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HIV-1–Mediated BAFF Secretion in Macrophages Does Not Require Endosomal TLRs, Type-I IFN, and Nef, but Depends on the Cellular Phenotype Status

Alejandro M. Gomez,* Michel Ouellet,* Alexandre Deshieres,* Yann Breton,* and Michel J. Tremblay*†

HIV-1 infection is characterized by persistent viral replication, chronic immune activation, and CD4+ T cell depletion. Moreover, several immune dysfunctions are observed in cells that are not targeted by the virus, such as B cells. Some B cell abnormalities include hypergammaglobulinemia, nonspecific B cell activation, class switching, increased cell turnover, breakage of tolerance, and a loss of the capacity to generate and maintain memory. Several cytokines and growth factors that are increased in the serum of HIV-1–infected individuals have been suggested to directly or indirectly trigger B cell activation, and one of these is BAFF. In this study, we investigate the ability of fully competent (R5-tropic) HIV-1 to induce BAFF production by monocyte-derived macrophages (MDMs). We demonstrate here that HIV-1 drives BAFF production in MDMs in a type-I IFN– and TLR-independent manner. Moreover, we determine that HIV-1 Nef accessory protein is dispensable in BAFF upregulation as a nef-deleted HIV-1 strain is still able to increase BAFF at levels similar to the wild type strain. Finally, we show that the macrophage phenotype status affects HIV-1 replication and BAFF induction, as both were abrogated in MDMs displaying a M1 phenotype. This study provides new useful information about the increased levels of BAFF observed during HIV-1 infection and highlights the importance of macrophages as a source of BAFF, a phenomenon that might contribute to B cell dysfunctions at inflammatory tissue sites in infected individuals. The Journal of Immunology, 2016, 196: 3806–3817.

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Abbreviations used in this article: ART, antiretroviral therapy; EFV, efavirenz; MDM, monocyte-derived macrophage; MVC, maraviroc; pDC, plasmacytoid dendritic cell; pol(E,LC), polynosinic-polycytidylic acid; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA.

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Infected macrophages display alterations in cytokine and chemokine production that contribute to the general state of immune activation in HIV-1 infection (34), which further supports a potential contribution of macrophages to BAFF production. Interestingly, macrophages directly regulate proliferation and activation-induced cytokine deaminase expression in B cells via BAFF (35, 36).

In the current study, we investigated the ability of fully competent R5-tropic HIV-1 to induce BAFF production by primary human monocyte-derived macrophages (MDMs). We report that HIV-1 upregulates BAFF production in MDMs independently of type-I IFN and TLR. We provide additional evidence that the accessory protein Nef is not involved in the virus-dependent BAFF production by MDMs. Finally, we show that the macrophage activation status influences BAFF secretion. Indeed, resting MDMs (M0 phenotype) are more permissive to HIV-1 replication and show a higher virus-mediated BAFF induction than M2a-activated (IL-4 treatment) and M1-activated MDMs do (LPS/IFN-γ treatment). Altogether our findings indicate that the HIV-1-driven induction of BAFF production by MDMs is TLR-, type-I IFN– and Nef-independent, but is modulated by the cell phenotype status.

Materials and Methods

Ethics statement and cell culture

The current study was approved by the Bioethics Committee from the Centre Hospitalier Universitaire de Québec-Université Laval (Pavillon du Centre Hospitalier de l’Université Laval). Peripheral blood samples were collected from healthy donors in accordance with the guidelines of the Institutional Bioethics Committee. PBMCs were isolated by Ficoll-Hyphaque gradient centrifugation. Monocytes were separated from other cells, mostly T lymphocytes, by adherence for 2 h at 37˚C in 150- by 20-mm tissue culture dishes, followed by washing with endotoxin-free PBS (Sigma-Aldrich, Oakville, ON, Canada). Monocytes were then allowed to differentiate for 2 d in RPMI 1640 culture medium supplemented with 5% human AB serum, 25 ng/ml M-CSF (Genscript, Piscataway Township, NJ) and antibiotics (100 U/ml Penicillin G, 100 µg/ml streptomycin), then washed and allowed to differentiate for 3–4 d more with the same supplemented culture medium. MDMs were recovered by scraping following 30 min incubation with Accutase (eBioscience, San Diego, CA). Experiments were performed with RPMI 1640 medium supplemented with 10% FBS and antibiotics in the absence of cytokines. In some experiments, MDMs were stimulated for 18 h with IFN-γ (20 ng/ml) and LPS (100 ng/ml) or with IL-4 (20 ng/ml), to obtain classically activated (or M1) and alternatively activated MDMs (or M2a), respectively. Following polarization, M1, M2a, and nonstimulated MDMs (or M0) were washed and incubated in cytokine-free complete culture medium as described above. Human embryonic kidney 293T cells used for transient transfections were cultured in complete DMEM. HEK-blue IFN-α/β indicator cells were purchased from InvivoGen (San Diego, CA) and maintained in complete DMEM culture medium supplemented with 100 µg/ml zeocin, 30 µg/ml blasticidin, and 100 µg/ml prinomuc (InvivoGen).

