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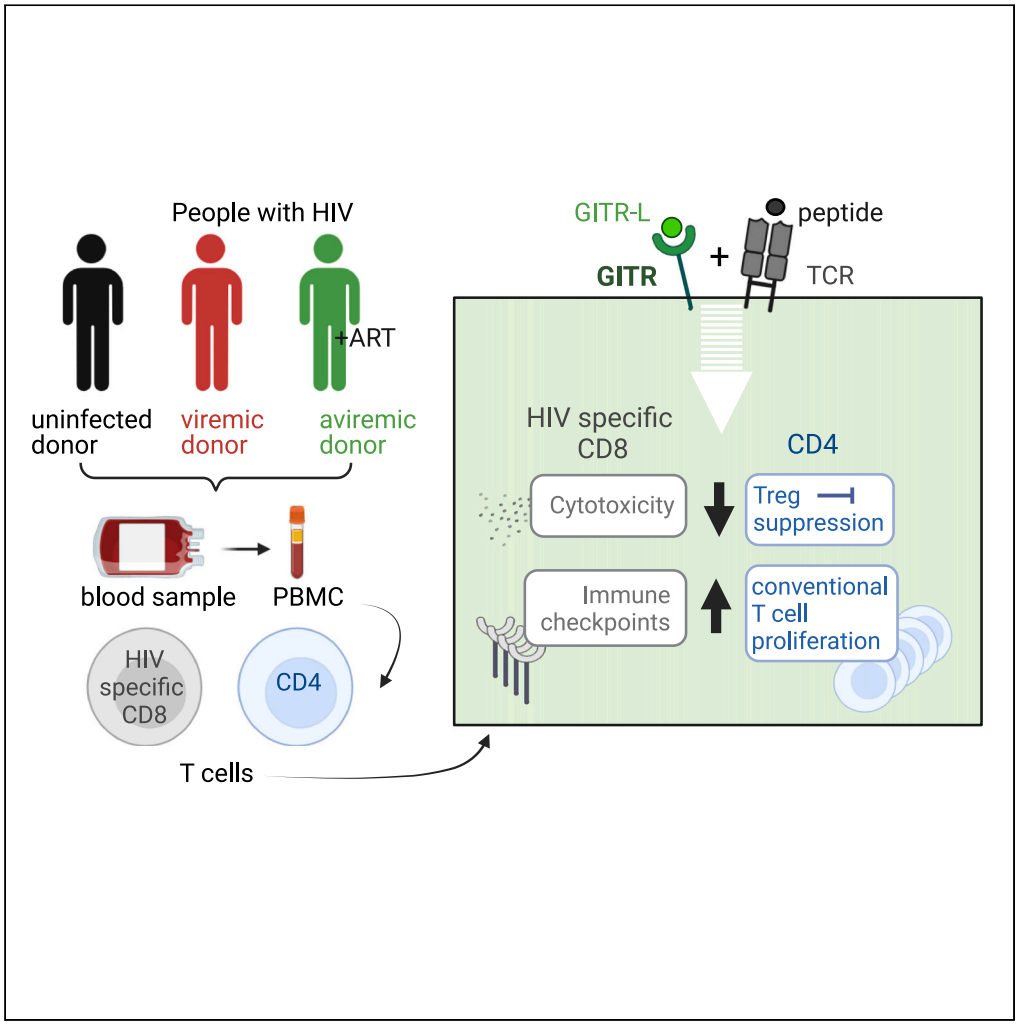

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Highlights

HIV-specific CD8 T cells expressed higher levels of GITR compared to total CD8 T cells

GITR stimulation decreased cytotoxicity and further exhausted HIV specific CD8 T cells

GITR stimulation abrogated T_{reg} suppression and increased CD4 T cell proliferation

GITR stimulation enhanced impaired HIV-specific immune responses in people with HIV

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GITR activation *ex vivo* impairs CD8 T cell function in people with HIV on antiretroviral therapy

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SUMMARY

Glucocorticoid-induced tumor necrosis factor related protein (GITR) is a co-stimulatory immune checkpoint molecule constitutively expressed on regulatory T cells (T_{regs}) and on activated T conventional cells (T_{conv}). In blood collected from PWH on suppressive ART, GITR expression was reduced in multiple activated CD4 and CD8 T cell subsets but was increased in Tregs. HIV specific CD8 T cells expressed higher levels of GITR and programmed cell death protein 1 (PD-1) compared to total CD8 T cells. Following stimulation with HIV peptides and GITR-ligand (L), we demonstrated a significant decrease in killing by HIV specific CD8 T cells and an increased exhausted profile. T cell receptor co-stimulation with GITR-L abrogated T_{reg} suppression and induced expansion of CD4 T_{conv}. We conclude that GITR activation is an additional factor contributing to an impaired HIV immune response in PWH on ART and that GITR agonist antibodies should not be pursued for HIV cure strategies.

INTRODUCTION

Antiretroviral therapy (ART) has significantly reduced morbidity and mortality for people with HIV (PWH); however, treatment is life long and there is no cure.[1] The main barriers to a cure are long-lived and proliferating, latently infected CD4 T cells containing intact integrated virus, which can rebound once ART is stopped. Despite control of virus replication on ART, immune dysfunction persists and is characterized by elevated expression of activation markers as well as immune checkpoint proteins, including programmed death-1 (PD-1)[2] reviewed by Gubser et al.[3] Reversing immune exhaustion is a key component of HIV cure strategies as functional cytolytic T cells are required to eliminate the HIV reservoir and also maintain long term control of virus replication once ART is stopped. Costimulatory signals generated by members of the tumor necrosis factor receptor superfamily such as glucocorticoid-induced tumor necrosis factor related protein (GITR) have long been described as important for the T cell immune responses and pose attractive candidates for the immunotherapy of HIV. [4–6] Here we aimed to determine the role of GITR in HIV-specific T cell function in PWH on ART and whether activation of GITR could enhance killing of HIV-infected cells.

GITR is a co-stimulatory receptor that is constitutively expressed on regulatory CD4 T cells (T_{regs}) and on activated CD4 and CD8 T conventional cells (T_{conv}).[7] The ligand for GITR (GITR-L) is temporarily expressed on activated antigen-presenting cells (APC) including dendritic cells, B cells, and macrophages.[8] The GITR-GITR-L interaction signals through tumor necrosis factor receptor-associated factor (TRAF) proteins with downstream activation of mitogen-activated protein kinases (MAPKs) Erk, p38, and JNK as well as nuclear factor kappa B (NF-kB) leading to B cell lymphoma (Bcl)-x(L) upregulation.[9,10] This ultimately results in increased interleukin-2 (IL-2) and interferon gamma (IFNγ) production as well as enhanced cellular proliferation following suboptimal T cell receptor (TCR) stimulation.[11]

Activation of GITR is currently being explored as an immunotherapy for cancer. An agonistic GITR antibody, used alone or in combination with anti-PD-1 or stereotactic radiosurgery, demonstrated depletion of tumor infiltrating T_{regs} and activation of tumor directed CD4 and CD8 T cells in a range of murine cancer models including intracranial glioma,[12] ovarian carcinoma,[13] and melanoma.[14] A pentamerized form of GITR ligand was shown to be effective against the growth of adenocarcinoma in a murine model when Tregs where depleted form the tumor tissue.[15] Activation of GITR can also enhance virus control and clearance, as demonstrated in mice infected with influenza,[9] Friend virus infection,[16] and chronic lymphocytic choriomeningitis virus (LCMV).[17] The beneficial effects of GITR activation on virus-specific CD8 T cell function is thought to be mediated through CD4 T cells, given the findings that in GITR-L transgenic mice infected with LCMV, depletion of CD4 T cells

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eliminated any antiviral effects of GITR.[8,18] The authors concluded that GITR activation mediated its effect on virus-specific CD8 T cells via an increase in autocrine production of IL-2 by CD4 T cells.[8,18]

Based on the encouraging data in pre-clinical mouse models, multiple agonist anti-GITR antibodies (TRX518, MK-1248, MK-4166, BMS-986156, MEDI1873, and AMG 228)[11] have now moved into clinical trials to test their effects against various malignancies (NCT01239134, NCT04021043, NCT02583165, NCT02553499, NCT02132754, NCT03707457, clinicaltrails.gov). While phase 1 human clinical trials demonstrated that agonist anti-GITR antibodies were safe and well tolerated, subsequent trials have failed to demonstrate clinical efficacy or reinvigoration of exhausted tumor directed T cells with MEDI1873, AMG228 or TRX518. [19–21] When MK-1248 or MK-4166 was used together with anti-PD-1, only limited antitumor responses were demonstrated.[22,23]

Despite extensive investigation of immune checkpoint proteins in PWH[24–29] there have been very few studies assessing the role of GITR in HIV infection, and no studies have specifically investigated the role of GITR on HIV persistence or HIV-specific T cell function in PWH on ART. GITR expression on the surface of CD4 T cells was shown to be higher in untreated PWH compared to uninfected controls.[30] Treatment of CD4 T cells from untreated PWH *ex vivo* with an agonistic GITR antibody triggered increased expression of TNFα and reduced expression of intracellular activated caspase-3 in HIV-specific $CD4^+$ T cells but there was no change in HIV-specific $CD8^+$ T cells.[30] GITR activation has also been explored as a mechanism to enhance responsiveness to an HIV vaccine. The administration of multimeric GITR-L with an HIV Gag DNA vaccine compared to the vaccine alone in a mouse model, resulted in increased T cell proliferative responses to Gag as well as increased anti-Gag antibody production.[31] Finally, a unique population of unconventional Tregs has been described in people with both HIV and tuberculosis which are thought to retain an ability to modulate T cell responses, due to the high expression levels of both PD1 and GITR on the same cell.[32] Taken together these data suggest that GITR activation may enhance HIV-specific T cell function and this approach could potentially be an alternative intervention to inhibition of PD-1, with either anti-PD1 or anti-PDL1, which are currently being explored as cure strategies.[3,33]

We therefore aimed to characterize GITR expression on T cells from PWH on and off ART and explored the impact of the GITR-GITR-L interaction on HIV-specific T cell function. We found that activation of GITR with GITR-L decreased lysis capacity, TNFα production, and degranulation of HIV specific CD8 T cells, with an increase in non-functional PD-1^+TIM-3^+GITR$^+$ T cells. In addition, GITR-L abrogated T_{reg} mediated suppression, increased expression of PD-1 and CD25 on T_{reg} cells and significantly expanded CD4 T_{conv} cells. Taken together, we conclude that GITR activation would have no beneficial role in HIV cure strategies.

