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Research paper

# Anti-Tat immunity defines CD4$^+$ T-cell dynamics in people living with HIV on long-term cART.

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ABSTRACT

*Background:* Low-level HIV viremia originating from virus reactivation in HIV reservoirs is often present in cART treated individuals and represents a persisting source of immune stimulation associated with sub-optimal recovery of CD4$^+$ T cells. The HIV-1 Tat protein is released in the extracellular milieu and activates immune cells and latent HIV, leading to virus production and release. However, the relation of anti-Tat immunity with residual viremia, persistent immune activation and CD4$^+$ T-cell dynamics has not yet been defined.

*Methods:* Volunteers enrolled in a 3-year longitudinal observational study were stratified by residual viremia, Tat serostatus and frequency of anti-Tat cellular immune responses. The impact of anti-Tat immunity on low-level viremia, persistent immune activation and CD4$^+$ T-cell recovery was investigated by test for partitions, longitudinal regression analysis for repeated measures and generalized estimating equations.

*Findings:* Anti-Tat immunity is significantly associated with higher nadir CD4$^+$ T-cell numbers, control of low-level viremia and long-lasting CD4$^+$ T-cell recovery, but not with decreased immune activation. In adjusted analysis, the extent of CD4$^+$ T-cell restoration reflects the interplay among Tat immunity, residual viremia and immunological determinants including CD8$^+$ T cells and B cells. Anti-Env immunity was not related to CD4$^+$ T-cell recovery.

*Interpretation:* Therapeutic approaches aiming at reinforcing anti-Tat immunity should be investigated to improve immune reconstitution in people living with HIV on long-term cART.

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**Research in Context**

*Evidence before this study*

Combination antiretroviral therapy (cART) suppresses HIV-1 replication, thus reducing hyper-immune activation and leading to immune reconstitution and $CD4^+$ T-cell recovery. However, residual levels of immune activation remain even after years of treatment, and represent a strong predictive factor for incomplete immune reconstitution and $CD4^+$ T-cell recovery. Several factors underlie persistent immune activation, including microbial translocation at the gut mucosal barrier, chronic co-infections, including Cytomegalovirus and Hepatitis C virus infection, and intermittent HIV-1 low-level viremia, which is often observed in otherwise virologically suppressed long-term treated individuals. HIV-1 low-level viremia, in turn, originates from continued, sporadic reactivation of latent HIV-1 from the so-called "virus reservoirs". Since the HIV-1 Tat protein is known to be pivotal in HIV-1 reactivation and in activation of immune cells, we hypothesized that anti-Tat immunity might be a predictive factor for reduced low-level viremia and immune activation, and for improved $CD4^+$ T-cell recovery in long-term treated HIV-1 individuals. We searched PubMed using the terms "cART" "persistent immune activation", "immune reconstitution", "Tat immunity" and "HIV low-level viremia" but found no studies examining the role of anti-Tat immunity in low-level viremia, immune activation or immune reconstitution upon cART.

*Added value of this study*

To our knowledge, this is the first study assessing the role of humoral and cellular immune responses against the HIV-1 Tat protein in modulating low-level viremia, T-cell activation and $CD4^+$ T-cell dynamics, in individuals undergoing long-term cART.

*Implications of all the available evidence*

The results of our study indicate that long-lasting recovery of $CD4^+$ T cells in individuals on long-term cART is defined by a comprehensive anti-Tat immunity mounted during natural infection, including production of anti-Tat antibodies and frequent cell-mediated immune responses against the Tat protein. In contrast, anti-Env immunity is not related to $CD4^+$ T-cell recovery. These data shed further light on the reduction of immune activation and improvement of immune reconstitution induced in individuals on cART by immunization with a Tat therapeutic vaccine. Hence, immunotherapies aimed at inducing or reinforcing anti-Tat immunity should be pursued to maximize cART-mediated immune reconstitution in people living with HIV.

## Introduction

Immune reconstitution in people living with HIV initiating cART occurs through a biphasic response, with an early fast increase of peripheral $CD4^+$ T-cell counts, mainly due to reduced cell apoptosis and redistribution from lymphoid tissues, followed by slower dynamics reflecting $CD4^+$ T-cell production and homeostatic proliferation [1]. The slow phase of $CD4^+$ T-cell gain can continue through several years of treatment, approaching asymptotically [2,3] or reaching [4–6] a plateau, which, however, may not match the normal $CD4^+$ T-cell number of uninfected individuals [2–7]. Immune activation persisting despite suppressive cART is a strong predictive factor for reduced $CD4^+$ T-cell gains along both phases of immune reconstitution [8–10]. Although several causes underlie persistent hyper-immune activation [11], low-level viremia (herein defined as transient residual viremia or viral "blips" when below or above the threshold of detection of common diagnostic assays, respectively) is frequently identified in cART-treated individuals and might conceivably represent a main cause of sustained immune stimulation. However, studies aimed at evaluating the impact of low-level viremia in persisting immune activation [12–15] and $CD4^+$ T-cell recovery [2,6,16–18] have produced conflicting results.

According to recent studies, low-level viremia is genetically non-evolving and appears to originate from archival provirus harboured in latently infected $CD4^+$ T cells and other yet unknown reservoirs [19–21]. Evidence indicates that HIV-1 latency reversal in individuals undergoing cART occurs sporadically at multiple tissue niches [22] driven by stochastic bursts of virus gene expression [23], which conceivably also contributes to immune stimulation. Recent studies have shown that the HIV-1 Tat protein plays a key role in the establishment and reversal of HIV latency by amplifying stochastic transcriptional fluctuations at the HIV-1 long terminal repeat (LTR) [23]. Furthermore, the Tat protein is released in the extracellular milieu and is internalized by neighbor immune cells, leading to activation of host cell genes [24]. In particular, extracellular Tat activates $CD4^+$ T cells through a non-classical pathway [25], up-regulates the HIV-1 chemokine co-receptor CCR5 while antagonizing CXCR4 signaling [26,27] and induces the production and release of pro-inflammatory cytokines [25,28]; in addition, it induces dendritic cell maturation and polarization towards a Th1 phenotype, an effect due to Tat-dependent induction of TNF-$\alpha$, a well know autocrine and paracrine activator of HIV-1 gene expression [29]. Moreover, extracellular Tat bound to the membrane of infected cells inhibits the effector phase of cytotoxic T cells (CTLs) [30]. Finally, upon cell internalization, Tat is translocated to the cell nucleus, where it activates latent HIV-1, eventually leading to production and release of viral progeny [31,32]. These findings suggest a key role for extracellular Tat in decreasing the probabilities of HIV-1 extinction at niches of virus reactivation and in sustaining ongoing immune stimulation. It is thus conceivable that anti-Tat immunity is pivotal in controlling residual viremia and persistent immune activation in long-term cART treated individuals, allowing for a more sustained and prolonged $CD4^+$ T-cell recovery. This conclusion is supported by a better control of viral replication, containment of $CD4^+$ T-cell losses and slower progression to AIDS in untreated infected individuals developing anti-Tat Antibodies (Abs) [33–35] and/or Tat-specific CTLs [36,37], as well as by the reduction of immune activation and improvement of immune reconstitution in individuals on cART immunized with a Tat therapeutic vaccine [38–41]. Furthermore, anti-Tat Abs are at low titres and infrequently found in people living with HIV but, when present, they appear to be associated with control of HIV infection and disease progression [33–35], whereas Abs against structural HIV-1 antigens, including Gag and Env, are invariably detected at high titers in people living with HIV but do not appear to protect from disease progression [42].

However, no studies have evaluated the role of anti-Tat immunity in residual viremia, immune activation and immune reconstitution in individuals undergoing long-term cART.

The present study aimed at assessing the relation of anti-Tat immunity with immune activation, residual viremia, and $CD4^+$ T-cell dynamics during the slow phase of immune reconstitution in 118 cART long-term treated individuals enrolled in a 3-year prospective observational study. Further, the impact of anti-Tat humoral and cell-mediated immune responses on $CD4^+$ T-cell recovery was compared to the effect of anti-Env responses. Several immunological parameters, including $CD8^+$ T cells, B cells, NK cells and $CD4^+$ and $CD8^+$ T-cell subsets, expression of the activation markers CD38 and HLA-DR on $CD4^+$ and $CD8^+$ T cells were included in the analysis. The results of this study indicate that comprehensive immunity to HIV-1 Tat including humoral and cell-mediated immune responses is associated with long-lasting kinetics of $CD4^+$ T-cell recovery, which, in turn, underlie the interplay among Tat immunity, residual viremia, $CD8^+$ T-cell and B-cell dynamics.

## Methods

### *Study design and participants*

The ISS OBS T-002 (ClinicalTrials.gov NCT01024556) was a prospective multicentre observational study directed at evaluating the frequency, quality and persistence of humoral and cellular anti-Tat immune responses in HIV-infected cART-treated individuals.

