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

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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: Thirty-eight HIV-infected treatment-naive, 35 HIV+/combination antiretroviral therapy, seven HIV+ long-term nonprogressors and 25 HIV control individuals were studied. Basal markers of glycolysis [e.g. glucose transporter-1 (Glut1) 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 Glut1 is up-regulated in CD4$^+$ T cells in HIV-infected patients compared with uninfected controls. The percentage of circulating CD4$^+$Glut1$^+$ T cells was significantly increased in HIV-infected patients and was not restored to normal levels following combination antiretroviral therapy. 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.

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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 combination antiretroviral therapy (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 human leukocyte antigen D-related (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 nonprogressors (LTNPs)], HIV-infected patients on cART, and HIV seronegative controls (see Table 1). Patients 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. Informed consent was obtained from all participants and the study was approved by the ethics committee at the participating institutions. Fresh blood samples from individuals recruited in Melbourne (45, 51, and 100% of the total study population of HIV-infected/treatment-naive, HIV+/cART, and HIV-negative individuals, respectively) were collected in EDTA, citrate, or heparin anticoagulant tubes and processed within 1 h of venipuncture; cryopreserved peripheral blood mononuclear cells (PBMCs) were shipped from University of Washington 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 3 weeks prior to participation. In some experiments, a representative subpopulation was analyzed in which there were no statistically significant differences between the subpopulation and the whole group in terms of sex, age, $CD4^+$ T-cell count, and viral load.

Peripheral blood mononuclear cell preparation

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Axis Shield, Dundee, Scotland), as previously described [26], and cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma–Aldrich, St Louis, Missouri, USA) and 90% autologous plasma.

Immunophenotyping

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

Glucose transporter-1 detection

Extracellular Glut1 expression was quantified on freshly isolated or cryopreserved PBMCs by flow cytometry using Glut1 antibody [MAB1418 clone (R&D Systems, Minneapolis, Minnesota, USA)] 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 ($Glut1_{c\text{-}term}$)

Table 1. Clinical characteristics of study groups.

Variables	*n*	Groups				*P* value		
		HIV− (A)	HIV+/naive (B)	HIV+/cART (C)	LTNP (D)[a]	A vs. B	B vs. C	A vs. C
Sex (M/F)	105	24/1	37/1	35/0	7	–	–	–
BMI (kg/m^2)	82	22.4 (22–25)	24.7 (22–27)	24.5 (21–27)	21.5 (16–29)	0.48	0.42	0.57
Age, years, mean (SD)	93	45.2 ± 20.5	42.3 ± 10.7	44.6 ± 11.5	54.0 ± 7.0	0.83	0.46	0.82
MSM (self-reported)	67	11/25	22/38	28/35	6/7	–	–	–
CD4$^+$ T-cell count (cells/μl)	63	–	400.0 (223–483)	479.0 (245–710)	493.0 (372–594)	–	0.08	–
Nadir CD4$^+$ T-cell count (cells/μl)	30	–	–	388 (38–500)	–	–	–	–
Time on cART, years, mean (SD)	30	–	–	6.1 (6.2)	–	–	–	–
% CD3$^+$CD4$^+$ T cells	105	54.5 (35–45)	27.5 (14–36)	39.2 (27–50)	35.0 (35–45)	<0.0001	0.0008	0.0001
CD4$^+$/CD8$^+$ ratio	94	1.7 (1–2)	0.5 (0.2–0.7)	0.8 (0.5–1.2)	0.6 (0.6–1)	<0.0001	0.001	<0.0001
Viral load (copies/ml)	72	–	86 000 (39 500–128 000)	<50 2385	(203–10963)	–	–	–
Plasma glucose (mmol/l)	80	4.7 (4.5–5.2)	4.7 (4.5–5.1)	4.9 (4.5–5.7)	5.8 (5.1–7.8)	0.64	0.32	0.26
Plasma insulin (μIU/ml)	48	5.9 (3.0–14.7)	4.4 (3.1–5.9)	3.7 (2.2–27.5)	–	0.50	>0.10	0.87
Plasma triglyceride (mmol/l)	64	0.9 (0.6–1.3)	1.0 (0.8–1.6)	1.4 (0.9–2.1)	0.7 (0.7–1.5)	0.30	0.22	0.03
Plasma HDLCH (mmol/l)	62	1.1 (0.8–1.4)	0.7 (0.6–1.0)	0.8 (0.7–1.0)	1.0 (0.8–1.1)	0.003	0.54	0.005
Plasma cholesterol (mmol/l)	60	4.2 (3.2–5.6)	4.0 (3.0–4.7)	3.3 (3.2–4.4)	4.2 (3.5–4.6)	0.40	0.34	0.06
Creatinine (μmol/l)	79	74.0 (67.0–84.0)	76.0 (66.0–88.4)	74.5 (69.0–80.2)	88.4 (79.6–97.2)	0.64	0.60	0.90
hsCRP (mg/l)	47	0.8 (0.5–1.4)	1.3 (0.7–4.4)	0.90 (0.5–4.7)	–	0.07	0.10	0.40
D-dimer (μg/ml)	48	0.8 (0.5–1.0)	0.9 (0.7–1.6)	0.77 (0.4–1.1)	–	0.06	0.16	0.38
Plasma vitamin D (nmol/l)	48	46.5 (34.0–99.0)	74.0 (41.3–88.5)	78.9 (36.8–99.7)	–	0.24	0.40	0.30
TNF (pg/ml)	48	6.0 (3.6–7.5)	8.7 (6.9–14.3)	8.2 (5.5–12.9)	–	0.005	0.28	0.02

