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Brief Conclusive Report

Plastic restriction of HIV-1 replication in human macrophages derived from M1/M2 polarized monocytes

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ABSTRACT

M1/M2 cytokine-dependent polarization of primary human MDMs has been shown to contain CCR5-dependent (R5) HIV-1 replication. In this study, a similar effect was achieved when monocytes were first polarized toward M1 or M2 and were infected 7 d after their differentiation into MDMs, regardless of whether the cytokines were removed 18 h after cell stimulation or were left in culture. Unlike polarized MDMs, no significant down-regulation of CD4 from the cell surface was observed in MDMs derived from M1/M2-polarized monocytes. A second stimulation of MDMs differentiated from M1/M2 monocytes with the opposite polarizing cytokines converted the virus replication profile according to the new stimuli. The expression of M1 and M2 markers (i.e., APOBEC3A and DC-SIGN, respectively) was induced by MDM stimulation with the opposite cytokines, although it also persisted in cells according to their first stimulatory condition. Thus, stimulation of monocytes with M1- and M2-inducing cytokines leads to a restriction of HIV-1 replication when these cells are infected several days later as differentiated MDMs. These observations imply that activation of circulating monocytes significantly influences their capacity to either support or restrict HIV-1 replication, once extravasated, and eventually to become infected as tissue macrophages.
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Introduction

In addition to $CD4^+$ T lymphocytes, MPs represent a main target of HIV-1 infection [1]. Unlike T cells, HIV-infected macrophages are essentially resistant to the cytopathic effects of virus replication in vitro and are not significantly depleted in vivo, supporting the hypothesis that MPs represent a major obstacle to the eradication of HIV-1 in individuals receiving cART [1, 2]. Like differentiated macrophages, circulating monocytes (expressing both CD4 and CCR5/CXCR4) are permissive of HIV-1 entry and have been described as infected in both viremic, therapy-naïve, and cART-treated patients [3–5]. However, virus replication in circulating monocytes is highly contained at or before the reverse transcription step before integration of the provirus, most likely as a consequence of high levels of expression of viral RFs of the APOBEC family such as APOBEC3G and, particularly, APOBEC3A that is down-regulated upon monocyte differentiation into MDMs [6–8].

Differentiated macrophages can be functionally polarized by exposure to microbial products and cytokines, such as IFN-γ and TNF-α, that can skew macrophage function toward a proinflammatory, M1-polarized phenotype. Conversely, anti-inflammatory and immunosuppressive signals, such as IL-4, IL-10, IL-13, TGF-β, and glucocorticoid hormones can drive alternative pathways of macrophage activation, collectively indicated as M2-polarization [9, 10], although these terms have been recently redefined [11].

In regard to the potential role of functional polarization of macrophages in HIV-1 infection, we have earlier shown that both M1 and M2 polarization of differentiated MDMs leads to a restriction of HIV-1 replication upon their in vitro infection in comparison to unpolarized cells, although with significant differences between the 2 conditions [12]. M1-dependent inhibition of virus replication was associated with a profound down-regulation of the primary HIV receptor CD4 and increased secretion of CCR5-binding chemokines resulting in decreased levels of HIV-1 DNA and protein synthesis, consistent with a profound impairment of viral entry [12]. However, we have also reported that M1-polarization of MDM inhibits virus production after infection by a VSV-g pseudotyped virus (that bypasses the

Abbreviations: APOBEC = apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like, BM = bone marrow, cART = combined antiretroviral therapy, CTR = control, DC-SIGN = dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin, MDM = monocyte-derived macrophage, MFI = mean fluorescence intensity, MP = mononuclear phagocytes, P = persistent protocol, PFA = paraformaldehyde, RF = restriction factors, RT = reverse transcriptase, VSV = vesicular stomatitis virus, W = washout protocol

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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requirement of CD4 and CCR5 for cell entry) [8]; furthermore, M1-polarization leads to re-expression of APOBEC3A [8], an HIV RF expressed by circulating monocytes and down-regulated upon their differentiation into MDMs, as discussed above [6]. This potent restriction was short-lived, however, in that it was lost if cells were infected 3 d after M1 polarization [12]. M2-MDM showed a less potent, but more durable inhibition of HIV-1 replication, despite undetectable impairment of HIV-1 DNA synthesis, implying interference at a postintegration step in the virus life cycle [12, 13]. M2-MDM, unlike M1 cells, showed an increased expression of DC-SIGN [14], a cell surface molecule capable of capturing HIV and facilitating its transfer to activated $CD4^+$ T lymphocytes [15].

