CONCISE COMMUNICATION

Human adipose tissue as a reservoir for memory CD4^{+} T cells and HIV

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Objective: The objective of this study is to determine whether adipose tissue functions as a reservoir for HIV-1.

Design: We examined memory CD4^{+} T cells and HIV DNA in adipose tissue–stromal vascular fraction (AT-SVF) of five patients [four antiretroviral therapy (ART)-treated and one untreated]. To determine whether adipocytes stimulate CD4^{+} T cells and regulate HIV production, primary human adipose cells were cocultured with HIV-infected CD4^{+} T cells.

Methods: AT-SVF T cells were studied by flow cytometry, and AT-SVF HIV DNA (Gag and Env) was examined by nested PCR and sequence analyses. CD4^{+} T-cell activation and HIV production were measured by flow cytometry and ELISA.

Results: AT-SVF CD3^{+} T cells were activated (>60% CD69^{+}) memory CD4^{+} and CD8^{+} T cells in uninfected and HIV-infected persons, but the AT-SVF CD4^{+}/CD8^{+} ratio was lower in HIV patients. HIV DNA (Gag and Env) was detected in AT-SVF of all five patients examined by nested PCR, comparably to other tissues [peripheral blood mononuclear cell (PBMC), lymph node or thymus]. In coculture experiments, adipocytes increased CD4^{+} T-cell activation and HIV production approximately two to three-fold in synergy with gamma-chain cytokines interleukin (IL)-2, IL7 or IL15. These effects were mitigated by neutralizing antibodies against IL6 and integrin-$\alpha1\beta1$. Adipocytes also enhanced T-cell viability.

Conclusion: Adipose tissues of ART-treated patients harbour activated memory CD4^{+} T cells and HIV DNA. Adipocytes promote CD4^{+} T-cell activation and HIV production in concert with intrinsic adipose factors. Adipose tissue may be an important reservoir for HIV. Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Eradication of HIV is challenging because the virus persists in cellular and anatomic reservoirs despite antiretroviral therapy (ART) [1]. Primary cellular reservoirs include memory $CD4^+$ T cells and macrophages; despite their low frequency (~1 per million), latently infected $CD4^+$ T cells are the primary source of viral rebound in patients whose ART is interrupted [2]. Physiological induction of latent HIV in $CD4^+$ T cells occurs via activation of $CD3^+$/T-cell receptor (TCR) cytokines [interleukin (IL)-2, IL7, IL15, IL6 and tumour necrosis factor-alpha (TNFα)], Toll-like receptor (TLR) ligands or free fatty acids [3]. Major tissue reservoirs include lymphoid tissue (lymph nodes, spleen, thymus and bone marrow), gut-associated lymphoid tissue (GALT) and the central nervous system.

Adipose tissue is a major endocrine organ with diverse functions and cellular composition. It is present mainly under the skin (subcutaneous adipose tissue) and around thoracoabdominal organs (visceral adipose tissue). The immune system is intimately associated with adipose tissue; every lymph node is encapsulated by adipose tissue, and adipocytes are abundant within bone marrow and in ageing thymus gland [4–6]. Adipose tissue is composed of mature adipocytes and the stromal-vascular fraction (SVF) that includes preadipocytes, mesenchymal stem cells, fibroblasts, endothelial cells and immune cells. Every type of leukocyte is found in adipose tissue, and adipose-resident $CD4^+$ T cells resemble those in other tissues in that they have an activated memory phenotype ($CD45RO^+CD69^+$) [7–9]. Stimuli for adipose $CD4^+$ T cells include cytokines (IL2, IL7, IL15, IL6, IL8 or TNFα) or interactions with adipose macrophages, dendritic cells or adipocytes [10,11]. Chemokines and receptors such as RANTES (regulated on activation, normal T-cell expressed and secreted) or CXCR3 (chemokine (C-X-C motif) receptor 3) are important for T-cell migration into adipose tissue, although the antigenic stimuli (microbial or lipids) and general functions (proinflammatory vs. anti-inflammatory) are still unclear [12,13]. Adipocytes themselves are unable to support HIV infection [14], but adipose cells could influence the pathogenesis of infected $CD4^+$ T cells within adipose depots. We hypothesized that adipose tissue could be a reservoir for HIV, and that adipocytes stimulate HIV production from $CD4^+$ T cells.

