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Human adipose tissue as a reservoir for memory CD4 T cells and HIV

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Abstract

Objective—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 5 patients (4 ART-treated and 1 untreated). To determine if adipocytes stimulate CD4 T cells and regulate HIV production, primary human adipose cells were co-cultured 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 5 patients examined by nested PCR,

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Conflicts of interest: None.

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comparably to other tissues (PBMC, lymph node, or thymus). In co-culture experiments, adipocytes increased CD4 T cell activation and HIV production ∼2-3 fold in synergy with gamma-chain cytokines IL2, IL7, or IL15. These effects were mitigated by neutralizing antibodies against IL6 and integrin-α1β1. Adipocytes also enhanced T cell viability.

Conclusions—Adipose tissues of ART-treated patients harbor 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.

Keywords

Adipose tissue; Memory CD4 T cells; Cytokines; HIV replication; HIV reservoir

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/TCR, cytokines (IL2, IL7, IL15, IL6, and TNFα), 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 (AT) is a major endocrine organ with diverse functions and cellular composition. It is present mainly under the skin (subcutaneous AT) and around thoracoabdominal organs (visceral AT). The immune system is intimately associated with AT - every lymph node is encapsulated by AT, and adipocytes are abundant within bone marrow and in aging thymus gland [4-6]. AT is composed of mature adipocytes and a stromal-vascular fraction (SVF) that includes pre-adipocytes, 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 or CXCR3 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 AT could be a reservoir for HIV, and that adipocytes stimulate HIV production from CD4 T cells.

Methods

Isolation of adipose tissue stromal-vascular-fraction (AT-SVF) 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, PA), or from live donors undergoing elective surgical procedures with informed consent. For isolation of AT-SVF cells, 1-2g adipose tissue was minced and digested with collagenase (Sigma), mesh-filtered, and SVF cells pelleted [15]. AT-SVF cells were centrifuged on Ficoll-Paque to further increase lymphocyte yield (typically resulting ~2-5×10^5 cells per gram of adipose tissue).

Flow cytometry and nested PCR of AT-SVF 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 or BD Biosciences), and analyzed with appropriate controls using a Gallios Flow Cytometer (Beckman-Coulter). For nested PCR's, DNA was first extracted from AT-SVF cells with QIAamp Microkit (Qiagen). Nested PCR reactions were performed by 2 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 PBMC of healthy donors via EasySep kits (Stemcell Technologies). Primary pre-adipocytes and adipocytes were obtained from Zen-Bio (Research Triangle Park, NC).

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

For co-culture of memory CD4 T cells with adipose cells, 2×10^5 uninfected or infected memory CD4 T cells were seeded into transwells (0.4μm pore) in 6-well plates with 2×10^5 preadipocytes or adipocytes in lower wells so that the cells did not touch. Cells were co-cultured for indicated periods with appropriate reagents (10-20ng/ml cytokines or 5-10μg/ml blocking abs) (R&D Systems or Biolegend). Blocking mabs included CD49a (clone TS2/7), CD29 (clone TS2/16), and CD126 (clone UV4) (Biolegend).

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). Extracellular IL6 in co-culture experiments was measured by ELISA (eBioscience), 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 24hrs, washed, then cultured +/- 20ng/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 $P<0.05$ was considered significant.

