Restriction of HIV-1 replication in macrophages and CD4⁺ T cells from HIV controllers

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How HIV controllers (HICs) maintain undetectable viremia without therapy is unknown. The strong CD8⁺ T-cell HIV suppressive capacity found in many, but not all, HICs may contribute to long-lasting viral control. However, other earlier defense mechanisms may be involved. Here, we examined intrinsic HIC cell resistance to HIV-1 infection. After in vitro challenge, monocyte-derived macrophages and anti-CD3-activated CD4⁺ T cells from HICs showed low HIV-1 susceptibility. CD4 T-cell resistance was independent of HIV-1 coreceptors and affected also SIVmac infection. CD4⁺ T cells from HICs expressed ex vivo higher levels of p21WAF1/Cip1, which has been involved in the control of HIV-1 replication, than cells from control subjects. However, HIV restriction in anti-CD3-activated CD4⁺ T cells and macrophages was not associated with p21 expression. Restriction inhibited accumulation of reverse transcripts, leading to reduction of HIV-1 integrated proviruses. The block could be overcome by high viral inocula, suggesting the action of a saturable mechanism. Importantly, cell-associated HIV-1 DNA load was extremely low in HICs and correlated with CD4⁺ T-cell permissiveness to infection. These results point to a contribution of intrinsic cell resistance to the control of infection and the containment of viral reservoir in HICs. (Blood. 2011;118(4):955-964)

Introduction

So-called HIV controllers (HICs), who represent ~0.25% of HIV-1–infected patients in France, have persistently undetectable viremia (<50 HIV RNA copies/mL) without antiretroviral therapy.¹,² Plasma viral load in HICs is similar to that observed during effective highly active antiretroviral therapy (HAART), whereas their total HIV DNA load in mononuclear blood cells is lower and stable over time.³,⁴ In many HICs, HIV-1–specific CD8⁺ T cells are able to suppress HIV-1 replication ex vivo through efficient granzyme B– and perforin-mediated killing of infected CD4⁺ T cells.⁵,⁶ In contrast, other HICs do not have high frequencies of HIV-specific CD8⁺ T cells, and their CD8 T cells do not strongly suppress HIV⁷,⁸ pointing to the existence of other mechanisms of viral control in these subjects.

Recent studies of HICs indicate that spontaneous viral control occurs during the first year after infection,¹¹ and low plasma viral load has been found during the acute phase of HIV infection in patients who are subsequently identified as HICs.¹² This suggests that the mechanisms of viral control come into play rapidly after infection. These mechanisms may include intrinsic restrictions that hamper HIV-1 replication in target cells, as shown in persons who are repeatedly exposed to HIV but remain uninfected.¹³,¹⁴ In particular, mechanisms that block HIV-1 integration in target cells may contribute to limiting the size of the viral reservoirs and to controlling the pathogenic effects of HIV.

Host genes influence the susceptibility of cells to HIV-1 infection. Whereas some factors are necessary to promote infection,¹³ in the past 10 years it has been shown that mammalian cells have devised an intrinsic immunity to block retroviral replication. Restriction factors reported so far act at different steps of viral replication, including uncoating (Trim5α), reverse transcription (Apolb3G), or viral release (Tetherin). However, HIV-1 has developed effective strategies to counteract these factors in human cells.¹⁵ Other factors that are not specific antiviral molecules may also inhibit viral replication. This is the case of p21WAF1/Cip1, a cyclin-dependent kinase inhibitor involved in regulating cell cycle and apoptosis,¹⁶,¹⁷ which we and others have shown to be able to block HIV replication in human macrophages and hematopoietic stem cells after viral entry and before genomic integration.¹⁸,¹⁹

We therefore investigated the susceptibility of CD4⁺ T cells and macrophages from HICs to HIV-1 infection in vitro. To our knowledge there are no data on the susceptibility of HIC macrophages to HIV infection, whereas several teams, including our own, have found that activated CD4⁺ T cells from HICs are as susceptible to HIV-1 infection as cells from healthy donors.⁴,⁵,⁷,²⁰ However, the conditions used in these CD4 T-cell experiments (mitogen activation or large viral inocula or both) might have overwhelmed mechanisms capable of inhibiting viral replication in vivo.²¹,²²

Here, we examined HIC CD4⁺ T-cell susceptibility to HIV-1 infection in various experimental conditions, as well as that of monocyte-derived macrophages (MDMs). Viral replication was lower in both cell types from HICs compared with controls.
Although p21<sup>Cip1/Waf1</sup> (p21) expression was higher in HIC CD4<sup>+</sup> T cells ex vivo than in cells from control groups, p21 did not appear to be directly responsible of HIV restriction in cells from HICs. CD4 T-cell permissiveness to HIV replication could be recovered by parameters enhancing viral infection and spreading, suggesting the implication of a saturable mechanism. Importantly, the level of cell-associated HIV DNA in HICs was inversely associated with the resistance of their CD4<sup>+</sup> T cells to HIV infection in vitro. These findings point to a contribution of intrinsic cell resistance to the persistent viral control observed in HICs.

