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Upregulation of IL-32 isoforms in virologically-suppressed HIV-infected individuals: Potential role in persistent inflammation and transcription from stable HIV-1 reservoirs

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Abstract

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Authors' Contributions

S. Z. and L. L., planned, performed the experiments and wrote the manuscript, E.L.A., R.B., I. T., M.S. and A.C. contributed to the measures of soluble cytokines and PCR assays, C.C.A., P.A., N.C., R.K., A.L., and M.D. contributed to the patient selection and study design, J.P.R., B.T., J.G.B., P.M., S.T. M.H., S.W., B.C., A.W., and R.T. provided patient samples. C.T. and M.E. designed and supervised the study, planned the experiments, analyzed data and wrote the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Background: Human IL-32 is a polyfunctional cytokine that was initially reported to inhibit HIV-1 infection. However, recent data suggest that IL-32 may enhance HIV-1 replication by activating the HIV-1 primary targets, CD4⁺ T-cells. Indeed, IL-32 is expressed in multiple isoforms, some of which are pro-inflammatory while others are anti-inflammatory.

Setting and Methods: Here, we aimed to determine the relative expression of IL-32 isoforms and to test their inflammatory nature and potential to induce HIV-1 production in latently-infected cells from virologically-suppressed HIV-infected individuals. IL-32 and other cytokines were quantified from plasma and supernatant of CD4⁺ T-cells by ELISA. Transcripts of IL-32 isoforms were quantified by qRT-PCR in PBMCs. The impact of recombinant human IL-32 isoforms on HIV-1 transcription was assessed in CD4⁺ T-cells from HIV-1⁺cART⁺ individuals by qRT-PCR.

Results: All IL-32 isoforms were significantly upregulated in HIV-1⁺cART⁺ compared to HIV^{neg} individuals with IL-32 β representing the dominantly expressed isoform, mainly in T-cells and NK-cells. At the functional level, while IL-32 γ induced typical pro-inflammatory cytokines (IL-6 and IFN- γ) in TCR-activated CD4⁺ T-cells, IL-32 α showed an anti-inflammatory profile by inducing IL-10 but not IL-6 or IFN- γ . However, IL-32 β showed a dual phenotype by inducing both pro- and anti-inflammatory cytokines. Interestingly, consistent with its highly pro-inflammatory nature, IL-32 γ , but not IL-32 α or IL-32 β , induced HIV-1 production in latently-infected CD4⁺ T-cells isolated from cART-treated individuals.

Conclusions: Our data report on the differential expression of IL-32 isoforms and highlight the potential role of IL-32, particularly the γ isoform, in fueling persistent inflammation and transcription of viral reservoir in HIV-1 infection.

Keywords

HIV-1; IL-32; Inflammation; HIV-1 reservoir

Introduction

Combined Anti-Retroviral Therapy (cART) has greatly diminished mortality and morbidity in people living with HIV (PLWH) by suppressing viral replication to undetectable levels. This remarkable control of viral infection underlies the significant decline in AIDS-related comorbidities and the increased life expectancy^{1,2}. However, non-AIDS co-morbidities remain common within the cART-treated PLWH, which clearly compromises the quality of life of individuals living with HIV³. Among key mediators of these non-AIDS co-morbidities are chronic immune activation and inflammation, which are not normalized even after prolonged therapy^{4,5}. Chronic perturbations in the cytokine network, particularly in proinflammatory cytokines such as TNF- α , IFN- α/β and IL-6, contribute to the compromised immunity in the cART era^{6,7,8}. While the contribution of these inflammatory mediators to HIV pathogenesis and disease progression is relatively well documented⁹, the role of novel cytokines known to be dysregulated during infection such as the human interleukin 32 (IL-32) remains elusive. IL-32 was initially reported as a pro-inflammatory mediator that induces the production of other inflammatory cytokines such as TNF- α , IL-8, IL-1 β , IFN- γ and IL-6^{10,11,12}. Early studies on IL-32 showed that this cytokine is upregulated in HIV infection and is associated with innate antiviral responses through the

induction of type I interferon (IFN- α/β) responses, which is supported by an increase in HIV replication upon inhibition of endogenous IL-32 expression^{13,14}. However, recent data revealed that certain isoforms such as the IL-32 γ may enhance HIV replication by increasing CD4⁺ T-cell activation and susceptibility to infection¹⁵.

Furthermore, in untreated HIV-infected individuals, high levels of IL-32 are associated with inflammation, immune suppression and disease progression^{11,16}. Interestingly, Smith et al.,¹⁶ showed that IL-32 is highly expressed in the lymphoid tissues and that the gamma isoform significantly induces the immunosuppressive enzyme Indoleamine 2,3-dioxygenase (IDO1)¹⁶. In turn, increased IDO1 expression is associated with impaired intestinal mucosal immunity, immune exhaustion and disease progression^{17,18}. This is in line with our recent data showing an association between IL-32 expression and disease progression in cART-naïve HIV-infected slow progressors¹¹. These studies suggest that IL-32 may indeed exert multiple functions in HIV infection that might be explained by the existence of multiple IL-32 isoforms (α , β , γ , δ , ϵ , D, ζ , η , sm and θ), which are produced by alternative splicing^{12,19,20}. Interestingly, while IL-32 γ induces a number of pro-inflammatory cytokines including TNF- α and IFN- γ ¹⁰, IL-32 β specifically induces the anti-inflammatory cytokine IL-10 by monocyte-derived DCs^{21,22,23}. Moreover, the functions of IL-32 γ on the induction of TNF- α through the activation of NF- κ B and p38 MAPK, is counteracted by the newly identified IL-32 θ isoform that diminishes these effects through the interaction and blockade of PKC δ , an upstream activator of both NF- κ B and MAPK²⁴. IL-32 θ also inhibits the PMA-induced expression of IL-1 β through the interaction with PKC δ ²⁵.

Considering the yet poorly documented expression and functions of IL-32 isoforms in the context of HIV infection, we sought in the current study to identify the dominantly expressed IL-32 isoforms and to investigate their functional consequences on persistent inflammation and residual HIV transcription during cART treatment.

Methods

Study population

Plasma and peripheral blood mononuclear cells (PBMCs) were collected from HIV-infected individuals and non-infected controls enrolled in the Canadian HIV and Aging Cohort Study (CHACS)²⁶. Samples from the baseline visits (study entry) of n=107 HIV^{neg} individuals, n=379 HIV⁺ virologically-suppressed individuals receiving cART and n=46 HIV⁺ viremic individuals were analyzed. Demographic and clinical parameters for the study participants are shown in Supplemental Table 1. Written informed consent was obtained from all participants included in this study. The study was approved by Institutional Review Boards (IRB) of the Centre Hospitalier de l'Université de Montréal Research Center (Ethical approval #: CE.11.063) and the participating centers.