cART, combination antiretroviral therapy; hsCRP, high-sensitivity C-reactive protein; LTNP, long-term nonprogressor; TNF, tumor necrosis factor. LTNP samples were obtained from the Clinical Research Core Repository at the University of Washington, Seattle, USA (UW). Continuous variables are expressed in median (interquartile range), unless otherwise indicated. Categorical variables are expressed in mean (±SD). The nonparametric Mann–Whitney test was used to evaluate significant difference between each group.

[a]Selected analysis of group D; BMI, $P = 0.40$ D vs. C; age, $P = 0.009$ D vs. B, $P = 0.03$ D vs. C; $P = 0.24$ D vs. A; CD4$^+$ T-cell count, $P = 0.30$ D vs. B; % CD3$^+$CD4$^+$ T cells, $P = 0.03$ D vs. B, $P = 0.81$ D vs. C; CD4$^+$/CD8$^+$ ratio, $P = 0.003$ D vs. A, $P = 0.08$ D vs. B, $P = 0.64$ D vs. C; viral load, $P = 0.0003$ D vs. B; plasma triglyceride, $P = 0.08$ D vs. C.

was detected using an unconjugated monoclonal antibody against $Glut1_{c\text{-}term}$ (Abcam) and a goat antimouse FITC-conjugated secondary antibody. Cells were surfaced stained as above and permeabilized using the Intra Stain kit (Dako, Campbellfield, Victoria, Australia). 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, Vancouver, British Columbia, Canada). mRNA extraction and quantitative real-time PCR were performed as previously described [31], using the following primer sets: β-actin – forward: 5′-3′: AGGCATCCTCACCCTGAAGT, reverse: 5′-3′: GCG TACAGGGATAGCACAGC; Glut1 – forward: 5′-3′: TCTGGCATCAACGCTGTCTTC, reverse: 5′-3′: CG ATACCGGAGCCAATGGT; and modified cycle conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of (95°C, 30 s; 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 Bioresearch Pty Ltd (Australia).

Glucose uptake assay

The fluorescently-labeled glucose analog, 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 24 h at 37°C, 5% CO_2 in supplemented RPMI-1640 medium. Cells were then treated with 2-NBDG, washed twice with 1× phosphate-buffered saline (PBS), stained for cell surface markers, resuspended in 1× PBS, and analyzed within 15 min on a FACSCalibur.

Glucose-6-phosphate assay

Briefly, 5×10^6 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, Thebarton, South Australia, Australia). Cell lysates were centrifuged at 10 000 r.p.m. at 4°C for 5 min and glucose-6-phosphate (G-6-P) levels were determined in the supernatant using a G-6-P assay kit (Biovision, San Francisco, California, USA).

L-lactate assay

Secreted L-lactate concentrations in cell-free culture supernatants were determined by using the Glycolysis Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, Michigan, USA). For intracellular L-lactate determination, cryopreserved cells were allowed to recover for 24 h 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 nonparametric Mann–Whitney test was used for comparison of unpaired data and the Wilcoxon matched-pairs signed-rank test was used to analyze paired data. Measures of central tendency are expressed as median and interquartile 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 less than 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 less than 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism (version 6.0; GraphPad Software, La Jolla, California, USA) or Stata (version 11; Stata Corp., College Station, Texas, USA).