### Methods

#### Subjects

HICs enrolled in the ANRS CO18 cohort are defined as patients infected by HIV-1 for ≥ 5 years who have never received antiretroviral treatment and whose last 5 consecutive plasma HIV RNA values are < 400 copies/mL. Frozen samples from 81 HICs enrolled in the cohort were used to determine total cell-associated HIV DNA levels with the use of an ultrasensitive method. Fifty-five HICs were enrolled in this study, and all agreed to provide a fresh blood sample of 30-50 mL. The patients are described in supplemental Table 1 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Blood samples from 50 healthy HIV-seronegative donors (HDs) were obtained from the French blood bank (Etablissement Français du Sang) as part of the Etablissement Français du Sang–Institut Pasteur Convention. Nine HIV-infected patients not on antiretroviral therapy and with HIV plasma viral load > 7500 RNA copies/mL (VIR) and 12 HAART-treated patients with viral load < 50 RNA copies/mL for ≥ 6 months (ART) were recruited among patients monitored at Hôpital Saint Louis and Hôpital Européen Georges Pompidou, Paris, France. The main characteristics of the 3 groups of HIV-1–infected patients are summarized in Table 1.

All the subjects gave their written informed consent in accordance with the Declaration of Helsinki, and the study was approved by the Ethics Review Committee (Comité de protection des personnes) Ile de France VII.

#### HIV DNA quantification

Total DNA was extracted from whole blood with QIAamp DNA minikits (QIAGEN), according to the manufacturer’s instructions. HIV-1 DNA was then quantified by ultrasensitive real-time PCR (long terminal repeat [LTR] amplification, ANRS, detection limit 10 copies/million leukocytes). Each extract was tested in 4 PCRs, each using 1 µg of total DNA.  

### Table 1. Characteristics of the groups of HIV-1–infected patients included in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Sex</th>
<th>Last CD4, median (range)*</th>
<th>Last viral load, median (range)†</th>
<th>Months on HAART, median (range)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV controllers</td>
<td>55</td>
<td>M:31 F:24</td>
<td>680 (266-1665)</td>
<td>&lt; 40 (&lt; 40-366)</td>
<td>—</td>
</tr>
<tr>
<td>HIV-1 viremics</td>
<td>9</td>
<td>M:6 F:3</td>
<td>378 (278-654)</td>
<td>42984 (8048-116 266)</td>
<td>—</td>
</tr>
<tr>
<td>HAART treated</td>
<td>12</td>
<td>M:10 F:2</td>
<td>633 (292-886)</td>
<td>&lt; 40 (&lt; 40-51)</td>
<td>45 (7-98)</td>
</tr>
</tbody>
</table>

*Last CD4<sup>+</sup> T-cell count/mm<sup>3</sup> of blood.

†HIV-1 RNA copies/mL of plasma with standard technique.

‡Months with successful HAART-related control of viremia before inclusion.

### Primary cell culture

CD4<sup>+</sup> and CD14<sup>+</sup> cells were purified (> 90%) from freshly isolated PBMCs by positive selection with antibody-coated magnetic beads in a Rosopset instrument (Stem Cell Technology). Monocytes were differentiated into macrophages as described elsewhere. Briefly, purified CD14<sup>+</sup> monocytes were cultured for 7-10 days in hydrophobic Teflon dishes (Lumox; D Dutcher) in macrophage medium (RPMI 1640 supplemented with 200 mM l-glutamine, 10 IU/mL penicillin, 10 µg/mL streptomycin, 10 mM HEPES, 10 mM sodium pyruvate, 50 µM β-mercaptoethanol, 1% minimum essential medium, vitamins, 1% nonessential amino acids) supplemented with 15% human AB serum. For experiments, macrophages were harvested and resuspended in macrophage medium containing 10% heat-inactivated FCS. Because of limiting amounts of MDM obtained from available blood samples, some experiments, such as p21 RNAi silencing, could be performed only in CD4 T cells.