The study was conducted between 2008 and 2012 in HIV-infected individuals, of either gender, $\geq$ 18 years-old, under effective cART (plasma viremia $<$ 50 HIV-1 RNA copies/mL for 6 months prior to screening).

Volunteers were enrolled in 9 clinical sites in Italy (San Raffaele University Hospital, Scientific Institute for Research, Hospitalization and Healthcare (IRCCS), Milan; L. Sacco University Hospital, Milan; A. di Savoia University Hospital, Turin; Spedali Civili University Hospital, Brescia; Arcispedale S. Anna University Hospital, Ferrara; Ospedale S. Maria Annunziata, Florence; Istituti Fisioterapici Ospitalieri San Gallicano, IRCCS, Rome; Policlinic University Hospital, Bari; Ospedale S.M. Goretti, Latina).

Participants were followed for up to 42 months (median 30 months; 25th percentile: 9 months, 75th percentile: 39 months; IQR= 30 months) with visits every 3 months. Immunological and virological tests were centralized at the Core Laboratory of Immunology and Virology (Ospedale S. Gallicano - IFO, Rome, Italy) and performed according to Standard Operating Procedures [38].

### *Ethics*

The study was approved by the competent authorities (General Directors) and the Ethics Committees of ISS and clinical centres [Comitato Etico Provincia of Ferrara (log. n. 36 of 29/11/2007), Comitato Etico IFO-Istituto San Gallicano IRCCS - Istituto Regina Elena IRCCS (log n. CE/567/02 of 5/10/2007), Rome; Comitato Etico AUSL of Latina (N. 273, log n. 111/206 of 20/07/2007); Azienda USL 10 of Florence S.S. Coordinamento Segreterie Comitati Etici (log n. 18/2007/CEL of 6/12/2007); Comitato Etico Azienda Ospedaliera Spedali Civili of Brescia (log n. SF of 7/11/2007); Comitato Etico Indipendente - Azienda Ospedaliera "Ospedale Policlinico Consorziale" of Bari (log n. 875/C.E of 12/09/2007); Comitato Etico Provinciale of Modena (log n. 2525/C.E. of 29/9/2007); Comitato Etico Interaziendale ASL of Turin (log Comitato n. 1017/25/07 of 11/12/2007); Comitato Etico Locale per la Sperimentazione Clinica, Azienda Ospedaliera Luigi Sacco of Milan (log n. 112/08/94/07/AP of 28/02/2008); Comitato Etico Fondazione Centro S. Raffaele Monte Tabor of Milan (06/09/2007)]. All participants signed the informed consent prior to enrolment. Personal data are protected according to the General Data Protection Regulation (GDPR) 2016/679 of the European Union.

### *Measurements of anti-Tat and anti-Env antibodies and cellular immune responses against Tat and Env*

Serum IgM, IgG and IgA against B clade Tat were assessed at each study visit by a homemade enzyme-linked immunosorbent assay (ELISA) as described previously [40,43]. The assay makes use of two cut-offs taking into account the baseline ("baseline cut-off") and the difference between signal and background noise ("delta cut-off"), respectively. To ensure a high specificity, the 2 cut-off values were set as 3 standard deviations above the mean of the optical density (OD) values obtained with sera from 89 Italian HIV-negative blood donors and 34 South African HIV-negative individuals [43]. Serum dilutions of 1:100 for IgG or 1:25 for IgM and IgA, respectively, identify the detection limit of the assay. Participants were considered anti-Tat Ab positive (anti-Tat $Ab^+$) when at least 1 anti-Tat isotype was detected at study entry and in follow-up visits. The same ELISA protocol and criteria for cut-off determination were applied for the measurement of anti-Env Abs and their titer definition [35]. HIV-1 SF162 gp120 (Novartis Vaccine and Diagnostics) was used. Only IgG Abs were tested, starting from a 1:100 dilution.

IFN-$\gamma$, IL-2 and IL-4 Elispot were performed as described previously [38,39] using commercial kits (EL285, EL202, EL204, R&D Systems), with 4 pools of overlapping 15mer-Tat peptides (5 mg/mL each) and 2 pools of Env peptides (5 mg/mL each). IFN-$\gamma$ Elispot was considered positive when spot-forming cells (SFC)/$10^6$ cells were $\geq$ 30 and SFC fold-increase over control was $\geq$ 3. The IL-2 and IL-4 Elispot were considered positive when SFC fold-increase was $\geq$ 3.

$CD4^+$ and $CD8^+$ T-cell proliferation was assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and expressed as fold-increase (FI) calculated as the ratio of antigen-induced proliferation index versus controls; FI $\geq$ 2 were considered positive [38,39].

Participants were considered positive for a cellular immune response if scored positive at least once during the study.

### *Lymphocyte phenotyping*

Freshly isolated peripheral blood lymphocytes (PBMC) were phenotyped with the BD Multitest 6-color TBNK reagent with BD Truecount tubes (BD Biosciences, San Jose, CA, USA). Samples were acquired with a FACSCanto flow cytometer (BD Biosciences) and data analyzed with a dedicated software (FACSCanto Clinical Software, BD Biosciences), as described [38,39]. $CD4^+$ T-cell counts determinations performed in parallel at each clinical center were highly concordant with those generated by the Core lab. For naïve, central and effector memory $CD4^+$ or $CD8^+$ T-cell subsets, freshly isolated PBMC were stained with anti-human CD3 (PerCP), CD8 (APC), CD45RA (FITC), CD62L (PE) monoclonal Abs (MultiTEST$^{TM}$ BD Biosciences) and anti-human CD4 (APC—Cy7) Ab (BD Biosciences), acquired by FACSCanto flow cytometer and analyzed with the BD FACSDiva Software (BD Biosciences), as described [38,39]. T-cell subsets were identified by hierarchical gating, as described [38,39]. For immune activation markers determination on $CD4^+$ and $CD8^+$ T cells, whole blood was stained with anti-CD8 FITC/CD38 PE/CD3 PerCP/HLA-DR APC (MultiTEST$^{TM}$ BD Biosciences) plus anti-CD4 APC—Cy7 Ab (BD Pharmingen). Collective quadrant gates, based on HLA-DR and CD38 expression on $CD4^+$ or $CD8^+$ T cells, were used [38].

### *Quantification of HIV-1 RNA*

The HIV-1 viral load (VL) in the plasma of HIV-1-infected participants was determined using a standardized RT-PCR assay with linear response from 20 to 10.000.000 HIV-1 RNA copies/mL (AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0; Roche Diagnostics), according

to the manufacturer's instructions; 850 $\mu$L of plasma were subjected to RT-PCR. Absence of threshold cycle (Ct) was categorized as "undetectable"; presence of Ct-values above that of the lower limit of quantitation (20 copies of HIV-1 RNA per mL) were categorized as "detected but not quantified"; presence of Ct-values equal or below that of the lower limit of quantitation as "quantified". Accordingly, participants were stratified into the following three classes:

VL= undetectable;

VL $\leq$ 40 copies HIV-1 RNA /mL [including participants with VL $<$ 20 copies/mL (i.e., HIV-1 RNA detected but not quantified) and participants with VL $\geq$ 20 copies/mL; $\leq$ 40 copies/mL];

VL $>$ 40 HIV-1 RNA copies/mL.

Values $\leq$ 40 copies HIV-1 RNA/mL were considered as "transient residual viremia", whereas values $>$ 40 HIV-1 RNA copies/mL were considered as "viral blips".

*Population stratification*

To determine the role of anti-Tat immunity in low-level viremia, persistent immune activation and CD4$^+$ T-cell recovery, participants were partitioned (stratified) according to anti-Tat humoral and cell-mediated immune responses. The partition procedure was done either by means of a single parameter (stratification by Tat serostatus OR frequency of anti-Tat cell-mediated immune responses), thus generating two sub-populations [i.e. anti-Tat Ab positive (anti-Tat Ab$^+$) and anti-Tat Ab negative (anti-Tat Ab$^-$) participants OR infrequent and frequent responders)] (single-stratification sampling), or by two parameters (Tat serostatus AND frequency of anti-Tat cell-mediated immune responses), thus generating four strata (i.e. anti-Tat Ab$^-$ infrequent responders, anti-Tat Ab$^-$ frequent responders, anti-Tat Ab$^+$ infrequent responders, anti-Tat Ab$^+$ frequent responders) (cross-stratification sampling).

*Statistical methods*

Descriptive statistics summarizing quantitative variables included mean, standard deviation, standard error, 50th, 25th and 75th percentile, interquartile range (IQR); qualitative variables were presented as number and percentage.

Comparison between anti-Tat Ab$^-$ and anti-Tat Ab$^+$ volunteers and volunteers with infrequent or frequent anti-Tat cell-mediated immune responses were performed using the Wilcoxon-Mann-Whitney Test for quantitative variables while the chi-square test (or Fisher's exact test) was performed to compare qualitative variables.

Longitudinal regression analysis for repeated measures was performed using a mixed model with random-effects to evaluate virological and immunological parameters over time both in the whole study population and upon stratification by anti-Tat humoral and/or cell-mediated immune responses.

