ORIGINAL ARTICLE

Differential transcriptional and functional properties of regulatory T cells in HIV-infected individuals on antiretroviral therapy and long-term non-progressors

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Abstract

Objectives. Regulatory T cells (Tregs) are widely recognised as a subset of CD4+CD25+FOXP3+ T cells that have a key role in maintaining immune homeostasis. The impact of HIV-1 infection on immunological properties and effector functions of Tregs has remained the topic of debate and controversy. In the present study, we investigated transcriptional profile and functional properties of Tregs in HIV-1-infected individuals either receiving antiretroviral therapy (ART, \(n=50\)) or long-term non-progressors (LTNPs, \(n=24\)) compared to healthy controls (HCs, \(n=38\)).

Methods. RNA sequencing (RNAseq), flow cytometry-based immunophenotyping and functional assays were performed to study Tregs in different HIV cohorts. Results. Our RNAseq analysis revealed that Tregs exhibit different transcriptional profiles in HIV-infected individuals. While Tregs from patients on ART upregulate pathways associated with a more suppressive (activated) phenotype, Tregs in LTNPs exhibit upregulation of pathways associated with impaired suppressive properties. These observations may explain a higher propensity for autoimmune diseases in LTNPs. Also, we found substantial upregulation of HLA-F mRNA and HLA-F protein in Tregs from HIV-infected subjects compared to healthy individuals. These observations highlight a potential role for this non-classical HLA in Tregs in the context of HIV infection, which should be investigated further in other chronic viral infections and cancer.

Conclusion. Our study has provided a novel insight into Tregs at the transcriptional and functional levels in different HIV-infected groups.

Keywords: gene expression, HIV infection, long-term non-progressors, regulatory T cells
INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) infection is characterised by the progressive depletion of CD4+ T cells and persistent immune activation resulting in gradual deterioration of the immune system.1,2 Persistent hyper-immune activation is linked to endothelial inflammation and subsequently increased rates of non-AIDS-associated comorbidities such as cardiovascular diseases.3 Furthermore, activated CD4+ T cells become an attractive target of HIV-1 infection by upregulating CCR5 and CXCR4 expression, two main HIV-1 co-receptors.4 Regulatory T cells (Tregs) are one of the main cornerstones responsible for controlling inappropriate HIV-1-associated hyper-immune activation.5 Tregs are a subset of CD4+ T cells that are characterised by the high surface expression of IL-2Rα (CD25) and the transcription factor FOXP3,6,7 Tregs constitute a heterogeneous population and can be divided into two main subsets of effector Tregs (eTregs) and central Tregs (cTregs).8,9 cTregs and eTregs can be distinguished based on the expression of CD45RA, CD62L and CCR7.10,11 While cTregs are mainly localised in secondary lymphoid tissues, eTregs are primarily found in non-lymphoid tissues and secondary lymphoid organs.9 Tregs exert their regulatory functions through several mechanisms including secretion of inhibitory cytokines or soluble factors (e.g. TGF-β, IL-35 and IL-10), the direct killing of target cells (through cytolytic molecules such as granzyme B (GzmB)), inhibition of dendritic cell maturation and function through engagement by co-inhibitory receptors (e.g. CTLA-4:CD80/CD86), metabolic deprivation (e.g. CD25-mediated IL-2 deprivation-induced apoptosis) and cell:cell interactions (e.g. PD-1/PDL-1 and Gal-9:Tim-3).12 Although Tregs may have potential benefits in HIV-1 infection by suppressing an inadvertent immune response, this could be counteracted by their detrimental effects on viral control through the suppression of HIV-1-specific immune response.

Long-term non-progressors (LTNPs) are a rare group of HIV-1-infected individuals with low viral load (< 10,000 copies mL⁻¹), high CD4+ T-cell count and antiretroviral therapy (ART) naïve.13 Several mechanisms have been proposed to explain the robust viral replication control in LTNPs such as the possession of certain HLA alleles, primarily HLA-B*27 and HLA-B*57 alleles.14 Previously, we showed that CD8+ T cells restricted by HLA-B27 and HLA-B57 evade Treg-mediated suppression and continue to proliferate and kill virally infected cells through secretion of lytic molecules such as GzmB.13 In contrast, CD8+ T cells restricted by non-HLA-B*27/B*57 alleles upon recognition of their cognate epitopes upregulate Tim-3 and therefore become suppressed by Tregs via Tim-3:galectin-9 (Gal-9) interactions.13 More recently, we reported that CD8+ T cells restricted by HLA-B*35Px and HLA-B*53 alleles, which are associated with rapid disease progression to AIDS, also evade Treg-mediated suppression by not upregulating Tim-3 upon cognate epitope recognition.15 Nevertheless, the phenotype and global function of Tregs in LTNPs versus patients on ART have not been well-characterised. Several studies have shown that LTNPs exhibit similar or decreased frequency of Tregs compared to healthy controls (HCs); however, the function of Tregs was not fully examined in these studies.16-18 The role of Tregs in HIV-1 infection has been the topic of debate and controversies.19 This becomes more complicated when there are conflicting data on the frequency of Tregs in HIV-infected individuals. For example, some studies have reported higher and others lower Treg percentages in HIV-1-infected patients.20-22

The present study was designed to conduct a comprehensive RNAseq analysis to better understand differences in Tregs at the transcriptome levels in LTNPs, HIV-infected individuals receiving ART (ART) and HCs. Furthermore, based on the RNAseq data, we performed additional studies to better characterise the functionality of Tregs in different study groups. Our results show that Tregs from ART patients upregulate pathways downstream of TCR and IL-2 stimulation, resulting in a more suppressive (activated) phenotype. In contrast, Tregs in LTNPs show the upregulation of pathways associated with less suppressive effector functions. We further confirmed our observations by showing that Tregs from patients on ART were more potent in suppressing the proliferation of effector T cells compared to Tregs from other groups. Thus, our studies provide a novel insight into the functionality of Tregs in different groups of HIV-infected individuals.
RESULTS

Differential gene expression profile of Tregs

For this study, we recruited 38 HCs, 50 HIV-infected individuals who have been on ART for a minimum of 2 years and 24 LTNPs defined as ART-naïve, infected with HIV-1 > 11 years, CD4+ T-cell count > 400 (cell mL–1 of blood) and plasma viral load < 10 000 copies mL–1 (Supplementary table 1). To determine possible differences between the transcriptional profiles of Tregs in ART, LTNPs and HCs, we conducted RNA sequencing (RNAseq) on the total RNA extracted from the enriched Tregs of five individuals per group (Supplementary table 2). Isolated Tregs had a purity of > 95% using CD25 and FOXP3 staining (Supplementary figure 1a).

