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Awatef Allouch, Institut Pasteur
Annie David, Institut Pasteur
Sarah M. Amie, Emory University
Hichem Lahouassa, Institut Cochin
Loïc Chartier, Institut Pasteur
Florence Margottin-Goguet, Institut Cochin
Françoise Barre-Sinoussi, Institut Pasteur
Baek Kim, Emory University
Asier Saez-Cirion, Institut Pasteur
Francois Pancino, Institut Pasteur

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p21-mediated RNR2 repression restricts HIV-1 replication in macrophages by inhibiting dNTP biosynthesis pathway

Awatef Alloucha,1,2, Annie David3, Sarah M. Amie3, Hichem Lahuassa1, Loïc Chartier2, Florence Margottin-Goguetc,e,f, Françoise Barré-Sinoussib,g, Baek Kimb,h, Asier Sáez-Ciriónb, and Gianfranco Pancinoa,g,1

Macrophages are a major target cell for HIV-1, and their infection contributes to HIV pathogenesis. We have previously shown that the cyclin-dependent kinase inhibitor p21 inhibits the replication of HIV-1 and other primate lentiviruses in human monocyte-derived macrophages by impairing reverse transcription of the viral genome. In the attempt to understand the p21-mediated restriction mechanisms, we found that p21 impairs HIV-1 and simian immunodeficiency virus (SIV)mac reverse transcription in macrophages by reducing the intracellular deoxyribonucleotide (dNTP) pool to levels below those required for viral cDNA synthesis by a SAM domain and HD domain-containing protein 1 (SAMHD1)-independent pathway. We found that p21 blocks dNTP biosynthesis by down-regulating the expression of the RNR2 subunit of ribonucleotide reductase, an enzyme essential for the reduction of ribonucleotides to dNTP. p21 inhibits RNR2 transcription by repressing E2F1 transcription factor, its transcriptional activator. Our findings unravel a cellular pathway that restricts HIV-1 and other primate lentiviruses by affecting dNTP synthesis, thereby pointing to new potential cellular targets for anti-HIV therapeutic strategies.

Macrophage infection by HIV-1 contributes to viral pathogenesis and progression to AIDS (1). Because of their longevity and relative resistance to the cytopathogenic effect of HIV-1, macrophages contribute to the establishment of viral reservoirs and are able to transmit the virus to other target cells (2–4). HIV-1 infection of macrophages also affects their functions in innate and adaptive responses to pathogens (5). However, macrophage susceptibility to HIV-1 infection varies according to their differentiation status and tissue localization (6, 7). Environmental stimuli such as cytokines, bacterial products, and immune complexes also influence macrophage permissiveness to HIV-1 infection (8). HIV-1 replication in macrophages is regulated by cellular factors that can either enhance or inhibit various steps of the viral replicative cycle (9). In particular, HIV-1 reverse transcription is much slower in monocyte-derived macrophages (MDMs) than in activated T cells. This difference is attributed mainly to the limited availability of nucleotide precursors in these nondividing cells, in which the average deoxynucleoside triphosphate (dNTP) concentration (∼26 nM) is about 200 times lower than in PHA-activated T cells (10–12). The dNTP triphosphohydrolase SAM domain and HD domain-containing protein 1 (SAMHD1) decreases dNTP levels by hydrolyzing dNTP into deoxynucleosides (dNs) and triphosphates, thereby limiting HIV-1 reverse transcription in dendritic cells, macrophages, and resting T cells (13–20). Contrary to HIV-1, SIV and HIV-2 have evolved a protein, Vpx, that relieves SAMHD1-mediated restriction by targeting SAMHD1 for proteasome-dependent degradation (21–23). In addition to SAMHD1, other cellular factors, in particular antiviral pathways induced by type I IFN, affect early postentry steps of HIV-1 replication in macrophages (24–27).

We have previously shown that p21Waf1/Cip1 (referred to hereafter as p21) restricts HIV replication in MDMs (28). p53-independent p21 induction by immune complex aggregation of FcγRs leads to a strong reduction in reverse transcripts of HIV-1, HIV-2, SIVmac, and SIVagm in MDMs whereas siRNA-mediated p21 depletion rescues viral replication (28). A further minor block of viral integration by p21 has been also observed (28) that would be a consequence of the major block of reverse transcription leading to the formation of incomplete reverse transcripts unable to integrate properly. The mechanism underlying p21-mediated HIV-1 inhibition in macrophages remains to be elucidated. In particular, we did not detect p21 interactions with HIV-1 proteins involved in reverse transcription/integration, including Viral protein R, matrix, reverse transcriptase, and Integrase (28). Although p21 causes a preintegrative HIV-1 blockade in human hematopoietic stem cells (29), it has been reported to either inhibit or enhance HIV-1 transmission in MDMs and T cells (30–32). However, we did not observe a significant impact of FcγR-mediated activation on HIV-1 transmission in MDMs (33).

