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## ENHANCEMENT OF HIV PERSISTENCE BY ADIPOSE TISSUE

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ENHANCEMENT OF HIV PERSISTENCE BY ADIPOSE TISSUE

by

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ENHANCEMENT OF HIV PERSISTENCE BY ADIPOSE TISSUE

A  
DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
MD Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by  
Jacob P. Couturier, B.S.  
Houston, Texas

Date of Graduation  
August 2015

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*Everything I achieve in life is devoted to them.*

*Achieving a PhD would not be possible without the help of countless people*

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## ENHANCEMENT OF HIV PERSISTENCE BY ADIPOSE TISSUE

Jacob P. Couturier, BS

Advisory Professor: Dorothy E. Lewis, PhD

A major challenge for HIV eradication is the persistence of cellular and anatomic viral reservoirs. The primary cellular reservoirs for HIV, memory CD4 T cells and macrophages, traffic into various tissues where most viral replication occurs, or in the case of virally-suppressed antiretroviral-treated patients, the virus becomes dormant and protected from drugs and immune responses. The major tissue reservoirs for HIV include lymphoid tissues such as lymph nodes, bone marrow, and gastrointestinal lymphoid tissues, but adipose tissues could potentially harbor HIV as well. Adipose tissues are ubiquitous connective tissues and major sites of infiltration and immune activity by T cells and macrophages. As adipose tissue regulates CD4 T cell migration and functions, we hypothesized that adipocytes may affect HIV replication and serve as a reservoir for infected immune cells. To study the role of adipocytes for HIV infection, HIV-infected memory CD4 T cells were cocultured with primary adipocytes in vitro to determine if adipocytes influence HIV replication. Adipocytes alone did not affect HIV replication, but via IL6 and integrin ligands, did enhance T cell activation and HIV replication in cooperation with the ubiquitous common gamma-chain cytokines, IL2, IL7, or IL15. Adipocytes further enhanced T cell viability and even mitigated the viral suppressive effects of some antiretroviral drugs. To determine if adipose depots are sites for HIV persistence in vivo, adipose tissues of infected humans and rhesus macaques were examined for memory CD4 T cells and viral DNA. Within the stromal-vascular-fraction cellular component of adipose tissues (AT-SVF), activated memory CD4 T cells and provirus were readily detectable in all infected humans (n=5) and rhesus macaques (n=8) so far examined, and at frequencies comparable to other major viral tissue reservoirs. Thus, adipocytes have the capacity for promoting HIV replication, and adipose tissues of humans and monkeys appear to be sites of chronic immune

activation and sanctuaries for HIV and SIV, which pose additional obstacles for viral eradication.

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# Chapter 1

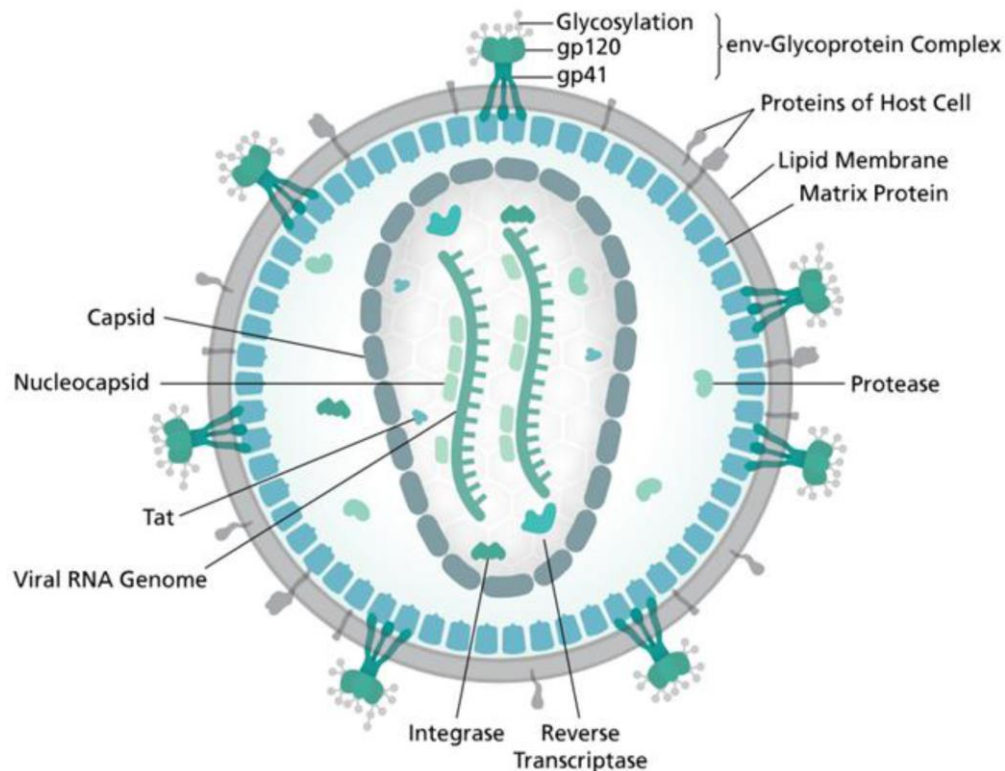
## General Introduction

### ***1.1. Overview of HIV-1 infection and pathogenesis***

Viruses are obligate intracellular microbes that persist by seizing control of protein synthesis and functions of a host cell, typically resulting in host cell dysfunction or death. Of such parasites, the Human Immunodeficiency Virus 1 (HIV-1) remains one of the most mystifying and pernicious during infection. HIV-1 is a lentivirus within the Orthoretrovirinae subfamily of the Retroviridae family [1]. In an infectious virion particle, the genome consists of 2 single strands of positive-sense RNA (~9,800 bps) containing 9 genes which encodes for ~15 proteins with unique functions during host cell infection (Fig. 1.1) [2]. This viral RNA is encapsidated by capsid proteins, and further enclosed with other critical enzymes such as reverse transcriptase, integrase, and protease by an outer membrane inserted with envelope glycoproteins gp120/gp41 which allow for binding and infection of target cells via CD4 and chemokine receptors. Based on the envelope protein sequence and geographic distribution, HIV-1 is classified into 4 major groups (M, N, O, and P) and subtypes, with group M being the most predominant infection leading to Acquired Immune Deficiency Syndrome (AIDS) [3]. HIV-2 is a related but less pathogenic form of HIV-1, is mostly endemic to West Africa, and more closely related to the primate counterpart of HIV-1, simian immunodeficiency virus (SIV) [4-5]. HIV-1 was determined to be the causative agent of AIDS in 1984 by Luc Montagnier, Françoise Barré-Sinoussi, and Robert Gallo, following the initial observations of AIDS signs and symptoms in patients in 1981. Despite substantial research conducted and virological and immunological knowledge acquired since its discovery, infection with HIV still remains incurable, although manageable control of viral replication has been achieved with antiretroviral therapy (ART).

**Figure 1.1. Composition of an infectious HIV-1 virion.**

A typical HIV-1 particle possesses RNA and several proteins and enzymes essential for infection and replication in a CD4 T cell or macrophage. The outer membrane is embedded with heavily glycosylated envelope gp120/gp41 glycoproteins (required for binding to the host cell CD4 receptor and CCR5 or CXCR4 coreceptors, and subsequent entry), which encapsulates the protease enzyme (required for posttranslational modifications of viral mRNA and virion maturation) and the inner capsid. The inner capsid contains 2 strands of single-stranded RNA, and the reverse transcriptase (required for conversion of viral RNA to DNA) and integrase (required for integration of viral DNA into the host genome) enzymes. (© Thomas Splettstoesser/Wikimedia Commons/ license CC-BY-SA-4.0).



## **1.2. Primary host cells for HIV-1 infection and replication**

CD4 T cells and macrophages, essential coordinators of adaptive and innate immunity, are the primary targets for HIV infection and replication (Fig. 1.2). HIV transmission occurs mostly by sexual contact through infectious fluids (semen), and less frequently by mechanical exposure (needlesticks), maternal transmission (during pregnancy or breast-feeding), or blood transfusions. In the case of sexual transmission within the vaginal mucosa, infectious virions or infected cells penetrate through the epithelium and into the lamina propria where mucosal CD4 T cells and macrophages become exposed to virus (either directly or through interactions

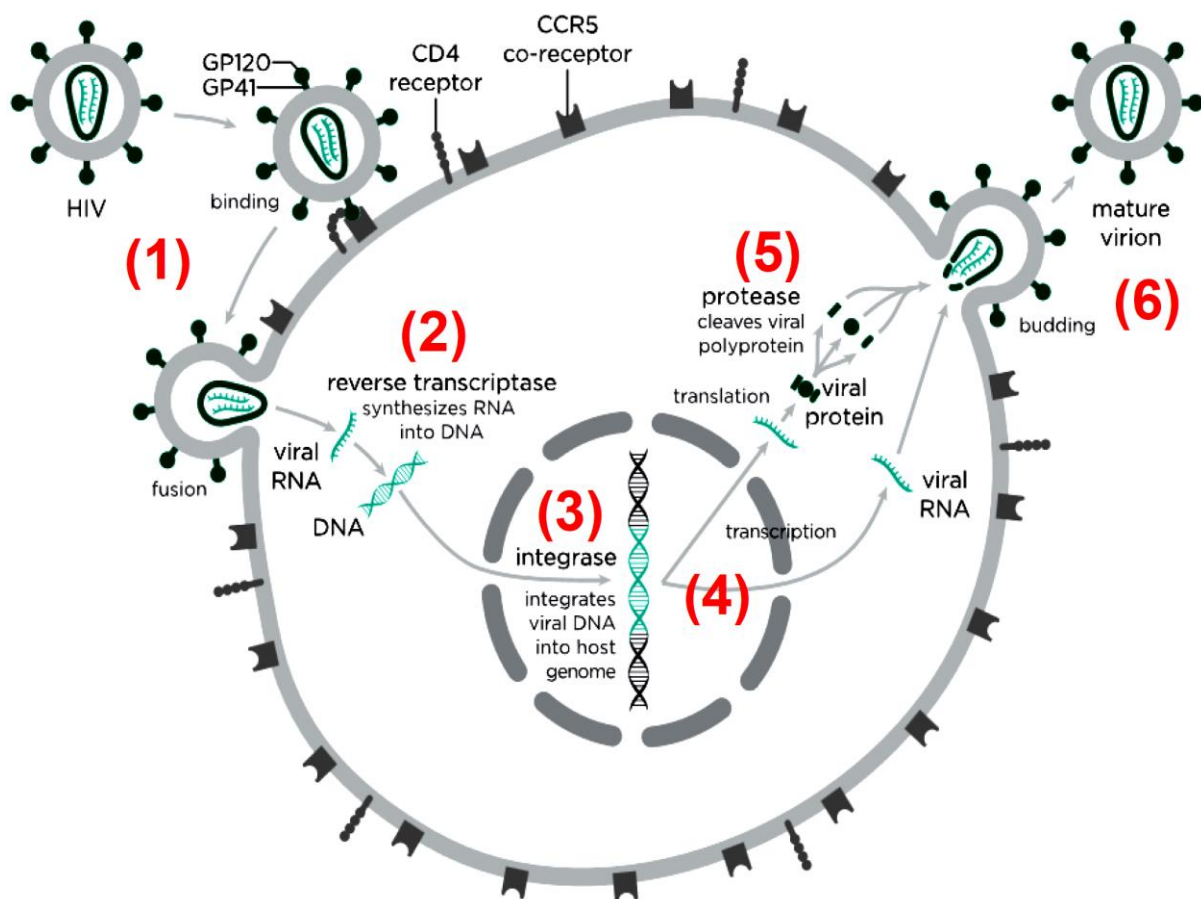
between CD4 T cells and dendritic cells) [6]. These infected cells migrate to lymph nodes where virus is further transmitted to additional target cells, which is then followed by widespread systemic dissemination. CD4 T cells and macrophages are the primary hosts firstly because they express the main receptors for virion binding and entry, CD4 and CCR5 or CXCR4. CD4 is first bound by the viral envelope gp120 protein, followed by additional binding of gp120 to the coreceptors CCR5 or CXCR4 (coreceptor specificities that are used to classify HIV strains as R5-, X4-, or R5X4 dual-tropic), which then induces further conformational changes and stabilization by gp41. R5 strains predominate during most infections, whereas X4 and R5X4 strains typically manifest during chronic infection and AIDS and are associated with greater disease progression [7]. The importance of the CCR5 coreceptor is best demonstrated in “elite suppressors”, individuals naturally resistant to HIV infection due to CCR5 homozygous mutation and lack of cell surface expression, as well as by the “Berlin patient”, the only patient cured of HIV in 2008 after receiving a bone marrow transplantation from a CCR5-Δ32 donor in conjunction with aggressive myeloablative chemotherapy [8]. Dendritic cells are also crucial players during HIV infection, but believed to be more important for binding and transmitting virus to CD4 T cells during primary infection [9].

HIV replication (with minor differences between CD4 T cells and macrophages) involves the general steps of reverse transcription of viral RNA into DNA and nuclear transport, integration of viral DNA into the host genome, proviral transcription of integrated DNA into mRNA and translation, and assembly and release of new virions, processes that hijack numerous host proteins leading to cellular dysfunction and death (Fig. 1.2). A variety of accessory or non-conventional hosts for HIV including epithelial cells (intestinal, renal, oral, mammary, or cervical), astrocytes, hepatocytes, keratinocytes, myocytes, osteoclasts, adipocytes, fibroblasts, endothelial cells, NK cells, CD8 T cells, B cells, or bone marrow stem cells have the capacity for being infected or sequestering virions, but are largely incapable of transcribing integrated provirus and producing new infectious particles as do CD4 T cells and macrophages (ie. being truly *productively infected*) [10-26]. These other cell types are unlikely

to be significant sources of virus in chronically-infected persons on virally-suppressive ART, or during viral rebound following ART interruption. Thus, CD4 T cells and macrophages remain the principal target cells for antiviral strategies.

**Figure 1.2. Overview of HIV-1 replication.**

A complete, productive infection of an activated CD4 T cell or macrophage by an infectious HIV virion occurs by several main steps. (1) Fusion and entry: the virion envelope glycoprotein (gp120/41) binds to the host cell CD4 and CCR5 or CXCR4 receptors, followed by fusion of the virion and host cell membrane and release of viral single-stranded RNA and reverse transcriptase enzymes into the cytoplasm. (2) Reverse transcription: viral RNA is reverse-transcribed into cDNA by reverse transcriptase. (3) Nuclear import and viral integration: viral cDNA is transported into the host cell nucleus and integrated into the host genome by the integrase enzyme. (4) Proviral transcription and mRNA export: induction of RNA transcription, mediated mainly by viral Tat protein, and export of transcripts into the cytoplasm. (5) Viral RNA translation and polyprotein processing: translation of RNA transcripts and cleavage and maturation of viral proteins mediated by protease enzymes. (6) Virion assembly and release: packaging of viral proteins and RNA, followed by budding from the host cell membrane. Replication requires the function of numerous host cell factors and usually results in host cell death. Antiretroviral drugs are designed to inhibit the function of critical proteins (envelope entry inhibitors) and enzymes (reverse transcriptase, integrase, and protease inhibitors). By contrast to the activated state, most steps of replication are impaired or blocked in resting CD4 T cells or macrophages, resulting in abortive and non-productive infection, or establishment of latency, and the host cell is typically spared from apoptosis until reactivation of latent provirus. (© Thomas Splettstoesser/Wikimedia Commons/ license CC-BY-SA-4.0).



