The CDK Inhibitor p21^{Cip1/WAF1} Is Induced by FcγR Activation and Restricts the Replication of Human Immunodeficiency Virus Type 1 and Related Primate Lentiviruses in Human Macrophages[▽]

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Macrophages are major targets of human immunodeficiency virus type 1 (HIV-1). We have previously shown that aggregation of activating immunoglobulin G Fc receptors (FcγR) by immune complexes inhibits reverse transcript accumulation and integration of HIV-1 and related lentiviruses in monocyte-derived macrophages. Here, we show that FcγR-mediated restriction of HIV-1 is not due to enhanced degradation of incoming viral proteins or cDNA and is associated to the induction of the cyclin-dependent kinase inhibitor p21^{Cip1/WAF1} (p21). Small interfering RNA-mediated p21 knockdown rescued viral replication in FcγR-activated macrophages and enhanced HIV-1 infection in unstimulated macrophages by increasing reverse transcript and integrated DNA levels. p21 induction by other stimuli, such as phorbol myristate acetate and the histone deacetylase inhibitor MS-275, was also associated with preintegrative blocks of HIV-1 replication in macrophages. Binding of p21 to reverse transcription/preintegration complex-associated HIV-1 proteins was not detected in yeast two-hybrid, pulldown, or coimmunoprecipitation assays, suggesting that p21 may affect viral replication independently of a specific interaction with an HIV-1 component. Consistently, p21 silencing rescued viral replication from the FcγR-mediated restriction also in simian immunodeficiency virus SIV_{mac}- and HIV-2-infected macrophages. Our results point to a role of p21 as an inhibitory factor of lentiviral infection in macrophages and to its implication in FcγR-mediated restriction.

Macrophages are targets of human immunodeficiency virus (HIV) infection and play crucial roles in viral dissemination and pathogenesis (23, 24, 70). HIV-infected macrophages contribute to HIV spread to CD4 T lymphocytes and to the establishment of cellular virus reservoirs (2, 25, 48, 60). Identification of the mechanisms controlling HIV-1 replication in macrophages may lead to new therapeutic strategies.

Microenvironmental stimuli can both enhance and inhibit HIV-1 replication in macrophages (29). Several cellular factors have been suggested to restrict HIV-1 infection in undifferentiated monocytes or to reduce macrophage permissivity to infection, including members of the APOBEC3 cytidine deaminase and TRIM families, the small isoform of the transcription factor C/EBPβ (CCAT enhancer-binding protein β), PPAR (for peroxisome proliferator-activated receptor), and more recently, microRNAs (8, 51, 61, 64, 71, 74). Despite these findings, no restriction factors that can be manipulated to render macrophages resistant to HIV-1 replication have clearly emerged.

We have previously shown that the engagement of activating immunoglobulin G Fc receptors (FcγR) by immune complexes

(IC) on monocyte-derived macrophages (hereafter called macrophages) restricts HIV-1 reverse transcription and integration, whereas viral entry, nuclear import, and gene expression from integrated proviruses are not inhibited (19, 52). Interestingly, FcγR-mediated inhibition is not limited to HIV-1 but also includes target related lentiviruses such as HIV-2, simian immunodeficiency virus (SIV)_{mac}, and SIV_{agm}, suggesting that this may be a common mechanism of lentivirus control (19). Engagement of activating FcγR on macrophages triggers signaling pathways, including phospholipase C, phosphatidylinositol-3 kinase, and mitogen-activated protein kinase/extracellular signal-regulated kinase (19). This leads to intracellular calcium augmentation, cytoskeleton remodeling and phagocytosis, as well as activation of transcription factors such as NF-κB, NFAT, and AP-1 (21, 33). Therefore, FcγR-mediated signaling could affect postentry steps of HIV-1 replication by modulating the expression of genes encoding molecules that interfere with reverse transcription or genome integration and/or by acting on the incoming virus through modifications of the cellular environment.

The present study was designed to test these hypotheses. We examined whether FcγR aggregation by IC could modulate the expression of host factors that can interfere with early postentry steps of HIV replication. We studied restriction factors of the APOBEC3 and TRIM families (43, 63, 65), as well as host proteins recruited in the HIV-1 reverse transcription and preintegration complexes (RTC/PIC). We found that the cyclin-

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dependent kinase inhibitor (CKI) p21^{Cip1/Waf1} (hereafter referred to as p21) is induced by FcγR aggregation. Interestingly, p21 has been recently involved in the resistance to HIV infection in primitive hematopoietic cells (81). We show here that p21 inhibits the replication of HIV-1 and related primate lentiviruses in macrophages.

MATERIALS AND METHODS

Monocyte-derived macrophages. Buffy coats from healthy HIV-seronegative donors were obtained through the French blood bank (Etablissement Français du Sang [EFS]) as part of the EFS-Institut Pasteur Convention. Written informed consent was obtained from each donor to use the cells for clinical research, in accordance with French law. Monocytes were isolated from buffy coats and differentiated into macrophages as previously described (19). Briefly, monocytes were separated from peripheral blood mononuclear cells by adherence to plastic and then detached and cultured for 7 to 11 days in hydrophobic Teflon dishes (Lumox; D. Dutscher) in macrophage medium (RPMI 1640 supplemented with 200 mM L-glutamine, 100 U of penicillin, 100 μg of streptomycin, 10 mM HEPES, 10 mM sodium pyruvate, 50 μM β-mercaptoethanol, 1% minimum essential medium, vitamins, 1% nonessential amino acids) supplemented with 15% of human AB serum. For experiments, macrophages were harvested and resuspended in macrophage medium containing 10% heat-inactivated fetal calf serum. Macrophage purity was assessed by flow cytometry, based on side and forward scattering and immunofluorescence staining. Cells obtained with this method are 91 to 96% CD14⁺ and express CD64, CD32, and CD16 FcγR.

For FcγR activation, macrophages were seeded in culture plates precoated with immune complexes formed by dinitrophenyl-conjugated bovine serum albumin (BSA-DNP) and anti-DNP. Briefly, the plates were coated with 0.1 mg of BSA-DNP/ml for 2 h at 37°C, saturated with 1 mg of BSA/ml in phosphate-buffered saline, and then incubated for 1 h at 37°C with 30 μg of rabbit anti-DNP antibodies (Sigma)/ml to form IC. All reagents were lipopolysaccharide (LPS)-free.

Cell treatment with MS-275 (Alexis) or MC 1568 (kindly provided by A. Mai and L. Altucci) was performed by adding the reagents at the indicated concentrations to culture medium 24 h before infection.