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*To whom correspondence may be addressed. E-mail: a.allouch@sns.it or gianfranco.pancino@pasteur.fr.

Present address: Institut National de la Santé et de la Recherche Médicale U1030, Institut Gustave Roussy, F-94805 Villejuif, France.

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p21 belongs to the Cip/Kip family of cyclin-dependent kinase inhibitors (CDKIs) (34, 35). As a cell-cycle inhibitor, p21 plays a critical role in the control of cell growth, but it also plays a role in monocyte differentiation and survival (36–38). p21 influences cell-cycle progression through cyclin/CDK inhibition, binding to PCNA, and control of E2F transcription and activity (39, 40). Although p21 is not a transcription factor, it may exert indirect effects on cellular gene expression. p21 induction in a fibrosarcoma cell line regulates the transcription of several genes, including genes involved in DNA replication, segregation, and repair, either repressing or enhancing their expression (41). Despite the fact that p21 is strongly expressed in terminally differentiated, nonreplicating MDMs, there is scarce information on its impact on gene expression in these cells.

Here, we addressed the mechanisms underlying p21-mediated restriction of the reverse transcription of HIV-1 and related lentiviruses in macrophages. Specifically, we investigated whether p21 might affect the dNTP pool in macrophages by interfering with genes involved in dNTP metabolic pathways.

Results

p21 Reduces dNTP Concentrations in MDMs. We have previously demonstrated that induction of p21 expression in MDMs through FcyR aggregation by immobilized Ig (IVIg) inhibits HIV-1 infection and intracellular dNTP biosynthesis (23). Although MDMs transduced with IVIg-VSV-G pseudotyped NL4-3-Luc HIV-1 virus (HIV-1-luc/VSVG) containing a luciferase reporter gene (28), Single-cell bioluminescence (42) showed that HIV-1 gene expression in infected living MDMs was almost abrogated in IVIg-coated wells with a significant (P < 0.001) reduction of the number of luciferase-positive cells (N = 0.6 ± 0.8 positive cells) in comparison with unstimulated (US) MDMs (N = 8.3 ± 2.2 positive cells) (Fig. 1A). The overall effect is reflected by a decrease (>95%) of luciferase activity in the lysates of IVIg-activated MDMs (IVIg) (Fig. 1B). This last measure, more easily quantifiable, was used in subsequent experiments.

We have previously shown that p21 blocks HIV-1 replication mainly by inhibiting the reverse transcription step (28). Exogenous deoxyinoscinucleosides (dNs) accelerate HIV-1 reverse transcription in MDMs by serving as dNTP precursors and thereby increasing the intracellular dNTP pool, which is normally a limiting factor (11, 45). Exogenous dNs are converted into dNTPs through the de novo dNTP biosynthesis pathway, and it has been reported that p21 can repress genes that encode enzymes involved in dNTP biosynthesis (41).

We therefore examined whether adding dNs to MDM cultures counteracted p21 inhibition of viral reverse transcription. Unstimulated and IVIg-stimulated MDMs were infected with HIV-1 luc/VSV-G and then treated with increasing amounts of pyrimidine and purine dNs (0.25 mM–1 mM). HIV-1 replication was inhibited by 70% in IVIg-stimulated MDMs in comparison with unstimulated MDMs (Fig. 1C). The addition of dNs increased HIV-1 replication in unstimulated MDMs and abrogated HIV-1 restriction in IVIg stimulated MDM in a concentration-dependent manner (Fig. 1C), suggesting that p21 inhibits HIV-1 replication by limiting dNTP pools available for reverse transcription.

We then investigated whether p21 expression modulates the intracellular dNTP pool. Intracellular dNTP concentrations were quantified in unstimulated and in IVIg-stimulated MDMs in which p21 had been silenced by transfection with a small interfering RNA (siRNA) targeting the p21 gene (siP21). Control cells were transfected with a nontargeting siRNA pool (siCTRL). Intracellular dNTP concentrations were measured with a highly sensitive single-nucleotide incorporation assay (11, 19). Both absolute and fold increase dNTP concentrations were determined for the siCTRL or sip21 RNA treated MDMs from three different donors. A representative experiment for donor 1 is depicted in Fig. S1, and the quantification of dATP and dTTP concentrations for the three donors is presented in Fig. 1D. As expected, dNTP levels in unstimulated and IVIg-stimulated MDMs treated with control siRNA (siCTRL) were very low (Fig. 1D and Fig. S1C) because of the low cellular dNTP level in macrophages, below or close to the linearity range of quantification. After p21 knockdown (sip21), dATP and dTTP intracellular concentrations increased in MDMs from the three donors (4.8-, 5.4-, and 4.3-fold for dATP and 1.5-, 2.2-, and 2.6-fold for dTTP in donors 1, 2, and 3, respectively) (Fig. 1D). In sip21-knockdown IVIg-stimulated MDMs, dATP concentrations increased of 3.6-, 2.6-, and 1.7-fold for donor 1, 2, and 3, respectively, and dTTP concentrations increased 1.9- and 2.8-fold in donors 1 and 3 and remained unchanged in donor 2 (Fig. 1D). These results indicate that p21 interferes with dNTP metabolism in MDMs, decreasing the dNTP pool.

