HIV-1-mediated insertional activation of STAT5B and BACH2 trigger viral reservoir in T regulatory cells

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HIV-1 insertions targeting BACH2 or MLK2 are enriched and persist for decades in hematopoietic cells from patients under combination antiretroviral therapy. However, it is unclear how these insertions provide such selective advantage to infected cell clones. Here, we show that in 30/87 (34%) patients under combination antiretroviral therapy, BACH2, and STAT5B are activated by insertions triggering the formation of mRNAs that contain viral sequences fused by splicing to their first protein-coding exon. These chimeric mRNAs, predicted to express full-length proteins, are enriched in T regulatory and T central memory cells, but not in other T lymphocyte subsets or monocytes. Overexpression of BACH2 or STAT5B in primary T regulatory cells increases their proliferation and survival without compromising their function. Hence, we provide evidence that HIV-1-mediated insertional activation of BACH2 and STAT5B favor the persistence of a viral reservoir in T regulatory cells in patients under combination antiretroviral therapy.

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Viruses have evolved strategies to exploit the host machinery for their own propagation at every step of their replication cycle. It has been demonstrated in animals that several retroviruses take advantage of insertional mutagenesis to activate proto-oncogenes, leading to cell transformation and ultimately cancer, thus favoring viral spread and persistence in the host.

For lentiviruses such as HIV-1, expe evidences and novel observations suggest that its integration in the host genome could actually result in insertional mutagenesis. In hematopoietic cells, HIV-1 and lentiviral vectors (LVs) preferentially integrate within the transcription unit of expressed genes and may induce aberrant RNA splicing mechanisms leading to the formation of chimeric transcripts harboring HIV sequences fused to cellular exon sequences. Moreover, LVs with active long terminal repeats (LTRs) are able to effectively activate cancer-related genes through promoter insertion and thus inducing neoplastic transformation. Finally, a significant enrichment of proviral integrations targeting some cancer-related genes, such as BACH2 and STAT5B, has been observed in peripheral blood mononuclear cells (PBMC) and CD4+ T lymphocytes isolated from HIV-infected individuals under combination antiretroviral therapy (cART). These data suggest that HIV-1, similarly to onco-retroviruses, could exploit insertional mutagenesis to activate or inactivate cancer-related genes, leading to the clonal expansion of the infected cell and thus favoring its persistence in the host. However, it is currently unknown how proviral integrations may cause the deregulation of these cellular genes and if the physiological consequences of this deregulation may result in oncogenesis or another phenotype that is selected in these specific conditions.

By retrieving HIV-1 insertion sites in a European cohort of HIV-1-infected patients, we found that BACH2 and STAT5B were the two most frequently targeted genes. Since most of the viral insertions within the transcriptional unit of BACH2 and STAT5B clustered in a small genomic window and were in the same orientation of the targeted gene transcription, we hypothesized that the HIV-1 LTR could directly control the expression of these genes by a mechanism known as promoter insertion and drive the formation of chimeric mRNA transcripts containing viral HIV-1 sequences fused by splicing to the first protein-coding exon of the targeted gene. By performing RT-PCR on the mRNA obtained from PBMCs of a large cohort of HIV patients, we found in 34% of patients the predicted chimeric transcripts, putatively encoding for unaltered full-length BACH2 or STAT5B proteins. Interestingly, these transcripts were specifically enriched in T central memory and T regulatory (Treg) cells, two cellular compartments that have been proposed to be the main reservoir of HIV virus. Finally, we demonstrated that forced expression of these two transcription factors in Treg cells, purified from healthy donors (HDs), increased their proliferation rate without impacting on their immune-suppressive function. In conclusion, our data indicate that in a large portion of HIV patients under cART, HIV/BACH2, or STAT5B chimeric transcripts are expressed in Treg cells and such expression could favor the persistence of this important HIV cellular reservoir.

**Results**

**BACH2** and **STAT5B** are highly targeted genes in HIV-1 patients. In order to investigate the biological role of HIV-1-mediated insertional mutagenesis, we first attempted to characterize the HIV-1 integration profile in PBMC from a cohort of 54 HIV-1-infected individuals under cART followed in our Institute and described in Tambussi et al. In this cohort 50% of patients under cART treatment had low levels of viremia and in 29 patients (54%) the cART treatment was supplemented by IL-2 administration for 12 months (Supplementary Table 1).

For integration site retrieval, linear amplification-mediated (LAM)-PCR was used to retrieve the viral/cellular genome junctions that were sequenced using the Illumina platform. Sequences were then mapped by a dedicated bioinformatics pipeline, previously used for the study of two LV-based gene therapy clinical trials. By this approach, a total of 13,671 HIV-1/cellular genomic junctions were retrieved, corresponding to 198 HIV-1 integration sites univocally mapped on the human genome (Supplementary Table 1). The genomic distribution of integration sites in our data set followed the known tendency of HIV-1 and replication-defective LVs to integrate within gene bodies and gene dense regions (Supplementary Fig. 1a, b). The genomic distribution in our integration data set was then compared to three previously published data sets from HIV-1-infected patients under cART and to five data sets of non-replicating HIV-1-derived LVs used to transduce ex vivo CD4+ T cells and hematopoietic stem cells (HSC) for gene therapy clinical trials. With the exception of the HIV-1 data sets from Maldarelli et al. and Wagner et al., the six remaining data sets were reanalyzed with our bioinformatics pipeline to harmonize the integration mapping data. The genomic distribution of integration sites of the different data sets was compared using intervals of 1 Mb by the Genomic Hyperbrowser analysis tool. No significant differences in the global genomic distribution of integration sites were found compared to Maldarelli et al., Wagner et al., data sets obtained from fully suppressed patients (Supplementary Table 2). In the data set from Ikeda et al., a region on chromosome 6 spanning from nt 900,000 to 1,000,000, containing BACH2, was significantly over targeted compared to our data set (false discovery rate, FDR 10% adjusted p-value = 1.6 × 10^-5).