Cell infection. For single-round infections we used HIV-1 particles containing the *luc* reporter gene and pseudotyped with the VSV-G envelope protein (HIV-1_{VSV-G}) that permits HIV receptor-independent entry into cells. HIV-1_{VSV-G} pseudotyped viruses were produced by transient cotransfection of HEK293T cells with proviral pNL4-3Nef⁻ Env⁻ Luc⁺ DNA and the pMD2 VSV-G expression vector. Supernatants containing pseudotyped viruses were harvested 48 h after transfection, passed through 0.45-μm-pore-size filters, and stored at -80°C. The viral stocks were titrated on HeLa P4P cells by measuring luciferase activity (relative light units per second), and HIV-1 p24 antigen was quantified with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Zeptometrix Corp.). Macrophages were seeded in untreated or IC-coated culture plates (10⁵ cells/well in 96-well plates or 0.5 × 10⁶ cells/well in 12-well plates) in HIV-1_{VSV-G} suspension (90 ng of p24 per 10⁶ cells) and infected by spinoculation (1 h of centrifugation at 1,200 × g at room temperature, followed by 1 h of incubation at 37°C). In experiments with PCR detection of HIV DNA, viral preparations were pretreated with DNase I (Roche Diagnostics) for 1 h at room temperature.

For productive infection we used the strains HIV-1_{Bal}, HIV_{mac251}, and HIV-2_{GH} propagated in phytohemagglutinin-activated human PBMC. Culture supernatants were collected at the times of peak p24 and p27 production, respectively. Viral stocks were titrated on human CD4⁺ T cells. p24 and p27 were measured in the stocks and supernatants by using commercial ELISA kits (Zeptometrix Corp.). In these experiments macrophages were infected by spinoculation with 0.1 or 0.05 50% tissue culture infective doses of HIV-1_{Bal} or HIV_{mac251}/10⁶ cells, respectively. HIV-2_{GH} was used at 230 ng of p27 per 10⁶ cells. Culture supernatants were harvested at various times after infection, and p24 and p27 were measured by ELISA.

Quantification of HIV-1 cDNA by quantitative PCR (qPCR). Total DNA in infected macrophages was purified 72 h postinfection (p.i.) by using a DNeasy kit as recommended by the manufacturer (Qiagen). Cytoplasmic DNA was selectively extracted with a mitochondrial/cytoplasmic viral DNA purification kit (V-GENE) as recommended by the manufacturer. Briefly, macrophages were collected in M-A lysis buffer and left for 5 min on ice in the presence of RNase. Nuclei were pelleted by centrifugation at 1,500 rpm for 5 min at 4°C, the cytoplasmic fraction was clarified by centrifugation at 5,000 rpm for 5 min at 4°C, and then the DNA was recovered by phase separation. Quantitative real-time

PCR analysis of late (U5-Gag) forms of viral DNA and two long terminal repeat (2-LTR) circles were carried on an ABI Prism 7000 sequence detection system as previously described (19). Standards for U5-Gag amplification products were generated by serial dilution of DNA extracted from HIV-1 8E5 cells containing one integrated copy of HIV-1 per cell. 2-LTR copies were quantified from standard curves generated by serial dilution of DNA extracted from CEM cells infected with HIV-1_{NL4-3}. Integrated HIV-1 DNA was quantified by real-time Alu-LTR nested PCR using the primers and probes described elsewhere with some modifications (19, 78). Briefly, the first round of amplification was performed on a GeneAmp PCR system 9700 (Applied Biosystems). Integrated HIV-1 sequences were amplified by using an Expand High Fidelity kit (Roche) using two Alu primers (Alu F and Alu R) and an LTR primer extended with an artificial tag sequence at the 5' end of the oligonucleotide (NY1R). Real-time nested PCR was run on the ABI Prism 7000 system using 10 μl of a 1/10 dilution of the first-round PCR product as a template (primers NY2F and NY2R; probe NY2Alu). The integrated HIV-1 DNA copy number was determined with reference to a standard curve generated by concurrent amplification of HeLa R7 Neo cell DNA (10). A nested PCR conducted in parallel without the Alu primers in the first round gave a very weak background signal. The number of integrated HIV-1 DNA copies was obtained by subtracting the copy number measured without the Alu primers in the first round from the copy number measured in the full reaction. The amount of viral DNA was normalized to the endogenous reference gene albumin (for total DNA extracts) or to mitochondrial DNA (for cytoplasmic DNA). Standard curves were generated by serial dilution of a commercial human genomic DNA (Roche).

We used a previously described qPCR method to measure HIV-1 cDNA degradation (79). Briefly, macrophages were transduced with strain HIV-1_{VSV-G} and refed with medium containing zidovudine (AZT) at 10 μM 30 h after infection. Total DNA from treated and untreated macrophages was extracted 30, 48, 72, and 96 h after infection. The number of cDNA molecules per cell treated with AZT was divided by the number of cDNA molecules per untreated cells (percentage of remaining cDNA).

siRNA transfection. Small interfering RNA (siRNA) duplexes for p21 were obtained as follows: siRNAs n.9 and n.12 and the SMARTpool for p21 were purchased from Dharmacon, and negative control siRNA was synthesized by Qiagen from the sequence proposed by Zhang et al. (81). A p21-specific siRNA sequence described by Zhang et al. (81) was also used in some experiments (not shown). The SMARTpool for p53 from Dharmacon was used for p53 silencing. Macrophages were plated in 12-well plates (0.5 × 10⁶ cells/well) in 500 μl of 10% fetal bovine serum-supplemented medium or in 96-well plates (10⁵ cells/well) in 100 μl of the same medium. siRNA transfection was then performed with InterferIN (PolyPlus Transfection), according to the manufacturer's instructions. Briefly, the siRNA was diluted in OptiMEM medium and mixed with InterferIN transfection reagent at a ratio of 0.15 nmol of siRNA/10 μl of transfection reagent. The siRNA reagent mixture was incubated for 10 min at room temperature and then added dropwise into wells at a final concentration of 100 nM siRNA. Macrophages were then incubated at 37°C for 24 h. The medium was replaced with fresh 10% fetal bovine serum medium before infection. Cell lysates were assayed for protein expression by Western blot and for mRNA expression by reverse transcriptase qPCR (RT-qPCR) to determine the efficiency of gene knockdown at the moment of infection.

RT-qPCR analysis. Total RNA from macrophages was extracted with the RNeasy kit (Qiagen) and treated with DNase according to the manufacturer's instructions. RNA was quantified by GeneQuant (Amersham), and equal amounts (1 μg) were reverse transcribed with SuperScript II RT (Invitrogen). We used custom RT² Profiler PCR arrays (SABiosciences) to quantify TRIM transcripts, allowing us to detect TRIM5, TRIM11, TRIM19/PML, TRIM22, TRIM26, TRIM31, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) simultaneously. PCRs were performed with the RT² Realtime SYBR green PCR mix (SABiosciences) according to the manufacturer's instructions on a Light-Cycler 480 (Roche Diagnostics). The amplification program consisted of 10 min at 95°C, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. For other transcripts, PCR amplification of cDNAs was carried out in duplicate in MicroAmp Optical 96-well reaction plates (30 μl/well), using 25 μl of TaqMan Universal Master Mix, 0.2 mM TaqMan, and 1.5 μl of Assays-on-Demand gene expression assay premade mix (GAPDH, Hs99999905_m1; p21, Hs00355782_m1; LEDGF/p75, Hs01045714_g1; BAF, Hs00427805_g1; Ini1, Hs00996890_m1; Gemin2, Hs01031721_m1; p53, Hs00153349_m1; and p27, Hs00153277_m1). The amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 90 s, on an ABI Prism 7700 sequence detector (Applied Biosystems). The data were analyzed with the cycle threshold (C_T) method, and the amount of target mRNA in each sample was normalized to GAPDH mRNA as an endogenous reference.