p21 Inhibits the Expression of Ribonucleotide Reductase Subunit R2 (RNR2). We then examined whether p21-mediated down-regulation of dNTP levels in MDMs was due to interference with dNTP biosynthesis pathways. We measured the expression of proteins involved in de novo dNTP biosynthesis [RNR1, ribonucleotide reductase subunit R2 (RNR2), and p53R2] by Western blot (WB) in unstimulated and IVIg-stimulated MDMs silenced or not for p21. The expression of PCNA, a p21-interacting protein, and of GAPDH, a housekeeping protein, was used to normalize each sample. As shown in Fig. 2, IVIg stimulation increased p21 protein levels, and p21 knockdown (sip21) reduced p21 protein levels relative to unstimulated and IVIg-stimulated MDMs transfected with control siRNA (siCTRL). RNR1 and p53R2 expression levels remained unchanged whether p21 levels increased or decreased (Fig. 2A). RNR2 protein was depleted upon p21 induction and strongly increased after p21 knockdown (sip21) (Fig. 2B). These data indicated that p21 down-regulates RNR2 protein expression in MDMs.

To determine whether p21 regulates RNR2 expression at the transcriptional level, RNR2 and p21 mRNAs were quantified by quantitative real-time PCR (qRT-PCR) in the same conditions as those described above. Results with MDMs from five different donors showed that RNR2 mRNA levels fell significantly following IVIg stimulation (by 60–90%; P = 0.005), in parallel with the increase in p21 mRNA levels (Fig. 2B). In contrast, p53R2 mRNA levels remained unchanged upon IVIg stimulation (Fig. 2C). Consistently with the lack of modulation of p53R2 protein levels (Fig. 2C), RNR2 mRNA levels increased significantly (5- to 30-fold; P = 0.005) in unstimulated MDMs depleted of p21 (sip21) (Fig. 2B). The RNR2 mRNA levels were rescued and increased by 2- to 15-fold in the IVIg-activated MDMs silenced for p21. The variations in p21 and RNR2 expressions corresponded closely to the effects of p21 induction/depletion on HIV-1 replication in all donor cells tested (Fig. 2C). HIV-1-luc/VSVG replication was inhibited in IVIg-activated MDMs (high p21 and low RNR2 levels) and enhanced in p21-knockdown MDMs (low p21 and high RNR2 levels) (Fig. 2C). These results suggested that p21 inhibits HIV-1 reverse transcription by inhibiting RNR2 expression and thereby blocking dNTP biosynthesis.

RNR2 Depletion Is Sufficient to Inhibit HIV-1 Replication at the Reverse Transcription Step. We detected both RNR2 and p53R2 expressions in MDMs, but only RNR2 expression was regulated by p21. We therefore investigated the specific role of RNR2 in p21-mediated inhibition of HIV-1 in macrophages. To evaluate the role of RNR2 in HIV-1 replication in MDMs, RNR2 was depleted by transfecting MDMs with a pool of four siRNAs targeting the RNR2 gene. Control cells were transfected with a pool of nontargeting siRNAs (siCTRL). As shown in Fig. 3A, RNR2 siRNAs (siRNR2) depleted the protein in MDMs as efficiently as IVIg stimulation. Given the high sequence homology of the RNR2 gene with the p53R2 gene (44), p53R2 protein expression...
was also checked by WB, which showed that RNR2 siRNAs did not affect p53R2 expression (Fig. 3A). RNR2 siRNAs reduced RNR2 mRNA levels in unstimulated MDMs by 65–98% in MDMs from seven different donors (P = 0.0008) (Fig. 3B). In contrast, p21 mRNA levels were not significantly affected by RNR2 knockdown (Fig. 3B). RNR2 knockdown significantly inhibited viral replication in unstimulated HIV-1.luc/VSVG-infected cells from all seven donors (−50% to −98% versus control unstimulated MDMs; P = 0.0008) (Fig. 3C), indicating that RNR2 is required for HIV-1 replication in MDMs. In parallel experiments, IVIg activation of MDMs infected HIV-1 replication to a similar extent (64–94%) (Fig. 3C). HIV-1 late reverse transcription products (LRTs) were quantified by qRT-PCR in MDMs from six of the seven donors. RNR2 knockdown