In our study, out of the 198 integrations retrieved, 3 targeted BACH2 and 4 targeted STAT5B (distributed in seven patients), a targeting frequency significantly higher than expected, as determined by Montecarlo simulations. Moreover, most of the insertions targeting these genes were in the same orientation of gene transcription and landed upstream of the first protein-coding exon (Supplementary Fig. 1c, d). Although the four insertions targeting STAT5B were retrieved only from patients that received the IL-2 treatment, its targeting frequency was not significantly different when compared to the control group without IL-2 treatment. Moreover, BACH2 and STAT5B were among the top three targeted genes in all the HIV-derived integration data sets studied (Supplementary Table 3), indicating that the level of viral replication and the cART regimen adopted did not significantly impact on the overall distribution of HIV insertion sites. The targeting frequency of BACH2 and STAT5B in our and the HIV-derived data sets was always significantly higher than in the LV data sets from ex vivo transduced CD4+ T cells and HSC gene therapy clinical trials (p < 0.0001 by Fisher exact test, Supplementary Table 1, Supplementary Fig. 1e, f). Only, the Ikeda et al. data set showed a significantly higher targeting frequency for BACH2 among the four data sets from HIV-infected patients under cART (Supplementary Table 4, Supplementary Fig. 1e, f), in agreement with the results obtained with the comparative analysis on the integration distribution at the genomic-wide level by the Genomic Hyperbrowser analysis tool (Supplementary Table 2). Moreover, in all the HIV data sets, viral insertions in BACH2 were always found in the same transcriptional orientation as the host gene, while they were randomly distributed in the LV-derived data sets (Supplementary Table 4). Differently, in our and Ikeda’s data sets almost all insertions targeting STAT5B were in the same orientation of gene transcription, while in the Maldarelli et al. and Wagner et al. data...
sets this bias was not observed (Supplementary Table 4). Overall, these data indicate that BACH2 and STAT5B insertions are enriched by a mechanism of insertional mutagenesis.

**Identification of chimeric mRNAs in HIV-1 patients.** The specific clustering of proviral integrations upstream the first protein-coding exon and the coherent orientation of the viral integrations in the same direction of BACH2 and STAT5B gene transcription resemble a pattern found in insertional mutagenesis screenings performed with retroviruses, transposon, and genotoxic LVs, in which the viral promoter drives the expression of oncogenes by read-through transcription and formation of aberrant splicing events. Therefore, we postulated that HIV-1 insertions targeting BACH2 or STAT5B could similarly drive the expression of chimeric transcripts containing HIV-1 sequences fused by splicing to STAT5B or BACH2 exons downstream the integration site. To address this hypothesis, we designed two oligonucleotide primers complementary to the HIV-1 LTR sequences paired to two oligonucleotides complementary to the coding exons 6 and 7 of BACH2 or to two different portions of the exon 4 of STAT5B. This nested RT-PCR strategy should allow to specifically amplify HIV/BACH2 or HIV/STAT5B chimeric transcripts that would be generated by HIV-1 insertions mapping in different positions of the targeted gene intron(s) (Fig. 1a, b and Supplementary Methods). We thus applied this RT-PCR strategy on total RNA from PBMCs of three patients in which we previously found HIV insertions in STAT5B (JID.63 and JID.29, Supplementary Fig. 1) and BACH2 (JID.87, Supplementary Fig. 1). In all patients, a RT-PCR product of the expected size (500 bp) was obtained (Fig. 1). Sequence analysis of the RT-PCR products showed that the transcripts contained the 5' HIV-1 LTR sequences extended until the canonical viral splice donor (SD) site and then correctly fused by splicing to the first protein-coding exon of STAT5B (exon 2) and BACH2 (exon 6) (Supplementary Fig. 2). Moreover, a HIV/STAT5B chimeric transcript was also amplified from a patient (JID.87) that did not show insertions targeting STAT5B in the previous integration site analysis, suggesting that the RT-PCR for this specific transcripts could better suited to reveal insertional mutagenesis events that are instead missed by integration site retrieval approach. We therefore expanded the RT-PCR for analysis to detect HIV/STAT5B and HIV/BACH2 chimeric transcripts on a second cohort of 87 patients, for which integration data was not available. This cohort was composed by 48 treatment-naive patients (NT) that received only Non-Nucleoside Reverse Transcriptase Inhibitors cART regimen and 39 multi-experienced cART-treated patients (EXP) who received both Nucleoside and Non-Nucleoside Reverse Transcriptase Inhibitors cART regimen (Supplementary Table 5, and Methods). From this analysis we found that 27 patients were positive for the presence of HIV/STAT5B transcripts (31%, Fig. 1c, N = 12 in NT and N = 15 in EXP), 8 patients were positive for HIV/BACH2 transcripts (9.2%, Fig. 1d, N = 4 in NT and N = 4 in EXP), and 5 patients (5.7%) expressed both (Fig. 1 and Supplementary Fig. 2, Supplementary Table 5). No biases for the presence of these chimeric transcripts were found in treatment-naive vs. multi-experienced patients by Fisher exact test comparison. Sequence analysis of the RT-PCR products amplified from this cohort of patients confirmed that chimeric transcript for both genes were generated by a mechanism of promoter insertion (Supplementary Fig. 2).

**Enrichment of HIV/STAT5B and HIV/BACH2 mRNAs in Treg cells.** We then investigated whether these chimeric transcripts could be preferentially expressed in different T lymphocyte subsets and/or monocytes. Therefore, nine patients that tested positive for HIV/STAT5B and/or HIV/BACH2 chimeric transcripts in the RT-PCR screening on PBMCs were selected for further molecular analyses. Specifically, RT-PCR was used to test the presence of HIV/STAT5B transcripts in Treg cells, T effector (Tefl), T naive, T stem cell memory (Tscm), T central memory (Tcm), T effector memory (Tem), total CD8+ T lymphocytes and monocytes purified by fluorescence activated cellsorting (FACS) sorting from the blood of two patients (ID_77 and 96) and in

