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Abstract

How HIV controllers (HIC) maintain undetectable viremia without therapy is unknown. The strong CD8+ T cell HIV suppressive capacity found in many, but not all, HIC may contribute to long lasting viral control. However, other earlier defence 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 HIC showed low HIV-1 susceptibility. CD4 T cell resistance was independent of HIV-1 coreceptors and affected also SIVmac infection. CD4+ T cells from HIC expressed ex vivo higher levels of $p21^{Waf1/Cip1}$, 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 to 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 HIC 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 HIC.

Introduction

So-called HIV controllers (HIC), who represent about 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 HIC is similar to that observed during effective HAART, while their total HIV DNA load in mononuclear blood cells is lower and stable over time [1,3,4]. In many HIC 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 [5-7]. In contrast, other HIC do not have high frequencies of HIV-specific CD8+ T cells, and their CD8 T cells do not strongly suppress HIV [8-10], pointing to the existence of other mechanisms of viral control in these subjects.

Recent studies of HIC 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 HIC [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 individuals 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. While some factors are necessary to promote infection [13], in the last ten 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 (Apobec3G) or viral release (Tetherin). However, HIV-1 has developed effective strategies to counteract these factors in human cells

[15]. Other factors which are not specific antiviral molecules may also inhibit viral replication. This is the case of p21$^{Cip1/Waf1}$, a cyclin-dependent kinase inhibitor involved in regulating cell cycle and apoptosis [16,17], which we and others have recently 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 HIC to HIV-1 infection *in vitro*. To our knowledge there are no data on the susceptibility of HIC macrophages to HIV infection, while several teams, including our own, have found that activated CD4+ T cells from HIC are as susceptible to HIV-1 infection as cells from healthy donors [4,7,20]. However, the conditions used in these CD4 T cell experiments (mitogen activation and/or large viral inocula) might have overwhelmed mechanisms capable of inhibiting viral replication in vivo [21-23].

Here we examined HIC CD4+ T cell susceptibility to HIV-1 infection in various experimental conditions, as well as that of monocyte-derived macrophages (MDM). Viral replication was lower in both cell types from HIC as compared to controls. Although p21$^{Cip1/Waf1}$ (p21) expression was higher in HIC CD4+ T cells *ex vivo* than in cells from control groups, p21 did not appear to be directly responsible of HIV restriction in cells from HIC. 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 HIVDNA in HIC was inversely associated to the resistance of their CD4+ T cells to HIV infection *in vitro*. These findings point to a contribution of intrinsic cell resistance to the persistent viral control observed in HIC.

MATERIALS AND METHODS

Subjects

HIV controllers enrolled in the ANRS CO18 cohort are defined as patients infected by HIV-1 for at least 5 years who have never received antiretroviral treatment and whose last 5 consecutive plasma HIV RNA values are below 400 copies/ml. Frozen samples from 81 HIC enrolled in the cohort were used to determine total cell-associated HIV DNA levels using an ultrasensitive method. Fifty five HIC were enrolled in this study, and all agreed to provide a fresh blood sample of 30-50 ml. The patients are described in Supplementary Table 1. Blood samples from 50 healthy HIV-seronegative donors (HD) were obtained from the French blood bank (Etablissement Français du Sang, EFS) as part of the EFS-Institut Pasteur Convention. Nine HIV-infected patients not on antiretroviral therapy and with HIV plasma viral load above 7500 RNA copies/ml (VIR) and 12 HAART-treated patients with viral load below 50 RNA copies/ml for at least 6 months (ART) were recruited among patients monitored at Hôpital Saint Louis and Hôpital Européen Georges Pompidou, Paris, France. Table 1 summarizes the main characteristics of the three groups of HIV-1-infected patients.

All the subjects gave their written informed consent, and the study was approved by the Ethics review committee CPP (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 (LTR

amplification, ANRS, detection limit 10 copies/million leukocytes). Each extract was tested in four PCRs, each using one microgram of total DNA [24].

Primary cell culture

CD4+ and CD14+ cells were purified (>90%) from freshly isolated peripheral blood mononuclear cells (PBMC) by positive selection with antibody-coated magnetic beads in a Robosep instrument (Stemcell Technology, France). Monocytes were differentiated into macrophages as described elsewhere [25]. Briefly, purified CD14+ monocytes were cultured for 7 to 10 days in hydrophobic Teflon™ dishes (Lumox™, D Dutscher) 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 fetal calf serum (FCS). Ought to 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+ cells were stimulated for 3 days with phytohaemagglutinin (PHA) at 1 μg/ml in the presence of interleukin-2 (IL-2) (Chiron, France) at 100 IU/ml. The culture medium was RPMI 1640 containing 10% FCS, penicillin (10 IU/ml) and streptomycin (10 μg/ml). CD4+ T cells (10^6 cells/ml) were super-infected with HIV-1 BaL (R5) in triplicate at a multiplicity of infection (m.o.i.) of $10^{-3.25}$ in 96-U-well

plates with a spinoculation protocol [26]. After challenge, the cells were washed and cultured in 96-U well plates (10^6 cells/ml in triplicate) for 14 days. Every 3-4 days the supernatant was recovered and replaced with fresh culture medium containing IL-2 (100 U/ml). Viral replication was monitored by measuring p24 in the supernatant with an ELISA method (Zetometrix, Gentaur, France).

Productive infection in vitro in suboptimal conditions

Purified CD4+ T cells were stimulated for 5 days with an anti-CD3 monoclonal antibody (X35 clone, Beckman Coulter)(0.5 ug/ml) and IL-2 (Chiron, France) at 50 IU/ml in RPMI 1640 medium supplemented with 10% FCS, penicillin (10 IU/ml) and streptomycin (10 ug/ml). The activated CD4+ T cells were then incubated with HIV-1 BaL (R5), NL 4.3 (X4) or SIVmac251 ($10^{-3.8}$, 10^{-3} and $10^{-3.8}$ m.o.i, respectively) for 4 h at 37°C. The cells were then washed twice and cultured in flat-bottom 48-well plates ($2.5x10^5$ 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 above. For experiments of saturation at high viral inoculum, serial dilutions of HIV-1 BaL concentrated by ultracentrifugation ($10^{17.5}$ $TCID_{50}$/ml) were used. All viral titers were calculated on PHA-activated CD4+ T cells using optimal conditions of infection.

