Altered adipose tissue macrophage populations in people with HIV on integrase inhibitor-containing antiretroviral therapy

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Objective: Antiretroviral therapy (ART) extends the life of people with HIV (PWH), but these individuals are at increased risk for obesity, dyslipidemia, diabetes, and cardiovascular disease. These comorbidities may be a consequence of HIV-related chronic inflammation and/or adverse effects of ART on tissue regulatory adipose tissue macrophages (ATMs). We sought to determine the effects of HIV/ART on metabolically beneficial ATM populations and functions.

Design: We examined subcutaneous ATMs from PWH on integrase inhibitor-containing ART (n = 5) and uninfected persons (n = 9). We complemented these studies with ex vivo and in vitro analyses of peripheral blood mononuclear cell (PBMC) and murine macrophage lipid metabolism and fatty acid oxidation gene expression.

Methods: ATM populations were examined by flow cytometry. Macrophage lipid metabolism and fatty acid oxidation gene expression were examined by Seahorse assay and quantitative PCR.

Results: Adipose tissue from PWH had reduced populations of metabolically activated CD9+ ATMs compared to that of uninfected controls (P < 0.001). PBMCs of PWH had lower fatty acid metabolism compared to those of uninfected controls (P < 0.01). Analysis of murine macrophages revealed that dolutegravir reduced lipid metabolism (P < 0.001) and increased expression of the fatty acid beta-oxidation enzyme enoyl-CoA hydratase, short chain 1 (P < 0.05).

Conclusions: We report the loss of metabolically beneficial ATM populations in PWH on ART, altered fatty acid metabolism of blood immune cells, and evidence that dolutegravir alters macrophage fatty acid metabolism. Future studies should examine direct or indirect effects and mechanisms of dolutegravir, and other integrase inhibitors and ART classes, on fatty acid beta-oxidation.

Keywords: adipose tissue, antiretroviral therapy, HIV, lipid metabolism, macrophage
Introduction

The life expectancy of people with HIV (PWH) has increased considerably due to the development of effective antiretroviral therapy (ART) over the past several decades [1,2]. However, PWH are at increased risk for several chronic, noninfectious comorbidities including obesity, dyslipidemia, diabetes mellitus, and cardiovascular disease [3–5]. As a result, metabolic and cardiovascular diseases are now some of the leading causes of death among PWH in developed countries [6]. Although etiologies for these comorbidities remain under investigation, associations have been identified between specific ART agents and worse metabolic outcomes [7–9]. Although protease inhibitors have many well known metabolic effects [10], they are used with decreasing frequency. In contrast, integrase inhibitor agents are now very widely used, but have been associated with considerable weight gain and increased adiposity [11]. Dolutegravir in particular, a second generation integrase inhibitor, is associated with greater weight gain as compared with first generation agents of the same class [12]. However, our limited understanding of the mechanistic basis for these associations limits our ability to prevent and/or treat HIV-associated metabolic diseases.

Over the past two decades, studies from the obesity field reveal that immune cells, and adipose tissue macrophages (ATMs) in particular, are central regulators of metabolic health. It is now appreciated that obesity is associated with the accumulation of ATMs in adipose tissue [13]. However, multiple ATM subsets with distinct cellular functions exist in obese adipose tissue [14]. For example, some obesity-associated ATMs undergo a metabolic activation that is characterized by increased lipid metabolism [15–19]. This functional state is likely an adaptive response to dead and dying adipocytes that have lost adequate blood supply due to tissue hypertrophy [20]. However, potentially as a natural progression of this process, ATM subsets have also been shown to increase secretion of tumor necrosis factor-alpha (TNFα) and other inflammatory mediators that contribute to adipose tissue insulin resistance [14,21,22]. It is the balance of these outcomes that ultimately contributes to the adipose tissue dysfunction that accompanies obesity [23].