#### Productive HIV-1 infection in vitro in optimal conditions

Purified CD4<sup>+</sup> cells were stimulated for 3 days with PHA at 1 µg/mL in the presence of IL-2 (Chiron) at 100 IU/mL. The culture medium was RPMI 1640 containing 10% FCS, penicillin (10 IU/mL) and streptomycin (10 µg/mL). CD4<sup>+</sup> T cells (10<sup>6</sup> cells/mL) were superinfected with HIV-1 BaL in triplicate at a MOI of 10<sup>-3.25</sup> in 96-U-well plates with a spinoculation protocol. After challenge, the cells were washed and cultured in 96-U-well plates (10<sup>5</sup> cells/mL in triplicate) for 14 days. Every 3-4 days the supernatant was replaced and recovered with fresh culture medium containing IL-2 (100 U/mL). Viral replication was monitored by measuring p24 in the supernatant with an ELISA method (Zeptometrix).

#### Productive infection in vitro in suboptimal conditions

Purified CD4<sup>+</sup> cells were stimulated for 5 days with an anti-CD3 monoclonal antibody (0.5 µg/mL; X35 clone; Beckman Coulter) and IL-2 (Chiron) at 50 IU/mL in RPMI 1640 medium supplemented with 10% FCS, penicillin (10 IU/mL), and streptomycin (10 µg/mL). The activated CD4<sup>+</sup> T cells were then incubated with HIV-1 BaL (R5), NL 4.3 (X4), or SIVmac251 (10<sup>-3.8</sup>, 10<sup>-3</sup>, and 10<sup>-3.8</sup> MOI, respectively) for 4 hours at 37°C. The cells were then washed twice and cultured in flat-bottom 48-well
plates (2.5 × 10⁴ cells/mL in triplicate) for 14 days. Every 3-4 days culture supernatants were recovered and replenished with fresh culture medium containing IL-2 (50 IU/mL). Viral replication was monitored in supernatants by p24 or p27 (SIVmac251) ELISA as described earlier. For experiments of saturation at high viral inoculum, serial dilutions of HIV-1 containing IL-2 (50 IU/mL) were used. Viral titers were calculated on PHA-activated CD4⁺ T cells from one representative HD and one HIC whose cells were resistant to infection. Means ± SDs of 3 independent infections are shown.

**Single-round infection**

Vesicular stomatitis virus glycoprotein (VSV-G)–pseudotyped HIV-1 particles were produced by cotransfecting (SuperFect; QIAGEN) 293T cells with the proviral pNL-Luc-E-R vector. CD4⁺ T cells and macrophages were challenged in triplicate with viral–pseudotyped particles (8.3 ng of p24/mL) in the suboptimal conditions described earlier. Luciferase activity was determined in cell lysates (Luciferase Reporter 1000 Assay System; Promega) with a Glomax plate reader (Promega) 48 hours after infection, as described.26

**PCR quantification of HIV-1 cDNA forms**

Twenty-four and 48 hours after challenge with VSV-G–pseudotyped HIV-1 particles the cells were washed in PBS, and total DNA was extracted with the DNaseasy Tissue kit (QIAGEN). Early and late reverse transcripts and viral DNA were quantified by real-time reverse transcriptase–polymerase chain reaction (RT-qPCR) as described earlier. Viral DNA copies from experiments conducted in parallel in the presence of Nevirapine 6.25μM (Boehringer Ingelheim) were subtracted to correct for DNA carryover from viral inoculum. HIC cells were studied in parallel, without in vitro challenge, to take into account the possible contribution of already-integrated HIV-1.

**Activation phenotyping**

The following antibodies were used: CD3-FITC (clone UCHT1), CD4-ECD (SFCI12T4D11), from Beckman Coulter, and CD25-V450 (M-A251) and CD69-PE(FN50) from BD Biosciences.

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**Figure 2.** CD4⁺ T cells from HICs have low susceptibility to HIV-1 infection. (A) PHA-activated CD4⁺ T cells from HICs and HDs were challenged with replicative HIV-1 Bal, using a protocol optimized to detect viral replication in vitro (left). HIV-1 replication in anti–CD3-activated CD4⁺ T cells from HICs and HDs (including all those depicted in the left panel) and HIV-1 viremic patients (VIR), after challenge with HIV-1 Bal, using a suboptimal in vitro infection protocol (right). Symbols represent the average (n = 3 independent infections) p24 values detected on day 7 after infection in culture supernatants for each donor. Horizontal lines indicate median values. (B) HIV-1 (Bal [R5], left), NL4.3 ([X4], center), and SIVmac251 (right) replication after infection, in suboptimal conditions, of CD4⁺ T cells from one representative HD and one HIC whose cells were resistant to infection. Means ± SDs of 3 independent infections are shown.