All results were expressed relative to unstimulated macrophages (nonactivated control macrophages) as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T\text{-sample}} - \Delta C_{T\text{-control}}$ and where $\Delta C_T = C_{T\text{-target gene}} - C_{T\text{-GAPDH}}$.

Measure of proteasome activity. Macrophages were cultured in 96-well plates with or without epoxomycin (50 nM) for 48 h. They were then washed once with phosphate-buffered saline and lysed in 150 μ l/well of lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100). The fluorogenic substrate Suc-LLVY-AMC was then added at 50 μ M to start the proteolysis reaction. The mixture was incubated at 37°C for 2 h, and AMC release was detected by measuring fluorescence emission at 450 nm (excitation 385 nm) with a Victor-2 fluorometer (Perkin-Elmer).

Western blot. Macrophages cultured in 12-well plates were lysed in 80 μ l of M-PER lysis buffer (Pierce) containing Complete protease and phosphatase inhibitor cocktail (Roche). Protein was quantified with the BCA kit (Pierce), and samples were then diluted to 1 μ g/ μ l with Laemmli buffer, boiled at 95°C for 5 min, and loaded in NuPAGE gel 4 to 12% (Invitrogen) for electrophoretic separation. Proteins were then blotted onto Immobilon-P membranes (Millipore). After blocking with 5% skimmed milk, the membranes were incubated with the primary antibodies as indicated, followed by secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Sigma). The proteins were revealed on Hyperfilms (Amersham) by using the ECL chemiluminescent substrate (GE Healthcare) and X-Omat films (Kodak). The anti-p21 mouse monoclonal antibody (1:500) was purchased from Santa Cruz, anti-GAPDH (1:5,000) was from Abcam, and mouse monoclonal anti- β -actin (1:2,000) was obtained from Sigma.

Plasmid construction. (i) Two-hybrid expression vectors. The complete cDNA of human p21 was ligated into the yeast two-hybrid prey vector pGad-GE, while the cDNA of proliferating cell nuclear antigen (PCNA) was cloned into the bait vector pLex10. Sequences encoding the HIV-1 proteins matrix p17 (MA), integrase (IN), Vpr, and RT p66 (RT) were fused to the LexA DNA-binding domain (LexABD) of the pLex10 vector.

(ii) Mammalian expression vectors. p21 was also ligated into the mammalian glutathione *S*-transferase (GST)-tagged expression vector pCMV-GST (GeneCopoeia). Vectors for the expression of hemagglutinin (HA)-tagged PCNA, IN, MA, and Vpr were constructed by inserting the corresponding cDNA into the pAS1b vector, as described elsewhere (59).

Pulldown assay. HeLa cells were seeded in 10-cm-diameter plates at a density of 1.5×10^6 cells/plate the day before transfection. Transfection was performed with the Lipofectamine reagent (Invitrogen) and 4 μ g of plasmid according to the manufacturer's instructions. Cells were then cultured for 48 h before being lysed for 10 min on ice in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, and an anti-protease cocktail (Sigma). Equal quantities of lysate were incubated with 25 μ l of glutathione-Sepharose beads (vol/vol) for 1 h at 4°C. The beads were extensively washed in lysis buffer and resuspended in 4 $\times$ LDS sample buffer (Invitrogen). Samples were loaded onto NuPAGE Bis-Tris gels (Invitrogen) and then blotted onto a nylon membrane (Hybond-P; GE Healthcare). The membrane was saturated for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.5% Tween 20 and then with the primary antibody (anti-HA [clone3F10 from Roche] or anti-GST [clone GST-2 from Sigma]) for 1 h in the blocking solution. The membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody in Tris-buffered saline-Tween, and proteins were detected with an ECL kit (GE Healthcare).

Yeast two-hybrid assay. The yeast reporter strain L40 containing the two LexA-inducible genes *HIS3* and *LacZ* was cotransformed with the indicated LexABD and Gal4AD hybrid expression vectors. Cotransformed yeasts were plated on selective medium lacking tryptophan and leucine. Double transformants were patched on the same medium and replica plated on selective medium lacking tryptophan, leucine, and histidine for auxotrophy analysis and on Whatman 40 filters for β -galactosidase (β -Gal) activity assay.

Statistical analyses. Analyses were performed by using the Mann-Whitney test and the Wilcoxon signed-rank test.

RESULTS

Fc γ R-mediated restriction of HIV-1 replication does not depend on increased degradation of incoming virus products. We have previously reported that macrophage stimulation by IC inhibits the accumulation of both viral reverse transcripts and integrated forms after HIV-1 infection (19). In this previous study, quantification of viral cDNA was performed on

whole-cell DNA extracts, and we were thus unable to precisely evaluate the degree of inhibition of reverse transcription before nuclear import of the viral cDNA. Here, we monitored the accumulation of newly synthesized viral cDNA in the cytoplasm of IC-activated and unstimulated macrophages in single-round infections with HIV-1_{VSV-G}. Full-length HIV-1 cDNA was measured by qPCR in purified cytoplasmic fractions, by comparison with total cell extracts. Viral cDNA accumulated in unstimulated macrophages, reaching maximal levels at 72 h in the cytoplasmic fraction and still increasing at 96 h in the whole-cell extract (Fig. 1A). As expected, HIV-1 cDNA levels were strongly decreased in total extracts of IC-activated macrophages (70% reduction at 72 and 96 h compared to unstimulated macrophages). A substantial reduction in HIV-1 cDNA was also observed in the cytoplasmic fraction of IC-activated macrophages (50% reduction at 72 h in comparison with unstimulated macrophages) (Fig. 1A). This confirms that a major block occurs during the reverse transcription process.