### **1.3. Stimulants of HIV replication**

HIV replication in CD4 T cells and macrophages is driven by a variety of stimuli, and replication occurs in both lymphoid and non-lymphoid tissues. Memory CD4<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> T cells are the primary hosts for replication and latency establishment, whereas naïve CD4 T cells are productively infected to a much lesser extent [27-28]. Particular subsets of effector and central memory CD4 T cells such as Th1/Th2, T regulatory (Treg), Th17, T follicular helper (Tfh), or CD4<sup>+</sup> T memory stem cells (TSCM) further differ in their capacities to support HIV replication and latency, but are generally comparable as HIV hosts, and replication in the pan-memory CD4 T cell pool accounts for the majority of the viral load in infected persons [29-31]. These infected memory CD4 T cells traffick into tissues such as lymph nodes and gastrointestinal lymphoid tissue (GALT) where replication and viral loads are highest [32-33]. HIV infects monocytes, but due to limiting factors such as low levels of dTTP's and Cyclin T1/P-TEFb transcriptional complexes, or antiviral activities of APOBEC3G enzymes, replication is restricted until the monocyte differentiates into macrophages in tissues [34]. The most important physiological stimuli for inducing HIV replication are believed to include CD3(TCR)/CD28 costimulation upon encounter with antigen-presenting cells (APC) such as dendritic cells or macrophages, cytokines such as IL2, IL7, IL15, IL6, IL8, and TNF $\alpha$ , and microbial products such as LPS that ligate toll-like receptors (TLR) expressed by T cells [35-41]. These stimuli activate signaling pathways such as protein kinase C (PKC- $\theta$ ), NF- $\kappa$ B, NF-AT and AP1, or MAP kinases (p38, ERK, or JNK) that positively regulate the key steps of HIV replication (reverse transcription, integration, transcription and translation, and virion assembly and release). A variety of accessory factors such as extracellular matrix proteins (collagens and fibronectin), certain chemokines (CCL5 and CCL19), metabolic intermediates (free fatty acids), or interactions with non-lymphoid cells (epithelial and endothelial cells) can further promote HIV replication alone or in conjunction with CD3/TCR activation or IL2/IL7/IL15 stimulation [42-54]. The diversity and ubiquitous nature of these stimuli all but ensures robust

HIV replication in CD4 T cells and macrophages in persons not receiving antiviral therapy (with the exception of elite suppressors), eventually leading to immune deficiency and AIDS.

#### **1.4. Detection methods of HIV infection and replication**

The viral load and replication capacity of HIV in patient cells and tissues are typically assessed by measurement of viral DNA and RNA. In ART-treated persons with low viral loads, nested PCR for HIV DNA tends to be more sensitive and reliable for detection compared to conventional PCR due to higher use of reaction cycles [55]. Additionally, PCR's that assay for particular DNA forms, such as 1- or 2-LTR circles (reverse-transcribed cDNA that fail to integrate into the host genome resulting in end-joining of LTR's), or the *Alu*-LTR real-time PCR (which distinguishes unintegrated viral cDNA from integrated provirus by amplifying genomic regions that span both the viral LTR and host *Alu* repeats), can better define particular steps of replication [56]. The detection of RNA is more indicative of active replication, and standard clinical assays utilize real-time PCR to measure viral RNA in plasma to monitor efficacy of antiviral treatments, with detection limits of ~50 copies/ml indicating viral suppression. DNA and RNA measurements usually target the Gag gene for PCR amplification and detection due to its conserved nature compared to other HIV genes such as Env. PCR products can further be purified, sequenced, and analyzed by phylogenetic methods to confirm assay specificity, study evolutionary patterns and mutations, or assess inter- or intra-patient differences of viral species. Newer technologies such as digital droplet PCR enhance quantitative and sensitive detection of HIV compared to traditional PCR assays [57]. HIV DNA and RNA levels are further associated with disease progression and correlated with inflammatory biomarkers and immune status, such as IL-6, TNF $\alpha$ , C-reactive protein (CRP), LPS, T cell activation markers (CD25, CD69, CD38, HLA.DR), and CD4 counts [58-59]. Measurement of the p24 capsid protein in vivo by ELISA is rarely conducted due to its limited expression compared to DNA/RNA levels, but is often used to assess HIV production during in vitro experiments.

An important limitation of DNA/RNA measurements is their inability to determine the replication-competence or infectiousness of integrated proviruses. Since the vast majority of proviruses are either non-inducible, or inducible but result in defective or non-infectious virions, quantitative viral outgrowth assays (Q-VOA) were developed to more accurately assess these viral parameters [60-62]. This method involves purification of resting CD4 T cells from patient tissues (usually peripheral blood), a small fraction of which harbors latent provirus, followed by strong activation such as with PHA+IL2 and allogeneic PBMC's to maximally induce replication of integrated proviruses. These activated patient cells are then cocultured with fresh allogeneic pre-activated CD4 T cells from healthy donors, and any patient CD4 T cells harboring inducible replication-competent provirus releases infectious virions that can subsequently infect the healthy donor feeder CD4 T cells, a 2-3 week process that results in increasing viral production and monitored by ELISA measurement of extracellular p24. HIV detection methods are constantly being improved, but current challenges include more rapid detection for early diagnosis during primary infection, enhancement of detection sensitivities, and more accurate determination of replication-competent versus defective viral gene expression.

### ***1.5. HIV latency, reservoirs and sanctuaries***

A principal reason underlying the difficulty of HIV eradication is latency. Viral latency occurs following infection in which productive replication ceases and the viral genome assumes a state of dormancy, but still retains the capacity for reactivation and replication upon optimal conditions [63]. For example, herpesviruses such as CMV or EBV are amongst the most widespread viruses in humans that establish latent, but mostly benign, infections during which latency is maintained as extra-chromosomal episomes [64-65]. These latent genetic elements can then be reactivated for productive replication upon exposure of the host cell to appropriate stimuli (ligation of the B cell receptor in the case of EBV reactivation). By contrast, HIV establishes proviral latency in which the reverse-transcribed cDNA becomes integrated as an intact provirus within the host cell genome. Latent infection occurs in CD4 T cells and

monocytes, but much less is understood regarding monocyte/macrophage latency, and reactivation of latently-infected CD4 T cells are believed to account most for viral rebound and influence HIV pathogenesis [66].

### ***1.6. HIV latency in resting CD4 T cells***

In resting memory CD4 T cells, HIV latency can occur as either pre-integration or post-integration latency. Pre-integration latency is a situation in which the reverse-transcribed DNA fails to be imported into the nucleus via the pre-integration complex (PIC), or fails genomic integration following nuclear import, and usually occurs in resting or minimally-activated cells due to low levels of critical metabolic products such as ATP and dNTP's [67]. These unintegrated products accumulate as linear cDNA or LTR circles, and are highly labile with half-lives of ~1 day. However, in an optimally stimulated cell, post-integration latency predominates in which nuclear import and genomic integration of the cDNA occurs, resulting in a stable, inducible provirus, and thus marking establishment of post-integration latency as the "point of no return" following infection. Numerous in vitro and in vivo models have been developed to better understand HIV latency (mostly post-integration), with the general goals of being able to quantify latently-infected cells and reservoir size, study signaling pathways and mechanisms of reactivation, and assess immune responses against latently-infected cells (such as by antiviral CD8 T cells) [68]. Amongst the important findings from latency studies are the following; post-integration latency becomes established when activated CD4 T cells return to a resting state following infection; latently infected memory CD4 T cells can survive for years and are present in multiple tissues (possibly most abundant in gastrointestinal lymphoid tissue); latent provirus is inducible by CD3/TCR costimulation, cytokines, and TLR ligands; and lastly, the reservoir size may be significantly larger (up to ~60-fold) than previously believed if non-inducible provirus are considered [61,69]. The frequency of latently infected CD4 T cells is only approximately 1 out of 1 million in ART-treated, virally-suppressed patients [70]. Additionally, newly released virions are highly labile with half-lives of less than 24hrs, and the vast majority

of these virions are defective or replication-incompetent, with estimates of only ~1-10% being infectious [71]. But despite the substantial obstacles confronting HIV replication, the virus still maintains a permanent, infectious presence in HAART-treated patients due to the presence of a very few latently-infected CD4 T cells.

### ***1.7. Antiretroviral compounds and combination drug (cART) therapies***

Once a person is infected with HIV, viral reservoirs become established and lifelong treatment with antiretroviral drugs is required to suppress viral replication and maintain normal levels of CD4 T cells and immune functions. The surprisingly quick establishment of the viral reservoir during acute infection was recently demonstrated in rhesus macaques, in which infected monkeys that were treated with ART as early as 3 days post-infection still demonstrated viral rebound following ART interruption, suggesting that a permanent reservoir can be established within a few days of infection [72]. Thus, due to the current inability to detect HIV so early after infection, subsequent HAART administration alone will never be a solution for HIV eradication.

Antiretroviral drugs are classified based on the viral replication step or enzyme being targeted such as binding/entry, reverse transcription, integration, or protease cleavage/virion maturation) [73]. Main classes include entry/fusion inhibitors (Maraviroc and Enfuvirtide, for example), nucleoside (Zidovudine and Tenofovir) or non-nucleoside (Rilpivirine and Efavirenz) reverse transcriptase inhibitors, integrase inhibitors (Dolutegravir and Raltegravir), or protease inhibitors (Atazanavir and Saquinavir). The systemic circulation and pharmacokinetics of antiretroviral drugs are highly complex, being influenced by factors such as drug half-life, binding by plasma proteins, or drug-drug interactions and synergies. These agents are most effective as 2-3 drug combinations (cART) and usually administered daily, with newer compounds continually under development. However, a major limitation of these therapies is their efficacy only during active replication (ie. when viral enzymes are actually synthesized and exposed to drugs for inactivation), and thus precluding any activity against latent HIV in resting

CD4 T cells [74]. Many of these drugs are also associated with numerous side effects, long-term toxicities, and development of antiviral resistance.

### ***1.8. Alternative antiretroviral strategies***

To overcome the limitations of cART therapy, other novel strategies to eradicate HIV are being explored. Although vaccine development remains a top priority due to its historical success, the extraordinary diversity of HIV strains and high mutation rate of proteins such as the envelope gp120/41, are amongst some of the many challenges preventing development of an effective HIV vaccine. Newer therapeutic strategies include the use of histone deacetylase inhibitors (HDACi) in conjunction with cART, as well as T cell immunotherapies. HDACi's are compounds that impair histone deacetylase activity (deacetylation of histone lysines which promote chromatin condensation and inhibition of gene expression), consequently relieving epigenetic suppression and allowing proviral transcription in an infected CD4 T cell. Examples of HDACi's under investigation as HIV latency-reversing agents (LRA's) include vorinostat, romidepsin, bryostatin, and panobinostat, with the goal of inducing viral replication (without concomitant activation of infected cells), followed by subsequent elimination by cART drugs or antiviral CD8 T cells [75]. Immunotherapeutic strategies involve genetic modification of patient T cells. For example, the harvest of autologous bone marrow hematopoietic stem cells or CD4 T cells, followed by genetic deletion of the CCR5 coreceptor ("CCR5-editing") and reinfusion into patients is being explored, in essence recapitulating the method of HIV eradication from the Berlin patient [76]. Additionally, the use of CAR-modified T cells (chimeric antigen receptor), which have demonstrated effectiveness against certain cancers, are being investigated in which CD4 and CD8 T cell CAR specificities are targeted toward HIV proteins such as gp120 [77]. The development of antiretroviral therapy has been one of the greatest achievements in combating HIV, but due to ineffectiveness against latently infected CD4 T cells, the development of alternative treatments towards a sterilizing or functional cure remains an ultimate priority for HIV research.

### **1.9. Anatomic compartmentalization of HIV**

Further compounding the difficulty of HIV eradication are the anatomic reservoirs or sanctuaries where infected CD4 T cells or macrophages reside. The term “reservoir” typically refers to infected CD4 T cells and monocytes/macrophages, whereas a “sanctuary” is considered to be an anatomic location where infected cells may be protected from ART drugs or immune responses such as antiviral CD8 T cells [78-79]. Virtually every type of major tissue or organ harbors HIV-infected cells in ART-treated persons, and some of these locations present unique obstacles, such as blood-tissue barriers or cellular drug transport mechanisms, for antiretroviral drug penetration and activity. A well-known sanctuary site, for example, includes CNS tissues such as the brain which hinder penetration of some ART drugs due to the blood-brain barrier [80]. Similarly, the male genital tissue may also represent a sanctuary site due to a “blood-testis” barrier, although this idea has been debated. Some sanctuaries may also obstruct immune responses as in the case of T follicular helper (Tfh) cells, CD4 subsets that enhance B cell activation. Tfh cells are recently reported to be more productive HIV hosts compared to other CD4 subsets, and further shown in ART-treated rhesus macaques to be inaccessible to antiviral CD8 T cell killing due to the inability of CTL’s to traffic into B cell follicles [81]. The ability to more precisely measure extracellular and intracellular concentrations of ART drugs in extravascular tissue has further enhanced our understanding of drug penetration and distribution. Recently reported comparative measurements of intracellular ART levels between peripheral blood and lymph node leukocytes showed significantly lower ART concentrations in lymph node cells in association with higher HIV RNA levels, suggesting that inadequate drug infiltration within lymphatic tissues contributes to viral persistence [82]. Lastly, a recently developed immunoPET method for examining whole body SIV localization in rhesus macaques (via Gp120 probes) has revealed additional surprises for HIV compartmentalization by demonstrating SIV accumulation within the nasal cavity of ART-treated monkeys [83]. Thus, dissemination of HIV by infected cells is likely more widespread

than previously believed with more novel sanctuaries yet to be identified, and many of these compartments pose tissue-specific challenges for antiretroviral drug efficacy.

#### **1.10. Recent developments in HIV cure research**

The importance of a better understanding of HIV latency and reservoirs and hideouts is perhaps emphasized by several highly publicized recent cases of viral remission and relapse in patients. In the case of the “Boston patients”, two HIV-infected adult cancer patients underwent allogeneic hematopoietic stem cell transplantation in conjunction with ART treatment in an attempt to replicate the treatment success of the Berlin patient [84]. However, these cases importantly differed from the Berlin patient in which the Boston patients received a less aggressive chemotherapeutic regimen compared to the Berlin patient, as well as the Berlin patient receiving CCR5-  $\Delta$ 32 donor cells, whereas the Boston patients received cells expressing normal wild-type CCR5. Although virus was undetectable in both patients for 3 and 5 years after HSCT and continued ART treatment (as determined by clinical assays measuring viral DNA/RNA in blood and rectal tissue, as well as Q-VOA assays), viral rebound occurred 12 and 32 weeks following ART interruption. Amongst the possible reasons cited for treatment failure were the presence of cellular reservoirs such as memory CD4 T cells and macrophages prior to HSCT that contributed to viral rebound. In the case of the “Mississippi baby”, a perinatally infected infant was administered ART within 30 hours of birth [85]. Mainly due to the lack of treatment adherence, ART was stopped at 18 months of age, but virus remained surprisingly undetectable for over 2 years after treatment interruption. However, at the age of 4, and over 2 years without ART treatment, viral rebound was observed. Based on the initial reports of the Mississippi baby, an infant in Italy was also administered ART within 12 hours of birth (the “Milan baby”) [86]. But when ART was stopped at the age of 3, viral rebound was observed within 2 weeks of ART interruption. Similarly, the unpublished cases of the “Canadian babies” involved early ART treatment, but viral rebound also occurred upon ART interruption. These adult and pediatric cases did demonstrate that ART initiation during a time

when the viral reservoir is low or weakly established can result in substantially prolonged periods for viral rebound (since detectable plasma RNA and viral rebound usually occurs within 2 months of ART interruption in adults) and lower viral loads [87]. But despite the undetectability of viral DNA and RNA in blood using sensitive detection methods and Q-VOA assays during ART treatment, viral rebound eventually occurred in these patients, possibly due to reactivation of latent HIV in lymphoid and non-lymphoid tissue reservoirs and sanctuaries. These cases have provided invaluable information for HIV cure research, but highlight that much more is still to be learned regarding HIV replication and systemic persistence. *However, a prevalent tissue that has been overlooked for its role in HIV persistence is adipose tissue and is the basis of the present thesis.*

### **1.11. Overview of adipose tissue**

Historically regarded as primarily connective tissue with important endocrine and thermoregulatory functions, research throughout the past decade have revealed adipose tissue to be far more dynamic in function and influence on other physiological systems. Broadly, the primary anatomic function of adipose tissue is to insulate and protect local organs and tissues, and the primary metabolic function is to accommodate energy requirements of cells and tissues mainly by storing triglycerides and releasing free fatty acids. Consistent with these roles, adipose tissue is ubiquitous throughout the human body, and a healthy lean adult typically possesses ~30-50 kilograms of total body fat. But beyond their fundamental physiological roles, the functions of adipose cells can be complex, exerting either ameliorative or exacerbative effects upon inflammation and disease.

