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The Engagement of Activating FcγRs Inhibits Primate Lentivirus Replication in Human Macrophages

Annie David,∗ Asier Sáez-Cirión,* Pierre Versmisse,* Odile Malbec,†‡ Bruno Iannascoli,†‡ Florence Herschke,* Marianne Lucas,§ Françoise Barré-Sinoussi,* Jean-François Mouscadet,¶ Marc Daëron,†‡ and Gianfranco Pancino2*∗

We previously reported that the stimulation of monocyte-derived macrophages (MDM) by plate-bound i.v. IgG inhibits HIV-1 replication. In this study, we show that IgG immune complexes also suppress HIV-1 replication in MDMs and that activating receptors for the Fc portion of IgG–FcγRI, FcγRIIA, and FcγRIIIA—arere responsible for the inhibition. MDM stimulation through FcγRs induces activation signals and the secretion of HIV-1 modulatory cytokines, such as M-CSF, TNF-α, and macrophage-derived chemokine. However, none of these cytokines contribute to HIV-1 suppression. HIV-1 entry and postintegration steps of viral replication are not affected, whereas reduced levels of reverse transcription products and of integrated proviruses, as determined by real-time PCR analysis, account for the suppression of HIV-1 gene expression in FcγR-activated MDMs. We found that FcγR-dependent activation of MDMs also inhibits the replication of HIV-2, SIVmac, and SIVagm, suggesting a common control mechanism for primate immunodeficiency lentiviruses in activated macrophages. The Journal of Immunology, 2006, 177: 6291–6300.

Macrophages play a major role in mounting innate and adaptive immune responses to pathogens. Macrophages react to HIV-1 infection by secreting cytokines, chemokines, and other molecules having antiviral activity or can directly control HIV-1 replication (15). However, HIV-1 infection may affect essential macrophage functions, such as Ag presentation, intracellular killing, and phagocytosis (16). Therefore, the regulation of HIV-1 and related lentivirus replication in monocytes and macrophages might affect the host susceptibility to infection and could help to control viral dissemination and pathogenesis in infected individuals. In a SCID mouse model, virus spread and pathology was abolished by suppressing macrophage infection with antineuron growth factor Abs (17).

We previously showed that the incubation of macrophages with i.v. Ig (IVIg) bound on culture plates potently inhibits HIV-1 replication independently of viral tropism (18). Inhibition was not observed when macrophages were incubated with IVIg-F(ab′)2, suggesting that it was mediated by receptor(s) for the Fc portion of IgG (FcγR) (18). FcγRs are a group of integral membrane proteins that bind to the Fc portion of IgG (19), which can either activate or inhibit cell activation when engaged by IgG immune complexes. Activating FcγRs have the high-affinity receptor FcγRI (CD64), which can bind monomeric IgG, and the low-affinity receptors FcγRIIA/C (CD32) and FcγRIIIA (CD16), which do not bind monomeric IgG but bind IgG aggregates and Ag-Ab immune complexes (ICs) with a high avidity. Activating FcγRs possess ITAMs that become phosphorylated upon FcγR clustering. ITAM phosphorylation promotes the recruitment of cytosolic protein tyrosine kinases. These kinases phosphorylate other proteins involved in signaling pathways, leading to the activation of PI3K and MAPKs (19). Inhibitory FcγRs consist of FcγRIIB, which contains an

Unlike other retroviruses, lentiviruses can integrate their DNA into the genome of nondividing cells and can therefore replicate in monocytes and macrophages. HIV and SIV infection of monocytes and differentiated tissue-resident macrophages may play a major role in viral transmission, dissemination, and persistence (1–4). The capacity of monocytes and macrophages to migrate in tissues makes them potential conveyors of HIV and SIV infections. Monocytes are thought to carry the virus to the CNS, and the expansion of subsets of activated monocytes has been associated with neurological diseases in AIDS (5, 6). Virions generated in infected macrophages are more efficient in establishing lymphocyte infection than cell-free virions (7). Macrophages may also favor cell-to-cell transmission to CD4+ T cells by producing chemotactic cytokines and by interacting with cells during Ag presentation (8). In addition, HIV-1-infected macrophages may also induce the apoptosis of uninfected bystander T cells and neuronal cells (9). Finally, infected monocytes and macrophages may act as viral reservoirs for HIV and SIV and be the main source of virus production during the late stages of disease in pathogenic infections when the numbers of CD4+ T cells are substantially reduced (10–14).

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Abbreviations used in this paper: IVIg, i.v. Ig; IC, immune complex; nPCR, real-time PCR; MDC, macrophage-derived chemokine; PLC, phospholipase C; m.o.i., multiplicity of infection; WN, West Nile; RT, reverse transcription; p.i., postinfection.
ITIM. This motif enables FcγRIIB to negatively regulate cell activation triggered by ITAM-containing receptors when coengaged with them.

In this study, we aimed at identifying which FcγR(s) is(are) involved in viral inhibition. We quantitatively analyzed FcγR-mediated inhibition of HIV-1 replication using real-time PCR (rt-PCR). We also determined whether FcγR-mediated inhibition was limited to HIV-1 or was a general antiviral mechanism by studying the effect of FcγR cross-linking on the replication of other primate lentiviruses in human macrophages.