Abs and reagents

Polyinosinic-polycytidylic acid [poly(I:C)] (TLR3 agonist), LPS (TLR4 ligand), and QUANTI-Blue reagent (alkaline phosphatase detection medium) were all purchased from InvivoGen. Human recombinant IL-4 (rIL-4) and human rIFN-α2a (called IFN-α hereafter) were obtained from R&D Systems (Minneapolis, MN). Human rIFN-γ was purchased from Biologend (San Diego, CA). PerCP-conjugated mouse anti-human BAFF (clone 137314) was purchased from R&D Systems, whereas the soluble human BAFF protein was obtained from R&D Systems (San Diego, CA). Lipofectamine RNAiMAX Transfection Reagent was purchased from Life Technologies, and efavirenz (EFV) and maraviroc (MVC) were obtained from R&D Systems, whereas the soluble human BAFF protein was purchased from Biolegend (San Diego, CA). Flow cytometry

Cells were incubated with Golgistop (BD Biosciences, Mississauga, ON, Canada) before fixation and permeabilization using Cytofix/Cytoperm kit (BD Biosciences, Mississauga, ON, Canada). Cells were incubated with PerCP-conjugated anti-B220 (5 µg) for 30 min at 4˚C and then washed twice with wash buffer. After acquisition on a FACSCantoA flow cytometer (BD Biosciences), data were analyzed with FCS Express 4 software (De Novo Software, Los Angeles, CA).

HIV-1 infection and stimulation assays

MDMs (2 × 105 cells) in 24-well plates were infected with NL4-3Balenv (20 ng of p24 per 1 × 105 cells), ADA (50 ng of p24 per 1 × 105 cells), ADAΔnef (50 or 200 ng of p24 per 1 × 105 cells) or left untreated for 2 h at 37˚C. Cells were then washed and cultured in complete RPMI 1640 culture medium. In some experiments, cells were stimulated with poly(I:C) (30 µg/ml) or IFN-α2a (2,500 U/ml) or incubated for 30 min before infection and maintained postinfection with EFV (50 nM) or MVC (200 nM) to abrogate productive HIV-1 infection in MDMs. HIV-1 production was estimated by measuring the p24 content at different time points following infection in the cell-free culture supernatant as described above.

Small interfering RNA gene knockdown in MDMs

Small interfering RNAs (siRNAs) targeting human TLR3 (s235), TLR7 (s27842), TLR8 (s27922), TLR9 (s28872), CD4, and SAMHD1 siRNAs (Supplemental Table I) were designed using the Designer of Small Interfering RNA software tool. It implements an algorithm based on previous publications (42). MDMs were transfected by reverse transfection using Lipofectamine RNAiMAX Transfection Reagent following the manufacturer’s protocol with slight modifications. For each transfection, 2 × 105 MDMs were transfected with 20 nM targeting siRNA and 1.5 µl lipofectamine reagent. Transfected MDMs were cultured in 24-well plates for 72 h before confirmation of gene knockdown by quantitative RT-PCR (qRT-PCR) analysis of gene expression with gene knockdown as described above. Given that the cell viability of our MDM cultures after the reverse transfection was good (data not shown), we did not count the number of cells before HIV-1 infection to avoid an additional detachment step that can be detrimental.

Analysis of mRNA expression and protein abundance

The total RNA was isolated from MDMs (2 × 105) using the illustra RNAspin Mini Kit (GE Healthcare, Mississauga, ON, Canada) and was reverse transcribed using Moloney murine leukemia virus enzyme (Promega, Madison, WI). Transcripts were quantified by real-time PCR using SYBR Green Master Mix on an ABI-PRISM 7500 Sequence Detector (Applied Biosystems, Carlsbad, CA). BAFF, TLR3, TLR7, TLR8, TLR9, CD4, SAMHD1, and ISG54 cDNAs were amplified using gene-specific primers (Supplemental Table II). The relative changes in gene expression were calculated using the 2−ΔΔCt method (43). This method was used after validation experiments demonstrated that the reaction efficiencies of the target and endogenous reference (i.e., ISG54) were approximately equal. DNA levels were normalized by quantifying the β2M gene. Results were expressed as fold increase in normalized values over those observed with uninfected or mock control cells. BAFF levels were quantified in cell-free supernatants with a commercially available ELISA kit according to the manufacturer’s protocol (Adipogen).