At the most general level, the two types of adipose tissue are white and brown adipose tissue (WAT and BAT), color-denoted as such due to the higher mitochondrial content of BAT. WAT dominates the human body and is the most familiar type when fat is mentioned, whereas BAT is present mostly during infancy with the main purpose of heat generation since infants lack adequate skeletal muscle for shivering thermogenesis. However, recent studies challenge

some traditional perceptions of BAT, notably the demonstration that adults possess more functional BAT than previously believed, mainly in the neck and supraclavicular regions [88]. Additionally, more specialized subsets of white adipose cells with developmental potential during cold exposure and possessing features of BAT cells (ie. higher mitochondrial content, and expression of critical BAT genes such as UCP1 and PGC-1 $\alpha$ ), have been described and denoted as “beige”, “brite”, or “inducible BAT” [89]. White adipose tissue is further sub-categorized as either subcutaneous (SAT) or visceral (VAT) adipose tissue in reference to the anatomic location. SAT underlies the dermal layer of the skin, and VAT surrounds the internal organs with omental and mesenteric VAT being in greatest abundance in the abdominal cavity. Due to its intimate relationship with the visceral organs and their energy demands, VAT is functionally more dynamic compared to SAT. For example, VAT is substantially more vascularized compared to SAT, facilitating more efficient circulation of metabolic mediators to visceral organs. However, VAT is also more strongly associated with metabolic disorders such as obesity, insulin resistance and diabetes, as well as a more abundant source of inflammatory cytokines and mediators, compared to SAT [90-92]. Less appreciated, but still physiologically important, are the minor deposits of adipocytes within lymphoid and non-lymphoid tissues such as in bone marrow and lymph nodes, interspersed throughout skeletal muscle, and surrounding the heart (pericardial). Thus, adipose tissues exhibit depot-specific functional differences, but such a prevalent nature of adipose cells highlights their influence on physiological systems.

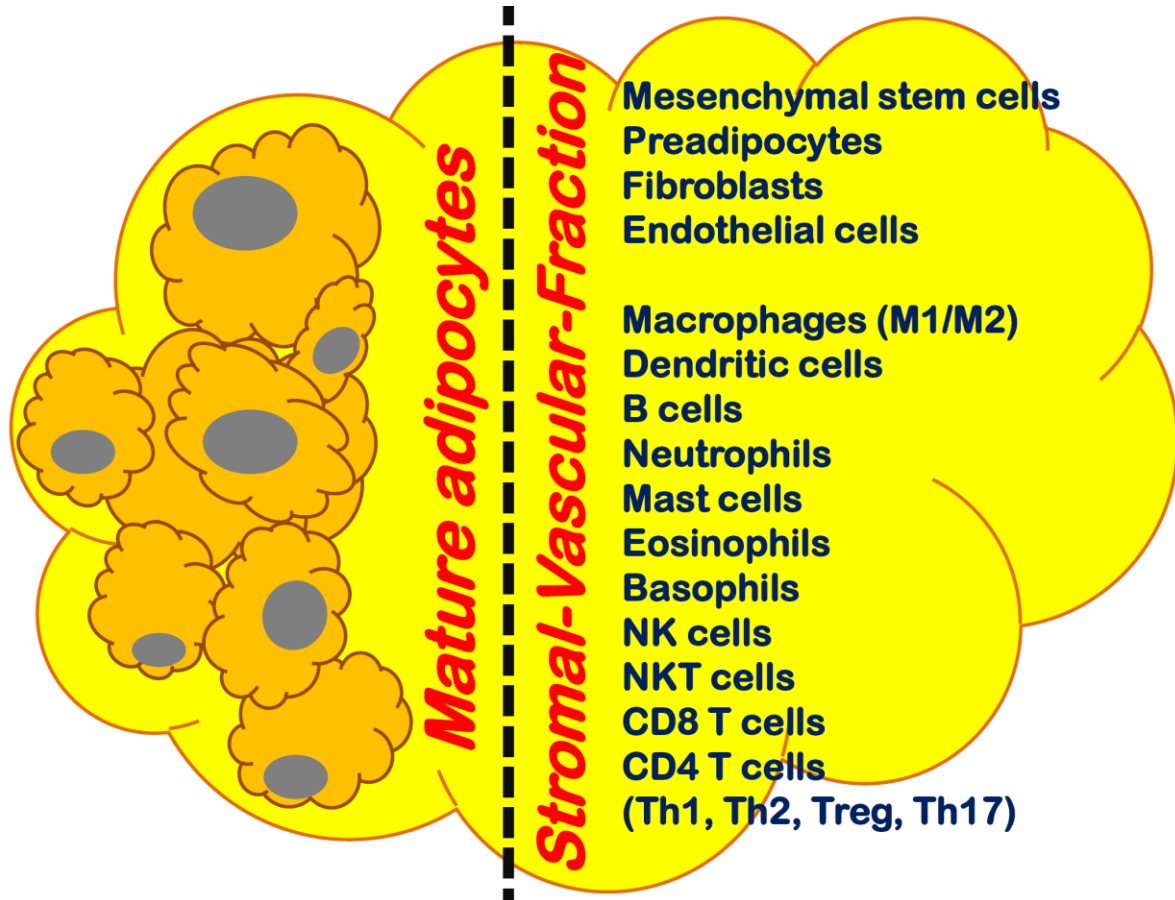
### ***1.12. Cellular composition of adipose tissue***

The cellular composition of adipose tissue is heterogeneous and includes various non-adipose cells, particularly leukocytes (Fig. 1.3). Mature adipocytes containing lipid droplets are by far the most abundant component, whereas the non-adipocytes are classified as stromal-vascular-fraction (SVF) cells and include the immature adipocytes (preadipocytes), multipotent mesenchymal stem cells (MSC), fibroblasts, endothelial cells of the adipose microcirculation, and immune cells [93]. Following collagenase digestion, the buoyancy of adipocytes allows for

these cells to be readily separated from the SVF cells by centrifugation for subsequent experimentation. Preadipocytes are derived from MSC's, and upon receiving appropriate stimuli and signals, such as insulin and glucocorticoids, differentiate into mature adipocytes. The terminal differentiation of preadipocytes (adipogenesis) is a transcriptionally and morphologically dramatic process that essentially transforms a fibroblast into a spherical mature adipocyte filled with triglyceride droplets [94]. This process can be studied in vitro and encompasses ~1-2 weeks depending on the species and cell model. Adipogenesis typically begins with a round of clonal division, immediately followed by expression of the transcription factors peroxisome proliferator-activated receptor gamma (PPAR $\gamma$  - the adipogenic "master regulator") and CCAAT-enhancer-binding protein alpha (C/EBP $\alpha$ ). These two transcription factors then induce expression of key enzymes and pro-adipogenic factors that regulate lipogenesis and triglyceride formation. Adipogenesis is tightly regulated and susceptible to various pro- and anti-adipogenic extracellular mediators. Whereas factors such as insulin, fatty acids, and glucocorticoids promote differentiation, other factors such as growth hormone, TGF $\beta$ , and proinflammatory cytokines such as TNF $\alpha$  can suppress adipogenesis [95]. In addition to adipocytes, the other main MSC lineages include chondrocytes (cartilage cells) and osteoclasts (bone cells), and MSC's have recently received much attention for their growth and regenerative capacity compared to bone marrow stem cells [96]. However, MSC's may be yet another source for a HIV cellular reservoir as MSC's differentiated along hematopoietic lineages into cells expressing CD4, CCR5, and CXCR4 can be productively infected [97]. The SVF leukocyte fraction has been most intensely studied in recent years and is discussed in the following section. The cellular organization of adipose depots is complex, and numerous in vitro coculture and rodent models have been developed to better understand the interactions amongst these adipose cells.

*Figure 1.3. Immune cells as major constituents of adipose tissue.*

Adipose tissue is comprised predominantly of mature adipocytes, whereas the non-adipocyte, stromal-vascular-fraction component, is comprised of adipocyte precursor cells, accessory cells, and immune cells. The presence of every type of immune cell has been determined in adipose tissue of humans or rodents during disease or inflammation, particularly during obesity, metabolic disorders, and infections.



### **1.13. Adipose tissue and HIV immunology**

Obesity is perhaps the most familiar and prevalent adipose-related health complication in western countries, and with the constantly increasing rates of obesity, particularly in the United States, biomedical research of adipose tissue and metabolic conditions has substantially increased as well. Consequently, the perception of adipose tissue functions, notably its relationship with the immune system, has dramatically changed based on research over just the past several years. Indeed, adipose tissues, especially within visceral depots, are now characterized as regions of considerable immune activity and regulation during obesity and

inflammation, and such studies have further given rise to a new sub-field of research termed “Immunometabolism” [98]. These investigations have further revealed adipose tissues to have potentially substantial impact for HIV pathogenesis.

The earliest studies directly addressing a relationship between adipose tissue and immune cells can be attributed to the work of Caroline Pond. Initial studies involved immune stimulation of guinea pig lymph nodes with LPS and examination of adipose activation, which resulted in altered morphology and increased rates of lipolysis by perinodal adipocytes [99]. Conversely, the accumulation of fatty acids by activated leukocytes in stimulated lymph nodes strongly correlated with the fatty acid composition of proximal perinodal adipocytes, but not with more distally located adipose tissue, demonstrating paracrine interactions between activated leukocytes and proximal adipose tissue [100]. In further attempts to better understand the etiology of adipose redistribution of HIV-infected persons, in vitro explants from these experimental systems were exposed to antiviral drugs such as zidovudine, lamuvidine, stavudine, and didanosine, resulting in drug-induced enhancement of lipolysis in perinodal adipose explants, but little effect by other nodeless adipose explants [101-102]. These studies demonstrated important functional relationships between adipose cells and immune cells, and proposed possible mechanisms for adipose redistribution during HIV infection. Shortly after the initial reports describing perinodal adipose tissue-immune cell interactions by Caroline Pond, studies by Anthony Ferrante described macrophage infiltration into adipose tissue of obese mice and humans, resulting in increased macrophage production of proinflammatory cytokines such as IL6 and TNF $\alpha$  [103]. This report would be followed by a significant number of studies by other groups mostly depicting the general theme of adipose inflammation mediated by activated macrophages and T cells. These studies by Pond and Ferrante are amongst the seminal reports which greatly heightened interest into adipose immunology.

The presence of virtually every type of leukocyte (CD4 and CD8 T cells, NK and NKT cells, B cells, monocytes and macrophages, dendritic cells, neutrophils, mast cells, eosinophils, and basophils) has been identified in adipose tissue of humans or rodents [104-105]. To lesser

extents, adipose leukocytes have been studied in other species as well, including non-human primate, bovine, porcine, rabbit, and canine. In general, these studies mainly focus on leukocyte interactions with adipose cells and regulation of obesity and inflammation, metabolic pathways, diabetes or insulin resistance. Amongst the adipose leukocytes, CD4 T cells and macrophages have been the most extensively investigated, and many of the findings have important implications for HIV pathogenesis. Firstly, in either lean or obese humans or mice, the CD4 T cells that traffic into adipose tissue are predominantly memory (CD4+CD45RO+) subsets, and upon entering adipose tissue upregulate various activation markers such as CD69, CD25, and HLA.DR [106-110]. The adipose CD4 T cells can receive stimulation from a variety of sources. The extracellular milieu is potentially stimulatory due to the presence of adipokines and cytokines. More surprisingly, adipocytes even have the capacity for activating CD4 T cells by direct contact via TCR/class II MHC interaction [111]. Additionally, other SVF cells including macrophages, dendritic cells, and MSC's have been demonstrated to activate adipose CD4 T cells [112-116]. Further characterization of adipose CD4 subsets includes Th1, Th2, Th17, and Tregs, all productive hosts for HIV [117]. Monocytes that migrate into adipose tissue differentiate primarily into either M1 (pro-inflammatory) or M2 (anti-inflammatory) subsets depending on the disease and metabolic state [118]. However, a macrophage differentiating from an infected monocyte would likely result in some level of viral replication. In vivo and in vitro coculture studies demonstrates crosstalk between macrophages and adipocytes that results in increased release of factors by adipocytes that could enhance T cell activation and HIV production, such as IL6, TNF $\alpha$ , and free fatty acids [119-120]. The question of whether monocyte/macrophage infiltration precedes or follows T cell infiltration is still conflicting, but these activities and phenotypes of CD4 T cells and macrophages in adipose tissue allude to favorable conditions for HIV infection and replication.