A transcript was considered differentially expressed (DE) if it had an FDR < 0.05 and a minimum of log2 fold change (L2FC) smaller than –1 or greater than +1. The greatest differences in transcriptions were observed when HCs and ART subjects were compared (Figure 1a and b, Supplementary figure 1b). Principal component analysis (PCA) based on Euclidean distances separated samples in HCs and ART groups in a two-dimensional plot, although one ART subject deployed a transcriptional profile similar to that of HCs (Supplementary figure 1c). More specifically, compared with HCs, 2956 and 2278 transcripts were found up- and downregulated in ART Tregs, respectively (Figure 1a). Among the most upregulated transcripts in the ART group were those belonging to genes in the human leucocyte antigen (HLA) complex, class I (22 transcripts) and class II (six transcripts), some of which expressed with an L2FC higher than 20. Thus, we concluded that the most upregulated transcripts in ART patients were transcripts belonging to the HLA class I and class II complexes. Nonetheless, nine of class I and two of class II genes were downregulated in the ART group. DDX39B was the second most upregulated HLA-associated transcript in the ART group as a component of the TREC complex and has been reported to stimulate viral RNA synthesis.23 TAP2 was the third upregulated transcript in ART Tregs compared to HC Tregs. TAP2 is involved in HLA-1 protein assembly and expression, which results in the generation of CD8+ T-cell-specific immune response against viral particles.24

Among the most downregulated transcripts were those from the splicing factor 1 (SF1) gene, which is a proto-oncogene involved in HIV-1 replication.25 Heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) was the next downregulated transcript in ART Tregs compared to HCs Tregs, which belongs to HIV-1 dependency factors (HDFs).26 Although HDFs are essential for HIV-1 replication, silencing of their expression is not lethal to the host cell.26,27 Moreover, 10 transcripts of the double bromodomain-containing protein (BRD2) were drastically downregulated in ART Tregs. BRD2 suppresses HIV-1 transcription in latent cells, contributing to HIV-1 dormant state in its reservoirs.28 The identities (IDs) of genes that contain the 10 most down- or upregulated transcripts in this comparison are shown in Supplementary figure 1f. When Tregs from HCs were compared to LTNP subjects, the observed transcriptional changes were less dramatic compared to the previous group (Figure 1b and c, Supplementary figure 1d). In this comparison, we identified 419 and 262 transcripts that were up- and downregulated, respectively. Although HCs and LTNPs were well-separated by the first two components of a PCA (Supplementary figure 1e), it was evident that LTNPs constituted two subgroups with three subjects deploying a transcriptional profile more similar to subjects in the HCs. The IDs of the genes that contain the 10 most down- or upregulated transcripts in this comparison are shown in Supplementary figure 1d.

Among the three most upregulated transcripts were those in particular HLA-F and genes associated with HLAs such as DDX39B, and to a lesser extent heat shock protein family A (Hsp70) member 1B (HSPA1B). Hsp70 binds to HIV-1 Gag polyprotein chains during transport to the plasma membrane and maintains their assembly-competent conformation.29,30 Besides, after the entry of virions to the target cells, virion-associated Hsp70 precipitates in the early phases of infection.31 Thus, Hsp70 proteins contribute to HIV-1 antigen presentation and promote viral replication inside the host cell.

SF1 was also the most downregulated gene in the LTNPs, as in the case of the ART group. The RNA-binding protein SMN1 and protein phosphatase 1 regulatory inhibitory subunit 11 (PPP1R11) were also among the most downregulated genes. PPP1R11 is a negative
Figure 1. Heat maps describing normalised abundance of differentially expressed transcripts. (a) Differentially expressed transcripts in HCs vs ARTs. (b) UpSet plots depicting transcripts DE in one or more comparisons. Upregulated (left panel) and downregulated (right panel) transcripts are shown separately. Sidebars represent number of transcripts DE in each comparison (red and green bars correspond to upregulated or downregulated DE transcripts, respectively). Dodger blue vertical bars represent the intersection size, that is the number of transcripts that were found DE in one or more comparisons. Blue dots represent comparisons in which transcripts were found DE. For instance, the left-most vertical blue bar in each panel indicates that 2665 and 2138 transcripts, respectively, were found up- or downregulated only in the comparison HC versus ART. Correspondingly, the right-most blue bar in each panel indicates that 19 and 26 transcripts, respectively, were found up- or downregulated in the comparisons of HCs versus ARTs, and LTNPs versus ARTs. Blue dots connected with a line indicate transcripts DE in more than one comparison. (c) Differentially expressed transcripts in HCs vs LTNPs. (d) Differentially expressed transcripts in LTNPs vs ARTs. Regularised logarithmic transformation was applied to the raw counts prior to heat map plotting. Only transcripts with an FDR < 0.05 and an absolute log2 fold change value > 1 are included. The number of upregulated or downregulated transcripts in each comparison is indicated with purple or dark-grey vertical bars on the right of each heat map and included in parentheses. Below each heat map, the magnitude of the log2 fold change is depicted in graph bar. As indicated, RNAseq was obtained from five human subjects in each group.
regulator of cytokine expression upon T-cell activation and might contribute to Treg polarisation. Finally, the comparison between LTNP and ART subjects revealed the upregulation and downregulation of 92 and 63 transcripts in ART compared with LTNP, respectively (Figure 1b and d, Supplementary figure 1f). PCA showed that although samples in the ART and LTNP groups were different, the latter one showed two subgroups (Supplementary figure 1g). A series of class I and class II HLA genes were found dramatically upregulated in the ART group compared to LTNP; however, a smaller number of class I HLA genes were found to be downregulated. The survival of motor neuron 1 (SMN2) and exocyst complex component 3 (EXOC3) were the next upregulated transcripts in ART compared to LTNP. EXOC3 involves in the targeted transfer of Nef between cells of the immune system, leading to the viral particle spreading between immune cells.

As mentioned before, a series of class I HLA genes were drastically downregulated in ART compared with LTNP Tregs. BRD2 and asparagine-linked glycosylation 13 (ALG13) were the next downregulated genes in ART Tregs. ALG13 heterodimerises with ALG14 to form UDP-GlcNAc glycosyltransferase. GlcNAcylation inhibits the activity of the HIV-1 promoter in infected host cells, resulting in decreased viral transcription. These analyses revealed substantial differences at the transcriptome levels between Tregs in HCs with patients on ART compared to the other group.