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**Fig. 1** Identification of the chimeric HIV/BACH2 and HIV/STAT5B transcripts in patients under cART. a, b Scheme of the RT-PCR strategy devised to amplify putative chimeric HIV/STAT5B (a) or HIV/BACH2 (b) transcripts; white and gray boxes represent non-coding and coding exons (Ex), respectively. The exon numbers and the position of the first ATG codon are indicated. The integrated provirus, the LTR, and the 5' major viral SD site are depicted in black. The arrows represent the position of the primer pairs used for RT-PCR. The black and gray bars indicate the amplified cDNA sequence from the provirus and host genome, respectively. The dashed lines indicate the splicing events. At the bottom of each panel, representative examples of the sequences of the RT-PCR products are shown. c, d Report agarose gel electrophorosis of RT-PCR products of either HIV/STAT5B or HIV/BACH2 obtained from PBMC of HIV-1-infected patients using the primers indicated in a, b; the amplicon size is ~500 bp (M molecular size markers). For each sample, RT-PCR for GAPDH was also performed as positive control of amplification (data not shown).
Treg, Teff, CD8+ T cells and monocytes purified by magnetic beads isolation from four patients (ID_25, 56, 63 and 76) (Fig. 2 and Supplementary Table 3). Moreover, the levels of the HIV/STAT5B and HIV/BACH2 chimeric transcripts were quantitatively measured by droplet digital (dd)-PCR on in vitro expanded Treg and Teff cells from six patients (ID_25, 77, 76, 32, 38 and 71) (Fig. 2). HIV/STAT5B transcripts were found in the purified Treg cells of six out of six patients and in Teff cells of two out six donors (Fig. 3a, b). Moreover, in two out of two patients tested, we detected HIV/STAT5B transcripts in Tcm cells (Fig. 3a),
Chimeric mRNAs are generated by multiple viral haplotypes. Since during infection the HIV-1 genome acquire mutations at relatively high frequency and generates a highly diverse viral population in patients, we reasoned that we could take advantage of the high genetic diversity of the integrated HIV-1 genome to get insights on the number of different cellular clones expressing HIV/STAT5B chimeric transcripts. In our rationale the presence of sequence variants (haplotypes) in the HIV-1 portion of the chimeric transcripts will indicate that these were generated by different viral haplotypes in independent infection events occurring in different cell clones. We thus performed Illumina sequencing of the amplified RT-PCR products of the chimeric transcripts from total PBMC of 10 patients, and from different T cell subsets of 6 patients (analyzed previously by RT-PCR, ID_25, 56, 63, 77, 76 and 96). Overall, we obtained an average of 5816 sequencing reads (ranging from 620 to 37010 reads) for each sample (Supplementary Table 6). The HIV-1 portion was then aligned to the reference HIV-1 sequence (HXB2 from the HIV database, www.hiv.lanl.gov) spanning from the U5 to the major SD signal (212 bp), while the STAT5B portion was aligned to the reference sequence spanning the exons 2, 3 and 4 (295 bp). The Propagating Dirichlet Process Mixture Model was then used to infer the number and frequency of the different HIV-1 haplotypes contained in each library\textsuperscript{33}. While, in the STAT5B portion only a

![Fig. 3](image-url)
silent nucleotide mutation was identified in a patient (ID_12), a significantly higher number of mutations (N = 170) were identified within the HIV-1 sequence (p < 0.0001 by Fisher exact test), indicating that the high rate of variability of the HIV-1 portion was not the result of sequencing errors (Supplementary Fig. 6 and Supplementary Table 6). In six patients multiple HIV-1 haplotypes were identified (from two up to four in a single patient), indicating that different HIV/STAT5B-expressing clones co-exist in patient’s blood cells. Phylogenetic relationships among the distinct haplotypes identified within each patient were constructed using ClustalW2 program employing a neighbor-joining-based procedure (Fig. 4 and Supplementary Fig. 6). This analysis revealed that for patients ID_77 and ID_96 related viral sequences were present, indicating that different cell clones with HIV integrations targeting STAT5B were generated by evolutionary-related viral variants over time.

**BACH2 and STAT5B overexpression in primary T cells.** To address the impact of the expression of HIV/STAT5B and HIV/BACH2 chimeric transcripts in Treg cells, we overexpressed BACH2 or STAT5B in in vitro-induced Treg (iTreg)24 and studied their immunophenotype and function. For this purpose, we designed bidirectional LVs allowing the coordinated expression of the Orange fluorescent protein (Or) with BACH2 (LV.BACH2) or STAT5B (LV.STAT5B) (Fig. 5a–c).

Naïve CD4+ T cells, purified from PBMC of HDs, were activated, transduced with LV.BACH2, LV.STAT5B, or with a GFP-expressing LV as control, and then cultured in Treg polarizing condition (Fig. 5d, e)24. After 2 weeks of culture, no significant differences (by one-way ANOVA) in the frequency of CD25+FOXP3+ cells or in their ability to suppress the proliferation of activated allogeneic CD4+CD25+ Teff cells were found between STAT5B-expressing or BACH2-expressing cells and GFP-transduced or untransduced control cells (Fig. 5f, g). Thus, overexpression of STAT5B and BACH2 in naïve CD4+ T cells neither affected their differentiation into iTreg cells nor their suppressive function. Interestingly, STAT5B-expressing iTreg cells were hyperproliferative upon IL-2 stimulation compared to GFP-transduced untransduced control cells (Fig. 5h).

We next investigated the effect of BACH2 or STAT5B expression in primary Treg cells purified from HDs (Fig. 6a). The transduction of Treg cells with LV.BACH2 and LV.STAT5B led to a 7.4-fold and 4.4-fold increase in the level of expression of BACH2 and STAT5B, respectively, when compared to GFP-transduced cells (Supplementary Fig. 7). After 10 days of culture, STAT5B-expressing and BACH2-expressing Treg cells showed a 2-fold increase in the total number of cells compared to controls (Mock) (Supplementary Fig. 7), but no significant differences in phenotype or function when compared to GFP-transduced or untransduced cells were observed (Fig. 6b and Supplementary Fig. 7).