The fact that macrophages are central to mammalian metabolism may be relevant to understanding HIV-associated metabolic disease. Monocytes and macrophages are important HIV reservoirs [24], and both HIV infection and ART can alter monocytes and macrophage activation and function during HIV infection [25–27]. Associations have also been made between monocyte expansion in HIV infection and insulin resistance [28]. There is also evidence that antiretrovirals can alter macrophage inflammatory functions in vitro [29]. However, studies to date have focused on the emergence of pro-inflammatory macrophage phenotypes among PWH on ART and have not investigated the metabolically-beneficial functions of ATMs in adipose tissue. In addition, studies of ATMs isolated from ART-treated PWH have not been performed. Addressing these knowledge gaps is the first step towards avoiding and/or specifically treating HIV-associated metabolic diseases.

Here, we investigated sub–cutaneous ATMs from samples of PWH being treated with integrase-containing regimens along with uninfected persons. We also investigated the effects specifically of dolutegravir on macrophage fatty acid metabolism. We show that PWH have a marked reduction in the proportion of ATMs that are metabolically active, altered lipid metabolism by peripheral blood mononuclear cells (PBMCs), and that dolutegravir specifically alters macrophage lipid metabolism.

Methods

Participant selection and recruitment
Potential participants with HIV infection were identified through the Penn Center for AIDS Research using relevant clinical data. Recruitment criteria included being on a stable integrase inhibitor-based ART regimen and HIV RNA < 200 copies/ml for at least 1 year. HIV uninfected participants were recruited through the Penn Human Metabolic Tissue Bank. This study was approved by the Institutional Review Board of the University of Pennsylvania (IRB 20-844427) and the Children’s Hospital of Philadelphia (IRB 18-015524).

Adipose tissue collection and isolation of stromal-vascular-fraction
Subcutaneous adipose tissue was collected from the anterior abdominal wall during planned surgical procedure or via biopsy and transported to the lab at 37°C within 30 min. The stromal vascular fraction (SVF, which contains adipocyte precursors, stromal cells, and immune cells) was isolated by incubating tissue samples in 5 ml of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 3 mg/1 ml collagenase IV at 37°C for 20 min in a rotating incubator [14]. SVF cells were pelleted at 800 × g and floating adipocyte fraction was removed and discarded. SVF were washed 3 times with FACS buffer [1 × phosphate buffered saline (PBS), 2% bovine serum albumin (BSA), 1 mmol/l ethylenediaminetetraacetic acid (EDTA), 0.1% sodium azide] before processing for flow cytometric analysis.

Flow cytometric analysis
SVF or bone marrow–derived macrophage (BMDM) cultures were treated with human Fc-receptor block (BD
mitochondrial respiration and mRNA levels. After 24 h, cells were harvested for analysis of peak serum levels observed in clinical studies [31,32]. Concentration was chosen as it is between trough and well plates (0.5–1 \( \mu \)M). Cells were subsequently transferred to six-well macrophage colony-stimulating factor (M-CSF) containing DMEM with 10% fetal bovine serum (FBS), 2 mmol/l GlutaMAX, 100 U/ml penicillin–streptomycin, 1 mmol/l sodium pyruvate, and 10 ng/ml macrophage colony-stimulating factor (M-CSF) (R&D Systems). On day 7 of culture, cells were isolated by scraping and BMDM purity was evaluated by flow cytometry. Cells were subsequently transferred to six-well plates (0.5–1 \( \times \) 10\(^6\) cells per well), and metabolically activated with palmitate (250 \( \mu \)mol/l) for 24 h as previously described [17]. Studies were performed in the presence or absence of dolutegravir (50 \( \mu \)mol/l). This concentration was chosen as it is between trough and peak serum levels observed in clinical studies [31,32]. After 24 h, cells were harvested for analysis of mitochondrial respiration and mRNA levels.