Results

Participant clinical characteristics

Demographic and clinical characteristics of participants are summarized in Table 1. A total of 105 participants including 38 HIV-infected treatment-naive (HIV+/naive), seven HIV-infected treatment-naive LTNP, 35 HIV-infected cART-experienced (HIV+/cART), and 25 HIV seronegative (HIV−) control individuals were recruited. LTNPs were infected with HIV for more than 10 years, and were not on cART. The median $CD4^+$ T-cell counts in the HIV+/naive and HIV+/cART groups were 400 and 479 cells/μl, respectively ($P = 0.08$). Plasma concentrations of tumor necrosis factor (TNF) were significantly elevated in the HIV+/naive ($P = 0.005$) 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 glucose transporter-1

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+/naive participants 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 LTNPs was only 11.6% (Fig. 1f, left panel). The

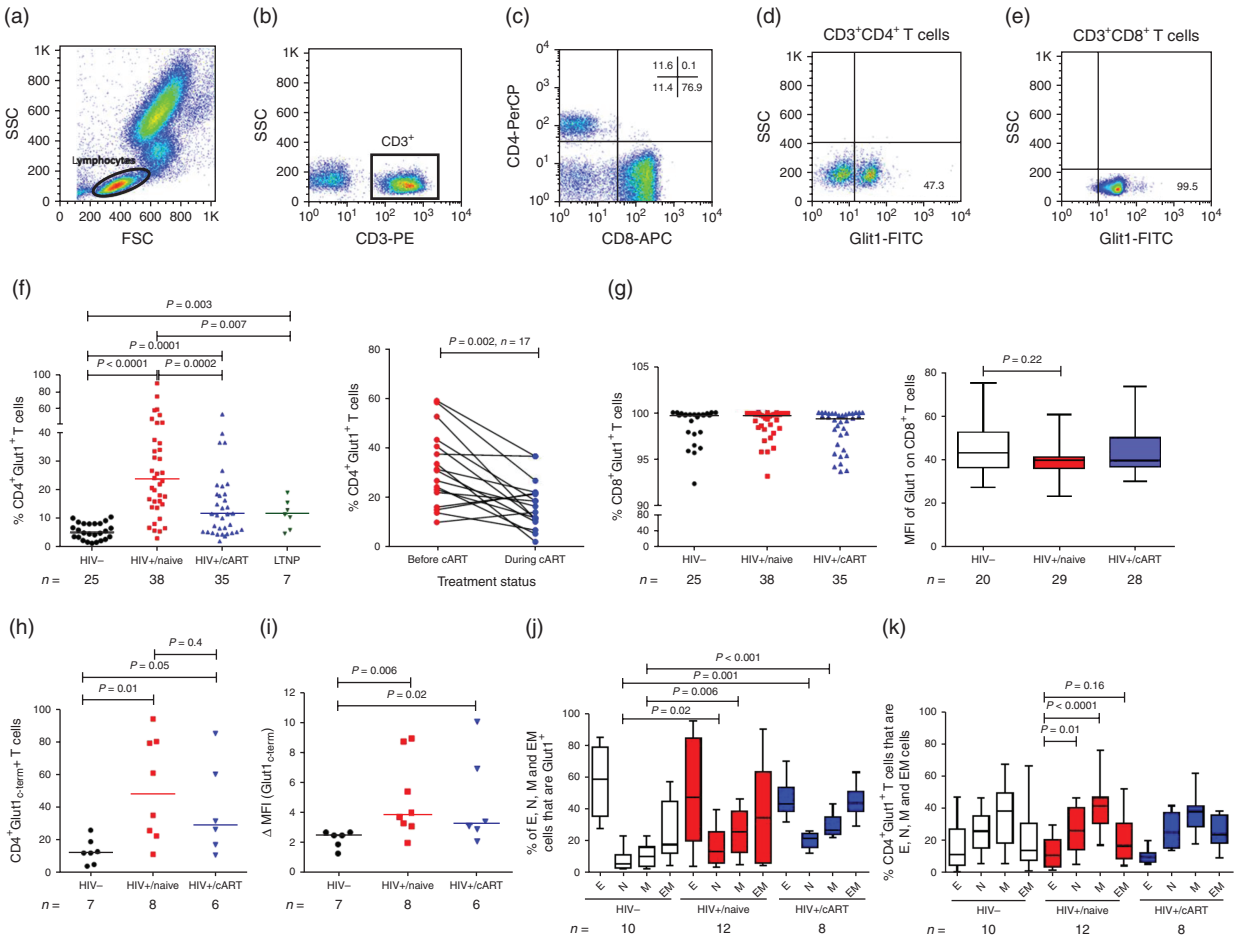

Fig. 1. Glut1 expression is increased on CD4$^+$ T cells from HIV+/naive patients and is not restored to baseline by cART. Within 1 h of collection, samples of whole blood were analyzed by flow cytometry for Glut1 expression on CD4$^+$ and CD8$^+$ T cells. (a) Lymphocytes (circled) were defined using 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+/naive patients. (f) Percentage of CD4$^+$Glut1$^+$ T cells in peripheral blood from HIV−, HIV+/naive, HIV+/cART, and LTNP patients (left panel), and percentage of CD4$^+$ T cells in peripheral blood of HIV+/naive patients 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 Glut1$_{c\text{-term}+}$ and (i) MFI of Glut1$_{c\text{-term}}$ 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$^-$), naive (N, CD45RA$^+$CD27$^+$), memory (M, CD45RA$^-$CD27$^+$), and effector-memory (EM, CD45RA$^-$CD27$^-$) cells. The nonparametric 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 analyze changes between paired data sets. Horizontal lines within histograms represent median value. Whiskers represent minimum and maximum values. cART, combination antiretroviral therapy; Glut1, glucose transporter-1; LTNP, long-term nonprogressor.

mean fluorescent intensity (MFI) of Glut1 on CD4$^+$ T cells from HIV+/naive individuals (median 13.3, range 5.8–45.6) was also significantly higher than that found on CD4$^+$ T cells from HIV− individuals (median 11.1, range 5.0–15.3; $P = 0.02$, data not shown). In a subgroup of 17 HIV+/naive individuals recruited at University of Washington (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). Figure 1g illustrates that Glut1 was expressed on virtually all CD8$^+$ T cells, 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+ patients irrespective of treatment status (Fig. 1h,i). Further, the level of Glut1 mRNA was also significantly higher in $CD4^+$ T cells from HIV+/naive compared to HIV− individuals ($P=0.03$; Fig. S1a, http://links.lww.com/QAD/A445). The Glut1 mRNA correlated significantly with the percentage of $CD4^+Glut1^+$ T cells ($P=0.0007$; Fig. S1b, http://links.lww.com/QAD/A445).