CD4⁺ T cells were incubated with the antibodies for 15 minutes then washed in PBS plus 1% FCS and fixed in 1% paraformaldehyde for flow cytometry on an LSRII device (BD Bioscience). The data were analyzed with FlowJo software (TreeStar Inc.).

**Quantitative RT-qPCR analysis**

Total RNA was extracted from macrophages and CD4⁺ T lymphocytes with the RNeasy kit (QIAGEN) and treated with DNase, following the manufacturer’s instructions. RNA was quantified with the GeneQuant method (Amersham) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). The amplification program consisted of 10 minutes at 25°C, 50 minutes at 42°C, and 15 minutes at 70°C. PCR amplification of cDNA was performed in duplicate in MicroAmp Optical 96-well reaction plates (30 μL/well), using 15 μL of TaqMan Universal Master Mix, 0.2 mM of Assays-on-Demand Gene Expression Assay premix (GAPDH, Hs99999905_m1; p21, Hs00357582_m1; APOBEC3G, Hs00222415_m1; TRIM5α, Hs01552559_m1; Applied Biosystems). The amplification conditions were as follows: 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The amount of target mRNA in each sample was normalized to GAPDH mRNA as an endogenous reference, and the data were analyzed with the cycle threshold (Ct) method. All results were expressed relative to a control cDNA, obtained from CD4⁺ T cells of a healthy donor, as 2⁻ΔΔCt, where ΔΔCt = ΔCSAMPLE – ΔCCONTROL, and ΔCt = CtTARGET – CtGAPDH.

**Western blot analysis**

Activated CD4⁺ T lymphocytes cultured in 24-well plates were lysed in 80 μL of M-PER lysis buffer (Pierce) containing the Complete Protease and Phosphatase Inhibitor Cocktail (Roche). Protein was quantified with the BCA kit (Pierce), and samples were then diluted with Laemmli buffer, boiled for 5 minutes, and loaded (30 μg) in NuPAGE gel 4%-12% (Invitrogen) for electrophoretic separation. Proteins were then blotted onto Hybond-P membranes (Amersham). After blocking with 5% skimmed milk, the membranes were incubated with the primary antibodies as indicated, followed by a secondary HRP-conjugated anti–mouse antibody (R&D Systems). Proteins were shown on Hyperfilms (Amersham) with the use of the ECL chemiluminescent substrate (GE Healthcare) and X-Omat film.
The anti-p21 mouse monoclonal antibody (1:1000) was from Upstate, and anti-GAPDH (1:5000) was from Abcam.

Small interfering RNA transfection

Small interfering RNA (siRNA) duplexes no. 9 and no. 12 from the SMARTpool for p21 were purchased from Dharmacon, and irrelevant negative control siRNA (5'-H11032-CTGCATCGTGCACAGGAGTATCA-3'-H11032) was synthesized by QIAGEN. siRNA transfection was performed with the AMAXA human T-cell Nucleofector kit (LONZA), following the manufacturer’s instructions. Briefly, 5 days after activation with anti-CD3, 2.5-4 x 10^6 CD4 T cells were centrifuged and resuspended in 100 μL of Nucleofector solution. siRNA was added at 200 pmoles per million cells. The Nucleofector program T-023 was used. The cells were then transferred into 2 mL of culture medium without antibiotics in 12-well plates and incubated at 37°C for 24 hours before infectious challenge. Cell lysates were assayed for mRNA by qualitative RT-PCR to determine the efficiency of gene knockdown at the moment of infection.

Statistical analyses

P values were calculated with the rank sum test. Correlations were identified with simple linear regression analysis and Spearman’s rank correlation test. SigmaStat 3.5 software was used (Systat Software Inc).