Isolation of peripheral blood mononuclear cells
Peripheral blood samples were obtained by venipuncture on the same day as adipose tissue, and PBMCs were isolated by Ficoll gradient.

Generation and metabolic activation of murine bone marrow-derived macrophages
BMDMs were generated from 6- to 8-week-old, C57Bl/6j mice as previously described [30]. Briefly, femurs were excised under sterile conditions and flushed with DMEM to isolate bone marrow cells. Cells were washed with PBS and incubated for 7 days at 37°C and 5% CO\(_2\) in BDMM differentiation medium containing DMEM with 10% fetal bovine serum (FBS), 2 mmol/l GlutaMAX, 100 U/ml penicillin–streptomycin, 1 mmol/l sodium pyruvate, and 10 ng/ml macrophage colony-stimulating factor (M-CSF) (R&D Systems). On day 7 of culture, cells were isolated by scraping and BMDM purity was evaluated by flow cytometry. Cells were subsequently transferred to six-well plates (0.5–1 \( \times \) 10\(^6\) cells per well), and metabolically activated with palmitate (250 \( \mu \)mol/l) for 24 h as previously described [17]. Studies were performed in the presence or absence of dolutegravir (50 \( \mu \)mol/l). This concentration was chosen as it is between trough and peak serum levels observed in clinical studies [31,32]. After 24 h, cells were harvested for analysis of mitochondrial respiration and mRNA levels.

Measurement of mitochondrial respiration
The analysis of mitochondrial respiration was performed on PBMCs of male subjects or murine BMDMs, due to limited human sample availability. Mitochondrial respiration was determined by monitoring the oxygen consumption rate (OCR) of cells in the presence of XF Palmitate – BSA (Seahorse Bioscience) and absence of exogenous glucose and glutamine. The proportion of respiration that is supported by exogenous fatty acids was determined using the fatty acid oxidation (FAO) inhibitor etomoxir (40 \( \mu \)mol/l). For quantifying mitochondrial respiration, cells were cultured at the density of 50 000 cells/well in XF96 cell culture plates in growth medium overnight. One hour before running the assay, cells were washed and incubated in XF assay medium containing palmitate at 37°C in a CO\(_2\) free atmosphere. Oligomycin (Oligo.), carbonyl cyanide 4 (trifluoromethoxy)phenylhydrazone (FCCP), and rotenone and antimycin A (R/A) were sequentially injected into each well to assess basal respiration, coupling of respiratory chain, and mitochondrial spare respiratory capacity, respectively. Data were collected on a Seahorse XF96 instrument and analyzed using XF Wave Software (Seahorse Bioscience). Cellular protein levels were determined using BCA protein assay kit (Pierce), and all data were normalized to the protein content of the assay well.

RNA isolation and cDNA synthesis
Total RNA was isolated from using TRIzol (Life Technologies) according to the manufacturer’s instructions. RNA quantity and quality were measured with the Nanodrop spectrophotometer, and cDNA was synthesized using Verso cDNA synthesis kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed with the Quant Studio 12K Flex System using the SYBR Green method. HPRT served as a housekeeping control. PCR primers used were: Acadl_F, TGCACACATACAGCGTGC; Acadl_R, GGGGCTGTA CTGGCTCATTCC; Cpt1a_L-R, ACCAGTGATGATGAAGCAGAACCGGAGT; Cpt1a_L-F, GACTCCGGGAAGCAGCAATGG; Cpt1a_R, TCTTGGATAACGAGTTC; Hadha_F, AGGACCTCGGTGTAAAGCAC; Hadha_R, TAGTGCAATGCCGATCACCTTC; Hprt_F, TCAGTCAGCAACGAGCAGAAA; Hprt_R, GGGGCTGTA CTGGCTCATTCC; Hprt_F, TCAGTCAGCAACGAGCAGAAA; Hprt_R, GGGGCTGTA CTGGCTCATTCC.