Materials and Methods
Monocyte-derived macrophages (MDM)
Human monocytes were isolated from buffy coats of healthy seronegative donors (Centre de Transfusion Sanguine Ile-de-France, Rungis and Hôpital de la Pitié-Salpêtrière, Paris, France) using lymphocyte separation medium (PA Laboratories) density gradient centrifugation and plastic adherence as previously described (18). Monocytes were then differentiated into macrophages by 7–11 day culture in MDM medium (RPMI 1640 medium supplemented with 200 mM-glutamine, 100 U of penicillin, 100 µg/mL streptomycin, 10 mM HEPES, 10 mM sodium pyruvate, 50 µM 2-ME, 1% MEM vitamins, and 1% nonessential amino acids) supplemented with 15% limited to HIV-1 or was a general antiretroviral mechanism by DH5α (Escherichia coli) as previously described (18). MDM were then harvested, washed, and resuspended in MDM medium containing 10% heat-inactivated FCS for experiments. The purity of CD14+ macrophages was usually >95% as assessed by immunofluorescent staining and flow cytometry analysis. FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) were all expressed on the MDM surface but the proportion of cells expressing each receptor varied with different donors and MDM preparations.

LPS content in the medium and all the reagents used for culture and stimulation of MDM were below the limit of detection of the QCL1000 Luminex amebocyte lysate test (BioWhittaker).

Antibodies
mAbs against FcγRs (CD64, clone 32.2; CD52, clones IV.3 and AT.10; CD16, clone 3G8) were purified from hybridoma supernatants. F(ab')2 from mAbs was generated by pepsin digestion (ImmunoPure F(ab')2 Preparation kit, Pierce). Anti-CD64 F(ab')2, 10.1 was from Ancell. Isotype-matched uncoupled or FITC- or PE-coupled irrelevant control mAbs or F(ab')2 were from Sigma-Aldrich. All F(ab')2 preparations used for stimulating MDM were passed through a polyethylene (P) column to elute contamination and were then verified as LPS-free by the Luminex amebocyte lysate test.

Human FcγRIIA-specific and FcγRIIB-specific polyclonal Abs were generated in rabbits immunized with GST fusion proteins containing the intracytoplasmic domain of either human FcγRIIA (ICIIA) or FcγRIIB (ICIIIB, Briefly). cDNAs encoding the intracytoplasmic domains of human FcγRIIA and FcγRIIB were amplified by PCR using the following primers:

FcγRIIA, forward: CGCGGATCCCGAATTCCCTAAATACGGTTCTGGTCATC.
Reverse: CGGAATTCCGTTAGTTATTACTGTTGACATGGTC.

FcγRIIB, forward: GCGGATCCGCGAATCCCACTAATCCTGATGAG.
Reverse: CGGAATTCCCTAAATACGGTTCTGGTCATC.

GAGG, reverse: CGGAATTCCGTTAGTTATTACTGTTGACATGGTC.

GAAG, reverse: CGGAATTCCGTTAGTTATTACTGTTGACATGGTC;

The above-described FcγRIIA- and FcγRIIB-specific primers were used to amplify the human transcripts in RNA preparations from MDM stimulation kit (Pierce). MDM were plated on 96-well plates and incubated with 100 ng/ml rabbit anti-DNP Abs in PBS-BSA (IC-beads) or with PBS-BSA alone (Ag-beads). MDMs were stimulated with the appropriate specific F(ab')2 per 10⁶ MDM and one-five hundredth of the volume of the reaction mixture were used for PCR amplification (30 cycles), using Taq (In vitroNig Life Technologies) in a GeneAmp PCR9700 (Applied Biosystems). The PCR products were analyzed by gel electrophoresis on 2% agarose gel.

Anti-FcγR-α rabbit IgG (gift from J.-M. Cavaillon, Institut Pasteur, Paris, France), anti-M-CSF goat IgG, or anti-macrophage-derived chemo-kine (MDC) chicken IgYs (both from R&D Systems) were used for TNF-α, M-CSF, or MDC neutralization, respectively. Isotypic controls were rabbit, goat, or chicken irrelevant Abs. Commercial Abs were detected by passage through polynynx B columns before use. TNF-α, M-CSF, and MDC levels in culture supernatants were measured by using quantikine ELISAs (R&D Systems).

The following Abs were also used: unconjugated mouse anti-phospho tyrosine mAb 4G10; purified from hybridoma supernatant on protein G-Sepharose; rabbit anti-phospho-phospholipase C (PLC)-γ1(tyrosine 783) Abs (Santa Cruz Biotecnology); rabbit anti-ERK1/2 and rabbit anti-phospho-ERK1/2 (Thr202/Tyr222; Ab (Cell Signaling); fluorochrome-conjugated CD11b-PE (clone Bear1) and CD4-PE (clone 13B8.2) (both obtained from Beckman Coulter); CD14-FTTC (clone Leu M3) and CD3-FTIC (clone Leu3) (both obtained from BD Biosciences).