Single-round infection

VSV-G pseudotyped HIV-1 particles were produced by co-transfecting (SuperFect, Qiagen) 293T cells with the proviral pNL-Luc-E-R+ [27] and the pMD2 VSV-G expression vector. CD4+ T cells and macrophages were challenged in triplicate with HIV-1 pseudotyped particles (8.3 ng of p24/ml) in the suboptimal conditions

described above. Luciferase activity was determined in cell lysates (Luciferase Reporter 1000 Assay System, Promega France) with a Glomax microplate luminometer (Turner BioSystems, Promega, France) 48 h after infection, as described [28].

qPCR quantification of HIV-1 cDNA forms

Twenty-four and forty-eight hours after challenge with VSV-G pseudotyped HIV-1 particles the cells were washed in PBS and total DNA was extracted with the DNeasy Tissue kit (Qiagen). Early and late reverse transcripts and integrated HIV-1 DNA were quantified by real-time R-U5 PCR, U5-Gag PCR and *Alu*-Gag nested PCR, respectively, on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as described elsewhere [25]. DNA loading was controlled by concurrently amplifying the albumin gene by rtPCR and quantifying with reference to a control human genomic DNA (Roche). HIV-1 DNA copies from experiments conducted in parallel in presence of Nevirapine 6.25 µM (Boehringer Ingelheim) were subtracted to correct for DNA carryover from viral inoculum. HIC cells were studied in parallel, without in vitro challenge, in order 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 (San Diego, CA); and CD25-V450 (M-A251), CD69-PE(FN50) from BD Biosciences (San Jose, CA).

CD4+ T cells were incubated with the antibodies for 15 min 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 (Tree Star, USA).

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 min at 25°C, 50 min at 42°C and 15 min at 70°C. PCR amplification of cDNA was carried out in duplicate in MicroAmp Optical 96-well reaction plates (30 µl/well), using 15 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; APOBEC3G, Hs00222415_m1; TRIM5α, Hs01552559_m1) (Applied Biosystems). The amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 60 s, 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 analysed with the cycle threshold (Ct) method [29]. All results were expressed relative to a control cDNA, obtained from $CD4^+$ T cells of a healthy donor, as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{SAMPLE} - \Delta Ct_{CONTROL}$, and $\Delta Ct = Ct_{TARGET\ GENE} - Ct_{GAPDH}$.

Western blot

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 min 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 HPR-conjugated anti-mouse antibody (R&D Systems). Proteins were revealed on Hyperfilms (Amersham) using the ECL chemiluminescent substrate (GE Healthcare) and X-Omat film (Kodak). 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 n°9 and n°12 from the SMARTpool for p21 were purchased from Dharmacon, and irrelevant negative control siRNA (5'-CTGCATCGTGCACAGGAGTATCA-3') was synthesized by Qiagen. siRNA transfection was performed with the AMAXA human T cell Nucleofector kit (LONZA) following the manufacturer's instructions. Briefly, five days after activation with anti-CD3, 2.5 to $4x10^6$ 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 h before infectious challenge. Cell lysates were assayed for mRNA by RT-qPCR 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 by using simple linear regression analysis and Spearman's rank correlation test. SigmaStat 3.5 software was used (Systat Software Inc-SSI, CA).

Results

Macrophages and CD4+ T cells from HIV controllers 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 HIC. 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 monocyte-derived macrophages (MDM) from 12 HIC and 11 HD with HIV-1 BaL. Viral replication was lower in HIC MDM than in HD MDM on day 3 post-challenge, and remained lower thereafter (Figure 1A). Overall, viral replication was far lower in HIC MDM (median 8.97 ng p24/ml) than in HD MDM (median 125 ng p24/ml, p=0.003) (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 HIC. Previous work, including our own, has suggested that CD4+ T cells from HIC and healthy donors are similarly susceptible to HIV-1 infection *in vitro* [4,7,20]. However, it is conceivable that the optimized viral challenge conditions used in these studies (potent stimuli and/or large viral inocula) masked restrictive mechanisms operating in vivo [21-23]. We therefore reassessed the susceptibility of CD4+ T cells from 31 HIC to HIV-1 superinfection, using CD3 cross-linking (a more physiological stimulus than PHA) and suboptimal conditions of cell activation and challenge [30]. CD4+ T cells from HIC and HD 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=0.35; Figure 2A, left). In suboptimal conditions, however, we noted clear differences in susceptibility between CD4+ T cells from HIC (median 3.43 ng p24/ml) and HD (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 HIC was observed with both R5-tropic and X4-tropic viruses (see Figure 2B for a representative example and Figure S1A for the summary of the experiments done with X4 HIV-1 and cells from 11 HIC and 7 HD). Moreover, CD4+ T cells from HIC showed a remarkable broad resistance to lentiviral infection as we found that they were also less susceptible to infection with SIVmac251 than CD4+ T cells from HD (Figure 2B shows a representative example and Figure S1B the summary of the experiments with cells from 8 HIC and 5 HD). Overall, our results show that both macrophages and CD4+ T cells from HIC have a marked reduced permissiveness to HIV replication in vitro.

Reduced HIV-1 susceptibility of cells from HIC is due to 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 HIC were also less susceptible than cells form HD to single cycle HIV-1 replication. A representative example of experiments with cells from 13 HIC and 11 HD is shown in figure 3A (right panel). Productive and single cycle HIV-1 infection were assessed in parallel in CD4+ T cells from 8 HIC and as many HD. With one exception, the relative levels of viral replication in HIC CD4+ T cells infected with HIV-1 replicative virus and with pseudotyped particles were correlated (Spearman 0.893 $p<0.00001$, n=7 HIC and 7 HD) (figure 3A for an example). As observed with CD4+ T cells, the HIV restriction in MDM 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 48h post infection the number of integrated provirus (*Alu*-LTR qPCR) in CD4+ T cells from 7 HIC and 7 HD. The number of integrated provirus in cells from HIC was very low compared to 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 24h and 48h post infection in the cells from 5 HIC and 5HD. 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 HD when infected in suboptimal conditions than what we have previously reported using PHA stimulation and optimal conditions of infection [14]. Whereas a sharp accumulation of early transcripts, and proportionate increase in late reverse transcripts, occurs between 24h and 48h in the cells from HD, the reverse transcripts did not accumulate in the cells from HIC. Thus, our results indicate that the block of HIV-1 replication in the cells from HIC occurs during reverse transcription.

CD4+ T cells from HIC express high levels of p21$^{Cip1/Waf1}$ 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 as we have found that p21 affects reverse transcription [18]. We quantified p21 mRNA levels in MDM at the time of infection and found no difference between MDM from HIC (median 2.53, n=7) and HD (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 (Figure S2A). In contrast, we found higher levels of p21 mRNA in freshly isolated CD4+ T cells from HIC (median 3.06 p21 mRNA relative expression, n=26) than in those from HD (median 1.13 p<0.0001, n=29) (Figure 4B, left panel). P21 levels were still higher in fresh CD4+ T cells

from HIC when compared to those in cells from HIV-1 viremic patients and HAART-treated patients with HIV-RNA <50 copies/ml(1.64 p=0.059, n=8; and 0.86 p=0.002, n=10; respectively for viremic and HAART-treated patients) (Figure 4B). However, we found no correlation between p21 levels in their freshly isolated CD4+ T cells and the susceptibility of activated CD4+ T cells to HIV-1 infection in HIC and HD for whom both parameters were available, (Figure S2B). Moreover, p21 mRNA levels dropped sharply upon anti-CD3 stimulation of CD4+ T cells from HIC and controls, and p21 mRNA levels at the time of infection were not higher in cells from HIC than in cells from individuals in control groups (Figure 4B, right panel). The reduced susceptibility of HIC CD4+ T cells to HIV-1 superinfection could not be explained either by higher p21 protein levels at the time of infection (Figure 4C). Finally, p21 knockdown with specific siRNA in anti-CD3-activated CD4+ T cells from HIC did not increase their susceptibility to HIV (n=4). One of these experiments is depicted in Figure 4D. Therefore, although freshly isolated HIC CD4+ 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+ T cells and MDM to HIV-1 infection that we observed in vitro.