Results

Macrophages and CD4 T cells from HICs have low susceptibility to HIV-1 infection

Intrinsic resistance of target cells to HIV-1 infection may contribute to control of infection and, in particular, to the small size of the viral reservoir in HICs. Macrophages are one of the main HIV-1 cell targets, and they play a crucial role in the spread and pathogenesis of HIV-1 infection. However, HIC macrophage susceptibility to HIV-1 infection has not previously been studied. We challenged MDMs from 12 HICs and 11 HDs with HIV-1 BaL. Viral replication was lower in HIC MDMs than in HD MDMs on day 3 after challenge and remained lower thereafter (Figure 1A). Overall, viral replication was far lower in HIC MDMs (median, 8.97 ng p24/mL) than in HD MDMs (median, 125 ng p24/mL; P = .003; Figure 1B). The reduced susceptibility of macrophages to HIV-1 infection prompted us to wonder whether restriction to infection extended to other cell targets in HICs. Previous work, including our own, has suggested that CD4 T cells from HICs and HDs are similarly susceptible to HIV-1 infection in vitro. However, it is conceivable that the optimized viral challenge...
However, we noted clear differences in susceptibility between conditions used in these studies (potent stimuli or large viral inocula or both) masked restrictive mechanisms operating in vivo. We therefore reassessed the susceptibility of CD4+ T cells from 31 HICs to HIV-1 superinfection, using CD3 cross-linking (a more physiologic stimulus than PHA) and suboptimal conditions of cell activation and challenge. CD4+ T cells from HICs and HDs showed similar susceptibility to HIV-1 in the conditions of activation/challenge used in our previous reports (median, 469.1 and 568.2 ng p24/mL of culture supernatant, respectively; \( P = .35 \); Figure 2A left). In suboptimal conditions, however, we noted clear differences in susceptibility between CD4+ T cells from HICs (median, 3.43 ng p24/mL) and HDs (median, 59.3 ng p24/mL; Figure 2A right). HIV-1 also replicated more efficiently in superinfected CD4+ T cells from HIV viremic patients (median, 21.3 ng p24/mL), although replication of endogenous virus may have been a confounding factor. Interestingly, this reduced susceptibility of CD4+ T cells from HICs was observed with both R5-tropic and X4-tropic viruses (see Figure 2B for a representative example and supplemental Figure 1A for the summary of the experiments done with X4 HIV-1 and cells from 11 HICs and 7 HDs). Moreover, CD4+ T cells from HICs showed a remarkable broad resistance to lentiviral infection because we found that they were also less susceptible to infection with SIVmac251 than CD4+ T cells from HDs (Figure 2B shows a representative example and supplemental Figure 1B the summary of the experiments with cells from 8 HICs and 5 HDs). Overall, our results show that both macrophages and CD4+ T cells from HICs have a marked reduced permissiveness to HIV replication in vitro.

Reduced HIV-1 susceptibility of cells from HICs is because of restriction occurring during reverse transcription

We performed one-round infection experiments with HIV-1 NL4.3Δenv particles carrying the luciferase reporter gene and pseudotyped with the pantropic VSV-G protein. CD4+ T cells from HICs were also less susceptible than cells form HDs to single-cycle HIV-1 replication. A representative example of experiments with cells from 13 HICs and 11 HDs is shown in Figure 3A right. Productive and single-cycle HIV-1 infections were assessed in parallel in CD4+ T cells from 8 HICs and as many HDs. With one exception, the relative levels of viral replication in HD CD4+ T cells infected with HIV-1 replicative virus and with pseudotyped particles were correlated (Spearman, 0.893; \( P < .00001; n = 7 \) HICs and 7 HDs; Figure 3A for an example). As observed with CD4+ T cells, the HIV restriction in MDMs was already operating at the first cycle of viral replication (Figure 3B for a representative example). These results ruled out the involvement of HIV coreceptors in viral restriction and pointed to a role of factors affecting early steps of the replicative cycle.

We analyzed whether the block-affected replication before or after viral integration by quantifying at 48 hours after infection the number of integrated provirus (Alu-LTR qPCR) in CD4+ T cells from 7 HICs and 7 HDs. The number of integrated proviruses in cells from HICs was very low compared with HD cells, and this reduction corresponded to the reduction in the level of viral replication measured by luciferase activity (Figure 3C), pointing to a restriction in preintegrative steps of viral replication. To find out if the block occurred during reverse transcription, we quantified early (R-U5) and late (U5-gag) reverse transcripts at 24 and 48 hours after infection in the cells from 5 HICs and 5 HDs. One representative example of these experiments is shown in Figure 3D. We found that the kinetics of viral replication were slower in the CD4+ T cells from HDs when infected in suboptimal conditions than what we have previously reported with PHA stimulation and optimal conditions of infection. Whereas a sharp accumulation of early transcripts, and proportionate increase in late reverse transcripts, occurs between 24 and 48 hours in the cells from HDs, the reverse transcripts did not accumulate in the cells from...
HICs. Thus, our results indicate that the block of HIV-1 replication in the cells from HICs occurs during reverse transcription.