Flow cytometry analysis
Cells were stained either with FITC-conjugated or PE-conjugated mAbs or with unconjugated mAbs or F(ab')2 followed by secondary FITC-goat anti-mouse IgG (Fab')2, or FITC-goat anti-mouse Fab (Fab')2 (Immunotech) and analyzed using a FACScan flow cytometer (Beckman Coulter).

Immunoblotting
For tyrosine phosphorylation analysis, cells were lysed by three cycles of incubation for 1 min in liquid nitrogen followed by 1 min at 37°C in lysis buffer at pH 8.0 (50 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, 1 mM NaVO₃, 5 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 1 mM PMSF). For FcγRII expression analysis, cells were lysed by boiling 5 min in 10 mM Tris (pH 7.4), 1% SDS. Proteins were quantified using a Bio-Rad protein assay. A total of 40 µg of proteins for tyrosine phosphorylation analysis or 10 µg of proteins for FcγRII analysis was boiled in sample buffer, fractionated by SDS-PAGE and then transferred onto Immobilon-P membranes (Millipore). The membranes were saturated with 5% BSA (Sigma-Aldrich) or 5% skimmed milk (Régilait) diluted in Western buffer (150 mM NaCl, 10 mM Tris, and 0.5% Tween 20 (Merck) (pH 7.4) and incubated with the indicated Abs and then incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse Ig Abs. Labeled Abs were detected using an ECL kit (Amersham Biosciences). Blots for FcγRII analysis were first probed with the anti-FcγRII Ab, then stripped with stripping buffer (reblo1; Chemicon International) as indicated by the manufacturer, and reprobed with the anti-FcγRIIA Ab.

MDM stimulation
MDM were stimulated using three different methods: 1) immobilized IVlg stimulation was conducted with human IgG for therapeutic use (IVlg) (Endobuline; Baxter) (0.1 mg/ml in PBS) as previously described (18). 2) Stimulation with preformed ICs was as follows: DNP groups were conjugated to LPS-free BSA (Sigma-Aldrich) using dinitrobenzene sulphone (Eastman Kodak) in alkaline medium and then dialyzed against PBS. Culture plates were coated with 0.1 mg/ml DNP-BSA Ag by incubation for 2 h at 37°C, washed with PBS, saturated by incubation with 1 mg/ml BSA in PBS for 30 min at 37°C and then incubated with 30 µg/ml rabbit anti-DNP Abs (Sigma-Aldrich) for 1 h at 37°C to form ICs. MDMs were stimulated by plating on IC-coated CFA plates. 3) Each FcγR was separately cross-linked on an MDM surface by incubating MDMs with the appropriate specific F(ab')2 (5 µg Fab')2 per 10⁶ MDM) for 30 min at 4°C. The MDMs were then washed with PBS and seeded on plates previously coated with 0.2 mg/ml goat anti-mouse Fab (FcγR) (Sigma-Aldrich) and saturated with 1 mg/ml MDMs were incubated in parallel with irrelevant mouse Fab(F(ab')2 as controls.

Cell viability was not affected in IgG or IC-stimulated MDM cultures, as evaluated by a WST-1-based colorimetric assay (data not shown).
Viruses and MDM infection

HIV-1 infections. The following viral strains were used for productive infections: HIV-1HXB, HIV-2_SIVm, SIVmac251, SIVagmGril. Strains were propagated in PHA-activated human PBMCs (except SIVagm, propagated on SupT1 cells) and the culture supernatants were collected at times of peak p24 (HIV-1) or p27 (HIV-2, SIV) production. p24 and p27 were measured with commercial ELISA kits (Beckman Coulter). Viral stocks were titrated on PHA-activated human PBMCs except SIVagm, which was titrated on SupT1 cells. Multiplicities of infection (m.o.i.) used in this study were between 10^-2 and 2 x 10^-2.

To assess covirus infections, HIV-1 particles containing the luc reporter gene and pseuotyped with the VSV-G envelope protein (HIV-1VSV-G), which allows HIV receptor-independent entry into cells were used. HIV-1VSV-G virions were produced by transient cotransfecting (SuperFect; Qiagen) 293T cells with the proviral pNL-Luc-E-R' + (20) vector and the VSV-G expression vector pCMV-G, as previously described (18). Supernatants were harvested 72 h after transfection, and p24gag levels were measured using a commercial ELISA kit (Beckman Coulter) with MDMs. Between 3 x 10^-3 and 3 x 10^-2 m.o.i. were used for MDM infection. Mock infections with equivalent amounts of p24 from supernatants from 293T cells transfected with pNL-Luc-E-R’ only were conducted in parallel as controls.

MDMs (0.8 x 10^5 – 1 x 10^5 cells/well in 96-well plates or 1 x 10^5 cells/well in 24-well plates) were infected either with viral strains or with pseuotyped particles by incubating cells with viral inoculum 1 h at 37°C, or by a spinoculation protocol (1 h centrifugation at room temperature at 1200 x g followed by 1 h incubation at 37°C), to increase the efficiency of infection (21). MDMs were then washed with PBS and cultured in MDM medium.