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, http://links.lww.com/QAD/A445). Therefore, at least in some patients 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 glucose transporter-1+ T cells are higher in effector $CD4^+$ T-cell subpopulations

In a subset of 10 HIV− controls, 12 HIV+/naive and 8 HIV+/cART patients, we measured Glut1 expression on $CD4^+$ effector, naive, 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 naive and memory $CD4^+$ T cells, irrespective of HIV or treatment status. The $CD4^+$ naive and memory subpopulations in HIV− individuals showed only a small fraction of $CD4^+Glut1^+$ T cells (median 5.2 and 10.3%, respectively); in HIV+/naive 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 naive and memory T cells remained significantly elevated ($P=0.001$ and $P<0.0001$, respectively) in HIV+/cART patients (Fig. 1j). The fraction of $Glut1^+$ cells was similar in each of the $CD4^+$ subpopulations measured from the different patient groups (Fig. 1k). The proportions of effector and effector-memory $CD4^+$ T cells were higher in HIV+/naive patients 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. S2a–c, http://links.lww.com/QAD/A445). Data were unavailable to determine the absolute number of $CD4^+Glut1^+$ T cells in HIV− individuals, but HIV+/naive patients had higher absolute $CD4^+Glut1^+$ T cells than did HIV+/cART patients (Fig. S2d, http://links.lww.com/QAD/A445). 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 glucose transporter-1 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, http://links.lww.com/QAD/A445). Using a different commercially available antibody, we confirmed by western blot that the cells were indeed overexpressing Glut1 (Fig. S3c, http://links.lww.com/QAD/A445). Interestingly, the R&D Glut1 antibody showed strong reactivity to permeabilized NIH3T3 cells transfected with Glut1-expressing lentivirus (Fig. S3d,e, http://links.lww.com/QAD/A445). However, no reactivity occurred using R&D Glut1 antibody on nonpermeabilized 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, http://links.lww.com/QAD/A445).

$CD4^+Glut1^+$ 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 $CD4^+CD38^+HLA\text{-}DR^+$ and $CD8^+CD38^+HLA\text{-}DR^+$ cells in HIV+/naive and HIV+/cART patients 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). Figure 2(c–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, http://links.lww.com/QAD/A445). 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, http://links.lww.com/QAD/A445).