Higher TCR and IL-2 signalling in Tregs of ART than in Tregs of HCs
We subsequently used the weighted IPA to computationally analyse the upstream regulators that govern the differential gene expression pattern in HCs vs the ART group. Our analysis revealed a higher expression of CD3, fibronectin 1 (FN1) and IL-2 upstream regulators in ART Tregs than in Tregs in HCs (Figure 2a). Since IL-2 signalling occurs through the phosphorylation of STAT5, we examined the phosphorylation of STAT5 in ART compared to HCs. Our observation revealed a higher expression of IL-2RA (CD25) transcript (Figure 2b) and the mean fluorescent intensity (MFI) of both CD25 and phospho-STAT5 in ART compared to HCs (Figure 2c–f). Although there was no significant difference in the expression of CD25 in ARTs versus LTNP, the MFI for phospho-STAT5 was significantly lower in Tregs of LTNP than in their counterparts in the ART group (Figure 2f). The FN1 works as a costimulatory molecule in T-cell receptor (TCR)-mediated T-cell activation, and TCR signalling is required for Treg effector differentiation and function, which is mediated through the phosphorylation of ZAP70. In agreement, we found higher phosphorylation of ZAP70 in Tregs of ART than in Tregs of HCs (Figure 2g and h). Similarly, the MFI of ZAP70 was significantly higher in Tregs of LTNP than in Tregs of HCs, but it was lower in Tregs of LTNP than in Tregs from patients on ART (Figure 2g and h). Thus, upstream analyses revealed higher TCR and IL-2 activity and upregulation of their downstream signalling pathways in Tregs of ART than in Tregs of HCs and LTNP.

Higher expression of HLA-F in Tregs from HIV-infected individuals than from HCs
Our RNaseq analysis revealed that Tregs from HIV-infected patients either ART or LTNP express significantly higher levels of mRNA for HLA-F than for HCs (Figure 2i). HLA-F is one of the non-classical HLA class Ib members with a tolerogenic role. The same pattern was observed at the protein level, and Tregs from HIV-infected individuals regardless of their status (ART or LTNP) expressed significantly higher surface of HLA-F than Tregs from HCs (Figure 2j and k). As reported elsewhere, we found that the activation of CD4 T cells was associated with the upregulation of HLA-F (Figure 2i and m). These observations suggest that HLA-F may play an important role in Treg effector functions in HIV patients, which merits further investigations.

Tregs in the ART group exhibit a greater effector phenotype than Tregs in HCs
We first quantified Treg frequency in HIV-infected individuals and HCs. However, we did not observe any significant difference in their percentages between groups (Supplementary figure 2a–c). We then used IPA to computationally translate the observed gene expression patterns into directional changes in canonical pathways in Tregs of HCs vs ART. As shown in Figure 3a, the mTOR pathway was the most upregulated pathway in Tregs from ART compared to Tregs from HCs. mTOR has a key
Figure 2. Higher TCR and IL-2 signalling in Tregs of ARTs than in those of HCs. (a) Upstream regulators predicted to account for transcriptional differences between HCs versus ART patients. (b) Log2 fold change in the CD25 gene in Tregs from ART compared to HCs (n = 5). (c) Representative histogram of CD25 expression, and (d) the mean fluorescence intensity (MFI) ± median with interquartile range (MIR) of CD25 expression in Tregs of different study groups (n = 13 HC, n = 17 ART, n = 9 LTNP). (e) Representative histogram, and (f) the MFI ± MIR of pSTAT5 expression in Tregs of different study groups (n = 10 HC, n = 11 ART, n = 8 LTNP). (g) Representative histogram, and (h) the MFI ± MIR of pZAP70 expression in Tregs of different study groups (n = 12 HC, n = 11 ART, n = 8 LTNP). (i) Log2 fold change in HLA-F gene in Tregs of ARTs and LTNP compared to HCs. (j) Representative histogram, and (k) the MFI ± MIR of HLA-F expression in Tregs of HIV-infected individuals versus HCs (n = 10 HC, n = 10 ART, n = 8 LTNP). (l) Representative histogram, and (m) the MFI ± MIR of HLA-F expression in unstimulated (n = 14 Unstim.) and stimulated CD4+ T cells from the ART group (n = 14 Stim.) CD4+ T cells with anti-CD3/CD28 for 5 h. Each dot represents a human subject either HC or HIV-infected. Data were obtained from a single RNAseq analysis or from 3 to 5 independent experiments.
role in many cellular processes in Tregs, including cell proliferation, metabolism and suppressive functions.\(^3\)\(^9\)\(^-\)\(^4\)\(^2\) Since the expression of mTOR has been reported to be higher in eTregs than in cTregs,\(^4\)\(^0\)\(^-\)\(^4\)\(^2\) we examined the expression of CD45RA and CCR7 in Tregs to compare their maturation and differentiation status in ART and HCs. Our results showed a significantly higher percentage of CD45RA\(^+\)CCR7\(^-\) Tregs in ART and LTNP than in HCs, compatible with a greater effector phenotype (Figure 3b and c). Moreover, we found a higher expression of other genes that are associated with eTregs in Tregs from ART than those from HCs (Figure 3d and Supplementary table 3). Among these genes, we identified the interferon regulatory factor 4 (IRF4), which is a downstream molecule of the TCR signalling pathway. It controls the expression of several genes that are related to activation, proliferation, differentiation and immunosuppression by Tregs. Then, we further analysed the expression of other genes that are under the control of IRF4 and found that many of IRF4 target genes were highly upregulated in Tregs from ART compared to HCs (Figure 3e and Supplementary table 4). It has been shown that IRF4\(^+\) Tregs express higher levels of genes related to the NF-κB members such as RELA and RELB and lower expression of transcripts associated with quiescent Tregs such as CCR7, LEF1 and TCF7.\(^4\)\(^3\)\(^,\)\(^4\)\(^4\) In agreement, we found higher expression of RELA and RELB but lower expression of CCR7, LEF1 and TCF7 in Tregs from ART than in Tregs from HCs (Figure 3f and Supplementary table 5).

To confirm our gene expression results at the protein level, we examined the expression of several markers that have been reported to be highly upregulated on the surface of eTregs, such as HLA-DR, Tim-3 and inducible costimulatory molecule (ICOS).\(^4\)\(^3\)\(^,\)\(^4\)\(^5\)\(^,\)\(^4\)\(^6\) Interestingly, we found that Tregs from ART expressed significantly higher levels of HLA-DR, Tim-3 and ICOS than Tregs from HCs (Figure 3g-k and Supplementary figure 2d). Moreover, Tregs from ART expressed significantly higher CTLA-4 protein than Tregs from HCs, confirming the gene expression analysis (Figure 3l and Supplementary figure 2e). In agreement with our gene expression analysis, Tregs from ART had a significantly lower surface expression of CCR7 than that from HCs (Supplementary figure 2f and g). Since mTOR contributes to a high proliferation rate in Tregs, we measured the proliferation of Tregs in ART and HCs using Ki67. Interestingly, we found higher Ki67 expression in Tregs from ART than in their counterparts in HCs (Figure 3m and n). Also, we compared the activation status of Tregs versus non-Treg-CD4\(^+\) T cells in each group by comparing the expression of Ki67 and HLA-DR. These analyses revealed that Tregs compared to non-Treg-CD4\(^+\) T cells express significantly higher Ki67 and HLA-DR in HCs and patients on ART, while this was the case only for HLA-DR in LTNP (Supplementary figure 3a and b). However, we did not find any significant difference in the expression of Ki67 and HLA-DR in non-Treg-CD4\(^+\) T cells between groups (Supplementary figure 3a and b). Finally, we compared Treg frequency using different FOXP3 clones such as the clone 150D/E4 versus the clone 235A/E7; however, we did not find any significant difference in FOXP3\(^+\) Treg frequency using these two different antibodies (Supplementary figure 3c and d). Therefore, our results show that Tregs from ART demonstrate a greater effector phenotype than Tregs from HCs.