To further characterize the selective advantage conferred by STAT5B and BACH2 expression, after the first 10 days of expansion, we performed a competitive proliferation experiment where primary Treg cells transduced with a GFP-expressing LV were mixed in a 1:1, 1:0.5 and 1:0.25 ratio with primary Treg cells transduced with BACH2-expressing or STAT5B-expressing LVs. The relative percentages of GFP+ and Orange+ cells were recorded over time after 10 and 20 days of culture. BACH2-expressing or STAT5B-expressing Treg cells significantly increased compared to GFP-expressing Treg cells, indicating that the expression of these transcription factors provides a significant selective advantage to Treg cells (Fig. 6c, d).

To assess if the selective advantage conferred by the overexpression of BACH2 or STAT5B was specific only for Treg cells, we tested the impact of their overexpression in primary CD4+ naïve T cells purified from the blood of HDs. Primary CD4+ naïve T cells were purified from six HDs, transduced with BACH2-expressing or STAT5B-expressing LVs at low MOI, to obtain a mixed population of transduced and non-transduced cells in the same well, and kept in culture in presence of low doses of IL-2 for 20 days. Orange-expressing cells at 10 and 20 days significantly increased compared to non-transduced cells, indicating that both transcription factors confer a proliferative advantage also to primary CD4+ naïve T lymphocytes (Supplementary Fig. 8).

Finally, given that C-terminally truncated STAT5 isoforms, frequently detected in patients’ PBMC25, can act as repressors on HIV-1 transcription26, we tested if the forced expression of STAT5B or BACH2 was able to decrease the transcriptional activity of an HIV-1 reporter genome in primary naïve CD4+ T cells. From this analysis, we did not observe any significant variation in the transcriptional activity of our HIV-1 reporter genome (Supplementary Fig. 9).

**Discussion**

Here, we show that in hematopoietic cells from a European cohort of HIV-1 patients under cART, BACH2 is significantly overtargeted by viral insertions oriented in the same direction of gene transcription. This finding is in agreement with three previous studies showing that BACH2 was highly targeted by viral insertions in PBMCs9, 10 or CD4+ T cells8 from HIV-1 patients under cART as the result of insertional mutagenesis. Thus, our data reinforce the notion that BACH2 is a culprit of HIV-1 insertional mutagenesis and that it is reproducibly found overtargeted in independent cohorts of HIV-infected patients worldwide.

On the other hand, the integration study performed on our patient’s cohort, together with the reanalysis of the integration data set published by Ikeda et al., candidates STAT5B as novel culprit of insertional mutagenesis in HIV patients under cART. Indeed, in our and the Ikeda’s integration site data sets, STAT5B was targeted at a significantly higher frequency than all other data sets and by viral insertions having almost in all the cases the same orientation of gene transcription. Maldarelli et al. proposed only BACH2 and MKL2 (targeted by 23 insertions, 22 of which in a single patient) as putative culprit of insertional mutagenesis9. However, MKL2 did not result to be significantly
targeted by HIV-1 insertions neither in our data set nor in previous two studies\(^8, 10\). The reason why different studies identify different culprits of insertional mutagenesis is currently unclear.

Based on the previous knowledge linking viral/vector insertion patterns near or within cancer genes to molecular mechanisms known to interfere with their expression levels and/or with their mRNA structure\(^1, 20\), we were able to “guess” the mechanism of insertional mutagenesis that is taking place when \(\text{STAT5B}\) and \(\text{BACH2}\) are targeted by HIV-1 insertions\(^3–^5\). Indeed, the clustering of the viral insertions upstream the first coding exon and the orientation bias of the viral integrations in the same direction of gene transcription suggested that both these transcription factors could be activated by a promoter insertion mechanism where the expression of oncogenes is under the direct control of the promoter of the viral LTR, leading to their over-expression and/or to the production of aberrant proteins with enhanced oncogenic activity\(^1, 20\). Based on these premises, as an alternative approach to the much more cumbersome study of genomic integrations, we devised a nested RT-PCR strategy to specifically amplify the predicted chimeric HIV/\(\text{STAT5B}\) and HIV/\(\text{BACH2}\) transcripts. The RT-PCR approach allowed to readily detect the HIV/\(\text{STAT5B}\) and/or HIV/\(\text{BACH2}\) transcripts in the mRNA obtained from PBMCs from 90 HIV patients. Interestingly, both chimeric transcripts were found in a relatively large proportion of patients (34%) and with similar frequencies in patients that experienced different cART regimens, thus suggesting that the treatment did not influence the rate of insertional mutagenesis events or the gene culprits implied in this phenomenon. Sequence analysis of the RT-PCR products showed that these mRNAs are predicted to encode for full-length wild-type \(\text{STAT5B}\) and \(\text{BACH2}\) proteins. Unfortunately, only the identification of these chimeric mRNAs in purified cell clones harboring these specific integration events would formally demonstrate that they are produced as the result of insertional mutagenesis events. Nonetheless, sequence analysis of the RT-PCR products showed that all chimeric mRNAs identified from the different patients shared the same structure: starting from the different patients shared the same structure: starting
Having addressed the feasibility of the RT-PCR to detect bona fide HIV/STAT5B and HIV/BACH2 chimeric transcripts, we decided to assess their expression in different hematopoietic cell subsets. Strikingly, the RT-PCR and quantitative ddPCR analysis revealed that these transcripts are enriched in Tcm and Treg cells but absent in CD8+ T cells or monocytes. Given that HIV/STAT5B and HIV/BACH2 transcripts were found at two different time points, collected 6 years apart from the same patients (nine out of nine tested), the HIV-mediated activation of STAT5B or BACH2 seems to provide a long-lasting selective advantage to Treg cells in HIV-infected patients. Moreover, the low level of expression of the HIV/STAT5B and HIV/BACH2 transcripts compared to that of the relative host gene may suggest that cell clones harboring such activating HIV integrations are relatively rare when compared to the whole population tested. This speculation is in agreement with the data from Maldarelli et al. in which the relative abundance of expanded clones harboring insertional mutagenesis events in patients under cART was reported to be on average 2.3% of the total number of HIV-infected clones. Finally, by analyzing the sequences of the HIV/STAT5B aberrant transcripts in PBMCs and sorted cell populations, we found that mutations were mostly present in the viral sequence and rare in the STAT5B portion, indicating that these transcripts must be generated by independent cell clones infected by different viral haplotypes rather than being a consequence of point mutations acquired by the cell after integration. Importantly, in two patients the observed haplotypes were phylogenetically related, indicating that cell clones expressing these transcripts are continuously produced during viral evolution and persist overtime.