The reduction in viral cDNA in the cytoplasm of IC-activated macrophages could result from enhanced degradation of incoming viral products, i.e., newly synthesized cDNA or viral proteins. We first compared the rate of degradation of HIV cDNA in unstimulated and IC-activated macrophages by determining HIV-1 cDNA stability after treatment with an RT inhibitor. Macrophages were infected with HIV-1_{VSV-G}, and AZT was added to the medium 30 h later in order to block further accumulation of reverse transcripts. The viral cDNA level showed a similar pattern of decline in IC-stimulated macrophages and in unstimulated macrophages (Fig. 1B). We then investigated whether IC stimulation could induce an increased degradation of incoming viral proteins by the proteasome, which is the main proteolytic complex operating in the cytosol (58, 73). The catalytic activity of the proteasome was evaluated by measuring hydrolysis of the fluorogenic peptide Suc-LLVY-MCA added to macrophage cell lysates. Degradation of the fluorogenic substrate was not significantly different between IC-stimulated and control macrophages (Fig. 1C), indicating that Fc γ R-mediated activation does not modulate proteasome activity. In the presence of epoxomycin, a selective irreversible inhibitor of chymotrypsinlike proteasome activity the catalytic capacity of the proteasome was strongly reduced (80%) in both activated and control macrophages (Fig. 1C), whereas cell viability measured at the same time was not affected (data not shown). Epoxomycin treatment of HIV-1_{VSV-G}-infected macrophages did not restore the loss of HIV-1 reverse transcription products and integrated forms (–55% and –84%, respectively) in IC-stimulated macrophages (Fig. 1D). Accordingly, HIV gene expression, reflected by luciferase activity in cell extracts, was not increased by epoxomycin in either IC-stimulated or control macrophages (data not shown). Altogether, these results strongly suggest that the decrease in viral cDNA induced by Fc γ R aggregation is not caused by increased degradation of reverse transcripts or viral proteins.

Fc γ R engagement triggers p21 gene expression. To determine whether Fc γ R engagement can induce factors that have been implicated in the restriction of early postentry steps of HIV-1 replication we examined gene expression of members of the APOBEC3 and TRIM families, including *APOBEC3A*, *APOBEC3F*, *APOBEC3G*, *TRIM5*, *TRIM11*, *TRIM19/PML*, *TRIM22*, *TRIM26*, and *TRIM31* (4, 6, 30, 41, 50, 67). We

FIG. 1. Degradation of incoming viruses does not account for the defective HIV-1 reverse transcription. (A) Macrophages plated in untreated (unstimulated, US) or IC-coated plates (stimulated, S) were infected with HIV-1_{VSV-G}. Late reverse transcription products (U5-Gag) in total DNA extracts or in cytoplasmic fractions of infected macrophages were analyzed by qPCR at 5, 24, 72, and 96 h p.i. U5-Gag copies in each sample were normalized to mitochondrial DNA. (B) Unstimulated (US) or IC-stimulated (S) macrophages were infected with HIV-1_{VSV-G}. At 30 h p.i., the medium was replaced, and 10 μ M AZT was added. The number of U5-Gag copies in infected cells was measured by qPCR at the indicated times, and the amount of remaining viral DNA (%) was calculated as the number of U5-Gag copies in AZT-treated macrophages divided by the number of U5-Gag copies in untreated macrophages. The error bars are the standard deviation of triplicate values of a representative experiment ($n = 3$). (C) Proteasome activity was measured in total cell extracts of unstimulated (US) or IC-stimulated (S) macrophages treated or not with 50 nM epoxomycin, as described in Materials and Methods. (D) Unstimulated or IC-stimulated macrophages, treated or not treated with epoxomycin, were infected with HIV-1_{VSV-G}. U5-Gag, and integrated forms of HIV-1 (Alu-LTR) were quantified 72 h p.i. by qPCR. Viral cDNAs were normalized to the albumin gene content in each sample. The error bars indicate the standard deviations of triplicate values of a representative experiment ($n = 3$).

measured their expression levels by RT-qPCR in IC-stimulated macrophages in comparison with unstimulated macrophages from three different donors. The target mRNA levels in IC-activated macrophages were normalized relative to the GAPDH mRNA level in each sample and were expressed as relative levels compared to unstimulated macrophages (Fig. 2A and B). APOBEC3A mRNA was very low or undetectable in unstimulated macrophages (data not shown) and its ex-

FIG. 2. Fc γ R aggregation induces p21 mRNA expression. (A and B) APOBEC3 and TRIM gene expression was measured in macrophages after 24 h of IC activation. Total RNA from macrophages was extracted and reverse transcribed, and APOBEC3 and TRIM expression was analyzed by RT-qPCR. The data are means $\pm$ the standard deviations of results obtained with macrophages from three different donors. (C) Macrophages were stimulated with IC, and after 24 h the total RNA was analyzed by RT-qPCR. The LEDGF/p75, Ini1, Gemin2, BAF, and p21 expression levels were normalized to the GAPDH gene and are presented relative to unstimulated macrophages (LEDGF/p75, $P = 0.08$; p21, $P = 0.04$, Wilcoxon signed-rank test). All results are expressed relative to unstimulated macrophages, as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T\text{-sample}} - \Delta C_{T\text{-control}}$ and $\Delta C_T = C_{T\text{-target gene}} - C_{T\text{-GAPDH}}$. The data are means $\pm$ the standard deviations of results obtained with macrophages from five different donors.

pression was not increased by IC stimulation (Fig. 2A). APOBEC3G and APOBEC3F mRNAs expression were downregulated by IC stimulation (Fig. 2A). None of the TRIM genes was upregulated by IC stimulation, and TRIM11, TRIM26, and TRIM22 expression was downregulated (Fig. 2B). Therefore, an increased expression of these restriction factors cannot account for the Fc γ R-mediated HIV-1 restriction.

Alteration of host cell components of RTC/PIC may affect the formation or the stability of these complexes and thereby have a negative impact on viral replication (22, 31). We therefore measured the expression of host factors associated with the RTC/PIC that might interfere with either reverse transcription or integration, including lens epithelium-derived growth factor (LEDGF)/p75 (12, 40), integrase interactor 1 (Ini1) (34, 45), barrier-to-autointegration factor (BAF) (39), Gemin2 (27), and p21(81), after IC stimulation. Although variations in basal gene expression were observed among the donors, IC

FIG. 3. Fc γ R aggregation induces p21 protein expression specifically and irrespective of p53 expression. (A) Uninfected and HIV-1_{VSV-G}-infected macrophages were either left untreated (US) or stimulated with IC or LPS (100 ng/ml) for 48 h before total protein extraction. Proteins were separated by electrophoresis, and p21 was revealed with a monoclonal anti-p21 antibody. β -Actin was used as a control. (B) Macrophages were activated with increasing concentrations of IC (3 μ g of anti-DNP/ml was added to wells coated with 0 to 30 μ g of DNP-BSA/ml). Total cell lysates were extracted 48 h later and analyzed by electrophoresis and Western blotting to reveal the p21 protein. (C) Macrophages were stimulated with IC and collected at the times indicated. p21 protein was then analyzed by Western blotting as reported above. (D) Macrophages were stimulated with IC, and total RNA was extracted 24 h later, reverse transcribed, and analyzed by RT-qPCR to determine the mRNA levels of p21, p27^{Kip1}, and p53. Values were normalized to the GAPDH gene and are reported relative to unstimulated macrophages. Macrophages from four different donors were analyzed. (E) Macrophages were transfected with p53-specific siRNA or a scrambled siRNA (si-Neg) or were mock treated (Ctrl) and seeded in the presence (S) or absence (US) of IC. After 24 h, p53 and p21 mRNAs were quantified in each sample by RT-qPCR, normalized to GAPDH, and expressed relative to US control macrophages (left and center). Proteins were analyzed by Western blotting. p21 and GAPDH (control) were detected with specific monoclonal antibodies (right). Consistent results were obtained with macrophages from three donors.