In the experiments for detecting HIV DNA by PCR, HIV-1VSV-G preparations were previously treated with DNase I (Roche Diagnostics). In cytokine/chemokine neutralization experiments, IVIg-stimulated or unstimulated MDMs were infected in triplicate with HIV-1HXB, and then cultured in 96-well plates in the presence of neutralizing concentrations of the each specific Ab (anti-TNF-α, 1/150 dilution; anti-M-CSF, 7.5 ng/ml; anti-MDC, 10 ng/ml) or equal concentrations of the appropriate control Ab. Half-culture supernatant was changed with fresh medium containing the appropriate concentrations of each Ab each 2 days.

West Nile (WN) virus infection. Production of WN virus strain IS-98-ST1 (GenBank accession no. AF481864) from mosquito Aedes pseudoscutellaris AP61 cell monolayers and virus titration on AP61 cells by focus formation. As a control for inhibition of WN virus replication, MDM were exposed to 10 IU/ml human recombinant IFN-α 2D (BioSource International), during and after infection.

Measure of luciferase activity in cell lysates

At various times after infection with pseuotyped HIV-1 virions, each well of MDMs was lysed with 100 μl of luciferase cell cycle lysis reagent (Promega). The luciferase activity was quantified in 10 μl of each lysate using the Promega Luciferase reporter 1000 Assay System and an LUMAT II and 1000 LUMAT II luminometer (Berthold Technologies).

rtPCR quantification of HIV-1 cDNA forms

At different times after infection, MDMs were washed in PBS and total DNA was extracted using the DEasy Tissue kit (Qiagen). The HIV-1 DNA forms R-U5, U5-Gag, and 2-LTR were quantified using rtPCR with an ABI PRISM 7000 instrument (Applied Biosystems Appler). For all the rtPCR, we used 100 ng of template DNA per reaction, corresponding to ~2 x 10^5 MDMs. DNA loading was controlled by concurrently amplifying the albumin gene by rtPCR and quantifying with reference to a control human gene (HBB, propried by Ron Neef; Roche). The real-time PCR master mixture contained 1 x TaqMan Universal PCR master mix, 300 nM of each primer (except R-U5 primers, 200 nM) and 200 nM of the appropriate fluorogenic probe, in a final volume of 30 μl. PCR cycle conditions were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Copy numbers of R-U5 and U5-Gag were determined with reference to a standard curve generated by concurrent amplification of serial dilutions of 5 x 10^2 cells containing one integrated copy of HIV-1 per cell (23). The copy number of 2-LTR was determined with reference to standard curves generated by serial dilutions of CEM cells infected with HIV-1NL4-3. The number of 2-LTR copies/pcr CEM cell was previously quantified against a standard curve generated by dilution of cloned DNA with matching sequences (pSSL-IIIb, gift of A. Brussel, Institut Pasteur, Paris, France) (24). R-U5 primers are described elsewhere (25), the probe was (FAM)-AGACGGGCACACACTA-(MGB). Primers and probes for U5-Gag (26), 2-LTR (27), and albumin (28) have been reported.

Integrated HIV-1 DNA was quantified by real time Alu-Gag nested PCR using primers and probes supplied by N. Yamamoto (Tokyo Medical and Dental University, Tokyo, Japan). The first round of amplification was conducted on a Gene Amp PCR system 9700 (Applied Biosystems). Integrated HIV-1 sequences were amplified with the expand high fidelity kit (Roche) using an Alu prim (NY1F) and a Gag prim (Fig. 1A). Replication-competent viruses, including HIV-1 Bal, were also inhibited by immobilized ICs (data not shown).

When equivalent concentrations of anti-DNP F(ab')2 were used to form DNP-anti-DNP ICs, no inhibition of HIV-1 replication was observed (Fig. 1D), indicating that the Fc portion of IgG is required for inhibition. Because complement is not present in incubation medium, this result implies that HIV-1 inhibition induced by ICs is mediated by FcγR.
Inhibition of HIV-1 replication is mediated by activating FcγRs

Human macrophages express several FcγRs. To identify which FcγR(s) account(s) for IC-induced inhibition of HIV-1 replication, we investigated first the effect of engaging separately the three activating receptors known to be expressed on macrophages, and for which specific Abs are available, i.e., FcγRII, FcγRIIA, and FcγRIIIA. MDMs were incubated with F(ab′)2 of mAbs specific for each receptor or with irrelevant mouse F(ab′)2, and seeded onto wells coated with F(ab′)2 of goat anti-mouse Fab Abs. MDMs were then infected with HIV-1 BaL, and viral replication was evaluated by measuring p24 production. The incubation of MDMs with each of the FcγR-specific F(ab′)2, but not with control F(ab′)2, decreased p24 production, compared with unstimulated MDMs. Anti-FcγRIIIA IV.3 F(ab′)2 induced the strongest inhibition (Fig. 2). These results indicated that cross-linking activating receptors can induce an inhibition of HIV-1 replication. The level of viral suppression induced by F(ab′)2 of Abs against activating FcγRs was, however, generally lower than that induced by either IVIg or ICs (Fig. 2 and data not shown).

