation of HIV-1 proteins, indicating that virus inhibition occurred at an early, preintegration step of the HIV-1 life cycle. This interpretation is supported by the detection of a clear-cut up-regulation of CCR5-binding chemokines (CCL3, CCL4, and CCL5) and a partial down-regulation of CD4 from the cell surface of M1 cells. M2a polarization, on the other hand, resulted in a less profound but more sustained inhibition of virus replication that was not associated with any impairment of virus entry, reverse transcription, or HIV protein accumulation, suggesting inhibition occurred during late events in the HIV-1 life cycle. Both M1 and M2a phenotypes were transient and reversible for most of the determinants investigated, including the capacity to support productive HIV-1 infection, despite a delayed wave of contra-regulatory effects observed at the chemokine/cytokine level.

Collectively, our results indicate that an impairment of early events in the HIV-1 life cycle is the main mechanism responsible for the inhibition of HIV-1 infection in M1-MDM. This finding is consistent with previous reports showing that both IFN-γ and TNF-α can down-regulate CD4, thereby limiting HIV-1 entry. In this regard, previous studies have also shown that IFN-γ decreases CD4 expression in monocytes, whereas TNF-α, either alone or in
combination with IL-13, down-regulates CCR5 and CXCR4, as well as CD4, from the surface of MDM (16, 44, 45, 48). Our study confirms and extends these observations to M1 MDM generated by the short-term costimulation with TNF-α plus IFN-γ. Although M1 polarization led to a marked reduction in CD4 and CXCR4, we did not observe a down-regulation of CCR5, despite increased secretion of its ligands, namely CCL3, CCL4, and CCL5. In addition to interfering with the entry of R5 HIV-1, it has been reported that IFN-γ up-regulates APOBEC-3G expression, which may further restrict infection at an early postentry level (63).

A partial CD4 down-regulation was also observed in M2a MDM, although to a lesser extent than in M1-MDM (Table III). As previously reported for monocytes that have been preincubated with IL-4 for 5 days (64), the decrease in CD4 on M2a MDM did not lead to a reduction in the accumulation of HIV-1 DNA at 48 h. In monocytes exposed to IL-4 before infection, virus production was found to be highly dependent on an inhibition of cell proliferation during in vitro differentiation into macrophages (64). In contrast, in mature macrophages already infected with HIV-1, IL-4 stimulation enhances HIV-1 transcription without affecting the levels of CCR5 expression (65). In our study, an IL-4-dependent increase in CCR5 expression was observed in some donors (Table I). This increase may have compensated for the decreased levels of CD4 expression on M2a cells consistent with the observation that macrophages expressing high levels of CCR5 but low levels of CD4 are fully susceptible to infection by R5 HIV-1 (66). Another feature that, according to the literature, could have contributed to the postentry restriction of HIV-1 expression in M2a MDM is the accumulation of transcriptional inhibitory NF-κB p50 homodimers in the cell nucleus (67–69). We investigated this hypothesis but found no evidence of such a phenomenon under our experimental conditions (data not shown). The detection of equivalent levels of cell-associated HIV-1 proteins in control and M2a MDM also renders it unlikely that HIV-1 replication was inhibited before transcription. Furthermore, the latter finding suggests that the inhibition of HIV-1 replication observed in M2a MDM involves late, posttranslational events in the virus life cycle. This is in contrast to a study published by Schuttemaker et al. (64) who reported that the IL-4-induced inhibition of HIV-1 replication in MDM occurs at the level of reverse transcription. This apparent discrepancy is most likely due to differences between the two models of MDM. In the previous study, monocytes were differentiated into MDM for 5 days in the presence of IL-4 before infection (64), whereas in our study, differentiated MDM were stimulated with IL-4 for only 18 h before infection.

Another salient feature of M1 polarization was the strong and sustained up-regulation of CXCL10 (for at least 7 days postpolarization), a chemokine that plays an important role in HIV-1 encephalitis (70, 71). Previous in vitro studies have shown that CXCL10 stimulates HIV-1 replication by down-regulating the secretion of CCR5-binding chemokines, thereby enhancing viral entry (49, 72, 73). CXCL10-associated enhancement of HIV-1 infection was not observed in our system, although the prolonged secretion of CXCL10 may have contributed to the rapid down-regulation of CCL3 secretion (and potentially CCL4 and CCL5) observed in M1 MDM (Fig. 6). In this regard, we observed that only 20% of MDM were induced to secrete CCL3 following M1-polarizing stimulation with TNF-α plus IFN-γ while most cells responded to lipid A (Fig. 4). This suggests that the MDM population consists of cells that differ in their ability to respond to M1 polarization (TNF-α and IFN-γ), at least in terms of CCL3 expression.

CCL2, a proinflammatory chemokine that was constitutively secreted by M1 and control MDM, was down-regulated in M2a MDM. CCL2 is involved in the early recruitment of monocytes to inflammatory lesions (14) and has been shown to enhance the replication of X4 HIV-1 strains in activated PBMC (54). Neutralization of this chemokine has been shown to inhibit the late phase of virion release in primary MDM (74). Thus, the reduction in CCL2 secretion may have contributed to the prolonged postentry inhibition of HIV-1 replication observed in M2a but not in M1 MDM.

A distinctive feature of M2a MDM polarization was the persistent up-regulation of CCL2 secretion, a finding that was not observed in either control or M1 MDM. The ability of IL-4 to up-regulate CCL2 has been described previously, whereas its lack of superinduction in M1 MDM is consistent with the known inhibitory effect of IFN-γ on the expression of this chemokine (11, 24). With respect to HIV-1, CCL2 was originally described as a suppressive factor released by activated CD8+ T cells as part of their repertoire of nonlytic soluble inhibitory factors (51). Although this observation was not confirmed by some laboratories (75, 76), we have reported that CCL2 inhibits HIV-1 replication in MDM but not in activated PBMC by acting at a postentry step of the viral life cycle (12). These results are consistent with the pattern of inhibition of HIV-1 replication observed in M2a MDM.

Both M1 and M2a phenotypes were reversible 3–7 days after removal of the inductive stimuli, although there were some exceptions. Of interest was the detection of a symmetrical wave of contra-modulatory effects that were temporally associated with the loss of M1 and M2a polarization, suggesting that this phenomenon may amplify and prolong the consequences of the initial M1 or M2a polarization program. These findings extend recent work by Porcheray et al. (33) who, by monitoring the differential expression of CD163, CD206, CCL3, and CCL18, observed that macrophage polarization was fully reversible and that differentially activated macrophages remained susceptible to further changes in the microenvironment. In our study, a strong correlation was observed between the return to a nonpolarized state and the loss of viral inhibition with M1 cells exhibiting an earlier recovery in both the expression of surface membrane CD4 and the capacity to support HIV replication. With the exception of CXCL10 (M1) and CCL22 (M2a), both M1 and M2a MDM showed a complete reversion to control levels 7 days after the removal of polarizing cytokines.

In conclusion, cytokine-mediated polarization of mature macrophages into M1 and M2a cells, as investigated in human MDM, may be an important regulator of macrophage susceptibility to HIV-1 infection and replication. The transient and reversible nature of the changes in MDM phenotype and activation status may represent a mechanism through which these cells, which are typically resistant to the cytopathic effect of HIV-1 (3–5), cycle between a state of resistance or latent infection and productive viral infection and spreading.

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Disclosures
The authors have no financial conflict of interest.
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