Fig. 1. Exogenous deoxynucleosides (dNs) rescue p21-mediated restriction of HIV-1 and p21 knockdown increases dNTP levels in MDMs. (A and B) Unstimulated (US) and IVIg-activated MDMs were infected with HIV-1.luc/VSVG 2 h after IVIg stimulation. HIV-1 replication was quantified at 5 d postinfection in living cells by bioluminescence imaging of the infected living MDMs (integration, 80 s) (A). The numbers of positive cells indicated above the images are means ± SD of 9 and 13 random fields for US and IVIg MDMs, respectively. In parallel samples, HIV-1 replication was quantified by measuring luciferase activity in lysates of infected MDMs (B). (C) Unstimulated (US) and IVIg-activated MDMs were infected with HIV-1.luc/VSVG 2 h after IVIg stimulation and were then treated with increasing amounts of dNs (0.25–1 mM) for 24 h. HIV-1 replication was determined by measuring luciferase activity 72 h postinfection (hpi). The data are means ± SD of triplicate wells. (D) Unstimulated (US) and IVIg activated MDMs from three donors were p21-silenced by transfection with p21 siRNAs (sip21) whereas control MDMs were transfected with a pool of nontargeting siRNAs (siCTL). At 24 h postactivation and 22 h postsiencing, MDMs were lysed for the quantification of intracellular dATP and dTTP using a single nucleotide primer extension gel analysis (11). The graphs represent the mean of duplicate absolute values in fmoles/million cells of intracellular dATP and dTTP in p21 knockdown (sip21), unstimulated (US), and IVIg-activated MDMs from three different donors.
reduced LRT levels by 80–93% in unstimulated MDMs (P = 0.002) (Fig. 3D). These results demonstrate that the suppression of RNR2 by p21 induction or by siRNA-mediated knockdown inhibits HIV-1 reverse transcription and indicate that RNR2 is an essential HIV-1 cofactor that promotes reverse transcription in MDMs.

However, p21 might interfere with other molecules involved in dNTP metabolism, such as kinases of the salvage pathway, which may contribute to p21 inhibition of reverse transcription. We reasoned that, in this case, the effect of RNR2 silencing on HIV reverse transcription could be modulated in cells depleted of p21. We then performed double knockdown experiments of p21 and RNR2 in MDMs and analyzed the effects on HIV-1 infection. Unstimulated and IVIg-activated MDMs were silenced for p21 alone or for both p21 and RNR2 by transfection with specific siRNAs (sip21 and siRNR2) before infection with HIV-1.luc/VSVG. Control MDMs were transfected with equal amounts of nontargeting siRNAs. Fig. 3E and F shows results obtained with MDMs from two different donors in which RNR2 could be successfully silenced in p21-knockdown cells. As already shown, induction of p21 expression in IVIg-treated MDMs coincided with repression of RNR2 transcription (Fig. 3E). Reciprocally, p21 knockdown up-regulated RNR2 expression in both unstimulated and IVIg-activated MDMs (Fig. 3E). Silencing of p21 alone enhanced or rescued HIV-1 reverse transcription in unstimulated and IVIg-activated MDMs, respectively (Fig. 3F) whereas p21 and RNR2 cosilencing (sip21 + siRNR2) did not rescue HIV-1 reverse transcripts either in unstimulated or in IVIg-activated MDMs (Figs. 3F). Collectively, these data confirm that RNR2 suppression is sufficient to account for the inhibition of HIV-1 reverse transcription mediated by p21.

**p21-Mediated Restriction Remains Active in the Absence of SAMHD1.**

SAMHD1 was recently demonstrated to restrict HIV-1 replication in macrophages by degrading the already existing intracellular pool of dNTPs (13, 14, 19). Moreover, SAMHD1 has been shown to be degraded by HIV-2 and SIV Vpx, which confers these two viruses the ability to escape the SAMHD1 restriction in macrophages and dendritic cells. Although we have previously demonstrated that p21 inhibits HIV-2 and SIV (28, 33), we wanted to verify whether SAMHD1 is involved in p21-mediated restriction of HIV-1 described above. We first controlled the expression of SAMHD1 by WB in unstimulated and IVIg-activated MDMs. As shown in Fig. 4A, the induction of p21 protein in IVIg-stimulated MDMs did not affect SAMHD1 protein levels compared with unstimulated MDMs. We then verified that SIVmac251, which expresses Vpx, induces the degradation of SAMHD1 after infection of MDMs (Fig. 4B). In contrast, p21 expression was not affected (Fig. 4B). Furthermore, we confirmed that SIVmac251 coinfection enhances HIV-1 infection, as previously reported (21) (Fig. 4C). We then examined whether, in the presence of Vpx that degrades SAMHD1, the induction of p21 by IVIg and the knockdown of RNR2 suppress SIV replication in MDMs. Unstimulated and IVIg-activated MDMs, silenced or not for RNR2 by transfection of RNR2 siRNAs (siRNR2), were infected with the same amounts of SIVmac251 used in Fig. 4B. SIVmac251 replication, determined by quantifying p27 antigen 7 d postinfection, was inhibited by 80% in IVIg-activated MDMs (P = 0.046) (Fig. 4D), confirming our previous results (28). Importantly, SIVmac251 re-
plication was inhibited to the same extent by RNR2-knockdown (Fig. 4D). As shown in Fig. 4E, the amount of SIVmac251 LRTs, quantified by qRT-PCR, was strongly reduced (94%) in RNR2-knockdown MDMs. These results indicate that SIVmac251 infection is inhibited at the level of reverse transcription by the suppression of RNR2 through p21 induction or siRNA-mediated knockdown, despite its ability to degrade SAMHD1. Thus, the inhibition of both HIV-1 and SIVmac is caused by a blockade of dNTP synthesis by p21 that is independent of SAMHD1 dNTP catabolic pathway.