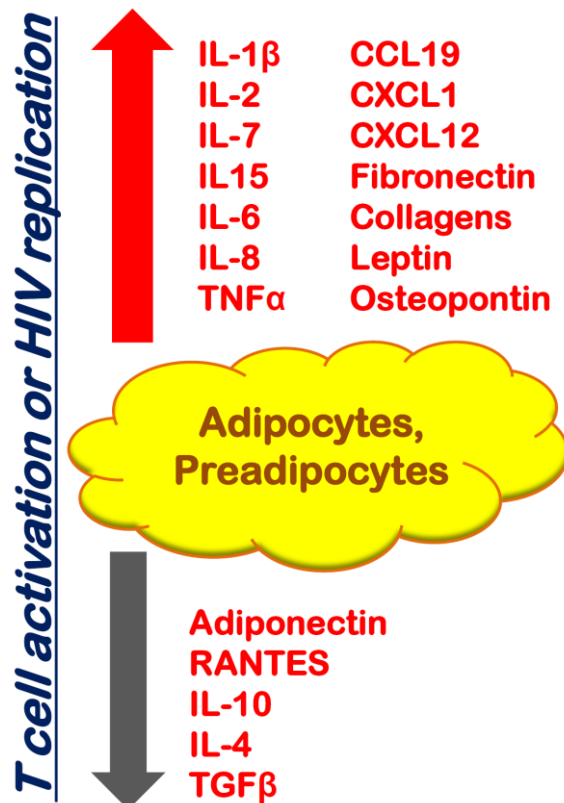
#### **1.14. Adipocyte endocrinology - adipokines and cytokines**

As adipocytes are major endocrine cells, these cells are rich sources of factors, referred to as adipokines, which regulate metabolic, as well as non-metabolic, physiological pathways. Importantly for HIV immunology and pathogenesis, a number of adipokines and adipose-derived factors regulate T cell and macrophage activation, differentiation, and functions (Fig. 1.4). Leptin and adiponectin are perhaps the best-known adipokines for their metabolic functions, but these factors also regulate T cell and macrophage functions. Activated human and murine T cells and monocytes upregulate leptin and adiponectin receptors, in which leptin can enhance immune cell activation (proliferation and cytokine production) and viability, but adiponectin can suppress T cell activation and proliferation [121-124]. Some free fatty acids, such as polyunsaturated free fatty acids, can function as signaling mediators that suppress T cell activation and proliferation [125]. In addition to these hallmark adipose factors, adipocytes also produce cytokines and chemokines that regulate immune function, and potentially HIV infection as well. The increased production of inflammatory cytokines (IL6, IL8, and TNF $\alpha$ ) and chemokines (CCR5, CCL19, MCP1/3, and MIP1 $\alpha$ ) by adipocytes are well-known events during HIV infection and obesity [126-131]. Whereas proinflammatory cytokines such as IL6 and TNF $\alpha$  enhance T cell activation and HIV replication, chemokines such as CCL19 can promote latency establishment in memory CD4 T cells [132]. Less appreciated is the evidence demonstrating adipocyte production of immunological cytokines such as IL2, IL7, and IL15, common  $\gamma$ -chain cytokines that can act as primary activation signals for T cells and HIV replication, and stimulation of which can be further augmented by proinflammatory cytokines [133-141]. Even components of the extracellular milieu of adipose tissue have the potential for regulating T cell activation and HIV replication. For example, the structural breakdown and reorganization of adipose tissue during HIV infection, and obesity and inflammation results in fibrosis and increased deposition of collagens, fibronectin, and metalloproteinases, factors of which are separately reported to regulate CD4 T cell activation and HIV replication [142-143]. Adipocytes are best known for their production of metabolic mediators, but these cells also

secrete numerous immune-regulatory factors with the potential to regulate HIV infection and latency.

*Figure 1.4. Production of adipokines and cytokines by adipose cells with immuno-regulatory functions and potential regulation of HIV infection.*

Adipose tissues are major endocrine organs that abundantly secrete not only adipokines important for metabolic regulation, but cytokines and chemokines as well. Many of these adipose factors also have the potential for regulating T cell activation and HIV replication.



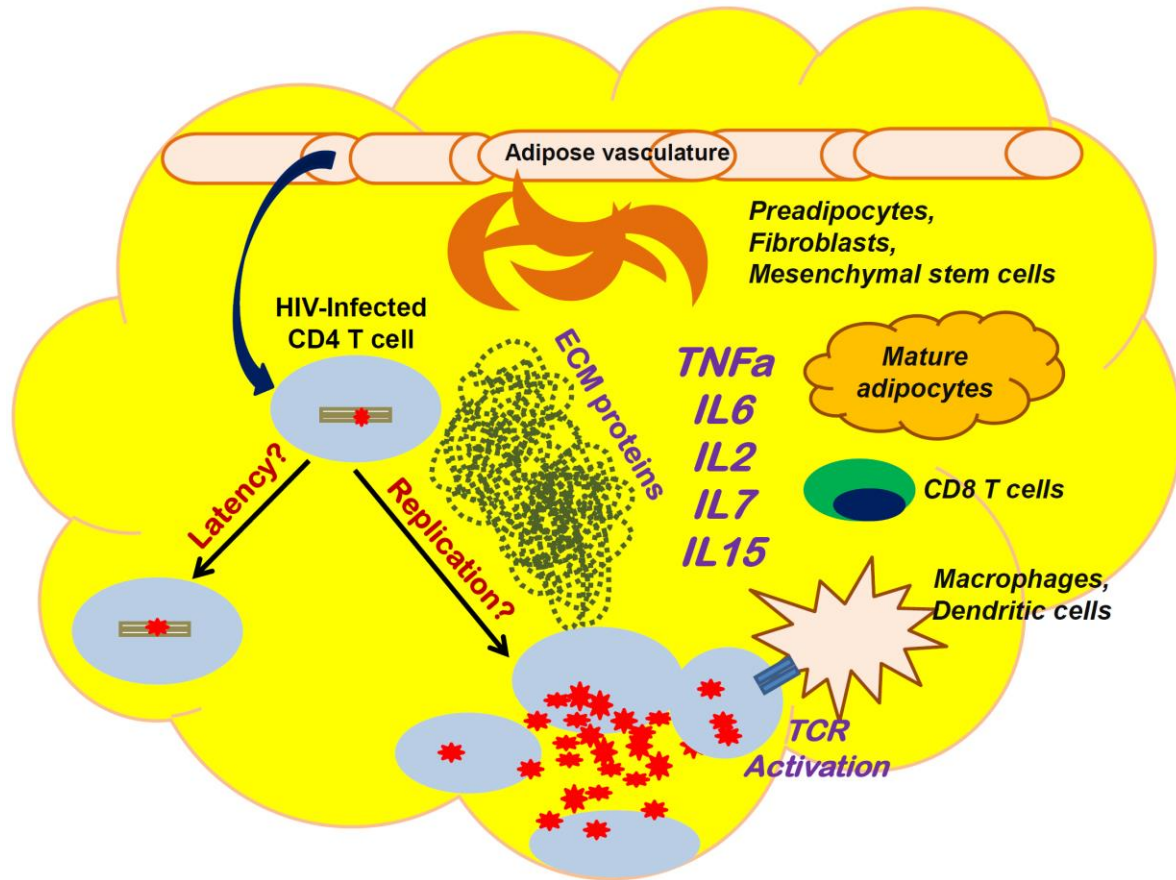
#### **1.15. Potential regulation of HIV-infected CD4 T cells or macrophages in adipose tissue**

The sum of these studies suggests a high probability that adipose tissue can serve as compartments that promote HIV replication, latency, and systemic persistence. A minimally plausible scenario would involve an infected resting CD4 T cell or monocyte trafficking into adipose tissue where it receives stimuli or signals that regulate its activation and differentiation (Fig. 1.5). However, whether virus becomes induced or remains dormant is a more complicated issue as the outcome would depend on the appropriate combination of factors and

stimuli. As in other tissues, the replication capacity of infected immune cells in adipose tissue would likely correlate with the virological and disease status of the patient, in which replication and viral load in adipose tissues would be expected to be significantly higher in acutely infected or untreated persons compared to chronically infected patients receiving ART. Adipose-resident CD4 T cells undergo activation (upregulation of CD69, CD25, and HLA-DR), produce cytokines (IL2 and IFN $\gamma$ ), and differentiate into various subsets (Th1, Th17, or Tregs), which resemble CD4 T cell phenotypes and functions in other tissues such as lymph nodes, spleen, and GALT of infected persons where HIV persists the most. The GALT of infected persons receiving ART therapy is a site of chronic inflammation, but adipose tissue of obese and infected patients is now considered to be a major site of chronic inflammation as well [104-105]. In the case of an infected monocyte, the monocyte would differentiate into a macrophage soon after extravasation into adipose tissue, but viral induction may depend on the differentiation pathway as HIV replication differs between M1 and M2 macrophages [144]. However, infected circulating monocytes are substantially less frequent than infected CD4 T cells in virally-suppressed ART-treated persons [145]. If adipose tissues are indeed demonstrated to be sites for infected immune cell infiltration, in which these cells subsequently produce infectious virus and proinflammatory factors, these events could contribute to adipose inflammation, dysfunctions, and lipodystrophies during HIV infection.

**Figure 1.5.** *The potential stimulatory environment in adipose tissue for HIV-infected CD4 T cells.*

Following extravasation from the adipose microcirculation, an infected CD4 T cell may be exposed to various cells (antigen-presenting cells or adipose cells) or soluble factors (cytokines, chemokines, or adipokines) that stimulate T cells and induce HIV replication.



### **1.16. HIV Lipodystrophy and adipose disorders during infection**

The term “Lipodystrophy” refers to disease or abnormalities of adipose tissue. A well-known condition during HIV infection is the manifestation of a variety of adipose physiological and metabolic dysfunctions, collectively referred to as “HIV-Associated Lipodystrophy Syndrome” (HALS), or “HIV-Associated Redistribution Syndrome” (HARS) [146-149]. These disorders were initially observed in the late 1990’s in patients treated with protease inhibitors such as indinavir, zidovudine, or zalcitabine. Other forms of lipodystrophies include Generalized Lipodystrophy (Congenital or Acquired), or Partial Lipodystrophy (Inherited or Acquired), and involve genetic, immunological, or infectious etiologies [150]. Unlike these relatively rare

lipodystrophies, and compared to many other HIV-associated pathologies, HIV-associated lipodystrophies are quite common in infected patients.

The primary diagnostic characteristics of HALS involve abnormal adipose morphology and functions, and systemic metabolic parameters. Fat loss or wasting usually occurs in the limbs, buttocks, and facial areas (peripheral lipoatrophy), whereas fat gain or accumulation occurs within the abdominal region (central adiposity or lipohypertrophy). A less frequent occurrence is the localized buildup of fat, called “Buffalo hump”, in the dorsocervical region [151]. The ectopic deposition of fat (ie. accumulation of adipose tissue in regions not normally associated with fat such as liver, pancreas, and skeletal muscles) is another aspect of HALS that further worsens a patient’s metabolic condition [152]. Metabolic pathways also become dysfunctional, contributing to dyslipidemias such as increased plasma triglycerides, cholesterol, and low-density lipoproteins (LDL), and decreased high-density lipoproteins (HDL). Additionally, HALS is associated with higher rates of metabolic disorders such as obesity, diabetes, insulin resistance, hypertension, cardiovascular disease, and metabolic syndrome [146-149]. These metabolic defects are further associated with immunological parameters such as increased inflammatory cytokines (IL6, IL8, and TNF $\alpha$ ) and immune cell activation. Since the signs and symptoms of HALS are diverse and complex, accurate diagnoses can be difficult, with estimates of HALS prevalence and frequency ranging widely from ~10-90% (depending on diagnostic criteria, ART treatment and virological status, and demographics), but the general consensus is that HIV infection dramatically impacts adipose physiology and metabolic pathways in many patients [148].

The underlying mechanisms of HALS are still mostly unclear, but many ART-induced metabolic defects have been described at the cellular level. The protease inhibitors were the first class of drugs to be associated with HALS, but other classes such as NRTI’s and NNRTI’s have been linked to HALS as well, whereas less is known regarding the impact of integrase and entry inhibitors. HIV patient studies comparing adipose tissue of ART-treated and ART-naïve or untreated patients have shown that ART-treated patients generally have higher levels

of lipolysis, increased expression of inflammatory cytokines, apoptosis, adipose breakdown and reorganization, and decreased expression of pro-adipogenic factors (PPAR $\gamma$  and C/EBP $\alpha$ ). Many of these in vivo findings are corroborated by in vitro experiments examining direct exposure of adipocytes to numerous ART drugs, also showing that they perturb metabolic pathways and adipokine production, impair mitochondrial function, induce inflammatory cytokines, increase apoptosis, and inhibit adipogenesis [153-157]. Additionally, the uptake and intracellular sequestration of drugs such as lopinavir and ritonavir by human adipose cells has been demonstrated in vitro [158]. Although the disruption of adipose and metabolic homeostasis is strongly associated with ART treatment, and the negative effects of drugs upon adipose physiology clearly demonstrated in vitro, the actual distribution and extracellular and intracellular concentrations of drugs within adipose tissue in vivo has yet to be studied. This is mainly due to the technical difficulties of obtaining such measurements in tissues compared to blood and plasma, but the improvement of methods such as high pressure-liquid chromatography with mass spectrometry are now allowing for accurate measurement of antiretroviral drugs in cells and tissues [159-160].

When the metabolic complications of HAART administration were initially recognized, these conditions were mainly believed to be side effects of the antiviral drugs, but later studies suggested a more complex etiology that attribute some of these abnormalities directly or indirectly to aspects of HIV infection itself. Many of the characteristics of HALS, including adipose redistribution, dyslipidemias, and metabolic disorders have been observed in patients not receiving ART as well [161-162]. Additionally, murine and in vitro studies have shown that activated T cells and macrophages are major regulators of numerous adipose defects such as insulin resistance, abnormal lipolysis, upregulation of inflammatory factors and downregulation of important adipokines, and impairment of adipogenesis. Chronic inflammation and the elevation of proinflammatory cytokines during HIV infection are important since TNF $\alpha$  is a potent inducer of lipolysis and inhibitor of adipogenesis [163]. Virion-free HIV proteins such as Vpr, Nef, and Tat have also been demonstrated to induce inflammatory factors, increase

lipolysis, promote insulin resistance, and suppress adipogenesis in vivo and in vitro [164-169]. For example, a murine model expressing freely soluble Vpr develops some of the metabolic dysfunctions resembling HALS, such as increased lipolysis and dyslipidemias [169]. Studies of adipose tissue and metabolism in non-human primate models of SIV infection have not been conducted, but a recent report demonstrated adipose inflammation and insulin resistance in rhesus macaques on high-fat diets, similarly to humans and mice [170]. Thus, while the metabolic disorders of HIV-infected persons can be generally attributable to ART treatment, studies clearly demonstrate similar adverse conditions in the absence of antiviral therapy.

### **1.17. *Microbes and adipose tissue***

Despite the recent explosion of research into adipose-related conditions such as HIV Lipodystrophy, obesity, and immunometabolism studies, relatively little attention has been given to the microbial composition of adipose tissue. Leukocyte hosts for particular microbes, especially T cells and macrophages, are likely to transport these pathogens into adipose tissue since fat depots are heavily-trafficked regions by immune cells. The role of microbial-derived signals for T cell infiltration is unclear, although clonotypic analyses of adipose-resident T cell TCR's in obese mice indicate relatively limited diversity compared to splenic T cells, suggesting that non-microbial signals and mechanisms may be more important mediators of adipose T cell migration [171]. Due to their close association with the intestine, the organ most abundant in microbial composition, the visceral omental and mesenteric adipose depots could be much more prone to bacterial infiltration compared to other adipose depots [172-175]. Microbial products, such as bacterial LPS or HIV Vpr, can circulate into adipose tissue as well. Adipocytes also express toll-like receptors that induce inflammatory responses upon exposure to microbial ligands. LPS (a TLR4 agonist), for example, upregulates inflammatory cytokines and chemokines, induces lipolysis, and blocks adipogenesis in human and murine adipocytes [176]. As summarized in Table 1, the presence of bacteria, parasites, and viruses has been demonstrated in adipose tissue of humans and mice.

Table 1. Detection of microbes in adipose tissue.

