Increased glucose metabolic activity is associated with CD4+ T cell activation and depletion during chronic HIV infection

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Abstract

Objectives: Glucose metabolism plays a fundamental role in supporting the growth, proliferation and effector functions of T cells. We investigated the impact of HIV infection on key processes that regulate glucose uptake and metabolism in primary CD4+ and CD8+ T cells.

Design and methods: 38 HIV-infected treatment naïve, 35 HIV+/cART, 7 HIV+ long-term non-progressors and 25 HIV- control subjects were studied. Basal markers of glycolysis (e.g., Glucose transporter-1 expression, glucose uptake, intracellular glucose-6-phosphate, and L-lactate) were measured in T cells. The cellular markers of immune activation, CD38 and HLA-DR were measured by flow cytometry.

Results: The surface expression of the glucose transporter 1 (Glut1) is upregulated in CD4+ T cells in HIV-infected subjects compared with uninfected controls. The percentage of circulating CD4+Glut1+ T cells was significantly increased in HIV-infected subjects and was not restored to normal levels following combination antiretroviral therapy (cART). Basal markers of glycolysis were significantly higher in CD4+Glut1+ T cells compared to CD4+Glut1- T cells. The proportion of CD4+Glut1+ T cells correlated positively with the expression of the cellular activation marker, HLA-DR, on total CD4+ T cells, but inversely with the absolute CD4+ T cell count irrespective of HIV treatment status.

Conclusion: Our data suggest that Glut1 is a potentially novel and functional marker of CD4+ T cell activation during HIV infection. In addition, Glut1 expression on CD4+ T cells may be exploited as a prognostic marker for CD4+ T cell loss during HIV disease progression.

Key words
Combination antiretroviral therapy; glucose-transporter-1; Glut1; HIV; inflammation; immune activation; lymphocytes; CD4 cells; metabolism; glucose
Introduction

Glucose is the major cellular fuel which supports T cell growth and survival [1]. Several immune functions with relevance to HIV infection depend on adequate glucose supply, including T cell activation [2], T cell-mediated antiviral responses, and other T cell effector functions [1, 3].

The pathogenesis of HIV disease in vivo is characterized by chronic immune activation, inflammation, and increased oxidative stress [4-6]. Even in the presence of effective cART, evidence of chronic immune activation may be observed and is associated with and predictive of incomplete CD4+ T cell recovery as well as increased morbidity and mortality [7-12]. Immune activation is characterized by high levels of T cell activation, measured by CD38 and HLA-DR expression on peripheral CD4+ and CD8+ T cells [13, 14].

Upon activation, the energy demands of T cells increase dramatically and they undergo a metabolic switch in glucose metabolism from oxidative phosphorylation to aerobic glycolysis so that growth, proliferation, and effector functions can be supported [15], (and as reviewed in references [16-19]). In peripheral tissues, glucose is transported into cells by glucose transporters (Gluts) that carry hexose sugars across the cell membrane. Gluts comprise a family of at least 13 members including the proton-myoinositol co-transporter, H+-coupled myoinositol co-transporter. Glucose transporter 1 (Glut1) is a Class 1 glucose transporter that has high affinity for glucose and is the primary glucose transporter on T cells [20, 21].

Few studies have evaluated the role of HIV infection on glucose metabolism in leukocytes and these have been conducted exclusively in vitro [22-24]. Given the sustained energy requirements of activated T cells (as reviewed in references [18] and [25]) we hypothesized that T cells would up-regulate Glut1 expression and increase glucose transport in the context of HIV infection. In the present study, we analyzed key steps of glucose metabolism in T cells from HIV-infected individuals (both treatment naive and cART-treated), including cell surface expression of Glut1 on lymphocyte subpopulations, glucose uptake, and glycolytic flux analysis. Thus far, our study represents the most comprehensive glucose metabolic analysis in T cells from HIV infected individuals. Identification of metabolic dysregulation of the immune system during HIV infection could uncover novel mechanisms and potential drug targets to reduce immune activation and to support CD4+ T cell recovery in some patients.
Methods

Study participants

The study population included untreated HIV-infected individuals (progressors and long-term non-progressors, LTNPs), HIV-infected subjects on cART, and HIV seronegative controls (see Table 1). Subjects were recruited from the community, the Infectious Diseases Unit at The Alfred Hospital in Melbourne Australia, and from the Clinical Research Core Repository at the University of Washington, Seattle, USA (UW). Informed consent was obtained from all participants and the study was approved by the ethics committee at the participating institutions. Fresh blood samples from subjects recruited in Melbourne (45, 51, and 100% of the total study population of HIV-infected/treatment naive, HIV+/cART, and HIV- subjects, respectively), were collected in EDTA, citrate, or heparin anticoagulant tubes and processed within 1h of venipuncture; cryopreserved peripheral blood mononuclear cells (PBMCs) were shipped from UW to Melbourne in liquid phase nitrogen. The main exclusion criteria included self-reported co-infection with hepatitis C virus (HCV), active malignancy, vaccination, physical trauma, or surgery within three weeks prior to participation. In some experiments a representative sub-population was analyzed in which there were no statistically significant differences between the sub-population and the whole group in terms of gender, age, CD4 T cell count, and viral load.