Human monocytes having been reported to express the inhibitory FcγRIIB, we investigated next the expression and the potential role of this receptor in HIV-1 inhibition. Because there are no available Abs that recognize specifically the extracellular domain of FcγRIIB, we generated FcγRIIB-specific and, as controls, FcγRIIA-specific polyclonal Abs by immunizing rabbits with peptides corresponding to the intracytoplasmic domain of each receptor. By Western blotting, anti-FcγRIIA Abs recognized proteins of the expected MW in lysates of cells stably transfected with cDNA encoding FcγRIIA but not in lysates of cells stably transfected with cDNA encoding FcγRIIB, but not in lysates of cells transfected with cDNA encoding FcγRIIA (Fig. 3A, left). We used these Abs to evaluate the expression of FcγRIIA and FcγRIIIB in monocytes and macrophages. As a control, we also examined B lymphocytes which express FcγRIIB. As expected, FcγRIIB was readily detected in B lymphocytes by the anti-FcγRIIB Ab (Fig. 3A, right). In contrast, FcγRIIB was undetectable in monocyte or macrophage lysates at concentrations that showed very strong FcγRIIA signals (Fig. 3A, right). The same

FIGURE 1. IgG-immune complexes inhibit HIV-1 replication in MDMs. A, MDMs infected with HIV-1VSV-G were plated on wells treated with BSA only (unstimulated, US) or coated with 30 μg/ml anti-DNP Abs (α-DNP) or with decreasing concentrations of DNP-BSA (10, 1, 0.1 μg/well) followed or not by 30 μg/ml anti-DNP Abs; B, MDMs were plated and incubated with medium (US) or with 3-μm polystyrene beads coated with DNP-BSA-anti DNP ICs or with DNP-BSA only (Ag) at a bead:cell ratio of 10:1 and 30:1 (10, 30) and infected with HIV-1VSV-G. C, HIV-1VSV-G infected MDMs from three different donors were stimulated with either immobilized IVIg or ICs. Results are expressed as percentage of inhibition of luciferase activity (means and SD of three independent wells) found in unstimulated MDMs plated on BSA or DNP-BSA. D, HIV-1VSV-G infected MDMs were plated on wells coated with 10 μg/well DNP-BSA or with complexes formed by DNP-BSA with 30 μg/ml of either anti-DNP IgG or anti-DNP Fab′, and infected with HIV-1VSV-G. In all the experiments, luciferase activity was measured in cell lysates 72 h p.i. Results are expressed as means and SD of three independent wells.
results were found with MDMs from four different donors. Thus, FcγRIIB was not detectably expressed in MDMs under our experimental conditions. When analyzed by RT-PCR with FcγRIIB- or FcγRIIA-specific primers, FcγRIIB transcripts were detected in MDM RNA, but in lower amounts than FcγRIIA transcripts (Fig. 3B). Indeed, when analyzing three 10-fold dilutions of MDM cDNA, FcγRIIB transcripts were clearly detected in the first dilution and barely detected in the second dilution, whereas FcγRIIA transcripts were detected in all three dilutions (Fig. 3B).

Altogether, Western blotting and RT-PCR results indicate that FcγRIIA is the predominant FcγRII in MDMs. To assess whether, although undetected by Western blotting, FcγRIIB could modulate FcγRIIA-mediated inhibition of HIV-1 replication, we compared the effect of IV.3 F(ab′)2, which recognize the extracellular domain of FcγRIIA but not that of FcγRIIB, and the effect of AT10 F(ab′)2, which recognize the extracellular domains of FcγRIIA, FcγRIIB, and FcγRIC. Similar percentages of positive cells and similar mean fluorescence intensities were found when MDM were stained with either AT10 or IV.3 F(ab′)2 (Fig. 3C). AT10 and IV.3 F(ab′)2 induced comparable HIV-1 inhibition in infected MDM (Fig. 3D).

Both expression and functional analyses altogether suggest that activating, rather than inhibitory FcγRs, account for the IC-induced inhibition of HIV-1 replication in MDM.
MDM stimulation through FcγR induces activation signals and cytokine secretion

We then investigated the signaling events induced by FcγRs in MDMs and their consequences on infection by HIV-1\textsubscript{VSV-G}. As expected, tyrosine phosphorylation of a number of intracellular proteins, including PLC-γ and ERK1/2, increased in IVIg-stimulated MDMs (Fig. 4A). PLC-γ phosphorylation was transient whereas ERK1/2 phosphorylation was sustained in noninfected MDMs (Fig. 4, A and C). HIV-1\textsubscript{VSV-G} infection did not detectably modify the phosphorylation patterns or kinetics (Fig. 4B), even when examined over an extended period of time (Fig. 4C).