RESULTS

Constitutive and inducible expression of GITR in T cell subsets in PWH on and off ART

We first compared GITR expression on T cells in blood collected from PWH on ART (aviremic; n = 10), PWH off ART (viremic; n = 11) and uninfected controls (n = 11) (details in Figure S1; Table S1). We examined GITR expression on CD4 and CD8 total T cells as well as memory subsets and T_{regs} at baseline and fold change in GITR expression after 16 h non-specific TCR stimulation with anti-CD3/anti-CD28 antibodies *ex vivo* (Figure 1A). For total CD4 T cells, there were no significant differences between the participant groups with respect to the frequency of cells expressing GITR with or without T cell stimulation, or the capacity to upregulate GITR in response to stimulation (Figure 1B, top left panels). In contrast, for total CD8 T cells, there was a decreased frequency of GITR expressing cells in aviremic PWH compared to uninfected donors (Figure 1B, top right panels).

We next analyzed constitutive and inducible GITR expression on T cell memory subsets (T_N = naive CD45RA+/CD62L+, T_{CM} = central memory CD45RA-/CD62L+/CD27+, T_{EM} = effector memory CD45RA-/CD62L-/CD27- T_{TM} = transitional memory CD45Ra-/CD62L-/CD27+, T_{TD} = terminally differentiated CD45RA+/CD62L-) as well as CD4 T_{regs} ($CD4^+CD25^+$CD127-) (Figure 1B lower panels; Figure S1).

In the CD4 T cell subsets, there was a higher frequency of GITR expressing cells in T_N cells of aviremic compared to viremic donors but not compared to HIV-uninfected individuals. Following stimulation with anti-CD3/CD28, CD4 T_N cells from both viremic and aviremic donors expressed less GITR compared to uninfected controls consistent with a defect in upregulation of GITR in these cells. In CD4 $T_{TD,}$ there was a higher frequency of GITR expressing cells and reduced capacity to upregulate GITR in response to TCR stimulation in aviremic compared to both HIV-uninfected and viremic donors. Finally, in CD4 T_{regs} there was decreased GITR expression in aviremic donors at baseline but an increased capacity to upregulate GITR in response to TCR stimulation in both viremic and aviremic donors compared to HIV-uninfected individuals (Figure 1B).

In CD8 T cell subsets, there was a higher frequency of GITR expressing T_N cells at baseline in aviremic donors compared to viremic donors. In T_{CM} there was a higher frequency of GITR expressing T_{CM} cells in aviremic compared to uninfected donors. In T_{CM} and T_{EM} there was a significantly reduced capacity to upregulate GITR in aviremic compared to viremic and uninfected donors. Within the T_{EMRA} population, there was a reduced frequency of both constitutive and inducible GITR expression in cells from aviremic donors compared to viremic donors (Figure 1B). Together these data demonstrate differences in the upregulation of GITR in response to TCR stimulation, that are seen in both aviremic and viremic donors compared to uninfected donors, suggesting that changes in GITR regulation are not normalized with ART.

Given that increased expression of PD-1 on HIV-specific CD8 T cells is associated with impaired effector functions of HIV cytotoxic CD8 T cells in PWH both off[26,34] and on ART [35,36], albeit at lower level in PWH on ART, and given our group has previously shown that PD-1 is co-expressed with a diverse range of other immune checkpoint molecules in PWH[2,37,38,39] we were keen to understand the interactions between GITR and PD1. We therefore next quantified the expression of PD-1 on GITR-expressing CD8 T cells. The frequency of GITR+ PD-1+CD8 T cells was lower in aviremic compared to uninfected donors both at baseline and after stimulation. However, there was no difference in the capacity to upregulate GITR in PD-1+ CD8 T cells in the three groups in the presence of TCR stimulation with aCD3/CD28 (Figure 1C).

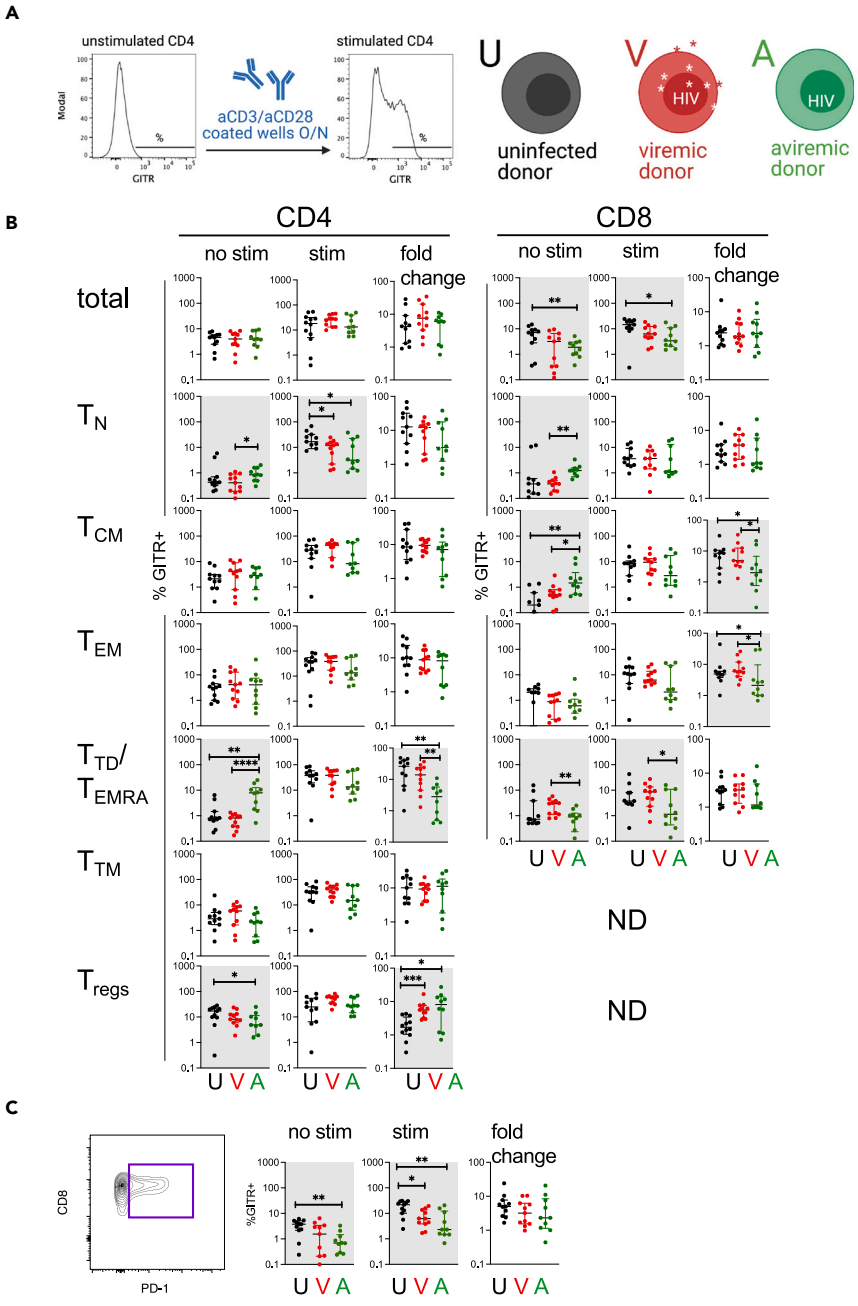

Figure 1. Constitutive and inducible expression of GITR following TCR stimulation of T cells isolated from blood from PWH on and off ART

(A–C) PBMC from HIV uninfected (U, black), PWH off ART (V, viremic; red), and PWH on ART (A, aviremic; green) were stimulated with plate-bound anti-CD3 and CD28 overnight (O/N) and expression of GITR was quantified by flow cytometry. A representative example of GITR positive CD4 T cells at baseline (unstimulated) and following stimulation with anti-CD3 and anti-CD28 (stimulated) is shown (A). The percentage of GITR positive cells (median +/− IQR) in total CD4 and CD8 T cells and T cell subsets (TEMRA and naive (TN) = naive; T_{CM} = central memory; T_{EM} = effector memory; T_{TD} = terminally differentiated; T_{TM} = transitional memory; T_{reg} = regulatory T cells) at baseline (no stim) or after stimulation with anti-CD3 and anti-CD28 overnight (stim) and the fold change expression of GITR in stimulated compared to unstimulated cells is shown in the three participant populations (B). The percentage of PD-1+CD8 T cells is shown as a contour plot and the percentage of GITR positive cells of the PD-1+CD8 T cells (median +/− IQR) at baseline (no stim), after anti-CD3 and anti-CD28 (stim) and fold change of expression of GITR in stimulated compared to unstimulated cells is shown for the three participant populations (C). Data were examined for normal distribution using and visual inspection of frequency histograms and quantile-quantile plots. Skewed data were log transformed to potentially achieve normal distribution. Parametric (t-test) or non-parametric test (Mann-Whitney-U test) was then applied as appropriate with statistical comparisons between experimental conditions performed by paired t test or signed-rank test. p value < 0.0001****, <0.001***, <0.1**, <0.05*, ≥0.05 ns.