Notably, LV-mediated forced expression of the wild-type form of STAT5B or BACH2 in primary Treg cells from HDs increased their proliferative capacity without affecting their phenotype and functions in vitro. Puzzlingly, forced expression of these transcription factors in primary CD4+ T cells appears to provide a selective advantage in vitro. However, the reasons why the enrichment of these aberrant transcripts occurs only in Treg and Tcm lymphocyte subsets but not in other T cell lineages are currently unknown.

Although this study confirm the role of BACH2 and candidates STAT5B as culprits of HIV-1-mediated insertional mutagenesis, shed light on the molecular mechanism used by HIV-1 insertions to alter their expression and identified the T cell subsets where these insertional mutagenesis events are enriched, several outstanding questions remain unanswered. It is unclear why no oncogenesis events were ever found associated to HIV-mediated insertional mutagenesis despite the high number of HIV-infected patients studied thus far, the huge number of integration events occurring during years of infection in each patient and the roles in cell survival and proliferation of BACH2 and STAT5B. Indeed, these transcription factors control the function, homeostasis and development of several cell types of the immune system, and the overexpression of their wild-type forms alone has never been associated to cancer. STAT5B can act as an oncogene only when specific activating mutations are present, and BACH2 is a tumor suppressor gene frequently found inactivated or silenced in human tumors, while been identified as CIS in murine insertional mutagenesis screenings. Thus, the activation of these genes could possibly prompt a specific functional state that favors the long-term persistence of Treg cells without inducing their uncontrolled proliferation or cell transformation. Moreover, beside BACH2 and STAT5B, it is unclear why genes such as LMO2, TAL1, CCND2, BMI1 and others, that are well-known players in spontaneously or vector-induced T cell leukemia in γ-retroviral vector-based HSC gene therapy patients, are not among the culprits of HIV-mediated insertional mutagenesis. We may speculate that several factors could be responsible for the specific outcome of HIV insertional mutagenesis. First, the occurrence and the type of tumor can be determined by the viral tropism and the cell-specific activity of the viral LTR, being
strongly modulated by the presence of cellular transcription factors that bind to the enhancer sequences contained within the LTR. For example, mice infected with replication-competent murine leukemia virus or mouse mammary tumor virus develop hematopoietic tumors or breast cancer, respectively, as the result of the different viral tropism and of the strong activity of the LTR in hematopoietic and epithelial progenitor/stem cells of these retroviruses. Second, the genetic and/or disease background may also modulate the risk of cell transformation. Indeed, in γ-retroviral vector-based HSC clinical trials for the treatment of X-linked severe combined immunodeficiency (SCID) and chronic granulomatous disease, and Wiskott Aldrich Syndrome, several patients developed leukemia as a consequence of vector-mediated insertional mutagenesis. However, the same vector type did not induce any transformation or clonal dominance events after 10 years of follow-up in a large cohort of HSC-transplanted patients enrolled in the gene therapy clinical trial for the treatment of adenosine deaminase SCID. Finally, in chronically infected HIV patients, transformed cells could suffer from cytoxicity caused by the viral infection, thus preventing the neoplastic transformation of the infected cell. However, it has been shown that FOXP3 expression in Treg cells represses the HIV LTR activity by targeting the NF-κB pathway, thus making these cells less sensitive to the cytopathic effects of the viral protein compared to other cell lineages. Given these considerations we may speculate that Treg cell clones harboring HIV insertions driving the expression of BACH2 and STAT5B could escape from the lytic activity of HIV-specific CTLs, thanks to their suppressive ability. Therefore, the persistence of Treg cells harboring BACH2 and STAT5B activating insertions could be favored, thanks to the intrinsic resistance of this cell type to HIV cytotoxicity and to their natural ability to tame the immune system.

BACH2 and STAT5B are transcription factors that have an important role in the development and function of regulatory T cells. Indeed, STAT5B specifically propagates IL-2-mediated signal favoring the development and proliferation of Treg cells. Homozygous STAT5B-deficient patients showed reduced FOXP3 expression level and impaired Treg cell number and function, while transgenic mice expressing a constitutive active form of Stat5b were characterized by hyperactive and highly proliferating Treg cells. Regarding BACH2 and its role in the biology of Treg cells, it has been shown that it promotes the development and survival of Treg lineage, restraints aberrant differentiation of Treg cells, and protects against immune-mediated diseases. Moreover, recent studies have highlighted important mechanistic roles for BACH2 in limiting terminal differentiation, preventing T cell exhaustion and potentially promoting the generation of long-lived, highly functional memory T cell population. In support of these notions, BACH2−/− mice were characterized by a severely decreased number of functional FOXP3+ Tregs that also displayed a terminally differentiated phenotype. Thus, considering the roles of BACH2 and STAT5B, the HIV-mediated overexpression of these genes could constitute part of the mechanism by which patients with HIV infection maintain a reservoir of infected cells in the Treg cell compartment. Treg cells are a relevant viral reservoir since they are long-lived, contain higher amounts of HIV DNA, and are able to produce more infectious viral particles upon activation compared to other T cell subsets. We did not specifically address if cell clones harboring BACH2 or STAT5B activating viral insertions could produce infectious HIV particles. Although as recently reported the integrated proviruses are frequently defective, rare expanded and persistent clones are able to produce infectious particles and contribute significantly to viral infection. Based on these reports, we may speculate that rare long-lived cell clones with BACH2 or STAT5B activating viral insertions and harboring an infectious viral genome could exist and sustain viral infection during years. Another fascinating and non-mutually exclusive possibility is that the enriched Treg cell clones may actually be specific for viral antigens, thus their expansion could favor the formation of an HIV immune-tolerant niche supporting viral persistence.