We then examined CD4+ T cell expression of Apobec3G and Trim5α, two other restriction factors that might interfere with early steps of viral replication. No difference was found between HIC, HD (Figure S3A) and HAART-treated patients (Figure S3B), either before or after CD3 stimulation.

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

We wondered whether the reduced susceptibility of CD4+ T cells from HIC might be associated with a different response (activation or proliferation) to anti-CD3 stimulation. CD4+ T cells from HIC and HD showed similar proliferation prior to HIV challenge (figure 5A) and expressed similar levels of CD25 and CD69 (figure 5B). In our previous work [7], in addition to PHA, we used spinoculation to enhance the binding of viral particles to CD4+ T cells [26], together with high cell densities to favor cell-to-cell HIV-1

transmission [31]. Here we explored the impact of each of these factors on HIC CD4+ T cell susceptibility to infection. Used separately, a higher cell density and spinoculation both enhanced viral replication in CD4+ T cells, especially in cells from HIC, in which viral replication was otherwise barely detectable (Figure 6A). These conditions diminished the difference between CD4+ T cells from HIC and HD (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+ 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+ T cells from 5 HIC and 6 HD using 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 HIC (median dilution for first positive p24 value 10^{-5} [10^{-3}-10^{-9}] and 10^{-11} [10^{-7}-10^{-13}] for HIC and HD, respectively). However, at higher viral inocula the level of viral replication in CD4+ T cells from HIC approached the one in cells from HD. This result further corroborates the presence of a saturable mechanism of HIV-1 restriction in the cells from HIC.

HIC CD4+ 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 [32]. We quantified total HIV-1 DNA in blood cells from 81 HIC enrolled in the ANRS CO18 cohort. PBMC-associated HIV-1 DNA levels ranged from undetectable (<11 HIV-DNA copies/10^6 PBMC or <1.04 log) to 728 copies/10^6 PBMC (2.86 log). Overall, HIV-1 DNA levels were far lower (1.45 log copies/10^6 PBMC [<1.34-1.65]) in HIC

than in patients with primary infection (ANRS PRIMO cohort: 3.30 log copies/10^6 PBMC [range: 1.84-4.93], n=674)[33]; patients with chronic infection (ANRS SEROCO cohort: 2.86 log copies/10^6 PBMC [IQR: 2.45-3.21], n=271)[34]; patients on effective HAART (SALTO study: 2.3 log copies/10^6 PBMC [IQR: 1.9-2.8], n=116) [35]; and long-term non progressors (ANRS ALT cohort: 2.29 log copies/10^6 PBMC [IQR: 1.53-2.89], n=50)[36]. Thus, our results on a large group of HIC extend previous observations and confirm that HIC have a remarkably small HIV reservoir.

We then compared blood cells-viral DNA load in 23 HIC 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=0.044; n=23; not shown). In other words, those HIC 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 HIC.

Discussion

Extraordinarily low levels of cell-associated HIV-DNA are found in HIV controllers, suggesting that these individuals possess mechanisms that hamper the formation of viral reservoirs. We and others have previously described a strong and effective cytotoxic T cell response that likely 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 be not yet fully developed [38]. HIV-1 DNA levels may already be low during primary infection in patients who are later identified as HIC [12]. In addition, a strong CTL response is found only in part of HIC [8-10](and our own unpublished results). 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 HIC than in the general population or in HIV-1-infected patients non controlling 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 HIC. We found that HIV-1 replication in target cells from HIC was restricted independently of viral tropism for cell receptors, that the mechanism is saturable and affects reverse transcription. Moreover, CD4+ T cells from HIC were also resistant to SIVmac251 infection.

Viral replication in MDM from most HIC 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 HIC 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 HIC in optimised conditions of HIV-1 challenge [4,7]. 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 Alireza Rabi et al, who found no differences in HIV-1 entry and early steps of viral replication between CD4+ T cells from HIC and uninfected donors [20]. In addition to other experimental differences (we used CD3-activated CD4+ T cells while Rabi et al 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 al showed that CD4+ T cells from HIC carry in vivo low levels of integrated HIV DNA [43] which is in agreement with our results showing a restriction before viral integration. However, the authors could not found a restriction of HIV-1 replication in vitro, but the use of spinoculation may have influenced these experiments. Graf et al also show an accumulation of 2-LTR nonintegrated forms in the cells from HIC. In our experiments restriction of viral replication occurred during reverse transcription, however, because of the limited number of individuals 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 HIC 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 HIC. This is also supported by the lack of modulation of TRIM5α expression in our experiments (Figure S3). The phenotype of HIV-1 restriction found in cells from HIC is reminiscent

of that induced by APOBEC3G, which also affects pre-integration steps [44,45]. A negative correlation between plasma viral load in HIV-1 infected patients and APOBEC3G mRNA levels has been reported [46], but more recent studies failed to confirm this result [47] or showed a positive correlation [48]. APOBEC3G-induced hypermutation of HIV-1 proviral DNA was not found to be higher in HIC than in HAART-treated patients [47]. 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 HIC than in those from HD (Figure S3). Overall, our results do not support the involvement of APOBEC3G in the restriction of viral replication in cells from HIC.

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 pre-integrative steps, leading to a strong reduction in the level of integrated proviruses [18,19]. Also while reviewing the manuscript Chen et al reported a resistance of CD4+ T cells from HIC to HIV-1 infection. These authors showed that resistance in their setting was due to elevated levels of p21 in the cells from HIC [49]. In our study p21 expression in MDM did not differ between HIC and uninfected controls, even though viral replication was also far lower in HIC MDM. Although we found that p21 expression was higher in CD4+ T cells from HIC than from healthy controls, it did not correlate with CD4+ T cell susceptibility to HIV-1 infection. Furthermore, upon CD3 activation, at infectious challenge, p21 mRNA levels in CD4+ T cells were downregulated and did not differ between HIC 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 MDM 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 [50]. Alternatively, the elevated levels of p21 observed in CD4+ T cells from HIC might be related to other activities of this protein such as its capacity to regulate cell survival or T cell homeostasis [51,52]. Along these lines, Grevenynghe et al have shown that memory CD4+ T cells from HIC are resistant to apoptosis [53]. Further work is needed to understand the possible influence of elevated p21 expression on HIC CD4+ T cell susceptibility to HIV.