p21 Represses RNR2 Transcription by Down-Regulating E2F1 Independently from RB. Induction of RNR2 transcription depends on the transcription factor E2F1 in fibroblast cell lines (45–47). To determine whether p21 represses RNR2 gene expression in MDMs by regulating E2F1, we first studied the effects of p21 induction by IVIg stimulation and of siRNA-mediated p21 knockdown on E2F1 protein and mRNA levels by using WB and qRT-PCR, respectively. E2F1 protein was depleted in IVIg MDMs (Fig. 5A). Importantly, p21 knockdown strongly enhanced the E2F1 protein level, indicating that p21 inhibits E2F1 expression (Fig. 5A).
MDMs from four different donors, and E2F1 mRNA levels fell by 65–80% after IVIg stimulation (P = 0.014) (similarly to RNR2 mRNA) while increasing 2.15– to 8.00-fold after p21 depletion (P = 0.014) (Fig. 5B). p21 knockdown increased E2F1 mRNA levels 1.33- to 4.37-fold (P = 0.014) in IVIg-activated MDMs (Fig. 5B). These data show that p21 represses E2F1 transcription. To assess the effect of E2F1 on RNR2 transcription, we silenced E2F1 by transfecting MDMs with four specific siRNAs (siE2F1) and then measured RNR2 and p21 mRNA levels by qRT-PCR. In E2F1-silenced MDMs (siE2F1) from four different donors, E2F1 mRNA levels were reduced by 50–80% compared with control cells transfected with nontargeting siRNAs (siCTL) (P = 0.014) (Fig. 5C). Importantly, E2F1 knockdown reduced RNR2 mRNA levels by 30–70% (P = 0.014) without affecting p21 mRNA levels, indicating that E2F1 specifically transactivates RNR2 transcription in MDMs (Fig. 5C). Finally, we examined whether E2F1 knockdown, like RNR2 knockdown, inhibited HIV-1 replication. Replication of HIV-1.luc/VSVG in MDMs from four different donors was indeed inhibited, by 73–92%, after E2F1 silencing (P = 0.014) (Fig. 5D). In MDMs from three of these four donors, E2F1 knockdown inhibited HIV-1 reverse transcription (LRTs) by 56–87% (P = 0.03) (Fig. 5E), indicating that E2F1 acts as a cellular cofactor that promotes HIV-1 reverse transcription in MDMs by transactivating RNR2 expression.

p21 has been shown to inhibit E2F1 transactivation activity via the Retinoblastoma protein (RB). Indeed p21 by inhibiting cyclin-dependent kinases (CDKs) maintains RB in an activated (unphosphorylated) state able to retain E2F1 and block its transactivation activity (48, 49). However, some reports showed that p21 binds E2F1 through the N-terminal region and is able to inhibit its transactivation activity independently from RB (40, 50). To assess the role of RB in E2F1 suppression by p21 in MDMs, we first investigated the expression of RB and found that RB mRNA levels, as measured by qRT-PCR, were unaffected by p21 up-regulation or knockdown in MDMs from three donors (Fig. S3A). We then knocked down RB by specific siRNAs (siRB1) transfection. Transfection with RB siRNAs efficiently silenced RB in both unstimulated MDMs (70%) and IVIg-activated MDMs (85%), as shown by WB (Fig. S3B) and mRNA quantification (Fig. S3C). In contrast to p21 knockdown, RB silencing increased neither E2F1 nor RNR2 gene expression (Fig. S3C), indicating that RB does not affect the transcription of RNR2 in MDMs. In keeping with these results, RB knockdown, contrary to p21 knockdown, failed to enhance HIV-1 replication in unstimulated MDMs or to rescue HIV-1 replication in IVIg-activated MDMs (Fig. S3D). These findings ruled out a relevant contribution of RB to either p21 suppression of E2F1 and p21-mediated restriction of HIV-1 replication.