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 SDs of the mean value of $Glut1^+CD4^+$ T-cell

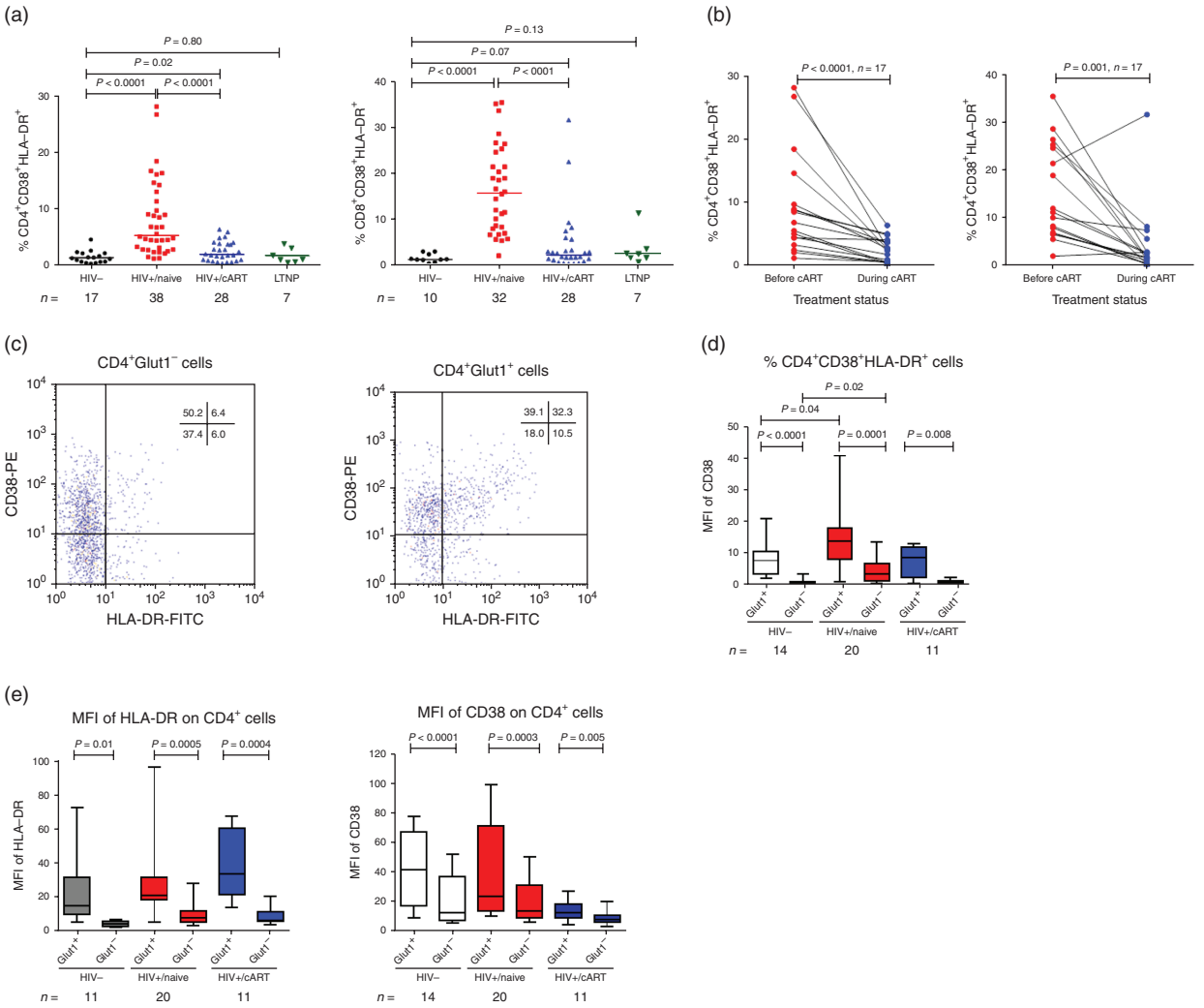

Fig. 2. Evaluation of T-cell activation and expression of activation markers on CD4$^+$Glut1$^+$ T cells. (a) The percentage of CD4$^+$ (left panel) and CD8$^+$ (right panel) T cells that co-express CD38 and HLA-DR was measured in whole blood from HIV−, HIV+/naive, and HIV+/cART patients using flow cytometry. (b) The percentage of CD4$^+$ (left panel) and CD8$^+$ (right panel) T cells that co-express CD38 and HLA-DR patients 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+/naive patient. (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 patients analyzed in (d). White histograms: HIV− ($n=14$); red histograms: HIV+/naive ($n=20$); and blue histograms: HIV+/cART patients ($n=11$). The nonparametric Mann–Whitney *t* test was used to evaluate significant differences between the median values of each group, whereas 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. cART, combination antiretroviral therapy; Glut1, glucose transporter-1; MFI, mean fluorescent intensity.

percentage from the respective groups. CD4$^+$ T cells from HIV+/naive individuals took up more glucose over time than do CD4$^+$ T cells from HIV− and HIV+/cART patients (Fig. 3a). After 60 min of incubation, the MFI of intracellular 2-NBDG was significantly higher in the CD4$^+$ T cells from HIV+/naive individuals than in cells from HIV− or HIV+/cART patients (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 patients took up more glucose than CD4$^+$Glut1$^+$ T cells from HIV− individuals (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 G-6-P and is catalyzed by hexokinases.