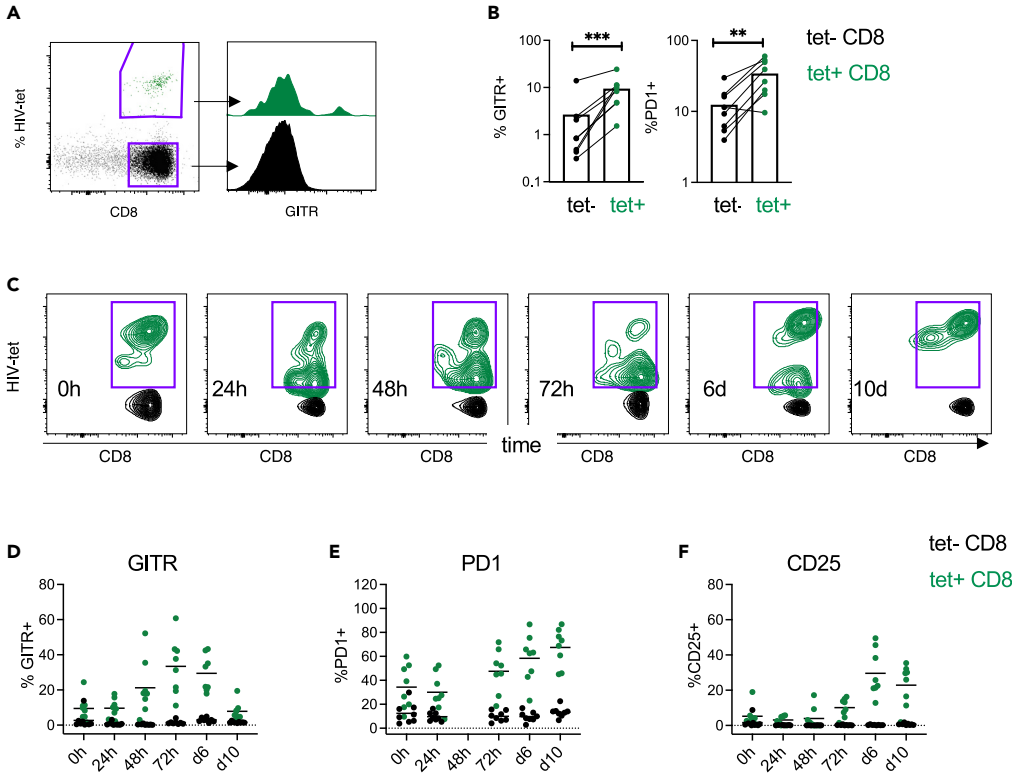

Figure 2. HIV tetramer-positive CD8 T cells express more GITR compared to total CD8 T cells in PWH on ART *ex vivo*

(A–F) Expression of GITR in tetramer-positive (green) and tetramer-negative (black) CD8 T cells from an HIV aviremic donor is shown as a contour plot and histogram (A). The percentage of cells expressing GITR (left) and PD-1 (right) among tetramer-negative (tet-, black) and tetramer-positive (tet+, green) CD8 T cells from seven HIV aviremic donors. Columns indicate mean (B). Contour plots showing tetramer-negative (black) and tetramer-positive (green) CD8 T cells in PBMCs stimulated with cognate HIV peptide over 10 days from one representative aviremic donor. Gate shows frequency of tetramer-positive cells (C). Percentage of cells expressing GITR (D), PD-1 (E), and CD25 (F) within tetramer-positive (green) and tetramer-negative (black) CD8 T cells over time course following HIV peptide stimulation of PBMC isolated from aviremic donors (n = 7) Horizontal line indicates mean. Data were examined for normal distribution and skewed data were log transformed to potentially achieve normal distribution. Paired t test or non-parametric test (Mann-Whitney-U test) were then applied. p value <0.0001****, <0.001***, <0.1**, <0.05*, ≥0.05 ns.

In summary, we identified multiple changes in GITR expression that were not normalized in aviremic participants, consistent with ongoing immune dysfunction despite ART. In aviremic participants compared to HIV-uninfected controls we found reduced capacity to upregulate GITR following TCR stimulation in CD4 T_N, CD4 T_{TD}, total CD8, CD8 T_{CM}, CD8 T_{EM} and PD-1+CD8 T cells. In contrast, in CD4 T_{regs} in aviremic participants compared to HIV-uninfected controls, we observed an increased capacity to upregulate GITR following TCR stimulation.

HIV-specific CD8 T cells compared to total CD8 T cells express increased levels of GITR and PD-1

To determine if GITR expression on HIV-specific CD8 T cells was different from total CD8 T cells in PWH on ART (aviremic donors), we next used a panel of MHC class I tetramers presenting dominant HIV epitopes in the context of HLA-A2, HLA-A3, HLA-B7, and HLA-B8 and assessed GITR expression on HIV tetramer-positive CD8 T cells (Figure 2A; Table S2). We found that HIV tetramer-positive CD8 T cells from aviremic donors expressed higher levels of both GITR and PD-1 compared to tetramer-negative CD8 T cells (Figures 2A and 2B).

We next assessed the kinetics of expression of GITR, PD-1, and CD25 (also known as the IL-2 receptor alpha subunit) following incubation of peripheral blood mononuclear cells (PBMC) from aviremic donors with cognate HIV peptides for 10 days. We gated on the tetramer-positive CD8 T cell population (green) (Figure 2C) and as expected observed TCR-induced down-modulation in response to cognate antigen recognition[40](Figure 2C) while upregulation of GITR, PD-1, and CD25 was only observed in antigen responding cells. GITR expression on tetramer-positive CD8 T cells following incubation with HIV peptides was highest at 72 h, declined by day 6 and was back to baseline by day 10 of culture (Figure 2D). In contrast, expression of PD-1 remained high and was still increasing after day 10 of culture (Figure 2E). The kinetics of CD25 upregulation was slower with a peak seen at day 6 (Figure 2F).

Taken together these results show that in aviremic PWH, tetramer-positive HIV-specific CD8 T cells express higher levels of GITR compared to tetramer-negative CD8 T cells and the kinetics of upregulation of GITR expression in the presence of cognate peptide was relatively transient, similar to CD25.