Discussion

Macrophages are among the cells targeted by HIV-1 and play a crucial role in the establishment of viral reservoirs and disease progression. We have previously shown that p21 inhibits the accumulation of reverse transcripts of HIV-1 and related lentiviruses in macrophages (28). Here, we examined the mechanism underlying this p21-mediated HIV-1 restriction. We show that p21 reduces the size of cellular dNTP pools required for HIV reverse transcription by suppressing the expression of RNR2, an enzyme indispensable for dNTP biosynthesis, through the down-regulation of the transcription factor E2F1. Therefore, the mechanism of p21-mediated lentiviral restriction affects the synthesis of dNTP and differs from the SAMHD1 catabolic pathway.

Lentivirus reverse transcription is much slower in macrophages than in activated CD4+ T cells (10), due in part to the low cellular dNTP levels, which create a rate-limiting step in viral cDNA synthesis (11). Although lentiviral reverse transcriptase has acquired the ability to catalyze DNA synthesis in the presence of limited dNTP pools (11), dNTP levels must still be above...
a critical threshold. Indeed, dNTP degradation by SAMHD1 impairs HIV-1 replication in noncycling myeloid cells and resting CD4+ T cells by reducing dNTPs to levels below those required for efficient reverse transcription (13–16, 19, 20). SIV and HIV-2 counteract SAMHD1 by the expression of Vpx, which targets SAMHD1 for proteosomal degradation, thus allowing these viruses to escape SAMHD1-mediated restriction in macrophages and dendritic cells (13, 14). We show here that p21 restricts HIV-1 reverse transcription by blocking the production of dNTP through the suppression of RNR2 expression, a key enzyme required for de novo pathway of dNTP synthesis. Importantly, this mechanism was also active against lentiviruses that express Vpx, such as SIVmac, despite Vpx-induced degradation of SAMHD1 in infected cells.

Interestingly, it has been reported that SAMHD1 phosphorylation by CDK1 prevents its antiviral activity without affecting its dNTP triphosphohydrolase activity (51). Because CDK1 is a probable target of p21, p21 might modulate SAMHD1 phosphorylation. In this case, p21 would have a double-negative effect on HIV-1 replication in macrophages, by blocking the dNTP synthesis on one side and by enhancing SAMHD1 antiviral activity by preventing its phosphorylation on the other side. Further studies are needed to assess the potential role of p21 in SAMHD1 phosphorylation and antiviral activity.

In mammalian cells, dNTP synthesis and degradation pathways are finely tuned to ensure a balance of the four dNTP pools (52). The predominant pathway of dNTP synthesis requires the reduction, by RNR, of ribonucleoside diphosphates (rNDP) to...
their corresponding deoxyribonucleoside diphosphates (dNDP), which are then phosphorylated to form the DNA precursor dNTP (53). In mammalian cells, RNR consists of two nonidentical subunits. The large subunit, RNR1, possesses binding sites for catalytic and regulatory activities whereas the small subunit, RNR2, provides a tyrosyl-free radical essential for nucleotide reduction. Another homologous small subunit, p53R2, also exists (44). Regulation of RNR activity has so far been studied in cell lines (mostly of murine origin) and not in terminally differentiated primary cells such as macrophages. In cycling cells, RNR2 is degraded during late mitosis, and quiescent fibroblast cell lines contain p53R2 but little or no RNR2 (54–56). Thus, it has been suggested that p53R2 serves as a partner of RNR1 in the RNR complex to supply noncycling cells that lack RNR2 with dNTP for DNA repair. However, p53R2 expression in response to DNA damage in mammalian cells did not lead to dNTP pool expansion (55). Moreover, recent reports suggest that, in quiescent cells, p53R2 is required primarily for mitochondrial DNA replication and repair (57, 58). Therefore, RNR regulation in noncycling cells is not yet fully elucidated. Surprisingly, we readily detected RNR2 mRNA and protein in terminally differentiated MDMs. The presence of RNR2, albeit at a low level, suggests that this subunit does contribute to the synthesis, by the RNR complex, of dNTPs required for DNA repair in human macrophages. As for the source of dNTP synthesis used by lentiviral reverse transcriptase in macrophages, we found, importantly, that RNR2 suppression (by p21 induction or siRNA knockdown) was sufficient to block HIV-1 and SIVmac251 reverse transcription. This result indicates that RNR2 plays a major role in providing dNTPs for HIV-1 reverse transcription in macrophages although p53R2 is also expressed in these cells. Elsewhere, increased RNR2 expression in differentiated macrophages infected with *Leishmania* has been found to enhance HIV-1 replication (59).