Microbe	Host	Adipose cellular fraction	Method of detection / In vitro studies	Disease association	Refs
Adenovirus 36	Human	Whole AT	Ad36 DNA by nested PCR	Massive AT deposition in thorax/abdomen; Lipomatosis	[177]
	Rhesus and Marmoset monkeys	Whole AT	Ad36 DNA by nested PCR	Obesity	[178]
Enterococcus faecalis	Human	Whole AT	Bacterial by confocal microscopy; Direct infection of human preadipocytes and mature adipocytes	Ulcerative colitis; Crohn's disease	[179]
Mycobacterium tuberculosis	Mouse	Fractionated mature adipocytes and AT-SVF	CFU assay/MGIT analysis; Bacilli immunohistochemistry; Direct ex vivo infection of primary mouse adipocytes	Tuberculosis/latent Tb.	[180]
	Human	Whole AT	IS6110 in situ-PCR and conventional PCR; Direct infection of human mature adipocytes and 3T3-L1 preadipocytes and mature adipocytes	Tuberculosis/latent Tb.	[181]
Neospora caninum	Mouse	AT-SVF	Nc5 DNA by real-time PCR; H/E staining and immunohistochemistry	Adipose immune cells in lean mice	[182]
Plasmodium berghei	Mouse, Rat	Whole AT	In vivo imaging	Cerebral malaria	[183]
Rickettsia prowazekii	Mouse	Whole AT	Bacterial DNA by PCR; OmpB immunohistochemistry; Direct in vitro infection of mature differentiated 3T3-L1 cells	Brill-Zinsser disease/ epidemic typhus	[184]
Trypanosoma cruzi	Mouse	Whole AT	Parasitic DNA by real-time PCR	Chagas disease	[185]
	Mouse	Whole brown and white AT	Parasitic DNA by real-time PCR; Direct infection of 3T3-L1 mature adipocytes	Chagas disease	[186]

The mode of adipose tissue localization and impact for adipose physiology and metabolism is unique for different pathogens. Bacterial pathogens such *M. tuberculosis*, *T. cruzi*, *R. prowazekii*, *S. aureus*, *C. pneumoniae*, and *E. faecalis* can be sequestered by human and murine adipocytes, generally resulting in inflammatory cytokine production or dysregulation of adipocyte functions [179-189]. *R. prowazekii*, *S. aureus*, and *C. pneumoniae* can further replicate in 3T3-L1 murine adipocyte cell lines, although *C. pneumoniae* replicates better in preadipocytes compared to mature adipocytes [188]. Since macrophages constitute a significant fraction of adipose SVF cells, *Mtb* may be the least surprising of pathogens that can establish a presence in adipose tissue. *Mtb* can additionally infect human and murine adipocytes by binding to adipocyte scavenger receptors, accumulate in lipid droplets, and become dormant and resistant to antibacterial compounds [180-181]. CD36, a scavenger

receptor for *P. berghei*, is also expressed by adipocytes and allows for the intracellular uptake of this parasite [183]. With respect to viruses, influenza virus, cytomegalovirus, respiratory syncytial virus, and adenovirus have been shown to infect adipocytes and upregulate inflammatory cytokine production [189]. However, by contrast to the other microbes, Ad36 is demonstrated to enhance adipogenesis and increase lipogenic gene expression by human and murine adipocytes, and further hypothesized as an etiological agent for obesity [177-178,190]. Additionally, human adipocyte expression of Coxsackie and Adenovirus Receptor (CAR) is reported to be upregulated during obesity [191]. Although HIV cannot productively replicate in adipose cells, adipocytes can still take up and sequester HIV virions or other soluble proteins such as Vpr, Nef, and Tat, disrupting adipose homeostasis. If HIV-infected immune cells do indeed infiltrate adipose tissue, it would be interesting to explore the virological and metabolic consequences of adipose-localized coinfections.

In summary, adipose tissue is a reservoir for some pathogens, which could include HIV in consideration of adipose immune cell infiltration during infection, inflammation, and obesity. In this respect, additional important questions pertaining to a potential adipose HIV reservoir include the adipose viral load (DNA and RNA levels), the main host cells (CD4 T cells, macrophages, or other SVF cells), the replication capacity (latency, inducibility, and infectiousness), the impact on adipose functions and morphology, the penetration and efficacy of antiretroviral drugs in adipose tissue, whether adipose HIV evolves into a distinct viral species, and how this viral reservoir compares to the major reservoirs such as lymphoid tissue and GALT. Due to the preponderance of adipose tissue, the overall viral reservoir would be substantially underestimated if it harbored infectious HIV.

### **Project Objectives and Specific Aims**

Based on these observations and the extensive interactions between adipose tissue and the immune system, there is a strong rationale for exploring the impact of these immune-metabolic relationships for HIV infection and pathogenesis. Since CD4 T cells in adipose tissue are predominantly of the memory phenotype and express high levels of activation markers, I hypothesized that adipocytes may promote HIV infection, and that adipose tissue of infected persons may harbor infected immune cells and potentially constitute a major anatomic reservoir. To study these hypotheses, the following in vitro and in vivo strategies were utilized:

#### ***Specific Aim 1: To determine if adipose cells influence HIV replication by CD4 T cells.***

To investigate how adipocytes affect HIV replication by CD4 T cells, coculture experiments (using transwells or direct contact) between HIV-infected CD4 T cells and primary adipose cells (preadipocytes or mature adipocytes) were conducted. Memory CD4 T cells were first purified from healthy donor blood, infected in vitro with HIV, then cocultured with adipose cells for approximately one week. At appropriate time points, T cell activation (CD69 and CD25), viability, and HIV production (p24) were measured mostly by flow cytometric and ELISA methods. The expression and role of crosstalk mediators such as proinflammatory cytokines or adipokines were also studied.

#### ***Specific Aim 2: To determine if adipose tissue harbors infected immune cells in vivo.***

To investigate if adipose tissue is a reservoir for HIV, adipose tissue samples of infected humans and rhesus macaques were examined for the presence of CD4 T cells, macrophages, and viral DNA in the stromal-vascular fraction. Immune cell phenotypes and function were characterized by flow cytometry, and viral DNA measured by nested pcr. Bands from positive PCR reactions were further sequenced and analyzed by phylogenetics to confirm specificity, and assess the possibility of adipose tissue virus comprising a unique species compared to virus in other tissues. Adipose tissue of uninfected humans and monkeys were also examined for comparison of immune cell phenotypes and functions.

## Chapter 2

### Results

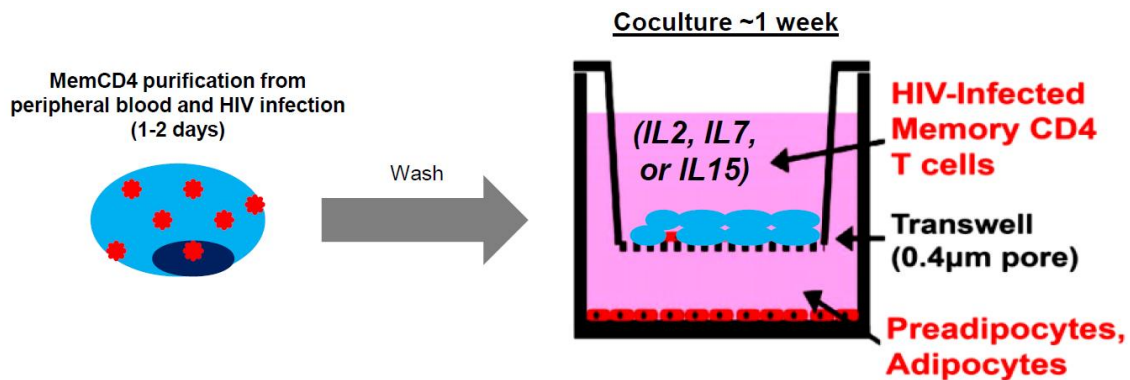
#### **Interactions between HIV-infected CD4 T cells and adipose cells in vitro**

##### ***2.1. Enhancement of T cell activation and HIV production by adipose cells in concert with IL2, IL7, or IL15***

Memory CD4 T cells, the predominant cellular reservoir for HIV, traffic into lymphoid and non-lymphoid tissues where most HIV replication occurs, or where virus becomes latent (but still inducible) in ART-treated persons. Infected CD4 T cells are known to migrate into and persist in tissues such as lymph nodes, GALT, and bone marrow, but adipose tissue has recently gained attention due to infiltration and metabolic regulation by T cells and macrophages, particularly during obesity or HIV infection. Additionally, adipose-resident CD4 T cells are primarily memory subsets and upregulate activation markers, similar to CD4 T cells in other tissues where HIV persists, suggesting that adipose cells could regulate T cell functions and HIV infection [192-194]. To first determine if adipose cells (preadipocytes and mature adipocytes) affect HIV replication, in vitro coculture experiments were conducted between HIV-infected memory CD4 T cells and adipose cells (Fig. 2.1). Memory CD4 T cells were purified from healthy donor peripheral blood and infected with R5-tropic HIV strains. Infected or uninfected memory CD4 T cells were then cultured in transwells with primary subcutaneous preadipocytes or differentiated adipocytes in lower wells for up to 7 days. T cell activation (CD69 and CD25 expression) and HIV production (intracellular and extracellular p24) were measured by flow cytometry and ELISA.

*Figure 2.1. General coculture method between HIV-infected memory CD4 T cells and primary human adipose cells.*

Human CD4<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> memory T cells were purified from peripheral blood of healthy donors, then infected for 24 hrs with HIV at 0.01-0.1 MOI's (mainly R5-tropic strains – SF162 or NSN-SX) in IL2 medium (20ng/ml). Cells were then washed, and seeded ( $\sim 2 \times 10^5$ ) into transwells (0.4 $\mu$ m pore size). Preadipocytes ( $\sim 1 \times 10^5$ ) or differentiated mature adipocytes were cultured in lower wells prior to coculture start. Cells were cocultured for approximately 7 days with appropriate agents (ie. cytokines or neutralizing antibodies), followed by appropriate assay measurements.

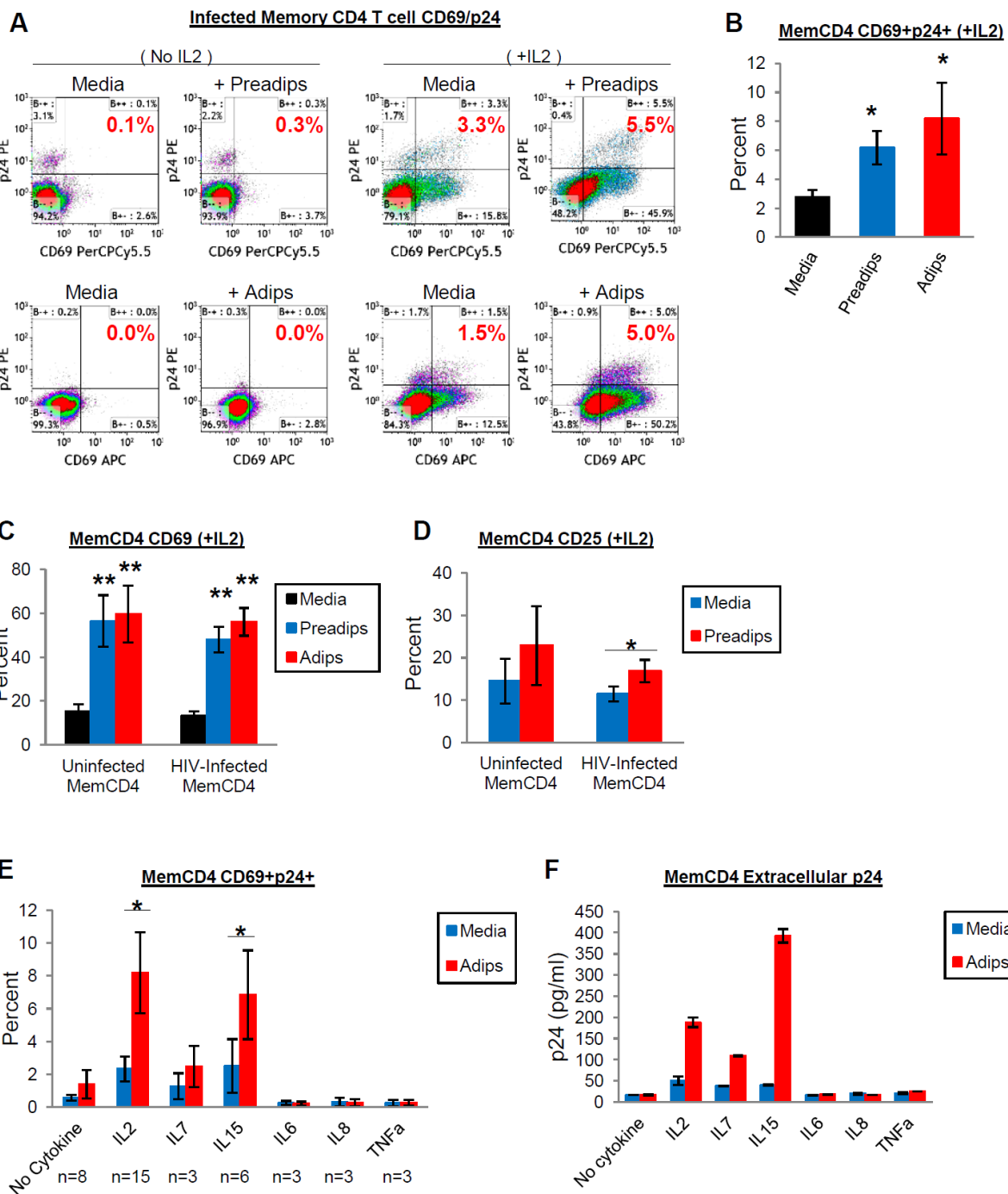


Preadipocytes or adipocytes alone did not affect memory CD4 T cell activation or HIV replication, but did enhance T cell activation and HIV production in the presence of exogenously added IL2, IL7, or IL15 during 7 days of coculture, an effect that resembles a previously reported synergy between IL2 and proinflammatory cytokines resulting in enhancement of T cell activation and HIV replication [38-39]. Fig.2.2A shows representative flow cytometry dotplots of CD69/p24 expression by HIV-infected memory CD4 T cells after 7 days coculture with preadipocytes or adipocytes, with or without IL2 (activation levels were also similar between uninfected and infected CD4 T cells during cocultures). In the absence of IL2, little to no HIV replication occurred during coculture. However, in the presence of IL2, preadipocytes and adipocytes increased T cell activation and HIV replication (CD69<sup>+</sup>/p24<sup>+</sup> double-positive expression) to  $\sim 6$ -8%, compared to  $\sim 3$ % in medium alone ( $p < 0.05$ ,  $n > 8$ , Fig. 2.2B). Total CD69 expression by uninfected and infected memory CD4 T cells in IL2 medium alone was  $\sim 13$ -15%, but increased to  $\sim 48$ -60% during 7 days coculture with preadipocytes or adipocytes ( $p < 0.05$ ,  $n > 4$ , Fig. 2.2C). CD25 expression by infected memory CD4 T cells was

also increased from ~11% in IL2 medium alone to ~17% during coculture with preadipocytes ( $p < 0.05$ ,  $n = 6$ , Fig. 2.2D). These enhancements of T cell activation and HIV replication during cocultures were also observed in the presence of IL7 or IL15. During 5 days coculture, adipocytes increased CD69+/p24+ expression by infected memory CD4 T cells ~2-3-fold compared to medium alone in the presence of IL2 or IL15, but not in the presence of proinflammatory cytokines such as IL6, IL8, or TNF $\alpha$  (Fig. 2.2E). Additionally, extracellular p24 from infected memory CD4 T cells was increased at least 2-fold by adipocytes in the presence of IL2, IL7, or IL15 (Fig. 2.2F shows one p24 ELISA representative of 3 separate experiments). Cocultures were also conducted with the addition of another common gamma-chain cytokine IL4 as a specificity control, but no effect was observed (data not shown). Lastly, treatment of infected memory CD4 T cells for 3 days with conditioned medium of preadipocytes (cultured in medium alone for 4 days) and IL2 also enhanced CD69 and p24 expression ~1-2-fold (data not shown). Direct contact experiments between infected memory CD4 T cells and adipose cells were also conducted, but results were inconsistent depending on culture conditions, and also potentially involved allogeneic stimulation by adipose cells (data not shown). Thus, adipose cells have the capacity for inducing HIV replication in CD4 T cells in the presence of some ubiquitous common gamma-chain cytokines such as IL2, IL7, or IL15, suggesting that latently infected circulating CD4 T cells that migrate into adipose tissue may induce viral reactivation.