To assess the effect of blocking activating FcγR-mediated signaling on HIV-1 infection in IC-stimulated MDMs, we used piceatannol, reported as an inhibitor of the tyrosine kinase Syk that is recruited by phosphorylated ITAMs in FcγR aggregates (30). Piceatannol concentrations of 20–40 μM partially but significantly (p = 0.001) removed viral suppression in IC-stimulated MDM infected with HIV-1\textsubscript{VSV-G} (data not shown). However, piceatannol treatment also caused a dose-dependent inhibition of HIV-1 replication in unstimulated MDM, which was almost complete at concentrations higher than 50 μM (data not shown), possibly because of the inhibition of phosphorylation pathways involved in HIV-1 replication. Indeed piceatannol can inhibit not only Syk but also numerous tyrosine and serine-threonine kinases (31–33).

MDM stimulation by either IVIg or ICs induced chemokine and cytokine secretion, including M-CSF, MDC, and TNF-α (Ref. 18 and data not shown). Cross-linking of activating FcγR with anti-FcγR F(ab')\textsubscript{2} on MDMs also induced the secretion of M-CSF (Fig. 4D) and other cytokines (data not shown). Whether using IVIg or ICs or anti-FcγR F(ab')\textsubscript{2}, in all cases the amounts of cytokines secreted by FcγR-activated MDMs, and particularly M-CSF, correlated with the magnitude of inhibition of HIV-1 replication (Figs. 2 and 4A, Ref. 18, and data not shown). However, we previously reported that HIV-1 suppression could not be induced by exposing MDMs to cytokine-containing supernatants from IVIg-stimulated MDMs and that MDC neutralization in IVIg-stimulated MDM cultures did not restore HIV-1 replication (18). These results indicate that neither MDC nor other secreted factors are responsible for FcγR-mediated HIV-1 inhibition. Supporting this conclusion, anti-M-CSF or anti-TNF-α neutralizing Abs reduced, rather than enhanced, HIV-1 infection in unstimulated MDMs, and increased IVIg-induced inhibition (data not shown).

**HIV-1 cDNA and integrated proviruses are decreased in FcγR-activated macrophages**

To identify the steps in the HIV-1 replicative cycle that are inhibited in FcγR-activated MDMs, we measured the intermediate products of HIV-1 replication using rtPCR in single-round infections with HIV-1\textsubscript{VSV-G} from entry to integration. We used primers and probes amplifying early (R-U5) and late (U5-Gag) products of reverse transcription (RT), 2-LTR circles (2-LTR), and integrated proviruses (Alu-LTR). We found similar HIV-1 replication inhibition profiles in MDMs activated either by IVIg or by ICs in MDMs from three different donors. A representative experiment is shown in Fig. 5. Luciferase activity in cell lysates was much lower in IC-activated MDMs (90% inhibition at 96 h) than in unstimulated MDMs (Fig. 5A). Similar levels of R-U5 products were found by rtPCR in unstimulated and in IC-stimulated MDMs at 4 and 24 h postinfection (p.i.) (Fig. 5B). At these early times, R-U5

![FIGURE 5. FcγR-mediated activation causes a reduction of HIV-1 retrotranscripts and of integrated proviruses in MDMs. MDMs were infected with DNase-treated HIV-1\textsubscript{VSV-G} and plated on DNP-BSA-coated plates (unstimulated (US)) or stimulated with ICs (S). Luciferase activity was monitored at 24, 48, and 96 h p.i. in MDM lysates (A). Early and late retrotranscription products and 2-LTR circles were analyzed by rtPCR using the appropriate primers (R-U5, U5-Gag, 2-LTR) and probes (B–D). Integrated copies were evaluated by Alu-LTR-nested rtPCR. Values are means of duplicate measures at the indicated times p.i. (E). Ratios between 2-LTR circles and integrated nuclear forms of HIV-1 at 48 and 96 h p.i. as measured by rtPCR in unstimulated or IC-activated MDMs (F). Results from a representative experiment of three performed with MDM from different donors are shown.](http://www.jimmunol.org/)

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Early postintegration steps are not inhibited in FcγR-activated macrophages

We then determined whether FcγR-mediated activation of macrophages could affect HIV-1 postintegration steps, including transcription. MDMs were infected with HIV-1\textsubscript{VSV-G} and cells were kept in suspension for 72 or 96 h before they were plated onto IV Ig-coated or uncoated wells. Under these conditions, MDMs activation was triggered after most of the viral DNA should be integrated (Fig. 5 and results not shown). Forty-eight hours after activation, similar levels of luciferase activity were found in lysates from unstimulated and from IV Ig-stimulated MDMs (Fig. 6). By contrast, the same MDM preparation activated at the same time as infection showed a luciferase activity 82% lower in IV Ig-stimulated MDMs than in unstimulated cells (Fig. 6). These results indicate that HIV-1 transcription and protein synthesis are not affected by FcγR-mediated activation in macrophages.