Univariate and multiple generalized estimating equations with adjustment for repeated measures in the same volunteers were used to evaluate the effects of time, anti-Tat humoral and/or cellular responses, immunological and virological parameters on CD4$^+$ T cells. First, univariate models were performed to evaluate statistically significant associations and then multiple models were developed using a stepwise approach. Only relationships that remained statistically significant after the mutual adjustment in the multiple model are shown.

Statistical analyses were carried out at two-sided with a 0.05 significance level, using SAS® (Version 9.4, SAS Institute Inc., Cary, NC, USA).

*Role of the funding source*

The funding source (Ministry of Health of Italy) of this study had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

## Results

*Study population*

One hundred and forty-two cART-treated HIV-infected volunteers were enrolled in this 3-year observational study and 118 participants with at least two visits were considered evaluable for statistical analysis and categorized for humoral and cellular immune responses to the HIV-1 Tat protein (**Supplementary Fig. 1**). Participants were also analysed for anti HIV-1 gp120 immune responses. In line with previous reports, [33–35] low titres anti-Tat Ab were detected in a minority of participants (19%, IgG mean titre = 232), but were highly stable throughout the duration of the study (**Supplementary Fig. 2**), with only 6 participants positive at baseline scoring subsequently negative in a minority (13–33%) of visits. Accordingly, volunteers negative at all visits were referred to as anti-Tat Ab$^-$ ($N$ = 95; 80.5%), whereas participants positive at study entry and during follow-up were categorized as anti-Tat Ab$^+$ ($N$ = 23; 19.5%) (**Supplementary Fig. 1**). Cellular immune responses to Tat, including cytokines production by PBMC (IFN-$\gamma$, IL-2 and IL-4) and CD4$^+$ or CD8$^+$ T-cell lymphoproliferative responses, were detected in most subjects (83%) but were scattered and intermittent along time; hence, volunteers were categorized based on the frequency of positive visits (median = 40%, IQR: 25). In particular, participants with a percentage of positive visits below the median were referred to as infrequent responders ($N$ = 40; 45.5%) and those with a percentage equal or above the median as frequent responders ($N$ = 48; 54.5%) (**Supplementary Fig. 1**); only participants with at least four visits ($N$ = 88) were included in this categorization. The occurrence of anti-Tat Abs and the frequency of cellular immune responses to Tat appeared to be independently distributed (**Supplementary Fig. 3**).

In contrast, immune responses to Env were found to be invariably present in all participants. In particular, anti-Env Abs were present in 100% of volunteers with titers considerably higher as compared to anti-Tat Abs (mean $Log_{10}$ IgG titers = 4.45). Anti-Env Abs titers were stable and comparably distributed among anti-Tat Ab$^-$ and anti-Tat Ab$^+$ individuals (**Supplementary Fig. 4).** Similarly, cell-mediated immune responses to Env were present in 117/118 participants ($>$ 99%) and were temporally more frequent as compared to anti-Tat cell-mediated responses. In particular, in volunteers with at least 4 visits the median of positive visits was 80% (IQR = 33.33), and only 7 of them had less than 50% positive visits. Notably, however, the median of Env positive visits was slightly higher for Tat frequent responders as compared to infrequent responders (**Supplementary Table 1)**, suggesting that cell mediated immunity against HIV structural and non-structural antigens may cluster to some degree. Overall, these data indicated that, unlike anti-Tat immunity, anti-Env immunity is both widespread and intense in individuals on long-term cART. The frequent occurrence of anti-Env humoral and cell-mediated immunity prevented stratifying the study population by Env serostatus and/or frequency of cell-mediated immune responses to Env. A comparison of baseline parameters of participants according to the Tat serostatus and frequency of anti-Tat cellular responses is presented in **Table 1a, b**. Participants were prevalently males (77%) and the median age was 44 years. The median time on cART was 5 years and the shortest time of therapy was 11 months, indicating that all volunteers had entered the late, slow phase of cART-driven immune reconstitution [1,2,4]. No differences were observed in terms of sex, age, years from HIV diagnosis, and years or type of cART between either anti-Tat Ab$^-$ and anti-Tat Ab$^+$ individuals or infrequent and frequent responders. Notably, however, being anti-Tat Ab$^+$ was associated with higher nadir CD4$^+$ T-cell counts ($p$ = 0.0005; **Table 1a**).

**Table 1**
Baseline characteristics of study participants, presented by Tat serostatus and frequency of anti-Tat cellular immune responses.Table 1a. Demography and baseline parameters.

| | | Anti-Tat Ab$^-$ (*N* = 95) | Anti-Tat Ab$^+$ (*N* = 23) | *p*-value | Infrequent responses (*N* = 40) | Frequent responses (*N* = 48) | *p*-value |
|---|---|---|---|---|---|---|---|
| **Gender** | *Male* | 72 (75.8%) | 19 (82.6%) | 0.4848* | 32 (80.0%) | 36 (75.0%) | 0.5773* |
| | *Female* | 23 (24.2%) | 4 (17.4%) | | 8 (20.0%) | 12 (25.0%) | |
| **Age (years)** | *Median (IQR)* | 44 (10) | 43 (8) | 0.8090** | 44 (16) | 43 (7) | 0.3904** |
| **Years of HIV** | *Median (IQR)* | 10 (11) | 6 (9) | 0.1768** | 7 (11) | 9 (13) | 0.7653** |
| **Years of HAART** | *Median (IQR)* | 5 (8) | 5 (7) | 0.4756** | 6 (7) | 5 (6) | 0.3677** |
| **Nadir** | *Median (IQR)* | 204 (198) | 309 (100) | 0.0006**,b | 239 (195) | 206 (189) | 0.3352** |
| | *<250* | 59 (66.1%) | 5 (21.7%) | 0.0005*,b | 21 (52.5%) | 32 (66.7%) | 0.1764* |
| | *250+* | 36 (37.9%) | 18 (78.3%) | | 19 (47.5%) | 16 (33.3%) | |
| **HAART treatment at baseline** | *NNRTI* | 55 (57.9%) | 10 (43.5%) | 0.1371* | 22 (56.4%) | 26 (54.2%) | 0.7014* |
| | *PI* | 34 (35.8%) | 12 (52.2%) | | 15 (38.5%) | 21 (43.7%) | |
| | *NRTI* | 5 (5.3%) | 0 (0.0%) | | 1 (2.6%) | 1 (2.1%) | |
| | *other* | 0 (0.0%) | 1 (4.3%) | | 1 (2.6%) | 0 (0.0%) | |
| **HIV RNA** | *0 copies* | 75 (78.9%) | 23 (100.0%) | 0.0122***,b | 33 (82.5%) | 41 (85.4%) | 0.7095* |
| | *1+ copies* [a] | 20 (21.1%) | 0 (0.0%) | | 7 (17.5%) | 7 (14.6%) | |

* Chi square test.
** Mann-Whitney test.
*** Fisher's exact test.
[a] 15 anti-Tat Ab$^-$ volunteers with 1–40 copies/mL; 5 with more than 40 copies/mL; 0 infrequent responders and 2 frequent anti-Tat cellular responders with more than 40 copies/mL.
[b] Statistical significance was retained after Bonferroni correction for multiple comparisons.

At study entry, 20 out of 95 (21%) anti-Tat Ab$^-$ volunteers presented low-level plasma viremia (15 with VL ≤ 40 copies/mL, and 5 with > 40 copies/mL), while all anti-Tat Ab$^+$ volunteers had undetectable VL ($p$ = 0.0122) (**Table 1a**). There was no significant association of low-level plasma viremia with the frequency of anti-Tat cellular responses ($p$ = 0.7095) (**Table 1a**).

Besides nadir CD4$^+$ T-cell counts, the other immunological parameters investigated (CD4$^+$ and CD8$^+$ T-cell count, CD4$^+$/CD8$^+$ T-cell ratio, NK cell number, B-cell count, CD4$^+$ T-cell and CD8$^+$ T-cell subsets, and activation markers CD38 and HLA-DR were not significantly different by Tat serostatus or frequency of cellular responses, except for naïve CD8$^+$ T-cell levels that were higher in frequent responders than in infrequent responders ($p$ = 0.0400), although statistical significance was lost upon correction for multiple comparisons (**Table 1b**). A non-significant, borderline association was observed for central memory CD4$^+$ Tcell percentages and infrequent responders ($p$ = 0.0736), and for CD8$^+$ T-cell percentages and anti-Tat Ab$^-$ subjects ($p$ = 0.0601) (**Table 1b**).

*Relation of anti-Tat and anti-Env immunity with immune reconstitution dynamics*

A regression analysis of CD4$^+$ T-cell number, CD8$^+$ T-cell number and CD4$^+$/CD8$^+$ T-cell ratio along the 42 months of follow-up evidenced ongoing slow immune reconstitution dynamics in the study population, with yearly changes comprising an increase of 10 CD4$^+$ T cells/$\mu$L, a decrease of 18 CD8$^+$ T cells/$\mu$L and an increase of 0.073 units in the CD4$^+$/CD8$^+$ ratio (**Fig. 1a, d and g**). The extent of these variations is consistent with what observed in other studies for the late cART immune reconstitution phase [2–8].