CD4+ T cells from HICs express high levels of p21\textsuperscript{Cip1/Waf1} ex vivo, but this does not appear to explain their reduced susceptibility

We wondered whether p21 might be responsible for the reduced susceptibility of HIC cells to HIV-1 because we have found that p21 affects reverse transcription.\textsuperscript{16} We quantified p21 mRNA levels in MDMs at the time of infection and found no difference between MDMs from HICs (median, 2.53; n = 7) and HDs (median, 2.91; n = 11; P = 1.0; Figure 4A), and no correlation was found either between p21 expression and MDM susceptibility to HIV-1 infection (supplemental Figure 2A). In contrast, we found higher levels of p21 mRNA in freshly isolated CD4\textsuperscript{+} T cells from HICs (median, 3.06 p21 mRNA relative expression; n = 26) than in those from HDs (median, 1.13 p21 mRNA relative expression; P < .0001; n = 29; Figure 4B left). The p21 levels were still higher in fresh CD4\textsuperscript{+} T cells from HICs than in cells from HIV-1 viremic patients and HAART-treated patients with HIV-RNA < 50 copies/mL (1.64, P = .059, n = 8; and 0.86, P = .002, n = 10, respectively; Figure 4B). However, we found no correlation between p21 levels in their freshly isolated CD4\textsuperscript{+} T cells and the susceptibility of activated CD4\textsuperscript{+} T cells to HIV-1 infection in HICs and HDs for whom both parameters were available (supplemental Figure 2B). Moreover, p21 mRNA levels dropped sharply on anti-CD3 stimulation of CD4\textsuperscript{+} T cells from HICs and controls, and p21 mRNA levels at the time of infection were not higher in cells from HICs than in cells from persons in control groups (Figure 4B right). In addition, the reduced susceptibility of HIC CD4\textsuperscript{+} T cells to HIV-1 superinfection could not be explained by higher p21 protein levels at the time of infection (Figure 4C). Finally, p21 knockdown with specific siRNA in anti-CD3-activated CD4\textsuperscript{+} T cells from HICs did not increase their susceptibility to HIV (n = 4). One of these experiments is depicted in Figure 4D. Therefore, although freshly isolated HIC CD4\textsuperscript{+} T cells carried high levels of p21 mRNA, we found no evidence of a direct role of p21 in the reduced susceptibility of HIC CD4\textsuperscript{+} T cells and MDMs to HIV-1 infection that we observed in vitro.

We then examined CD4\textsuperscript{+} T-cell expression of Apobec3G and Trim5\alpha, two restriction factors that might interfere with early steps of viral replication. No difference was found between HICs, HDs (supplemental Figure 3A), and HAART-treated patients (supplemental Figure 3B), either before or after CD3 stimulation.

Reduced HIV-1 susceptibility of cells from HICs is overcome by high viral inocula

We wondered whether the reduced susceptibility of CD4\textsuperscript{+} T cells from HICs might be associated with a different response (activation or proliferation) to anti-CD3 stimulation. CD4\textsuperscript{+} T cells from HICs and HDs showed similar proliferation before HIV challenge (Figure 5A) and expressed similar levels of CD25 and CD69 (Figure 5B). In our previous work,\textsuperscript{7} in addition to PHA, we used spinoculation to enhance the binding of viral particles to CD4\textsuperscript{+} T cells,\textsuperscript{26} together with high cell densities to favor cell-to-cell HIV-1 transmission.\textsuperscript{31} Here, we explored the effect of each of these factors on HIC CD4\textsuperscript{+} T-cell susceptibility to infection. Used separately, a higher cell density and spinoculation both enhanced viral replication in CD4\textsuperscript{+} T cells, especially in cells from HICs, in which viral replication was otherwise barely detectable (Figure 6A). These conditions diminished the difference between CD4\textsuperscript{+} T cells from HICs and HDs (Figure 6A). This effect was even more pronounced when spinoculation and a high cell density were used simultaneously. These results confirmed that the reduced susceptibility of HIC CD4\textsuperscript{+} T cells to HIV-1 may be overcome by favoring binding of virus to the target cells or cell-to-cell transmission, thus pointing to a saturable mechanism. To confirm this point, we performed infections of CD4\textsuperscript{+} T cells from 5 HICs and 6 HDs with the use of serial dilutions of HIV-1 BaL and suboptimal conditions of infection (a representative experiment is shown in Figure 6B). In all cases more virus was necessary to detect viral replication in the cells from HICs (median dilution for first positive p24 value, $10^{-5}$ [10\textsuperscript{-3} to 10\textsuperscript{-7}] and 10\textsuperscript{-11} [10\textsuperscript{-7} to 10\textsuperscript{-13}] for HICs and HDs, respectively). However, at higher viral inocula the level of viral replication in CD4\textsuperscript{+} T cells from HICs approached the one in cells from HDs. This result further corroborates the presence of a saturable mechanism of HIV-1 restriction in the cells from HICs.