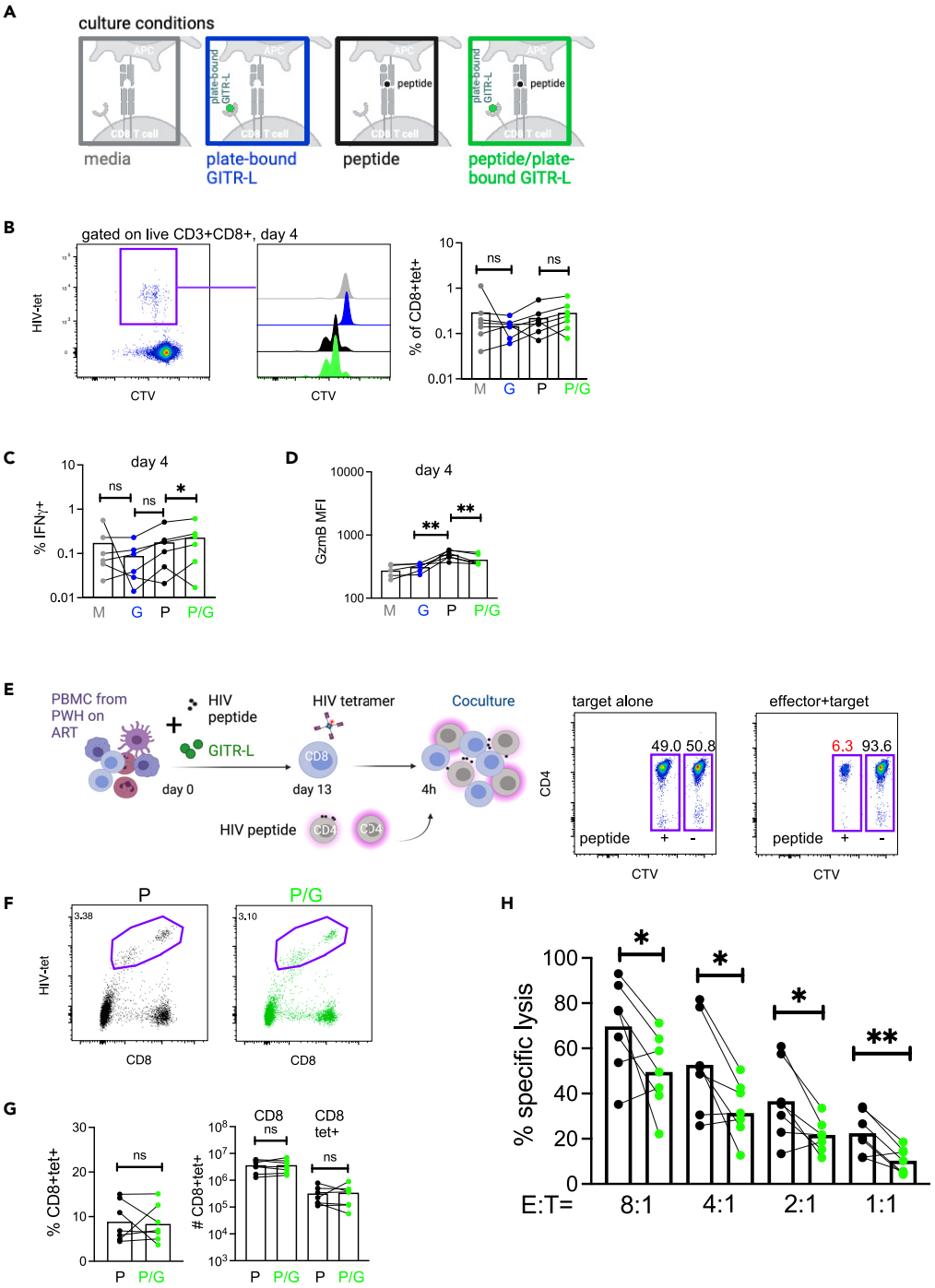

Figure 3. GITR stimulation decreases cytotoxicity of tetramer-positive CD8 T cells in PWH on ART *ex vivo*

(A–H) Graphical depiction of PBMC culture conditions including media (gray), GITR-L (blue), HIV peptide (black) or GITR-L plus HIV peptide (bright green) (A). The proportion of cell trace violet (CTV) and tetramer-positive CD8 T cells after stimulation with anti-CD3 and anti-CD28 for 4 days in different culture conditions. Contour plot (left) and histograms (right) from a representative aviremic donor in different culture conditions, i.e., media (M, gray), GITR-L (G, blue), peptide (P, black), or GITR-L plus peptide (G/P, bright green). Bar graph shows percentage of tetramer-positive CD8 T cells in the different culture conditions. Height of the bar indicates mean (B). The percentage of IFNγ+ (C) and mean fluorescence intensity (MFI) of Granzyme B (GzmB) (D) of tetramer-positive CD8 T cells after 4 days in the different culture conditions. Height of the bar indicates mean (C and D). Graphical depiction of HIV-specific CD8 T cell killing assay. PBMC from PWH on ART (aviremic) were expanded with cognate HIV peptides in presence or absence of GITR-L for 13 days. On day 13, purified autologous CD4 T cells were CTV labeled with a high and low concentration of CTV. CTV-low cells were pulsed with same peptide used to recall CD8 T cell for 13 days and then mixed with CTV-high, unloaded CD4 T cells at a 1:1 ratio. The 1:1 pool of CD4 "target" T cells was cocultured with HIV-peptide-expanded and -purified

Figure 3. *Continued*

CD8 "effector" T cells at various effector:target ratios for 4 h at 37°C. A representative pseudocolor plot shows the "target" only readout (left) and the "effector:target" readout (right) demonstrating decreased frequency of the CTV-low CD4 population (red number) consistent with target specific lysis (E). Dot plots show tetramer-positive CD8 T cells expanded with peptide alone (black) or peptide+ GITR-L (green) on day 13 of culture (F). The frequency (left) and absolute cell numbers (right) of tetramer-positive CD8 T cells in those cultures is shown. Height of the bar indicates mean (G). The percentage specific lysis of HIV specific CD8 T cells cultured with CTV-stained target CD4 T cells with peptide alone (black) versus peptide plus GITR-L (bright green) at various effector:target ratios. Height of the bar indicates mean (H). Data were examined for normal distribution and skewed data were log transformed to potentially achieve normal distribution. Paired t test or non-parametric test (Mann-Whitney-U test) was then applied. p value <0.0001****, <0.001***, <0.1**, <0.05*, ≥0.05 ns.

GITR stimulation with GITR-L *ex vivo* decreases cytotoxicity of HIV-specific CD8 T cells

Multiple prior studies in mice and human cells have shown that agonist antibodies for GITR can stimulate CD8 T cells to increase effector functions.[11] Given our findings that GITR was highly upregulated on HIV-specific CD8 T cells in aviremic participants and followed similar kinetics of another co-stimulatory molecule, i.e., CD25, we examined the effects of recombinant GITR ligand (GITR-L) and an agonist GITR antibody (MK-1248) to determine if we could improve effector functions of these cells *ex vivo*.

We cultured PBMC from aviremic donors with cognate peptides alone (Figure 3A, black), peptides and plate-bound GITR-L (Figure 3A, green) or negative controls i.e., media or plate-bound GITR-L (Figure 3A, gray and blue, respectively) and assessed proliferation capacity and effector functions of tetramer-positive CD8 T cells on day 4 of culture. We saw no significant difference in proliferation between conditions (Figure 3B). Following treatment with peptide and plate-bound GITR-L compared to peptide alone, there was a modest increase of IFNγ producing tetramer-positive CD8 T cells on day 4 (Figure 3C) but a significantly decreased expression of granzyme B expression (Figure 3D).

We further investigated the cytotoxicity of GITR stimulated HIV-specific CD8 T cells in an *ex vivo* killing assay (Figure 3E). We cultured PBMC from aviremic donors with either cognate HIV peptide alone or in the presence of plate-bound GITR-L for 13 days to obtain maximal cell numbers. On day 13, CD8 T cells were purified, and tetramer staining was performed to determine the frequency of tetramer-positive CD8 T cells (Figure 3F). We found no difference in the frequency or the absolute number of tetramer-positive CD8 T cells between the two culture conditions (Figure 3G).

Autologous CD4 T cells were then purified and loaded with the identical HIV peptide used for HIV-specific CD8 T cell recall. We labeled the peptide-loaded CD4 T cells with a low concentration of CellTrace Violet (CTV) and peptide-negative CD4 T cells with a high concentration of CTV. The CTV bright, peptide-negative CD4 T cells served as an internal control for non-specific cytotoxicity. CD8 T cells ("effectors") were then cocultured with CTV-dim peptide-loaded and CTV-bright peptide-negative CD4 T cells ("targets") at various "effector":"target" (E:T) ratios and quantified the percentage specific lysis capacity (Figure 3H). At all E:T ratios we saw a significant decrease in the specific lysis capacity of HIV-specific CD8 T cells that had been expanded in the presence of peptide/GITR-L compared to peptide alone (Figure 3H, green and black, respectively).

We also had the opportunity to evaluate the anti-GITR agonist antibody MK-1248 (kind gift from Merck) in the same functional assays. We were able to demonstrate specific GITR binding (Figure S3A), but no functional effect on either CD8 T cell proliferation, production of cytokine or cytotoxicity from PWH on ART (Figures S3B–S3D). Therefore, further studies only included the human recombinant GITR-L.

Taken together these data show that co-stimulation of GITR with plate-bound GITR-L in addition to antigen specific TCR stimulation reduced the cytotoxic capacity of HIV-specific CD8 T cells from aviremic participants resulting in reduced lysis of target cells *ex vivo*. This decreased cytotoxicity may in part result from a reduction in granzyme B production, in the context of combined TCR and GITR stimulation.

GITR stimulation with GITR-L *ex vivo* increases non-functional PD-1+TIM-3+GITR+ HIV tetramer-positive CD8 T cells

We next used multicolour flow cytometry to further understand the functional and phenotypic differences of HIV-specific CD8 T cells cultured in the presence of peptide alone or peptide and plate-bound GITR-L for 13 days (Figure 4A). Comparing stimulation with peptide/GITR-L and peptide alone, we observed a significant decrease in tetramer-positive CD8 T_{EM} cells (Figure 4A) and an increase in tetramer-positive CD8 T_{CM} (Figure 4A) by day 13. Using an intracellular cytokine assay, stimulation with peptide/GITR-L compared with peptide alone resulted in a significant decrease in degranulation, measured by CD107a, and TNFα but no significant change in IFNγ or GzmB (Figure 4B).

To better visualize the flow cytometry data, we used dimensional reduction of tetramer-positive CD8 T cells in the different culture conditions, i.e., peptide or peptide/GITR-L. The tSNE plots showed one main cluster of functional cells, defined by expression of CD107a, TFNα and IFNγ and two smaller clusters with non-cytokine producing cells (Figure 4C). Interestingly, one of the two smaller clusters expressed higher levels of GITR, PD-1, and TIM-3 compared to the main functional cluster (Figure 4C). This PD-1+TIM-3+GITR+ population was significantly increased when tetramer-positive CD8 T cell were treated with peptide/GITR-L (Figure 4D).