Materials and methods

Isolation of adipose tissue stromal-vascular fraction cells

Protocols were approved by the Baylor College of Medicine Institutional Review Board. Solid adipose tissue samples were procured from cadavers through National Disease Research Interchange (Philadelphia, Pennsylvania, USA), or from live donors undergoing elective surgical procedures with informed consent. For isolation of AT-SVF cells, 1–2 g adipose tissue was minced and digested with collagenase (Sigma, St Louis, Missouri, USA), mesh-filtered and SVF cells pelleted [15]. AT-SVF cells were centrifuged on Ficoll-Paque to further increase lymphocyte yield (typically resulting in $\sim 2-5 \times 10^5$ cells per gram of adipose tissue).

Flow cytometry and nested PCR of adipose tissue stromal-vascular fraction cells

For flow cytometry of AT-SVF T cells, isolated AT-SVF cells were stained for CD3-Pacblue, CD4-PerCPCy5.5, CD45RO-FITC and CD69-APC mAbs [Biolegend (San Diego, California, USA) or BD Biosciences (San Jose, California, USA)], and analysed with appropriate controls using a Gallios Flow Cytometer (Beckman-Coulter, Miami, Florida, USA). For nested PCRs, DNA was first extracted from AT-SVF cells with QIAamp Microkit (Qiagen, Valencia, USA) Nested PCR reactions were performed by two rounds of 35–40 cycles using primers spanning HIV LTR-Gag (RU5+SK39 outer and US5+US3 inner primers) [16], and Envelope C2V3C3 (PCR5+R344 outer and EN1+EN4B inner primers) regions [17]. PCR products were gel-purified and sequenced by the BCM DNA Sequencing Core.

In-vitro studies of memory $CD4^+$ T cells and adipose cells

Human memory $CD4^+CD45RO^+$ T cells were purified from peripheral blood mononuclear cells (PBMC) of healthy donors via EasySep kits (Stemcell Technologies, Vancouver, British Columbia, Canada). Primary preadipocytes and adipocytes were obtained from Zen-Bio (Research Triangle Park, North Carolina, USA).

For in-vitro infections, HIV viral stocks were generated by the BCM/UTHSC-Houston CFAR Virology Core. Most experiments first involved noninfection or infection of memory $CD4^+$ T cells with R5-tropic HIV strains at 0.01–0.1 MOI for 24 h in IL2 medium (complete RPMI and 20 ng/ml IL2), washing, then coculture with adipose cells and indicated agents.

For coculture of memory $CD4^+$ T cells with adipose cells, 2×10^5 uninfected or infected memory $CD4^+$ T cells were seeded into transwells (0.4 μm pore) in six-well plates with 2×10^5 preadipocytes or adipocytes in lower wells so that the cells did not touch. Cells were cocultured for indicated periods with appropriate reagents (10–20 ng/ml cytokines or 5–10 μg/ml blocking antibodies) [R&D Systems (Minneapolis, Minnesota, USA) or Biolegend]. Blocking mAbs included CD49a (clone TS2/7), CD29 (clone TS2/16) and CD126 (clone UV4) (Biolegend).

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T-cell activation and viability were measured by flow cytometry staining for CD69 and CD25 (Biolegend), or viability dye (Life Technologies). HIV production was measured by flow cytometry staining for intracellular p24 (KC57-PE; Beckman-Coulter) with Cytofix/Cytoperm solutions (BD Biosciences), and extracellular p24 by ELISA (Advanced BioScience Laboratories, Rockville, Maryland, USA). Extracellular IL6 in coculture experiments was measured by ELISA (eBioscience. San Diego, California, USA). and IL6 mRNA of fractionated human adipose tissue was measured by real-time PCR.