In conclusion, our data suggest that HIV target cells in HIV controllers 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 optimising the conditions of viral challenge in vitro, it is likely to be 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 HIC.

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AUTHORSHIP AND CONFLICT OF INTEREST STATESMENTS

All authors declare no conflict of interest.

Authors' contributions: performed research: AS-C, CH, AB, AD, PV, AM; analyzed and interpreted data: AS-C, CH, AB, CR, GP; coordinated inclusion of patients: FB, OL; collected clinical data from patients: FB; discussed results: FB-S, OL; designed research: AS-C; GP; wrote the manuscript: AS-C, GP. All the authors commented and approved the final version of the manuscript.

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

Group	N	Sex[A]	Last CD4[B]	Last viral load[C]	months on HAART[D]
HIV controllers	55	M: 31 F:24	680 [266-1665]	<40 [<40-366]	-
HIV-1 Viremics	9	M:6 F:3	378 [278-654]	42984 [8048-116266]	-
HAART treated	12	M:10 F:2	633 [292-886]	<40 [<40-51]	45 [7-98]

[A]M: Male; F: Female

[B]CD4+ T cell counts/ml of blood: median [range]

[C]HIV-1 RNA copies/ml of plasma with standard technique, median [range]

[D]months with successful HAART-related control of viremia prior to inclusion, median [range]

Figure legends

Figure 1. Macrophages from HIC have low susceptibility to HIV-1 infection. A. Kinetics of HIV-1 BaL replication after infection of monocyte-derived macrophages (MDM) from one representative HD (triangle) and one HIC (circles). Mean and standard deviation of three independent infections are shown. **B.** HIV-1 replication on day 10-11 p.i. in MDM from HIC and HD, after challenge with HIV-1 BaL. Symbols represent the average (n=3 independent infections) p24 values detected in culture supernatants for each donor. Horizontal lines indicate median values.

Figure 2. CD4+ T cells from HIC have low susceptibility to HIV-1 infection. A. PHA-activated CD4+ T cells from HIC and healthy donors (HD) were challenged with replicative HIV-1 BaL, using a protocol optimized to detect viral replication in vitro (left). HIV-1 replication in antiCD3-activated CD4+ T cells from HIC and HD (including all those depicted in 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 post-infection (p.i.) in culture supernatants for each donor. Horizontal lines indicate median values. **B.** HIV-1 (BaL (R5), left, and 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. Mean and standard deviation of three independent infections are shown.

Figure 3. The lower susceptibility of cells from HIC is apparent during the first cycle of infection and related to blockade of HIV-1 during reverse transcription. A. Comparison of HIV-1 BaL replication on day 7 p.i. (left panel) and single-cycle infection with HIV-1_{VSV-G}, measured 48 h p.i. in terms of luciferase activity (central panel) in CD4+ T cells from a representative HIC and a HD. Suboptimal conditions were

used for infection. Mean and standard deviation of three independent measurements are shown. **B.** HIV replication in MDM from a HIC and a HD on day 10 p.i. with productive HIV-1 BaL (left panel) and 48 h p.i. with single-cycle VSV-G pseudotyped HIV-1 (right panel). Two additional experiments were performed with MDM from two other HIC and HD with similar results. Mean and standard deviation of three independent measurements are shown. **C.** Levels of luciferase activity and integrated HIV-1 copies (Alu-LTR) in CD4+ T cells from HIC and HD 48h post-infection. Mean and SD of experiments with cells from 7 HIC and 7 HD are shown. **D.** Number of early (R-U5) and late (U5-gag) reverse transcripts determined by qPCR 24h and 48h post infection of CD4+ T cells from one HIC (grey bars) and one HD (black bars) with VSV-G pseudotyped HIV-1 particles. One representative example of experiments with cells from 5 HIC and 5 HD is shown.

Figure 4. High p21 expression in HIC CD4+ T cells is not directly involved in HIV restriction. A. Levels of p21 mRNA in MDM from HIC and HD at the time of infection (after 7-10 days of differentiation from monocytes). Each symbol represents one individual. Median values for each group are shown. **B.** Levels of p21 mRNA in non activated CD4+ T cells from HIC, HD, viremic HIV-1-infected patients (VIR) and patients on effective HAART (ART) (left). Levels of p21 mRNA in CD4+ T cells from HIC, HD, VIR and ART after 5 days of activation with anti-CD3 (right). In A and B, the results are expressed as relative expression levels compared to cDNA in CD4+ T cells from one HD who was used as a common reference throughout the study. **C.** Levels of HIV-1 replication 7 days p.i. with replicative HIV-1 BaL (black bars) in CD4+ T cells from 2 HIC, 1 VIR and 1 HD (top); and levels of p21 protein at the time of infectious challenge (bottom). The ratio of the optical densities of the p21 and GAPDH signals were calculated for each sample with Photoshop CS3 software (Adobe, USA), and reported in the graph (grey bars) relative to the HD control. **D.** Levels of p21 mRNA in anti-CD3 activated CD4+ T cells from one HD and one HIC 24

h after nucleofection of irrelevant and p21-specific siRNAs (top panels). Luciferase activity 48 h after challenge of nucleofected cells with single-cycle HIV-1_{VSV-G} (bottom panels). Cells were challenged 24 h after nucleofection. Means of three independent measures and standard deviations are shown.

Figure 5. Similar levels of activation and proliferation of CD4 T cells from HIC and HD in response to anti-CD3 stimulation A. Proliferation index of CD4+ T cells from HIC and HD, calculated as the ratio between the cell density before (10^6 cells/ml) and after 5 days of antiCD3 stimulation. Each symbol represents one individual. Medians for each group are shown. **B.** Expression of the CD25 and CD69 activation markers was measured by flow cytometry on the surface of CD4+ T cells from HIC and HD five days after stimulation with anti-CD3.

Figure 6. HIC CD4 T cell resistance is overcome by increasing viral inoculum or cell-to-cell transmission. **A.** HIV-1 BaL replication on day 7 p.i. in antiCD3-activated CD4+ T cells from one representative HIC (top panel), and the logarithmic fold reduction in viral replication when compared to cells infected in parallel from a HD (bottom panel) in various experimental conditions. Cells were challenged as follows: in suboptimal conditions (1st column), with a higher cell density (10^6 cells/ml) (2nd column), using a spinoculation protocol to facilitate viral binding to the cell surface (3rd column), and using both a higher cell density and spinoculation (4th column). Mean and standard deviations are shown (in the bottom panel, standard deviations are relative to the mean infection level measured in cells from HD). **B.** Levels of p24 in culture supernatants of CD4+ T cells from one HIC (circles) and one HD (triangles) at day 9 p.i. in suboptimal conditions with serial dilutions of HIV-1 BaL.