**Low ceramide expression contributes to the activation of mTOR and protein synthesis in Tregs from ART**

Next, we examined the role of other pathways that show differential expression in Tregs of ART vs HCs. The ceramide pathway was the most downregulated in Tregs of ART compared to HCs (Figure 3a). Ceramide is an endogenous activator of protein phosphatase 2A (PP2A), the core enzyme of the ceramide pathway.\(^4\)\(^7\) Ceramide is generated inside the cell through enzymatic cleavage of sphingomyelins in the cell membrane by the acid or neutral sphingomyelinas.\(^4\)\(^8\) The levels of both acid and neutral sphingomyelinas were lower in Tregs from the ART group than those from HCs (Figure 4a), which is reflected in the downregulation of the sphingomyelin metabolism pathway observed in the ART group (Figure 3a). PP2A inhibits mTORC1 and EIF2 pathways,\(^4\)\(^9\) which may contribute to the upregulation of both EIF2A and mTOR pathways observed in Tregs of ART patients. The second highly upregulated pathway after the mTOR pathway in Tregs of ART patients was the EIF2 pathway (Figure 3a). The EIF2 pathway contributes to protein synthesis following the TCR stimulation.\(^5\)\(^0\)\(^,\)\(^5\)\(^1\) As such, among 26 ribosomal proteins whose expression was assessed in our data set, 23 had higher expression in Tregs from
Figure 3. Higher effector phenotype in Tregs from the ART group than in those of HCs. (a) Canonical pathways enriched in differentially regulated genes in Tregs from HCs vs ARTs using IPA. (b) Representative flow cytometry plots, and (c) cumulative data of the expression of CD45RA and CCR7 in Tregs of different study groups (n = 19 HC, n = 19 ART, n = 10 LTNP). (d) Log2 fold change in associated genes with eTreg phenotype of ARTs compared to HCs. (e) Log2 fold change in the genes directly controlled by the IRF4 binding to the genome in Tregs from ARTs compared to HCs. (f) Log2 fold change in NF-kB family member genes in Tregs of ARTs compared to HCs. (g) Representative flow cytometry plots, and (h) cumulative data of HLA-DR expression in Tregs of different study groups (n = 20 HC, n = 21 ART, n = 8 LTNP). (i) Representative flow cytometry plots, and (j) cumulative data of Tim-3 expression in Tregs of different study groups (n = 26 HC, n = 26 ART, n = 8 LTNP). (k) Cumulative data of the expression of ICOS in Tregs of different study groups (n = 13 HC, n = 24 ART, n = 13 LTNP). (l) Cumulative data of CTLA-4 expression in Tregs of different study groups (n = 26 HC, n = 23 ART, n = 8 LTNP). (m) Representative flow cytometry plots, and (n) cumulative data of Ki67 expression in Tregs of different study groups (n = 25 HC, n = 24 ART, n = 14 LTNP). Data were obtained from a single RNAseq analysis, from 3 to 6 independent experiments.
ART than those in HCs (Figure 4b and Supplementary table 6). Moreover, the expression of several enzymes associated with protein synthesis, including ribosomal protein S6 kinases B1 (p70S6 kinase), was higher in Tregs from ART than in Tregs from HCs (Figure 4c and Supplementary table 7). The P70S6 kinase as a target of mTOR increases the activity of ribosomal protein S6 through its phosphorylation.52 Thus, we assessed the activity of P70S6 kinase by examining the phosphorylation of ribosomal protein S6 at S235 and S236 serine residues. Our results showed significantly higher levels of p-S235-236 in Tregs from ART than in those from HCs and LTNPs (Figure 4d and e). These data suggest enhancement of protein synthesis in Tregs of ART patients, which is required for synthesising necessary materials for Treg activation and proliferation.

Tregs from the ART group exhibit a more suppressive phenotype

The hippo pathway was the third highly upregulated pathway in Tregs of ART compared to Tregs of HCs. The hippo pathway mainly controls the size of animal organs with well-recognised roles in the differentiation and function of Tregs.53,54 MST-1, the key enzyme of the hippo pathway, enhances FOXP3 acetylation through the activity of histone acetyltransferase p300 (HAT), resulting in higher stability of FOXP3.55 We found a higher expression of both MST-1 and HAT transcripts in Tregs from ART than in Tregs from HCs (Figure 4f). Moreover, the upstream IL-2 signalling facilitates STAT5 phosphorylation and activation through MST-1. As we showed in Figure 2a–f, IL-2 signalling and pSTAT5 levels were upregulated in Tregs of ART compared to HCs. TCR signalling and STAT5 pathways control distinct sets of genes that contribute to Treg-suppressive activity.56,57 Therefore, we investigated the expression of genes that were STAT5 activation-dependent in our RNAseq data sets. Our analysis showed the upregulation of genes associated with STAT5 activation in Tregs of ART, whereas genes indicative of STAT5 inhibition were downregulated in ART Tregs (Figure 4g and Supplementary table 8). Although CD25 enhances the stability of the FOXP3 gene through the activation of pSTAT5, there seems to be a positive feedback loop between CD25 and FOXP3, in which FOXP3 binds to several binding sites in CD25 locus, leading to increased expression of CD25.58 The FOXP1 gene was another highly upregulated gene in ART Tregs from ART patients, which enhances FOXP3-mediated expression of several Treg lineage genes including CD25.58 FOXP1 also represses the IL-7ra locus, resulting in decreased IL-7-dependent proliferation in Tregs.59 Our analysis of the expression level of FOXP1 and IL-7ra genes in our data set revealed higher and lower levels of FOXP1 and IL-7ra genes in ART Tregs than those in HCs, respectively (Figure 4h). Collectively, the comparison of Tregs from HCs with ART revealed upregulation of the pathways that are associated with more suppressive phenotype in ART Tregs. Also, we observed the upregulation of MAPK signalling and elevated levels of pERK in Tregs of ARTs and LTNPs compared to HCs (Figure 4i and j), which are associated with a more suppressive phenotype in ART Tregs.60

Higher glycolysis but lower fatty acid oxidation in Tregs of LTNPs than in those of HCs