In light of the recent demonstration of a BM–independent origin of tissue-resident macrophages, it has been stated that "monocytes should now be further investigated as distinct precursors of only newly recruited monocyte-derived cells and as effector cells in their own right." [16]. Therefore, we investigated the potential effect of M1/M2 cytokine-dependent polarization on human monocytes freshly isolated from peripheral venous blood of healthy HIV-seronegative donors, in terms of their susceptibility to R5 HIV-1 infection once they have become differentiated several days later as MDMs. M1/M2 monocyte polarization led to the inhibition of HIV-1 replication when they were differentiated and infected 7 d later as MDMs, with profiles superimposable on those of MDMs polarized and infected after their in vitro differentiation. Polarized cells maintained full plasticity, rapidly acquiring features typical of the opposite subset, including a more or less profound inhibition of virus replication, according to the new M1/M2 polarization profile, under the proper environmental stimulation, although they also maintained signatures of their first polarization (*i.e.,* APOBEC3A and DC-SIGN expression) for several days of culture.

MATERIALS AND METHODS

Reagents

The human, endotoxin-free recombinant cytokines IL-4 and IFN-γ (both used at 20 ng/ml) and TNF-α (2 ng/ml) were purchased from R&D Systems (Minneapolis, MN, USA); Ficoll-Hypaque and Percoll media were purchased from Amersham Biosciences Europe (Milan, Italy) and from GE Healthcare (Pittsburgh, PA, USA), respectively. DMEM, PBS, FBS, normal human serum, penicillin/streptomycin, and glutamine were purchased from Lonza (Cologne, Germany).

Isolation of human monocytes from PBMCs and differentiation into MDMs

PBMCs were isolated from buffy coats of healthy HIV-seronegative blood donors by Ficoll-Hypaque centrifugation. The monocytes were then obtained by Percoll density gradient centrifugation, reaching 80–90% purity [17], and seeded into 48-well plastic plates at 5×10^5 cells/ml in DMEM containing penicillin/streptomycin (1%), glutamine (1%), heat-inactivated FBS (10%), and heat-inactivated normal human serum (5%) (complete medium) [18]. Nonadherent cells (mostly lymphocytes) were then removed, thus increasing the purity of monocyte preparations. Monocytes were then either left unstimulated or were stimulated with polarizing cytokines (i.e., IFN-γ plus TNF-α for M1 or IL-4 for M2) immediately after isolation or 7 d later as MDMs. In the case of monocyte polarization, the cytokine-containing medium was either removed after 18 h of culture (W protocol) or maintained for 7 d (P protocol) before infection. In the case of polarized MDMs, the cytokine-enriched supernatant was removed 18 h after cell stimulation before infection.

R5 HIV-1 infection of MDM

Human MDMs were infected with the macrophage-tropic, laboratory-adapted R5 HIV-1_{BaL} strain at a multiplicity of infection of 0.1, without removing the excess virus after infection. The virion content of cell-free culture supernatants was determined by measuring the levels of Mg^{2+}-dependent RT activity [19].

Western blot analysis

The levels of expression of the HIV restriction factors APOBEC3A, APOBEC3G, and SAMHD1 were determined in monocytes and MDMs by Western blot analysis. The cells were lysed in NP40 buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% NP40, and 0.5% w/v deoxycholate) containing protease inhibitors. Proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane by electroblot analysis, and probed with rabbit anti-APOBEC3A/G Ab kindly provided by Dr. Klaus Strebel [Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA] or with rabbit anti-SAMHD1 Ab (Sigma-Aldrich, Milan, Italy); anti-GAPDH rabbit mAb (14C10 clone; Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the control.

Flow cytometry

Cells were detached from the culture plates with a rubber policemen, washed with Perm/Wash buffer (BD Italia, Milano, Italy), and stained with the different mAbs for 20 min at room temperature. After staining, the cells were washed again with Perm/Wash buffer and resuspended in 2% PFA, to be analyzed by FACSCanto (BD Italia). The acquired events were analyzed with FloJo, version 8.8.7 (Tree Star, Ashland, OR, USA).