*Figure 2.2. Enhancement of memory CD4 T cell activation and HIV production by adipose cells in the presence of IL2, IL7, or IL15.*

(A-D) Memory CD4 T cells were purified from peripheral blood, infected with HIV, then cocultured with preadipocytes or mature adipocytes for 5 days with or without IL2. T cell activation (CD69 or CD25) and HIV production (p24) were then measured. (A and B) Shown are representative CD69/p24 flow cytometry dotplots and mean $\pm$ sem CD69+/p24+ expression by HIV-infected memory CD4 T cells after coculture with preadipocytes or adipocytes (\* $p$ <0.05 compared to medium alone,  $n$ >8). (C) Mean $\pm$ sem total CD69 expression by uninfected or HIV-infected memory CD4 T cells after coculture with preadipocytes or adipocytes and IL2 (\*\* $p$ <0.001 compared to medium alone,  $n$ >5). (D) Mean $\pm$ sem CD25 expression by uninfected or HIV-infected memory CD4 T cells after coculture with preadipocytes and IL2 (\* $p$ <0.05,  $n$ >4). (E and F) Coculture between HIV-infected memory CD4 T cells and adipocytes in the presence of 20ng/ml of either IL2, IL7, IL15, IL6, IL8, or TNF $\alpha$ . Shown are flow cytometry CD69+/p24+ expression (\* $p$ <0.05) and extracellular p24 (one p24 ELISA representative of 3 separate experiments).



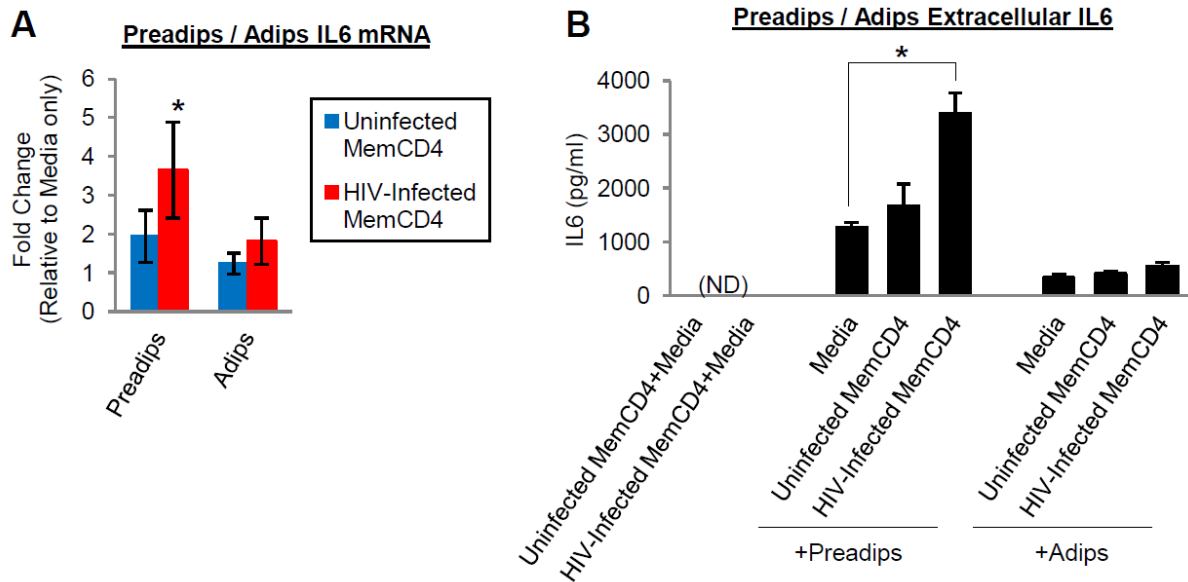
## **2.2. Adipose IL6 and extracellular matrix proteins as potentiators of HIV replication**

Since proinflammatory cytokines such as IL6 and TNF $\alpha$  augment T cell activation and HIV replication in conjunction with IL2 or polyclonal stimulants, we studied a role for IL6 due to its abundant expression by adipose cells during inflammation and HIV infection [195-197].

Preadipocytes or mature adipocytes were cocultured with uninfected or HIV-infected memory CD4 T cells and IL2 for 5 days, then IL6 mRNA and extracellular IL6 examined. IL6 mRNA was constitutively expressed by preadipocytes in medium alone, which was increased ~3-4-fold during coculture with infected CD4 T cells ( $p < 0.001$ ,  $n = 3-4$ ), and non-significantly increased during coculture with uninfected CD4 T cells (Fig. 2.3A). Adipocytes also constitutively expressed IL6 mRNA, but expression was non-significantly increased during coculture with uninfected or infected CD4 T cells. Extracellular IL6 levels as measured by ELISA showed similar patterns of expression, and consistent with other reports, preadipocytes produced more IL6 than adipocytes (Fig. 2.3B). Extracellular IL6 from preadipocytes increased from ~1,200pg/ml in medium alone to ~3,300pg/ml during coculture with infected CD4 T cells ( $p < 0.01$ ,  $n = 3$ ). However, extracellular IL6 from adipocytes was not affected during coculture with memory CD4 T cells, and memory CD4 T cells in medium alone did not secrete IL6. These findings suggest that preadipocytes could be an important source of IL6 in adipose tissue during HIV infection.

**Figure 2.3. Upregulation of adipose IL6 by HIV-infected memory CD4 T cells.**

Preadipocytes or mature adipocytes were cocultured with uninfected or HIV-infected memory CD4 T cells and IL2 for 6 days. (A) Mean±sem IL6 mRNA expression (fold changes relative to medium alone) by preadipocytes and adipocytes (\*p<0.05 compared to medium alone, n=4). (B) Mean±sem extracellular IL6 production by preadipocytes or adipocytes (\*p<0.05, n=3).



To determine if adipose IL6 mediates the increase of T cell activation and HIV production, cocultures were conducted with anti-IL6 neutralizing antibodies. Blocking abs against IL6 or IL6 receptor (CD126) partially mitigated adipose-induced increases of CD4 T cell CD69 expression and HIV production by ~30% (Fig. 2.4A and B shows representative flow cytometry dotplots of CD69 expression, and 2.4C and D shows one p24 ELISA representative of at least 3 separate experiments). To further determine the role of other mediators, blocking abs were tested against factors expressed by adipocytes, or cytokines that regulate T cell activation or HIV replication. Such candidate factors included other proinflammatory cytokines (TNF $\alpha$  and IL8), chemokines (RANTES, MCP-1/2/3, and MIP-1/2), integrin receptors ( $\alpha$  and  $\beta$  chains), and ECM proteins (collagens I/IV and fibronectin). Cocultures in the presence of these neutralizing abs demonstrated that the combination of blocking IL6 and integrins  $\alpha$ 1 $\beta$ 1 (VLA-1) most consistently, and near completely, mitigated the adipose-induced increases of T cell activation and HIV replication (Fig. 2.4A-D). However, during HIV infection and obesity, the

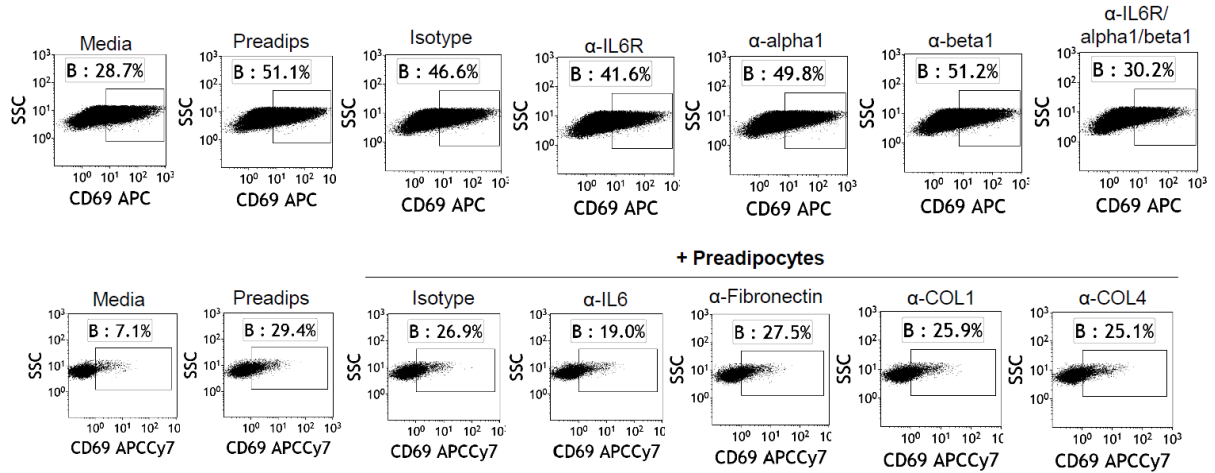
breakdown and reorganization of adipose tissue is frequent event, and is associated with fibrosis and increased deposition of extracellular matrix protein such as collagens and fibronectin [142-143]. VLA-1 is also a late indicator of T cell activation, and expressed by memory CD4 T cells in inflamed tissues [198-200].

*Figure 2.4. IL6 and integrin signaling as mediators of increased T cell activation and HIV replication by adipose cells.*

Memory CD4 T cells were purified from peripheral blood, infected with HIV, then cocultured with preadipocytes or mature adipocytes and neutralizing antibodies for 5 days in IL2 medium. (A and B) Memory CD4 T cell CD69 expression during coculture with preadipocytes or adipocytes in the presence of blocking abs against soluble IL6, IL6 receptor, and integrin  $\alpha 1/\beta 1$  (blocking abs against soluble fibronectin and collagens 1/4 during coculture with preadipocytes were also tested for comparison). Shown are CD69 flow cytometry dotplots representative of 3-4 separate experiments. (C and D) Extracellular p24 levels during memory CD4 T cell coculture with preadipocytes or adipocytes in the presence of blocking abs against soluble IL6, or against IL6 receptor and integrin  $\alpha 1/\beta 1$  (shown are one p24 ELISA representative of 3-4 separate experiments).

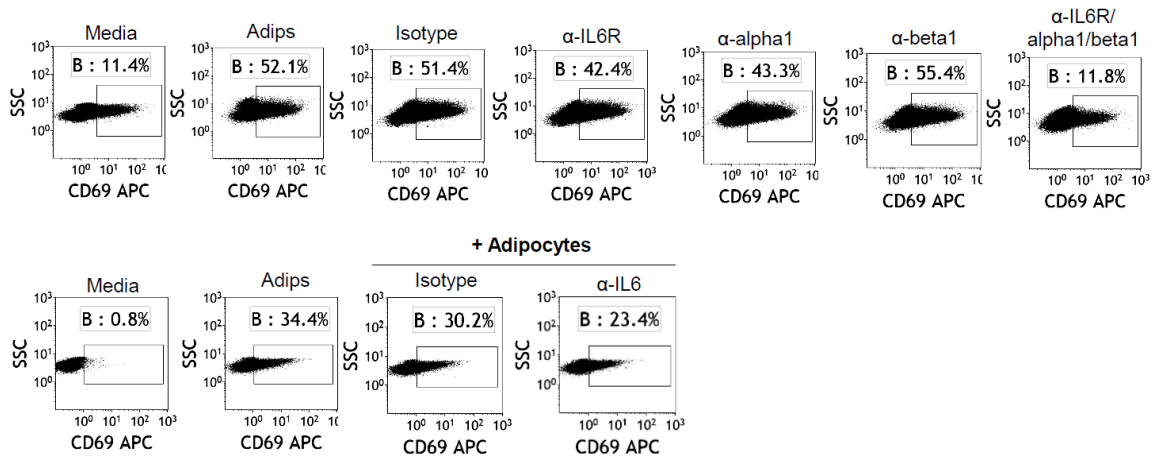
**A**

**+ Preadipocytes**



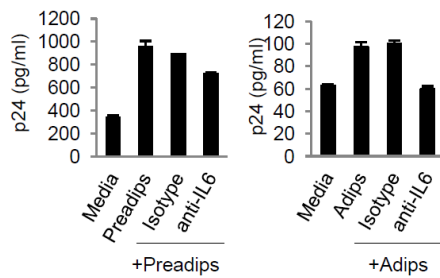
**B**

**+ Adipocytes**



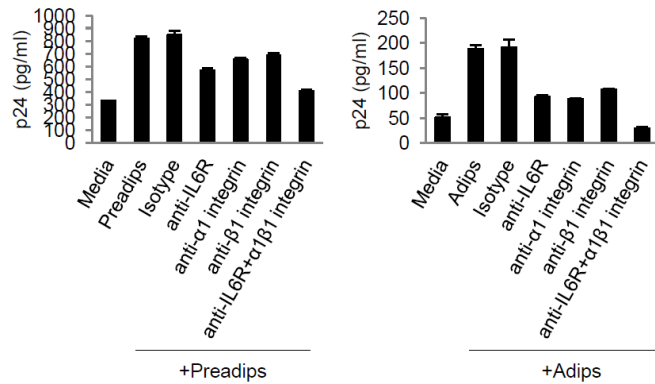
**C**

**HIV Production + anti-IL6 (+IL2)**



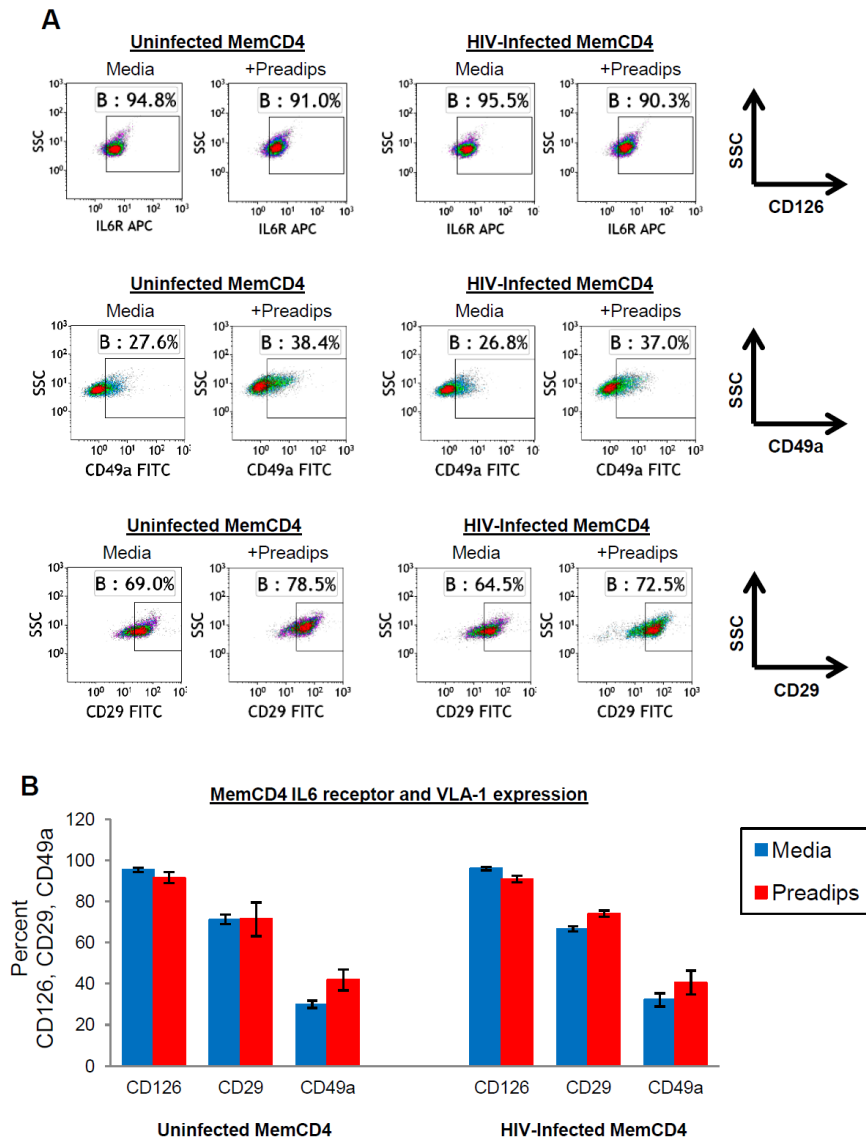
**D**

**HIV Production + anti-IL6R, Integrin  $\alpha$ 1 $\beta$ 1 (+IL2)**



IL6 receptor and integrins  $\alpha 1$  and  $\beta 1$  were also highly expressed by memory CD4 T cells. Uninfected and HIV-infected memory CD4 T cells expressed similar levels of IL6R (~90-95%), integrin  $\alpha 1$  (~25-40%), and integrin  $\beta 1$  (~60-80%), and expression was unaffected during coculture with preadipocytes (Fig. 2.5). In summary, these results suggest that IL6 and ECM proteins in adipose tissue may be important mediators in promoting T cell activation or HIV replication.