Measurements of soluble IL-32, IL-6, IL-10 and IFN γ proteins

Plasma levels of human IL-32 (total pool of isoforms) were quantified using the human IL-32 ELISA kit (R&D System Inc., Cat # DY3040-05) as per manufacturer's recommendations on the available samples from baseline visits (study entry) of HIV⁺ and

HIV^{neg} individuals. Samples of both HIV⁺ and HIV^{neg} were inactivated using disruption buffer (DB) [PBS; 0.05%, Tween-20, 2.5% Triton X-100, 0.02% thimerosal and 1% Trypan blue] with a ratio of 1:4 (DB:Sample). IL-10, IFN γ and IL-6 were measured using human ELISA sets from R&D and PeproTech (Cat #DY-217B-05, 900-M27 and 900-K16, respectively) according to the supplier's protocol. Cell-associated IL-32 protein was measured as previously described¹¹.

Quantification of IL-32 by Real-time quantitative SYBR Green PCR

Total RNA was isolated from PBMCs using the RNeasy plus mini kit from Qiagen (Catalog No. 74134) as per manufacturer's protocol. Quantification of IL-32 isoforms (α , β , γ , δ , ϵ and θ) was carried out using one-step SYBR Green quantitative real-time PCR, which was performed on the LightCycler 480 II (Roche) apparatus using QIAGEN reagents (RNase-Free DNase Set (50); Cat. No. 79254). Real-time qRT-PCR was performed in duplicates using 25ng RNA per reaction. No-template controls were included in parallel for each gene master mix. Gene expression for each individual isoform was normalized to the housekeeping gene β -glucuronidase. Primer sets for IL-32 α , β , ϵ (C) and β -glucuronidase have been described earlier²⁷, while primer sets for IL-32 γ and θ were designed based on γ and θ transcript reference sequences as summarized in Supplemental Table 2.

Cell sorting

To study the expression of IL-32 isoforms in different cell types, we sorted T-cells, B-cells, NK cells and monocytes from total PBMCs of HIV positive and HIV negative subjects using flow cytometry (BD FACSAria; BD Biosciences). Briefly, cell Surface staining was performed with fluorochrome-conjugated antibodies from BD Biosciences; Pacific BlueTM Mouse Anti-Human CD3 (Clone UCHT1, Cat # 558117), Alexa Fluor[®] 700 Mouse Anti-Human CD4 (Clone SK3, Cat # 566318), APC-H7 Mouse anti-Human CD8 (Clone SK1, Cat # 560179), APC Mouse Anti-Human CD14 (Clone M5E2, Cat # 555399), PerCP-CyTM5.5 Mouse Anti-Human CD19 (Clone HIB19, Cat # 561295), PE Mouse Anti-Human CD56 (Clone B159, Cat # 561903) and FITC Mouse Anti-Human CD16 (Clone 3G8, Cat # 555406). Average purity of sorted cells was of >95% as determined by post sorting FACS quality control analysis.

Isolation and stimulation of CD4⁺ T-cells

Total CD4⁺ T-cells were isolated from PBMCs by negative selection using EasySep Human CD4⁺ T-cell Enrichment Kit (StemCell[®] Cat # 19052) according to the supplier's protocol. Purity of isolated CD4⁺ T-cells were on average 99%. Stimulation of purified cells took place in pre-coated plates with anti-CD3 antibodies (1 μ g/ml) (BD biosciences[®], Cat # 555329) and soluble anti-CD28 antibodies (0.5 μ g/ml) (BD biosciences[®], Cat # 5555726) with or without 500ng IL-32 isoforms from R&D (IL-32 α # 3040-IL-050, IL-32 β cat# 6769-IL-025 and IL-32 γ cat# 4690-IL-025/CF). Cells were cultured at 37°C for 2 days and secreted cytokines in the supernatant were measured by ELISA.

HIV-1 production in primary CD4⁺ T-cells from cART-treated individuals

CD4⁺ T-cells were isolated from PBMCs of virologically-suppressed individuals by negative magnetic selection (StemCell). Cells (4-5x10⁶ cells per experimental condition) were cultured in the presence of antiretrovirals (ARVs) (200nM lamivudine, 200nM raltegravir). Cells were stimulated with 250-500 ng/mL of IL-32 α , IL-32 β or IL-32 γ or with Dynabeads Human T-expander CD3/CD28 (Invitrogen) at a concentration of 1 bead per cell as a positive control. Medium was harvested at day 6 post-stimulation and used for quantification of viral particles and replaced with fresh medium containing ARVs and IL-32 when appropriate. Freshly collected cell-culture supernatants were centrifuged for 1 hour at 25,000 g to pellet HIV particles. Viral RNAs were extracted using the Qiaamp viral RNA kit (Qiagen) and quantified using an ultrasensitive semi-nested real-time reverse transcription–polymerase chain reaction (RT-PCR) with a detection limit of a single copy of HIV RNA *per* PCR reaction. Briefly, extracted viral RNA was reverse transcribed and subjected to 16 cycles of amplification followed by dilution and a second amplification by a nested real-time PCR for 40 cycles on the Rotor-Gene Q using primers and probes summarized in Supplemental Table 3. In all experiments, serial dilutions of HIV particles (LAI strain) in culture medium were processed in parallel of experimental samples and used as quantification standards.

Statistical analysis

Differences between groups were considered statistically significant at values of $p < 0.05$. Kruskal-Wallis and Dunn's subtest were used to analyze more than one group, non-parametric Mann-Whitney was used for unpaired samples, Wilcoxon for paired samples and nonparametric Spearman test was used for correlation studies. All analyses were performed using Prism 7 software (GraphPad, San Diego, CA, USA).