Detection of type-I IFN

Levels of type-I IFN in cell-free supernatants were determined through the use of HEK-Blue IFN-α/β indicator cells, according to the manufacturer’s protocol (InvivoGen). This indicator cell line allows the detection of bioactive IFN-α and IFN-β by monitoring the activation of the ISGF3 pathway. HEK-Blue IFN-α/β cells are stably transduced with a secreted embryonic alkaline phosphatase promoter gene under the control of the IFN-α/β-inducible ISG54 promoter. Levels of secreted embryonic alkaline phosphatase in cell-free supernatants were determined with QUANTI-Blue (Invirogen) and reading the OD at 655 nm.

Flow cytometry

Cell-associated BAFF was analyzed in MDMs. In brief, cells were treated with Golgistop (BD Biosciences, Mississauga, ON, Canada) for 6 h and then MDMs were recovered by scraping following incubation with PBS containing 5 mM EDTA. Before staining, cells were incubated with 20% pooled human sera to block nonspecific binding sites and washed once with PBS supplemented with 0.5% BSA. After fixation and permeabilization using Cytofix/Cytoperm kit (BD Biosciences), cells were stained with PerCP-conjugated anti-B220 (5 µg) for 30 min at 4˚C and then washed twice with wash buffer. After acquisition on a FACSCantoA flow cytometer (BD Biosciences), data were analyzed with FCS Express 4 software (De Novo Software, Los Angeles, CA).
Statistical analysis

Means of raw data were compared using either Student t test or one-way ANOVA, followed by Dunnett–Tukey multiple comparisons when more than two means were considered. The p values < 0.05 were deemed statistically significant. Calculations were performed with the GraphPad PRISM 6 for Windows software (GraphPad Software, La Jolla, CA).

Results

HIV-1 increases BAFF expression and secretion in MDMs

We previously reported that the release of type-I IFN by pDCs following exposure to HIV-1 drives BAFF expression in human monocytes (30). These results motivated us to investigate whether HIV-1 could also modulate BAFF production in macrophages, because they represent another important cellular source of BAFF (35, 36, 44–47) and are, contrary to monocytes, permissive to productive HIV-1 infection (48). As shown in Fig. 1A, BAFF mRNA levels were not increased in MDMs early postinfection (i.e., 24 to 72 h) with fully infectious R5-tropic NL4-3Balenv viruses compared with uninfected control cells. The TLR3 agonist poly(I:C) and IFN-α were used as positive controls in the current studies due to their known capacity to upregulate BAFF expression in different cell types, including macrophages (25, 30, 49, 50). Next, we assessed BAFF secretion in MDM supernatants at similar time points using a commercial ELISA kit. As depicted in Fig. 1B, BAFF secretion was almost undetectable at 24 h, and only poly(I:C) and IFN-α were able to increase BAFF secretion at 48 and 72 h after treatment.

HIV-1 replication typically reached a peak at 9–12 d postinfection in MDMs (Fig. 1C). Therefore, we pursued the analysis of BAFF expression and secretion at later time points. As depicted in Fig. 1D, a statistically significant increase in BAFF mRNA levels was provoked by HIV-1 from day 6 postinfection when compared with uninfected control cells. However, the virus-mediated augmentation in BAFF protein secretion was statistically significant only at day 12 postinfection (Fig. 1E).

Cell-associated BAFF in MDMs

BAFF is a type-II protein of the TNF superfamily that is expressed initially as a membrane-bound form that can be cleaved by a putative furin family protease to generate its soluble form (22, 23). Interestingly, membrane-bound BAFF has been reported to be increased on mature dendritic cells and monocytoid precursors in HIV-1–infected patients (17). Moreover, we established that HIV-1 increases cell-associated BAFF in classical and intermediate monocytes (30). Therefore, we analyzed cell-associated BAFF (both membrane-bound and intracellular protein) by flow cytometry in MDMs after infection with HIV-1 (Fig. 2A). Although the mean fluorescence intensities between HIV-1–infected and uninfected control cells were not statistically significant (data not shown), higher percentages of BAFF+ cells were seen in virus-infected MDMs starting at day 3 and reaching statistical significance at day 6 postinfection (Fig. 2B). Interestingly, the percentage of BAFF+ cells diminished at later time points (i.e., 9 and 12 d postinfection).