HIC CD4\textsuperscript{+} T-cell permissiveness to HIV-1 replication correlates with the size of the viral reservoir in vivo

Finally, we examined whether the reduced susceptibility of HIC cells to HIV-1 infection in vitro might contribute to limiting the infection in vivo and, particularly, the size of their HIV-1 reservoir. The HIV-1 DNA level in blood cells is a stable parameter reflecting the size of the total HIV-1 reservoir, being proportional to and correlating with HIV-DNA levels in gut-associated lymphoid tissue.\textsuperscript{32} We quantified total HIV-1 DNA in blood cells from 81 HICs enrolled in the ANRS CO18 cohort. PBMC-associated HIV-1 DNA levels ranged from undetectable (< 11 HIV-DNA copies/10\textsuperscript{6} PBMCs or < 1.04 log) to 728 copies/10\textsuperscript{6} PBMCs (2.86 log). Overall, HIV-1 DNA levels were far lower (1.45 log copies/10\textsuperscript{6} PBMCs; range, < 1.34-1.65) in HICs than in patients with primary infection (ANRS PRIMO cohort: 3.30 log copies/10\textsuperscript{6} PBMCs; range, 1.84-4.93 log copies/10\textsuperscript{6} PBMCs; n = 674),\textsuperscript{35} in patients with chronic infection (ANRS SEROCO cohort: 2.86 log copies/10\textsuperscript{6} PBMCs; interquartile range [IQR], 2.45-3.21 log copies/10\textsuperscript{6} PBMCs; n = 271),\textsuperscript{34} in patients on effective HAART (SALTO study: 2.3 log copies/10\textsuperscript{6} PBMCs; IQR, 1.9-2.8 log copies/10\textsuperscript{6} PBMCs; n = 116),\textsuperscript{35} and om long-term nonprogressors (ANRS...
ALT cohort: 2.29 log copies/10^6 PBMCs; IQR, 1.53-2.89 log copies/10^6 PBMCs; n = 50).38 Thus, our results on a large group of HICs extend previous observations and confirm that HICs have a remarkably small HIV reservoir.

We then compared blood cell viral DNA load in 23 HICs who have been studied for susceptibility to infection in vitro and the level of HIV-1 replication in their CD4^+ T cells and found a positive correlation (Spearman, 0.42, P = .044; n = 23; not shown). In other words, those HICs whose CD4^+ T cells were least susceptible to HIV-1 infection ex vivo were also those with the lowest levels of total cell-associated HIV-1 DNA in vivo (Figure 7). These results support a role of target cell resistance in the control of HIV-1 infection in HICs.

Discussion

Extraordinarily low levels of cell-associated HIV DNA are found in HICs, suggesting that these persons possess mechanisms that hamper the formation of viral reservoirs. We and others have previously described a strong and effective cytotoxic T-cell response that probably plays a role in maintaining viral load at low or undetectable levels.5-7,37 However, other mechanisms may participate in the observed viral control. Simian studies show that the viral reservoir is constituted very early during primary SIV or SHIV infection, when adaptive T-cell responses may not yet be fully developed.38 HIV-1 DNA levels may already be low during primary infection in patients who are later identified as HICs.15 In addition, a strong CTL response is found only in part of HICs8-10 (A.S.-C., G.P., unpublished data on > 150 HIC followed up in the ANRS CO18 cohort since 2009). Here, we show that the main cellular targets of HIV-1, namely CD4^+ T lymphocytes and macrophages, exhibit far lower susceptibility to HIV infection in HICs than in the general population or in HIV-1–infected patients noncontrolling infection. Importantly, low CD4^+ T-cell permissiveness in vitro was associated with low levels of viral DNA in vivo, suggesting that this intrinsic cell resistance contributes to restricting the size of the viral reservoir in HICs. We found that HIV-1 replication in target cells from HICs was restricted independently of viral tropism for cell receptors and that the mechanism is saturable and affects reverse transcription. Moreover, CD4^+ T cells from HICs were also resistant to SIVmac251 infection.

Viral replication in MDMs from most HICs was severely impaired. Macrophages contribute to viral dissemination in body tissues, including “sanctuaries” such as the brain, and to the establishment of viral reservoirs.39-41 A major role of macrophages in HIV-1 spread and pathogenesis is suggested by numerous studies, including one showing that infection of macaques with an SIV(SM) PBj virus unable to replicate in macrophages is associated with milder infection and low viral load.42 Therefore, inhibition of HIV-1 replication in macrophages might have important consequences for the human infection.