Peripheral blood mononuclear cell (PBMC) preparation

PBMCs were isolated by density gradient centrifugation (Lymphoprep, Axis Shield), as previously described [26], and cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) and 90% autologous plasma.

Immunophenotyping

Fresh PBMCs were prepared and stained on ice for 30 min as previously described [27], using the following pre-titrated antibodies: CD3-PE, CD4-PerCP, CD8-APC, CD27-APC, CD45-RA-PE, CD38-PE, and HLA-DR-FITC (BD Biosciences). Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software, version 8.8 (Tree Star Inc, USA). Cryopreserved PBMCs (>90% viability) were rested for 24h in supplemented RPMI-1640 medium [(10% human serum, penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen)] prior to staining.

Glucose transporter-1 (Glut1) detection

Extracellular Glut1 expression was quantified on freshly isolated or cryopreserved PBMCs by flow cytometry using Glut1-antibody [MAB1418 clone (R&D Systems)] conjugated with FITC or APC to
detect cell surface Glut1 [28-30]. A pilot analysis of Glut1 expression on T cells revealed that the cryopreservation and thawing process did not affect its expression on T cells. Intracellular Glut1 (Glut1c-term) was detected using an unconjugated monoclonal antibody against Glut1c-term (Abcam) and a goat anti-mouse FITC conjugated secondary antibody. Cells were surfaced stained as above and permeabilized using the Intra Stain kit (Dako). Cells were acquired on a FACSCalibur.

Gene expression analysis

Total CD4+ T Cells were isolated from cryopreserved PBMCs using magnetic bead-based negative selection (Stemcell Technologies). mRNA extraction and quantitative real-time PCR were performed as previously described [31], using the following primer sets,

β-actin:
Forward: 5 'AGGCATCCTCAAGCTGAAGT 13
Reverse: 5 'GGTACAGGGATAGCAGGC 14

Glut1:
Forward: 5 'TCTGGCATCAAGCTGTCTTC 15
Reverse: 5 'CGATACCGGAGCCAATGGT 16

and modified cycle conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of (95°C, 30 sec; 60°C, 1 min).

Plasma cytokine and biochemical analysis

Cytokines, 25-hydroxy Vitamin D, Insulin, high sensitivity C-reactive protein (CRP), D-dimer and biochemical analyses were conducted by Cardinal Bio-research Pty Ltd (Australia).

Glucose uptake assay

The fluorescently-labelled glucose analogue, 2-N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2 deoxyglucose (2-NBDG) (Invitrogen), was used to measure glucose uptake. Cryopreserved PBMCs were thawed and recovered for 24h at 37°C, 5% CO₂ in supplemented RPMI-1640 medium. Cells were then treated with 2-NBDG, washed twice with 1×PBS, stained for cell surface markers, resuspended in 1×PBS, and analyzed within 15 min on a FACSCalibur.

Glucose-6-phosphate (G-6-P) assay

Briefly, 5 × 10⁶ cryopreserved cells were rested in the supplemented RPMI-1640 medium with or without glucose (glucose-limiting media) for 4 h. The cells were then pelleted, washed once in ice-cold 1×PBS, and resuspended in 50 µl of ice-cold 1×PBS. Cells were sonicated for 30 min on “high
setting” (Soniciean PTY Ltd). Cell lysates were centrifuged at 10,000 rpm at 4°C for 5 min and G-6-P levels were determined in the supernatant using a G-6-P assay kit (Biovision).

L-lactate assay
Secreted L-lactate concentrations in cell-free culture supernatants were determined by using the Glycolysis Cell-Based Assay Kit (Cayman Chemical). For intracellular L-lactate determination, cryopreserved cells were allowed to recover for 24h in supplemented RPMI-1640 medium and suspensions were stained using the Glycolysis Cell-Based Assay Kit (Cayman Chemical). The cells were washed once in wash buffer (0.5% FCS/1×PBS), stained with cell surface markers, and resuspended in 1×PBS prior to analysis. The highly colored intracellular formazan was detected in the FL3 channel on a FACSCalibur.

Statistical analysis
The non-parametric Mann Whitney test was used for comparison of unpaired data and the Wilcoxon matched-pairs signed rank test was used to analyse paired data. Measures of central tendency are expressed as median and inter-quartile range (IQR 25th, 75th percentile), unless otherwise stated. Linear regression was applied to assess the relationship between different covariates. Markers with a significant value of <0.05 in univariate analyses were entered in a multivariate linear regression model and the final model was derived through a process of backward elimination. Spearman Rank test was used for correlation analyses. P-values <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism (version 6.0) or Stata (version 11).
Results

Subject clinical characteristics

Demographic and clinical characteristics of subjects are summarized in Table 1. A total of 105 participants including 38 HIV-infected treatment naïve (HIV+/naïve), 7 HIV-infected treatment naïve LTNP, 35 HIV-infected cART-experienced (HIV+/cART), and 25 HIV seronegative (HIV-) control subjects were recruited. LTNP were infected with HIV for >10 years, and were not on cART. The median CD4+ T cell count in the HIV+/naïve and HIV+/cART groups was 400 and 479 cells/µl, respectively (p=0.08). Plasma concentrations of TNF were significantly elevated in the HIV+/naïve (p=0.005) and HIV+/cART (p=0.02) groups relative to the HIV- group.