For direct infection of AT-SVF cells isolated from adipose tissues of healthy donors, cells were infected with HIV (R5-tropic NSN-SX at 0.1 MOI) for 24 h, washed, then cultured with or without 20 ng/ml IL2 or IL7 for up to 8 days. AT-SVF memory $CD4^+$ T cells were stained by flow cytometry, and HIV production measured by p24 ELISA.

Statistics

Analyses were performed using SAS and Excel. Differences were compared by paired or unpaired Student's *t*-test, and *P* values less than 0.05 were considered significant.

Results

Detection of memory $CD4^+$ T cells and HIV provirus in human adipose tissue

$CD4^+$ T cells and macrophages reside in adipose tissue of healthy humans in which they are activated, undergo polarization and regulate adipose physiology and metabolism [8,9,11,18]. During HIV infection, infected $CD4^+$ T cells and monocytes could traffic into adipose tissue to establish reservoirs [19,20]. To determine whether HIV provirus is present in AT of patients, adipose tissue samples (subcutaneous and visceral) were acquired from five patients (three live donors undergoing surgery, and two recently deceased patients), and from uninfected healthy control donors. Four patients were ART-treated (three with undetectable plasma viral load, Fig. 1a). AT-SVF cells were examined by flow cytometry and HIV DNA detected by nested PCR.

Memory $CD4^+$ ($CD3^+CD4^+CD45RO^+$) and memory $CD8^+$ ($CD3^+CD4^+CD45RO^+$) T cells, as well as $CD69^+$ expression, were examined in peripheral blood and AT-SVF of healthy control and HIV patients (gating scheme in Fig. 1b). Within the $CD3^+$ T-cell population of healthy controls, AT-SVF contained predominantly memory $CD4^+CD45RO^+$ cells ($63.1 \pm 5.4\%$, $n = 4$), whereas in peripheral blood, these comprised $39.9 \pm 4.5\%$ of $CD3^+$ T cells ($P < 0.05$, Fig. 1c), which were greater than memory $CD8^+$ proportions ($15.4 \pm 5.5\%$ in AT-SVF and $18.2 \pm 2.7\%$ in peripheral blood). However, the distribution of $CD4^+$ to $CD8^+$ T cells was reversed in AT-SVF of HIV patients (reduced $CD4^+CD45RO^+$ cells to $34.9 \pm 7.9\%$, and $CD4^+CD45RO^+$ cells increased to $46.0 \pm 8.3\%$, $P < 0.05$ compared with healthy control AT-SVF). AT-SVF memory T cells CD69 expression were 61–72% in healthy control AT-SVF and 60–67% in HIV patient AT-SVF (Fig. 1d), similar to $CD69^+$ expression levels in lymphoid and intestinal tissues [7].

HIV DNA in AT-SVF cells was determined by nested PCR using primers targeting LTR-Gag and Envelope. About $1-2 \times 10^5$ cell equivalents of DNA was used per reaction, and sensitivity determined using ACH2:PBMC ratios with detection limits of nearly one HIV copy per 1×10^5 uninfected PBMC (data not shown). HIV DNA was detectable in AT-SVF cells from different adipose depots (visceral, subcutaneous or deep neck) of all five HIV patients with both primer sets (Fig. 1e), and detection frequencies were comparable to those in PBMCs or memory $CD4^+$ T cells (purified from peripheral blood), thymus or mesenteric lymph nodes. The second round PCR products were gel-purified and sequenced to assess HIV diversity, but phylogenetic analyses of Gag and Env sequences indicated no significant intrapatient differences among different tissues (data not shown). Thus, adipose tissue of HIV patients on ART harbours HIV, but the source of the virus ($CD4^+$ T cells or macrophages) remains to be determined.