However, when the analysis was stratified for anti-Tat immunity, anti-Tat Ab$^-$ volunteers and infrequent cellular responders exhibited only non-significant, marginal increases of CD4$^+$ T cells (≤ 4 cells/$\mu$L per year), whereas anti-Tat Ab$^+$ participants and frequent cellular responders showed an overall gain of about 30 ($p$ < 0.0001) and 25 ($p$ = 0.0242) CD4$^+$ T cells/$\mu$L per year, respectively (**Fig. 1b and c**).

**Table 1b**
Baseline virological and immunological parameters.

| | Anti-Tat Ab$^-$ | | Anti-Tat Ab$^+$ | | *p* value | Infrequent responders | | Frequent responders | | *p* value |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | Median (IQR) | N | Median (IQR) | | N | Median (IQR) | N | Median (IQR) | |
| **HIV RNA (if >0 copies/mL)** | 20 | 23 (34) | 0 | – | na | 7 | 8 (8) | 7 | 25 (38) | 0.1094 |
| **CD4$^+$ (cells/$\mu$L)** | 95 | 624 (378) | 23 | 661 (336) | 0.4130 | 40 | 626 (380) | 48 | 669 (358) | 0.5242 |
| **CD8$^+$ (cells/$\mu$L)** | 93 | 864 (492) | 23 | 886 (478) | 0.9283 | 40 | 925 (429) | 46 | 872 (545) | 0.6936 |
| **CD4/CD8 T-cell ratio** | 93 | 0.76 (0.47) | 23 | 0.89 (0.36) | 0.3886 | 40 | 0.74 (0.54) | 46 | 0.73 (0.47) | 0.5473 |
| **NK (cells/$\mu$L)** | 93 | 206 (159) | 23 | 206 (167) | 0.3120 | 40 | 203 (203) | 46 | 223 (182) | 0.3131 |
| **B (cells/$\mu$L)** | 93 | 256 (211) | 23 | 224 (109) | 0.2770 | 40 | 257 (189) | 46 | 239 (174) | 0.8118 |
| **Naive CD4$^+$ (%)**[a] | 59 | 26.40 (23.78) | 19 | 21.60 (18.61) | 0.7621 | 35 | 23.48 (21.45) | 35 | 28.90 (19.63) | 0.1169 |
| **Central Memory CD4$^+$ (%)**[a] | 59 | 51.60 (17.52) | 19 | 55.60 (20.10) | 0.2739 | 35 | 56.23 (12.70) | 35 | 50.56 (18.84) | 0.0736 |
| **Effector Memory CD4$^+$ (%)**[a] | 59 | 18.29 (12.84) | 19 | 15.27 (9.20) | 0.2045 | 35 | 18.63 (14.00) | 35 | 18.06 (10.81) | 0.7032 |
| **Naive CD8$^+$ (%)**[a] | 59 | 25.90 (19.39) | 19 | 25.60 (15.20) | 0.8386 | 35 | 21.60 (18.90) | 35 | 26.62 (14.75) | 0.0400[c] |
| **Central Memory CD8$^+$ (%)**[a] | 59 | 23.20 (15.38) | 19 | 20.30 (9.46) | 0.0601 | 35 | 24.19 (15.96) | 35 | 22.37 (10.47) | 0.5658 |
| **Effector Memory CD8$^+$ (%)**[a] | 59 | 46.00 (21.26) | 19 | 51.66 (19.20) | 0.1696 | 35 | 50.60 (22.45) | 35 | 46.05 (17.63) | 0.3071 |
| **CD4$^+$/CD38$^+$/HLA-DR$^+$**[b] | 69 | 58.65 (17.52) | 19 | 58.22 (26.83) | 0.9112 | 37 | 54.36 (15.57) | 45 | 59.98 (18.55) | 0.5052 |
| **CD8$^+$/CD38$^+$/HLA-DR$^+$**[b] | 69 | 49.05 (24.23) | 19 | 51.58 (37.03) | 1.0000 | 37 | 51.59 (22.54) | 45 | 47.97 (25.27) | 0.4731 |

Median (IQR) of virological and immunological parameters of study participants at baseline. N indicates the number of individuals tested for each parameter. Comparison between anti-Tat Ab$^-$ and anti-Tat Ab$^+$ participants and infrequent and frequent anti-Tat cellular responders was performed using the Wilcoxon-Mann-Whitney Test.
[a] Baseline values are the first available determination and were not necessarily collected at study visit 1.
[b] Sum of the percentages of CD38$^+$/HLA-DR$^-$, CD38$^-$/HLA-DR$^+$ and CD38$^+$/HLA-DR$^+$ cells; baseline values are the first available determination and were not necessarily collected at study visit 1.
[c] Statistical significance was not retained after Bonferroni correction for multiple comparisons.