Taken together these data indicate that *ex vivo*, plate-bound GITR-L treatment of PBMC from aviremic donors in the presence of HIV antigen, reduced the function of HIV-specific CD8 T cells and concurrently expanded PD-1+TIM-3+GITR+ non-functional HIV-specific CD8 T cells.

GITR stimulation with GITR-L *ex vivo* increases CD4 T_{conv} proliferation and abrogates T_{reg} suppression

Given our finding that in aviremic donors compared to HIV-negative donors, TCR stimulation led to significantly greater upregulation of GITR on CD4 T_{regs} (Figure 1B), we next assessed the suppressive capacity of these cells in a classical T_{reg} suppression assay.[41] In brief, we

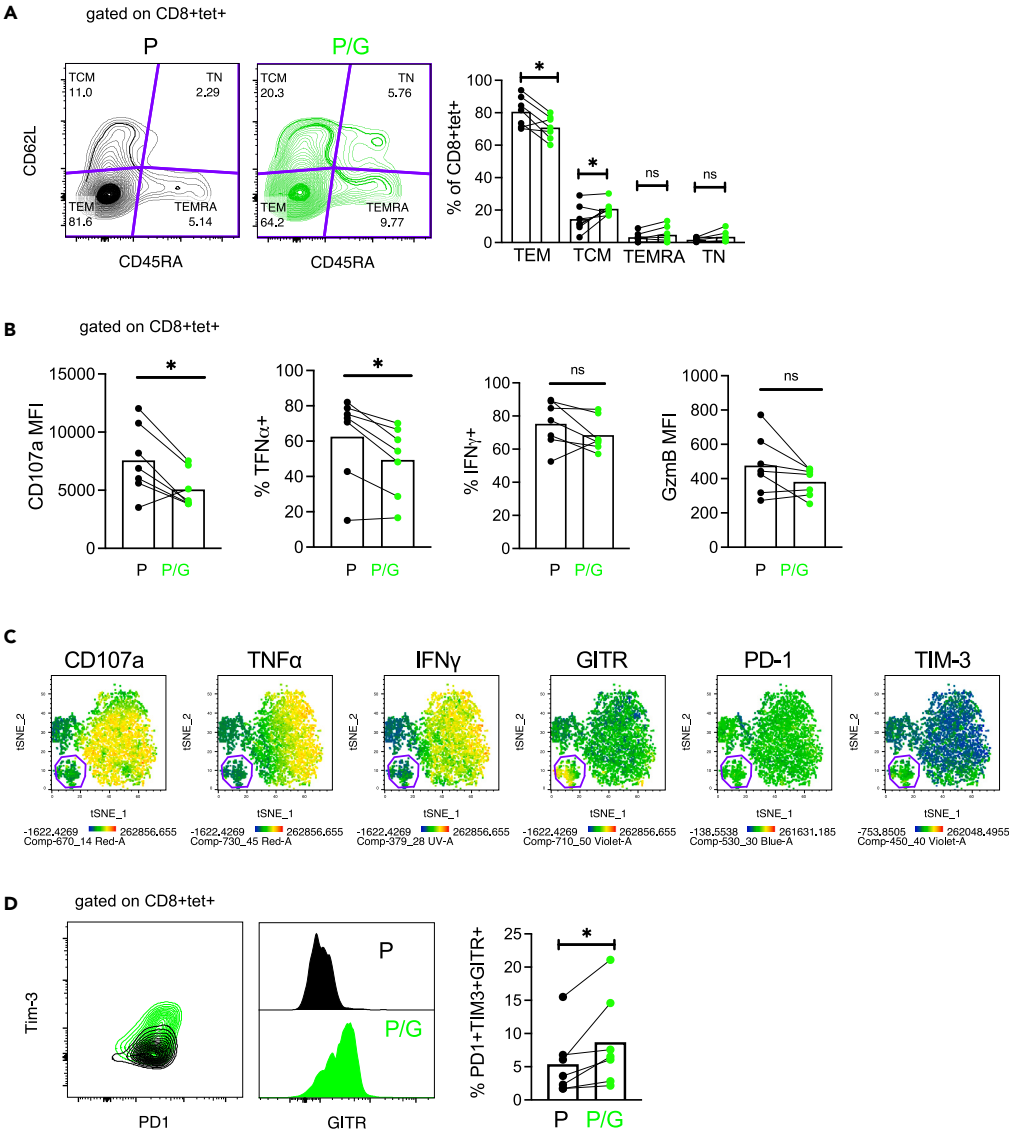

Figure 4. Stimulation with GITR-L increases PD-1+TIM-3+GITR+ non-functional tetramer-positive CD8 T cells in PWH on ART *ex vivo*

(A–D) PBMC from PWH on ART were cultured with peptide alone (P, black) or peptide plus GITR-L (P/G, bright green) and the phenotype of tetramer-positive CD8 T cells were analyzed by flow cytometry on day 13. A representative contour plot showing expression of CD45RA and CD62L in tetramer-positive CD8 T cells is shown. Bar graphs show the percentage of T cell effector memory (T_{EM}), central memory (T_{CM}), T_{EMRA} and naïve (T_N). Height of the bar indicates mean (A). Expression of CD107a, TNFα, IFNγ, and GzmB in tetramer-positive CD8 T cells following culture with peptide alone (P, black) or peptide with GITR-L (P/G, bright green) was quantified using intracellular cytokine staining. Height of the bar indicates mean (B). T-SNE plots of tetramer-positive CD8 T cells that have been cultured with peptide alone or peptide plus GITR-L. Color gradient depicts protein expression level. Gate highlights a group of cells that have low expression of CD107a, TNFα, and IFNγ and have upregulated GITR, PD-1, and TIM-3 (C). A representative contour plot showing expression of PD-1 and TIM-3 and histograms showing GITR in tetramer-positive CD8 T cells cultured with peptide (black) or peptide plus GITR-L (bright green) are shown. Bar graph shows percentage of PD-1+TIM-3+GITR+ tetramer-positive T cells in those cultures. Height of the bar indicates mean (D). Data were examined for normal distribution and skewed data were log transformed to potentially achieve normal distribution. Paired t test or non-parametric test (Mann-Whitney-U test) was then applied. p value <0.0001****, <0.001***, <0.1**, <0.05*, ≥0.05 ns.

co-cultured purified CD4 T_{regs} from aviremic donors with autologous CTV-labelled CD4 T conventional (T_{conv}) cells at a 1:1 ratio and on day 4 assessed the number of cells with reduced CTV expression using flow cytometry, indicating proliferation of CD4 T_{conv}, (Figure 5A). To control for antiproliferative effects resulting from nutrient competition in the well we also co-cultured autologous CD4 T_{conv} cells with CTV labeled CD4 T_{conv} cells (Figure 5A, middle panels). Following stimulation with plate-bound anti-CD3 antibody and suboptimal amounts of soluble anti-CD28 antibody (Figure 5B, black), CTV-labelled CD4 T_{conv} with or without autologous CD4 T_{conv}, proliferated to a similar extent indicating there was no nutrient competition in the wells.