To better understand the upstream regulators of the differential gene expression pattern in Tregs of HCs vs LTNPs, we compared the gene expression profile in these two groups. Our results showed upregulation of the MYC, IL-15, TCR and hypoxia-inducible factor-1A (HIF1A) upstream regulators and downregulation of IL-10 regulator in Tregs of LTNPs vs HCs (Figure 5a). We further verified increased TCR signalling in LTNP Tregs by showing higher pZAP70 expression than that of HCs (Figure 2g and h). IL-15 and IL-10 mitigate and increase the suppressive function of Tregs, respectively.61–63 Moreover, HIF-1α is involved in intracellular glycolysis pathway64 and C-Myc is a general transcription factor that regulates the expression of several genes involved in cell metabolism and glycolysis.64 We then analysed the canonical pathways that were differentially expressed in Tregs of HCs vs LTNPs. Our analysis revealed that the EIF2 signalling was the most upregulated pathway in Tregs of LTNPs followed by glycolysis and gluconeogenesis (Figure 5b). In contrast, fatty acid oxidation (FAO) and PPAR signalling were downregulated in Tregs of LTNPs compared to HCs. As shown in Figure 5c, we found a significantly higher expression of transcripts of several enzymes that are involved in
Figure 4. Low ceramide expression contributes to the activation of mTOR and protein synthesis in Tregs from ARTs with a more suppressive phenotype. (a) Log₂ fold change in the acid and neutral sphingomyelinase genes in Tregs of ARTs compared to HCs. (b) Log₂ fold change in the ribosomal protein genes in Tregs of ARTs compared to HCs. (c) Log₂ fold change in enzymes associated with protein synthesis in Tregs of ARTs compared to HCs. (d) Representative histogram, and (e) the MFI ± MIR of p-S235-236 expression in Tregs of different study groups ($n = 6$ HC, $n = 9$ ART, $n = 9$ LTNP). (f) Log₂ fold change in MST-1 and HAT genes in Tregs of ARTs compared to HCs. (g) Log₂ fold change in FOXP1 and IL-7R genes in Tregs of ARTs compared to HCs. (h) Log₂ fold change in the genes controlled by pSTAT5 binding to the genome in Tregs of ARTs compared to HCs. (i) Representative histogram, and (j) the MFI ± MIR of pERK expression in Tregs of different study groups ($n = 13$ HC, $n = 15$ ART, $n = 8$ LTNP). Each dot represents a human subject either HC or HIV-infected. Data were obtained from a single RNAseq analysis and from three independent experiments.
glycolysis in Tregs of LTNPs. However, we identified lower expression of multiple genes belonging to the aldehyde dehydrogenase family in Tregs of LTNPs than in Tregs of HCs, possibly resulting in lesser oxidation of long-chain aldehydes into fatty acids (Figure 5d). FAO involves the generation of acetyl-CoA through sequential removal of two-carbon units from the acyl chain as it enters the tricarboxylic acid (TCA) cycle in mitochondria to regulate mitochondrial OXPHOS.65 Interestingly, glycolysis and OXPHOS have reciprocal effects on Treg-mediated suppression. In contrast to glycolysis that reduces FOXP3 suppression. In contrast to glycolysis that reduces FOXP3 phosphorylation (OXPHOS) increases the suppressive function of Tregs.65,66 Thus, the observed changes in the glycolysis and FAO suggest a lower suppressive function for Tregs in LTNPs than those in HCs. We also observed a higher activation of gluconeogenesis pathway in Tregs of LTNPs than those in HCs (Figure 5b). Therefore, the increased glucose production through gluconeogenesis may fuel the necessary glucose required for glycolysis in Tregs.

**High PPAR signalling results in a lower STAT3 in Tregs of LTNPs**

Our results showed the downregulation of PPAR signalling in Tregs of LTNPs vs HCs (Figure 5b). The PPAR signalling results in the activation of FAO and the suppression of glycolysis.67 Moreover, the loss of PPAR-α in Tregs is associated with a decline in their suppressive activity.68–70 Since PPAR-γ leads to the inactivation of STAT3,71 we analysed the expression of STAT3 transcript in our data set. We observed a higher expression of the STAT3 gene in Tregs of LTNPs than those in HCs (Figure 5e). We also noted a higher expression of IL-6 signal transducer (IL-6 ST) in Tregs of LTNPs (Figure 5e), which is responsible for STAT3 phosphorylation and activation.72 Accordingly, we found higher phosphorylation of STAT3 in Tregs of LTNPs than those of HCs (Figure 5f and g). STAT3 binds to STAT5 binding sites on FOXP3 loci, resulting in Treg destabilisation.73,74 The loss of function in Tregs is accompanied by higher production of inflammatory cytokines such as IL-2 and IFN-γ.75,76 In agreement, we found a significantly higher expression of the IL-2 gene and a lower expression of the TGF-beta gene in Tregs of LTNPs than those of HCs (Figure 5e). Also, we investigated the expression of other genes that are associated with Treg stability and functionality. Interestingly, we observed a lower expression of TNF receptor superfamily member 25 (TNFRSF25), TNF receptor superfamily member 1A (TNFRSF1A) and IL-3 receptor IL-3RA genes that are associated with the stability of Tregs (Figure 5h).77–79 Finally, we found a higher activity for IFN-α and IFN-β, IFN regulatory factor 7 (IRF7) and CD40L upstream regulators in Tregs from ART than in Tregs from LTNPs (Figure 5i). IFN-α/β signalling promotes Treg development and function, which occurs through key transcriptional regulators such as IRF7 and IRF9.80 Similarly, CD40L ligation results in Treg activation.81 Taken together, these results revealed an increase in glycolysis but a decrease in the suppressive activity of Tregs in LTNPs compared to their counterparts in HCs. Moreover, our data support the enhanced activity of Tregs in the ART group compared to LTNPs.