Statistical analyses
Graphical data are presented as mean ± SEM of at least three independent experiments. Data were analyzed by a Student’s \( t \) test for comparison between PWH and uninfected persons (GraphPad Prism). Analysis of associations between %CD9\(^+\) ATMs and participant characteristics were performed by simple regression. Differences were considered statistically significant at \( P \)-values (alpha level) < 0.05.

Results
Metabolically-activated adipose tissue macrophages are reduced in sub-cutaneous adipose tissue of people with HIV
The demographic and clinical characteristics of PWH and uninfected participants are shown in Table 1. Participants were recruited to represent a diverse BMI range from normal weight (18.5–24.9) to obese class III (≥40). All HIV+ participants were on integrase-containing ART regimens (either bictegravir or dolutegravir) consistent with current ART treatments. ATMs were examined by flow cytometry (Figure 1, Supplemental Digital Content, http://links.lww.com/QAD/C538), and surface expression of the metabolic activation marker CD9 was compared between the groups.
As a proportion of non-B, non-T (NBNT) immune cells, ATMs were reduced in subcutaneous adipose tissue of PWH as compared with uninfected individuals (35 ± 3.0% vs. 11 ± 1.6%; P = 0.0001; Fig. 1a). We also observed a marked and consistent reduction in the surface expression of CD9 on subcutaneous adipose tissue ATMs of PWH as compared with uninfected individuals (82 ± 6.9% vs. 28 ± 9.5%; P = 0.0006; Fig. 1b, c). Because all PWH participants were African American (AA) while uninfected participants were more racially diverse, we carried out a subgroup analysis of AA participants and found a similar result (Figure 2, Supplemental Digital Content, http://links.lww.com/QAD/C538). There was no significant relationship between %CD9+ ATMs and BMI, and both obese and nonobese HIV/ART subjects showed reduced CD9+ ATMs compared to uninfected participants (Tables 1 and 2, Supplemental Digital Content, http://links.lww.com/QAD/C538, and Figure 3, Supplemental Digital Content, http://links.lww.com/QAD/C538).

One hallmark of ATM metabolic activation is a shift towards lysosome-mediated fatty acid metabolism [15–19]. Because the number of ATMs isolated from adipose tissue was too low for metabolic studies, we investigated metabolic function of PBMCs. We observed that PBMCs isolated from a subset of PWH participants had markedly lower basal and maximal respiration when metabolizing fatty acid compared with uninfected participants (5 ± 0.2 vs. 33 ± 3.4, and 21 ± 0.5 vs. 37 ± 2.7; P = 0.0018 and P = 0.0429, respectively; Fig. 1d). Treatment of PBMCs with etomoxir, an inhibitor of fatty acid metabolism, reduced mitochondrial respiration of palmitate as the only cellular fuel source. We observed that dolutegravir resulted in a significant and reproducible reduction in mitochondrial maximal (33 ± 1.1 vs. 63 ± 5.1; P = 0.0008) and spare (19 ± 1.1 vs. 45 ± 2.9; P = 0.0010) respiratory capacity (Fig. 2a, b). Treatment of BMDMs with etomoxir reduced mitochondrial respiration, indicating that the measured oxygen consumption was primarily from metabolism of palmitate (Figure 5, Supplemental Digital Content, http://links.lww.com/QAD/C538). There was no change in CD9+ expression as a result of dolutegravir (DTG) treatment of metabolically activated BMDMs (Figure 6, Supplemental Digital Content, http://links.lww.com/QAD/C538).