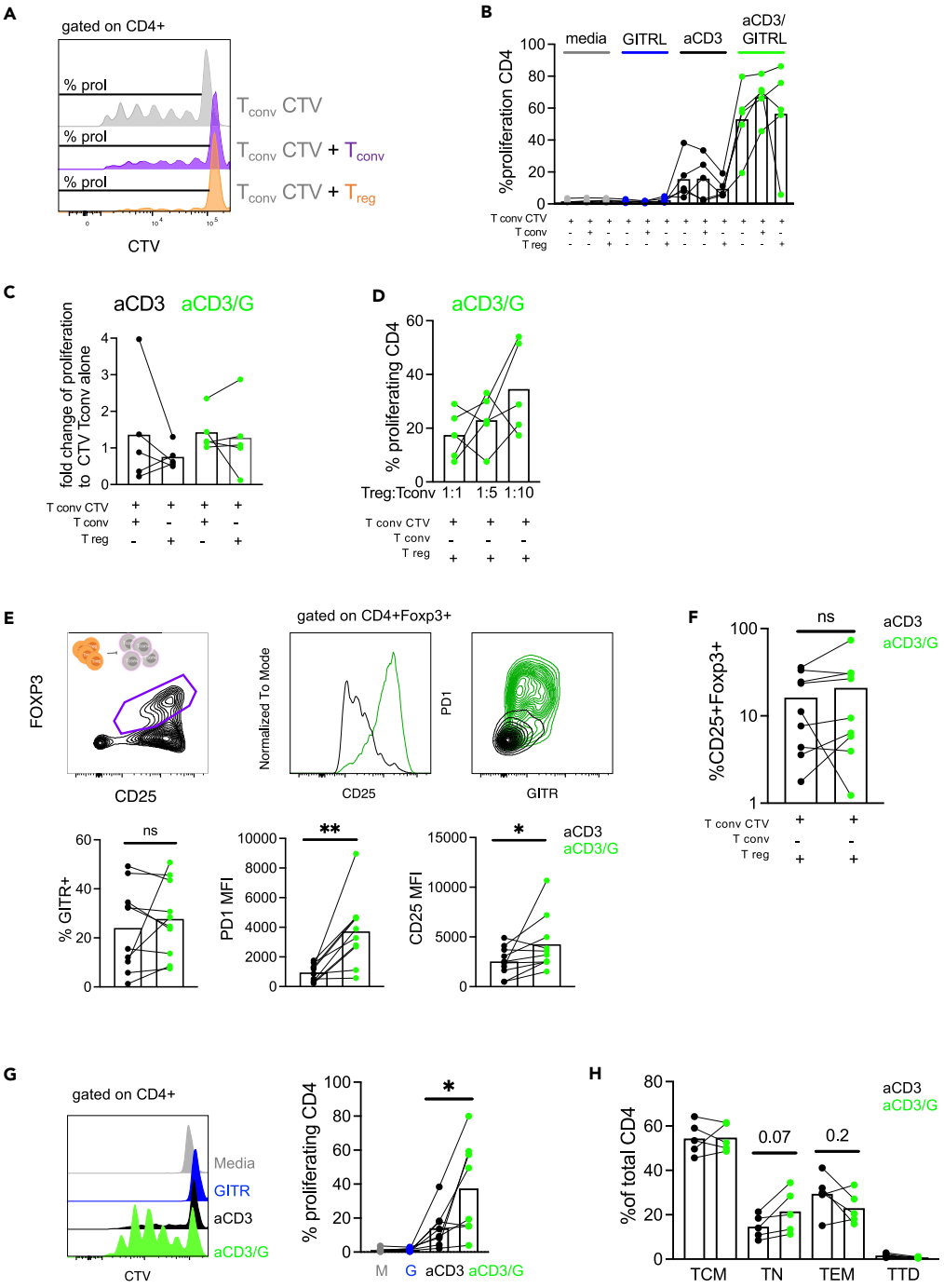

Figure 5. Stimulation with GITR-L of PBMC from PWH on ART *ex vivo* increases proliferation of CD4 T_{conv} and abrogates T_{reg} suppression

(A–H) Representative histogram overlay shows proliferation peaks of CTV labeled CD4 T_{conv} cells from PWH on ART (top histogram, gray) either cocultured with autologous, non-CTV labeled CD4 T_{conv} (middle histogram, purple) or autologous CD4 T_{regs} (bottom histogram, orange) (A). The percentage of proliferating CD4 T cells in co-cultures with different culture conditions, i.e., media (gray), GITR-L (blue), anti-CD3 antibody (black) or anti-CD3 antibody plus GITR-L (bright green). Height of the bar indicates mean (B). The fold change of proliferating CD4 T_{conv} cells co-cultured with either autologous CD4 T_{conv} or T_{regs} in the presence of anti-CD3 antibody (black) or anti-CD3 antibody plus GITR-L (bright green), normalized to the same culture conditions containing CD4 T_{conv} only. Height of the bar indicates mean (C). The percentage of proliferating CD4 T cells in co-cultures that have been stimulated with anti-CD3 antibody plus GITR-L and contain various T_{reg}:T_{conv} ratios. Height of the bar indicates mean (D) Contour plot shows CD4 T cells from T_{reg}:T_{conv} co-cultures, gate indicates CD4+Foxp3+T_{regs}. Histogram overlay shows CD25 MFI (middle) of T_{regs} in T_{conv} co-cultures stimulated with anti-CD3 antibody (black) or anti-CD3 plus GITR-L (bright green). Contour plot overlay (left) shows PD-1 and GITR expression on T_{regs} from those co-cultures. Bar graphs show percentage of GITR expressing Tregs and MFI of PD-1 and

Figure 5. *Continued*

CD25 (E). Bar graph shows frequency of CD25+Foxp3+ T_{regs} from "suppressive" T_{conv} co-cultures (F). Histograms show proliferation peaks of CTV labeled, purified CD4 T_{conv} cells that have been cultured in media (gray), with GITR-L (blue) anti-CD3 antibody (black) and anti-CD3 antibody plus GITR-L (bright green). Bar graph shows percentage of proliferating CD4 T_{conv} from those cultures (G). Bar graph depicts memory subsets (T_{CM}, T_N, T_{EM}, and T_{TD}) of proliferating CD4 T_{conv} cells (H). Data were examined for normal distribution and skewed data were log transformed to potentially achieve normal distribution. Paired t test or non-parametric test (Mann-Whitney-U test) was then applied. p value <0.0001****, <0.001***, <0.1**, <0.05*, ≥0.05 ns.

Co-culture of autologous CD4 T_{regs} in the presence of anti-CD3 alone, reduced CD4 T_{conv} proliferation, as expected. The addition of anti-CD3 with plate-bound GITR-L abrogated T_{reg} mediated suppression (Figure 5B black and green; Figure 5C black and green conditions). However, a low level of T_{reg} suppression was maintained as we still observed increased proliferation of CTV labeled CD4 T_{conv} at reduced T_{regs}:T_{conv} ratios (Figure 5D). When we compared the phenotype of T_{regs} in the "suppressive" co-cultures, we observed that the addition of anti-CD3/GITR-L compared to anti-CD3 alone, resulted in a greater increase in expression of PD-1 and CD25 (Figure 5E) with no difference in GITR expression (Figure 5E) or the frequency of T_{regs} (Figure 5F). Finally, the addition of anti-CD3/GITR-L compared to anti-CD3 alone resulted in a greater increase in proliferation of purified (CD25-depleted) CTV labeled CD4 T_{conv} (Figures 5B and 5G), and a trend toward increased proliferation of CD4 T_N cells (Figure 5H).

Taken together, these results demonstrate that the addition of plate-bound GITR-L to anti-CD3 abrogated T_{reg} mediated suppression and increased expression of PD-1 and CD25 on T_{regs} from PLWH on ART. Plate-bound GITR-L with anti-CD3 compared to anti-CD3 alone, induced a significant expansion of purified CD4 T_{conv} cells. These effects of plate-bound GITR-L on CD4 T cells would be unfavourable for eliminating the HIV reservoir and could potentially enhance clonal expansion of infected cells as well as impairing anti-HIV immune responses.

DISCUSSION

Boosting HIV-specific T cell function is one strategy being actively pursued to achieve an HIV cure or remission off ART. While blocking negative IC receptors like PD-1 have shown promising results with expansion of HIV-specific T cells[2,26,42,43] the immunomodulatory effects of activating co-stimulatory IC receptors like GITR have thus far not been assessed in PWH on ART. Here we provide evidence that GITR is another key marker of T cell exhaustion in PWH on ART.

We identified changes in the constitutive and inducible expression of GITR in blood from aviremic participants, consistent with ongoing immune dysfunction. Contrary to our hypothesis, GITR activation resulted in a decrease in HIV-specific CD8 T cell cytotoxic function with lower levels of cytokine producing effector memory cells and expansion of a dysfunctional PD-1+TIM+GITR+ population. Furthermore, GITR activation increased proliferation of CD4 T_{conv} and abrogated T_{reg} mediated suppression. We conclude that GITR activation would have a deleterious outcome in the setting of chronic HIV and that GITR agonistic antibodies should not be pursued for HIV cure strategies.

CD4 T_{regs} in blood from aviremic participants compared to HIV-uninfected controls showed an increased capacity to upregulate GITR following TCR stimulation, while multiple CD4 and CD8 T_{conv} cell subsets, including PD-1+CD8 T cells demonstrated reduced upregulation of GITR. Lahey et al. previously showed higher GITR expression on CD4 T cells in PWH off ART, although clinical details of the cohort enrolled in this study were not described. Interestingly, activation of GITR resulted in enhanced production of TNF-a and IFN-g and a reduction in caspase 3 in HIV specific CD4 T cells.[30] These findings argue for an immune stimulatory and protective effect of GITR on HIV-specific CD4 T cells from PWH off ART. It is important to highlight that in our study, we only assessed HIV-specific CD8 T cells and not HIV-specific CD4 T cells and only in PWH on ART.

To our knowledge our study is the only one to analyze the direct role of GITR on HIV specific CD8 T cell function in PWH on ART. We found that in PWH on ART, HIV tetramer-positive compared to tetramer-negative CD8 T cells expressed higher levels of GITR, similar to previous reports of increased expression of other immune checkpoints, including PD-1.[26] In a kinetic analysis with cognate peptide stimulation, we found that expression of GITR on tetramer-positive cells peaked at 72 h whereas PD-1 expression continued to increase over time in culture, likely marking exhaustion. These data are consistent with GITR expression on tetramer-positive CD8 T cells following the pattern of a TCR costimulatory molecule with rapid up- and down regulation upon TCR triggering. Therefore, increased expression of GITR on CD8 T cells from PWH on ART *ex vivo* (in the absence of TCR stimulation) could indicate persistent activation *in vivo* from low level expression of viral antigen in tissue, as has been shown to occur in PWH on ART.[44] Other data supporting the consistent expression of viral antigen despite long-term suppressive ART, includes the persistent recognition and response to certain viral proteins, such as Nef, by HIV-specific T cells.[45]

Contrary to what we anticipated, we found that TCR co-stimulation in the presence of GITR-L impaired cytolytic function of HIV specific CD8 T cells from PWH on ART. Therefore, we propose that GITR upregulation and consequent activation contributes to T cell exhaustion in PWH on ART. The reduced cytotoxicity may in part result from a reduction of CD107a and TNFα and a concurrent expansion of PD-1+TIM-3+GITR+ non-functional tetramer-positive CD8 T cells in the context of TCR and GITR co-stimulation. Overcoming immune exhaustion of antigen experienced cells by blocking negative signaling of TCR co-receptor, for example with anti-PD-1 antibodies, has shown tremendous success and has led to significant clinical advances in the context of cancer.[46,47] Our findings demonstrate that triggering positive signaling of the TCR with activation of GITR further enhanced rather than reversed immune dysfunction, despite demonstrating abrogation of T_{reg} suppressive function in the presence of GITR-L.