HIV infection is associated with an increased percentage of circulating CD4+ T cells expressing Glut1

Figure 1A- E illustrates the gating strategy used to evaluate Glut1 expression on T cells. The percentage of CD3+CD4+ T cells that expressed Glut1 (referred to as CD4+Glut1+ T cells) in HIV+/naïve subjects was significantly higher (median: 23.8%) than that found in HIV- controls (median: 5.2%; p<0.0001) and remained so after commencing cART (median: 11.7%; p=0.0002). The median percentage of CD4+Glut1+ T cells in LTNP was only 11.6% (Fig 1F, left panel). The mean fluorescent intensity (MFI) of Glut1 on CD4+ T cells from HIV+/naïve subjects (median: 13.3, range: 5.8-45.6) was also significantly higher than that found on CD4+ T cells from HIV- subjects (median: 11.1, range: 5.0-15.3; p=0.02, data not shown). In a subgroup of 17 HIV+/naïve individuals recruited at UW, Seattle, USA commencing cART and analyzed 2.1±1.3 years after initiation of therapy, the proportion of CD4+Glut1+ T cells decreased significantly from a median of 30.9 to 16.5% (p=0.002) (Fig. 1F, right panel). Over this time, their CD4+ T cell count increased from a median of 233 cells/µl (range: 11-488) to 433 cells/µl (range: 123-1090). Fig. 1G illustrates that Glut1 was expressed on virtually all CD8+ T, irrespective of HIV or treatment status; there were no significant differences in the levels of Glut1 expression on CD8+ T cells between the groups.

In a subset of representative samples (based on CD4 cell count), we observed increased intracellular Glut1 in CD4+ T cells from HIV+ subjects irrespective of treatment status (Fig. 1H-I). Further, the level of Glut1 mRNA was also significantly higher in CD4+ T cells from HIV+/naïve compared to HIV- subjects (p=0.03, Fig. S1A). The Glut1 mRNA correlated significantly with the percentage of CD4+Glut1+ T cells (p=0.0007, Fig. S1B).
These data suggest that transcription, synthesis, and cell membrane trafficking of Glut1 in CD4+ T cells from HIV-infected individuals are higher compared with cells from uninfected controls. Additional data on intracellular Glut1 and mRNA expression in a larger sample size will be required to confirm this. Noteworthy, there was a weak inverse relationship between the percentage of CD4+Glut1+ T cells and time on cART (r=-0.40, p=0.02, Fig. S1C). Therefore, at least in some subjects on cART, Glut1 expression on CD4+ T cells might be a function of duration of viral suppression and/or CD4+ T cell count.

The frequencies of Glut1+ T cells are higher in effector CD4+ T cell subpopulations

In a subset of 10 HIV- controls, 12 HIV+/naïve and 8 HIV+/cART subjects, we measured Glut1 expression on CD4+ effector, naïve, memory and effector-memory cells, as defined by their expression of CD45RA and CD27. Glut1 was expressed on a higher percentage of effector and effector-memory CD4+ T cells than of naïve and memory CD4+ T cells, irrespective of HIV or treatment status. The CD4+ naïve and memory subpopulations in HIV- subjects showed only a small fraction of CD4+Glut1+ T cells (median: 5.2%, 10.3%, respectively); in HIV+/naïve individuals, by contrast, these populations showed significantly increased Glut1 expression (median: 13.3%, p=0.02 and 25.5%; p=0.006, respectively). The expression of Glut1 on naïve and memory T cells remained significantly elevated (p=0.001 and p<0.0001, respectively) in HIV+/cART subjects (Fig. 1J). The fraction of Glut1+ cells was similar in each of the CD4+ subpopulations measured from the different subject groups (Fig.1K). The proportions of effector and effector-memory CD4+ T cells were higher in HIV+/ naïve subjects than in HIV- controls and there was a positive correlation between the percentage of circulating CD4+Glut1+ T cells and the frequency of these subpopulations (Fig. S2, A-C). Data were unavailable to determine the absolute number of CD4+Glut1+ T cells in HIV- subjects, but HIV+/ naïve subjects had higher absolute CD4+Glut1+ T cells than did HIV+/cART subjects (Fig. S2, D). In sum, increased percentages of circulating CD4+Glut1+ T cells during HIV infection might not only be attributed to increased fractional representation of effector and effector-memory CD4+ T cells but may also reflect an absolute increase in the number of these cells in blood.