stimulation induced no significant change in the expression of Ini1, Gemin2, or BAF relative to control macrophages (Fig. 2C). LEDGF/p75 mRNA expression was slightly reduced in IC-activated macrophages from four out of five donors, but the difference did not reach significance ($P = 0.08$). In contrast, p21 expression was significantly upregulated by IC in all of the donors ($P = 0.04$, Fig. 2C).

Fc γ R engagement upregulates p21 specifically and irrespective of p53 modulation. We then examined the impact of IC stimulation on p21 protein expression compared to LPS stimulation that also inhibits early steps of HIV-1 replication by reducing reverse transcription (83) but via signaling pathways different from those activated by Fc γ R engagement. We found that p21 protein was strongly induced by IC stimulation (Fig. 3A). In contrast, LPS reduced p21 expression (Fig. 3A). Macrophage infection with HIV-1_{VSV-G} did not further modulate p21 expression in either IC-stimulated or unstimulated macrophages (Fig. 3A). p21 induction by IC stimulation was concentration dependent (Fig. 3B), and the protein level was clearly increased 6 h after IC stimulation and then accumulated during the time of monitoring (Fig. 3C).

Usually, p21 expression is transcriptionally regulated by p53 (20), although it can be modulated by p53-independent mechanisms (7). We therefore examined the effect of IC stimulation

on p53 expression. We also examined whether IC stimulation could induce other members of the Cip/Kip family of CDK inhibitors, to which p21 belongs, such as p27^{Kip1} (5). We measured p53 and p27^{Kip1} transcript levels in IC-stimulated macrophages from four different donors, in parallel with p21. In contrast to p21, neither p53 nor p27 mRNA levels were increased by IC stimulation (Fig. 3D), and they were even downregulated in some donors, suggesting that Fc γ R engagement induces p21 specifically and irrespective of p53 modulation. To gain further insight on the role of p53 in the Fc γ R-mediated induction of p21, we knocked down p53 expression in macrophages by siRNA transfection. We then analyzed the effect of p53 reduction on p21 expression in unstimulated macrophages and in macrophages activated by IC. In siRNA-untreated macrophages, IC stimulation resulted in an induction of p21 and a decrease of p53 expression (a representative example of experiments with macrophages from three donors is shown in Fig. 3E). At 24 h posttransfection with specific siRNA, p53 mRNA levels were reduced of 51 and 49% in unstimulated and IC-stimulated macrophages, respectively, compared to macrophages transfected with nonspecific siRNA (Fig. 3E left). p53 silencing led to a decrease in p21 expression both at mRNA and protein levels (Fig. 3E middle, right). However, p21 induction by IC was not modified by p53 silencing: the p21

FIG. 4. p21 silencing enhances HIV-1 replication in macrophages. (A) Macrophages were seeded in the presence (S) or absence (US) of IC and immediately transfected with p21-specific siRNA duplexes n.9 and n.12 or SMARTpool for p21 (Dharmacon), or a scrambled siRNA (si-Neg) or were mock treated (Ctrl). Cells were cultured for 48 h and then lysed. p21 mRNA was quantified in each sample by RT-qPCR, normalized to GAPDH, and expressed relative to US control macrophages (left). Proteins were analyzed by Western blotting. p21 and GAPDH (control) were detected with specific monoclonal antibodies (right). (B) Unstimulated macrophages were transfected with p21 si-RNA or an irrelevant siRNA (si-Neg) and infected with HIV-1_{VSV-G} 24 h later. The luciferase activity was measured at 24, 48, and 72 h p.i. The data are means $\pm$ the standard deviations of triplicate wells. (C) Macrophages were transfected with a p21-specific siRNA or an irrelevant siRNA (si-Neg) in the presence (S) or absence (US) of IC. Cells were infected with HIV-1_{VSV-G} 24 h after siRNA transfection. At 72 h p.i. the cells were lysed for luciferase assay and RNA extraction. Consistent results were obtained with macrophages from five different donors. (D) Macrophages were seeded in the presence (S) or absence of IC (US) and were transfected with p21-specific siRNA duplexes (si-p21) or mock treated (Ctrl). At 24 h after siRNA transfection, the macrophages were infected with HIV-1_{Bal}, and p24 capsid protein was quantified in the supernatants at the indicated times. Consistent results were obtained with macrophages from three donors.

mRNA in IC-stimulated macrophages was increased 2.4- and 2.6-fold, respectively, after p53 and nonspecific siRNA transfection with a corresponding increase in protein levels (Fig. 3E, middle, right). With the caution that p53 knockdown was not complete, these results suggest that p21 expression is partially regulated by p53 both in unstimulated and in IC-stimulated macrophages, but other pathways may contribute to its induction by IC.

Fc γ R-mediated induction of p21 restricts HIV-1 replication in macrophages. To determine whether p21 expression exerts antiviral activity in macrophages, we knocked down p21 expression and monitored HIV-1 replication in p21 silenced mac-

rophages. Transfection with specific siRNAs reduced both mRNA and protein levels of p21 in both unstimulated and IC-activated macrophages (Fig. 4A). p21 mRNA and protein levels consistently remained slightly higher in siRNA-treated IC-stimulated macrophages than in siRNA-treated unstimulated macrophages, suggesting an increase in their stability in activated macrophages (Fig. 4A). Unstimulated p21 knockdown macrophages were infected with HIV-1_{VSV-G} 24 h after siRNA transfection, and the luciferase activity was then measured at various times. p21 silencing enhanced HIV-1 replication (Fig. 4B). Compared to cells treated with nonspecific siRNA, the median increase in luciferase activity in p21 si-

lenced macrophages from five donors was 4.8-fold (range, 1.3 to 14.9, $P = 0.016$). Remarkably, p21 silencing also rescued HIV-1 replication in IC-stimulated macrophages to the levels seen in siRNA-untreated unstimulated macrophages (Fig. 4C). Compared to macrophages treated with control siRNA, the median increase of luciferase activity in IC-activated macrophages from five donors was 4.5-fold (range, 3.3 to 19.6, $P = 0.016$). Similar results were obtained using three different sequences of p21-specific siRNAs for p21 depletion (data not shown).