Our findings *ex vivo* are also consistent with data from human clinical trials with agonist-GITR antibody monotherapy in the context of solid tumors, which have thus far not demonstrated clinical efficacy.[19–23] While candidate agonist anti-GITR antibodies including TRX518, MEDI1873, or AMG228 had favorable effects on T_{regs} in animal models, the antibodies failed to overcome exhaustion of the tumor directed

CD8 T cells.[19–21] The combination of a GITR agonist antibody with anti-PD-1 did not improve performance of candidate molecules, MK-1248 or MK-4166.[22,23] As a result, plans to explore agonistic GITR antibodies further clinically, have been ceased. It remains unclear why the human clinical trial data in cancer was not consistent with pre-clinical studies in mouse models; however, our data in the context of HIV infection demonstrate a clear adverse impact of GITR-L on CD8 T cell function. Our findings may also have implications for future HIV vaccine strategies. While the administration of multimeric GITR-L with an HIV Gag DNA vaccine increased Gag specific T cell responses an anti-Gag antibodies in a mouse model[31] it is unclear if similar results will be found in a clinical trial in people.

Consistent with prior studies in mouse models,[41] we demonstrated that the addition of GITR-L to anti-CD3 abrogated T_{reg} mediated suppression, which could potentially be a favourable outcome. The T_{regs} expressed high levels of PD-1 and CD25 following addition of GITR-L indicating strong activation which could potentially result in activation-induced-apoptosis. The downside of this effect on Tregs was our observation of significant CD4 T_{conv} proliferation following GITR-L with anti-CD3, which could potentially expand, and not contract the HIV reservoir.

In conclusion we show that in blood from PWH on ART, GITR activation impaired rather than improved HIV-specific T cell function, abrogated the inhibitory effects of T_{regs} on CD4 T cell proliferation and directly expanded CD4 T_{conv}. Our findings are consistent with the disappointing results of GITR-agonist antibodies *in vivo* in clinical trials in the setting of cancer. We conclude that GITR activation has unfavourable effects on antigen experienced HIV specific CD8 T cells and further exhausts the HIV immune response resulting in reduced cytotoxicity. In addition, GITR stimulation could potentially enhance persistence of the HIV reservoir through clonal expansion on CD4 T cells.

Limitations of the study

A clear limitation of our study was that we did not directly assess the effects of GITR activation on the HIV reservoir itself, including effects on latency reversal or proliferation, nor on HIV-specific $CD4^+$ T cell function; however, these activities should be explored in future studies. In addition, we used a low concentration of IL-2 in our killing experiments, and given that IL-2 functions in an autocrine manner to enhance GITR function, [48–50] adjustments in the IL-2 concentration *ex vivo* should be considered. While this manuscript represents the first comprehensive analysis of constitutive and inducible expression of GITR on T cells in PWH, we did not quantify the expression of GITR-L on APC and therefore our work is unable to provide insights as to whether activity of GITR *in vivo* could also be affected by the expression of GITR-L in PWH on or off ART.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

C.G. and S.R.L. conceived the study and designed all the experiments. C.G., R.D.P., A.S., and R.C. performed and analyzed all the experiments. J.M. and J.L. recruited the patient cohorts. S.R.L., R.D.P., T.A.R., J.C., and C.C. provided intellectual input on data interpretation. C.G. and T.A.R. performed statistical analysis. C.G. and S.R.L. drafted the manuscript. All authors approved the final manuscript.

DECLARATION OF INTERESTS

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INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor® 488 anti-human FOXP3	Biolegend	320111: RRID: AB_430882
Brilliant Violet 421™ anti-human FOXP3	Biolegend	320123: RRID: AB_2565972
Brilliant Violet 605™ anti-human CD25	Biolegend	356141: RRID: AB_2800963
Brilliant Violet 785™ anti-human CD25	Biolegend	356139: RRID: AB_2750205
PE/Cyanine7 anti-human CD25	Biolegend	985808; RRID: AB_2894589
PerCP anti-human CD45RA	Biolegend	304155; RRID: AB_2616997
Alexa Fluor® 700 anti-human CD3	Biolegend	300324; RRID: AB_493738
Brilliant Violet 785™ anti-human CD3	Biolegend	317329; RRID: AB_11219196
APC/Cyanine7 anti-human CD62L	Biolegend	304813; RRID: AB_493583
Brilliant Violet 711™ anti-human CD357 (GITR)	Biolegend	371211; RRID: AB_2687161
PE/Cyanine7 anti-human CD357 (GITR)	Biolegend	371223; RRID: AB_2687170
Alexa Fluor® 488 anti-human CD357 (GITR)	Biolegend	371209; RRID: AB_2650623
Brilliant Violet 421™ anti-human CD366 (Tim-3)	Biolegend	345007; RRID: AB_10900073
Purified anti-human CD3	Biolegend	317301; RRID: AB_571926
Purified anti-human CD28	Biolegend	302901; RRID: AB_314304
FITC anti-human/mouse Granzyme B	Biolegend	515403; RRID: AB_2114575
BD PE-CyTM7 Mouse Anti-Human CD45RA	BD Bioscience	337167; RRID: AB_647424
BD APC Mouse Anti-Human CD25	BD Bioscience	340939; RRID: AB_400551
BD PharmingenTM Alexa Fluor® 700 Mouse Anti-Human CD3	BD Bioscience	561027; RRID: AB_10561682
BD HorizonTM BUV395 Mouse Anti-Human CD4	BD Bioscience	563550; RRID: AB_2738273
BD HorizonTM BUV737 Mouse Anti-Human CD4	BD Bioscience	612748; RRID: AB_2870079
BD HorizonTM BUV737 Mouse Anti-Human CD27	BD Bioscience	612830; RRID: AB_2870152
BD HorizonTM BV421 Mouse Anti-Human CD127	BD Bioscience	562437; RRID: AB_11153481
BD OptiBuildTM BUV661 Mouse Anti-Human CD279 (PD-1)	BD Bioscience	750260; RRID: AB_2874457
BD PharmingenTM PE Mouse anti-Human CD279 (PD-1)	BD Bioscience	560795; RRID: AB_2033989
BD HorizonTM BUV737 Mouse Anti-Human CD8	BD Bioscience	612755; RRID: AB_2870086
BD OptiBuildTM BUV805 Mouse Anti-Human CD8	BD Bioscience	749366; RRID: AB_2873737

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
BD Human CD8 PharmingenTM PerCP-CyTM5.5 Mouse Anti- Human CD8	BD Bioscience	560662; RRID: AB_1727513
BD Horizon™ BUV395 Mouse Anti-Human IFN-γ	BD Bioscience	563563; RRID: AB_2738277
BD Pharmingen Alexa Fluor® 700 Mouse Anti-Human TNF	BD Bioscience	561023; RRID: AB_10563768
BD PharmingenTM Human BD Fc BlockTM	BD Bioscience	564219; RRID: AB_2728082
BD PharmingenTM APC Mouse Anti-Human CD107a	BD Bioscience	560664; RRID: AB_1727417
Biological samples		
Human peripheral blood mononuclear cells	Alfred Hospital, Melbourne, Australia and the Zuckerberg San Francisco General Hospital, San Francisco, CA	This manuscript
Chemicals, peptides, and recombinant proteins		
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation	Thermo Fischer	L34957
eBioscience™ Fixable Viability Dye eFluor™ 780	Thermo Fischer	65-0865-14
Streptavidin, R-Phycoerythrin Conjugate (SAPE) - 1 mg/mL	Thermo Fischer	S866
CellTrace™ Violet Cell Proliferation Kit, for flow cytometry	Thermo Fischer	C34557
Recombinant Human TNFSF18 (GITRL) (carrier-free)	Biolegend	559202
True-Nuclear™ Transcription Factor Buffer Set	Biolegend	424401
Brefeldin A	Sigma Aldrich	20350-15-6
Monensin	Sigma Aldrich	M5273
IL-2 Recombinant Human Protein	R&D Systems	BT-002
SLYNTVATL	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
ILKEPVHGV	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
KIRLRPGG	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
QVPLRPMTYK	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
RLRPGGKKK	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
FPRTWLHGL	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
TPGPGVRYP	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
TPQVPLRPM	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
FLKEKGGL	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
EIYKRWII	Genscript	https://www.genscript.com/peptide-services.html?src=google_sitelinks&gclid=CjwKCAjw3oqoBhAjEiwA_UaLtiCGu6V4iM0_C-VxNjAxOp4k33sxNyDzw5x6_INytcxk0JUfmTL6GhoCYl4QAvD_BwE
Biotinylated A2-SL9 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated A2-IV9 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated A3-KK9 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated A3-RK9 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated A3-QK10 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biotinylated B7-FL9 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated B7-TM9 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated B7-TL10 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated B8-EI8 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Biotinylated B8-FL8 HLA:peptide monomers	Professor Andrew Brooks at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia	This manuscript
Critical commercial assays		
$CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human	Miltenyi	130-091-301
EasySep™ Human $CD8^+$ T Cell Isolation Kit	StemCell Technologies	17953
EasySep Human $CD4^+$ T-cell Isolation Kit	StemCell Technologies	17952
Software and algorithms		
GraphPad Prism (version 9.1.2)		
BioRender	(BioRender.com)	
FlowJo version 10.8.1		
Other		
Anti-Human GITR antibody MK-1248	Merck	N/A
Anti-Human RSV IgG4 isotype control	Merck	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sharon Lewin (Sharon.lewin@unimelb.edu.au).