The specificity of Glut1 detection

Given published concerns about the specificity of the R&D Glut1 antibody [32], we conducted two independent Glut1 overexpression experiments and confirmed increased cell surface reactivity of the R&D Glut1 antibody on HEK293T cells overexpressing Glut1 (Fig. S3A-B). Using a different commercially available antibody, we confirmed by Western blot that the cells were indeed overexpressing Glut1 (Fig. S3C). Interestingly, the R&D Glut1 antibody showed strong reactivity to
permeabilized NIH3T3 cells transfected with Glut1-expressing lentivirus (Fig. S3D-E). However, no
reactivity occurred using R&D Glut1 antibody on non-permeabilized NIH3T3 cells that were
overexpressing Glut1, presumably due to defects in Glut1 trafficking in these cell lines. In addition
we demonstrated significant cell surface reactivity of R&D Glut1 antibody on the highly metabolically
active and paraformaldehyde-fixed Jurkat cell and N2a cells (positive control for cell surface
Glut1[33]) (Fig. S4).

**CD4+Glut1+ T cells have high expression of activation and proliferation markers**

HIV infection is associated with immune activation [9, 11, 12], as is reflected in this study by elevated
plasma concentrations of TNF (Table 1) and by an increased frequency of peripheral blood
CD4+CD38+HLA-DR+ and CD8+CD38+HLA-DR+ cells in HIV+/naive and HIV+/cART subjects compared
with HIV- controls (Fig. 2A). In subgroup of 17 HIV+/naive individuals commencing cART and
analyzed 2.1±1.3 years after initiation of therapy, the proportion of CD4+ and CD8+ T cells co-
expressing CD38 and HLA-DR appeared to have more rapidly declined than the percentage of
CD4+Glut1+ T cells (Fig. 2B vs Fig 1F, right panel). Fig. 2C-E shows that markers of T cell activation
were significantly higher in the Glut1+ population than in the Glut1- population in all study groups.
Time course experiments showed that Glut1 expression occurred early during the activation of CD4+
T cells (Fig. S5A). In contrast to the other activation markers and as expected [34], there was a rapid
increase in percentage of CD4+Glut1+ T cells expressing CD69, followed by a time dependent
decrease in expression of CD69 on these cells (Fig. S5B-C).

**HIV infection increases glucose uptake and glycolytic activity in CD4+ T cells**

To associate Glut1 expression with glucose metabolic activity in CD4 cells, we selected samples that
were within 2 standard deviations of the mean value of Glut1+CD4+ T cell percentage from the
respective groups. CD4+ T cells from HIV+/naive subjects take up more glucose over time than do
CD4+ T cells from HIV- and HIV+/cART subjects (Fig. 3A). After 60 min of incubation, the MFI of
intracellular 2-NBDG was significantly higher in the CD4+ T cells from HIV+/naive subjects than in
cells from HIV- or HIV+/cART subjects (Fig. 3B), and this correlated significantly with Glut1 expression
on CD4+ T cells (r=0.70, p=0.005, n=24, data not shown). Notably, CD4+Glut1+ T cells from
HIV+/naive and HIV+/cART subjects took up more glucose than CD4+Glut1+ T cells from HIV-
subjects (Fig. 3C). Confirmatory activation experiments showed that the presumably blast cells that
expressed more Glut1, were also highly positive for 2-NBDG (Fig. 3D-E).
Intracellular retention of glucose occurs by phosphorylation of glucose to glucose-6-phosphate (G-6-P) and is catalysed by hexokinases. We therefore measured the intracellular concentrations of G-6-P in purified unstimulated CD4+ T cells. Jurkat cells were used as positive controls (Fig. 3F). The levels of intracellular G-6-P were significantly higher in CD4+ T cells from HIV+/naive subjects compared to HIV- (p=0.0009) and when compared to HIV+/cART (p=0.005) (Fig. 3G), consistent with an increased transport of glucose in these cells.

We extended the above observations to show that CD4+ T cells from HIV+/naive subjects secreted significantly more L-lactate into the culture medium than HIV- subjects (Fig. 3H). Using Jurkat cells and anti-CD3/CD28-stimulated PBMCs as positive controls (Fig. 3I-J), we confirmed by flow cytometry that the intracellular concentration of L-lactate was significantly higher in Glut1+ compared with Glut1- cells from HIV+/naive subjects (p=0.01) (Fig. 3K).

A high frequency of Glut1-expressing CD4+ T cells is associated with markers of HIV disease progression

A significant inverse correlation was found between the percentage of CD4+Glut1+ T cells and the percentage of CD4+ T cells (p<0.0001) and absolute CD4 count (p=0.0002) in peripheral blood of HIV+/naive subjects (Fig. 4A). This was also true when HIV+/cART subjects were analyzed separately (r=-0.53, p=0.001, n=35 for percentage CD4+ T cells and r=-0.50, p=0.004, n=27 for absolute CD4 count; data not shown). There were no significant correlations between plasma concentrations of glucose and insulin and the percentage of CD4+Glut1+ T cells, suggesting that peripheral glucose homeostasis is an unlikely factor influencing Glut1 expression on CD4+ T cells in this setting.