To determine the impact of p21-mediated restriction on productive HIV-1 infection, we treated unstimulated and IC-stimulated macrophages with p21 siRNA or control siRNA and then infected them with HIV-1_{Bal} and monitored supernatant p24 levels. HIV-1_{Bal} infection was strongly suppressed in IC-activated macrophages (2-log reduction on day 14), and p21 silencing rescued HIV-1 replication by 1.5 log in these macrophages (Fig. 4D). A small and transient increase (0.2 log) was observed in p21 siRNA-treated unstimulated macrophages on day 4 p.i., suggesting that p21 silencing in unstimulated macrophages that have low levels of p21 expression and are permissive to HIV-1 infection may not have significant effects on multiple cycles of infection. Nonspecific siRNA did not affect either p21 expression or HIV-1 replication in either unstimulated or IC-stimulated macrophages (data not shown).

Together, these results strongly suggest that p21 is a limiting factor for HIV-1 replication in macrophages and that it largely accounts for FcγR-mediated HIV-1 inhibition.

p21 restricts HIV-1 reverse transcription and integration.

To go further in the characterization of the mechanisms of HIV-1 restriction mediated by p21 in macrophages, we investigated which steps of HIV-1 replication are affected by p21. We measured reverse transcripts and integrated DNA by qPCR in unstimulated and IC-activated p21 knockdown macrophages infected with HIV-1_{VSV-G}. p21 silencing increased the level of late transcription products in both unstimulated and IC-stimulated macrophages (by two- and fourfold, respectively, in the experiment shown in Fig. 5A). The integrated forms of HIV-1 increased 2.6-fold in unstimulated macrophages and were rescued in activated macrophages, from an undetectable level to levels higher than those in untreated unstimulated macrophages (Fig. 5B). These results therefore indicate that p21 affects the same steps of HIV-1 replication as those restricted by FcγR engagement in macrophages.

p21 induction in macrophages by other stimuli is also associated with reduced permissivity to HIV-1 infection. To assess whether the effect of p21 on HIV-1 replication was specific to FcγR-mediated restriction, we induced p21 expression in macrophages by treatment with phorbol myristate acetate (PMA) and the histone deacetylase (HDAC) inhibitor MS-275, both of which have been reported to induce p21 (54, 56, 57). As expected, PMA increased p21 expression in macrophages (Fig. 6A, inset), and treatment of macrophages with PMA before HIV-1_{VSV-G} infection strongly inhibited viral replication, as reflected by luciferase activity decrease (Fig. 6A). Of note, we have previously shown that PMA treatment after macrophage infection, when HIV-1 integration is completed, leads to an enhancement of viral gene expression, owing to stimulation of HIV-1 transcription (55). These results suggest

FIG. 5. p21 restricts HIV-1 reverse transcription and integration in macrophages. Macrophages were transfected with a p21-specific siRNA or an irrelevant siRNA (si-Neg) in the presence (S) or absence (US) of IC. Cells were infected with HIV-1_{VSV-G} 24 h after siRNA transfection. Late reverse transcription products (U5-Gag) (A) and integrated forms (Alu-LTR) (B) were quantified by qPCR in DNA extracted from infected macrophages at 72 h p.i. The data were normalized to the albumin gene. These results are from the experiment shown in Fig. 4C. The error bars represent standard deviations of triplicate wells. Similar data were obtained with macrophages from four donors.

that the viral inhibition caused by PMA treatment before infection occurs at a preintegration step.

Treatment of macrophages with increasing concentrations of MS-275 increased p21 expression by up to fourfold compared to untreated macrophages in a concentration-dependent manner (Fig. 6B). Remarkably, when MS-275-treated macrophages were infected with HIV-1_{VSV-G}, viral replication fell as p21 expression rose (Fig. 6B). Since HDAC inhibitors have several effects on cell biology and may thus affect HIV-1 replication next to their effect on p21, we also used a class II HDAC inhibitor, MC 1568 (42, 47) that does not induce p21. Macrophage treatment with MC 1568 did not upregulate p21 and did not affect HIV-1 replication (Fig. 6C). MC 1568 activity in macrophages was assessed by measuring the acetylated tubulin (Fig. 6C, inset). Cell viability was not reduced by the chosen concentration ranges of either MS-275 or MC 1568 (data not shown). The association between p21 upregulation by different stimuli and reduced permissivity to HIV-1 further supports a negative effect of p21 on HIV-1 replication in macrophages.

Since the inhibition of HIV-1 replication by MS-275 may affect different steps from those targeted upon FcγR engagement, we measured HIV-1 cDNA in MS-275-treated macrophages at 96 h after HIV-1 infection. MS-275 caused a dose-dependent reduction of viral cDNA, concomitant to p21 increase, corroborating evidence for the implication of p21 in a preintegration block (Fig. 6D).

FIG. 6. PMA and the HDAC inhibitor MS-275 induce p21 expression and inhibit HIV-1 replication in macrophages. (A) Macrophages were treated with PMA (30 and 100 ng/ml) and infected with HIV-1_{VS-V-G}. The luciferase activity was measured 72 h p.i. For the inset, macrophages were treated with PMA (30 ng/ml) for 48 h before lysis and Western blot detection of p21. β -Actin was used as a control. (B and C) Macrophages were treated with the HDAC inhibitors MS-275 (B) or MC 1568 (C) at the indicated concentrations for 24 h and then infected with HIV-1_{VS-V-G}. Total RNA was extracted at 48 h after treatment and p21 mRNA expression was measured by RT-qPCR. Values were normalized to the GAPDH gene and expressed relative to untreated macrophages. The luciferase activity in cell lysates of infected macrophages was measured at 72 h p.i. The data are means $\pm$ the standard deviations of triplicate wells. Similar data were obtained with macrophages from three donors. (D) Macrophages were treated with MS-275 at the indicated concentrations for 24 h and then infected with HIV-1_{VS-V-G}. Total RNA was extracted at 48 h after treatment, and p21 mRNA expression was measured by RT-qPCR. Values were normalized to the GAPDH gene and are expressed relative to untreated macrophages. Total HIV-1 DNA was measured 96 h p.i. in DNA extracts from infected macrophages and normalized to the albumin gene. (C) Macrophages were treated with the indicated concentrations of MC 1568. At 24 h after treatment, macrophages were lysed, and the amount of acetylated tubulin was measured by Western blotting. β -Actin protein was used as a control.