Results

Persistent upregulation of IL-32 under cART

Our earlier longitudinal studies on a limited number of HIV⁺ subjects from the Montreal Primary HIV-infected Cohort showed that IL-32 is highly expressed during the primary phase of HIV infection and is not normalized after a year of treatment with cART¹¹. In the current study, we investigated the expression of IL-32 in HIV-infected individuals receiving cART for extended periods of time (average: 12.7 \pm 6.7 years) by measuring plasma IL-32 levels (total pool of all IL-32 isoforms). IL-32 levels remained significantly elevated ($p < 0.0001$) in HIV-infected individuals ($n = 379$) (IQR: 437.4pg/ml, Q1-Q3: 229.9-667.3 pg/ml) compared to non-infected controls ($n = 107$) (IQR: 254.14 pg/ml, Q1-Q3: 162.5-416.7 pg/ml). However, these levels were significantly lower compared to HIV⁺ viremic individuals ($n = 46$) (IQR: 783, Q1-Q3: 301.6-1085.2pg/ml), Figure 1A. As we have previously shown¹¹, IL-32 levels positively correlated with HIV viral load ($r = 0.488$, $p = 0.0006$) and negatively with the ratio CD4/CD8 ($r = -0.316$, $p = 0.032$) in the HIV⁺ viremic individuals (Figure 1B, left and middle panels, respectively). However, this association between IL-32 and CD4/CD8 ratio was lost in virologically-suppressed individuals (Figure 1B, right panel).

Dominant expression of IL-32 β isoform

Our data showed that circulating levels of total IL-32 remain elevated in HIV⁺ cART-treated individuals. However, as IL-32 is expressed in different isoforms for which specific antibodies are currently unavailable and since these isoforms have distinctive functions¹⁹, it was important to identify the expression of these individual transcripts. Therefore, we developed isoform-specific RT-PCRs to quantify individual IL-32 isoforms (α , β , γ , ϵ , and θ in addition to the new uncharacterized isoform D, NCBI Reference # NM_001012636.1) in PBMCs from both HIV⁺ and HIV^{neg} individuals (amplification of the rest of the reported isoforms was unsuccessful). Our RT-PCR strategy is depicted in Supplemental Figure 1. As shown in Figure 2A (left panel), the amplified complementary DNA corresponded to the expected spliced transcripts of the different IL-32 isoforms and was further confirmed by DNA sequencing (data not shown). Similar to the total pool of IL-32 protein, the total pool of IL-32 mRNA (a sum of relative expression of all isoforms amplified in PBMCs) was significantly higher in HIV⁺cART⁺ aviremic participants (n=10) compared to non-infected controls (n=10) (Figure 2A, middle panel, p<0.0001). However, we observed a high variability in IL-32 mRNA expression in HIV⁺ viremic participants (n=7) (likely due to the high level of cell death of activated and infected cells). As expected, total IL-32 mRNA levels positively and significantly correlated with the total pool of cell-associated IL-32 protein, measured by ELISA from total cell lysates (Figure 2A right panel, n=20, r=0.46, p=0.04). At the single isoform level, IL-32 β was the dominant isoform in HIV^{neg} (in green) and both HIV⁺ virologically-suppressed individuals (in blue) and HIV⁺ viremic participants (in red) (Figure 2B). Interestingly, with the exception of the IL-32D, all of the IL-32 isoforms were expressed at significantly higher levels in HIV⁺ cART treated individuals compared to HIV^{neg} controls (p=0.023, p<0.0001, 0.0068, 0.089, p<0.0001, and <0.0001 for α , β , γ , D, ϵ , and θ , respectively). Furthermore, in the HIV⁺ viremic participants, these isoforms positively correlated with the HIV viral load (Figure 2C).

As these measures were performed in total PBMCs, we next aimed to determine the expression of individual IL-32 isoforms in different cell subsets to identify the major cellular source of IL-32. We used classical phenotypic lineage markers for T-cells (CD3⁺CD56^{neg}), NK cells (CD3^{neg}CD19^{neg}CD56^{hi/lo}), B-cells (CD3^{neg}CD56^{neg}CD19⁺), and monocytes (CD3^{neg}CD4^{lo}CD14⁺CD16^{+/-}) to isolate these cell subsets by flow cytometry-based cell sorting from n=4 individuals (Figure 3A). Quantification of IL-32 isoforms in these cells showed that IL-32 β was the dominant isoform in the former two cell types followed by IL-32 ϵ and IL-32 γ then IL-32 α , IL-32D and IL-32 θ (Figure 3B). However, IL-32 γ , the most pro-inflammatory isoform, was the dominant one in monocytes and B cells. Finally, by pooling all IL-32 isoforms together, T-cells and NK cells showed the highest expression levels of IL-32 compared to B cells or monocytes (Figure 3C). Of note, within the T-cell population, both CD4 and CD8 cells showed similar levels of IL-32 isoform expression (data not shown).

Differential functions of IL-32 isoforms

Since we identified T-cells as the major source of IL-32, we aimed to investigate the response of this subset to IL-32 isoform proteins (IL-32 α , β and γ ; the only three available recombinant proteins for IL-32). Primary CD4⁺ T-cells isolated from uninfected controls

were activated through the TCR in the presence of IL-32 α , β , or γ as described earlier¹¹ and anti- and pro-inflammatory cytokines were quantified in the supernatants. IL-32 α and IL-32 β but not IL-32 γ induced IL-10 production in TCR-activated CD4⁺ T-cells (Figure 4A, left panel, n=22, p=0.0096, data are normalized to the levels of IL-10 induced by TCR stimulation alone from each donor). Paired analysis from the same donor cells showed that IL-32 α induced higher levels of IL-10 compared to IL-32 β and IL-32 γ (Figure 4A, left and middle panels, p=0.011 and p=0.0029, respectively) and that IL-32 β had a higher capacity to induce IL-10 compared to IL-32 γ (Figure 4A, right panel, p=0.0043). In contrast to IL-10, IL-6 and IFN γ productions were induced by IL-32 β and IL-32 γ but not by IL-32 α (Figure 4B, n=14, P=0.0008 and Figure 4C, n=11, p=0.01, respectively). Altogether, these results are in agreement with earlier reports describing IL-32 γ as a potent inducer of proinflammatory cytokines compared to IL-32 α ²⁸, and show for the first time that IL-32 α , and to a lower extent IL-32 β , may favor an anti-inflammatory response by inducing IL-10 from activated CD4⁺ T-cells.