Overall, our molecular and functional studies provide novel compelling evidence that, as other retroviruses, HIV-1 takes advantage of insertional mutagenesis to favor its persistence in the host by infecting long-living and self-renewing cellular reservoir endowed with the ability to diminish the immune surveillance against infected cells. Our findings imply that HIV-1 does not only infect Treg cells but also favors their persistence over time, thereby “carving” its own reservoir. Given the role of STAT5B and BACH2 in promoting HIV-1 persistence, we propose that new targeted therapies aimed at interfering with their activity could provide the general means for the immunological re-sensitization toward HIV-1-infected cells and reducing long-lived cells carrying integrated, infectious proviruses with the ultimate goal of achieving a significant curtailment of latently infected cell reservoirs in ART-treated patients.

Methods

Study participants. HIV-1 integration site analysis has been performed from total DNA extracted from PBMC of 54 patients that received a 3-drug cART regimen based on a virus protease inhibitor plus two nucleoside reverse-transcriptase inhibitors. Twenty-nine patients out of 54 (54%) received the same ART treatment plus IL-2 administration, as described in Tambussi.

For RNA studies, samples were obtained from 87 HIV-1-infected individuals enrolled in ART studies. Forty-eight subjects were NT who started cART based on their CD4+ T cell counts, HIV-1 RNA levels in plasma (viremia) and the presence or absence of genotypic resistant mutations to antiretroviral agents, enrolled in previously published studies (MAIN study, EUDRACT number: 2008-007004-29; VEMAN study, EUDRACT number 2008-006287-11). Under cART, all patients achieved undetectable viremia levels (<50 copies of RNA/mL). Thirty-nine subjects were adult multi-experienced cART-treated HIV-1-infected patients who showed resistance or previous failures to all antiretroviral classes (NRTIs, NNRTIs, and PIs) and were enrolled into the MK0158-023, A4001050, and TMC125-C214 expanded access programs. A new cART regimen was prescribed based on genotypic resistant mutations, phenotypic viral tropism result, and previous failure history. All patients received raltegravir; new regimen was first-long term regimen, with achievement and maintenance of undetectable viremia levels. Informed consent has been signed from all patients. MAIN, VEMAN, MK0158-023, A4001050, and TMC125-C214 studies have been approved by the San Raffaele Hospital ethical committee.

Genomic analysis. Genomic DNA was extracted from total PBMC or cultured cells using the Qiagen-midi or micro DNA-Kit. For LV-transduced cells, vector copies per genome were determined by quantitative (q)PCR, as previously described. LAM-PCR was executed as previously described and used to retrieve HIV-1 genomic DNA junctions starting from 1 µg of DNA extracted from total PBMC. LAM-PCR products were sequenced by 454-Roche or Illumina MiSeq platforms and the sequences mapped on the human genome using a dedicated bioinformatics pipeline.

Cell purification, sorting, and flow cytometry. To isolate hematopoietic cell subsets from HIV-1-infected patients, PBMC were isolated by Ficol-Hypaque gradient from 90 ml of blood. Monocyte (CD14+ CD3−), CD8+, T-effector (CD4+ CD28−), and Treg (CD4+ CD25+ CD127+) cells were isolated by FACs sorting procedure after staining of PBMC with fluorescent anti-CD3 (cod. 345765, 1:100 dilution, BD Biosciences), anti-CD4 (PRNA392654), anti-CD8 (558116, 1:100 dilution, BD Biosciences), anti-CD14 (345784, 1:100 dilution, BD Biosciences), anti-CD25 (340907, 1:100 dilution, BD Biosciences), and anti-CD127 (25 12 78 42, 1:100 dilution, BD Biosciences). T stem cell memory, T central memory, T naive, and T effector memory cells were isolated by FACs sorting procedure starting from an enriched fraction of CD4+ cells purified using lineage-specific kit (Miltenyi Biotech). Next, CD4+ cells were labeled with anti-CD8 (555368, 1:100 dilution, BD Biosciences), anti-CD45RA (560362, 1:100 dilution, BD Biosciences), anti-CD4 (371418, 1:50 dilution, BD Biosciences), anti-CD25 (305608, 1:100 dilution, Biolegend), anti-CD45RO (1A-449-T100, 1:100 dilution, Biolegend), and anti-CD45RA (1A-449-T100, 1:100 dilution, Exbio) fluorescent mAbs. The different T cell subsets were identified by the following markers: Tscm, CD4+CD45RA+.
CD62L CD95+; T naïve, CD4+CD45RA-CD62L-CD95+; Tcm, CD4+CD45RA-CD62L+; and Tem, CD4+CD45RA+CD62L-.

Cells were amplified by MoFlo-XDP cell sorter (BeckmanCoulter) and resulted in >97% purity, as determined by repeating flow cytometric analysis of some populations of the sorted cells. For other HIV-1-infected patients and healthy subjects, monocytes, CD8, Teff, and Treg cells were purified from total PBMCs by immune-magnetic separation using lineage-specific kits (Miltenyi Biotec). The purity of each fraction was determined by flow cytometric analysis.

Vector production and T cell transduction. The bidirectional SIN.LV.PGK.BACH2 and SIN.LV.PGK.STAT5B plasmid constructs were generated using Gateway Technology (for details refer to Supplementary Methods). All concentrated VSV.G-pseudotyped LV stocks were produced by transient transfection of 293 T cells (CRL-3216, ATCC), as previously reported7. Gateway Technology (for details refer to Supplementary Methods). All BACH2 and SIN.LV.PGK.STAT5B plasmid constructs were generated using Vector production and T cell transduction previously reported24, 56, 57. Briefly, human naive CD4+ T cells were cultured from total PBMC from the blood of HIV-infected patients or from HD by immuno-magnetic cell purification. Purified cells were expanded in culture for 10 days by using the Dynabeads Human Treg Expander beads (Life Technologies) and RIL-2 (100 U/ml IL-2, Novartis and 5 ng/ml TGF-B, R&D Systems, Minneapolis, MN) to promote Treg polarization (iTreg condition). After 2 weeks of culture, cell phenotype was evaluated. The suppressive activity of ex vivo expanded Treg and iTreg cells were tested in an allogeneic setting. Indeed, allogeneic CD4+CD25+ T cells or total PBMC were purified from normal donor, stained by e-flour (Invitrogen), activated with plate-bound anti-CD3 (10 µg/ml, Dynal), 130-093-387, Miltenyi) and soluble anti-CD28 mAbs (1 µg/ml, cd. 555725, BD Pharmingen) and cultured in presence of absence of different concentrations of iTreg and Treg. After 4–5 days of activation, proliferation was assessed by dilution of e-flour staining by flow cytometry. The proliferation of iTreg was evaluated on CD25+ cells purified by magnetic immune-selections from transduced cells after 2 weeks of polarizing culture cells and activated, as described above, in presence or absence of IL2 (100 U/ml). After 72 h of culture, cells were pulsed for 16h with [3H] thymidine (1 mCi/well) (Amersham Biosciences).