Interactions between p21 and viral proteins of the HIV-1 RTC/PIC were not detected by yeast two-hybrid and pulldown assays. Coimmunoprecipitation assays in human megakaryoblastic leukemia and ACH2 cell lines have suggested that p21 is associated with the HIV-1 PIC (75, 81). To determine whether p21 could inhibit preintegration steps of HIV-1 replication by direct interaction with viral proteins of the RTC/PIC we used a yeast two-hybrid assay to test p21 binding to

HIV-1 IN, MA, RT, and viral protein R (Vpr). p21 protein was fused to Gal4AD and tested for interactions with PIC-associated viral proteins fused to LexABD in the L40 yeast strain, which contains the two LexA-inducible reporter genes *LacZ* and *HIS3*. We used LexA-PCNA fusion as a positive control, since the interaction with p21 has been previously reported using a similar system (72). None of the tested viral proteins reacted with p21, as revealed by the absence of growth on

FIG. 7. p21 protein interaction with viral components of the HIV-1 PIC is not detected in yeast two-hybrid or in vitro. (A) Two-hybrid assay between p21 and viral components of the PIC. The yeast reporter strain L40, expressing the indicated pairs of hybrid proteins, was plated in medium with histidine (+His) or without histidine (−His) or replica plated on Whatman filters and tested for β-Gal activity (β-Gal). Growth in the absence of histidine and development of a blue color in the β-galactosidase assay both indicate interaction between hybrid proteins. (B and C) In vitro binding analysis of the interaction between p21 and viral proteins of the PIC. HeLa cells were cotransfected with 2 μg of plasmids for expression of GST (lanes 1 to 3, 7, and 8), GST-p21 (lanes 4 to 6, 9, and 10), HA-tagged MA (lanes 1 and 4), IN (lanes 2 and 5), Vpr (lanes 7 and 9), or PCNA (lanes 3, 6, 8, and 10). Lysates of transfected cells were incubated with equal amounts of glutathione-Sepharose beads. Bound proteins and cell lysates (3% of the total cellular extract) were resolved by NuPAGE 10% Bis-Tris gel and immunoblotted with anti-HA or anti-GST. Protein markers are shown in kilodaltons on the left (PageRuler; Fermentas).

medium without histidine (−His) and expression of β-Gal activity (Fig. 7A). The fusion Gal4AD-p21 protein was efficiently expressed, since it yielded positive signals, growth on medium without histidine, and expression of β-Gal activity in the presence of the LexA-PCNA fusion protein. Expression of the viral proteins in yeast cells was checked by using specific partners of each viral protein (data not shown). We confirmed the lack of p21 interaction with HIV-1 proteins by using a Gal4BD-p21 fusion protein and Gal4AD-fused IN, MA, and Vpr (not shown).

We further investigated the possible p21 interaction with HIV-1 proteins in pulldown assays after coexpression of each viral protein with GST-p21 in HeLa cells. GST-tagged proteins (GST alone or GST-p21) were precipitated from cell lysates with glutathione-Sepharose beads, and the precipitates were analyzed by Western blotting with an anti-HA antibody. MA, IN (Fig. 7B, lanes 4 and 5), Vpr (Fig. 7C, lane 9), and RTp66 (not shown) were not precipitated with GST-p21, whereas PCNA was efficiently precipitated (Fig. 7B, lane 6, and Fig. 7C, lane 10). Analysis of cellular lysates indicated that all of the proteins (MA, IN, Vpr, RTp66, and PCNA), were correctly expressed in the transfected cells (Fig. 7B and C, bottom panels, and results not shown). Moreover, no signal was detected after coimmunoprecipitation of Flag-tagged IN or Vpr and

HA-tagged p21 coexpressed in HeLa cells, whereas p21 was immunoprecipitated with Flag-PCNA (data not shown). Therefore, in keeping with the yeast two-hybrid assay results, we detected no interaction between p21 and IN, Vpr, or MA in pulldown assays nor between p21 and IN or Vpr in coimmunoprecipitation experiments. These results suggest that p21 may interfere with RTC/PIC functions independently of a specific interaction with HIV-1 proteins.

p21-mediated inhibition affects HIV-1-related primate lentiviruses in macrophages. To determine whether p21-mediated restriction was or not virus specific, we examined the effect of p21 silencing on the replication of HIV-1 related lentiviruses SIV_{mac} and HIV-2. Macrophages were transfected with p21-specific siRNAs and then challenged with SIV_{mac251} or and HIV-2_{GH} 24 h later. The levels of viral replication, evaluated by measuring supernatant p27 levels at day 7 p.i., were reduced by 80 and 48% for SIV_{mac} and HIV-2, respectively, in IC-activated macrophages (Fig. 8). p21 silencing increased SIV_{mac} and HIV-2 replication 3.4- and 2.8-fold, respectively, in IC-activated macrophages compared to macrophages treated with nonspecific RNA (Fig. 8). Thus, FcγR-mediated inhibition of SIV_{mac} and HIV-2 was substantially reversed by p21 silencing.

FIG. 8. p21 silencing rescues SIV_{mac} and HIV-2_{GH} replication in IC-stimulated macrophages. (A and B) Macrophages were seeded in the presence (S) or absence of IC (US) and were transfected with p21-specific siRNA duplexes (si-p21) or with an irrelevant siRNA (si-Neg) or mock treated (Ctrl). At 24 h after siRNA transfection, macrophages were infected with SIV_{mac251} or HIV-2_{GH}, and p27 capsid protein was quantified in the supernatants at 7 days p.i. Consistent results were obtained with macrophages from three donors.

Together, these results show that p21 inhibits not only HIV-1 but also other primate lentivirus replication in macrophages restricting preintegration steps of viral cycle, a finding consistent with its implication in FcγR-mediated restriction (19).

DISCUSSION

We have previously shown that IC activation of human macrophages through FcγRs inhibits the replication of HIV-1 and other primate lentiviruses, reducing both reverse transcript and provirus levels (19). We show here that this antiviral activity involves the CDK inhibitor p21. The main findings that support a role of p21 in FcγR-mediated lentiviral restriction are as follows: (i) the inhibition of HIV-1 replication induced by IC activation of macrophages was accompanied by increased p21 mRNA and protein expression and (ii) siRNA silencing of p21 rescued HIV-1, SIV, and HIV-2 replication in IC-activated macrophages by increasing reverse transcript and integration levels in IC-activated macrophages. Our results also suggest that p21 is a limiting factor to HIV infection in macrophages. Its depletion enhanced HIV-1 replication not only in IC-activated macrophages but also in unstimulated macrophages. In addition, p21 induction in macrophages by different stimuli, including PMA and the HDAC inhibitor MS-275, was associated with preintegration restrictions of HIV-1 replication. The degree of viral inhibition exerted by p21 in macrophages depends on its intracellular concentration and might thus vary according to macrophage cellular microenvironments in body tissues, including cytokine patterns, and other stimuli (76).

p21 belongs to the Cip/Kip family of CKIs (26, 28, 77). Although it was first described as a cell cycle inhibitor, blocking cell cycling at the G₁/S interface and playing a critical role in the control of cell growth, p21 has also been shown to be involved in the regulation of apoptosis and differentiation (16, 62). It has been reported that p21 exerts a protective role against apoptosis in macrophages and that the antiapoptotic activity of p21 in monocyte differentiation is determined by its cytoplasmic localization (3, 76). In fact, the activities of p21 depend on the cell type, its subcellular (nuclear or cytoplasmic) location, and its expression level and phosphorylation status (3, 13, 46). p21 expression is regulated by both p53-dependent and p53-independent mechanisms (20, 82). The increase in p21 expression induced by FcγR cross-linking in macrophages was not accompanied by an induction of p53, since p53 expression was either unaffected or downregulated by IC stimulation. siRNA-mediated p53 silencing decreased p21 expression in both unstimulated and IC-activated macrophages but did not block p21 induction by IC. Altogether, these results suggest that while p21 expression in macrophages is modulated by p53, other pathways may contribute to its induction by FcγR cross-linking.