Differential impact of IL-32 isoforms on HIV production in CD4⁺ T-cells from cART-treated individuals

Our results showed that IL-32 isoforms modulate the production of pro and anti-inflammatory cytokines by CD4⁺ T-cells. Since this compartment harbors the major HIV reservoir under cART²⁹ and given the recent reports showing that IL-32 enhances HIV replication *in vitro*¹⁵, we investigated the potential of IL-32 at inducing the residual HIV production in latently infected CD4⁺ T-cells. Residual HIV production under cART may further contribute to the persistent immune activation and inflammation seen in HIV-1 infection^{30,31}. We investigated the potential impact of the dominantly expressed IL-32 β isoform together with IL-32 α and IL-32 γ . CD4⁺ T-cells were isolated from peripheral blood of cART successfully treated HIV⁺ individuals with undetectable viremia (n=8) and exposed to the three IL-32 isoforms. Quantification of HIV particles was performed in culture supernatant after 6 days of activation. As shown in Figure 5A, HIV-1 production was readily detected under conditions of T-cell receptor stimulation with anti-CD3 and anti-CD28 antibodies. Both IL-32 α and IL-32 β treatment enhanced baseline levels of HIV production in cells from only 3 out of 8 individuals (Figure 5B, **upper right and lower left panels**). In contrast, IL-32 γ significantly increased HIV production in samples from 7 out of 8 individuals (Figure 5B, **lower right panel**, matched pair analysis, p=0.023). Together, these data showed that consistent with its proinflammatory nature, IL-32 γ has a significant potential to sustain HIV production in CD4⁺ T-cells from cART-treated individuals.

Discussion

IL-32 is expressed in different isoforms with a multitude of functions that can promote either pro- or anti-inflammatory milieu^{21,22}. The balance between these individual isoforms may dictate the overall impact of IL-32 on persistent inflammation in HIV infection. For instance, dominant expression of IL-32 γ , the most active and pro-inflammatory isoform²⁸ could be deleterious for the immune response. Therefore, IL-32 γ may undergo multiple splicing into shorter and less inflammatory isoforms such as IL-32 α & β to prevent deleterious inflammatory effects^{19,32}. Furthermore, smaller isoforms such as IL-32 θ were also shown to

counteract the inflammatory nature of IL-32 γ by specifically inhibiting TNF α production²⁴. In the current study, we observed that IL-32 expression, both protein and RNA, remained higher in HIV-infected individuals receiving cART compared to non-infected controls. All IL-32 isoforms were upregulated in cells from HIV-infected individuals with IL-32 β being the dominant isoform followed by IL-32 ϵ and IL-32 γ . Interestingly, in a clear contrast to IL-32 γ that plays a potent proinflammatory role, the dominant IL-32 β isoform was shown to have the potential to dampen inflammation as it induces expression of the anti-inflammatory cytokine IL-10 in monocytes and DCs^{11,22,23,11,28}.

Furthermore, IL-32 β was also shown to limit the expression of IL-12 in monocyte-derived DCs²³, which may diminish the inflammatory responses as IL-12 is a key mediator for Th1 cell development³³. However, the impact of IL-32 β on IL-10 and other cytokines production by T-cells (the major source of IL-32) was not yet known. Here we demonstrated that both IL-32 β and IL-32 α isoforms can induce IL-10 by activated T-cells, however IL-32 α had a more profound effect. This effect of IL-32 α combined with its moderate induction of IL-6 and IFN γ , compared to the two other isoforms, is consistent with earlier reports describing IL-32 α as the least activatory isoform for proinflammatory cytokines^{11,28}. This also suggests that IL-32 α may function as an anti-inflammatory isoform, at least in the context of T-cell activation.

The specific impact of IL-32 α and IL-32 β on the induction of IL-10 is of particular interest given the key roles of IL-10 in regulating and balancing inflammatory responses and tolerance *in vivo*³⁴. These later functions are dysregulated in HIV infection with higher and persistent levels of IL-10 likely contributing to immune suppression and disease progression. In this regard, we have previously shown that triggering of programmed cell death-1 (PD-1) on monocytes negatively impacts HIV-specific CD4 T-cell proliferative responses in an IL-10-specific fashion³⁵. Therefore, expression of IL-10 must be fine-tuned in order to maintain a balance between efficient Ag-specific responses and control of inflammation. Indeed, the impact of at least IL-32 β on IL-10 production seems to be tightly regulated by other isoforms of IL-32 such as the IL-32 δ . This regulation is mediated by direct binding of IL-32 δ to IL-32 β leading to inhibition of IL-32 β binding to PKC δ , a prerequisite for the induction of IL-10^{36,37}. However, whether IL-32 α -mediated IL-10 production is similarly regulated by other IL-32 isoforms and whether it correlates with or counteracts disease progression remains to be investigated. In this regard, one limitation of the current study is the use of only three IL-32 isoforms (α , β and γ ; the only commercially available recombinant IL-32 proteins) in the activation assays. However, a better understanding for the IL-32 effects on cell activation and inflammation may require cell activation with a combination of the different isoforms to reflect the physiological conditions where all of these isoforms are expressed together and isoform-isoform interaction may likely impact the overall IL-32 functions^{36,37}. Further studies are warranted to generate the different IL-32 isoform recombinant proteins to address these questions.

Another characteristic that differentiated IL-32 isoforms at the functional level was their capacity to modulate HIV production in primary CD4⁺ T-cells from successfully treated individuals. While IL-32 γ showed a significant potential to enhance viral production from latent HIV-1 reservoirs, IL-32 α and IL-32 β failed to show consistent and significant impact.

The variability in response to IL-32 α and IL-32 β may indicate an indirect impact of IL-32 on the production of HIV from latent reservoirs. In support of this hypothesis, recent reports by Palstra et al.,¹⁵ suggested that IL-32 might enhance HIV transcription and replication indirectly by promoting a proinflammatory environment conducive for HIV infection through the activation of CD4⁺ T cells. Meanwhile, CD4⁺ T-cell activation by IL-32 (as measured by cytokine production) shows donor-to-donor variability (Figure 4) that is likely mediated by donor-dependent activation threshold and/or differential expression of IL-32 receptor(s). Given the fact that IL-32 receptors are yet to be identified, these last probabilities remain hypothetical and further studies are warranted to validate them. It remains intriguing that while both IL-32 β and IL-32 γ can induce pro-inflammatory cytokines such as IL-6 and IFN γ in primary CD4⁺ T-cells in culture, they may exert opposite effects on viral transcription. A partial explanation for these observations may arise from the ability of IL-32 β to induce IL-10, a cytokine associated with viral latency and inhibition of HIV-1 replication^{38,39}. Although the ultimate goal towards an HIV-1 cure in the era of cART is the elimination of persistent viral reservoirs, exploiting the reactivation potential of proinflammatory cytokines such as IL-32 γ is unlikely to be part of future cure strategies, given its relatively modest ability at inducing viral production from latently infected cells. In contrast, this modest, but detectable impact on HIV-1 transcription and production by IL-32 γ may have detrimental clinical consequences *in vivo* as it may contribute to persistent inflammation under therapy⁴⁰. This is supported by earlier reports clearly showing that cell-associated HIV RNA levels correlate with immune activation⁴¹. Furthermore, IL-32 γ was shown to favor an inflammatory milieu with a high propensity to sensitize CD4⁺ T-cells for *de novo* HIV-1 infection¹⁵, which may further suggest a vicious cycle between HIV-1 infection, IL-32 expression, activation of HIV-1 production by cellular reservoirs and persistent inflammation. This persistent inflammation is likely to increase the risk for cardiovascular diseases, which are highly prevalent in the HIV⁺ population⁴². In addition, IL-32 γ was shown to suppress proper HIV-specific immune responses through the induction of IDO-1 expression in macrophages in gut and lymph nodes from HIV-infected individuals¹⁶. Interestingly, our data on the expression of IL-32 isoforms in different cell types showed that IL-32 γ was the dominant isoform in monocytes; cells with highly plastic nature and potential to differentiate into inflammatory macrophages⁴³. However, whether IL-32 can directly impact the differentiation of monocytes to inflammatory macrophages and whether the multiple isoforms of IL-32 can mediate distinctive differentiation phenotypes is not yet known.