Our findings indicate that p21 regulates RNR2 expression at the transcriptional level in macrophages. These data concur with results from previous studies using a p21-inducible fibrosarcoma cell line (41). However, contrary to these latter cells, we found that p21 did not modulate RNR1 expression in macrophages. As p21 does not appear to contain a DNA-binding domain, it must interact indirectly with the RNR2 promoter, through another transcription factor or cofactor. The RNR2 promoter contains E2F elements (60), and RNR2 transcription in fibroblast cell lines is dependent on the transcription factor E2F1 (45–47). We found that p21 depletion strongly induced both E2F1 and RNR2 expressions in macrophages. Importantly, siRNA-mediated depletion of E2F1 down-regulated RNR2 expression and blocked HIV-1 replication by inhibiting the reverse transcription step. Similar effects were observed upon p21 induction by FcγR aggregation. Although it has been reported that RNR1 is also activated by E2F1 in cell lines (45), RNR1 expression in macrophages was not modulated upon p21 induction or knockdown. p21 can control E2F transcriptional activity through an RB-dependent pathway (48, 49) or by direct interaction with E2F1 (40, 50). RB-sensitive E2F activity is induced during the G1 phase in proliferating cells (50, 61). p21, by inhibiting CDK2 kinase, maintains RB in the active (hypophosphorylated) form, which negatively regulates the activity of E2F transcription factors during the transition through G1 in cycling cells (48, 49). In addition, RB has been reported to inhibit the expression of dNTP metabolic enzymes, including RNR2, in cell lines (62). However, these studies are not directly transposable to primary macrophages. We found that RB silencing did not significantly affect either RNR2 expression or HIV-1 replication in macrophages. Thus, RB does not appear to be involved in p21-mediated RNR2 regulation in macrophages or to play a determining role in p21-mediated HIV-1 restriction. Together, these data strongly support the following mechanism of lentiviral restriction in macrophages (Fig. 6): p21 represses E2F1 transcription, possibly by blocking E2F-binding sites in the E2F1 promoter and, therefore, E2F1 autoactivation (63, 64). The repression of E2F1 leads to down-regulation of RNR2 gene expression, which blocks de novo dNTP biosynthesis, thus impairing reverse transcription. In addition to impairing HIV cDNA synthesis, dNTP depletion following RNR2 down-regulation may also favor misincorporation of cellular rNMPs that are efficiently incorporated into HIV-1 DNA in macrophages (65, 66).

Our results suggest that HIV-1 cDNA synthesis could be inhibited by targeting RNR2, either directly or indirectly through p21 or E2F1. Interestingly, a transient reduction in residual plasma viremia and viral reservoirs has been reported in patients treated with high-dose IVIg in addition to effective highly active antiretroviral therapy (HAART) (67). It is conceivable that IVIg-mediated p21 induction in macrophages may contribute to this effect. Likewise, the ribonucleoside reductase inhibitor, hydroxyurea (HU) was shown to have a synergistic effect with conventional HAART on suppressing HIV (68, 69). However, HU inhibits both RNR2 and p53R2 subunits of RNR (70). The identification of new molecules that specifically inhibit RNR2 might lead to an improved anti-HIV-1 activity.

**Materials and Methods**

**Monocyte-Derived Macrophages.** Buffy coats from healthy donor blood were obtained from the French blood bank (Etablissement Français du Sang) as part of a convention with the Pasteur Institute. In accordance with French law, written informed consent to use the cells for clinical research was obtained from each donor. Monocytes were isolated from buffy coats and were differentiated into macrophages by using human AB serum in macrophage medium, as previously described (28, 33). For experiments, MDMs were harvested and suspended in macrophage medium containing 10% (vol/vol) heat-inactivated FBS, yielding 91–96% of CD14+ cells that also expressed FcyR5 (CD16, CD32, and CD64), differentiation markers (CD11b and CD71), and M2 macrophage polarization markers (CD163 and CD206). For Fcγ receptor stimulation, 96-well, 24-well, and 12-well plates were precoated for 2 h at 37 °C with respectively 100 μL, 250 μL, and 500 μL of 1 mg/mL human IgG for therapeutic use (IVig) (Endobuline; Baxter) in PBS. We have previously shown that immobilized IVig and preformed immune complexes have the same impact on HIV-1 replication in MDMs (33). MDMs (1 × 10^6/mL of macrophage medium + 10% FBS) were seeded in uncoated or IVIg-precoated wells (6.5 × 10^6 MDMs per well of 96-well plates, 2.5 × 10^6 MDMs per well of 24-well plates, and 0.5 × 10^6 MDMs per well of 12-well plates). Unstimulated and IVig-activated MDMs were allowed to attach to the plates for 2 h at 37 °C before infection or siRNA transfection.
sirRNA Transfection. Small interfering RNAs (siRNAs) were all purchased from Dharmacon. The siRNA against the p21 gene (si21) was on-target plus siRNA n.12. The siRNAs and the control siRNAs (siRNR2, siRNR3, and RB (siRIR) genes were siGENOME smart pool, each composed of a pool of four siRNAs. Control siRNAs were a pool of four on-target plus non-targeting siRNAs. The siRNAs used in knockdown experiments had the following sequences: si21, 5′ AGA CCA GCA UGA CAG AUU U3; siRNR2, 5′-GCA CUC UAA UGA ACG AUA A 3′; 5′-GAA GAG UAG GGA GUA U 3′; 3′-G5 UAG AAG ACC CAU UUG A 3′; and 5′-GGU UCA GCC UGU UGG ACA A 3′; siE2F1, 5′-GAA CAG GGC CAC UAG CUC U3; 5′-UGG ACC ACC UGA AUA AUU U 3′; 3′-5′ CCC AGG AGG UCA CUU CUG A 3′; and 5′-4′-5′ GGG UGG UCC AAA CUG A 3′ and siRB, 1′-3′ GAA AGA ACA UGU GAA CUA U3′; 2′-3′ GAA GAA UUA UUA G 5′ S, 3′-3′ GAA AUG ACU UCU AGA C A 5′ and 4′-3′-GGU UCA ACA CGU GGU GUA A 5′.