Multivariate analysis was conducted to determine which covariates were associated with the percentage of circulating CD4+Glut1+ T cells. Only the total percentage of CD4+ T cells and MFI of HLA-DR on CD4+ T cells were independently associated with the percentage of circulating CD4+Glut1+ T cells in the peripheral blood of subjects (Supplement Table 1). In HIV+/naive subjects, the percentage of CD4+Glut1+ T cells and known correlates of CD4+ T cell activation had a comparably inverse relationship with the percentage of CD4+ T cells (Fig. 4B), but the percentage of CD4+Glut1+ T cells in HIV+/cART subjects showed the strongest correlation with total CD4+ T cell percentage (Fig. 4C). On the other hand, the percentage of CD4+CD38+HLA-DR+ T cells had the strongest correlation with viral load in HIV+/naive subjects (Fig. 4D). The relationship between CD8+ T cell activation and total CD4+ T cell percentage were relatively weak in HIV+/naive and HIV+/cART
subjects (Fig. S6A-B). Conversely, there was a strong correlation between the levels of CD38 expression on CD8+ T cells and viral load in HIV+/naive subjects (Fig. S6C).

Multivariate analysis (described in Supplemental methods) was used to compare the strength of CD4+Glut1+ percentage with established predictors in predicting the absolute number, and percentage of CD4+ T cells. Compared with known variables of T cell activation, the percentage of CD4+Glut1+ T cells, and the MFI of CD38 on CD4+ T cells were the only independent predictors of CD4 cell count in HIV+/treatment naïve subjects. However, the percentage of CD4+Glut1+ T cells was the only predictor of CD4 T cell count in subjects on cART (Supplemental Table 2A-B). Likewise, CD4+Glut1+ T cell percent was the only independent predictor of absolute CD4+ T cell percentage in both therapy naïve and cART treated subjects (Supplemental Table 2C-D). In contrast, the percentage of CD8+CD38+HLA-DR+, the MFI of HLADR on CD4+ T cells, and the MFI of Glut1 on CD4+ T cells were independently associated with viral load in HIV+/naïve subjects.
Discussion:

We report here that HIV infection is associated with increased glucose metabolism in T cells. In HIV+/naïve subjects compared to uninfected controls, there is a substantial increase in the percentage of circulating CD4+ T cells that express the glucose transporter, Glut1, and this percentage remained elevated despite virologic suppression on cART. The percentage of CD4+Glut1+ T cells correlates inversely with the percent and absolute CD4+ T cell count, irrespective of treatment status. HIV+/naïve and HIV+/cART subjects have an increased proportion of Glut1-expressing naive and memory CD4+ T cells compared with HIV- controls. The expression of Glut1 on total CD4+ T cells reflects their activation status as demonstrated by significantly higher expression levels of both CD38 and HLA-DR in the Glut1+ versus Glut1- population in all patient study groups, supporting a critical role for Glut1 in activated T cells, and confirming and extending in vitro reports [32, 35, 36]. Multivariate analysis indicates that the percentage of circulating CD4+Glut1+ T cells is independently associated with both the percentage and the levels of activation of CD4+ T cells. In HIV+/cART subjects, the percentage of CD4+Glut1+ cells has a broader dynamic range and correlates more strongly with CD4+ T cell loss than the percentage of CD4+ or CD8+ T cells co-expressing CD38 and HLA-DR. Finally, CD4+Glut1+ T cells take up more glucose and have higher glycolytic activity than do CD4+Glut1- T cells, a metabolic phenomenon characteristic of other viral responses in different cell types [37]. In contrast to CD4+ T cells, there were no significant changes in the cell surface expression Glut1 and glucose uptake by CD8+ T cells in HIV infected subjects.

Conflicting reports have been published concerning the specificity of the R&D antibody that was used in our experiments to detect Glut1 [32, 38]. In one case, that antibody failed to detect endogenous Glut1 in cells, including Jurkat cells known to express abundant levels of Glut1 [32]. However, we observed strong immune reactivity of the R&D Glut1 antibody on paraformaldehyde-fixed Jurkat cells and on unfixed N2a cells, suggesting that in some situations the Glut1 epitope may be masked by post-transcriptional modifications [32, 39]. We also observed that, under the conditions of our experiments, this antibody detected a dramatic increase in Glut1 levels following T cell activation that was highly correlated with increased glucose uptake. Although we cannot fully explain the discrepancy between our observations and those reported in ref. 32, we speculate that they may be related to different protocols to achieve T cell activation (e.g., 24h versus 2 to 4 days) and/or to subtle differences in staining protocols. Interestingly, the R&D Glut1 antibody detected intracellular but not cell surface Glut1 in NIH3T3 cells that were over-expressing Glut1, an observation that is not consistent with the suggestion that the antibody interacts with a different cell surface protein that is associated with Glut-1 overexpression in transformed cells [32]. More
recently, this antibody has been shown by others to be specific for Glut1 [40] and has been used to evaluate Glut1 expression on cell surfaces [28, 29] including T cells in a cohort of HIV-infected individuals [30]. We have also clearly shown increased intracellular Glut1 (using a Glut-1cterm antibody), increased Glut1 mRNA and increased glucose uptake in CD4+ T cells in HIV+/naïve subjects, all of which is consistent with increased glucose metabolic activity.