Statistical analysis

The Prism GraphPad software v. 5.0 (GraphPad Software, San Diego, CA, USA) was adopted. Results are reported as means ± SE. Comparison between groups was performed with the parametric 1-way ANOVA; $P < 0.05$ denoted significant differences. To control for interdonor variability, all assays were performed in triplicate in MDMs derived from independent donors.

RESULTS AND DISCUSSION

Polarization of human monocytes into M1 or M2 cells leads to a long-lasting restriction of R5 HIV-1 replication in MDMs

Human monocytes purified from PBMCs of 5 healthy donors were exposed (or not) to IFN-γ plus TNF-α or to IL-4 alone to induce their polarization into M1 or M2 cells, respectively, according to a published protocol optimized for MDMs [12]. The cytokines were either removed by cell centrifugation after 18 h or were not removed. Seven days later, the differentiated MDMs were infected with the R5 strain HIV-1_{BaL} (multiplicity of infection = 0.1). Unpolarized (CTR) MDMs and MDMs obtained from the same donors and polarized according to M1 or M2 protocols after 7 d of differentiation were infected in parallel (**Fig. 1**).

As expected, cytokine-driven polarization restricted HIV-1 replication in fully differentiated MDMs (Fig. 1, left), as reported [12]. In addition, inhibition of virus replication was observed when cell polarization was applied to freshly isolated monocytes 7 d before infection. In this regard, M1 polarization caused a stronger and more persistent inhibition of virus replication than that observed in cells stimulated in the M2 condition when it was applied to monocytes before their differentiation and infection (Fig. 1, middle and right). M1 polarization caused a stronger