CD4^+ T cells from HICs were also less susceptible to HIV-1 infection in vitro. However, restriction of viral replication in HIC CD4^+ T cells was overcome by spinoculation, by increasing the target cell density or by high virus inoculum. This “saturability” may explain why previous studies, including our own, failed to show reduced susceptibility of CD4^+ T cells from HICs in optimized conditions of HIV-1 challenge.4,5 This may also account for the apparent discrepancy between our results that suggest a restriction during reverse transcription in HIC and the results reported very recently by Rabi et al,20 who found no differences in HIV-1 entry and early steps of viral replication between CD4^+ T cells from HICs and uninfected donors. In addition to other experimental differences (we used CD3-activated CD4^+ T cells, whereas Rabi et al20 used unstimulated CD4^+ T cells), they used spinoculation to infect cells with pseudotyped HIV-1 viruses, a technique that increases viral concentration on the cell surface and can overcome viral restriction, as we show here. While we were reviewing the manuscript, a report by Graf et al43 showed that CD4^+ T cells from HICs carry in vivo low levels of integrated HIV DNA, which is in agreement with our results showing a restriction before viral integration. However, the investigators could not find a restriction of HIV-1 replication in vitro, but the use of spinoculation may have influenced these experiments. Graf et al43 also show an accumulation of 2-LTR nonintegrated forms in the cells from HICs. In our experiments restriction of viral replication occurred during reverse transcription; however, because of the limited number of persons in which this aspect could be assessed, we cannot discard that viral replication may be blocked at different stages of viral replication in other HIC.

Our results suggest that HIV-1 restriction mechanisms in HICs can be saturated, as can TRIM5α-mediated restriction.22,23 However, TRIM5α is species specific and cannot account for the inhibition of the HIV-1 replicative cycle in cells from HICs. This is also supported by the lack of modulation of TRIM5α expression in our experiments (supplemental Figure 3). The phenotype of HIV-1 restriction found in cells from HICs is reminiscent of that induced...
by APOBEC3G, which also affects preintegration steps. A negative correlation between plasma viral load in HIV-1–infected patients and APOBEC3G mRNA levels has been reported, but more recent studies failed to confirm this result or showed a positive correlation. APOBEC3G-induced hypermutation of HIV-1 proviral DNA was not found to be higher in HICs than in HAART-treated patients. In addition, APOBEC3G-mediated inhibition is effective in the absence of Vif, and we used Vif– replicative HIV-1 strains and pseudotypes. Finally, APOBEC3G expression was not significantly different in CD4+ T cells from HICs than in those from HDs (supplemental Figure 3). Overall, our results do not support the involvement of APOBEC3G in the restriction of viral replication in cells from HICs.

Another candidate factor possibly explaining the relative resistance of HIC cells to HIV-1 is p21. Indeed, p21-mediated inhibition of HIV-1 replication in macrophages and hematopoietic stem cells affects preintegration steps, leading to a strong reduction in the level of integrated proviruses. In addition, while reviewing the manuscript, Chen et al. reported a resistance of CD4+ T cells from HICs to HIV-1 infection. Those investigators showed that resistance in their setting was because of elevated levels of p21 in the cells from HICs. In our study p21 expression in MDMs did not differ between HICs and uninfected controls, even though viral replication was also far lower in HIC MDMs. Although we found that p21 expression was higher in CD4+ T cells from HICs than from healthy controls, it did not correlate with CD4+ T-cell susceptibility to HIV-1 infection. Furthermore, on CD3 activation, at infectious challenge, p21 mRNA levels in CD4+ T cells were down-regulated and did not differ between HICs and controls. Neither p21 protein expression correlated with the level of HIV-1 replication. Finally, siRNA-mediated p21 knock-down did not restore HIV replication in HIC CD4+ T cells. Overall, the results of the present study argue against a direct role of p21 in the HIV-1 restriction observed here and indicate that additional cell restriction factors are responsible for the resistance of HIC MDMs and CD4 T cells that we found. Our results do not exclude, however, that p21 may have played an indirect role by modulating cellular factors involved in HIV-1 replication or that p21 may play a role in regulating HIV-1 replication in CD4+ T cells in vivo.

In conclusion, our data suggest that HIV target cells in HICs are intrinsically resistant to infection, possibly contributing to the observed control of viral replication in vivo. In particular, mechanisms blocking viral DNA integration in CD4+ T cells and macrophages appear to hinder the formation of the viral reservoir, as suggested by the negative correlation between the degree of CD4+ T-cell resistance and the level of cell-associated HIV-1 DNA. Although the restriction observed in vitro can be overcome by optimizing the conditions of viral challenge in vitro, it is probably an effective barrier to viral spread in vivo. This intrinsic resistance could cooperate with specific T-cell responses in controlling HIV infection and might have a prominent role in HICs.

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A list of the members of the ANRS CO18 Cohort is available in the supplemental Appendix.

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Restriction of HIV-1 replication in macrophages and CD4+ T cells from HIV controllers

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