Recent metabolomics analyses of HIV-infected primary CD4+ T cells in vitro have shown a profound increase in intracellular levels of key glycolytic metabolites with a concomitant increase in glucose uptake when compared with HIV uninfected cells in the same culture [23], suggesting that direct HIV infection of CD4+Glut1+ T cells may contribute at least in part to the increased glycolytic activity in CD4+Glut1+ T cells in some HIV+ subjects. Remarkably, in HIV+ subjects, we observed a paradoxical increase in the frequency of Glut1+ T cells in the naïve and memory subpopulations. This could potentially allow these “resting” cells to be more permissive for productive infection, as shown directly by experiments demonstrating that IL-7-induced Glut1 expression enabled HIV infection in naïve CD4+ T cells in the absence of activating stimuli [24]. The origin of the heightened glucose metabolism in CD4+ T cells in HIV+ subjects is unknown but may be a result of elevated cytokines such as IFNγ, IL-2 and IL-7 [19, 41], and/or persistent inflammatory signals such as translocated microbial products [42]. However, direct HIV infection of CD4+ T cells may be an additional contributor of increased glucose metabolic activity, especially in untreated subjects, supporting the observation of increased glucose metabolic activity by HIV in vitro [23].

It has been suggested by several groups that Glut1 is a T cell activation marker based on its increased expression on T cells activated in vitro [32, 35, 36]. However, none of these investigators evaluated the expression of established activation markers on these cells nor did they examine co-expression of activation markers with Glut1. Our data suggest that Glut1 is a potential marker of CD4+ T activation in the context of HIV infection, although it might be expressed in a small proportion of cells independently of the activation markers evaluated here. Interestingly, HIV/cART subjects with low CD4+ T cell count have elevated percentages of CD4+Glut1+ T cells even when their CD38 and HLA-DR levels on CD4+ T cells returned to almost normal. It is possible that CD4+ T cells may lose CD38 and HLA-DR with the suppression of HIV in cART treated subjects, but retain metabolic activation markers like Glut1. This may be interpreted as a homeostatic response to drive the increase of CD4+ T cell. Compared with the activation markers CD38 and HLA-DR, Glut1 is unique because it is upregulated on CD4 but not CD8+ T cells in HIV+ subjects. It will also be interesting to
determine whether subpopulations of CD4+Glut1+ T cells preferentially contain HIV viral DNA, especially in those cells that lack the expression of the traditional activation markers.

What are the biological consequences of increased glucose metabolic activity in CD4+ T cells in HIV+ subjects? Glut1-mediated glucose metabolic pathways are proposed as critical regulators of HIV infection in human primary CD4+ T cells and T cell lines in cell culture [22, 24]. In recent reviews, hyper-activation of aerobic glycolysis in CD4+ T cells during HIV infection has been hypothesized to foster the apoptosis and destruction of such cells [19, 43]. Indeed, a high rate of glycolysis in cells increases the concentrations of metabolites such as L-lactate which induce acidosis and can trigger apoptosis, either through the p53 pathway or by acid-induced collapse of the transmembrane H(+) gradient [44, 45]. In addition, Glut1 is recognized as a key transporter for vitamin C [46], and under oxidative stress, it can be oxidized to ascorbate free radical which may also contribute to cell death [47].

In conclusion, our study identifies the glucose metabolic machinery as component of HIV-associated T cell activation and provides a rationale for exploratory approaches for therapeutic interventions. We also identified Glut1 as a potentially novel marker of CD4+ T cell activation of HIV disease progression. One limitation of this study is the small sample size and cross sectional analysis design. Longitudinal analysis using a larger sample size will shed more light on the role of glucose metabolism in HIV disease progression. Another limitation of the study is that we had access to cells from only a limited number of subjects within each group for several experiments, raising the possibility that some interpretations may be affected by subject selection bias. In addition, in vitro studies to assess the effects of targeted pharmacological and genetic inhibition of glycolysis in CD4+ T cells may help to clarify a mechanism and direct link between glucose metabolism and CD4+ T cell activation. The maturation of different functional subsets of T cells such as Tregs, Th1, Th2 and Th17 are dependent on distinct metabolic programming [48]. It will be of interest to evaluate how changes in glucose metabolic activity affect the functions of these cells during HIV infection and the course of HIV disease progression.
Figure legends