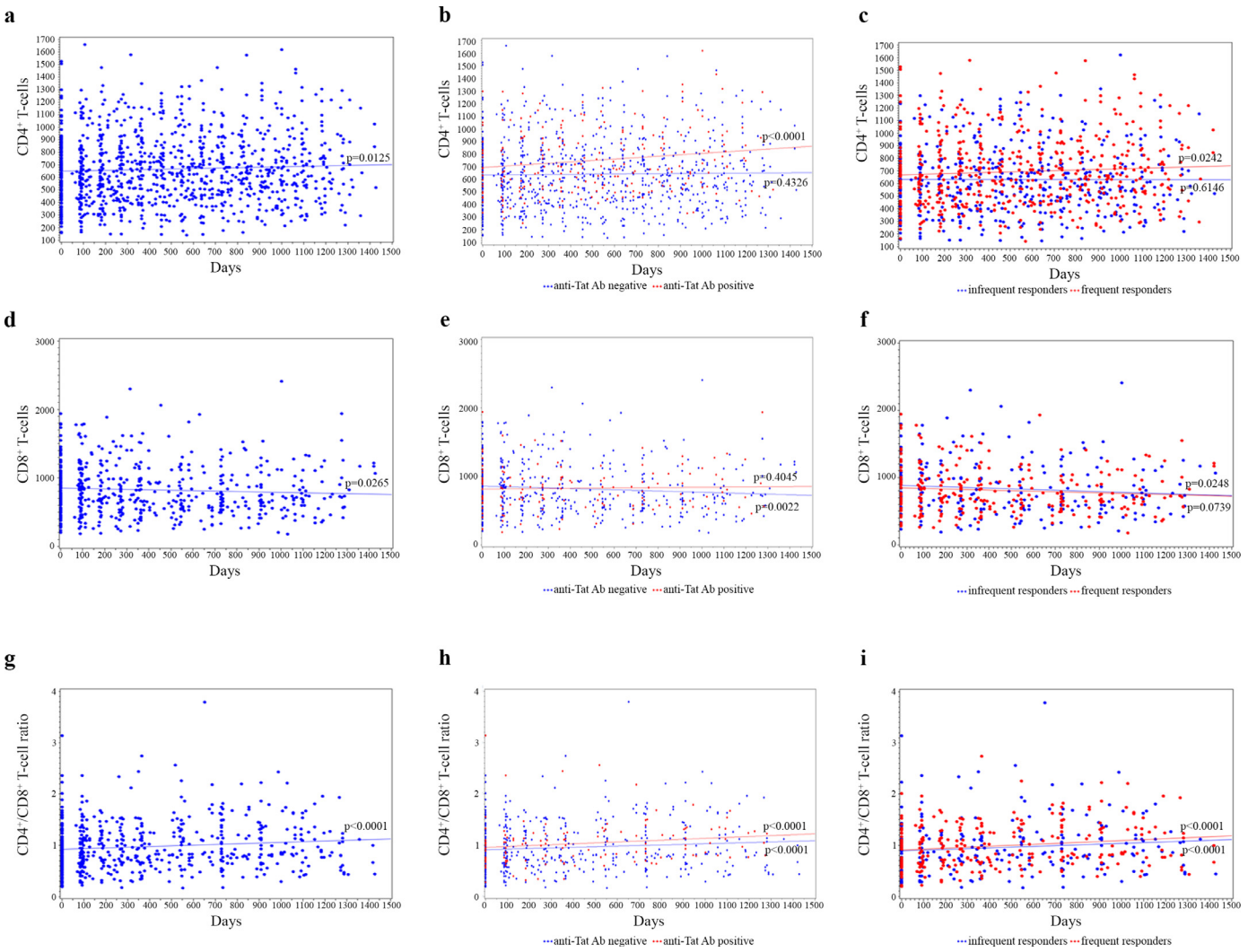


**Fig. 1.** *Dynamics of immune parameters over 3 years of follow up in participants stratified by anti-Tat antibodies or cellular immune responses to Tat.* CD4$^+$ T cells, CD8$^+$ T cells and CD4$^+$/CD8$^+$ T-cell ratio over time were analysed according to anti-Tat humoral and cellular responses in a longitudinal regression analysis for repeated measures using a mixed model with random-effects; $N$ = 95 anti-Tat Ab$^-$, 23 anti-Tat Ab$^+$, 40 infrequent responders, 48 frequent responders. **(a, b, c)** CD4$^+$ T-cell dynamics over time. (**a**) Regression analysis of the whole study population ($\beta$ = 0.028 cells/day, $p$ = 0.0125); (**b**) Participants stratified by humoral responses to Tat (anti-Tat Ab$^+$: $\beta$ = 0.09 cells/day, $p < 0.0001$; anti-Tat Ab$^-$: $\beta$ = 0.01 cells/day, $p$ = 0.4326); (**c**) Volunteers stratified by cellular immune responses to Tat (frequent responders: $\beta$ = 0.03 cells/day, $p$ = 0.0242; infrequent responders: $\beta$ = 0.01 cells/day, $p$ = 0.6146). **(d, e, f)** CD8$^+$ T-cell kinetics. (**d**) Regression analysis of the whole study population ($\beta$ = -0.051 cells/day, $p$ = 0.0265) (**e**) Regression analysis according to anti-Tat humoral responses (anti-Tat Ab$^+$: $\beta$ = 0.04 cells/day, $p$ = 0.4045; anti-Tat Ab$^-$: $\beta$ = -0.08 cells/day, $p$ = 0.0022); (**f**) Analysis according to cellular immune responses to Tat (frequent responders: $\beta$ = -0.06 cells/day, $p$ = 0.0739; infrequent responders: $\beta$ = -0.07 cells/day, $p$ = 0.0248); **(g, h, i)** CD4$^+$/CD8$^+$ T-cell ratio dynamics over time. (**g**) Regression analysis of the whole study population ($\beta$ = 0.0002 cells/day, $p < 0.0001$) (**h**) Participants stratified by anti-Tat humoral responses (anti-Tat Ab$^+$: $\beta$ = 0.0002/day, $p < 0.0001$; anti-Tat Ab$^-$: $\beta$ = 0.0001/day, $p < 0.0001$); (**i**) Participants stratified by cellular immune responses (frequent responders: $\beta$=0.00015/day, $p < 0.0001$; infrequent responders $\beta$ = 0.00016/day, $p < 0.0001$). $\beta$: slope of the regression line; $p$-values: probability $\beta \neq 0$.

Thus, in this analysis the CD4$^+$ T-cell dynamics of the study population appear to be driven by anti-Tat immunity.

Conversely, CD8$^+$ T cells showed significant decreases in anti-Tat Ab$^-$ individuals (-30 cells/$\mu$L/year, $p$ = 0.0022) but not in anti-Tat Ab$^+$ volunteers. A significant decrease was detected also in infrequent responders (-25 cells/$\mu$L/year, $p$ = 0.0248), whereas a non-significant, borderline decrease was present in frequent responders (-22 cells/$\mu$L/year; $p$ = 0.0739) (**Fig. 1e and f**). Hence, CD8$^+$ T-cell decrease appears to be related to a restricted anti-Tat immunity characterized by the absence of humoral immunity or the presence of infrequent cell-mediated immune responses.

The CD4$^+$/CD8$^+$ T-cell ratio increased in all groups ($p < 0.0001$), reflecting the specific combined dynamics of CD4$^+$ and CD8$^+$ T cells. Specifically, the increase was in the range of 0.06 to 0.07 units per year for anti-Tat Ab$^+$ subjects as well as frequent and infrequent responders, whereas a lower increase (0.035 units/year) was observed in the anti-Tat Ab$^-$ subjects (**Fig. 1h and i**).

CD4$^+$ and CD8$^+$ T-cell subsets (naïve, central memory and effector memory) did not show any consistent variation in any of the groups. Noticeably, however, frequent responders showed a significant increase of about 15 NK cells per $\mu$L per year ($p$ = 0.0203) (**Supplementary Table 2**).

These data were obtained by sorting participants independently for anti-Tat humoral or cell-mediated immunity (single-stratification analysis, see Methods). To confirm these findings, participants were simultaneously sorted for both Tat serostatus and anti-Tat cellular responses (cross-stratification analysis). Although this procedure doubled the subset groups reducing their size, significant variations were observed that confirmed the previous analysis. In particular, CD4$^+$ T-cell increases were detected in anti-Tat Ab$^+$ frequent

responders (47 cells/$\mu$L/year, $p$ = 0.0001), but not in anti-Tat Ab$^+$ infrequent responders nor in anti-Tat Ab$^-$ participants, irrespectively of the frequency of cell-mediated responses (**Supplementary Table 3**). These data confirmed that a comprehensive anti-Tat immunity, comprising both humoral and frequent cell-mediated responses, underlies the late phase of CD4$^+$ T-cell recovery over long-term cART. In the cross-stratification approach, CD8$^+$ T-cell reductions remained significant for anti-Tat Ab$^-$ frequent responders ($p$ = 0.0047); furthermore, a non-significant, borderline decrease was observed in anti-Tat Ab$^-$ infrequent responders ($p$ = 0.0788) (**Supplementary Table 3**), confirming that CD8$^+$ T-cell decreases might be related to a restricted anti-Tat immunity. A significant CD4$^+$/CD8$^+$ T-cell ratio increase ($p$ = 0.0001) and a substantial stability of CD4$^+$ and CD8$^+$ T-cell subsets was observed in all groups also in this analysis. Notably, anti-Tat Ab$^-$ frequent responders showed a significant increase of about 15 NK cells per $\mu$L per year ($p$ = 0.0239), confirming the results obtained with the single-stratification analysis, whereas an increase of 21 B cells per $\mu$L per year was detected in anti-Tat Ab$^+$ frequent responders ($p$ = 0.0364) (**Supplementary Table 3**). Therefore, these data confirmed that anti-Tat immunity is associated with immune reconstitution upon long-term cART underlined by long-lasting CD4$^+$ T-cell, NK-cell and B-cell recovery.

The impact of anti-Tat immunity on immunological dynamics, as examined by both single and cross-stratification analysis, is summarized in **Fig. 2.** The presence of a comprehensive immunity to Tat (i.e., humoral immunity combined with high frequency of cellular responses) appeared to be associated with a significant CD4$^+$ T-cell ($p$ = 0.0001) and B-cell ($p$ = 0.0364) increases with stable CD8$^+$ T-cell counts over time. In contrast, an immune background characterized by a negative Tat serostatus (with or without frequent cellular responses) was generally associated to a decrease of CD8$^+$ T cells; in this category, frequent responders showed a significant increase of NK cells ($p$ = 0.0239). All groups experienced an increase of CD4$^+$/CD8$^+$ T-cell ratio reflecting their distinctive CD4$^+$ and CD8$^+$ T-cell dynamics over time ($p < 0.0001$).

Finally, to gain insights on the role of comprehensive anti-HIV immunity in CD4$^+$ T-cell dynamics, anti-Tat Ab$^-$ and anti-Tat Ab$^+$ participants were categorized according to the median value of anti-Env Ab titers (**Supplementary Table 4**). In this analysis, anti-Tat Ab$^-$ participants failed in gaining significant CD4$^+$ T-cell increases, irrespectively of anti-Env antibody titers. In contrast, anti-Tat Ab$^+$ volunteers, either with anti-Env Ab titers below or above the median, showed similar gains of about 37 ($p$ = 0.0239) and 32 ($p$ = 0.0027) CD4$^+$ T cells/$\mu$L per year, respectively. Thus, anti-Env humoral immunity does not appear to affect the recovery of CD4$^+$ T cells, which, in contrast, appears to be related to immunity against the HIV-1 Tat protein.

*Relation of nadir CD4$^+$ T-cell counts and immune activation with immune reconstitution dynamics*

Analyses were then focused to evaluate the impact of nadir CD4$^+$ T-cell counts and immune activation on CD4$^+$ T-cell dynamics since they are known to affect the immune reconstitution driven by cART [8,10,44].

A longitudinal regression analysis of CD4$^+$ T-cell counts over time according to nadir CD4$^+$ T cells showed non-significant, borderline gains of 9 and 12 CD4$^+$ T cells per $\mu$L per year for participants with nadir either below or above 250 cells per $\mu$L, respectively (**Supplementary Table 5**). However, when participants in the upper nadir stratum (i.e. $\geq$ 250 cells per $\mu$L) were stratified according to anti-Tat Abs or frequency of cell-mediated responses to Tat, a significant increase was observed for anti-Tat Ab$^+$ (27 cells/$\mu$L/year, $p$ = 0.0163) (**Supplementary Table 5**). These data, and the association of the anti-Tat seropositive status with higher nadir CD4$^+$ T cells (**Table 1a**), suggest that anti-Tat humoral responses rather than nadir CD4$^+$ T cells are a main predictive factor of CD4$^+$ T-cell increases during the late, slow phase of cART immune reconstitution.