Figure 7. Resistance of HIC CD4+ T cells to HIV-1 is associated with low total cell-associated HIV-1 DNA levels. Total cell-associated HIV-1 DNA levels were compared between HIC whose CD4+ T cells yielded ≤ 1 ng of p24/ml of culture supernatant on day 7 p.i. with HIV-1 BaL, and HIC whose CD4+ T cells yielded > 1 ng of p24/ml of culture supernatant. The samples used to calculate cell-associated HIV-1 DNA load and susceptibility to infection were obtained at the same time. Each symbol represents one individual. Medians for each group are shown.

Figure 1

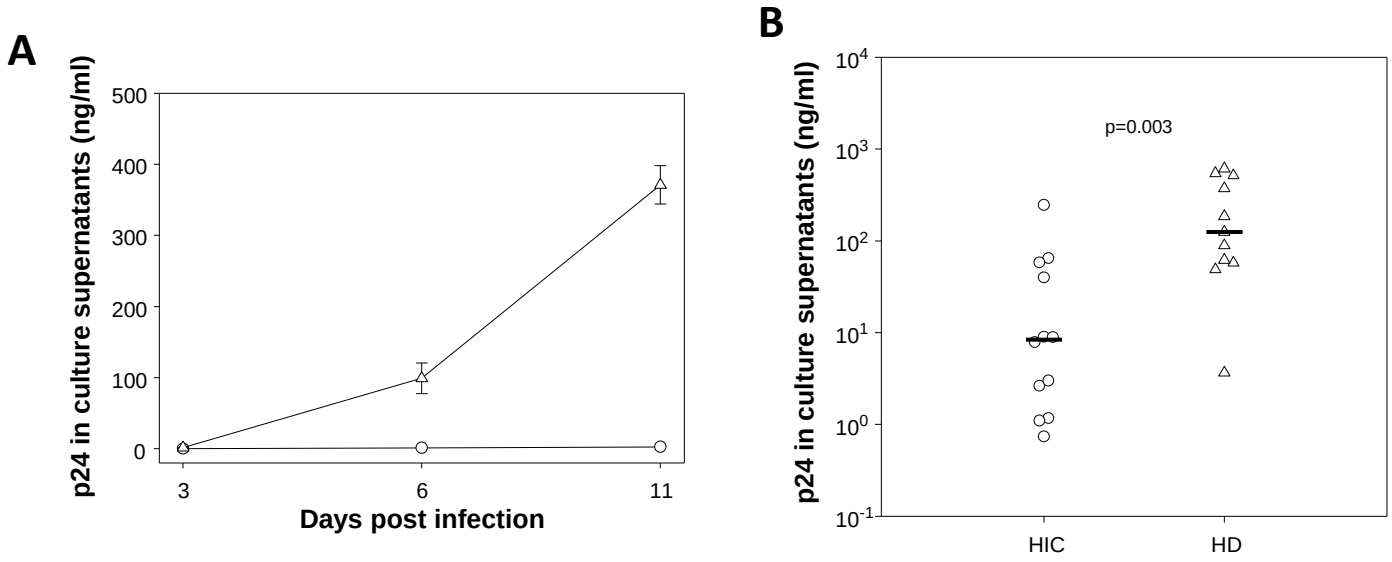

A
p24 in culture supernatants (ng/ml)
500
400
300
200
100
0
3
6
11
Days post infection
B
p24 in culture supernatants (ng/ml)
10^4
10^3
10^2
10^1
10^0
10^{-1}
p=0.003
HIC
HD