Figure 1. Glut1 expression is increased on CD4+ T cells from HIV+/naïve subjects and is not restored to baseline by cART. Within 1h of collection, samples of whole blood were analyzed by flow cytometry for Glut1 expression on CD4+ and CD8+ T cells. (A) Lymphocytes (circled) were defined using side scatter (SSC) and forward scatter (FSC) characteristics. (B-C) Gating strategy showing CD3+ T cells within the lymphocyte-gated population which were then further defined based on CD4 and CD8 surface expression. (D-E) Representative flow cytometric dot plot of Glut1 expression on CD4+ T and CD8+ T cells in peripheral blood from HIV+/naïve subjects. (F) Percentage of CD4+Glut1+ T cells in peripheral blood from HIV-, HIV+/naïve, HIV+/cART and LTNP subjects (left panel), and percentage of CD4+ T cells in peripheral blood of HIV+/naïve subjects before and during cART that express Glut1 (right panel). (G) Percentage of CD8+ T cells that are Glut1+ (left panel) and MFI of Glut1 on CD8+ T cells (right panel). (H) Percentage of CD4+ T cells that are Glut1c-term+ and (I) MFI of Glut1c-term on CD4+ T cells. (J) Median percentage of each CD4+ T cell subpopulation that is Glut1+. (K) Median percentage of CD4+Glut1+ T cells expressing markers of functional subpopulations. Subpopulations were defined by their expression of CD45RA and CD27 to identify effector (E, CD45RA+CD27-), naïve (N, CD45RA+CD27+), memory (M, CD45RA-CD27+) and effector-memory (EM, CD45RA-CD27-) cells. The non-parametric Mann-Whitney T test was used to evaluate significant differences between the median values of each group. Wilcoxon matched-pairs sign rank T test was used to analyse changes between paired data sets. Horizontal lines within histograms represent median value. Whiskers represent minimum and maximum values.

Figure 2. Evaluation of T cell activation and expression of activation markers on CD4+Glut1+ T cells. (A) The percentage of CD4+ (left panel) and CD8+ (right panel) T cells that co-express CD38 and HLA-DR was measured in whole blood from HIV-, HIV+/naïve, and HIV+/cART subjects using flow cytometry. (B) The percentage of CD4+ (left panel) and CD8+ (right panel) T cells that co-express CD38 and HLA-DR subjects before and during cART. (C) Flow cytometric dot plot showing the expression of CD38 and HLA-DR on CD4+Glut1- and CD4+Glut1+ T cells from a representative HIV+/naïve subject. (D) The percentage of CD4+Glut1+ and CD4+Glut1- cells that co-express CD38 and HLA-DR was measured by flow cytometry on whole blood. (E) MFI of HLA-DR (left panel), and CD38 (right panel) expressed on CD4+Glut1+ and CD4+Glut1- T cells from the subjects analyzed in (D). White histograms: HIV- (n=14); red histograms: HIV+/naïve (n=20); and blue histograms: HIV+/cART subjects (n=11). The non-parametric Mann-Whitney T test was used to evaluate significant differences between the median values of each group while significant differences between Glut1+ and Glut1- subsets were evaluated using the Wilcoxon matched-pairs sign rank T
test. Horizontal lines within histograms represent median value. Whiskers represent minimum and maximum values.

**Figure 3. Effects of HIV status on glucose uptake and glycolysis in CD4+ T cells.** (A) The kinetics of glucose uptake were compared using three representative HIV-, HIV+/naïve, and HIV+/cART subjects who had low (3.6 ± 1.6, black symbols), high (55.8 ± 17.1, red symbols), and moderate (15.6 ± 2.2, blue symbols) percentages of CD4+Glut1+ T cells, respectively. PBMCs were treated with 15 µM 2-NBDG for the indicated times (n=3 per group). Cells were washed and internalised 2-NBDG was detected by flow cytometry, as described in Methods. (B) Uptake of 2-NBDG in CD4+ T cells present in PBMCs incubated for 60 min with 15 µM 2-NBDG (n=8 per group). (C) Uptake of 2-NBDG by CD4+ T cells in the same donors analyzed in (B), stratified for Glut1 expression. (D) Dot plot of cells gated within the CD4+ T population to identify Glut1-expressing CD4+ T cells present in unstimulated (left panel) or anti-CD3/CD28 stimulated (right panel) PBMCs from a representative HIV- subject. (E) CD4+ blast cells (red) and CD4+ cells (black) present within PBMCs stimulated with anti-CD3/CD28 beads and incubated for 60 min with 2-NBDG were overlayed onto the SSC versus 2-NBDG dot plot. Data are from PBMCs from the same representative HIV-uninfected donor sample used in D. (F) Concentrations of intracellular glucose-6-phosphate (G-6-P) in Jurkat cells cultured in glucose-containing (11 mM) and glucose-deprived (0 mM) RPMI-1640 medium for 4h. (G) Basal concentrations of intracellular G-6-P in purified CD4+ T cells incubated in glucose containing RPMI-1640 for 4h. (H) Basal secretion of L-lactate into culture medium by 1 × 10^6 purified viable CD4+ T cells incubated for 24h in glucose (11 mM) containing RPMI-1640. (I) Representative histogram showing intracellular L-lactate levels determined by flow cytometry in Jurkat cells cultured for 24h in the absence (blue) or presence (brown) of 11 mM glucose. (J) Representative histogram showing intracellular L-lactate levels in CD4+Glut1+ (red) and CD4+Glut1- (black) cells in PBMCs from a representative HIV- control subject and stimulated for 24h with anti-CD3/CD28 microbeads. (K) Intracellular L-lactate staining in Glut1- (black) and Glut1+ (red) cells present in CD4+ T cells purified from a representative HIV+/naïve subject. Horizontal lines within histograms represent median value and whiskers represent minimum and maximum values. The non-parametric Mann-Whitney T test was used to evaluate significant differences between the median values of each group while significant differences between Glut1+ and Glut1- subsets were evaluated using the Wilcoxon matched-pairs sign rank T test.