We then asked whether the dolutegravir effect on murine macrophage cellular metabolism might be linked to changes in expression of genes relevant to fatty acid beta-oxidation. Dolutegravir resulted in a significant (threefold) up-regulation of BMDM Ech1 expression, which encodes the protein which functions in the second step of the mitochondrial fatty acid beta-oxidation pathway (Fig. 2c). In contrast, expressions of other genes in the fatty acid beta-oxidation pathway (Acd1, Cpt1a, and Hadha) were not significantly altered. Thus, dolutegravir...
Discussion

PWH are at increased risk for metabolic dysfunction and disease [3–5]. It is therefore important that we improve our understanding of the etiology of these comorbidities to reduce morbidity and mortality in this population [6]. Although HIV infection itself results in immune activation that may influence metabolic homeostasis [25–27], there is growing evidence that various ART agents may contribute to metabolic dysregulation [7]. For example, integrase inhibitors are associated with more weight gain than other antiretrovirals, and dolutegravir (a second generation integrase inhibitor) is associated with greater weight gain as compared with first generation agents of the same class [11,12]. Together, these observations have led to concern that specific ART regimens are contributing to HIV-associated obesity and metabolic diseases [33].

The adipose tissue immune system, and ATMs in particular, are critical regulators of adipose tissue function and metabolic health. It is therefore essential to examine the impact of HIV and ART on ATM activation and functions. A few studies have examined effects of HIV/ART on ATMs, but they have focused on inflammatory outcomes. For example, elevated monocyte numbers have been shown to correlate with worsening HIV immune outcomes [28], and treatment with protease inhibitors and reverse transcriptase inhibitors has been shown to enhance monocyte production of monocyte chemoattractant protein-1 and interleukin-6 [29]. In contrast, we chose to examine the key activity that ATMs have in maintaining normal adipose tissue functions through the uptake and metabolism of free lipid [15–19].

Fig. 1. Total and CD9+ ATMs are reduced in sub-cutaneous adipose tissue of PWH. (a) Frequency of total CD14+ ATMs as a percentage of NBNT immune cells in subcutaneous adipose tissue of uninfected (control) or PWH participants. Mean ±SEM shown, n = 9 and n = 5, respectively. (b) Flow-cytometric analysis of CD9+ adipose tissue macrophages in sub-cutaneous adipose tissue samples from representative uninfected control or PWH participants. Gated on alive, CD45+, CD3−, CD20−, CD14+ cells. Numbers indicate percentage of parent gate. (c) Frequency of CD9+ ATMs in subcutaneous adipose tissue of uninfected (control) or PWH participants. Mean ±SEM shown, n = 9 and n = 5, respectively. (d) Respiration of PBMCs from uninfected control or PWH participants as measured by oxygen consumption rate (OCR) in palmitate-containing assay media. During measurements, cells were treated with the complex V inhibitor oligomycin (Oligo.), the uncoupler fluoro-carbonyl cyanide phenylhyrazone (FCCP), and the complex I and III inhibitors rotenone/antimycin A (R/A) at the times indicated (black arrows). Basal and maximal (max) respiration are shown. Calculations were corrected for nonmitochondrial respiration and normalized to the cellular protein content of the assay well. Data points are represented as mean ± SEM from ≥3 biologic replicates and data are representative of at least three independent experiments. Statistics by unpaired or paired t test, (*) P < 0.05; (**) P < 0.01; (***) P < 0.001. ATM, adipose tissue macrophage; PWH, people with HIV; SEM, standard error of mean.

alters macrophage fatty acid metabolism and expression of a key gene involved in fatty acid metabolism.
We observed that subcutaneous adipose tissue of PWH have a reduced ATM frequency as well as reduced frequency of CD9\(^+\) ATMs, a beneficial ATM subpopulation that are metabolically activated as evidenced by upregulation of lipid receptors, phagocytosis of lipid, and activation of lipid metabolic pathways [14,19]. The loss of CD9\(^+\) ATMs in mice results in systemic metabolic dysregulation highlighting the beneficial roles that these cells play [19]. It is therefore possible that the impairment of CD9\(^+\) ATMs development and/or function contributes to the adipose tissue dysfunction and metabolic disease observed among PWH.