Results

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

CD4 T cells and macrophages reside in AT of healthy humans where they are activated, undergo polarization, and regulate adipose physiology and metabolism [8,9,11,18]. In HIV infection, infected CD4 T cells and monocytes could traffic into AT to establish reservoirs [19,20]. To determine if HIV provirus is present in AT of patients, AT samples (subcutaneous and visceral) were acquired from 5 patients (3 live donors undergoing surgery, and 2 recently deceased patients), and from uninfected healthy control (HC) donors. Four patients were ART-treated (3 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 HC and HIV patients (gating scheme in Fig. 1b). Within the CD3 T cell population of HC, AT-SVF contained predominantly memory CD4+CD45RO+ cells (63.1±5.4%, n=4), whereas in peripheral blood these comprised 39.9±4.5% of CD3 T cells ($p<0.05$, Fig. 1c), which were greater than memory CD8 proportions (15.4±5.5% in AT-SVF and 18.2±2.7% in PB). However, the distribution of CD4 to CD8 T cells was reversed in AT-SVF of HIV patients (reduced CD4+CD45RO+ cells to 34.9±7.9%, CD4-CD45RO+ cells increased to 46.0±8.3%, $p<0.05$ compared to HC AT-SVF). AT-SVF memory T cells with CD69 were 61-72% in HC 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. $1\text{-}2\times10^5$ cell equivalents of DNA was used per reaction, and sensitivity determined using ACH2:PBMC ratios with detection limits of ~1 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 5 HIV patients with both primer sets (Fig. 1e), and detection frequencies were comparable to those in PBMC's or memory CD4 T cells (purified from peripheral blood), thymus, or mesenteric lymph nodes. The 2nd round PCR products were gel-purified and sequenced to assess HIV diversity, but phylogenetic analyses of Gag and Env sequences indicated no significant intra-patient differences among different tissues (data not shown). Thus, AT of HIV patients on ART harbors 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 if adipose cells (pre-adipocytes or adipocytes) affect HIV replication, primary adipose cells were co-cultured with infected memory CD4 T cells in transwell dishes

(purified from healthy donor blood and infected in vitro prior to co-culture). T cell activation (CD69 expression) and HIV production (p24) were measured.

Pre-adipocytes 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. Fig. 2a shows representative CD69/p24 dot plots of infected (R5-tropic) memory CD4 T cells after 7 days co-culture with pre-adipocytes or adipocytes +/- IL2. Compared to medium alone, adipocytes increased CD69 and p24 in infected memory CD4 T cells ∼2-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 co-cultures had no effect (Fig. 2b). Extracellular HIV production by memory CD4 T cells also increased 2-3 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 AT IL6 in enhancing HIV replication by memory CD4 T cells. Adipose cells 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 AT of HIV patients compared to those from HC (Fig. 2e, n=3), consistent with previous reports [19,23,24]. In co-culture experiments, HIV-infected memory CD4 T cells increased pre-adipocyte IL6 secretion ∼3-fold ($p<0.01$, n=3, Fig. 2f). Blocking antibodies against IL6 and other candidate factors in co-culture experiments showed that blocking IL6 and integrin-α1β1 (VLA-1) signaling 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 ∼10-30% (Fig. 2g), and blocking both IL6R and integrin α1β1 abolished HIV production (Fig. 2h). These data suggest a role for IL6 and integrin α1β1 signaling 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' co-culture (Fig.2i).

We lastly determined if AT-SVF cells supported productive HIV infection ex vivo (Fig. 2j-k). AT-SVF cells were isolated from healthy AT, then uninfected or infected with HIV (R5-tropic) for 24hrs, washed, and cultured +/- IL2 or IL7 for up to 8d. CD69 expression by untreated, uninfected AT-SVF memory CD4 T cells declined to 28-35% (compared to >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 AT of HIV-infected persons.

Discussion

AT is a likely sanctuary site for HIV in ART-treated patients. AT 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 5 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 AT 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 $\sim 1\times10^5$ 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 AT, comparable to HIV DNA levels 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 non-lymphoid tissues, including AT, 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 AT and these cytokines has important implications for HIV persistence in lymphoid tissues such as bone marrow, thymus, and GALT, which are intimately associated with adipocytes [4-6]. IL6 expression is increased in AT in obesity and HIV-associated lipodystrophy [23,24], and expression of VLA-1 by 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]. Additionally, AT reorganization during HIV lipodystrophy is partly due to breakdown of extracellular matrix, and increased expression of collagens and fibronectin leading to fibrosis [19,35].