Figure 2

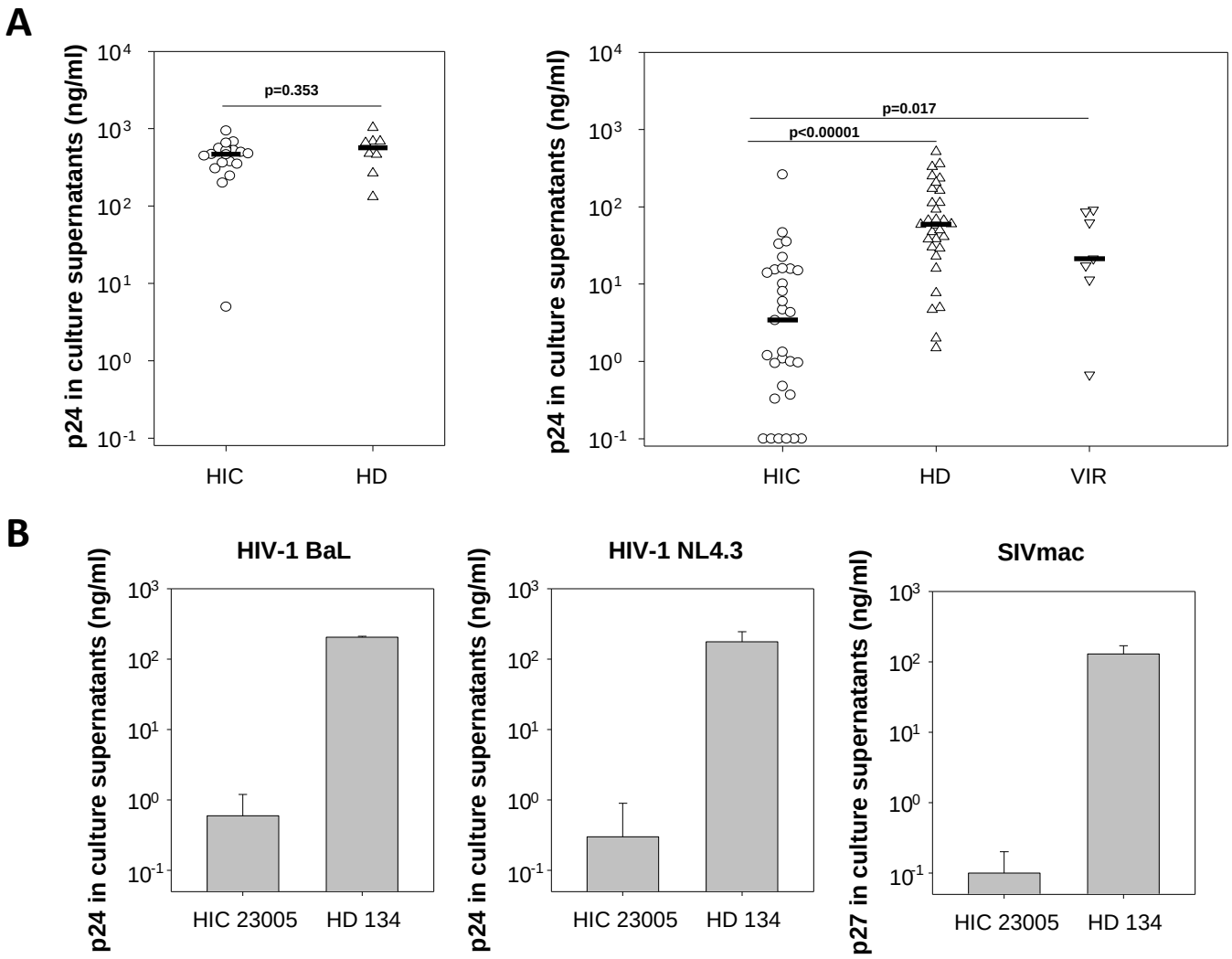

A
p24 in culture supernatants (ng/ml)
p=0.353
HIC
HD
p=0.017
p<0.00001
HIC
HD
VIR
B
HIV-1 BaL
HIV-1 NL4.3
SIVmac
p24 in culture supernatants (ng/ml)
p27 in culture supernatants (ng/ml)
HIC 23005
HD 134

Figure 3

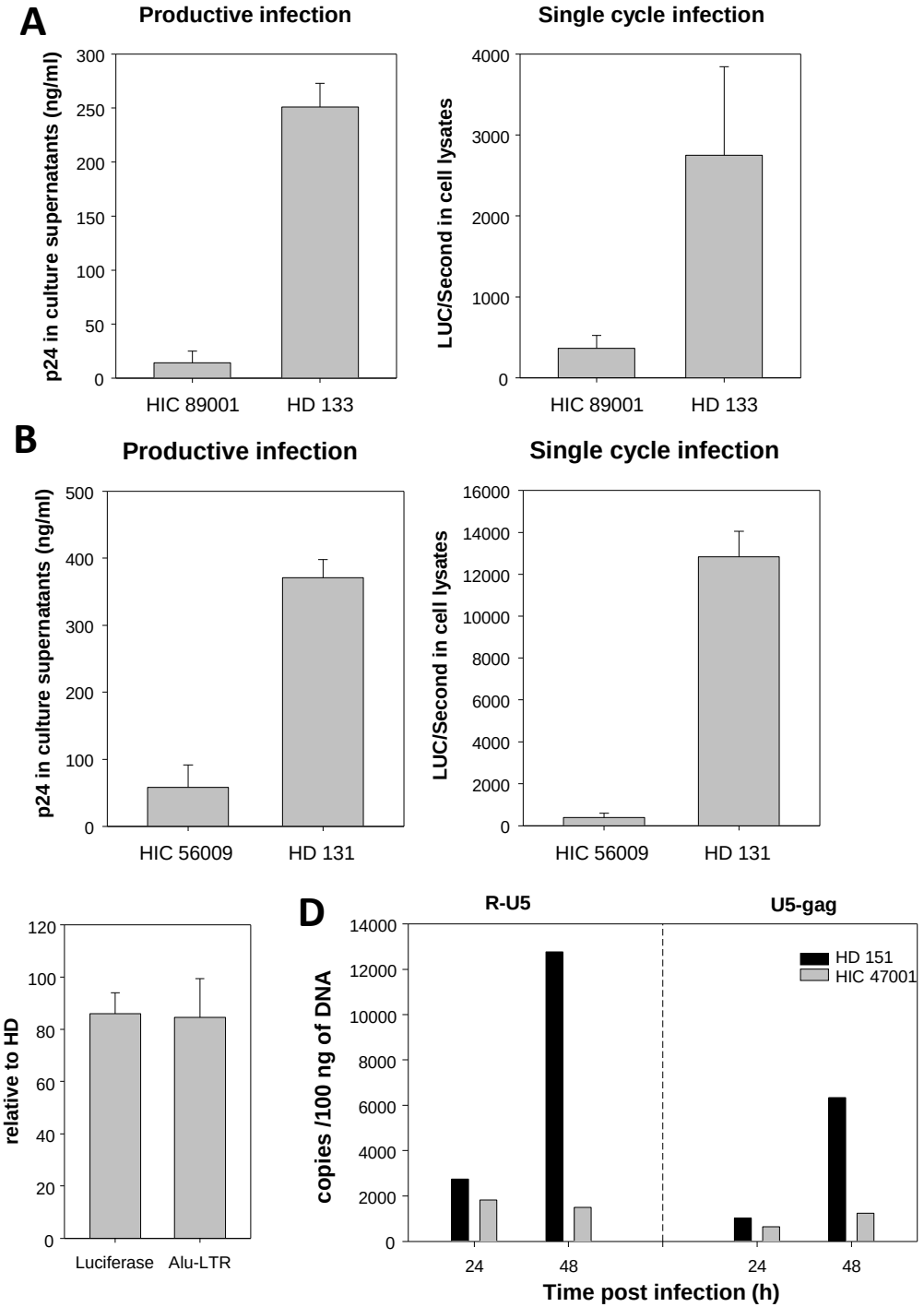

A
Productive infection
p24 in culture supernatants (ng/ml)
300
250
200
150
100
50
0
HIC 89001
HD 133
Single cycle infection
LUC/Second in cell lysates
4000
3000
2000
1000
0
HIC 89001
HD 133
B
Productive infection
p24 in culture supernatants (ng/ml)
500
400
300
200
100
0
HIC 56009
HD 131
Single cycle infection
LUC/Second in cell lysates
16000
14000
12000
10000
8000
6000
4000
2000
0
HIC 56009
HD 131
C
% of inhibition in HIC CD4+ T cells relative to HD
120
100
80
60
40
20
0
Luciferase
Alu-LTR
D
R-U5
U5-gag
copies /100 ng of DNA
14000
12000
10000
8000
6000
4000
2000
0
HD 151
HIC 47001
24
48
24
48
Time post infection (h)