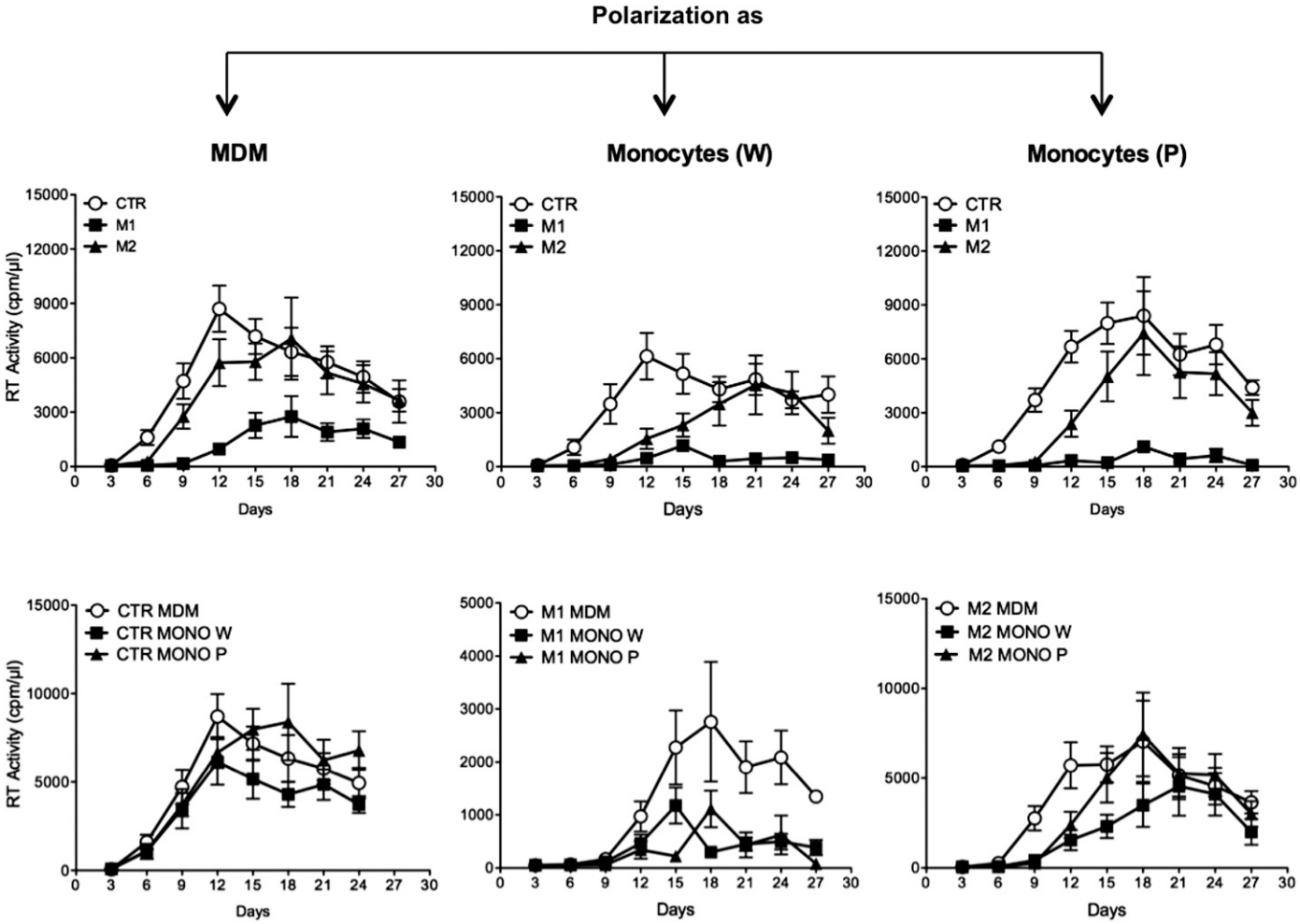

Figure 1. Monocyte polarization restricts HIV-1 replication upon infection of differentiated MDMs. HIV-1 replication was monitored over a 27 d period for supernatant-associated reverse transcriptase activity. Top: cell polarization resulted in superimposable profiles of restricted virus replication, regardless of whether the cytokines were removed after 18 h (W) or were left in culture (P), with M1 cytokines inducing a stronger inhibition of virus replication than IL-4, both in cells polarized as MDMs (left) or as monocytes (middle and right). Bottom: different replication profiles in unpolarized, M1- and M2-MDMS were grouped according to the stimulatory conditions, showing a stronger (although not statistically significant) inhibition by M1 cytokines when applied to monocytes rather than MDMs. The results represent the mean ± SE of MDMs isolated from 5 independent donors.

impairment of virus replication when the cells were stimulated as monocytes rather than as differentiated MDMs (Fig. 1, middle), although the differences were not statistically significant, most likely because of interdonor variability. In contrast, no obvious differences in virus replication were observed in CTR or M2 cells obtained according to the different protocols described herein.

Thus, unlike "short-memory" differentiated MDMs [12], polarized monocytes "remembered" their functional condition also 7 d later, once differentiated and infected as MDMs. As we did not observe a significant variability of results according to the P and W protocols, the following experiments were conducted only by this latter condition.

Plasticity of cell polarization-dependent inhibition of HIV-1 replication

We next assessed the potential plasticity of MDMs derived from polarized monocytes to determine their capacity to contain HIV-1 propagation. Seven-day-old MDMs obtained from either M1- or M2-stimulated monocytes of 7 independent donors were exposed to the opposite polarizing cytokines for 18 h before HIV-1 infection (**Fig. 2A**). These new stimulatory conditions caused a switch in the observed HIV-1 replication profiles, in that MDMs derived from M1-polarized monocytes partially lost their ability to restrict HIV-1 production when stimulated with IL-4 and, vice versa, MDM originating from M2-stimulated monocytes acquired a stronger ability to contain HIV-1 spreading after incubation with M1 cytokines (Fig. 2B). These findings underscore the well-known plasticity of MPs [20], even in a condition in which they are already polarized as M1 or M2 cells.

Phenotype of polarized and repolarized MDMs

When the expression of the primary HIV receptor CD4 was investigated immediately after monocyte polarization, no significant differences were observed in CTR vs. M1 or M2 cells, both