In summary, our current data demonstrate that IL-32 levels remain significantly higher in HIV-1 infected and treated individuals with the IL-32 β isoform being dominantly expressed. At the functional level, IL-32 γ , as expected, shows a typical proinflammatory nature by inducing IL-6 and IFN γ , whereas IL-32 α and IL-32 β induce the anti-inflammatory cytokine IL-10 in activated T-cells. The proinflammatory nature of IL-32 γ was consistent with its capacity to enhance HIV production from stable HIV reservoirs in CD4⁺ T-cells from individuals receiving cART. These data suggest that targeted blockade of IL-32 inflammatory isoforms, especially the IL-32 γ , may represent an attractive approach to limit persistent inflammation and potentially decreasing HIV-1 residual viremia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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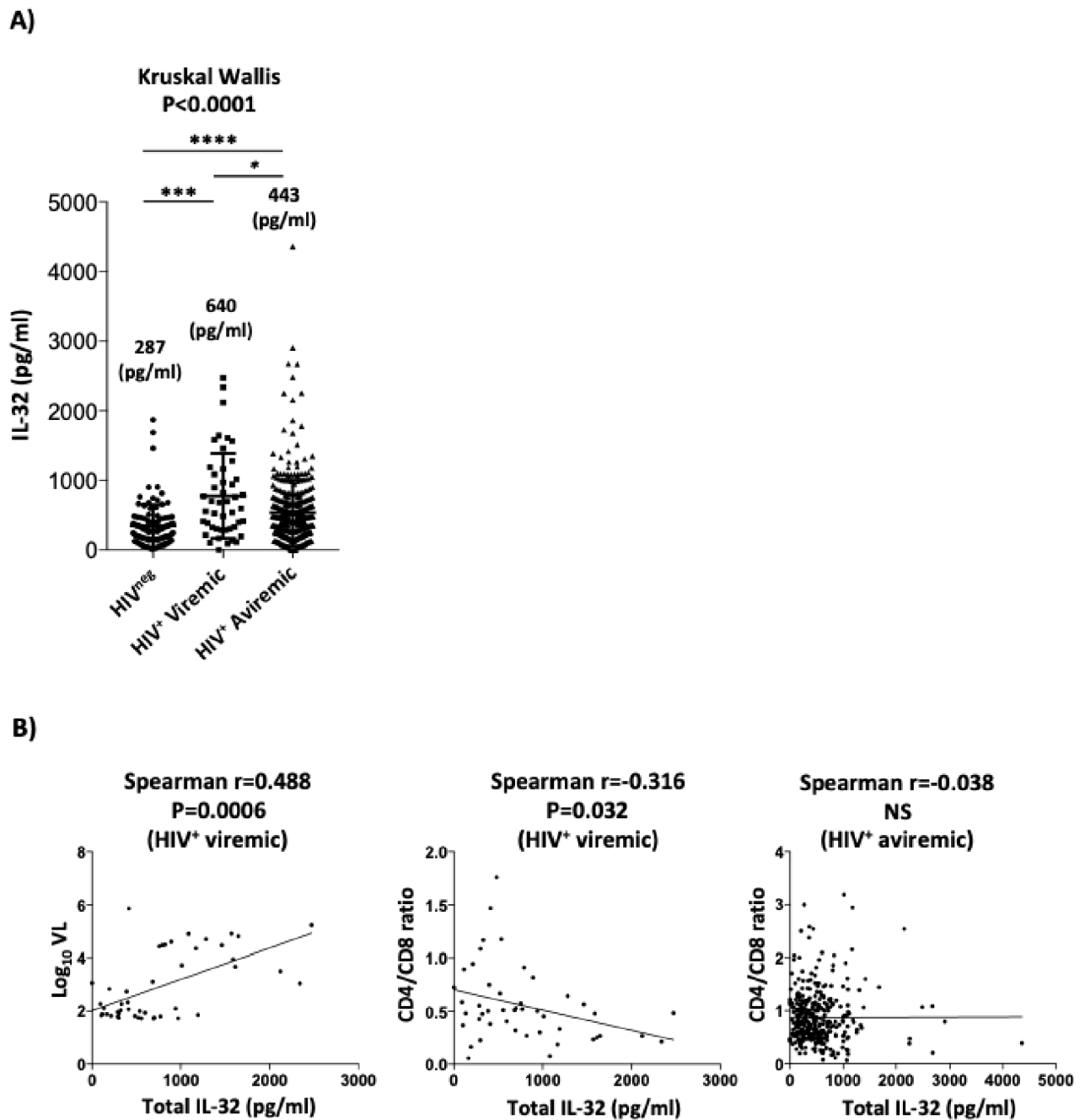


Figure 1: High levels of IL-32 in plasma from cART-treated HIV⁺ individuals.

Shown are (A) plasma levels of total IL-32 in HIV^{neg} (n=107), HIV⁺ viremic individuals (n=46), and HIV⁺ virologically-suppressed on cART individuals (n=379). Numbers on the graph refer to the median levels of IL-32 in plasma of each group expressed as pg/ml. (B) correlation between total IL-32 levels and Log₁₀ viral load (VL) (left panel), CD4/CD8 ratios (middle panel) in viremic individuals (n=46) and CD4/CD8 ratios in cART-treated individuals (right panel). Data were analyzed by the non-parametric Kruskal-Wallis and Dunn's sub-test for multiple comparisons in A. Spearman correlation test was used to assess

the significance of correlations between IL-32 and HIV VL or CD4/CD8 ratio in B. NS, non-significant ($p>0.05$).