Enhancement of T cell activation, HIV production and viability by adipocytes

To determine whether adipose cells (preadipocytes or adipocytes) affect HIV replication, primary adipose cells were cocultured with infected memory $CD4^+$ T cells in transwell dishes (purified from healthy donor blood and infected *in vitro* prior to coculture). T-cell activation ($CD69^+$ expression) and HIV production (intracellular or extracellular p24) were measured.

Preadipocytes or adipocytes alone did not affect memory $CD4^+$ T-cell activation or HIV production, but enhanced T-cell activation and HIV production in the presence of IL2, IL7 or IL15. Figure 2a shows representative CD69/p24 dot plots of infected (R5-tropic) memory $CD4^+$ T cells after 7 days coculture with preadipocytes or adipocytes with or without IL2. Compared with medium alone, adipocytes increased CD69 and p24 in infected memory $CD4^+$ T cells nearly two–fold with IL2, IL7 or IL15 ($P < 0.05$ for IL2 and IL15, $n = 3$), whereas addition of proinflammatory cytokines IL6, IL8 or TNFα into cocultures had no effect (Fig. 2b). Extracellular HIV production by memory $CD4^+$ T cells also increased two to three-fold by adipocytes with IL2, IL7 or IL15 (Fig. 2c). Thus, IL2, IL7 or IL15 in adipose depots may be important for HIV persistence.

We assessed the role of adipose tissue IL6 in enhancing HIV replication by memory $CD4^+$ T cells. Adipose cells

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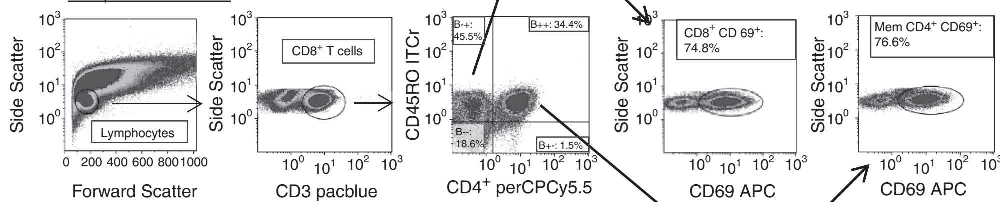
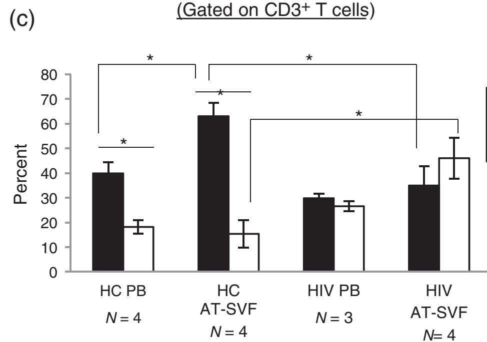
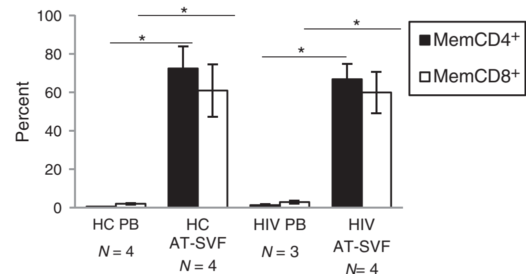
(a) HIV patient characteristics

Patient	Gender, age	Viral load (RNA copies/ml)	CD4+ count (cells/μl)	ART	BMI
01 (Live)	M, 39	<50	288	Yes	26
02 (Cadaver)	M, 60	ND	ND	Yes	ND
03 (Live)	F, 36	<50	142	Yes	37
04 (Live)	F, 50	<50	623	Yes	32
05 (Cadaver)	M, 39	>500,000	53	No	16