Figure 4

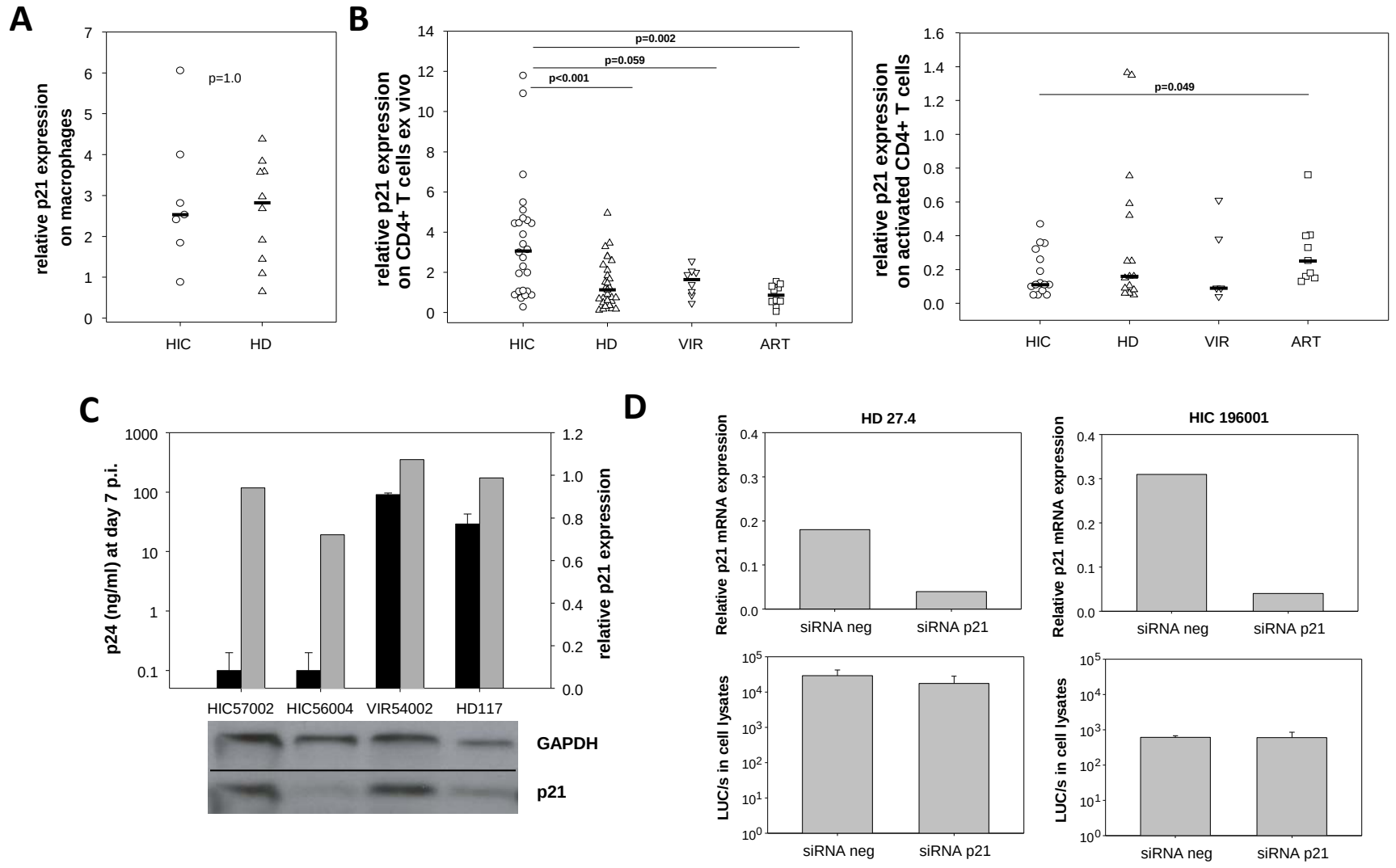

A
relative p21 expression on macrophages
p=1.0
HIC
HD
B
relative p21 expression on CD4+ T cells ex vivo
p=0.002
p=0.059
p<0.001
HIC
HD
VIR
ART
relative p21 expression on activated CD4+ T cells
p=0.049
HIC
HD
VIR
ART
C
p24 (ng/ml) at day 7 p.i.
relative p21 expression
HIC57002
HIC56004
VIR54002
HD117
GAPDH
p21
D
HD 27.4
HIC 196001
Relative p21 mRNA expression
LUC/s in cell lysates
siRNA neg
siRNA p21

Figure 5

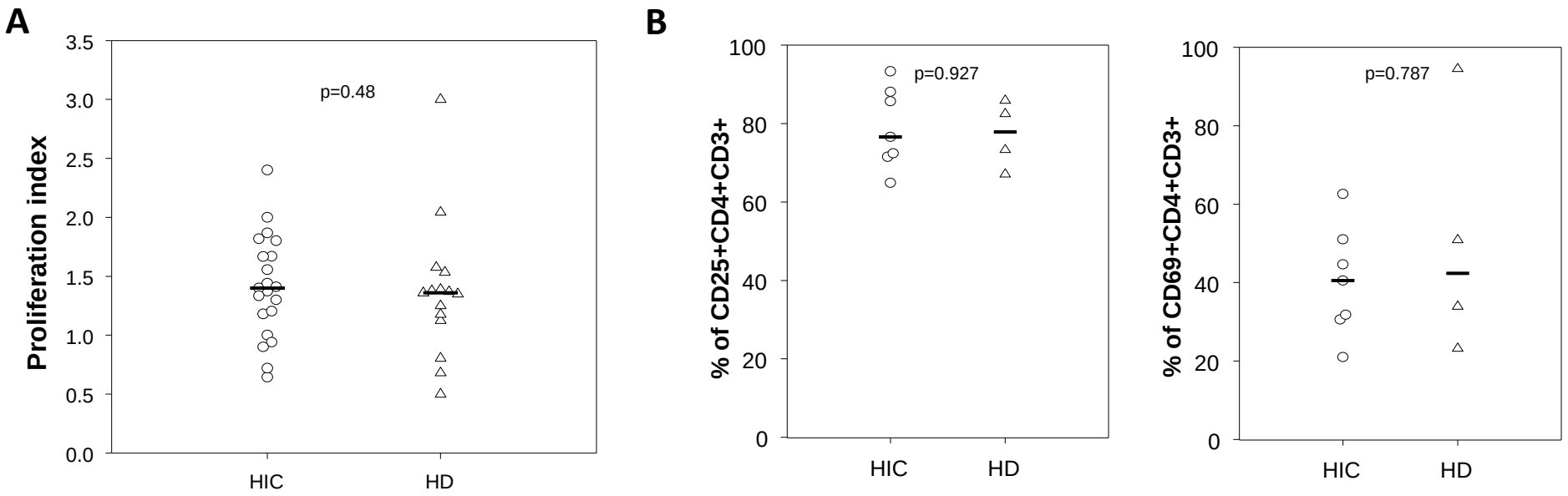

A
Proliferation index
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p=0.48
HIC
HD
B
% of CD25+CD4+CD3+
100
80
60
40
20
0
p=0.927
HIC
HD
% of CD69+CD4+CD3+
100
80
60
40
20
0
p=0.787
HIC
HD