**Tregs from ART exhibit more suppressive properties compared to their counterparts in HCs and LTNPs**

To further evaluate the functionality of Tregs in our different study cohorts, we measured effector T-cell proliferation in the presence/absence of Tregs (at 1:1 ratio). Interestingly, we observed higher suppression of the responder T cells to Tregs from ART than that to HCs and LTNPs (Figure 6a and b). However, we did not find any significant difference between the proliferative capacities of effector T cells in the presence of Tregs from HCs or LTNPs (Figure 6a and b). We and others have previously shown that Tregs express Gal-1, Gal-9 and PD-L1 and exert some of their regulatory functions through the interaction of these ligands with their corresponding receptors on effector T cells.13,82,83 Thus, we decided to investigate the expression of the above ligands in Tregs of our different study cohorts. We observed higher expression of Gal-1, Gal-9 and PD-L1 transcripts in Tregs from ARTs than those in HCs (Figure 6c). We also found a higher gene expression of Gal-8 in Tregs of ART subjects (Figure 6c), which is associated with enhanced IL-2 signalling in Tregs.84 However, we did not observe any difference in the gene expression of the above molecules between Tregs of LTNPs and HCs. We further found that Tregs in ARTs expressed significantly higher levels of Gal-9 on their surface than those in HCs (Figure 6d and e). It has been shown that apoptotic Tregs have more suppressive...
functions than non-apoptotic Tregs. As such, we assessed the apoptosis of Tregs in our different study groups. Interestingly, we found that Tregs from ARTs had significantly higher expression of Annexin V than Tregs from HCs and LTNPs (Figure 6f and g). This was accompanied by the upregulation of several genes related to apoptosis in Tregs from ARTs, such as BCL-2-interacting protein 3 (BNIP3), XIAP, BCL-2-associated transcription factor 1 (BCLAF1), FAS and caspases 2, 7, 8, 10 and 14 (Figure 6h). Apoptotic Tregs release ATP that is converted to adenosine through the tandem function of CD39 and CD73, two ectoenzymes that are expressed on the surface of Tregs. Contrary to previous reports, we did not find any significant difference in the expression of CD39 on Tregs of different study groups (Figure 6i and Supplementary figure 4a). However, we observed decreased frequency of CD73+ Tregs and hence CD39+CD73+ Tregs in ART compared with HCs and LTNPs (Figure 6i–k). It has been shown that in contrast to murine Tregs, CD73 is mainly expressed intracellularly in human Tregs. However, we did not observe any significant difference in the percentage of intracellular CD73+ cells in our different study cohorts (Supplementary figure 4b and c). These data suggest that a higher apoptosis rate in ART Tregs may provide more ATP for the adenosine production in Tregs from the ART group.

DISCUSSION

It has been reported that infection of Tregs with HIV-1 impairs their suppressive potential through DNA methylation of the FOXP3 gene, but ART reverses this impaired function. In contrast, some studies have reported that Treg functions are not altered in the setting of HIV infection. However, the potential differences in Tregs of different HIV subgroups at the transcriptional level or functionality have not been fully elucidated.

In this report, we show that Tregs from the ART group demonstrate a more suppressive phenotype than Tregs from LTNPs and HCs. Our IPA revealed the upregulation of IL-2 and TCR signalling in Tregs of ARTs compared to HCs. Also, we observed the upregulation of mTOR, hippo and MAPK signalling pathways that are associated with Treg activation in patients on ART. The mTOR pathway has a key role in the activation and differentiation of cTregs to eTregs and is crucial for the peripheral T-cell tolerance and homeostasis. The TCR signalling induces IRF4 and contributes to the acquisition of an effector phenotype in Tregs. The mTOR controls the expression of IRF4 at the post-transcriptional level and induces the expression of ICOS, which derives eTreg differentiation. Therefore, a higher expression of IRF4 and its controlled genes in Tregs of ARTs than those in HCs may support their suppressive effector phenotype. A higher mTOR activity contributes to the higher expression of the large neutral amino acid transporter CD98 (LAT1) gene and the transferrin receptor (CD71) in Tregs of ARTs, which are required for Treg activation.

We also observed the hippo pathway upregulation in Tregs of patients on ART. The hippo pathway controls the size of animal organs through tight regulation of cell proliferation and apoptosis. The core enzyme of the hippo pathway, MST-1, has several key roles in Treg stability. For example, it mediates IL-2-induced STAT5 phosphorylation, resulting in the expression of the genes that are associated with the inhibitory activity of Tregs. Also, MST-1 increases FOXP3 acetylation. While FOXP3 is expressed transiently in effector T cells, stable expression of FOXP3 is required for the development and establishment of a Treg phenotype. Moreover, the interaction of MST-1 with the DOCK8-LRCH complex controls T-cell migration. Interestingly, both DOCK8 and LRCH were upregulated in Tregs of ARTs compared to HCs (Supplementary figure 4d). This suggests a higher migratory ability for Tregs in the ART group, which may enhance their access to the effector site.

In contrast, we observed a lower ceramide signalling in Tregs of the ART group. Protein phosphatase 2A (PP2A), the core enzyme of the ceramide pathway, is a highly conserved and ubiquitously expressed serine–threonine phosphatase, which regulates many cellular functions. This enzyme has three different subunits, with the Ac subunit being the catalytic and Aa subunit as the scaffold subunit. The abundance of catalytic PP2Ac is similar in different cells. However, its activity is regulated through the phosphorylation of the carboxy-terminal end of PP2Ac at the Tyr307 residue. The SET oncoprotein inhibits PP2A activity through its phosphorylation. Besides, ceramide interacts with the SET oncoprotein and inhibits its function (Supplementary figure 4e), thus preventing its inhibitory effect on PP2A. It has been shown that the inhibition of acid sphingomyelinase...
increases the suppressive activity of Tregs through the increased CTLA-4 turnover. Therefore, decreased ceramide levels and subsequent decline in PP2A activity in Tregs of ART may enhance their suppressive activity through the upregulation of the mTOR pathway. However, increased ceramide level in CD4+ T cells and monocyte reduces their infectivity to HIV-1. As such, decreased ceramide levels in Tregs of ARTs may enhance their susceptibility to HIV-1 infection and make them a favorite target for the virus.

Our further analysis revealed an increase in MYC, IL-5, TCR and HIF1A but a decrease in IL-10 signalling in LTNP Tregs versus HC Tregs. The upregulation of genes involved in IL-15 and the downregulation of genes involved in IL-10 signalling suggest the decreased suppressive function of Tregs in LTNPs. MYC and HIF1A can increase the glycolysis in Tregs, which was demonstrated by the upregulation of the glycolysis pathway in Tregs of LTNPs. HIF-1α also reduces the suppression role of Tregs through the degradation and destabilisation of FOXP3 gene.

Compared to other subsets of T cells such as Th1, Th2 and Th17 cells, Tregs rely less on glycolysis but mainly on mitochondrial metabolism and oxidative phosphorylation (OXPHOS) as their source of energy because glycolysis inhibits Treg-suppressive function. As such, FOXP3 and CTLA-4 suppress glycolysis and enhance OXPHOS, resulting in reprograming of Treg metabolism.