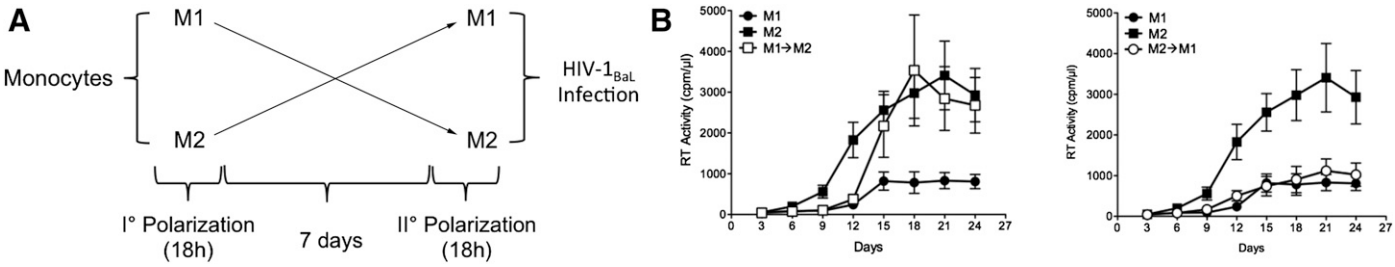

Figure 2. M1/M2 polarization is a reversible process. (A) Experimental scheme. After monocyte isolation, the cells were either polarized or not by M1 and M2 cytokines for 18 h, the stimuli were then removed, and the cells were allowed to differentiate into MDMs for 7 additional days before being exposed to a second round of stimulation with the opposite cytokines for 18 h and finally infected with R5 HIV-1. (B) Reversible profiles of HIV-1 replication in repolarized MDMs. Exposure of M1-MDMs and M2-MDMs to the opposite polarizing cytokines resulted in a conversion of the HIV-1 replication profiles according to the last stimulatory condition. The results represent the mean ± SE of MDMs isolated from 7 independent donors.

in percentage of positive cells (~20% in all conditions) and MFI, as shown in **Fig. 3A**. In contrast with their significant capacity to restrict virus replication, similar levels of CD4 expression were observed in polarized and CTR cells 7 d after monocyte stimulation (with the partial exception of M2 cells that showed a moderate, though not significant reduction of the percentage of positive cells and MFI vs. CTR and M1 cells; Fig. 3B, left middle; 3C). However, stimulation of MDMs derived from polarized monocytes with the opposite M1/M2 cytokines resulted in a significant reduction in the number of $CD4^+$ cells and MFI that was comparable to that observed in control MDMs exposed to the same stimuli (Fig. 3B, left bottom and Fig. 3C).

DC-SIGN was barely detectable on the surface of both CTR and M1-MDMs, but it was clearly expressed by M2-MDM (Fig. 3A), as reported [14]. A strong up-regulation of DC-SIGN was observed in MDMs derived from M2-monocytes 7 d after cytokine stimulation (Fig. 3B, right middle; 3C) as well as in M1-MDM restimulated with IL-4 (Fig. 3B, right bottom; and C). DC-SIGN, however, remained clearly expressed after incubation of M2 cells with M1 cytokines (Fig. 3B, right bottom; and C), likely as a consequence of its relative stability on the plasma membrane. No significant differences in DC-SIGN expression were observed in MFI in these different stimulatory conditions (Fig. 3A, C).

APOBEC3A expression is inducible in MDMs derived from M2-monocytes by stimulation with M1 cytokines

APOBEC3A expression was still detectable by Western blot in CTR and M2 monocytes after 18 h of culture, although at lower levels than observed in M1-monocytes (**Fig. 4**, lanes 1 and 3 vs. lane 2, respectively). As expected, APOBEC3A was barely detectable in CTR and M2-MDM after 7 d of culture (Fig. 4, lanes 4 and 6, respectively), but it was strongly expressed by M1-MDMs (Fig. 4; lane 5). Of interest, exposure of MDM derived from M2-polarized monocytes to M1-polarizing conditions promptly induced APOBEC3A expression (Fig. 4; lane 8). However, APOBEC3A remained highly expressed in MDMs derived from M1-monocytes upon incubation with IL-4 (Fig. 4, lane 7). This observation was unexpected on the basis of the (partial) loss of inhibition of HIV-1 replication observed in these experimental conditions (Fig. 2). Thus, the plasticity of M1/M2-MDMs in response to opposite polarizing cytokines does not appear to fully erase all the acquired phenotypic and functional features, in that the cells maintained "signatures" of their previous activation state (i.e., APOBEC3A expression in M1-derived M2 cells and DC-SIGN expression in M2-derived M1 cells) despite the new vice versa stimulatory conditions.

A moderately increased expression of APOBEC3G was observed in MDMs established from both M1 and M2 monocytes vs. CTR cells, without significant differences according to polarizing conditions (Supplemental Fig. 1). No significant modulation of SAMHD1 was observed in the different experimental conditions (data not shown).