Figure 6

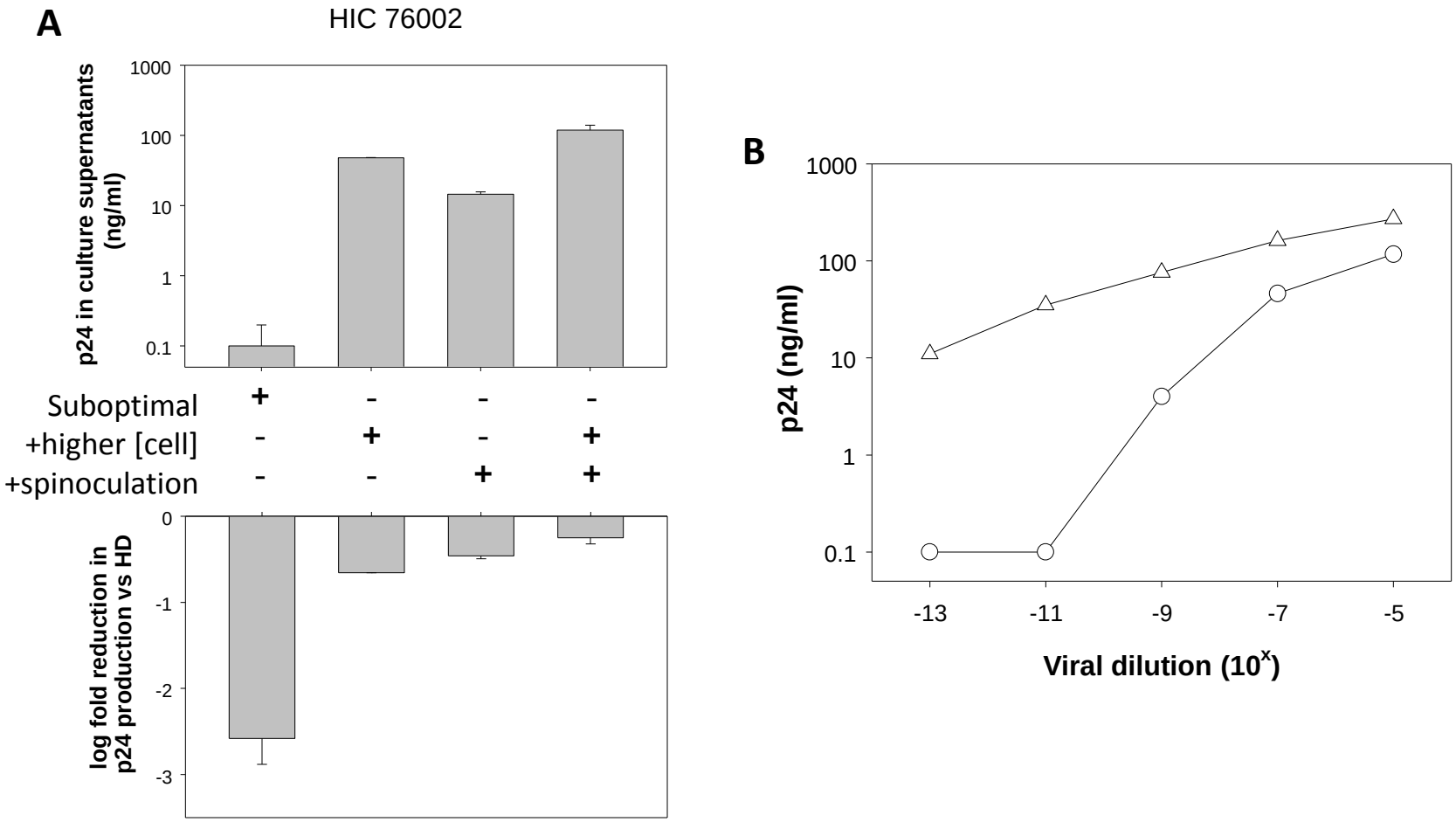
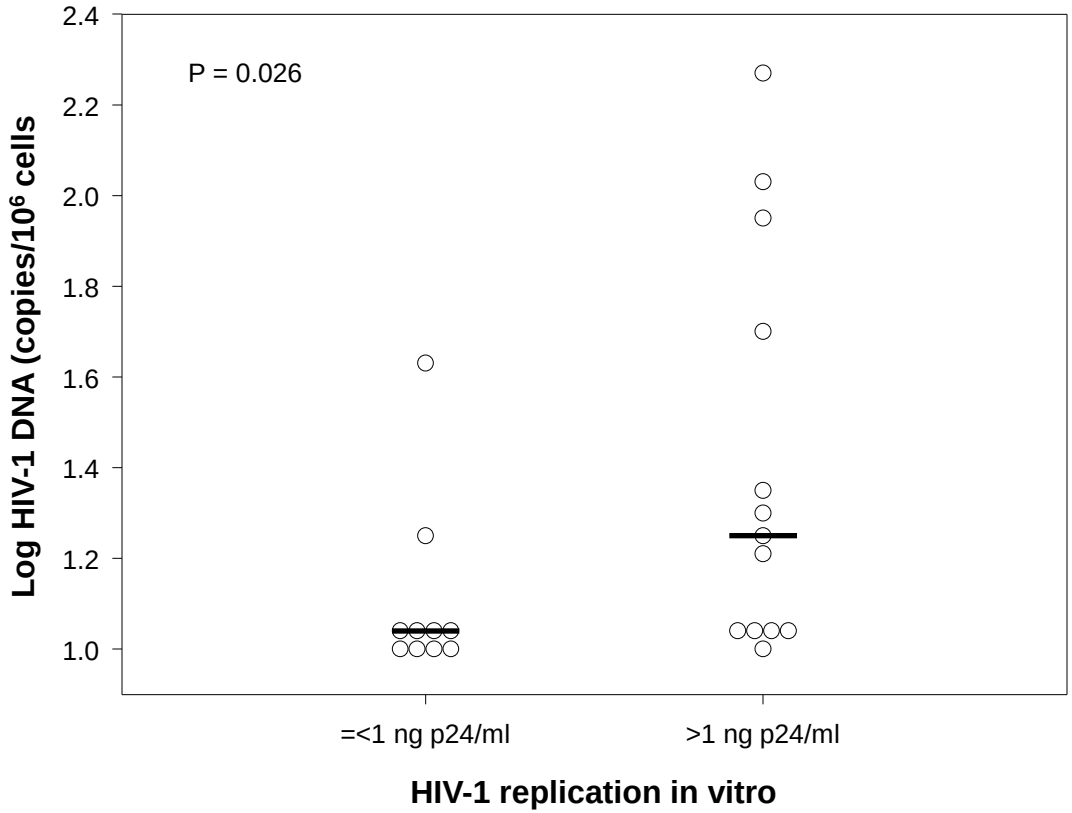

A
HIC 76002
p24 in culture supernatants (ng/ml)
1000
100
10
1
0.1
Suboptimal
+higher [cell]
+spinoculation
+ - - -
- + - +
- - + +
log fold reduction in p24 production vs HD
0
-1
-2
-3
B
p24 (ng/ml)
1000
100
10
1
0.1
-13
-11
-9
-7
-5
Viral dilution (10^x)

Figure 7


P = 0.026
Log HIV-1 DNA (copies/10⁶ cells
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
=<1 ng p24/ml
>1 ng p24/ml
HIV-1 replication in vitro