**Figure 4. Relationship between percentage CD4+Glut1+ T cells and total CD4+ T cell percentage, CD4+ T cell count and HIV viral load in HIV-infected subjects.** (A) Spearman’s correlations between
the percentage (left panel) and absolute number (right panel) of CD3+CD4+ T cells and the percentage of CD4+Glut1+ T cells in the peripheral blood of HIV+/naïve and HIV+/cART subjects. (B and C) Comparative relationship between the percentage of CD4+Glut1+ T cells and markers of CD4+ T cell activation, and the percentage of CD3+CD4+ T cells in (B) HIV+/ naïve and (C) HIV+/cART subjects. (D) Comparative relationship between the percentage of CD4+Glut1+ T cells and markers of CD4+ T cell activation, and HIV viral load in HIV+/naïve subjects.

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References


Figure 3

A. MFI of 2-NBDG on CD4+ T cells over time (min)

B. MFI of 2-NBDG in CD4+ T cells

C. MFI of 2-NBDG

D. Unstimulated PBMCs and Anti-CD3/CD28 stimulated PBMCs

E. 2-NBDG uptake

F. Glucose-6-phosphate (μM/250,000 cells)

G. Glucose-6-phosphate (μM/10^6 viable cells)

H. L-lactate (μM) secreted

I. Unstained and stained Jurkat cells

J. Count for anti-CD3/CD28 stimulated PBMCs

K. L-lactate (μM)
Figure S1

A

Relative Glut1 mRNA levels

HIV-  HIV+/naive  HIV+/cART

p=0.5  p=0.03  p=0.34

B

r=0.73, p=0.0007, n=16

% CD4+Glut1 T cells

Glut1 mRNA

C

r=-0.40, p=0.02, n=32

% CD4+Glut1 T cells

Years on cART
Figure S2

A

Percentage of CD4+ T cell subpopulation

p=0.05

HIV- HIV+/naive

E N M EM

B

Percentage of CD4+ T cell subsets

N (r=-0.3, p=0.21)

M (r=-0.5, p=0.02)

%CD4+Glut1+

C

Percentage of CD4+ T cell subsets

E (r=0.78, p<0.0001)

EM (r=0.58, p=0.0068)

%CD4+Glut1+

D

Absolute CD4+Glut1+ T cell count

p=0.03

HIV+/naive HIV+/cART

n= 30 26
(A) % of Max vs. FITC for Isotype and Glut1 Ab

(B) % of Max vs. FITC for Isotype and Glut1 Ab

(C) Western blot showing Glut1 at 40-55 KDa and β-actin

(D) Flow cytometry scatter plots for Vector-only and Glut1 Over-expression

(E) Flow cytometry histogram for Glut1 over-expression compared to Vector-only control
Figure S6

A. HIV+/naïve subjects

- MFI 38 on CD8+ T cells (n=39, r=-0.40, p=0.01)
- MFI HLA-DR on CD8+ T cells (n=38, r=-0.33, p=0.04)
- % CD4+Glut1+ T cells (n=45, r=-0.71, p<0.0001)
- % CD8+CD38+HLA-DR+ (n=39, r=-0.46, p=0.002)

B. HIV+/cART subjects

- MFI 38 on CD8+ T cells (n=28, r=0.05, p=0.80)
- MFI HLA-DR on CD8+ T cells (n=28, r=-0.15, p=0.42)
- % CD4+Glut1+ T cells (n=35, r=-0.53, p=0.001)
- % CD8+CD38+HLA-DR+ (n=28, r=-0.07, p=0.70)

C. MFI CD38 on CD8+ T cells (n=34, r=0.55, p=0.0007)
- MFI HLA-DR on CD8+ T cells (n=34, r=0.21, p=0.22)
- % CD4+Glut1+ T cells (n=37, r=0.46, p=0.004)
- % CD8+CD38+HLA-DR+ (n=34, r=0.53, p=0.001)