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

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(a) HIV patient characteristics

Patient	Gender, Age	Viral load (RNA copies/ml)	CD4 count (cells per 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

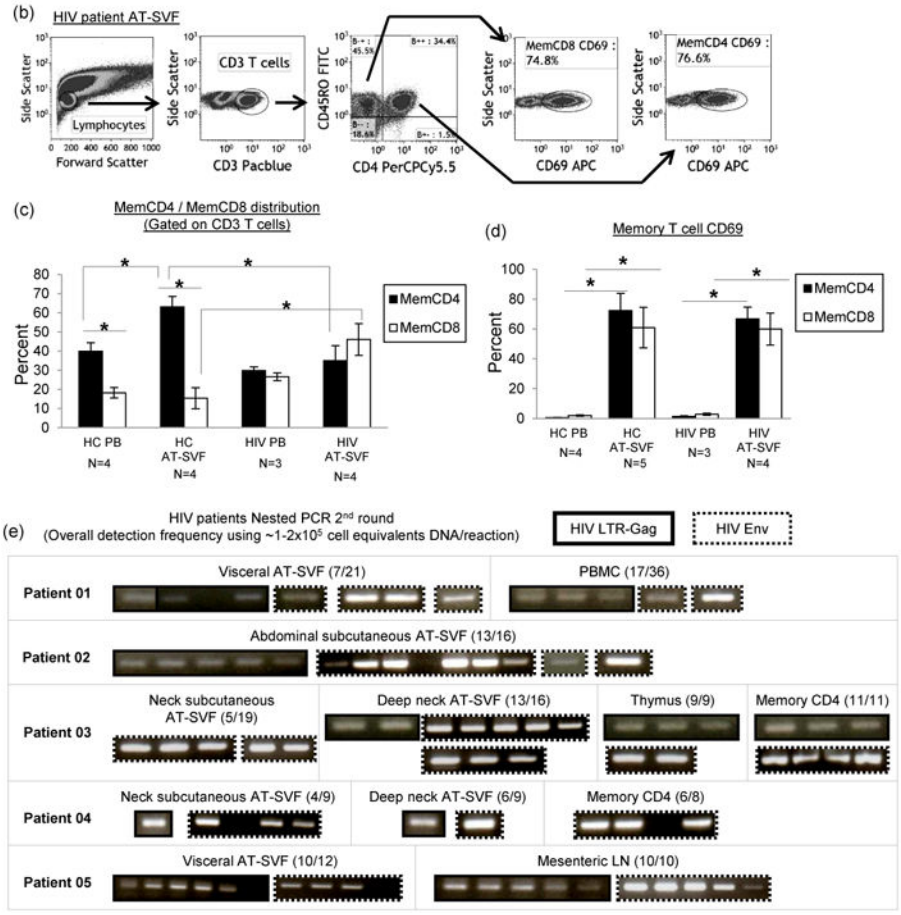

Figure 1. Memory CD4 T cells and HIV DNA in AT of HIV patients

(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) HIV DNA in patient AT-SVF cells by nested PCR. AT-SVF cells were extracted from adipose (visceral, subcutaneous, or neck regions), followed by DNA extraction from ~1-2×10^5 AT-SVF cells. DNA was also extracted from other tissues (PBMC or memory CD4 T cells from peripheral blood, thymus, or mesenteric lymph node). Nested PCR was conducted with primers for LTR/Gag and Env. Shown are representative 2nd round bands of nested PCR reactions for

tissues with overall detection frequency (total positive bands observed/total reactions tested) indicated in parentheses.