A major breakthrough in our understanding of the ontogeny and function of MPs has been the recent demonstration that most tissue-resident macrophages do not originate from the BM, but rather from yolk sac, fetal liver, or hematopoietic fetal cells [21], as reviewed [22–24]. Tissue-resident macrophages are credited with self-renewal capacity and are likely responsible for maintaining tissue homeostasis in physiologic conditions. However, upon tissue injury or infection, monocytes are rapidly recruited and differentiate in loco to become "tissue monocytes" eventually maturing to cells indistinguishable from tissue-resident macrophages of BM-independent origin [22–24].

The concept of macrophage polarization has been introduced to underscore the differential capacity of MPs to cope with different microenvironmental signals, including microbial products and host-derived cytokines. Whereas proinflammatory signals induce an M1 phenotype (also referred to as "classic activation") with increased cell surface expression of MHC class II molecules and an arginine metabolism geared toward production of the antimicrobial gas NO, anti-inflammatory stimuli lead to the M2 phenotype (or "alternative activation") more prone to tissue repair, quenching inflammation, and modifying their arginine metabolism accordingly [9–11].

Human MPs are, together with $CD4^+$ T cells, a major target of HIV-1 infection [1, 25]. That circulating monocytes, and particularly the $CD14^+CD16^+$ subset that is expanded in HIV-1^+ subjects [7, 26–28], are relevant targets for HIV-1 infection in vivo has been suggested by some studies [3, 5, 29–31], but not confirmed by others [32, 33]. In vitro, infection of freshly isolated monocytes is poorly productive unless the cells are allowed to differentiate for several days to become MDMs, a process associated with an increased expression of CD4 and CCR5 and down-regulation of APOBEC3A [6, 32, 34, 35]. This