siRNAs transfection was performed using INTERFERin kits (Polyplus Transfection). Various amounts of siRNAs were prediluted in 1 mL of Opti-MEM, to which 20 μL of INTERFERin was added, and the transfection mix was left at room temperature for 10 min. Transfection mix volumes of 32 μL, 125 μL, and 250 μL, respectively, were added to 6.5 × 10^5, 2.5 × 10^6, and 1 × 10^6 MDMs at final concentrations of 50 nM siP21, siRNR2, or siE2F1 siRNAs, and 25 nM siRIR siRNAs. Equal amounts of on-target plus non-targeting siRNAs were added to control MDMs. MDMs were then incubated at 37 °C for 24 h. Transfection was performed with fresh macrophage medium supplemented with 10% FBS before infection. Cell lysates were assayed for protein expression by Western blot and by mRNA expression by real-time quantitative PCR (RT-qPCR) to determine the efficiency of gene knockdown at the time of infection. Western blot analysis of RB-knockdown MDMs was performed 44 h post-siRNA transfection.

Cell Infections. NL4-3-Luc HIV−1 (pseudotyped with VSVG−G HIV−1.Luc(VSVG)) was produced and quantified as previously described (28, 33). Replication-competent SIVmac251 was produced in CEM × 174 T-cell line. knockout MDMs were infected 24 h after IVg stimulation and 22 h after siRNA transfection. MDMs were infected with 4−5 ng of HIV−1.Luc(VSVG) p27 per 1 × 10^6 cells during 1 h of spinoculation (1,200 × g, room temperature) followed by 1 h at 37 °C. The cells were then washed with PBS and incubated at 37 °C in fresh macrophage medium supplemented with 10% FBS for 24 h to control SAMHD1 degradation by WB by anti-mouse antibodies (Cell Signaling). Proteins were revealed on Hyperfilm (Amersham) using the SuperSignal West picochemiluminescent substrate (Pierce). The primary antibodies—anti-PCNA (PC10), anti-RNR2 (N-18), anti-E2F1 (KH95), anti-RB (F8), and anti-RNR1 (H-300)—were all used at 1:200 dilution in 1% skimmed milk in PBS-0.05% Tween.

MDMs (0.5 × 10^5) were lysed in 100 μL of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing a protease and phosphate inhibitor mixture (Roche). Cell extracts (30−50 μg) were hybridized with the primary antibodies, followed by secondary horseradish peroxidase anti-goat, anti-rabbit (Sigma), or anti-mouse antibodies (Cell Signaling). Proteins were revealed on Hyperfilms (Amersham) using the SuperSignal West picochemiluminescent substrate (Pierce). The primary antibodies—anti-PCNA (PC10), anti-RNR2 (N-18), anti-E2F1 (KH95), anti-RB (F8), and anti-RNR1 (H-300)—were all used at 1:200 dilution in 1% skimmed milk in PBS-0.05% Tween. The primary polyclonal anti-p53R2 (1:500), monoclonal anti-SAMHD1 (1:2,000), and monoclonal anti-GAPDH (1:1,000) antibodies were from Abcam. The monoclonal anti-p21 (1:1,000) was from Millipore, and anti-actin (1:5,000) was from Sigma.

Statistical Analysis. Fold change is calculated as the ratio of the mean value from the “treated” samples (IVg activated, knockdowned MDMs) to the mean value from the control sample (unstimulated and unsilenced MDMs). The mean value is obtained from the three replicate values. Differences among groups were analyzed with the Kruskal–Wallis nonparametric test. When a significant difference was observed, the two-by-two comparisons were performed with the Mann–Whitney test. Data were analyzed with STATA software version 12.0 (Stata Corporation). Experiments with less than three values among groups were analyzed with the Kruskal–Wallis nonparametric test.

Note Added in Proof. During the submission process of this work, Diaz-Griﬃero’s and Benkiran’s laboratories reported that the anti−HIV−1 activity of SAMHD1 is modulated by its phosphorylation by Cyclin A2/CDK1 (51, 72).

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