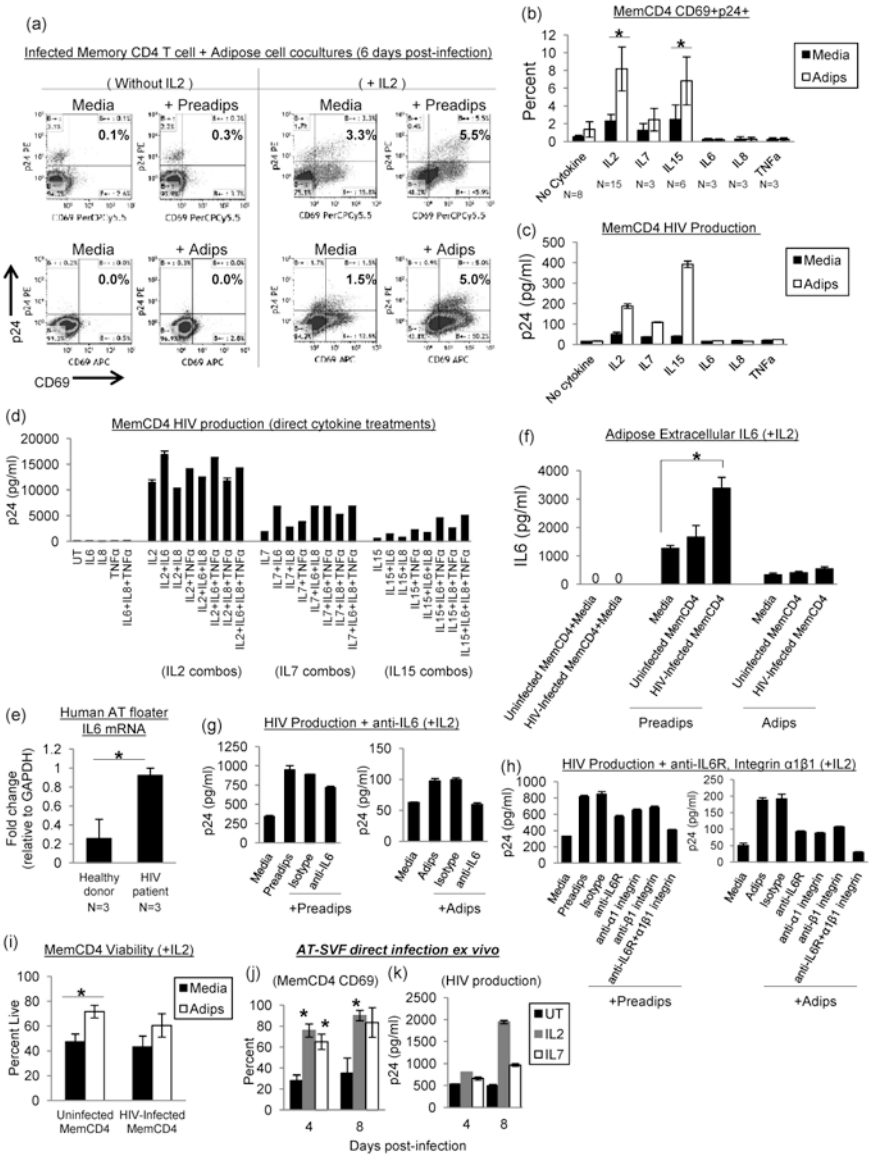

Figure 2. Adipocytes enhance CD4 T cell activation, viability, and HIV production

(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 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 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 peripheral blood and infected with HIV (NSN-SX), followed by treatment of 2×10^5 cells with 10ng/ml cytokines for 5 days (shown are p24 ELISA's of 3 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 increased by infected memory CD4 T cells. Pre-adipocytes or adipocytes (2×10^5) were cultured with IL2 medium,, or with uninfected or HIV-infected memory CD4 T cells (5×10^5) and IL2 for 6 days, then IL6 measured. 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 pre-adipocytes or adipocytes and IL2 for 5 days with blocking abs (5-10μg/ml) against soluble IL6 (g), or IL6 receptor, integrin α1, and integrin β1 (h). Shown are p24 ELISA's of 3-4 experiments. (i) Viability of memory CD4 T cells during co-culture with adipocytes. Uninfected or HIV-infected memory CD4 T cells (5×10^5) were co-cultured with adipocytes (2×10^5) and IL2 for 7 days, then viability measured. (*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 memory CD4 T cells during culture with IL2 or IL7. AT-SVF cells from healthy control donor adipose, then 5×10^5 cells cultured without or with 20ng/ml IL2 or IL7. CD69 expression of AT-SVF CD3+CD4+CD45RO+ cells (*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 (R5-tropic strain SF162 at 0.1 MOI for 24hrs), washed, then 5×10^5 cells cultured 4-8 days without or with 20ng/ml IL2 or IL7 (shown are p24 ELISA ‘s representative of 2-3 experiments).