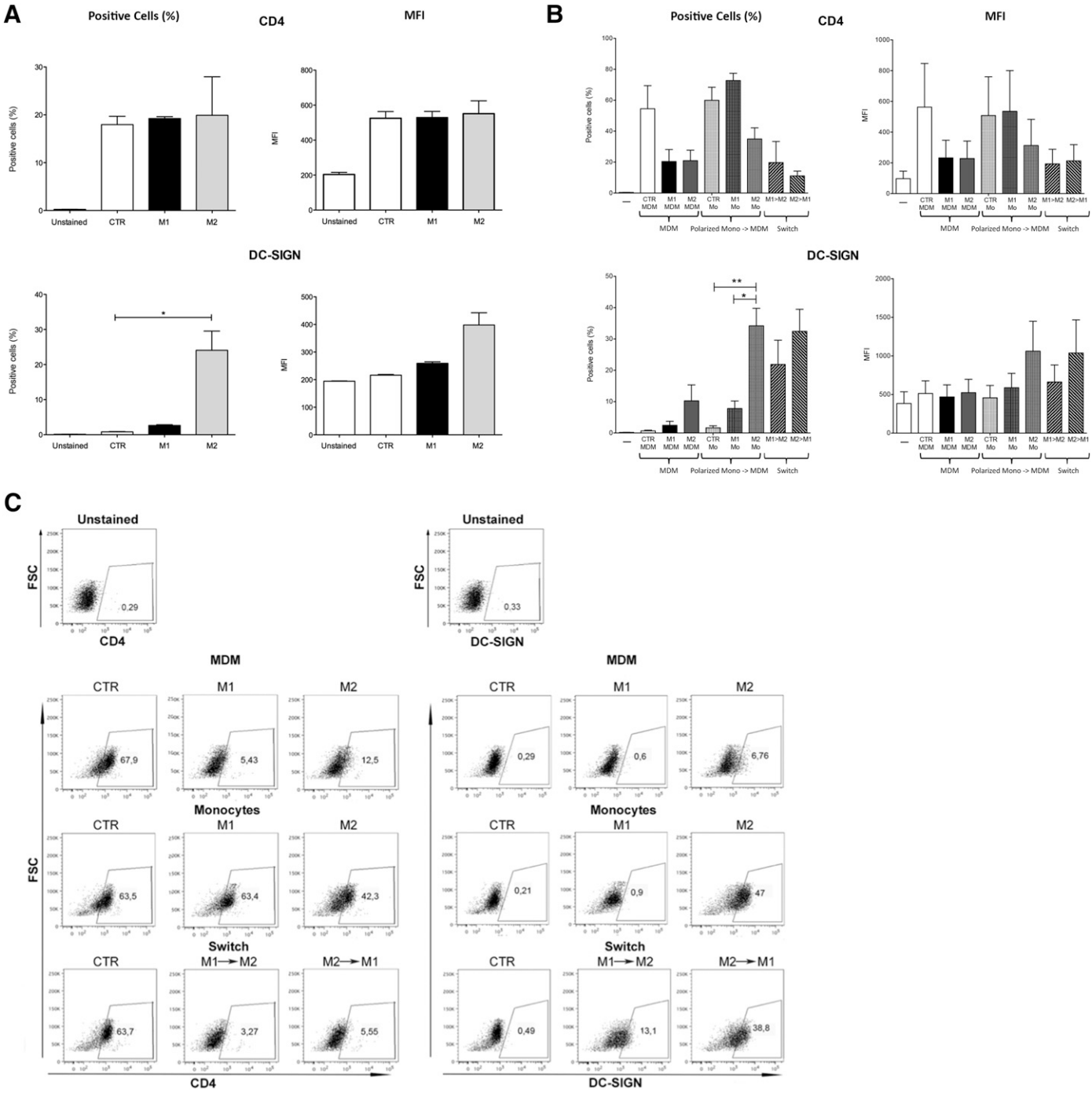

Figure 3. Expression of CD4 and DC-SIGN in polarized and unpolarized monocytes and MDMs. (A) No significant differences were observed in cells expressing CD4 in the different experimental conditions, either in cell percentages (left) or MFIs (right). In contrast, DC-SIGN expression was strongly induced in a percentage of positive cells in M2a vs. CTR and M1 cells ($P < 0.05$; $n = 3$). (B) CD4 expression was down-regulated upon M1 or M2 polarization of MDM (top) as reported [12]. Its expression, however, returned to control levels after 7 d of culture (middle), but it was down-regulated if these cells were re-exposed to polarizing cytokines (bottom) according to the scheme illustrated in Fig. 2A. DC-SIGN was not expressed by CTR and M1-cells, but was clearly detectable in M2-monocytes 18 h after stimulation (upper panels). Its expression was further boosted by IL-4 stimulation when this marker was evaluated 7 d later (middle). DC-SIGN was also induced by IL-4 in MDMs derived from M1 monocytes, although it was maintained in MDMs derived from M2 monocytes that were then stimulated with M1 cytokines (bottom). The results shown were obtained with cells of 1 donor representative of 5 independently tested. Pol-Mono: polarized monocytes; Switch: inversion of polarizing conditions (18 h). (C) A down-regulation of CD4, in both percentage of positive cells and MFI, was observed in MDMs exposed to polarizing cytokines, consequent to either a first or a second polarization, but was not observed in MDM derived from polarized (Pol) monocytes. DC-SIGN expression was strongly induced in terms of percentage of positive cells upon stimulation with IL-4. No significant differences in MFI were noted (right) ($n = 5$). *$P < 0.05$; **$P < 0.001$.