FcγR-mediated activation of macrophages inhibits the replication of primate lentiviruses

All lentiviruses can complete integration in nondividing cells and can thus replicate in macrophages. Therefore, we wondered whether other primate lentiviruses would also be susceptible to FcγR-mediated inhibition in human macrophages. MDMs were infected with HIV-2SBL, SIVmac, or SIVagm, plated onto wells coated with ICs, and viral p27 levels were measured in culture supernatants every 3 days for 23 days (Fig. 7, A–C). All three lentiviruses replicated efficiently in control MDMs, with p27 production becoming >1 \(\mu\text{g/ml}\). p27 levels were markedly reduced in the cultures of MDMs infected with each of the three viruses and stimulated with ICs (Fig. 7, A–C). FcγR-mediated inhibition of viral infection is therefore not limited to HIV-1 but affects other primate lentiviruses.

FcγR-mediated inhibition affects HIV-1 reverse transcription and integration that are peculiar features of retroviral replication. Therefore, we investigated whether FcγR-mediated activation of MDMs could affect other viruses. For this experiment, we used the WN virus, an unrelated macrophagotropic Flaviviridae virus. WN virus replication in unstimulated MDMs was compared with that in
FcγR-stimulated MDMs (Fig. 7D). As a control for inhibition, MDMs were treated with IFN-α (34–36). MDMs were infected with WN virus IS-98-ST1 strain at 1 m.o.i. No cytopathic effect was observed in any condition (not shown). After 72 h, titers of virus produced in cell culture supernatants were determined. No difference was observed in the virus titer between unstimulated and stimulated MDMs (Fig. 7D). However, as expected, virus titer was strongly decreased in IFN-α-treated MDMs. These results suggest therefore that FcγR-mediated inhibition selectively affects lentiviruses.

Discussion

In the present study, we show 1) that the inhibition of HIV-1 replication that we previously reported in macrophages plated onto immobilized IVIg (18), can also be induced by IgG immune complexes; 2) that activating FcγRs account for HIV-1 inhibition, but that FcγR-induced cytokines are not responsible for HIV-1 inhibition; 3) that inhibition affects neither viral entry nor postintegration steps, but causes a reduction of viral cDNA and blocks viral integration; 4) that FcγR-mediated suppression is not only limited to HIV-1 but also affects other primate lentiviruses.

We showed that the three known ITAM-bearing FcγRs can mediate HIV-1 replication inhibition. Although the levels of HIV-1 inhibition varied depending on the MDM preparation, it was consistently higher upon FcγRIIA cross-linking than upon FcγRI or FcγRIIA cross-linking (Fig. 2). Whether this difference is due to a higher expression of FcγRIIA on MDMs, to specific properties of this receptor, or to a higher affinity of the anti-FcγRIIA mAb used is not known. Inhibition of HIV-1 correlated with MDM activation, as judged by M-CSF and MDC secretion. Accordingly, the partial recovery of HIV-1 replication by piceatannol in IC-stimulated MDM may suggest that blocking signaling pathways downstream activating FcγRs can remove HIV-1 inhibition. However, the significance of this result was obscured by a direct inhibitory effect of piceatannol on HIV-1 replication. Both inhibition of HIV-1 and MDM activation were generally lower when induced by anti-FcγR (F(ab')2) than when induced by IVIg or ICs. This is consistent with previous studies showing that FcγR cross-linking with immobilized IgG induces TNF secretion by human monocytes, whereas cross-linking with anti-FcγR Abs does not (37). It also indicates that ICs or IVIg are more efficient at aggregating FcγRs than anti-FcγR Abs.

We found no evidence that FcγRIIB contributes to IC-induced HIV-1 inhibition in MDMs. On possible reason is the low expression of FcγRIIB in these cells. We did not detect FcγRIIB proteins by Western blotting, but we found relatively low levels of FcγRIIB transcripts by RT-PCR, in MDMs. These results are consistent with previous reports where FcγRIIB detection by Western blotting required very high amounts of monocytes (38, 39). FcγRIIB is highly regulated by culture conditions and the presence of cytokines, including IL-4 (38–40). Culture conditions used for macrophages differentiation, i.e., culture medium supplemented with human serum but with no added cytokines, may not be optimal for FcγRIIB expression. Our data, however, do not exclude that, if expressed at sufficient levels, FcγRIIB could negatively regulate HIV-1 replication inhibition by activated FcγRs. The coengagement of FcγRIIA/C and FcγRIIB by AT10 F(ab')2, however, had the same effect on HIV-1 replication as the engagement of FcγRIIA alone by IV.3 F(ab')2.

FcγRs have been involved in either enhancement or inhibition of HIV-1 infection when engaged by anti-HIV-1 Abs. FcγR1 has been suggested to contribute to the control of infection in HIV-1-infected patients by favoring the internalization of HIV-1-IgG complexes and the degradation of the virus in macrophages (41). Likewise, bispecific Abs that could target HIV-1 to macrophage activating FcγRs inhibited HIV-1 infection (42). On the contrary, FcγR1 or FcγRII have been suggested to enhance the entry of Ab-opsonized HIV-1 virions into macrophages (43–45). Whatever the effects of activating FcγRs when engaged by HIV-anti-HIV immune complexes, we consistently found that activating FcγRs inhibited HIV-1 infection when engaged by irrelevant IgG immune complexes. It was recently reported that FcγRIIA/IIIA polymorphisms which confer higher avidity binding to ICs are associated with protection against HIV infection, but, on the contrary, these same polymorphisms are associated with the likelihood of infection in HIV gp120-vaccinated individuals (46). Based on these data, one may speculate that in the presence of preexistent HIV-gp120-specific Abs induced by vaccination, higher avidity for ICs may be deleterious favoring Ab-dependent enhancement of HIV infection. In contrast, in the absence of preexistent HIV-1 Abs, higher avidity of FcγRs for circulating ICs may favor protection against incoming infection by limiting viral replication in IC-activated macrophages.