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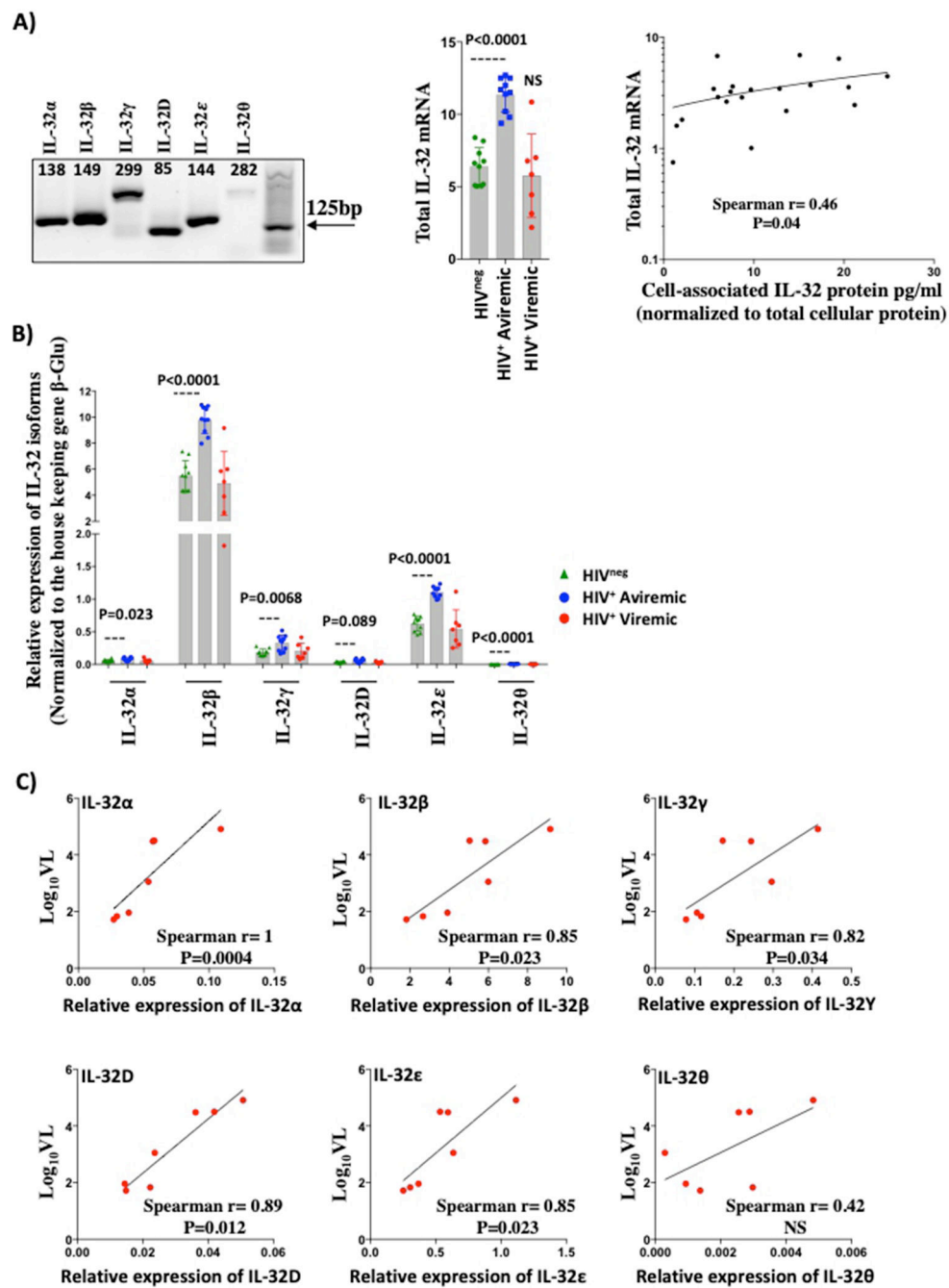


Figure 2: Expression of IL-32 isoforms in HIV⁺ individuals.

A) PCR products (amplicons) for each individual isoform analyzed on agarose gel (1.5%) (left panel). Numbers above the DNA fragments represent the size, in base pairs, of each isoform. Middle panel: Relative expression of total IL-32 mRNA (sum of relative expression of 6 IL-32 isoforms) in PBMCs from HIV^{neg} (n=10, in green), HIV⁺ cART-treated (n=10, in blue) and HIV⁺ viremic participants (n=7 in red). Right panel: Correlation between cell-associated total IL-32 mRNA and cell-associated IL-32 protein (measured by ELISA on total PBMCs lysate) (n= 20). B) Relative expression of individual IL-32 isoforms in total

PBMCs from HIV^{neg} (n=10, in green), HIV⁺ cART-treated individuals (n=10, in blue) and HIV⁺ viremic participants (n=7, in red). C) Correlations between Log₁₀ HIV viral load (Log₁₀VL) and relative expression of individual IL-32 isoforms in viremic individuals (n=7). Data were analyzed by non-parametric Mann-Whitney test in A (middle panel) and B, whereas Spearman correlation was used for data in A (right panel) and C. β-glu: β-glucuronidase.