Interestingly, the expression of glucose transporter Glut-1, the rate-limiting step in glycolysis, was higher in Tregs of LTNPs than in those of HCs. It has been shown that the overexpression of Glut-1 decreases the expression of FOXP3 in Tregs. In contrast to glycolysis, OXPHOS results in the production of reactive oxygen species (ROS) in T cells preventing nuclear transport of BTB domain and CNC homolog 2 (Batch2) through Bach2 deSUMOylation. DeSUMOylation of BACH2 prevents its nuclear export, which leads to a stable FOXP3 expression and stability of Tregs. Another important observation was the upregulation of gluconeogenesis in Tregs of LTNPs. Although gluconeogenesis mainly occurs in the liver and kidney, it has been reported to occur in memory T cells. The similarity of the metabolic pathway in memory T cells and Tregs may account for the observed gluconeogenesis pathway in Tregs. Overall, the metabolic pattern of Tregs in LTNPs and the activation of the IL-6/STAT3 pathway suggest impaired suppressive properties of Tregs in this group. This was further demonstrated when we noted a significant deficiency in Treg-mediated suppression of effector T-cell proliferation by Tregs of LTNPs versus those in the ART group. We further found that Tregs in ARTs were more apoptotic and showed higher expression of surface co-inhibitory receptors (e.g. CTLA-4 and ICOS), which might contribute to their enhanced suppressive functions. Apoptotic Tregs exhibit more suppressive function through the release of ATP and its conversion to adenosine by the enzymatic activity of CD39 and CD73 ectoenzymes. Of note, we observed significant elevation of HLA-F mRNA and HLA-F protein in Tregs from HIV-infected individuals (both ART and LTNP) compared to HCs. Upregulation of HLA-F in Tregs of HIV-infected individuals might be related to their hyper-activation status. HLA-F is largely located in the endoplasmic reticulum, and its expression is tightly regulated. This suggests that HLA-F surface expression on Tregs of HIV-infected patients might serve as a marker of cell stress including endoplasmic reticulum stress. Additionally, HIV-1 infection of CD4+ T cells can increase the transcription of HLA-F mRNA but decrease the binding of its ligand (KIR3DS1) as a potential mechanism of avoiding recognition by NK cells. Thus, it is possible to speculate that the ability of KIR3DS1 to bind to HLA-F expressed on Tregs in HIV-infected patients provides a mechanistic link between Tregs and NK cells. This hypothesis merits further investigation and might also have relevance in other infectious diseases, autoimmune disorders and cancers. Taken together, we have provided a novel insight into the differential properties of Tregs in HIV-infected individuals. In agreement with these observations, we have previously reported that CD8+ T cells restricted by HLA-B*27 and HLA-B*57 upon recognition of their cognate epitopes do not upregulate TIM-3 and therefore evade Treg-mediated suppression. In contrast, CD8+ T cells restricted by non-protective HLA alleles upon encounter with their cognate epitopes upregulate TIM-3 and therefore become suppressed by Tregs. Thus, T-cell evasion of Treg-mediated immune regulation and lower suppressive properties of Tregs in LTNPs may explain the higher propensity of autoimmune diseases in
Figure 5. High glycolysis and low FAO in Tregs of LTNPs compared to HCs. (a) Upstream regulators predicted to account for transcriptional differences between Tregs in HCs versus LTNPs. (b) Canonical pathways enriched in differentially regulated genes between Tregs of HCs and LTNPs, as determined using IPA. (c) Log2 fold change in transcripts of several enzymes that are involved in glycolysis in Tregs of LTNPs compared to HCs. (d) Log2 fold change in transcripts of several aldehyde dehydrogenase family members in Tregs of LTNPs compared to HCs. (e) Log2 fold change in TGFB1, IL-2, STAT3 and IL-6 ST genes in Tregs from LTNPs compared to HCs. (f) Representative histogram, and (g) cumulative data of the MFI ± MIR of pSTAT3 in Tregs of LTNPs (n = 6) vs HCs (n = 7). (h) Log2 fold change in IL-3RA, TNFRSF25 and TNFRSF1A in Tregs of LTNPs compared to HCs. (i) Upstream regulators predicted to account for transcriptional differences between Tregs in the ART group versus LTNPs. Data were obtained from a single RNAseq analysis. Each dot represents a human subject either HC or HIV-infected, and pSTAT3 studies are representative of two independent experiments.
individuals possessing HLA-B*27 and HLA-B*57. In contrast, it is possible to speculate that the robust immune response against HIV infection in LTNPs may in part be associated with impaired Treg functions. More recently, we demonstrated that CD8+ T cells restricted by HLA-B*35Px were not suppressed by Tregs in HIV-infected progressors. Although because of the cell limitation, we were unable to conduct RNAseq analysis on Tregs from HIV-infected progressors, we have shown that Tregs in these individuals exhibit impaired suppressive properties. This hypothesis can be further supported by the higher susceptibility of individuals restricted by HLA-B*35Px to autoimmune diseases such as subacute thyroiditis.

We are aware of several study limitations. For example, we performed limited functional studies on Tregs in vitro. Isolating a large number of Tregs was not practical for functional assays, mainly because of the cell limitation. Similarly, the discrepancy in the sample size in some experiments was related to the same issue. There were no selection criteria for the samples in each set of experiments, and samples were randomly selected; however, we tried to use the same subset of samples for experiments that were related to each other (e.g. phospho assays). The period of HIV infection may influence the functionality of Tregs, and we were unable to obtain accurate information for our patients on ART, but they have been on ART beyond 2 years. The sex of HIV-infected individuals is also an important factor to be taken into consideration, but our patients were a combination of men and women. The activation status of T cells in HCs versus HIV patients may influence the results. As such, higher activated T cells in one group than in the other may influence the transcriptional levels. However, our observations do not support this concept since non-Treg-CD4+ T cells exhibited similar activation status as measured by Ki67 and HLA-DR in our cohort. In this study, we were unable to examine the effects of ART on Treg transcriptional profile/functionality. Nevertheless, future studies comparing Treg transcriptional profiles and effector functions pre- and post-treatment are warranted.

Taken together, we demonstrated differential Treg phenotype and function in different HIV subgroups versus HCs. In particular, by conducting RNAseq analysis, we provided a novel insight into the mechanism underlying differential Treg properties in HIV-infected individuals.

### METHODS

#### Study population

We recruited 112 human subjects for our study, consisting of (1) 38 HCs, who were HIV-1, hepatitis C virus (HCV) and hepatitis B virus (HBV) seronegative; (2) 50 HIV-infected individuals on ART; and (3) 24 LTNPs, who were ART-naive, had been infected with HIV-1 > 11 years with CD4+ T-cell count > 400 (cell mL⁻¹ of blood) and plasma viral lead < 10 000 copies mL⁻¹. Some of the LTNPs were recruited through the University of Washington/Fred Hutch Centre for AIDS Research (CFAR). The remaining patients were recruited from the cohort of the Northern Alberta HIV programme.

#### Cell isolation

We isolated the peripheral blood mononuclear cells (PBMCs) from the fresh blood of either healthy subjects or HIV-infected patients using Ficoll-Paque gradients. PBMCs were cultured in RPMI 1640 (Sigma-Aldrich, Toronto) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). In some studies, Tregs were isolated from fresh PBMCs according to the manufacturer’s instruction (STEMCELL Technologies, Vancouver), with a purity exceeding 95% (Supplementary figure 1a).