A similar analysis was performed to evaluate the impact of immune activation on CD4$^+$ T-cell dynamics. To this purpose, the longitudinal counts of CD4$^+$ and CD8$^+$ T cells expressing the activation markers CD38 and HLA-DR were used to determine, for each volunteer, the intra-individual mean percentage of activated cells. The volunteers were then stratified according to the median of the intra-individual means. Unexpectedly, a significant increase of CD4$^+$ T cells over time was observed for participants expressing CD4$^+$ or CD8$^+$ T-cell activation markers above the median (**Supplementary Table 6**). These data are counterintuitive, given the known association of persistent immune activation, particularly CD8$^+$ T-cell activation, with suboptimal CD4$^+$ T-cell gains in cART-treated individuals [6,7]. The only exception, however, was represented by CD4$^+$/CD38$^-$/HLA-DR$^+$ cells, which were predictive of CD4$^+$ T-cell increase when their mean percentage was below the median ($p$ = 0.0089) (**Supplementary Table 6**).

Notably, the above activation markers were independently distributed irrespectively of anti-Tat humoral or cell-mediated immune responses (chi-square test; $p > 0.05$), indicating that anti-Tat immunity is not associated with a decreased immune activation in individuals on long-term cART.

*Relation of low-level viremia with anti-Tat immunity, immune activation and CD4$^+$ T-cell dynamics*

The dynamics of HIV residual viremia and viral blips were evaluated along the 42 months follow-up study. A higher percentage of

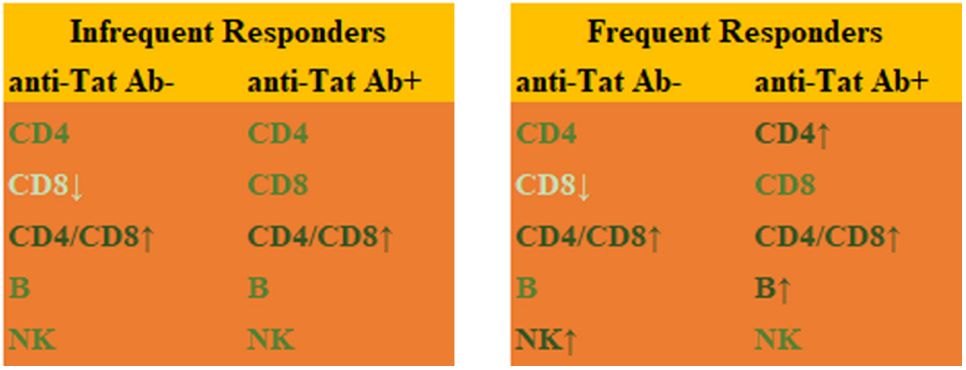


**Fig. 2.** *Volunteers' immunological dynamics according to Tat humoral and cellular responses.* Summary of the results of longitudinal regression analysis of immune-cell variations over time in volunteers cross-stratified for Tat serostatus and frequency of cellular responses to Tat. Dark green with arrow pointing upwards: significant increase over time; light green with arrow pointing downwards: significant decrease along time; intermediate green and absence of arrow: non-significant variations. CD4$^+$ and CD8$^+$ T cells in anti-Tat Ab$^+$ infrequent responders showed non-significant increases and decreases, respectively, resulting in a significant increase of the CD4$^+$/CD8$^+$ T-cell ratio over time (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

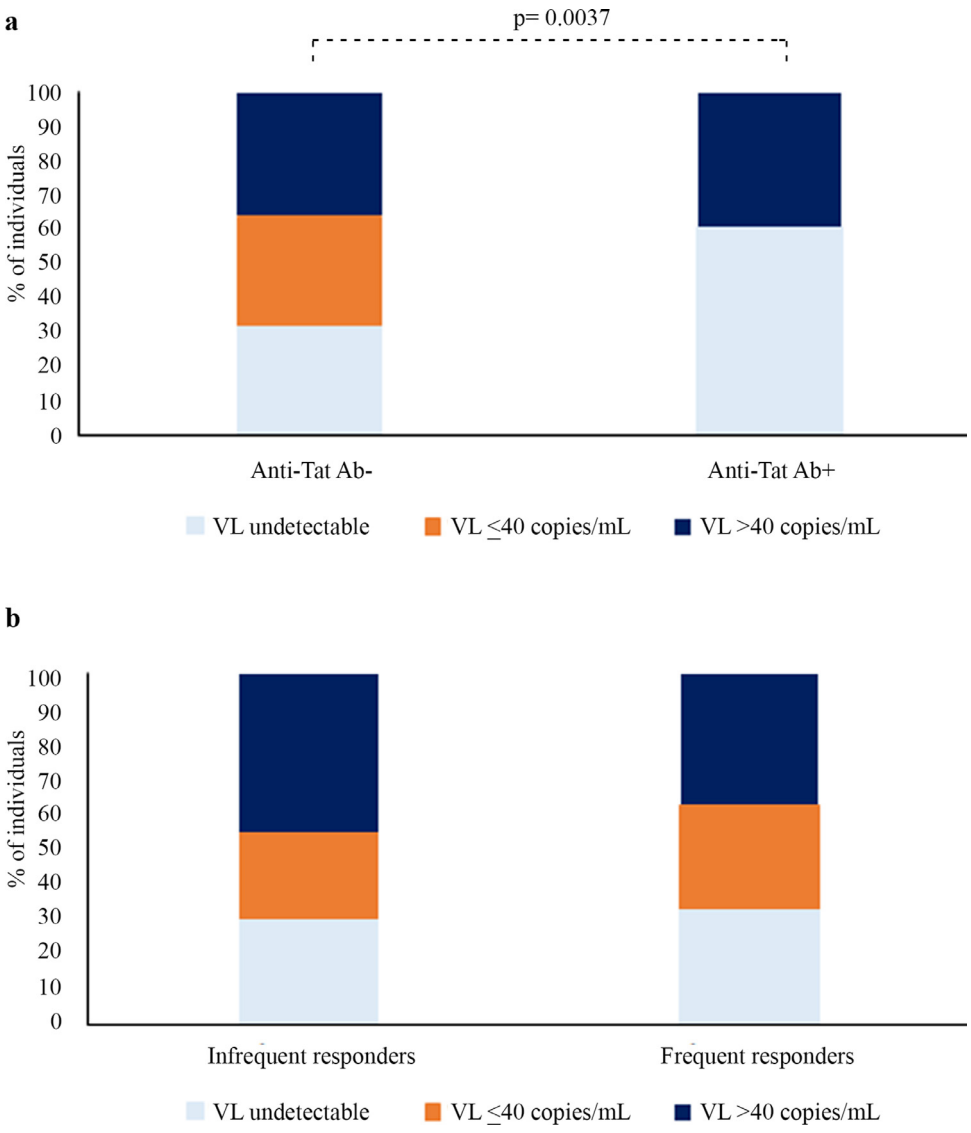


**Fig. 3.** *Residual viremia over 3 years of follow up in participants stratified by anti-Tat antibodies or cellular immune responses to Tat.* Percentages of individuals that remained persistently aviremic (VL = undetectable) or had episodes of transient residual viremia (VL ≤ 40 HIV-1 RNA copies/mL) or viral blips (VL > 40 HIV-1 RNA copies/mL) during the study. (A) Participants stratified by anti-Tat humoral response ($p$ = 0.0037) (anti-Tat Ab$^-$: VL = undetectable: n. 31; VL ≤ 40: n. 30; VL > 40: n.34; anti-Tat Ab$^+$: VL = undetectable: n. 14; VL ≤ 40: n. 0; VL > 40: n. 9); (B) Participants stratified by frequency of cellular responses to Tat ($p$ = 0.7730) (infrequent responders: VL = undetectable: n.12; VL ≤ 40: n. 10; VL > 40: n.18; frequent responders: VL = undetectable: n. 16; VL ≤ 40: n. 14; VL > 40: n. 18). A chi-square test was utilized.

anti-Tat Ab$^+$ volunteers resulted persistently aviremic over the entire study as compared to anti-Tat Ab$^-$ subjects (**Fig. 3a**). In fact, although viral blips (VL > 40 HIV-1 RNA copies/mL) were observed in both groups (39% and 36%, respectively), anti-Tat Ab$^+$ participants did not show episodes of sporadic residual viremia (VL ≤ 40 HIV-1 RNA copies/mL), which was detected only in anti-Tat Ab$^-$ subjects (chi-square test, $p$ = 0.0037) (**Fig. 3a**). In contrast, no significant differences were observed for infrequent versus frequent responders, who showed a comparable percentage of sporadic viremic episodes over time (chi-square test, $p$ = 0.773, **Fig. 3b**). These data indicated that anti-Tat Abs, known to neutralize extracellular Tat [45], are associated with control of residual viremia in otherwise virologically suppressed individuals undergoing long-term cART.

A complex relation of low-level viremia was detected with CD8$^+$ T-cell activation. In particular, the aviremic status was significantly associated with increased HLA-DR expression ($p$ = 0.0147), whereas viral blips with increased, simultaneous expression of CD38 and HLA-DR ($p$ = 0.0364) **Supplementary Table 7**).