To better understand the etiology of reduced CD9\(^+\) ATMs in PWH on ART, we examined immune cell lipid metabolism. As the number of ATMs recovered from subcutaneous adipose tissue biopsies were too small for cellular-metabolic analysis, we studied PBMCs from uninfected and PWH participants. We observed that metabolism of palmitate, a 16-carbon saturated fatty acid that is a fuel source for metabolically activated macrophages, was lower in PWH compared with uninfected individuals. Although PBMCs contain mainly lymphocytes and as such are not a direct indication of ART-induced changes in ATM metabolism, this observation does suggest that immune lipid metabolism is altered by HIV infection/ART, and raises the possibility that such effects are not limited to the adipose tissue.

Finally, we sought to determine if integrase inhibitor ART alters macrophage lipid metabolism, focusing on dolutegravir since it is associated with more weight gain than other agents [11,12]. We observed that dolutegravir significantly reduced BMDM palmitate metabolism, which is consistent with our studies of ATMs from PWH, all of whom were receiving integrase inhibitors. This result also suggests that, in addition to inflammatory effects of HIV infection on myeloid cells [25–27], antiretrovirals may contribute to ATM dysfunction and HIV-associated metabolic disease. We also observed significant up-regulation of Echs1 expression by BMDMs treated with dolutegravir, indicating effects on macrophage expression of metabolism genes. Echs1 encodes enoyl-CoA hydratase, short chain 1, an enzyme that forms the second step of the mitochondrial fatty acid beta-oxidation pathway.
Upregulation of Echs1 expression could represent a compensatory response to dolutegravir’s negative effects on fatty acid metabolism. The effects of DTG on BMDM metabolism occurred in the absence of any alterations in cell surface CD9 expression. This may be due to the acute nature of the DTG exposure in our experimental system and suggests that cellular-metabolic effects of acute DTG exposure may mechanistically precede changes in CD9 protein or surface expression. Future studies should interrogate this hypothesis, as well as any direct and/or indirect effects of dolutegraviron enoyl-CoA hydratase, short chain 1 function.

There are some limitations to our study. Firstly, we examined a relatively small participant number and as such our ability to detect age, sex, or race-dependent effects was limited. There were also differences in the sex and racial distribution of our control and experimental groups that could bias our findings. This work should therefore be expanded in a larger cohort that includes individuals of diverse demographic groups. We studied individuals on integrase inhibitor-containing ART, so our in vitro results cannot be ascribed to integrase inhibitors specifically, though they are concordant with our in vivo observations. Thus, it will be important to study PWH on non-integrase inhibitor ART regimens. Similarly, our in vitro analyses study the effects of a single integrase inhibitor (dolutegravir) on mitochondrial respiration and gene expression in murine cells. As these studies were not performed using other integrase inhibitors we cannot generalize the observed effects to the entire class of integrase inhibitors. As such, future studies should examine the effects of other integrase inhibitors, and other antiretroviral classes, on ATMs and human macrophage mitochondrial respiration. In addition, we examined sub-cutaneous adipose tissue but did not have access to visceral or other adipose tissue depots. Although there may be important similarities between the effects of HIV-infection/ART on beneficial macrophage functions between these depots they are also functionally distinct and should be examined independently. Finally, future studies with larger participant numbers should examine relationships between these outcomes and BMI, diabetes, and other features of metabolic dysfunction.

In sum, we find a previously unreported dysregulation of adipose tissue macrophage populations in PWH on integrase inhibitor-containing regimens regardless of BMI, and a detrimental effect of dolutegravir on beneficial macrophage functions related to adipose tissue homeostasis. This effect may act synergistically with known inflammatory effects HIV infection on myeloid cells to promote adipose tissue dysfunction and metabolic disease in some individuals with HIV infection. Future studies should investigate additional ATM functions and ART drugs, which will contribute to understanding of the mechanisms and role of macrophage functions in obesity and metabolic disease among PWH.

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Conflicts of interest

There are no conflicts of interest.

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