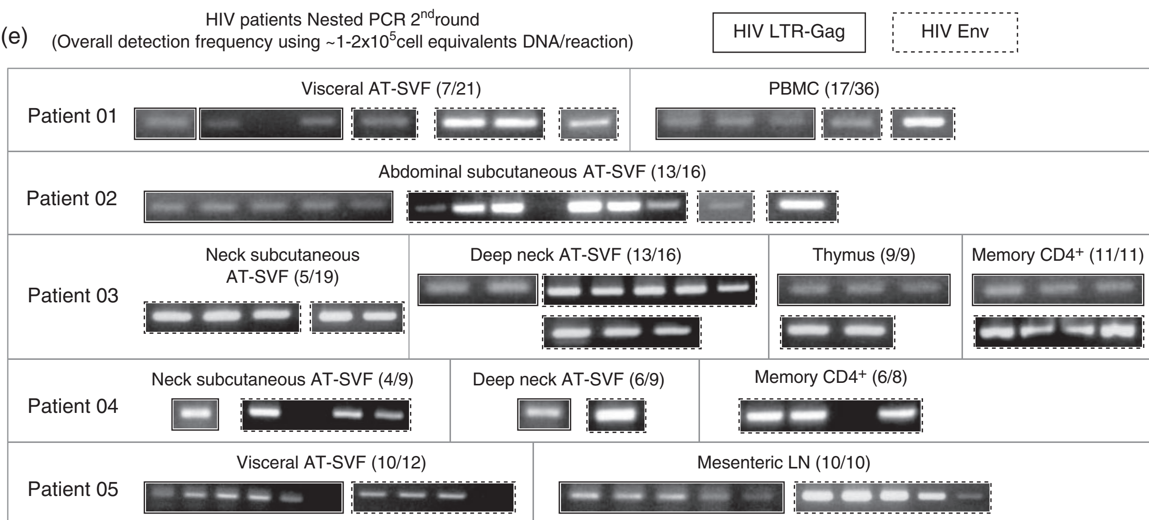


Fig. 1. Memory CD4^{+} T cells and HIV DNA in AT of HIV patients. (a) HIV patient characteristics. (b) Flow cytometry gating for measurement of adipose-tissue-stromal-vascular-fraction (AT-SVF) memory CD4^{+} (CD3^{+}CD4^{+}CD45RO^{+}) and memory CD8^{+} (CD3^{+}CD4^{+}CD45RO^{+}) T cells, and CD69 expression. (c) Mean ± SEM peripheral blood (PB) and AT-SVF CD4^{+}CD45RO^{+} or CD4^{-}CD45RO^{+} expression (gated on CD3^{+} T cells) of uninfected healthy control donors (HC, $n = 4$) and HIV patients ($n = 3$–4, $^{*}P < 0.01$). (d) Mean ± SEM CD69 expression by peripheral blood and AT-SVF memory CD4^{+} and memory CD8^{+} T cells of HC donors and HIV patients ($^{*}P < 0.01$). (e) Detection of HIV DNA in patient AT-SVF cells and other tissues by nested PCR. Shown are representative second round bands of nested PCR reactions for tissues with overall detection frequency (total positive bands observed/total reactions tested) indicated in parentheses.