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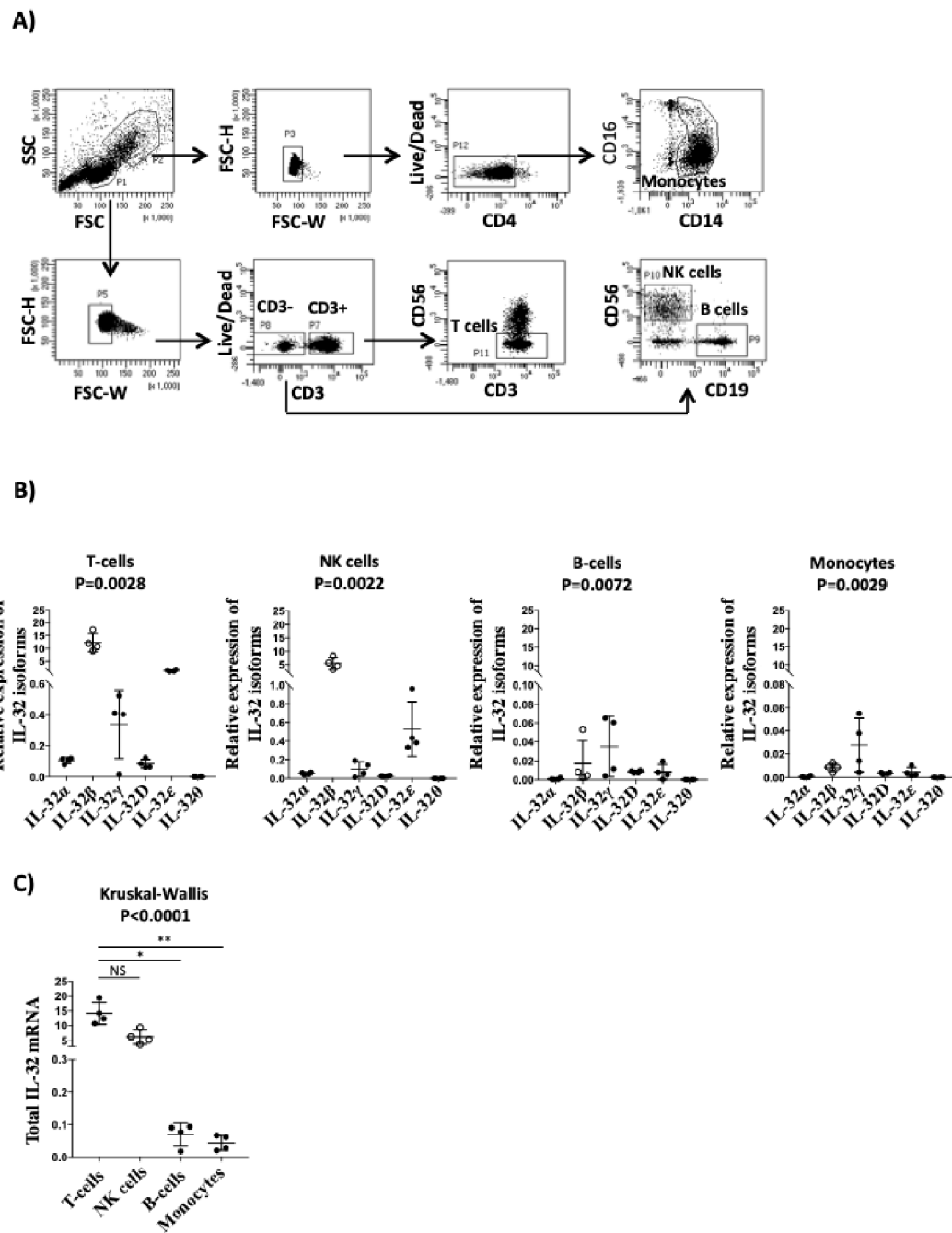


Figure 3: Expression of IL-32 isoforms in primary T-cells, NK, B-cells and monocytes.

A) Shown is the flow cytometry-based cell sorting gating strategy using lineage markers to isolate the different cell types from PBMC as follows: T-cells ($CD3^+CD56^{neg}$), B-cells ($CD3^{neg}CD56^{neg}CD19^+$), NK cells ($CD3^{neg}CD19^{neg}CD56^{(hi/lo)}$) and monocytes ($CD3^{neg}CD4^{lo}CD14^+CD16^{+/-}$). B) Shown is the *ex vivo* relative expression of the individual IL-32 isoforms (normalized to the house keeping gene β -glucuronidase) in T, NK, B-cells and monocytes sorted from PBMCs of $n=4$ ($n=2$ HIV⁺ and $n=2$ HIV^{neg} individuals). C) Comparison between the relative expression of IL-32 mRNA (sum of all IL-32 isoforms) in

the different cell types described in A. Data were analyzed by the non-parametric Kruskal-Wallis for multiple comparisons and Dunn's sub test in B and C.

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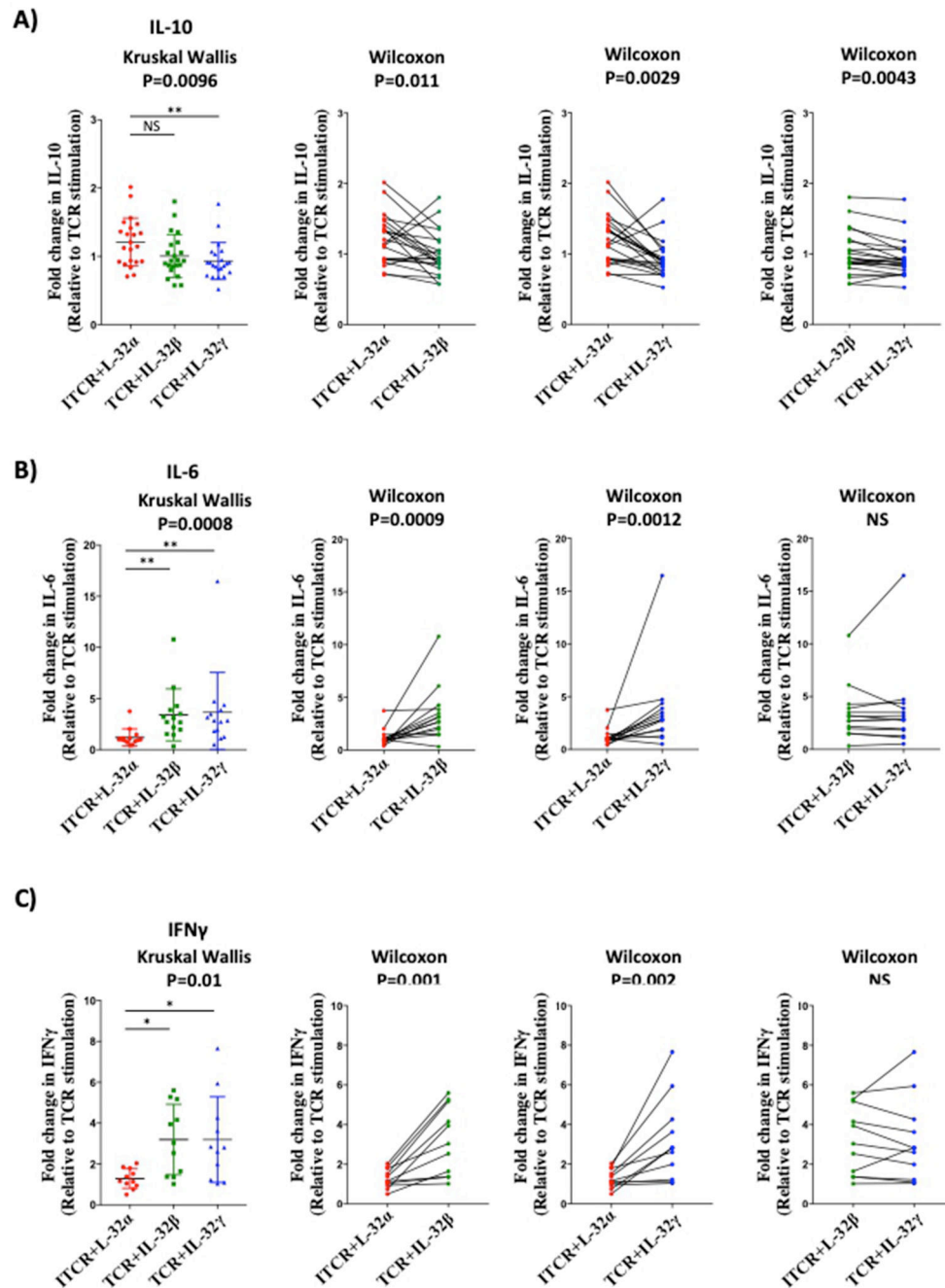


Figure 4: Differential induction of pro and anti-inflammatory cytokines by recombinant human IL-32 α , β , and γ isoforms.