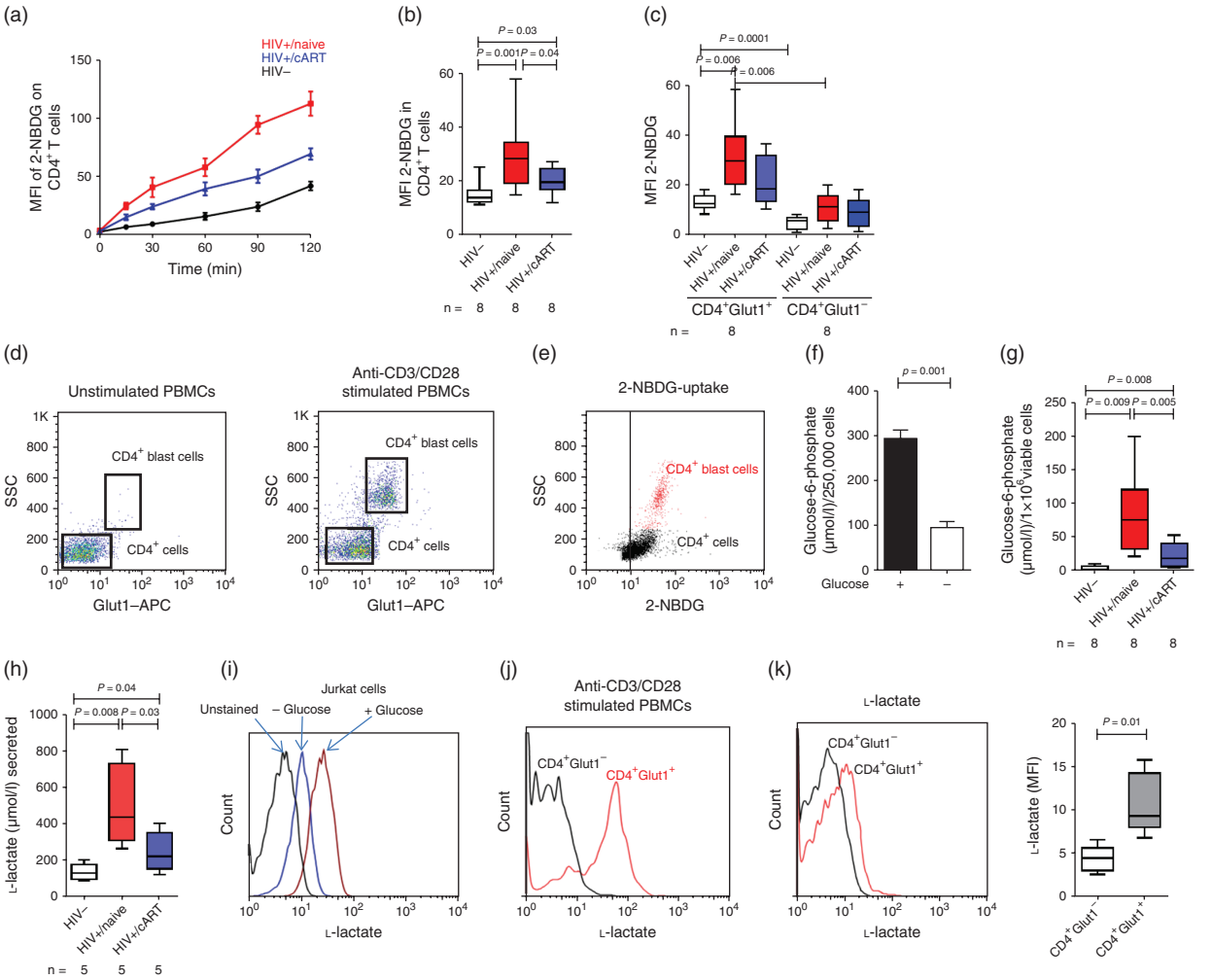

Fig. 3. Effects of HIV status on glucose uptake and glycolysis in CD4$^+$ T cells. (a) The kinetics of glucose uptake were compared using three representative HIV−, HIV+/naive, and HIV+/cART patients who had low (3.6 ± 1.6, black symbols), high (55.8 ± 17.1, red symbols), and moderate (15.6 ± 2.2, blue symbols) percentages of CD4$^+$Glut1$^+$ T cells, respectively. PBMCs were treated with 15 µmol/l 2-NBDG for the indicated times ($n = 3$ per group). Cells were washed and internalized 2-NBDG was detected by flow cytometry, as described in the Methods section. (b) Uptake of 2-NBDG in CD4$^+$ T cells present in PBMCs incubated for 60 min with 15 µmol/l 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− individual. (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 vs. 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 mmol/l) and glucose-deprived (0 mmol/l) RPMI-1640 medium for 4 h. (g) Basal concentrations of intracellular G-6-P in purified CD4$^+$ T cells incubated in glucose containing RPMI-1640 for 4 h. (h) Basal secretion of L-lactate into culture medium by 1×10^6 purified viable CD4$^+$ T cells incubated for 24 h in glucose (11 mmol/l) containing RPMI-1640. (i) Representative histogram showing intracellular L-lactate levels determined by flow cytometry in Jurkat cells cultured for 24 h in the absence (blue) or presence (brown) of 11 mmol/l glucose. (j) Representative histogram showing intracellular L-lactate levels in CD4$^+$Glut1$^+$ (red) and CD4$^+$Glut1$^-$ T (black) cells in PBMCs from a representative HIV− control individual and stimulated for 24 h 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+/naive individual. Horizontal lines within histograms represent median value and whiskers represent minimum and maximum values. The nonparametric Mann–Whitney *t* test was used to evaluate significant differences between the median values of each group, whereas significant differences between Glut1$^+$ and Glut1$^-$ subsets were evaluated using the Wilcoxon matched-pairs sign rank *t* test. 2-NDBG, 2-N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2 deoxyglucose; cART, combination antiretroviral therapy; Glut1, glucose transporter-1; PBMCs, peripheral blood mononuclear cells; SSC, side scatter characteristic

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 individuals 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 individuals secreted significantly more L-lactate into the culture medium than HIV− individuals (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 individuals ($P=0.01$) (Fig. 3k).

A high frequency of glucose transporter-1-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$^+$ cell count ($P=0.0002$) in peripheral blood of HIV+/naive individuals (Fig. 4a). This was also true when HIV+/cART patients 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$^+$ cell 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 patients (Supplement Table 1, http://links.lww.com/QAD/A444). In HIV+/naive individuals, 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