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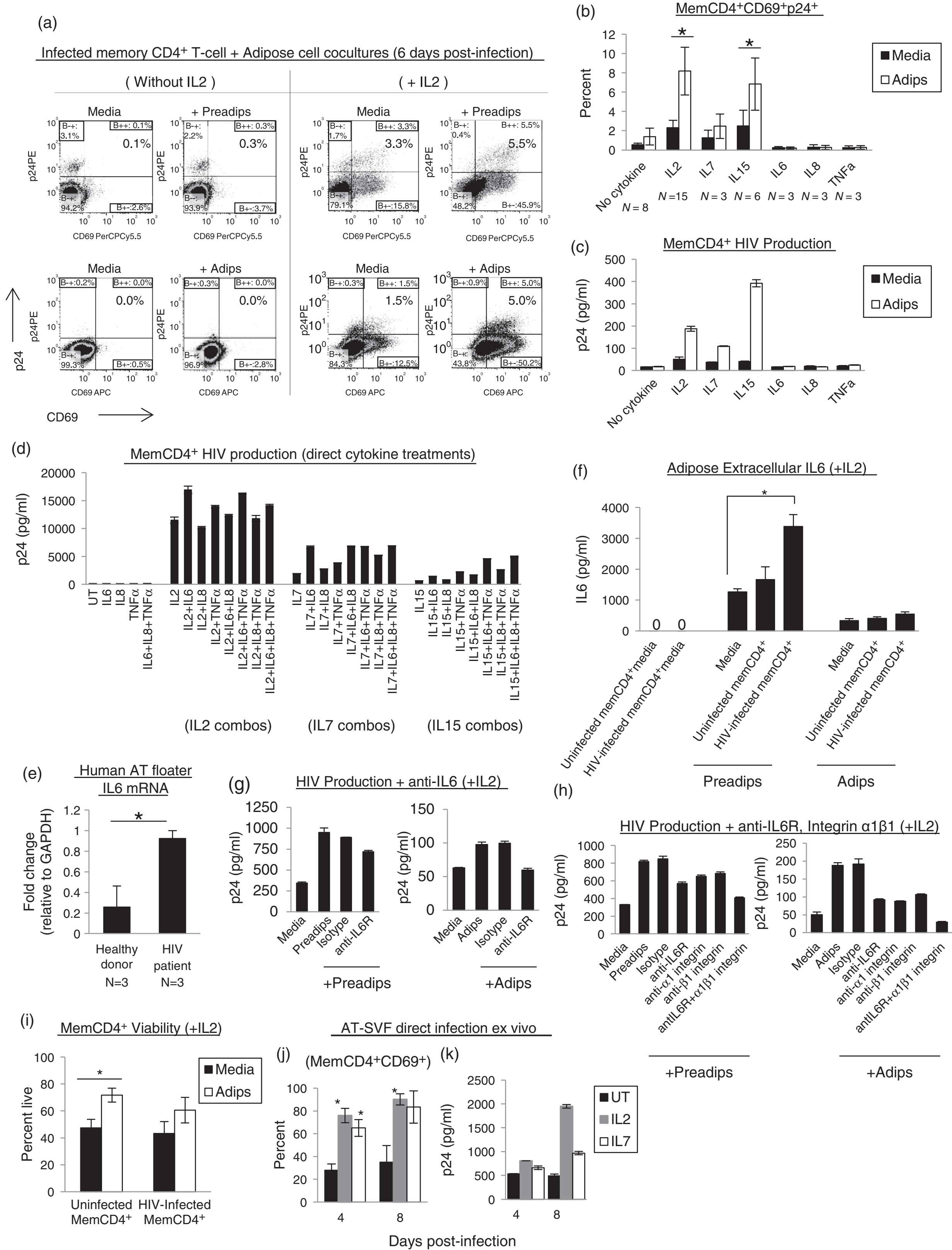

Fig. 2. Adipocytes enhance CD4$^+$ T-cell activation, viability, and HIV production. (a–c) Memory CD4$^+$ T cells from healthy donor peripheral blood were infected with HIV (strain NSN-SX), then 5×10^5 cells co-cultured with 2×10^5 pre-adipocytes or adipocytes for 5 days with or without 20 ng/ml IL2, IL7, or IL15. (a) CD69/p24 flow cytometry dot plots ($n = 6$) of HIV-infected memory CD4$^+$ T cells after 7 days co-culture with pre-adipocytes or mature adipocytes. (b) CD69$^+$p24$^+$ double-positive