**Figure 2.5.** Expression of IL6 receptor and integrin  $\alpha 1/\beta 1$  (VLA-1) by memory CD4 T cells. Uninfected or HIV-infected memory CD4 T cells were cocultured with preadipocytes for 5 days in IL2 medium, then flow cytometry stained for IL6 receptor (CD126) and integrin  $\alpha 1/\beta 1$  (CD49a and CD29). Shown are representative flow cytometry dotplots (A) and mean $\pm$ sem (B) CD126, CD49a, and CD29 expression by memory CD4 T cells (n=3).



### ***2.3. Synergism between adipose common gamma-chain and proinflammatory cytokines as potential mediators of HIV replication***

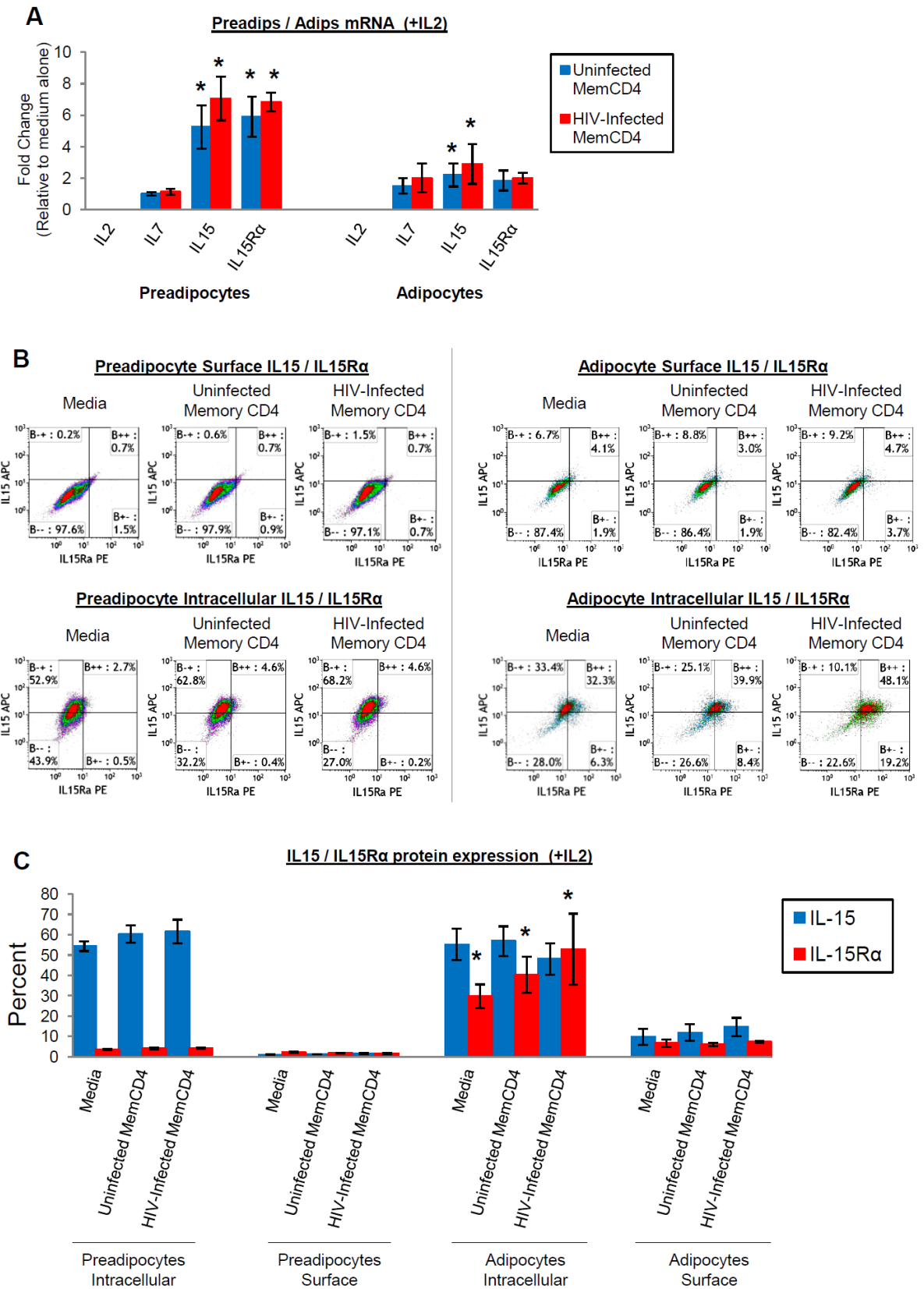
Since adipocytes increased T cell activation and HIV production in the presence of either IL2, IL7, or IL15, as shown in Fig. 2.2, the expression of these common gamma-chain cytokines (mRNA and protein) by adipose cells was also examined. Preadipocytes and adipocytes constitutively expressed mRNA for IL7, IL15, and IL15R $\alpha$ , but not for IL2. However, memory CD4 T cells increased adipose expression of IL15 and IL15R $\alpha$  (Fig. 2.6A, n=3). In preadipocytes, uninfected memory CD4 T cells increased preadipocyte IL15 mRNA ~5-fold ( $p<0.001$  compared to medium alone) and IL15R $\alpha$  mRNA ~6-fold ( $p<0.01$ ), and infected CD4 T cells increased IL15 mRNA ~7-fold ( $p<0.0001$ ) and IL15R $\alpha$  mRNA ~6-fold ( $p<0.001$ ). In adipocytes, IL15 was increased ~2-fold by both uninfected ( $p<0.05$ ) and HIV-infected ( $p<0.01$ ) memory CD4 T cells, whereas IL15R $\alpha$  expression was not significantly affected.

Despite IL15 mRNA expression by adipose cells, secreted IL15 protein was not detected in any coculture condition by ELISA, suggesting that adipocytes might trans-present IL15 via IL15R $\alpha$  conjugation, in similar fashion to IL15/IL15R $\alpha$  trans-presentation by monocytes, macrophages, dendritic cells, and epithelial cells [201-204]. To examine this possible IL15/IL15R $\alpha$  trans-presentation expression pattern by adipose cells, surface and intracellular IL15/IL15R $\alpha$  protein expression was measured by flow cytometry, and extracellular IL15 measured by ELISA, during 5 days coculture with memory CD4 T cells and IL2 (Fig. 2.6B and C). Preadipocytes cultured in medium alone did not express surface IL15 or IL15R $\alpha$  (1-2% for IL15 and IL15R $\alpha$ ), but expressed intracellular IL15 and IL15R $\alpha$  (~50% IL15, and ~3% IL15R $\alpha$ ), and non-specific binding by isotype controls was minimal (<2%). Additionally, coculture with memory CD4 T cells did not affect preadipocyte expression of IL15 or IL15R $\alpha$ . Adipocytes cultured in medium alone expressed modest levels of surface IL15 (~10%) and IL15R $\alpha$  (~5%), and similar to preadipocytes, expressed more intracellular IL15 (~50%) and IL15R $\alpha$  (~30%), which were also unaffected by coculture with memory CD4 T cells. These patterns of IL15/IL15R $\alpha$  expression resemble those of monocytes, dendritic cells, and epithelial

cells, suggesting that adipocytes could also trans-present IL15 to CD4 and CD8 T cells in adipose tissues. These data further suggest that adipocytes could be self-sustaining sources of both common gamma-chain and proinflammatory cytokines that regulate T cell activation and HIV induction in vivo.

*Figure 2.6. Adipose expression of IL2, IL7, and IL15/IL15R $\alpha$ .*

(A) mRNA expression of IL2, IL7, IL15, and IL15R $\alpha$  by preadipocytes and adipocytes after 5 days coculture with uninfected or HIV-infected memory CD4 T cells and IL2. Graph shows mean $\pm$ sem fold changes relative to preadipocytes or adipocytes in medium alone (\* $p$ <0.01 compared to medium alone,  $n$ =3). (B and C) Potential IL15/IL15R $\alpha$  trans-presentation expression pattern by adipose cells. Shown are representative flow cytometry dotplots (B) and mean $\pm$ sem (C) surface and intracellular IL15/IL15R $\alpha$  protein expression by preadipocytes and adipocytes after 5 days coculture with memory CD4 T cells and IL2 (\* $p$ <0.05 compared to preadipocyte intracellular IL15R $\alpha$  expression,  $n$ =3). Extracellular IL15 was measured by ELISA but not detected in any condition.

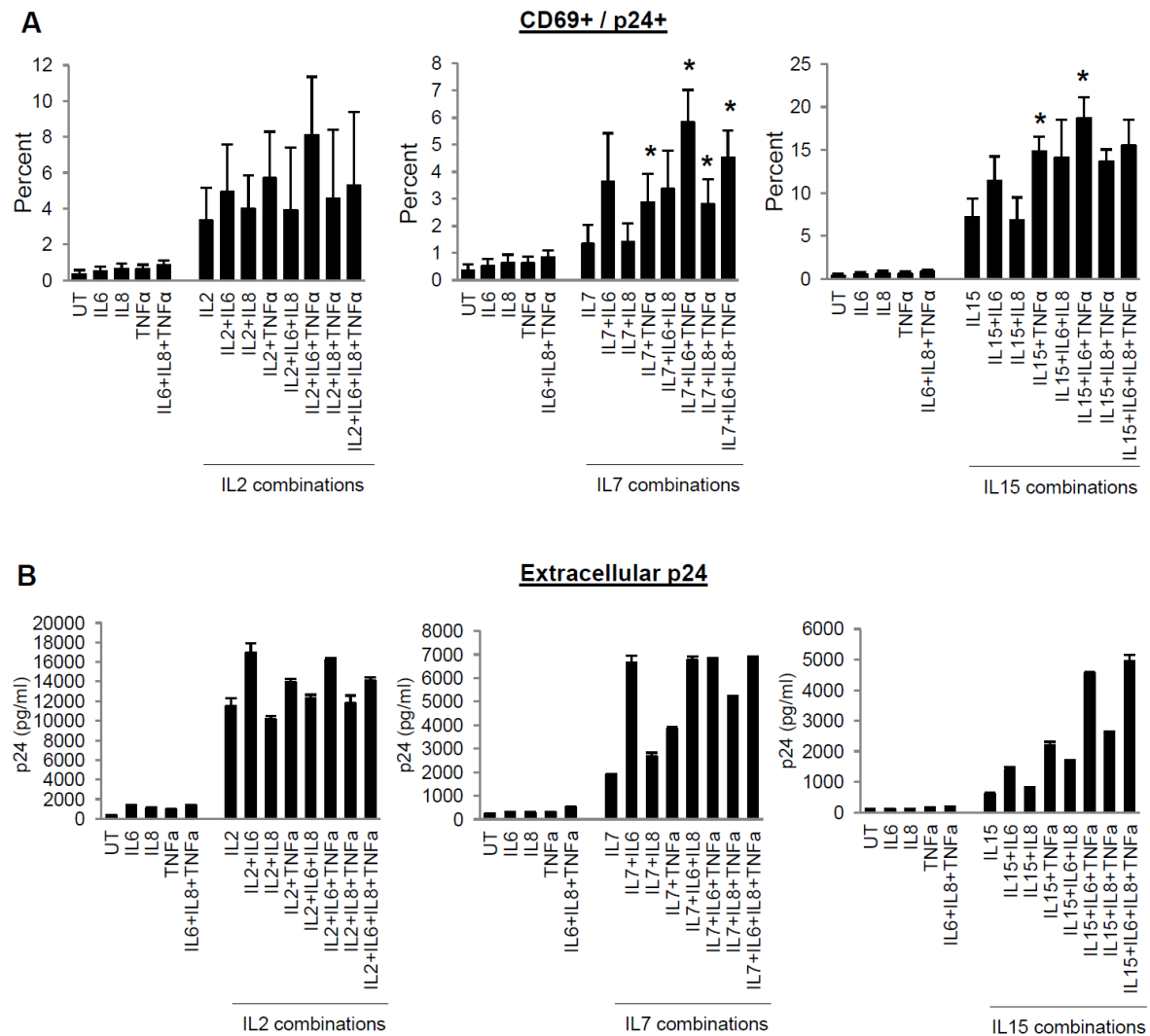


(Undetectable extracellular IL15 by ELISA)

To further corroborate the synergistic role of common  $\gamma$ -chain cytokines and adipose inflammatory mediators for increasing T cell activation and HIV replication, HIV-infected memory CD4 T cells were directly treated with combinations of recombinant IL2, IL7 or IL15 with IL6, IL8 or TNF $\alpha$  (in the absence of adipose cells). Compared to treatment with IL2, IL7, or IL15 alone, treatment with combinations of IL2, IL7 or IL15 with IL6, IL8 or TNF $\alpha$  generally increased CD69 and extracellular p24, whereas treatment with IL6, IL8, or TNF $\alpha$  alone did not affect T cell activation or HIV replication (Fig. 2.7A shows mean $\pm$ sem CD69+/p24+ expression, and 2.7B shows one p24 ELISA representative of 3 separate experiments). These data show that proinflammatory cytokines are important enhancers of HIV replication, but first require a primary signal such as IL2, IL7, or IL15 to exert their effects.

**Figure 2.7. Synergism between common gamma-chain and proinflammatory cytokines increase T cell activation and HIV replication.**

Memory CD4 T cells were purified from peripheral blood and infected with HIV in the presence of 20ng/ml of either IL2, IL7, IL15, or no cytokine (UT) for 24 hrs. Cells were washed, then cultured with indicated cytokines for 5 additional days, followed by measurements of T cell activation and HIV production. Shown are (A) mean±sem flow cytometry CD69+/p24+ percentages (n=3), and (B) extracellular p24 (one p24 ELISA representative of 3 separate experiments).