To evaluate whether low-level viremia had a significant impact on CD4$^+$ T-cell dynamics over time, a longitudinal regression analysis was performed by stratifying volunteers according to VL cut-off intervals (aviremic, transient residual viremia, viral blips). In this analysis, a non-significant, borderline increase of CD4$^+$ T cells was associated with the aviremic status ($p$ = 0.0614) and not with residual viremia or blips (**Table 2**), suggesting that low-level viremia may hamper long-term immunological recovery under cART. However, when participants were simultaneously stratified by both viral load cut-off intervals and anti-Tat humoral or cellular responses, a significant increase of CD4$^+$ T cells was observed in anti-Tat Ab$^+$ subjects irrespectively of low-level viremia (aviremic volunteers: 27 CD4$^+$ T cells/$\mu$L/year, $p$ = 0.0473; volunteers with viral blips: 38 CD4$^+$ T cells/$\mu$L/year, $p$ = 0.0007) (**Table 3a**). As to what concerns cellular immune responses to Tat, a significant increase of CD4$^+$ T cells was observed in infrequent responders with transient residual viremia (35 cells/$\mu$L/year, $p$ = 0.0069) as well as in frequent responders with viral blips (25 cells/$\mu$L/year, $p$ = 0.0037), but not in aviremic participants with either infrequent or frequent cellular responses (**Table 3b**).

Thus, residual viremia does not behave as an independent determinant of CD4$^+$ T-cell recovery in long-term cART-treated subjects, but it does appear to be related to anti-Tat humoral immunity.

**Table 2**
Impact of residual viremia on CD4$^+$ T-cell dynamics.

| | N | Estimate[a] | 95% CI[b] | | *p*-value |
|---|---|---|---|---|---|
| **Aviremic status (VL = undetectable)** | 45 | 0.0434 | -0.0021 | 0.0889 | 0.0614 |
| **Transient residual viremia (VL ≤ 40 RNA copies/mL)** | 30 | 0.0186 | -0.0216 | 0.0587 | 0.3634 |
| **Viral blips (VL ≥ 41 RNA copies/mL)** | 43 | 0.0258 | -0.0067 | 0.0583 | 0.1191 |

Longitudinal regression analysis of CD4$^+$ T cells over time according to residual VL. A longitudinal regression analysis for repeated measures was performed using a mixed model with random-effects in order to evaluate CD4$^+$ T cells over time stratified by classes of viral load.
[a] Variation of CD4$^+$ T-cell number per day.
[b] Confidence Interval.

*Multiple regression analysis of CD4$^+$ T-cell counts*

A multiple generalized estimating equation with adjustment for repeated measures was used in a stepwise approach (see Methods) to explore the interplay of time, anti-Tat humoral and cellular immunity, residual viremia/blips, immunological parameters (including CD8$^+$ T-cell count, B-cell count, NK-cell count, and naïve, central memory and effector memory CD8$^+$ T-cell subsets), and immune activation parameters on CD4$^+$ T-cell counts over time (**Table 4**).

In this adjusted analysis, the only explanatory co-variates that remained significant were residual viremia/blips, CD8$^+$ T-cell counts and B-cell counts (see **Table 4**).

The loss of significance of the variate time after mutual adjustments in this model indicates that the late, slow phase of CD4$^+$ T-cell recovery is dependent upon the complex interplay over time of anti-Tat immunity, low-level viremia, CD8$^+$ T-cell and B-cell dynamics.

## Discussion

The primary hypothesis of the present study was that anti-Tat immunity mounted in the course of natural infection (and/or potentially reconstituted upon cART) might blunt latent HIV reactivation, with consequent reduction of persistent low-level viremia and T-cell activation, allowing for long-lasting CD4$^+$ T-cell recovery in the late phase of cART-mediated immune reconstitution. Such an assumption is supported by several observations including (i) the key role of the Tat protein in determining the fate of HIV life cycle toward latency or productive infection [23,32], (ii) the know effects of extracellular Tat, released upon virus reactivation, on immune activation [25,28,29], (iii) the containment of CD4$^+$ T-cell losses and the lower progression to AIDS observed in untreated individuals developing anti-Tat Abs and Tat-specific CTLs [33–37] and (iv) amelioration of immune homeostasis, including increase of CD4$^+$ T cells, stabilization of CD8$^+$ T-cell number, increase of CD4$^+$/CD8$^+$ T-cell ratio, B cells and NK cells, as well as decline of some markers of T-cell activation and re-equilibration of T-cell memory subsets, in long-term-treated individuals immunized with a Tat vaccine [38–41].

In support of this hypothesis, we now provide evidence that continued recovery of peripheral CD4$^+$ T cells in the late, slow phase of cART-mediated immune reconstitution is predicted by comprehensive immunity against the HIV-1 Tat protein including both humoral responses and a high frequency of cell-mediated responses. Individuals with this signature showed a continued increase of CD4$^+$ T cells and of the CD4$^+$/CD8$^+$ T-cell ratio, which appears to be mainly due to CD4$^+$ T-cell increases driven by humoral responses to Tat, in a background of substantial stability of CD8$^+$ T-cell counts, as found in Tat vaccinees [38–41]. Anti-Tat cell-mediated immunity, alone or combined to humoral immunity, was also found to predict a significant increase of NK cells or B cells, respectively. In contrast, anti-Env immunity was invariably present and intense in all participants, and humoral responses against the gp120 subunit were found to be associated with CD4$^+$ T-cell reconstitution only in the presence of anti-Tat Abs, as previously described for the association with reduced risk of initiating therapy in individuals naïve to treatment [35]. We have also observed that the frequency of anti-Env and anti-Tat cell-mediated responses tend to cluster to some extent. Additional studies are required to clarify whether this is related to a more complex and comprehensive immunity against HIV or to amelioration of immunity against structural HIV antigens by anti-Tat immunity, as observed in individuals on long-term cART vaccinated with the Tat vaccine [38].

Concurrently, we provide evidence that anti-Tat humoral immunity is associated with control of low-level viremia, a finding that might reflect a block of HIV reactivation from virus reservoirs by Abs

**Table 3**
Impact of anti-Tat Abs and cellular responses to Tat on CD4$^+$ T-cell dynamics according to plasma viremia.

| a. Anti-Tat Abs | N | Estimate[a] | 95% CI[b] | *p*-value | |
|---|---|---|---|---|---|
| **Aviremic status (*n* = 45)** | | | | | |
| ***Anti-Tat Ab$^-$*** | 31 | 0.0245 | -0.0332 | 0.0822 | 0.4040 |
| ***Anti-Tat Ab$^+$*** | 14 | 0.0734 | 0.0009 | 0.1458 | 0.0473 |
| **Transient residual viremia (*n* = 30)** | | | | | |
| ***Anti-Tat Ab$^-$*** | 30 | 0.0186 | -0.0216 | 0.0587 | 0.3634 |
| **Viral blips (*n* = 43)** | | | | | |
| ***Anti-Tat Ab$^-$*** | 34 | -0.0040 | -0.0415 | 0.0334 | 0.8326 |
| ***Anti-Tat Ab$^+$*** | 9 | 0.1056 | 0.0451 | 0.1660 | 0.0007 |
| **b. Cellular responses** | | | | | |
| **Aviremic Status (*n* = 28)** | | | | | |
| ***Infrequent responders*** | 12 | 0.0371 | -0.0337 | 0.1080 | 0.3028 |
| ***Frequent responders*** | 16 | 0.0415 | -0.0145 | 0.0976 | 0.1452 |
| **Transient residual viremia (*n* = 24)** | | | | | |
| ***Infrequent responders*** | 10 | 0.0954 | 0.0265 | 0.1643 | 0.0069 |
| ***Frequent responders*** | 14 | -0.0194 | -0.0677 | 0.0290 | 0.4309 |
| **Viral blips (*n* = 36)** | | | | | |
| ***Infrequent responders*** | 18 | -0.0298 | -0.0740 | 0.0145 | 0.1870 |
| ***Frequent responders*** | 18 | 0.0690 | 0.0226 | 0.1154 | 0.0037 |

A longitudinal regression analysis for repeated measures was performed using a mixed model with random-effects in order to evaluate CD4$^+$ T-cells changes over time upon stratification by anti-Tat Ab status or frequency of cell-mediated responses to Tat and classes of viral load.
[a] Variation of CD4$^+$ T-cell number per day.
[b] Confidence Interval.