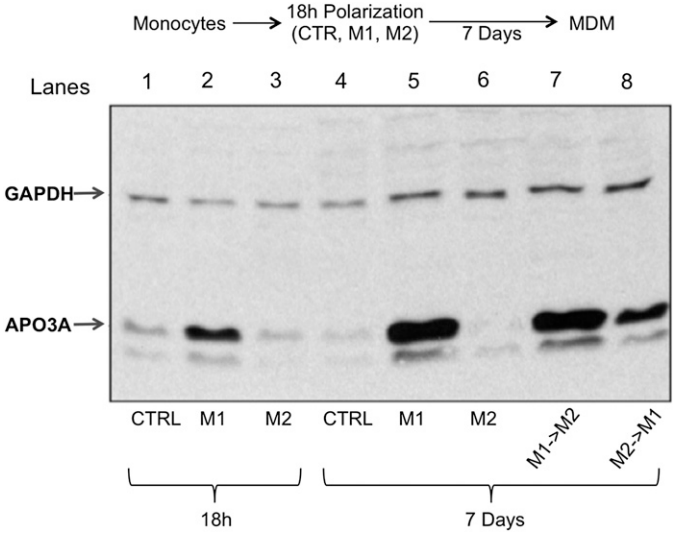

Figure 4. APOBEC3A (APO3A) expression in polarized monocytes and MDMs. Monocytes were either left unpolarized or were polarized toward M1 and M2 phenotypes for 18 h, immediately after their isolation from PBMCs. APOBEC3A expression was still detectable in CTR and M2 monocytes and was increased in M1 cells. After 7 d, APO3A was barely detectable in CTR and M2-MDM, but was strongly expressed in MDMs derived from M1 monocytes. APO3A expression was maintained in M1-MDMs incubated with IL-4 for 18 h (lane 7), but was also induced in M2-MDMs exposed to M1 cytokines for the same time, although at lower levels (lane 8). The results shown were obtained with the cells of 1 donor representative of 3 independently tested.

RF is a member of DNA-editing enzymes, including APOBEC3G and APOBEC3F, known to prevent the reverse transcription of the HIV-1 genome in "nonpermissive" primary cells and cell lines [36, 37]. In this regard, the role of APOBEC3A in macrophage infection was recently linked to neutralization of CCL2, a chemokine endogenously secreted by these cells and responsible for the tonic release of HIV-1 particles from infected MDMs [38]. In the same study, and consistent with our present findings, the potential role of another potent RF for myeloid cells—namely, SAMHD1—was ruled out. For these reasons, we did not investigate the potential effects (presumably inhibitory) of M1 or M2 polarization on monocyte infection shortly after their purification from peripheral blood.

Exposure of freshly isolated monocytes to M1- and M2-polarizing cytokines induced phenotypic and functional profiles very similar to MDM in marker expression and, more important, control of HIV-1 replication, once these cells were allowed to mature into fully differentiated MDMs and were then infected. However, a major difference observed in comparing the consequences of M1/M2 polarization of monocytes and MDMs is that only the latter condition induced a strong down-regulation of CD4 at the time of infection (Fig. 3), even though very similar levels of inhibition of virus replication were observed in the 2 experimental settings. This observation implies that neither M1- nor M2-associated restriction of virus replication is the likely consequence of a decreased HIV-1 entry, as originally interpreted [12], also suggested by our previous report of a strong restriction of virus expression after infection of M1-MDM with VSV-g pseudotyped virus bypassing CD4 and CCR5 for entry [8].

The observation that APOBEC3A expression is maintained in M1-MDM repolarized to M2 cells by IL-4 stimulation (that partially lose control of virus replication) suggests that the expression of this putative RF per se is an unlikely explanation for the poorly permissive replication profile imposed by M1 polarization of these cells. In this regard, IL-4 was long ago credited with both inhibitory and up-regulatory effects on virus replication in MPs as a function of their stage of differentiation [39]. Furthermore, a recent study showed that inhibition of HIV-1 replication by IL-4 is not associated with the up-regulation of known RF, including cyclophilin A, TRIM-5α, APOBEC3G, TREX-1, and SAMHD1, or of microRNAs known to interfere with HIV-1 replication [40].

In summary, monocytes appear to be fully responsive to M1/M2-polarizing cytokines, resulting in the differentiation of cells capable of restricting HIV-1 replication, even when infected several days after their stimulation. This finding highlights a disparity with differentiated polarized MDMs exposed to the same stimulatory conditions that lose their capacity to restrict virus replication with as little as 3 d of cell polarization in the case of M1 cells [12], and suggest a stronger "memory" of monocytes to become committed M1- or M2-polarized macrophages. This observation also fits with the recent notion of "trained monocytes" or "innate memory" believed to rely on epigenetic modifications of cells induced by their original polarization [22]. The long-lasting expression of APOBEC3A and DC-SIGN in M1 and M2 cells exposed to vice versa stimulatory conditions reported in the present study further supports this hypothesis.

Finally, functional polarization of circulating monocytes may bear relevance to their potential fate as HIV-target cells, once differentiated in tissue in terms of productive vs. restricted virus infection and, ultimately, to the composition and accessibility of the viral reservoir in infected individuals receiving cART.

AUTHORSHIP

F.G. designed and executed most of the experiments, participated in the interpretation of the results, and contributed to writing the manuscript; E.V. contributed to the experimental design, participated in the interpretation of the results, and helped write the manuscript; and G.P. supervised the experimental design, participated in the interpretation of the results and was responsible for writing the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
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