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are major sources of IL6, and the combination of IL2 and proinflammatory cytokines induces HIV replication from latently infected $CD4^+$ T cells [21,22]. Combinations of IL7 or IL15 with pro-inflammatory cytokines increased both T-cell activation (data not shown) and HIV replication (Fig. 2d). More IL6 mRNA was found in fractionated mature adipocytes from adipose tissues of HIV patients compared with those from healthy controls (Fig. 2e, $n=3$), consistent with previous reports [19,23,24]. In coculture experiments, HIV-infected memory $CD4^+$ T cells increased preadipocyte IL6 secretion nearly three-fold ($P<0.01$, $n=3$, Fig. 2f). Blocking antibodies against IL6 and other candidate factors in coculture experiments showed that blocking IL6 and integrin-$\alpha1\beta1$ very late antigen-1 (VLA-1) signalling mitigated adipocyte-mediated increases in T-cell activation (data not shown) and HIV production (Fig. 2g,h). Blocking soluble IL6 reduced adipocyte-mediated enhancement of HIV production by nearly 10–30% (Fig. 2g), and blocking both IL6R and integrin $\alpha1\beta1$ abolished HIV production (Fig. 2h). These data suggest a role for IL6 and integrin $\alpha1\beta1$ signalling for adipose-induced T-cell activation and HIV replication (although promiscuous interactions with other integrin chains could also be involved). Adipocytes further enhanced viability of T cells from 43–47% to 61–72% after 8 days' coculture (Fig. 2i).

We lastly determined whether AT-SVF cells support productive HIV infection *ex vivo* (Fig. 2j,k). AT-SVF cells were isolated from healthy donor adipose tissues, then uninfected or infected with HIV (R5-tropic) for 24 h, washed and cultured with or without IL2 or IL7 for up to 8 days. $CD69^+$ expression by untreated, uninfected AT-SVF memory $CD4^+$ T cells declined to 28–35% (compared with >60% from fresh AT-SVF, Fig. 1d), but remained at 65–90% if cultured with IL2 or IL7 ($P<0.05$ compared to untreated, $n=2–4$, Fig. 2j). HIV production (p24 ELISA) by infected AT-SVF cells occurred without addition of IL2 or IL7, but the cellular source of productive infection was not determined (Fig. 2k). Thus, adipose cells can induce HIV replication, mediated by factors elevated in adipose tissue of HIV-infected persons.

Discussion

Adipose tissue is a likely sanctuary site for HIV in ART-treated patients. Adipose tissue contains activated memory $CD4^+$ T cells, the major cellular reservoir for HIV [1]. Adipose memory $CD4^+$ T-cell numbers declined relative to $CD8^+$ T-cells in HIV patients. Adipocytes potentiated $CD4^+$ T-cell activation and HIV replication *in vitro* in the presence of IL2, IL7 or IL15, cytokines known to be produced in adipose depots [25–27].

HIV provirus was detectable in AT-SVF from different fat depots (subcutaneous, abdominal visceral, deep neck) of all five patients studied, in association with decreased memory $CD4^+$ and increased $CD8^+$ T cells (Fig. 1). Inversion of the $CD4^+/CD8^+$ ratio is also observed in peripheral blood and GALT of HIV patients [28]. Memory T cells in adipose tissue of healthy donors and HIV patients expressed high levels of $CD69^+$, indicating activation. $CD69^+$-high expression typically distinguishes resting memory T cells in peripheral blood from T cells in tissues [7], suggesting that blood contamination of AT-SVF samples was unlikely. Precise determination of viral copy number was limited due to cell numbers, but each nested PCR replicate contained approximately 1×10^5 AT-SVF cell equivalents of DNA, of which 1–10% were memory $CD4^+$ T cells. Assuming one HIV copy per positive PCR product in AT-SVF $CD4^+$ T cells, there could be one copy per 1×10^4 $CD4^+$ T cells in adipose tissue, comparable to HIV DNA levels