#### Flow cytometry analysis

Fluorophore antibodies with specificity to antigens and cytokines of human cells were purchased mainly from BD Biosciences (San Jose), Thermo Fisher Scientific (Waltham) and BioLegend (San Diego). The following antibodies were used specifically in our study: anti-CD3 (SK7), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-TIGIT (MBSA43), anti-TIM-3 (7D3), anti-CD39 (U66), anti-Gal-9 (9M1-3), anti-CD25 (M-A251), anti-CD127 (HL-7R-M21), anti-pSTAT5 (pY694), anti-CD45RA (HL100), anti-FOXp3 (150D/E4), anti-FOXP3 (PCH101), anti-HLA-DR (LN3), anti-CD62L (DREG-56), anti-pZap70 (J34-602), anti-Ki67 (20Raj1), anti-pERK1/2 (MILAN8R), anti-CD73 (AD2), anti-CTLA-4 (BNI3), anti-CCR7 (2-L1-A), anti-ICOS (C398.A4), anti-p5235/5236 (N7-548) and anti-HLA-F (3D11). For stimulation, mouse anti-human CD3 (UCHT1) was purchased from BD Biosciences. The apoptotic assay was performed using the PE Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s protocol. Surface staining and intranuclear staining were performed as we previously described elsewhere. To assess cell viability, LIVE/DEAD Kit (Thermo Fisher Scientific) was used. Stained cells were fixed with paraformaldehyde (2%) and were acquired on a Fortessa X-20 or LSRFortessa SORP (BD Biosciences). Data were analysed using FlowJo software (version 10). For Treg staining, PBMCs or isolated T cells were subjected to surface staining for the viability dye (Thermo Fisher Scientific) and anti-CD3, CD4, CD25 and CD127 antibodies followed by washing. Then, cells were permeabilised using the permeabilisation buffer (Thermo Fisher Scientific) as per the manufacturer’s instruction.
Figure 6. Greater suppressive properties of Tregs in ARTs than in their counterparts in HCs and LTNPs. (a) Representative plots, and (b) cumulative data of % suppression of proliferation by Tregs from different groups ($n = 9$ HC, $n = 6$ ART, $n = 6$ LTNP). (c) Log$_2$ fold change in Gal-1, Gal-8, Gal-9 and CD274 in Tregs of ART compared to HCs. (d) Representative flow cytometry plots, and (e) cumulative data of Gal-9 expression in Tregs of ART patients ($n = 12$) vs HCs ($n = 21$). (f) Representative histogram, and (g) cumulative data of the MFI ± MRI of Annexin V expression in Tregs of different study groups ($n = 14$ HC, $n = 10$ ART, $n = 6$ LTNP). (h) Log$_2$ fold change in several genes associated with apoptosis in Tregs of ART compared to HCs. (i) Representative flow cytometry plots of CD39 and CD73 expression in Tregs of different study groups. (j) Cumulative data of % of CD73$^+$ Tregs in different study cohorts ($n = 29$ HC, $n = 31$ ART, $n = 8$ LTNP). (k) Cumulative data showing % of CD39$^+$/CD73$^+$ Tregs in different study cohorts ($n = 29$ HC, $n = 31$ ART, $n = 8$ LTNP). Each dot represents a human subject either HC or HIV-infected. Data were obtained from a single RNAseq analysis and from 2 to 6 independent experiments.
before staining for the FOXP3 as we have reported elsewhere. For comparing surface staining versus intracellular staining for CD73, we first stained the cells with the anti-CD73 antibody for surface staining followed by fixation/permeabilisation and intracellular staining using the same clone but a different fluorophore.

**Phospho-flow assays**

Phospho-STAT5, phospho-ERK, phospho-Zap70 and pS235/pS236 1/2 staining were performed according to the manufacturer's instructions (BD Biosciences). In brief, PBMCs were cultured in RPMI media containing (0.5% FBS) in a 96-well plate and were stimulated for 30 min with IL-2 (100 IU) for STAT5, with PMA (2 μL/mL) for ZAP70 and pERK, and with IL-6 (100 ng/mL) for STAT3. Then, cells were stained for surface antigens followed by fixation with 2% PFA. Cells were incubated for 30 min at room temperature in the dark, and then, the cell pellet was resuspended in 100 μL of ice-cold 90–100% methanol. Next, the plate was incubated at 2–8°C for 30 min and washed with 2% FBS in PBS. The cell pellet was resuspended in 50 μL of 2% FBS in PBS followed by the addition of appropriate concentration of conjugated phospho-antibodies for 45-min incubation at room temperature. Finally, the cell pellet was washed and resuspended in 2% FBS in PBS for analysis by the flow cytometer.

**Proliferation assay**

For functional assays, T cells were isolated (STEMCELL Technologies) and labelled with CFSE dye (Thermo Fisher Scientific) and cultured in 96-well plates in the absence or presence of Tregs at 1:1 ratio. Cell stimulation was performed using anti-CD3 (1 μg/mL) and mitomycin-treated whole PBMCs as we have reported elsewhere. The suppression of proliferation was calculated by (% CFSEloCD3 without Tregs – % CFSEloCD3 with Tregs)/CFSEloCD3 w/out Tregs x100 as reported elsewhere.

**Library construction and sequencing**

Total RNA was extracted with TRIzol reagent (Invitrogen, Waltham) as per the manufacturer's instructions. RNAseq libraries were made from 100 ng of total RNA using the TruSeq RNA Library Prep Kit v2 (Illumina). Polyadenylated mRNAs were pulled down with oligo dTs conjugated to paramagnetic beads by sequential ETOh washes that removed non-polyadenylated transcripts. Recovered mRNAs were chemically fragmented and used for first- and second-strand cDNA synthesis. cDNAs were blunted and A-tailed; T-A ligation was used to add; and finally, Illumina adapters containing multiplexing barcodes were incorporated by 12 cycles of PCR. Sequencing was done on a HiSeq 2500 instrument, with a paired-end 150 cycles protocol. Demultiplexing was carried out in instrument. Data generated are publicly available from the SRA portal of NCBI under Accession Number PRJNA671810.

**Bioinformatic analyses**

Fragments were aligned to the human cDNA database (GRCh38) using Kallisto, with 100 permutations during pseudo-alignments and bias correction. Differential expression (DE) analysis of count data was conducted using negative binomial generalised linear models with the DESeq2 R package. Gene abundance differences with a corrected P-value (Padj) < 0.05 and a log2 –1 < fold change > +1 were considered differentially expressed. Plots were generated with R scripts.

**Statistical analysis**

The P-values shown in the graphs were determined by the non-parametric Mann–Whitney U-test. Statistical analysis was performed using the Prism software, and non-parametrical measures are expressed as median with interquartile range (MIR) and a P-value < 0.05 was considered to be statistically significant.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

Shima Shahbaz: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing-original draft. Juan Jovel: Software; Validation. Shokrollah Elahi: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Validation; Writing-review & editing.

**ETHICS STATEMENT**

The institutional review boards at the University of Alberta approved our study with the ethics # Pro00070528 and Pro000064046. All study participants gave written informed consent to participate in the study.
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Supporting Information

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