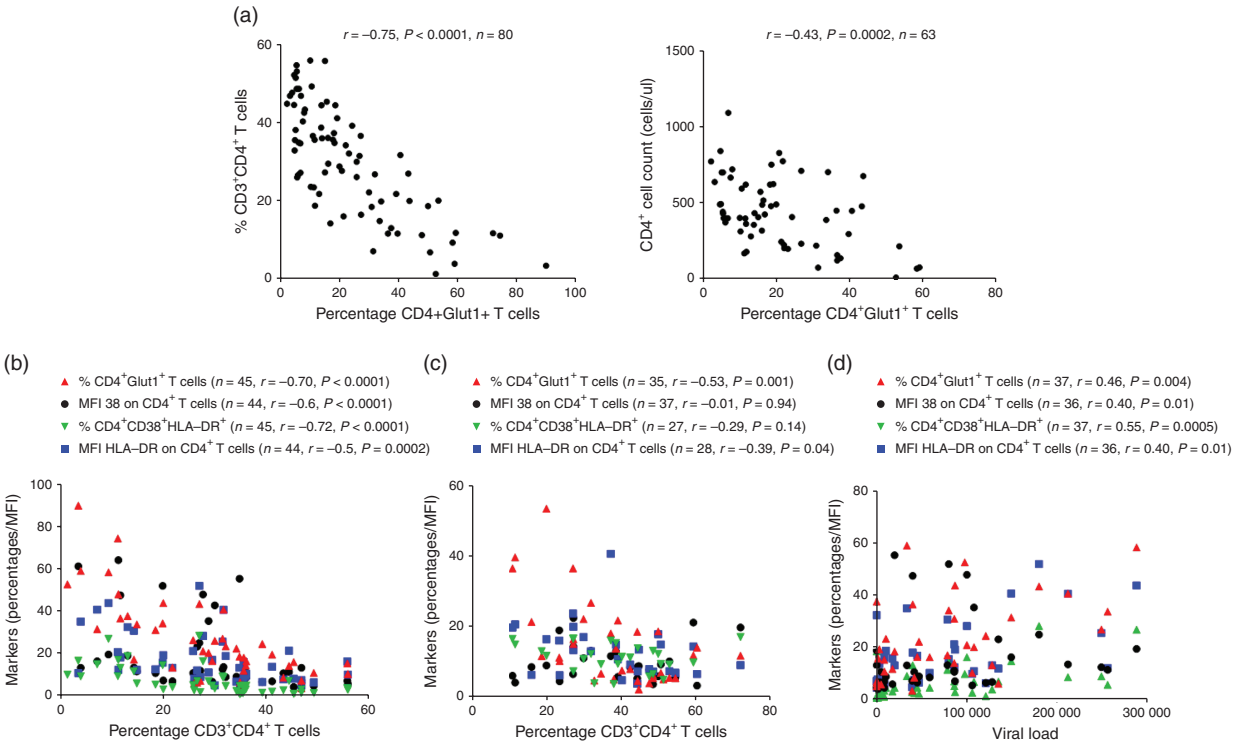

Fig. 4. Relationship between percentage CD4$^+$Glut1$^+$ T cells and total CD4$^+$ T-cell percentage, CD4$^+$ T-cell count, and HIV viral load in HIV-infected patients. (a) Spearman's correlations between the percentage (left panel) and absolute number (right panel) of CD3$^+$CD4$^+$ T cells and the percentage of CD4$^+$Glut1$^+$ T cells in the peripheral blood of HIV+/naive and HIV+/cART patients. (b, 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+/naive and (c) HIV+/cART patients. (d) Comparative relationship between the percentage of CD4$^+$Glut1$^+$ T cells and markers of CD4$^+$ T-cell activation, and HIV viral load in HIV+/naive patients. cART, combination antiretroviral therapy; Glut1, glucose transporter-1.

(Fig. 4b), but the percentage of $CD4^+Glut1^+$ T cells in HIV+/cART patients showed the strongest correlation with total $CD4^+$ T-cell percentage (Fig. 4c). On the contrary, the percentage of $CD4^+CD38^+HLA\text{-}DR^+$ T cells had the strongest correlation with viral load in HIV+/naive individuals (Fig. 4d). The relationship between $CD8^+$ T-cell activation and total $CD4^+$ T-cell percentage was relatively weak in HIV+/naive and HIV+/cART patients (Fig. S6a,b, http://links.lww.com/QAD/A445). Conversely, there was a strong correlation between the levels of CD38 expression on $CD8^+$ T cells and viral load in HIV+/naive individuals (Fig. S6c, http://links.lww.com/QAD/A445).

Multivariate analysis (described in Supplemental methods, http://links.lww.com/QAD/A446) was used to compare the predictive strength of $CD4^+Glut1^+$ percentage with established predictors of HIV disease progression. Compared with known variables of T-cell activation, the percentage of $CD4^+Glut1^+$ T cells was the only independent predictor of $CD4^+$ cell count and $CD4^+$ percentage (Supplemental Table 2a,b, http://links.lww.com/QAD/A444). In contrast, the percentage of $CD8^+CD38^+HLA\text{-}DR^+$, 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+/naive individuals.

Discussion

We report here that HIV infection is associated with increased glucose metabolism in T cells. In HIV+/naive individuals 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 percentage and absolute $CD4^+$ T-cell count, irrespective of treatment status. HIV+/naive and HIV+/cART patients 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 $Glut1^+$ vs. $Glut1^-$ 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 patients, 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 patients.

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. 24 h vs. 2–4 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 overexpressing 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 $Glut\text{-}1_{cterm}$ antibody), increased Glut1 mRNA, and increased glucose uptake in $CD4^+$ T cells in HIV+/naive individuals, 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+ patients. Remarkably, in HIV+ patients, we observed a paradoxical increase in the frequency of $Glut1^+$ T cells in the naive 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 naive $CD4^+$ T cells

in the absence of activating stimuli [24]. The origin of the heightened glucose metabolism in CD4$^+$ T cells in HIV+ patients is unknown but may be a result of elevated cytokines such as interferon (IFN)γ, interleukin (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 patients, 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-cell 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 patients 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 individuals, 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 up-regulated on CD4$^+$, but not CD8$^+$ T cells in HIV+ patients. 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+ patients? 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, hyperactivation 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 a 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 patients within each group for several experiments, raising the possibility that some interpretations may be affected by patient 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.

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Conflicts of interest

The authors declare no competing financial interest.

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