Fig. 2. (*Continued*)
expression and (c) HIV production (one p24 ELISA, representative of 3–4 experiments) by infected memory $CD4^+$ T cells after 5 days co-culture with adipocytes and either IL2, IL7, IL15, IL6, IL8, TNFα, or without cytokines. (d) Induction of HIV replication by combinations of gamma-chain and proinflammatory cytokines. Memory $CD4^+$ T cells from healthy donor blood were infected with HIV (NSN-SX), followed by treatment with 10 ng/ml cytokines for 5 days (shown are p24 ELISAs of three experiments). (e) IL6 mRNA expression by the mature adipocyte fraction ('floaters') of AT from uninfected healthy control donors or HIV patients. IL6 expression levels relative to GAPDH ($^*P<0.05$, $n=3$). (f) Adipose IL6 production increase by infected memory $CD4^+$ T cells. Preadipocytes or adipocytes were cultured with IL2 medium alone, or with uninfected and HIV-infected memory $CD4^+$ T cells and IL2 for 6 days, followed by IL6 measurement. Shown are mean ± SEM extracellular IL6 ($^*P<0.05$, $n=3$). (g–h) Adipose IL6 and soluble ECM proteins induce HIV replication. Memory $CD4^+$ T cells were infected, then co-cultured with preadipocytes or adipocytes and IL2 for 5 days with blocking abs (5–10 μg/ml) against soluble IL6 (g), or IL6 receptor, integrin α1, and integrin β1 (h). Shown are p24 ELISAs of 3–4 experiments. (i) Viability of memory $CD4^+$ T cells during co-culture with adipocytes. Uninfected or HIV-infected memory $CD4^+$ T cells were co-cultured with adipocytes and IL2 for 7 days, followed by viability measurement. ($^*P<0.05$, $n=5–6$). (j–k) Healthy control donor AT-SVF cells treated with gamma-chain cytokines and HIV infected ex vivo. (j) CD69 expression by AT-SVF $CD3^+CD4^+CD45RO^+$ cells during culture with IL2 or IL7 ($^*P<0.05$ compared to UT, $n=2–4$). (k) HIV production by healthy control donor AT-SVF cells. AT-SVF cells were infected with HIV and cultured 4–8 days without or with IL2 or IL7 (shown are p24 ELISAs representative of 2–3 experiments).

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in other reservoirs [2,29]. However, the precise cellular source of this AT-SVF HIV, and contribution of infected macrophages, is still to be determined.

Adipose cells enhanced $CD4^+$ T-cell activation and HIV replication with gamma-chain cytokines and inflammatory factors (Fig. 2). IL2, IL7 and IL15 are expressed in lymphoid and nonlymphoid tissues, including adipose tissue, and regulate T-cell homeostatic stimulation, proliferation and HIV infection [25–27]. A systemic elevation of these cytokines is also observed during primary infection or following ART interruption [30]. Synergy between adipose tissue and these cytokines has important implications for HIV persistence in lymphoid tissues such as bone marrow, thymus and gut-associated lymphoid tissue, which are intimately associated with adipocytes [4–6]. IL6 expression is increased in adipose tissue in obesity and HIV-associated lipodystrophy [23,24], and expression of VLA-1 by memory CD4 T cells is increased during activation in inflamed tissues [31,32]. VLA-1 ligands include collagens and fibronectin, which enhance $CD4^+$ T-cell activation and HIV production [33,34]. In addition, adipose tissue reorganization during HIV lipodystrophy is partly due to breakdown of extracellular matrix and increased expression of collagens and fibronectin leading to fibrosis [19,35].

Adipose tissue may be a widespread sanctuary for HIV, and ongoing studies are investigating the replication-competence and infectiousness of AT-SVF virus, and whether adipose tissue presents a barrier to ART drugs. A better understanding of adipose tissue as a potential HIV reservoir and its mechanisms of viral induction will be important for effective viral eradication strategies.

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J.C. conducted experiments and wrote the manuscript. J.W.S. procured adipose tissue samples from study participants. D.J.L. and N.A. performed experiments and analysed data. D.I., X.Y., C.N., C.A.K., P.A.O., M.L.M. and J.E.B provided technical expertise and analysed data. A.B. and D.E.L. supervised the study and reviewed the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts of interest.

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