Materials availability

This study did not generate new unique reagents. There are restrictions to the availability of Anti-Human GITR antibody MK-1248 and Anti-Human RSV IgG4 isotype control as these are not commercially available and have been a kind gift from Merck.

Data and code availability

- All data reported in this paper have been deposited on lab archives and will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

PWH on suppressive ART underwent leukapheresis for collection and storage of peripheral blood mononuclear cells (PBMC). Inclusion criteria were being HIV antibody positive and on suppressive ART with viral load <50 copies/ml for at least 3 years. Participants were also negative for HBsAg, HCV antibodies and HCV RNA. HIV-negative donors and PWH off ART underwent standard blood collection. These participants were matched with PWH on ART for age (median aviremic: 51.5 yrs, viremic: 41 yrs, uninfected: 44yrs) sex (male) and ethnicity (caucasian) (Figure S1)

and were also negative for HBsAg, HCV antibodies and HCV RNA. Participants were enrolled at the Alfred Hospital, Melbourne, Australia and the Zuckerberg San Francisco General Hospital, San Francisco, CA. The study was approved by Human Research Ethics Committees at The Alfred in Melbourne, the University of Melbourne and the Institutional Review Board at University of California San Francisco. Each participant provided written informed consent under institutional guidelines. PBMC were isolated by Ficoll-Paque density gradient centrifugation, resuspended in FBS with 10% DMSO and stored in liquid nitrogen for later use.

METHOD DETAILS

Peptides and tetramers

Tetramers were folded with 4-6 consecutive additions of 2ul streptavidin-PE (1mg/ml) to 10ug monomers every 5min at room temperature and in the dark. After folding, tetramers were stored up to 4 weeks at 4 degrees. Staining was performed in presence of Fc-block in FACS buffer for 25 minutes at 37°C and 5% CO_2.

HLA-typing

HLA typing for HLA-A -B -C -DPB1 -DQA1 -DQB1 and - DRB1345 was performed on some participants, using the Illumina next generation sequencing (NGS) technology at the Institute for Immunology and Infectious Diseases, Murdoch University, Perth, Australia.

Surface and intracellular staining with and without activation

Twenty four well plates were coated with anti-CD3 antibody (1ug/ml) for 2h at 37°C. Thawed PBMC were cultured at 2 million cells/ml in RF10 (500ml Gibco RPMI 1640 culture media plus 10% heat inactivated foetal bovine serum (FBS) and 100 U/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL glutamine (Gibco), raltegravir (1mM/ml; Selleck Chem) and soluble anti-CD28 antibody (2ug/ml) overnight at 37°C and 5% CO_2. The following day, 0.5-1 million PBMC were stained in a 96-well U-bottom plate and washed with PBS before live/dead staining and Fc-block was performed at room temperature for 10-15 minutes in the dark. Cells were washed in FACS buffer (PBS, 1%FBS, 1mM EDTA) and then surface stained for 10 minutes on ice and in the dark. Cells were then washed twice with FACS buffer and fixed for 20 minutes at room temperature and in the dark using the True-Nuclear Transcription Factor Buffer Set (Biolegend). Intracellular staining in permeabilization buffer was performed on fixed cells for 45-60 minutes on ice and in the dark. Cells were then washed twice in FACS buffer. All flow data was acquired with a LSRFortessa cytometer (BD Bioscience). Data was further analysed using FlowJo V10 with a tSNE plug-in and graphs were generated with GraphPad Prism (version 9.1.2, GraphPad LLC).

Kinetics of GITR-expression in tetramer-positive CD8 T-cells

HLA-typed PBMC from PWH on ART were thawed and one third of the cells were pulsed with peptides in RF10+raltegravir. The peptides SLYNTVATL and ILKEPVHGV (presented in context of HLA-A2) or FLKEKGGL and EIYKRWII (presented in the context of HLA-B8) were incubated at 1ug/peptide/ml for 60 minutes at 37°C and 5% CO2. Peptide pulsed PBMC were then washed in big volumes twice and added back to the remaining PBMC. Total cells were resuspended in RF10+raltegravir and cultured at 2 million cells/ml in a 6 well plate format for 10 days. On days 1, 2, 3, 6 and 10, aliquots of 1 million cells were assessed with live/dead- tetramer- and surface staining, according to the protocol described above.

T cell proliferation and intracellular cytokine staining

Six well plates were coated with GITR-L (10ug/ml) (Biolegend, San Diego, CA), anti-GITR antibody MK-1248 (10ug/ml) (Merck, Darmstadt, Germany) or IgG4 Isotype control (Merck) in PBS and left at 37°C and 5% CO2 for 24 hours. HLA-typed PBMC from PWH on ART were thawed and CellTrace Violet (CTV) labelled according to the manufacturer's instructions (Invitrogen, Waltham, MA). One third of PBMC was then peptide pulsed with either SLYNTVATL, FPRTWLHGL, EIYKRWII or QVPLRPMTYK as described above and then added back to the unpulsed PBMC. Cells were cultured at 2 million cells/ml in RF10+raltegravir for 10 days. Aliquots of cultured cells were analysed on day 4 and day 10 for tetramer binding and phenotype using flow cytometry. In addition, 1 million cultured PBMC were re-stimulated with cognate peptide (2ug/ml) and in the presence of brefeldin A (5ng/ml) plus monensin (5ng/ml) for 6 hours at 37°C and 5% CO2. Cells were collected for live/dead- tetramer- and surface and intracellular staining with anti-IFNγ, -TNFα, -Granzyme B (GzmB), as described above.

CD8 T cell killing assay

We assessed CD8 T-cell killing as previously described[51] with some modifications. In brief, we coated 75ml culture flask with GITR-L, anti-GITR antibody MK-1248 or IgG4 Isotype control as described above. HLA-typed PBMC from PWH on ART were thawed, peptide pulsed and cultured at two million cells/ml in RF10+raltegravir at 37°C and 5% CO2 for 13 days. Half media changes were performed at day 3 or 4, with addition of Il-2 (10U/ml). On day 12, autologous $CD4^+$ T-cells were purified from a new aliquot of thawed PBMC using the EasySep $CD4^+$ T-cell isolation kit (StemCell). CD4 T-cells were rested overnighting in RF10+raltegravir. On day 13, peptide expanded CD8 T-cells were purified from cultures using the EasySep $CD8^+$ T-cell isolation kit (StemCell), counted and stained with tetramers to assess the frequency of HIV peptide specific CD8 T-cells. In parallel the rested, autologous CD4 T-cells were split in half and labelled with a low and high concentration of CTV. CTV low CD4 T-cells were pulsed with the corresponding peptide that was used to stimulate the same donor on day 0. CTV high CD4 T-cells were not pulsed with peptides. Peptide pulsed (low CTV) and unpulsed (high CTV) CD4 T-cells were then co-cultured with

CD8 T-cells at various ratios for 6 hours. Live/dead- and surface staining was performed. Cells were then fixed with 1% paraformaldehyde and acquired by LSRFortessa cytometer (BD Biosciences). In addition, phenotypic surface staining and intracellular cytokine staining of the expanded HIV-specific CD8 T-cells was performed on day 13, as described above. Specific lysis capacity was calculated as the % specific lysis = [100 - (CTV low/CTV high) in the presence of effector cells] / (mean of the three wells containing target cells alone in the absence of effector cells) x100).

CD4 T cell suppression and proliferation assay

We assessed CD4 T_{reg} and T conventional cell interactions in the context of GITR agonistic antibodies as previously described[41] with some modifications. In brief, we coated U-bottom 96-well plates with GITR-L (10ug/ml) and anti-CD3 antibody (1ug/ml) overnight at 37°C and 5% CO2. PBMC from PWH on ART were thawed and T_{reg} cells were isolated using the Miltenyi $CD4^+CD25^+$ T_{reg} isolation kit. Negative isolated (untouched) CD4 T-cells were CTV labelled and co-cultured with either 1) CD25 positive isolated autologous T_{regs} or 2) CTV negative, autologous CD4 T-cells in RF10+raltegravir for 4 days. Soluble anti-CD28 antibody (0.1ug/ml) was added to wells containing anti-CD3 antibody. Live/Dead-, surface and intracellular staining was performed as described previously.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data was examined for normal distribution using GraphPad Prism's normality algorithms (D'Agostino-Pearson, Shapiro-Wilk, Anderson-Darling and Kolmogorov-Smirnov), visual inspection of histograms and quantile-quantile plots by GraphPad Prism (version 9.1.2). Skewed data was log transformed to potentially achieve normal distribution. Parametric (t-test) or non-parametric test (Mann-Whitney-U test) were then applied as appropriate with statistical comparisons between experimental conditions performed by paired t-test or signed-rank test.

ADDITIONAL RESOURCES

Illustrations were made with BioRender (BioRender.com).