HIV-1–mediated BAFF secretion in MDMs can be reduced by antiretroviral drugs

Circulating levels of BAFF in sera from HIV-1–infected patients receiving ART were reported to be lower than those observed in untreated patients but remained greater than in healthy subjects (20). Another study reported that plasma BAFF concentrations decreased slightly upon initiation of ART in HIV-1 rapid progressors, whereas membrane BAFF on blood myeloid dendritic cells (mDCs) and monocyte precursors remained high regardless of ART (17). Therefore, we analyzed the effect of two antiretroviral drugs on the HIV-1–dependent induction of BAFF in MDMs. We used the nonnucleoside reverse transcriptase inhibitor EFV and the CCR5 receptor antagonist MVC. As shown in Fig. 3A, treatment of HIV-1–exposed MDM cultures with EFV or MVC completely abrogated viral production. BAFF secretion in these samples was thus analyzed 12 d after exposure, at which time point a statistically significant difference in BAFF secretion was observed between infected and uninfected cells (Fig. 1E). In the absence of EFV or MVC, infection with HIV-1 led to an increased secretion of BAFF, whereas drug treatment at doses that inhibit viral replication returned the levels of BAFF secretion by MDMs at levels similar to those of uninfected controls (Fig. 3B).

HIV-1–mediated BAFF production in MDMs is independent of type-I IFN production

Increased BAFF levels seen in certain chronic autoimmune disorders and chronic inflammatory diseases were reported to be associated with an elevated type-I IFN response (51–53). Moreover, we reported that HIV-1–directed release of type-I IFN by pDCs plays a determinant role in BAFF production by monocytes (30). As shown in Fig. 1A and 1B, IFN-α induces BAFF production in MDMs. Consequently, we suspected that type-I IFN production could also play a role in the HIV-1–mediated BAFF induction in MDMs. As shown in Fig. 4, secretion of biologically active type-I IFN was not detected at any time following virus infection of MDMs. In contrast, poly(I:C) treatment induced secretion of type-I IFN in the first 3 d after treatment, and it returned to basal levels at later time points. These results reveal that type-I IFN does not play a role in the HIV-1–driven BAFF production in MDMs.

Endosomal TLR sensing of HIV-1 is not involved in BAFF upregulation

HIV-1, like other single-stranded RNA viruses, triggers innate immune receptors, including endosomal TLRs. Recognition of HIV-1 by TLR7 and, to a lesser extent, by TLR9 in pDCs results in type-I IFN production (54). Uridine-rich oligonucleotides derived from HIV-1 RNA also activate TLR7/8 in human and mouse macrophages and induce innate immune responses (55, 56). Moreover, the stimulation of TLR8 by HIV-1 single-stranded RNA has been reported to promote TNF release in human macrophages (57). Although we did not observe a type-I IFN response in virus-infected MDMs (data not shown), it has been shown that HIV-1 can activate the inflammasome in monocytes and macrophages via endosomal TLRs without induction of type-I IFN, a process leading to secretion of proinflammatory cytokines such as IL-1β and IL-18 (58). Therefore, we investigated whether endosomal TLRs could be involved in the HIV-1–mediated induction of BAFF secretion by MDMs. To achieve this goal, we used specific siRNAs to silence all four endosomal TLRs that were detected by qRT-PCR in MDMs (i.e., TLR3, TLR7, TLR8, and TLR9) (Fig. 5A). Western blot analyses aimed at assessing the efficiency of the tested siRNAs at reducing TLR protein levels were unsuccessful. It is likely that TLR3, TLR7, TLR8, and TLR9 proteins in MDM preparations are present in amounts too low to be detected by western blotting. This possibility is supported by our qRT-PCR analyses of SAMHD1, CD4, TLR3, TLR7, TLR8, and TLR9 at the mRNA level (data not shown). For example, comparative quantitative analyses of mRNA levels using the 2−ΔΔCt method indicate that the mRNA level for TLR3 is 40-fold lower than for SAMHD1, whereas the mRNA level for TLR9 is 140-fold lower than for SAMHD1. Next, we infected the TLR-silenced MDMs with HIV-1 and monitored BAFF production at 12 d postinfection. As depicted in Fig. 5B, siRNA control and TLR-silenced cells showed a statistical increase of BAFF.
production upon infection compared with uninfected controls. However, no statistical differences in BAFF production were observed between control and TLR-silenced MDMs, thus suggesting that stimulation of endosomal TLRs by HIV-1 is not driving BAFF upregulation in MDMs. For controls of silenced MDMs, we transfected siRNAs targeting CD4 or SAMHD1 in MDMs (Fig. 5C). This is based on the notion that the cell surface CD4 molecule plays a critical role in HIV-1 entry in macrophages (59) and SAMHD1 is a host protein expressed in myeloid cells that blocks the infection with retroviruses, including HIV-1, at the reverse transcription step (60). As expected, HIV-1 replication was reduced in CD4-silenced MDMs whereas it was increased in SAMHD1-silenced cells when compared with controls (Fig. 5D). Next, BAFF secretion was analyzed in these samples (Fig. 5E). Results show that HIV-1–mediated BAFF secretion was reduced to levels observed in uninfected MDMs in CD4-silenced infected MDMs. Conversely, and as expected, HIV-1–mediated BAFF secretion was increased in SAMHD1-silenced infected MDMs. Altogether, these results suggest that the HIV-1–induced BAFF production is independent of endosomal TLR sensing and stems from viral replication itself as altering viral replication in MDMs affects the levels of HIV-1–mediated BAFF secretion.