**Table 4**
Multiple longitudinal regression analysis of CD4$^+$ T cells.

| a. Infrequent cellular responses | | Estimate[a] | 95% CI[b] | | *p* value |
|---|---|---|---|---|---|
| *Ant-Tat Ab$^-$* | *HIV RNA (copies/mL)* | -0.004 | -0.01 | 0.00 | 0.0139 |
| | *CD8$^+$ (cells/μL)* | 0.066 | -0.05 | 0.19 | 0.2772 |
| | *B (cells/μL)* | 0.491 | 0.14 | 0.84 | 0.0057 |
| *Anti-Tat Ab$^+$* | *HIV RNA (copies/mL)* | -0.088 | -0.11 | -0.06 | < 0.0001 |
| | *CD8$^+$ (cells/μL)* | 0.274 | 0.11 | 0.44 | 0.0012 |
| | *B (cells/μL)* | 0.021 | -0.19 | 0.23 | 0.8426 |
| b. Frequent cellular responses | | | | | |
| *Ant-Tat Ab$^-$* | *HIV RNA (copies/mL)* | -0.001 | 0.00 | 0.00 | 0.5079 |
| | *CD8$^+$ (cells/μL)* | 0.188 | 0.09 | 0.27 | < 0.0001 |
| | *B (cells/μL)* | 0.262 | -0.01 | 0.53 | 0.0599 |
| *Anti-Tat Ab$^+$* | *HIVRNA (copies/mL)* | 1.932 | 1.81 | 2.06 | < 0.0001 |
| | *CD8$^+$ (cells/μL)* | 0.130 | -0.01 | 0.27 | 0.0754 |
| | *B (cells/μL)* | 0.680 | 0.23 | 1.13 | 0.0032 |

Longitudinal analysis for repeated measures by the generalized estimating equation method (GEE) was used in order to evaluate the effect of time, residual viremia, CD8$^+$ T-cell counts, CD4$^+$/CD8$^+$ T-cell ratio, percentages of CD8$^+$ T-cell subsets (naïve, central and effector memory), percentages of activated CD4$^+$ T cells and CD8$^+$ T-cell, NK and B-cell counts on CD4$^+$T-cell counts in participants with infrequent or frequent anti-Tat cellular responses with or without anti-Tat Abs. Only the immuno-virological parameters with significant variations are shown (see Methods).
[a] Variation of CD4$^+$ T-cell number.
[b] Confidence Interval.

targeting extracellular Tat [45]. In agreement with this finding, anti-Tat Abs predicted higher $CD4^+$ T-cell gains irrespectively of plasma HIV RNA levels, confirming that low-level viremia is not an independent determinant of $CD4^+$ T-cell dynamics. The identification of anti-Tat immunity as a main determinant of $CD4^+$ T-cell dynamics in the late, slow phase of cART-mediated immune reconstitution is further underlined by the finding that higher nadir $CD4^+$ T cells are associated with anti-Tat Abs and that anti-Tat Abs predict $CD4^+$ T-cell gains among volunteers populating the same nadir stratum. A multiple regression analysis to adjust for the impact of all explanatory co-variates examined in this study indicated that $CD4^+$ T-cell recovery is due to the complex interplay of anti-Tat immunity, low-level viremia, $CD8^+$ T-cell and B-cell dynamics.

Previous studies have shown that effective immune reconstitution occurring within the first 48 weeks of cART treatment is characterized by a net gain of naïve $CD4^+$ T cells re-equilibrating the naïve/memory compartments, which appears to be inversely related to nadir $CD4^+$ T cells [44]. We did not observe significant changes of $CD4^+$ or $CD8^+$ T-cell subsets over time, a discrepancy that may be related to the relatively high median nadir $CD4^+$ T-cell value and to the longer time on cART of our study population.

Our working hypothesis is, nevertheless, partially disproved by the lack of convincing evidence of a relation between anti-Tat immunity and decreased $CD4^+$ or $CD8^+$ T-cell activation. This finding might be related, at least in part, to the complex interplay of T-cell activation and low-level viremia found in this study, namely, the association of $CD8^+/CD38^+/HLA\text{-}DR^+$ cells with viral blips and the contemporaneous association, as found in HIV controllers [46], of $CD8^+/CD38^-/HLA\text{-}DR^+$ cells with the aviremic status. Such a complexity is also evident in previous studies showing conflicting results with regard to the impact of residual viremia on persistent immune activation [12–15]. It should be noted, however, that a clear relationship of anti-Tat immunity and reduced immune activation was seen in Tat vaccinees [38,39,41], most likely due to the higher efficacy of Tat immunization, as compared to natural infection, in eliciting high anti-Tat Ab titres and strong cell-mediated immunity to Tat [38–41].

A striking finding of our study is that high levels of persistent T-cell activation, and not low levels, were predictive of $CD4^+$ T-cell recovery, a result contradicting most studies so far. It should be noted, however, that this analysis could not be cross-stratified for anti-Tat immunity due to the (relatively) low numbers of determinations for immune activation parameter. In this regard, we have also found that activated $CD4^+$ and $CD8^+$ T cells slowly decreased over time despite a median of 5 years of treatment before enrolment (data not shown). Given that the majority of volunteers was undergoing cART since several years before enrolment, and that increased HLA-DR expression on $CD8^+$ T cells is associated with the aviremic status as in HIV controllers [46], it is tempting to speculate that immune exhaustion was partially reversed in a fraction of our study population, and that activated $CD4^+$ and $CD8^+$ T cells might reflect, at least in part, effective immune responses that would be not detrimental for T-cell dynamics, as also suggested by others [15]. In this regard, it is noteworthy that HIV-infected individuals on long-term cART immunized with a Tat vaccine showed increased co-expression of HLA-DR and CD38 on $CD8^+$ T cells, a pattern possibly related to immature, rapidly cycling T cells in a context of immune homeostasis [38]. In line with these data, growing evidence indicates, indeed, that monocyte/macrophage-related inflammation, and not T-cell activation, is predictive of HIV disease progression [11].

It should also be noted that low-level viremia was shown to have a complex relationship with $CD4^+$ T-cell recovery in cART-treated individuals in previous studies, where low-level plasma HIV-1 RNA and/or low frequency of sustained plasma viremia episodes were associated with increased $CD4^+$ T-cell gains, and, by contrast, high-frequency residual viremia/blips were associated with decreased $CD4^+$ T-cell recovery [2,6]. Noticeably, we have similarly detected significant increases of $CD4^+$ T cells in association with transient residual viremia or viral blips in volunteers with infrequent or frequent cellular immune responses to Tat. Additional studies are required to ascertain whether these findings underlie the association of T-cell immune activation with increased $CD4^+$ T-cell gains observed in the present study.

Some limitations of our study should be evidenced. First, data related to cell-mediated immunity, $CD4^+$ and $CD8^+$ T-cell subsets (naïve, central and effector memory), and activation parameters (CD38 and HLA-DR) were collected only for a subset of participants and/or a subset of visits, introducing a potential limitation in the statistical power of our study. Secondly, the assessment of the interplay of anti-Tat immunity and low-level viremia in $CD4^+$ T-cell dynamics was restricted by the lack of simultaneous cross-stratification of the participants according to the Tat serostatus, cellular immune responses to Tat and low-level viremia, which was hampered by the size of the study population. Another limitation is the underrepresentation of the female gender at enrolment, reflecting the routine rate of presentation of the two genders at the clinics, which prevented drawing a firm conclusion on whether the present data can be fully generalized to women living with HIV. This issue, and whether populations of non-Caucasian genetic backgrounds exposed to non-B HIV-1 clades may be represented by the present study, is object of further investigations in our center.

In conclusion, although larger prospective or retrospective studies with a longer time of follow-up should be undertaken to confirm these data, the results of our study indicate that a comprehensive anti-Tat immunity positively impacts the long-lasting kinetics of immune reconstitution in long-term cART treated individuals. These findings support the hypothesis that blocking extracellular Tat may be key to intensify cART, particularly for poor responders and individuals initiated too late on treatment, thus advocating new immunotherapies to boost anti-Tat immunity, or new treatments to block extracellular Tat, to maximize immune reconstitution in people living with HIV.

## Declaration of Competing Interest

M. Di Pietro reports grants received from the Azienda Sanitaria of Florence during the conduct of the study. The other authors declares no conflict of interest.

## Contributors

AT, coordination of the experimental work, data collection and manuscript drafting; OP, clinical study management, data interpretation and statistical analysis, manuscript drafting. SM, clinical study management and manuscript preparation; CS, clinical study management supervision, data interpretation and manuscript drafting; AC data and manuscript critical review; VF, AA, GP, MC, MR P-C, experimental data production and collection; LS, AL, VS M, M DP, FC, AS, G DP, MG, SN, study conduct at the clinical sites; CM data and manuscript critical review; FE supervision of the experimental work and data and manuscript critical review; PM, data analysis and interpretation, manuscript conceptualization and preparation; BE study concept and design, supervision of the experimental work, data analysis and interpretation, and manuscript conceptualization and revision. AT, OP, SC, PM and BE have verified the underlying data. All contributors have read and approved the revised version of the manuscript.

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## Data sharing statement

Individual participants' data are protected for confidentiality reasons and can be made available only upon approval of a collaborative research proposal by the corresponding author, the ISS Ethical Committee and the ISS Data Protection Officer. The research proposal must adhere to the Informed Consent signed by the participants.

## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103306.

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