Gene expression analysis. Total RNA was extracted from total PBMC or sorted cells with RNeasy purification kits (Qiagen) and reverse-transcribed with High Capacity cDNA Reverse Transcription Kit (Invitrogen). cDNA was used as template for qualitative PCR.

Amplification of the chimerical HIV/STAT5B and HIV/BACH2 transcripts was obtained by two sequential nested PCR reactions using oligonucleotides complementary to the U3-HIV LTR and STAT5B exons or BACH2 exons 6 and 7, see Supplementary Methods for sequence details.

Nested PCR was performed using 300 ng of cDNA isolated from 3 × 10^6 total PBMC using 300 ng of cDNA isolated from 3 to 5 × 10^6 total PBMC. The amplification was executed in a total volume of 25 µl and using GO Taq polymerase (Promega). The second nested PCR amplification was performed using 8 µl of the first PCR reaction. When PCR was performed on cDNA obtained from sorted cells, the final amplification was executed using from 4 to 5 ng of starting cDNA. Template-free controls and HIV negative samples were always used in our PCR testing and the presence of the specific chimERIC bands in a sample was confirmed by repeating the PCR on newly retro-transcribed RNA from the same collection or on cDNA obtained from PBMC collected at a different time point. Furthermore, each amplified band was cloned and sequenced to confirm its viral/cellular chimERIC nature (Supplementary Fig. 1). Finally, laboratory contaminations were excluded by looking for the presence of different point mutations among the different patients in the HIV portion of the chimeric sequence. Conversely, and as expected, the cellular genomic portion of BACH2 and STAT5B was conserved among the different patient samples.

The levels of the HIV/BACH2 and HIV/BACH2 chimERIC transcripts were measured by ddPCR using the QuantaLife ddPCR system. Custom-made ddPCR assays were designed for the detection of the chimeric HIV/BACH2 and HIV/BACH2 transcripts (see Supplementary Methods for sequence details). TaqMan® Gene Expression Assays (Applied Biosystems) were used to detect gene expression of STAT5B (Hs00723500_m1, exon boundary 16-17), BACH2 (Hs00223634_m1, exon boundary 8-9), and HPRT (Hs99999909_m1, exon boundary 6-7). Ten nanograms of cDNA were used for PCR amplification performed in duplicate and in a final volume of 20 µl. Approximately up to 20,000 monodispersized droplets for each sample were prepared using the QuantaLife droplet generator. Plates were quantified in a QuantaLife droplet reader, and the concentrations of the targets in the samples were determined using QuantaSoft software. The copies of the chimeric transcripts and the host genes were normalized to HPRT copies. Significance was calculated using paired t-tests. See Supplementary Methods for further details.

On selected samples, Illumina barcodes were attached to the amplified products using TruSeq Nano DNA LT Sample Prep Kit (Illumina), and the different libraries were pooled and sequenced using an Illumina MiSeq platform. Sequence reads were then aligned and a previously described approach was used to identify the number and frequency of the different viral haplotypes contained in each library (see Supplementary Methods).

Statistical analysis. Statistical analyses were performed as indicated and using GraphPad Prism 5.0 software.

Data availability. The sequence of the RT-PCR products used to identify viral haplotypes and the raw sequencing data used to resolve viral insertion sites are available from NCBI sequence archives under accession code PRJNA392654. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or are available from the authors upon request.

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References

1. Coffin, J. M., Hughes, S. H. & Varmus, H. E. Retroviruses (Cold Spring Harbor Laboratory Press, 1997).
2. Sander, A. R. et al. HIV-1 integration in the human genome favors active genes and local hotspots. Cell 110, 521–529 (2002).
3. Cesana, D. et al. Whole transcriptome characterization of aberrant splicing events induced by lentivector integrations. J. Clin. Invest. 122, 1667–1676 (2012).
4. Moiani, A. et al. Lentiviral vector integration in the human genome induces alternative splicing and generates aberrant transcripts. J. Clin. Invest. 122, 1653–1666 (2012).
5. Sherrill-Mix, S., Ocwieja, K. E. & Bushman, F. D. Gene activity in primary T cells infected with HIV89.6: intron retention and induction of genomic repeats. Retrovirology 12, 79 (2015).
6. Ranzanli, M. et al. Lentiviral vector-based insertional mutagenesis identifies genes associated with liver cancer. Nat. Methods 10, 155–161 (2013).
7. Cesana, D. et al. Uncovering and dissecting the genotoxicity of self-inactivating lentiviral vectors in vivo. Mol. Ther. 22, 774–785 (2014).
8. Ikeda, T., Shibata, J., Yoshimura, K., Koito, A. & Matsuishi, S. Recurrent HIV-1 integration at the BACH2 locus in resting CD4+ T cell populations during effective highly active antiretroviral therapy. J. Infect. Dis. 195, 716–725 (2007).
9. Malldorff, F. et al. HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. Science 345, 179–183 (2014).
10. Wagner, T. A. et al. Proliferation of cells with HIV integrated into cancer genes contributes to persistent infection. Science, doi:10.1126/science.1256304 (2014).
11. Tambussi, G. et al. Gacyfety of low-dose intermittent subcutaneous interleukin (IL)-2 in anti-tumor drug-experienced human immunodeficiency virus-infected persons with detectable virus load: a controlled study of 3-il2 regimens with antiviral drug therapy. J. Infect. Dis. 183, 1476–1484 (2001).
12. Calabria, A. et al. VISP: a computational pipeline for the identification and analysis of genomic vector integration sites. Genome Med. 6, 67 (2014).
13. Aiuti, A. et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341, 1233151 (2013).
14. Biffi, A. et al. Lentiviral hematopoietic stem cell gene therapy benefits with better clinical outcome. Science 341, 1233153 (2014).
15. Brady, T. et al. HIV integration site distributions in resting and activated CD4+ T cells infected in culture. AIDS 23, 1461–1471 (2009).
16. Hartig, N. et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science 326, 818–823 (2009).
17. Wang, G. P. et al. Analysis of lentiviral vector integration in HIV+ subjects undergoing autologous blood transplantation. Blood 119, 725 (2007).
18. Abu, A. et al. Analysis of the number of common integration sites of viral vectors—new methods and computing programs. PLoS ONE 8, e24247 (2013).
19. Collier, L. S., Carlson, C. M., Ravivomoh, S., Dupuy, A. J. & Langesaas, D. A. Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. Nature 436, 272–276 (2005).
20. Jonkers, J. & Berns, A. Retroviral insertional mutagenesis as a strategy to identify cancer genes. Biochem. Biophys. Acta 1287, 29–57 (1996).