Nef is dispensable for HIV-1–mediated BAFF upregulation

Because TLR and type-1 IFN signaling pathways are not involved in HIV-1–driven BAFF secretion, we were interested to investigate whether the HIV-1 accessory protein Nef could play a role in BAFF upregulation. Indeed, several studies have shown that Nef is involved in HIV-1 disease progression, boosting viral replication by enhancing virus infectivity and spread (61–63). Moreover, Nef expression in macrophages induces the release of inflammatory factors such as MIP-1α, MIP-1β, IL-6, TNF, and IL-1β, among others (64–67). Of high relevance to the present work, one study has reported also that recombinant soluble Nef increases BAFF expression in monocyte-derived DCs (28). As the biological effects of recombinant proteins are sometimes dictated by factors such as dosage and purity, we chose a different approach in which MDM cultures were infected with fully replicative molecular clones of HIV-1 that differ only in their expression of Nef. As shown in Fig. 6A, the Nef-deleted ADA mutant virus (HIV-1/ΔNEF) displays an expected lower infectivity per unit of p24 and reduced kinetics of viral replication compared with the wild-type ADA virus (HIV-1/WT NEF). By increasing the viral input of HIV-1/ΔNEF, higher (donor 1) or similar (donor 2) levels of viral replication were achieved when compared with the parental virus. Secretion of BAFF in these samples and uninfected controls was then analyzed 12 d postinfection. As depicted in Fig. 6B, different amounts of HIV-1/ΔNEF were still able to induce a statistically significant increase in BAFF secretion when compared with uninfected cells. These results suggest that Nef is not responsible for the HIV-1–mediated BAFF secretion in MDMs. The lower levels of BAFF production in presence of Nef were only seen with donor #1 and not with donor #2 (not statistically significant for donor #2). Nonetheless, these results do not jeopardize our overall conclusion indicating that Nef is not required for the induction of BAFF in macrophages because the Nef-deleted virus is still able to mediate BAFF production.

HIV-1–mediated BAFF induction is modulated by the macrophage phenotype status

Monocytes and macrophages are characterized by a high level of plasticity and ability to respond to a wide range of environmental stimuli. By analogy to Th1/Th2 polarization observed in T cells, macrophages can also undergo functional polarization in response to microbial products and host cytokines (68). These polarization pathways have been designated M1 and M2 (69) in which M1 or classically activated macrophages are highly microbicidal, secrete proinflammatory mediators such as TNF, IL-1, IL-12, and IL-6, and are important producers of reactive nitrogen and oxygen intermediates, whereas M2 or alternatively activated macrophages are involved in the resolution of inflammation and tissue repair and secrete anti-inflammatory mediators such as IL-10 (70). M1 macrophages are generally induced by IFN-γ and microbial products such as LPS (71), whereas different stimuli can lead to M2-activated macrophages. For example, M2a macrophages are induced by exposure to IL-4 or IL-13, M2b macrophages are induced by immune complexes, TLR stimulation or by the IL-1ra, and M2c macrophages are induced by IL-10 or glucocorticoid hormones (68). Therefore, we evaluated the effects of M1 (treated with IFN-γ and LPS) or M2a (treated with IL-4) polarization on BAFF production in virus-infected and uninfected MDMs. As depicted in Fig. 7A, constitutive BAFF levels were statistically increased in supernatants from uninfected M1 MDMs when compared with uninfected M0 (nonstimulated) or M2a MDMs at 3 d poststimulation. Although a slight reduction in BAFF secretion was observed in M2a when compared with M0 MDMs, it was not statistically significant. Polarized MDM cultures or controls were then infected with HIV-1, and virus production was monitored in cell-free culture supernatants. HIV-1 replication was lower in M2a cells compared with M0 MDMs and was almost totally inhibited in M1 MDMs (Fig. 7B), which is in agreement with previous observations (72). Analysis of BAFF secretion in infected or uninfected polarized or control MDM supernatants following 12 d of incubation revealed that HIV-1 stimulates BAFF production in M0 and M2a MDMs but not in M1 cells (Fig. 7C). Interestingly, BAFF levels in supernatants of uninfected M1 and M2a cells were not statistically different from those of uninfected M0 cells at day 12 poststimulation. Thus, although basal BAFF levels were higher in M1 MDMs at 3 d poststimulation (treatment with