Purified CD4⁺ T-cells were stimulated with plate-coated anti-CD3 (1 μ g/ml) and soluble anti-CD28 (0.5 μ g/ml) in the presence or absence of IL-32 α , IL-32 β or IL-32 γ (500ng/ml) for 48h. A) IL-10 production by TCR-stimulated cells in the presence of IL-32 α , IL-32 β or IL-32 γ (Left panel, n=22). Middle and right panels: matched pair analysis for IL-10 production by IL-32 isoforms. B) IL-6 production (n=14) and C) IFN- γ production (n=11) as in A. Data were analyzed by the non-parametric Kruskal-Wallis for multiple comparisons

(A, B and C left panels) and Wilcoxon for matched pair analysis (A, B and C, middle and right panels).

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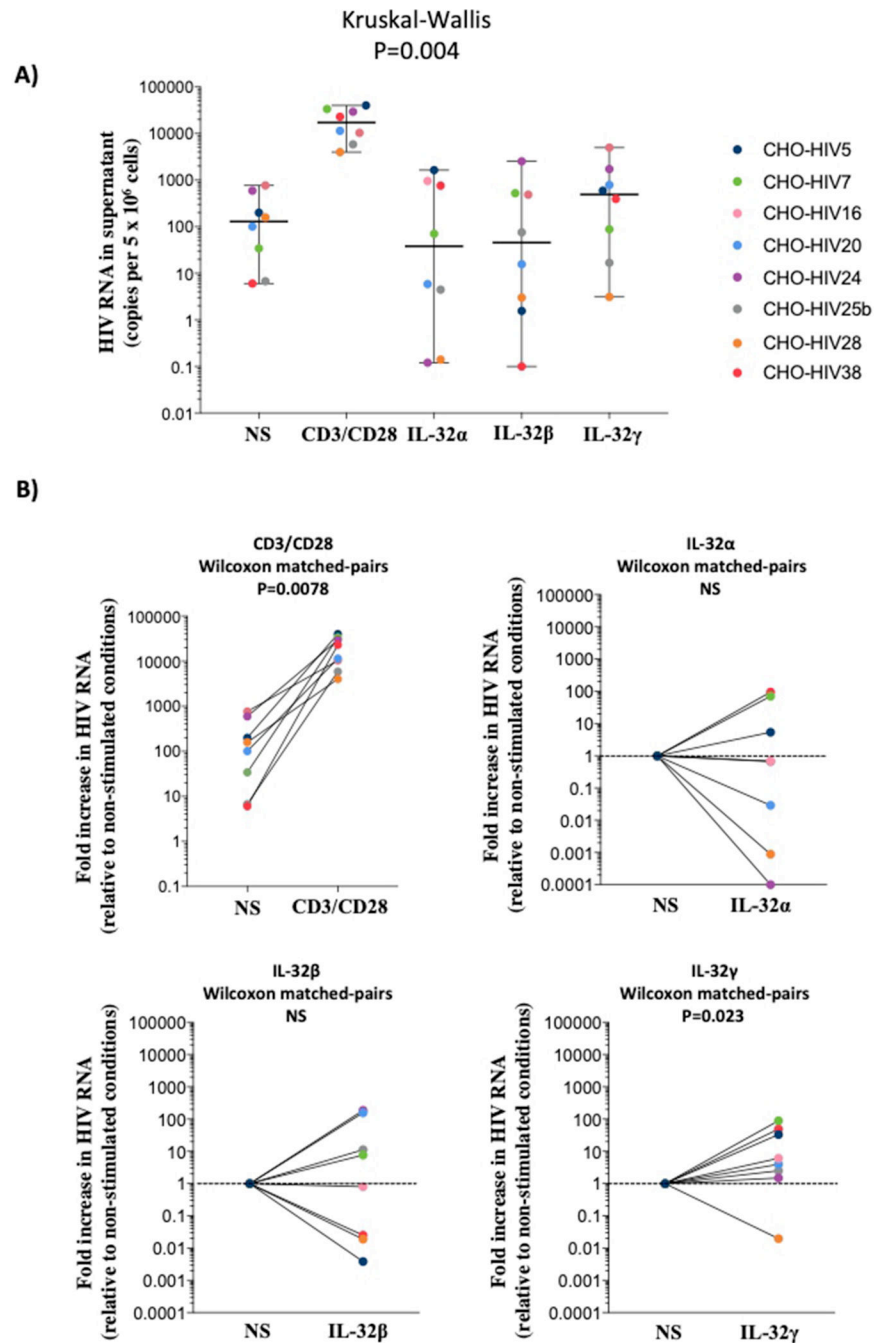


Figure 5: HIV-1 production in CD4⁺ T-cells from HIV-1⁺ cART-treated aviremic individuals (n=8) in response to IL-32 isoforms.

Cells were activated with either Dynabeads Human T-expander CD3/CD28 at a concentration of 1 bead per cell (used as positive control for the efficacy of HIV-1 reactivation assay) or with IL-32 recombinant proteins (250-500ng/ml). A) HIV-1 RNA quantified by qRT-PCR from supernatant of CD4⁺ T-cells (expressed as HIV-1 copies per 5×10^6 treated or untreated cells) at day 6 post stimulation. NS represents non-stimulated control cells from the same individuals. B) Matched pair analyses for cells from the same individual shown in A, stimulated with TCR (upper left panel), IL-32 α (upper right panel),

IL-32 β (lower left panel) and IL-32 γ (lower right panel) relative to non-stimulated (NS) cells. Data were analyzed by the non-parametric Kruskal-Wallis for multiple comparisons in A and matched pair analysis was done by Wilcoxon test in B.

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