21. Akkar, A. N., Vukmanovic-Stijic, M., Taams, L. S. & Macallan, D. C. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. Nat. Rev. Immunol. 7, 231–237 (2007).

22. Zhang, X. et al. CD4+CD62L(+) central memory T cells can be converted to Foxp3(+) T cells. PLoS ONE 8, e77322 (2013).

23. Prabhakaran, S., Rey, M., Zagordi, O., Beerenwinkel, N. & Roth, V. HIV haplotype inference using a propagating dirichlet process mixture model. IEEE/ACM Trans. Comput. Biol. Bioinform. 11, 182–191 (2014).

24. Levings, M. K. et al. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. J. Immunol. 166, 5530–5539 (2001).

25. Bovolenta, C. et al. Constitutive activation of STATs upon in vivo human immunodeficiency virus infection. Blood 94, 4022–4029 (1999).

26. Crotti, A. et al. Naturally occurring C-terminally truncated STAT5 is a negative regulator of HIV-1 expression. Blood 109, 5380–5389 (2007).

27. Kim, E. H. et al. Bach2 regulates homeostasis of Foxp3+ regulatory T cells and protects against fatal lung disease in mice. J. Immunol. 192, 985–995 (2014).

28. Kanai, T., Jenks, J. & Nadeau, K. C. The STAT5b pathway defect and autoimmune. Front. Immunol. 3, 234 (2012).

29. Yamada, K. et al. Constitutively active STAT5A and STAT5B in vitro and in vivo: mutation of STAT5 is not a frequent cause of leukemogenesis. Int. J. Hematol. 71, 46–54 (2000).

30. Rajala, H. L. et al. Discovery of somatic STAT5B mutations in large granular lymphocytic leukemia. Blood 121, 4541–4550 (2013).

31. Haam, K. et al. Epigenetic silencing of BTB and CNC homology 2 and transcription factors for memory CD8(+) T-cell development. Front. Immunol. 5, 234 (2014).

32. Kuwahara, M. et al. The Menin-Bach2 axis is critical for regulating CD4 T-cell senescence and cytokine homeostasis. Nat. Commun. 5, 3554 (2014).

33. Swaminathan, S. et al. Bach2 mediates negative selection and p53-dependent tumor suppression at the pre-B cell receptor checkpoint. Nat. Med. 19, 1014–1022 (2013).

34. Liu, J. et al. Identification of novel Bach2 transcripts and protein isoforms through tagging analysis of retroviral integrations in B-cell lymphomas. BMC Mol. Biol. 10, 2 (2009).

35. Chomont, N. et al. Integration sites and host genomic elements drive the beta-actin promoter activity in vivo. Blood 121, 4022–4029 (2013).

36. Braun, C. J. et al. Gene therapy for Wiskott-Aldrich syndrome-long-term efficacy and genotoxicity. Sci. Transl. Med. 6, 227ra233 (2014).

37. Hacein-Bey-Ahina, S. et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J. Clin. Invest. 118, 3132–3142 (2008).

38. Howe, S. J. et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J. Clin. Invest. 118, 3143–3150 (2008).

39. Stein, S. et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. Nat. Med. 16, 198–204 (2010).

40. Aiuti, A. et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. N. Engl. J. Med. 360, 447–458 (2009).

41. Cicala, M. P. et al. Update on the safety and efficacy of retroviral gene therapy for immunodeficiency due to adenosine deaminase deficiency. Blood 128, 45–54 (2016).

42. Grant, C. et al. Foxp3 represses transcription by targeting both NF-kappaB and CREB pathways. PLoS Pathog. 2, e33 (2006).

43. Selliah, N. et al. Foxp3 inhibits HIV-1 infection of CD4 T-cells via inhibition of LTR transcriptional activity. Virology 381, 161–167 (2008).

44. Jenks, J. A. et al. Differentiating the roles of STAT5B and STAT5A in human CD4+ T cells. Clin. Immunol. 148, 227–236 (2013).

45. Roychoudhuri, R. et al. BACH2 regulates CD8(+) T cell differentiation by controlling access of AP-1 factors to enhancers. Nat. Immunol. 17, 851–860 (2016).

46. Hu, G. & Chen, J. A genome-wide regulatory network identifies key transcription factors for memory CD8(+) T-cell development. Nat. Commun. 4, 2830 (2013).

47. Tran, T. A. et al. Resting regulatory CD4 T cells: a site of HIV persistence in patients on long-term effective antiretroviral therapy. PLoS ONE 3, e3305 (2008).

48. Cohn, L. B. et al. HIV-1 integration landscape during latent and active infection. Cell 160, 420–432 (2015).

49. Simonetti, F. R. et al. Clonally expanded CD4+ T cells can produce infectious HIV-1 in vivo. Proc. Natl. Acad. Sci. USA 113, 1883–1888 (2016).

50. Roychoudhuri, R. et al. BACH2 regulates CD8(+) T cell differentiation by controlling access of AP-1 factors to enhancers. Nat. Immunol. 17, 851–860 (2016).