Macrophage activation by FcγRs affects the mechanisms that eventually lead to proviral integration. Previous qualitative PCR analysis of IVIg-stimulated MDMs infected with a replication competent virus detected a reduction in integrated proviral DNA but not in RT products (18). Using one-round infections, which avoid overlapping replication cycles, and quantitative PCR, we now show that, whereas reverse transcription was not affected at the earliest times p.i., the levels of both early and late cDNA products were eventually reduced in activated MDMs (Fig. 5). Levels of integrated proviruses were further inhibited in either IVIg or IC-stimulated MDMs. By contrast, the ratio of circular 2-LTR forms to integrated forms was higher in activated macrophages than in controls (Fig. 5F). 2-LTR circles are unintegrated nuclear forms of HIV-1 DNA (47–49) and thus reflect the translocation of viral transcripts into the nucleus of infected cells. Our results suggest that unintegrated HIV-1 DNA accumulates because of an inhibition of integration, as observed with anti-integrase drugs (50).

The level of HIV-1-integrated forms in FcγR-activated MDMs decreased to levels similar as viral replication inhibition levels, as shown by reduced luciferase activity (Fig. 5 and data not shown), suggesting that the postintegration steps of replication are unaffected. We confirmed this hypothesis by showing that FcγR-stimulation did not alter viral gene expression, once integration was achieved (Fig. 6).

The activation of PI3K or of the MAPK pathways has been shown to be essential for an efficient replication of HIV-1 (51–53). Therefore, one expects early signaling events triggered by activating FcγR cross-linking to favor HIV-1 replication rather than to exert an antiviral effect. We thus suggest that FcγR-induced late signaling events, which need to be identified in future work, are involved in HIV-1 infection. These might include the mobilization, the neosynthesis or the suppression of molecules that are critical for RT and/or integration. FcγR-activated MDMs secrete several cytokines, such as M-CSF, TNF-α, and MDC, which either up- or down-regulate HIV-1 infection in macrophages (54–56). Neutralization of these cytokines in MDM cultures showed that the inhibitory mechanisms induced by FcγR-aggregation overcome the enhancing effects of M-CSF and TNF-α on HIV-1 replication and are not linked to MDC (data not shown and Ref. 18). The preintegration inhibition induced by FcγR stimulation is reminiscent of the inhibitory effects of IFN-αβ (57). IFN-αβ was, however, not detected in FcγR-activated MDM supernatant (18). In addition, WN virus infection, which is inhibited by IFN-α and β (Fig. 7B and Refs. 34 and 35), was not affected in FcγR-activated
MDM. The participation of type I IFNs in HIV-1 inhibition in FcγR-activated MDM is therefore unlikely.

Two distinct inhibition mechanisms may be operating: one affecting the retrotranscription process, and another inhibiting viral integration after nuclear translocation of HIV-1 cDNA. A single mechanism may however inhibit both steps of the viral cycle. An increased degradation of reverse transcripts by endonucleases, as suggested for APOBEC3G (58), or of incoming viral proteins by the proteasome (59, 60), would reduce both the levels of reverse transcripts and the RT products available for integration. Alternatively, mechanisms that hinder HIV-1 integrase activity and/or affect the preintegration complex stability would have a negative impact on both integration and reverse transcription. Indeed, although reverse transcription and integration occur in distinct cellular compartments, they take place in the same molecular environment formed by the preintegration complex (61). Moreover, HIV-1 integrase was involved in different steps of the HIV-1 life cycle, including RT (62, 63).

Remarkably, FcγR-mediated antiviral activity is not limited to HIV-1 as it also affects other primate lentiviruses. By contrast, unrelated macrophage-tropic viruses such as the WN virus were not affected, suggesting that FcγR-mediated antiviral activity is not a general antiviral defense mechanism. If it targets highly conserved lentiviral proteins and/or their functions, such as RT and integration, one would expect inhibition to affect other HIV-1-related lentiviruses. It would be interesting to study the effect of FcγR-mediated activation of macrophages on the replication of lentiviruses in their natural hosts in more distant animal systems. This would be especially relevant for diseases caused by lentiviruses having a restricted tropism for macrophages, such as the caprine arthritis and encephalitis virus or the Maedi-Visna virus.

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References


The authors revised the Acknowledgments to include the origin of the SIVagm strain used in the study. The corrected section is shown below.

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In Figure 8, the time duration bars should have indicated 20 seconds. The corrected figure is shown below.


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