FcγR cross-linking activates several signaling pathways in macrophages, including PKC and ERK1/2 (19), both of which have been implicated in PMA induction of p21 in myeloid cells (18, 49, 57). Although further studies are needed to precisely identify the signals involved in p21 induction by IC, they are likely to occur at the transcriptional level, as p21 mRNA expression increased after IC stimulation. In addition to transcriptional activation, stabilization of p21 mRNA and/or protein may contribute to the IC-mediated enhancement of p21 expression (32, 49) since, after p21 siRNA treatment, p21 mRNA and protein levels remained higher in IC-activated macrophages than in unstimulated macrophages. In keeping with this observation, p21 silencing did not restore HIV-1 gene expression or cDNA levels in IC-activated macrophages to the levels achieved by p21 silencing in unstimulated macrophages (Fig. 4C and 5A). We cannot, however, rule out the possibility that the residual inhibition of HIV-1 replication observed in IC-stimulated macrophages after p21 silencing was due to additional factors. The decrease in LEDGF/p75 expression after IC stimulation in macrophages from some donors might, for example, contribute to reducing viral integration (68).

Conflicting results have been reported with respect to p21/HIV-1 interaction. HIV-1 infection of T lymphocytes was found to be associated with a loss of p21 expression (15), while two studies based on transcriptome analysis showed either an increase or no change in p21 expression in HIV-1-infected macrophages (9, 69). Vazquez et al. reported that p21 enhance HIV-1 infection in macrophages 12 to 14 days after viral challenge (69). Our data seem to be at odds with these results. Whereas Vazquez et al. detected no change in HIV-1 DNA levels during the first 2 days of infection, our knockdown experiments showed an inhibitory effect of p21 on the reverse transcription and integration steps of HIV-1 replication. However, we used one-cycle infection and real-time PCR, while Vazquez et al. used a replicative strain of HIV-1 and nonquantitative PCR, which are not ideally suited to analyzing the first steps of viral replication. The increase of p21 expression at late times (14 days) after HIV-1 infection reported by Vazquez et

al. may be linked to the accumulation of Vpr that stimulates p21 gene expression in infected cells (1, 14, 17, 69) or may be a cell response against stress and apoptotic stimuli associated to infection (3, 76). p21 might also have different impacts on HIV-1 infection of macrophages depending on the time since infection: a block of early stages of HIV-1 replication in acute infection, as we show here, or an activation of HIV-1 gene expression, synergistically with Vpr, in chronic infection (17). However, we did not observe an inhibitory effect of p21 depletion on productive HIV-1 infection of unstimulated macrophages. Methodological differences, including monocyte differentiation into macrophages (fetal bovine serum in Vazquez's study versus human serum in our study), might also account, at least in part, for the discrepancy between the results of the two studies.

Zhang et al. reported that p21 knockdown enhances human hematopoietic stem cell (HSC) sensitivity to transduction with pseudotyped HIV-1 vectors (80). More recently, they showed that p21 knockdown permits productive HIV-1 replication in human HSC by enhancing HIV-1 integration (81). Whereas p21-mediated restriction targeted only viral integration in HSCs, our results indicate that p21 interferes already with the cytoplasmic phases of viral replication in macrophages inhibiting the accumulation of reverse transcripts. This could be related to p21 translocation from the nucleus to cytoplasm during monocyte differentiation (3). Differences in p21 localization and cellular context might subtend the observed differences in the restriction phenotypes in macrophages and HSCs. p21 restriction in macrophages was not mediated by either enhanced expression of APOBEC3 or TRIM restriction factors or increased degradation of nascent reverse transcripts or incoming viral proteins. Since reverse transcription, as well as integration, depend on a functional RTC/PIC, we examined potential interactions between p21 and viral components of the RTC/PIC that could inhibit their activity. It has previously suggested that p21 may inhibit HIV-1 integration in HSC by complexing with IN (81). However, we did not detect p21 interactions with RTC/PIC-associated HIV-1 proteins, including IN and Vpr, either in yeast two-hybrid assays, in pulldown or in immunoprecipitation experiments. Although an interaction of p21 with Vpr has been previously reported (17), we did not detect it by any of the experimental approaches that we used. Using the same yeast two-hybrid system, interactions of Vpr with three different cellular partners: the nucleoporin hCG1, DCAF1/VprBP, and the uracyl DNA glycosylase UNG have been detected in previous studies (37, 38). In addition, these interactions have also been visualized by pulldown experiments with HA-tagged Vpr (11, 38), confirming that Vpr protein is well folded in our assay. Although we cannot formally exclude interactions either in the context of the RTC/PIC, since viral proteins were analyzed for their binding to p21 separately, or in vivo in infected cells, our results suggest that p21 may interfere with HIV-1 RTC/PIC activities independently of a direct interaction with its viral components. p21 might modify the cellular context of the PIC, affecting either its stability or its interactions with other host factors important for its function. An antiviral activity independent of a specific interaction with an HIV-1 protein may underlie the inhibitory effect of p21 on other lentiviruses. Indeed, p21 affected not only HIV-1 but also SIV_{mac} and HIV-2 replication in macro-

phages, in line with our previous results on the FcγR-mediated restriction of primate lentiviruses (19). On the contrary, p21 silencing did not modify HSC restriction of SIV_{mac} replication (81). However, macrophages are susceptible to HIV and SIV infections, whereas HSCs are resistant to both viruses: different host cell-virus interactions might subtend the differences in the spectrum of lentiviral restriction in the two cell types.

Further work will be required to determine the precise mechanisms responsible for p21-mediated restriction of HIV-1 replication in macrophages and other cells. Our findings point to a role of p21 as an inhibiting factor of primate lentivirus replication in macrophages, suggesting its relevance in viral control in a cellular compartment that is critical to HIV infection and pathogenesis. p21 has already been proposed as a target for anticancer therapy (36, 44, 66) and can be induced by pharmacological compounds, including HDACi, that are studied as adjuvants to highly active antiretroviral therapy to eradicate HIV-1 cellular reservoirs (35, 53). If p21 acts indeed as an inhibitor of HIV infection, this could have implications for antiretroviral therapy research.

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