FIGURE 1. HIV-1 drives BAFF expression and secretion in MDMs. MDMs were first differentiated for 5–6 d and then infected with NL4-3Balenv (Bal) for 2 h and washed extensively to remove unbound virus. In some conditions, cells were either left untreated (CTRL) or treated with poly (I:C) or IFN-α (used as positive controls). (A) Thereafter, total RNA was extracted at the indicated time points and BAFF mRNA was evaluated by qRT-PCR. Results are expressed as fold increase relative to control cells for five different donors. Each symbol represents a different donor. The horizontal line represents the mean of all donors. Statistical analysis was made using one-way ANOVA, followed by Dunnett multiple comparisons test. *p < 0.01, ***p < 0.001. (B) Cell-free supernatants were harvested, and BAFF protein levels were evaluated by a commercial ELISA kit. Results shown are expressed as picograms per milliliter of BAFF protein for five or six different donors. Statistical analysis was completed using one-way ANOVA, followed by Dunnett multiple comparisons test. *p < 0.05, ***p < 0.001. (C) Virus replication was monitored in MDMs by quantifying the p24 content in cell-free supernatants at the indicated time points. Data shown represent the mean ± SD of duplicates for a representative donor. (D) BAFF mRNA was evaluated as described in (A) for five different donors. Statistical analysis was made using Student t test. *p < 0.05. (E) BAFF protein levels were evaluated as described in (B) for six different donors. It should be noted that separate randomized donors were used for the different experiments. Statistical analysis was made using Student t test. *p < 0.05.
IFN-γ/LPS; Fig. 7A), this difference was not maintained in M1 MDMs following 12 d of incubation (Fig. 7C).

**Discussion**

HIV-1 immunopathogenesis involves major perturbations in both innate and adaptive immunity, including B cell responses. Several proinflammatory cytokines and soluble factors that are found to be increased in sera from HIV-1-infected patients have been proposed to have an influence on the functionality of the B cell compartment (5). One of them, BAFF, has been postulated to contribute in a significant manner to the numerous HIV-1-associated B cell dysfunctions (17–20). Our group has established recently that HIV-1 increases BAFF production in monocytes through a type-I IFN–dependent process involving pDCs (30). Monocytes are a subset of circulating leukocytes that can further differentiate into a range of tissue macrophages and dendritic cells (73). Macrophages have a broad role in the maintenance of tissue homeostasis, through the clearance of senescent cells and the remodeling and repair of...
tissues after inflammation (74). They are also considered an important source of BAFF, and they could play an important role in the regulation of B cell responses at inflamed tissue sites. In addition, macrophages support HIV-1 replication, contributing to viral spread and may also harbor or produce infectious virus for extended periods of time (31). Therefore, we were interested to investigate whether HIV-1 could also drive BAFF production in this important cellular target. In the present work, we show that HIV-1 induces BAFF mRNA and protein expression and secretion by MDMs. BAFF upregulation was observed after infection, when HIV-1 replication is high. Interestingly, HIV-1–induced upregulation of BAFF mRNA and cell-associated protein occurred concurrently, whereas BAFF secretion showed a slight delay. The diminution in the percentages of BAFF+ cells at late postinfection times compared with early postinfection times may indicate that BAFF is already cleaved in a soluble form. These findings reinforce the idea that other regulatory steps might be involved in the release of BAFF, such as the control of a putative furin protease activity responsible for its cleavage from the membrane. In addition, the HIV-1–mediated induction of BAFF was statistically significant when we analyzed the percentages of BAFF+ cells, but not at the level of mean fluorescence intensities. Similar observations were made previously in human monocytes inoculated with HIV-1 (30), thus suggesting that HIV-1 increases the number of BAFF-expressing cells without affecting BAFF production at the cell level.

We then proceeded to show that blocking HIV-1 infection in vitro using antiretroviral drugs can restore BAFF secretion to normal levels. These studies were prompted by the fact that although ART can improve some B cell responses and reduce BAFF levels in sera from HIV-1–infected patients, circulating BAFF concentrations remain somewhat higher than those found in healthy individuals (5, 17, 20). It is thus possible that ART penetration and bioavailability in deep tissues are significantly lower than in peripheral blood. In fact, low levels of viral replication could still be detected in certain tissue macrophages (75–77) of long-term treated patients, and such viral replication could lead to a chronic, higher than normal, BAFF secretion. Moreover, chronic immune activation caused by bacterial translocation in disrupted mucosal gut tissues could activate local macrophages to sustain high levels of BAFF, affecting surrounding B cell responses.

We then investigated the mechanism of HIV-1–mediated BAFF upregulation in MDMs and reveal that this process is independent of type-I IFN production. This result is in agreement with previous reports showing that HIV-1 infection of macrophages neither triggered IRF3 pathways nor induced type-I IFN gene expression (78–81). Moreover, a recent report showed that mDCs and macrophages fail to induce expression of all known type-I and type-III IFN genes in response to HIV-1 (82). It was further shown that accessory viral proteins Vpr and Vif block...
FIGURE 5. HIV-1–dependent BAFF production in MDMs does not rely on TLR signaling. Functional knockdown of endosomal TLRs (i.e., TLR3, TLR7, TLR8, and TLR9), CD4, and SAMHD1 were generated by RNA interference. MDMs were transfected by reverse transfection using lipofectamine. In brief, MDMs (2 × 10⁵) were transfected with 20 nM of the targeting siRNA or siRNA control (siCTRL). (A) MDMs were mock-transfected (only lipofectamine) or transfected with siRNA targeting TLR3, TLR7, TLR8, and TLR9 or a nontargeting scramble sequence (siCTRL). After 72 h, cell lysates were generated, total RNA was extracted, and efficacy of TLR-silenced was confirmed by qRT-PCR using specific primers. Results are expressed as fold change relative to mock-transfected cells for four different donors. Statistical analysis was completed using one-way ANOVA, followed by Dunnett multiple comparisons test. (B) TLR-silenced and siCTRL cells were infected with NL4-3Balenv (Bal) or left untreated. Cell-free supernatants were harvested at day 12 postinfection, and BAFF protein levels were evaluated by a commercial ELISA kit. Results shown are expressed as picograms per milliliter BAFF protein for eight different donors. Statistical analysis was completed using Student t test. (C) MDMs were mock-transfected or transfected with siCTRL or siRNA targeting CD4 or SAMHD1. The efficacy of knockdown was determined as described in (A). Results are...
induction of IFN in myeloid cells via a physical interaction with TANK-binding kinase 1 (TBK1), which will in turn prevent autophosphorylation of TBK1 (82). The main subset of PBMCs that is responsible for the production of type-I IFN in response to HIV-1 in humans was instead found to be pDCs (83), and we recently showed that HIV-1–triggered release of type-I IFN by pDCs increases BAFF production by monocytes (30). A similar cross-talk between pDCs and mDCs–macrophages at inflammation sites could also upregulate BAFF production in the latter.

A previous study reported that HIV-1 can activate the inflammasome in monocytes and macrophages via endosomal TLRs without the induction of type-I IFN (58). We postulated that HIV-1 could drive BAFF secretion in macrophages by exploiting this pathway. However, our results using siRNA silencing of endosomal TLRs suggest that HIV-1–mediated BAFF upregulation is independent of TLR stimulation. However, we cannot totally exclude the possibility that the virus-directed BAFF induction could be mediated by both TLR7 and TLR8. BAFF might be upregulated in TLR8-silenced MDMs by TLR7 sensing of HIV-1 RNA and vice versa. Nevertheless, one study reported that HIV-1 RNA-mediated release of TNF by human alveolar macrophages is dependent of TLR8 signaling but silencing TLR7 had no effect on this response (57). Furthermore, TLR8 is the primary inflammasome sensor of HIV-1 in monocytes (58). Therefore, these findings suggest that TLR8 could play a more important role than TLR7 in sensing HIV-1 RNA in human macrophages. Nevertheless, our results obtained in TLR-silenced MDMs suggest that TLR8 is not involved in HIV-1–mediated BAFF upregulation in MDMs. Alternatively, HIV-1 RNA could be detected by viral sensors present in the cytosol of infected cells such as RIG-I–like receptors. The engagement of these sensors is known also to lead to inflammasome activation and production of proinflammatory cytokines (84). The possible contribution of RIG-I–like receptor–sensing of HIV-1, or other pattern recognition receptors, in the virus-dependent BAFF upregulation in MDMs remains to be investigated. On the other hand, macrophages are susceptible to productive HIV-1 infection, but restrict or limit the levels of virus replication to protect their function through different cellular processes; however, these mechanisms can also allow these infected cells to evade detection and escape an effective antiviral immune response (85). In this study, we show that reducing the expression levels of the host restriction factor SAMHD1 in MDMs increases viral replication and consequently HIV-1–mediated BAFF production. Interestingly, a recent study reported that SAMHD1 prevents autoimmunity by maintaining genome expression as fold change relative to mock-transfected cells for three to four different donors. Statistical analysis was completed using one-way ANOVA, followed by Dunnett multiple comparisons test. (D) CD4-silenced, SAMHD1-silenced, or siCTRL-treated cells were infected with NL4-3Balenv (Bal). Virus replication was estimated in MDMs by quantifying the p24 content in cell-free supernatants at the indicated time points. Data shown represent the mean ± SD of duplicates for two different donors. (B) Cell-free supernatants were harvested 12 d postinfection, and BAFF protein levels were evaluated with a commercial ELISA kit. Results shown are expressed as picograms per milliliter of BAFF protein for two different donors. Error bars indicate the SD. Statistical analysis was made using one-way ANOVA, followed by Dunnett multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001.
stability (86). SAMHD1 is required for genome integrity by maintaining balanced dNTP pools. dNTP imbalances caused by SAMHD1 deficiency cause DNA damage, leading to intrinsic activation of IFN signaling. Indeed, mutations of SAMHD1 cause Aicardi–Goutiéres syndrome, an infancy-onset inflammatory encephalopathy that phenotypically mimics congenital viral infection and shows overlap with systemic lupus erythematosus (87). Both disorders are characterized by the formation of anti-nuclear Abs and constitutive activation of type-I IFN (88, 89). Furthermore, BAFF plasma levels are increased in systemic lupus erythematosus (27) and could be increased in Aicardi–Goutiéres syndrome. The possible interplay between SAMHD1, type-I IFN, and BAFF in the context of HIV-1 infection and other autoimmune and inflammatory diseases need further investigation.

The HIV-1 accessory protein Nef was another possible candidate that could have a role in the noticed virus-dependent BAFF secretion in MDMs because of its known capacity to induce production of several soluble factors in monocyte–macrophages (65), including BAFF in mDCs (28). However, our results using a Nef-deleted variant suggest that such virus is still able to upregulate BAFF production in MDMs. Although Nef might be dispensable for BAFF secretion in this cell type, this regulatory protein could affect B cell responses during HIV-1 infection through different divergent mechanisms. On the one hand, Nef protein shuttled from macrophages to B cells via intracellular conduits could inhibit Ig class switching from IgM to IgG and IgA, thus limiting the repertoire of Abs produced by B cells (90). On the other hand, ferritin (91) and proinflammatory cytokines (65) induced by Nef in macrophages could promote polyclonal B cell activation.

Finally, we investigated how the macrophage phenotype status could influence HIV-1–mediated BAFF production. In such studies, monocytes were subjected to an initial exposure to M-CSF and LPS and IL-4 to generate M1 and M2a MDMs, respectively, to derive macrophages before an additional treatment with IFN-γ/IL-4 to generate M1 and M2a MDMs, respectively. To derive macrophages before an additional treatment with IFN-γ/IL-4 to generate M1 and M2a MDMs, respectively.

FIGURE 7. HIV-1–mediated BAFF production is influenced by the cell phenotype status. MDMs were first differentiated for 5–6 d and next left untreated (M0 phenotype), treated with IFN-γ (20 ng/ml) and LPS (100 ng/ml) (M1 phenotype), or treated with IL-4 (20 ng/ml) (M2a phenotype). (A) Cell-free supernatants were harvested 3 d poststimulation, and BAFF protein levels were evaluated with a commercial ELISA kit. Results shown are expressed as picograms per milliliter of BAFF protein for seven different donors. Statistical analysis was completed using one-way ANOVA, followed by Tukey multiple comparisons test. (B) M0, M1, and M2a MDMs were infected with NL4-3Balenv (Bal). Virus replication was estimated in MDMs by quantifying the p24 content in cell-free supernatants at the indicated time points. Data shown represent the mean ± SD of duplicates for a representative donor. (C) BAFF secretion levels were evaluated 12 d postinfection as described in (A). Results shown are expressed as picograms of milliliter of BAFF protein for five different donors. Statistical analysis was completed using Student t test. *p < 0.05, **p < 0.01, ***p < 0.001.

In summary, our study provides new insights for the increased BAFF levels observed during HIV-1 infection. Although the exact mechanism responsible for the HIV-1–dependent BAFF secretion by MDMs is still unidentified, we provide evidence that this phenomenon relies on productive virus infection, which is itself influenced by the cell phenotype status and is independent of TLR and type-I IFN signal transduction and the action of Nef.

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Disclosures
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