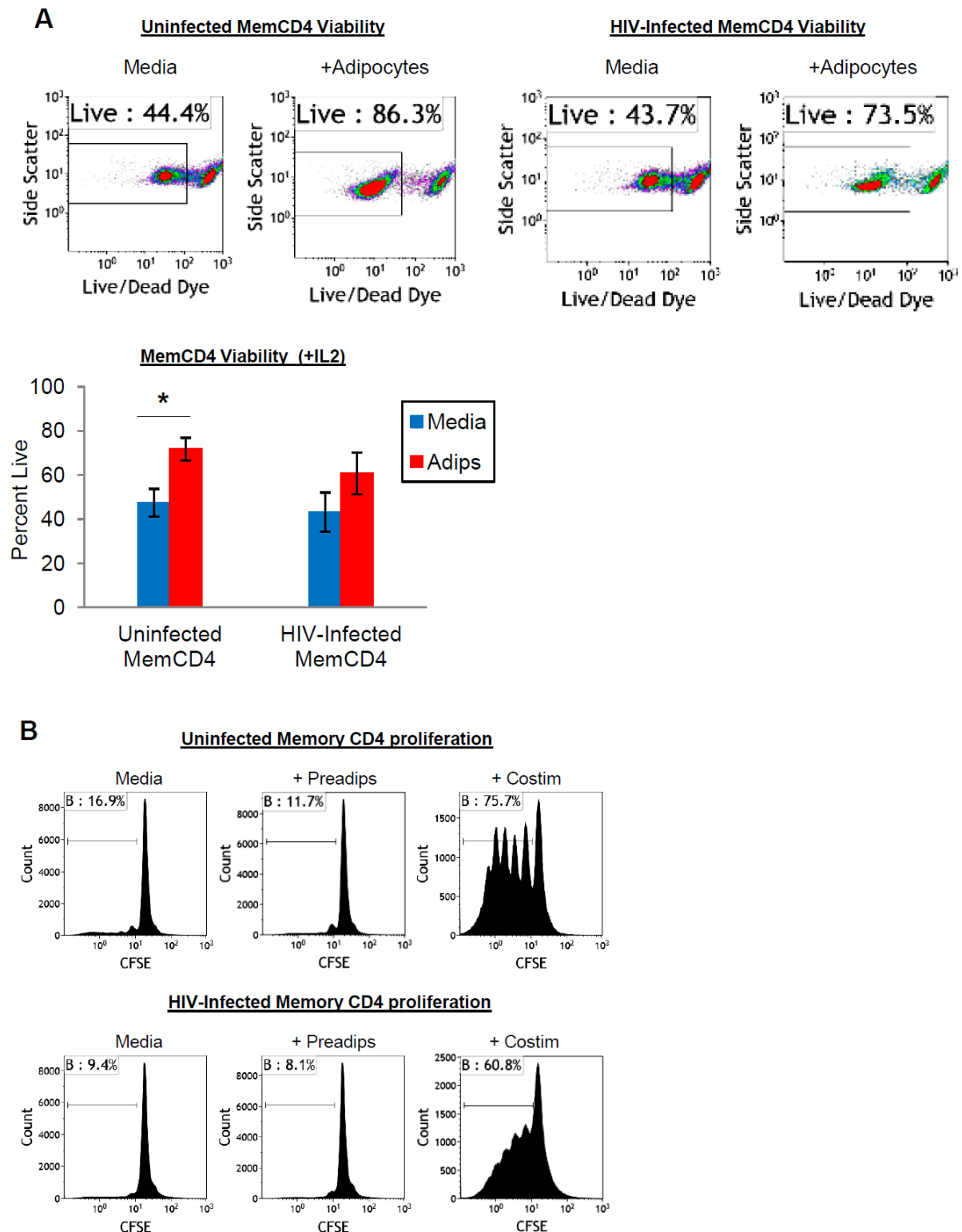


#### **2.4. Enhancement of T cell viability by adipocytes**

Adipose cells can influence T cell viability and proliferation in mouse and human studies. For example, adipose IL7 and leptin enhances T cell viability, polyunsaturated free fatty acids and adiponectin suppress T cell proliferation, and mesenchymal stem cells inhibit T cell proliferation and promote apoptosis [115,122-125]. To determine if adipocytes affect viability and proliferation of HIV-infected CD4 T cells, adipose cells were cocultured with memory CD4 T cells and IL2 for up to 8 days. After 8 days culture, viability of uninfected and HIV-infected memory CD4 T cells in medium alone was 43-47%, but was modestly increased to 61-72% during coculture with adipocytes (\* $p < 0.05$  comparing HIV-infected CD4 T cells in medium alone to coculture with adipocytes,  $n=5-6$ , Fig. 2.8A). To examine whether adipocytes affect T cell proliferation, memory CD4 T cells were labeled with CFSE and cocultured with preadipocytes for 6 days and IL2. Cell division, as indicated by CFSE dye dilution, was not observed by uninfected or HIV-infected memory CD4 T cells in medium alone or during coculture with preadipocytes. CD3/CD28 costimulation was used as a positive control, and proliferation of HIV-infected CD4 T cells was reduced compared to uninfected cells as expected (Fig. 2.8B). Thus, adipocytes may further promote HIV persistence by enhancing T cell survival, but not proliferation.

**Figure 2.8. Enhancement of T cell viability by adipocytes.**

(A) Uninfected or HIV-infected memory CD4 T cells were cocultured with adipocytes and IL2 for 7 days. Shown are representative flow cytometry dotplots and mean $\pm$ sem T cell viability (\* $p$ <0.05,  $n$ =5-6). (B) Lack of adipose influence for T cell proliferation. Memory CD4 T cells were labeled with CFSE and cocultured with preadipocytes and IL2 for 7 days. Shown are representative CFSE flow cytometry histograms ( $n$ =3).



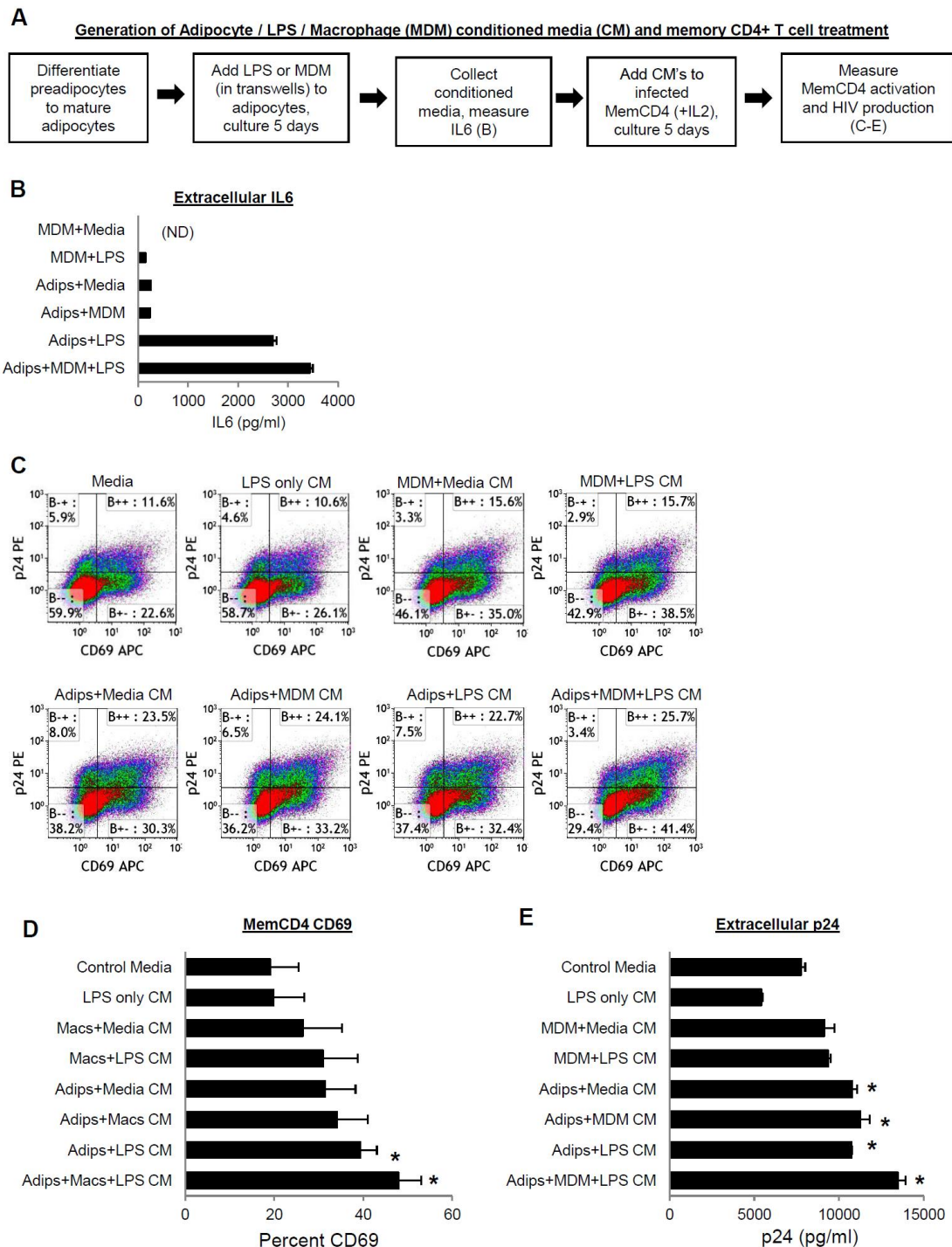
## **2.5. Activated macrophages as additional potentiators of adipocyte-mediated increases of HIV replication**

Adipose tissues of persons with HIV infection and obesity, and in conditions associated with increased circulating lipopolysaccharide (LPS), are characterized by infiltrating monocytes and macrophages, as well as elevated markers of inflammation such as IL6. To determine if activated macrophages exacerbate crosstalk between adipocytes and activated CD4 T cells, conditioned media (CM) of adipocytes cultured with pure LPS or monocyte-derived macrophages (MDM) for 5 days were prepared (Fig. 2.9A). Cell-free culture supernatants were harvested and transferred to HIV-infected memory CD4 T cells, and T cell activation and HIV production measured after an additional 5 days of culture.

Coculture with MDM, LPS treatment, or both, enhanced IL6 secretion from adipocytes (Fig. 2.9B). CM from these cultures were transferred to HIV-infected memory CD4 T cells and cultured for an additional 5 days with IL2. Fig. 2.9C shows representative flow cytometry dotplots of CD69/p24 expression by HIV-infected memory CD4 T cells treated with these CM's (n=3). Fig. 2.9D shows total CD69 expression by HIV-infected memory CD4 T cells, which increased from  $19.0 \pm 9.5$  in medium alone to  $34.1 \pm 9.5$  in CM from Adipocytes+MDM ( $p < 0.05$ ); to  $39.4 \pm 9.5$  in CM from Adipocytes+LPS ( $p < 0.01$ ); and to  $47.9 \pm 9.5$  in CM from Adipocytes+MDM+LPS ( $p < 0.001$ ). Fig. 2.9E shows a representative p24 ELISA (n=3) of infected memory CD4 T cells cultured in these CM's, indicating highest levels of HIV production by CD4 T cells during culture in Adipocytes+MDM+LPS CM. These results suggest that adipocyte inflammation mediated by macrophages or adipose microbial translocation may further augment T cell activation and HIV induction in adipose tissue.

**Figure 2.9. Upregulation of T cell activation and HIV replication by adipocytes is further potentiated by activated macrophages.**

(A) Steps for preparation of conditioned media (CM) of adipocytes cultured with LPS or monocyte-derived macrophages (MDM), and subsequent treatment of HIV-infected memory CD4 T cells. (B) Mean $\pm$ sem extracellular IL6 production by adipocytes (+/- MDM and LPS) and MDM's (+/- LPS) after 5 days culture (n=3). (C-E) Memory CD4 T cell activation and HIV production during 5 days culture in CM's and IL2 (n=3). Shown are (C) representative CD69/p24 flow cytometry dotplots, (D) mean $\pm$ sem CD69 expression, and (E) extracellular p24 (one p24 ELISA representative of 3 separate experiments).



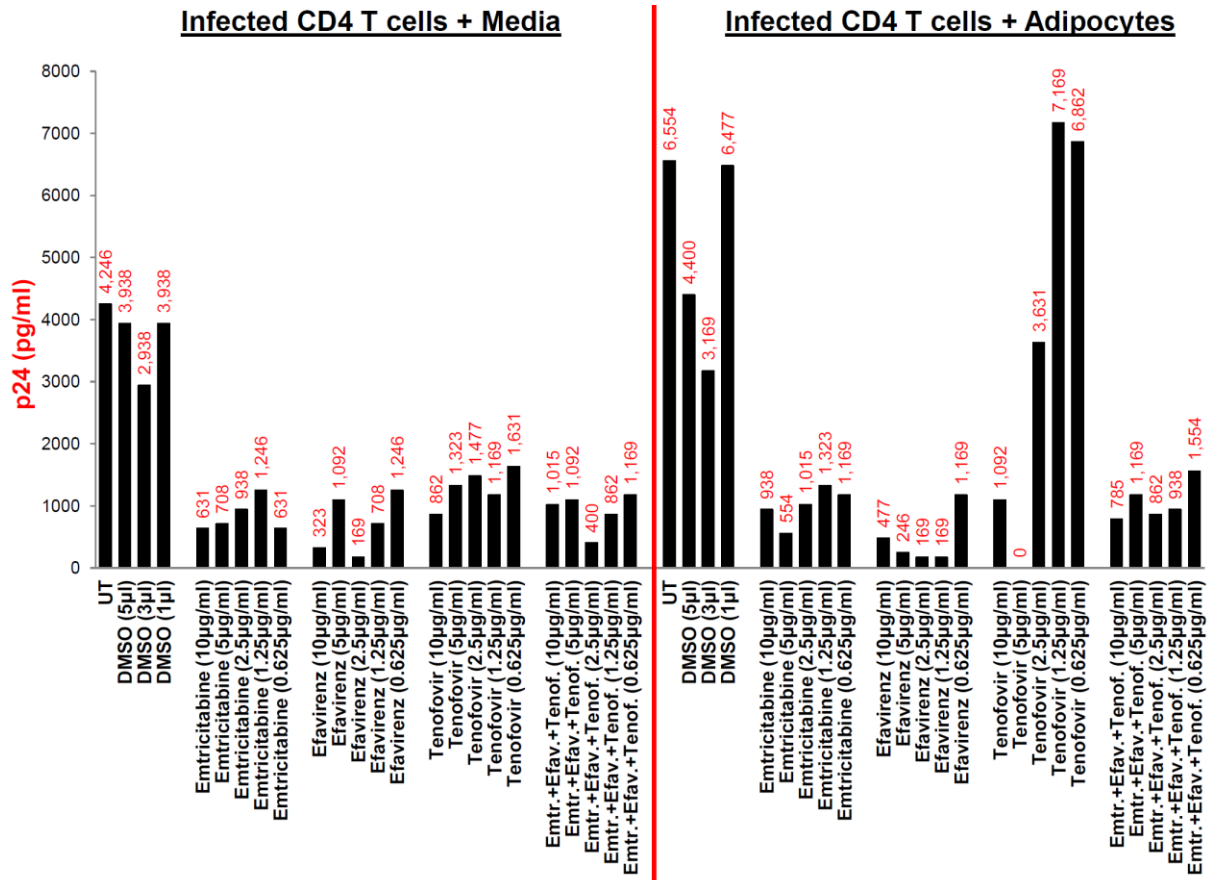
## **2.6. Potential impairment of antiretroviral efficacy by adipocytes**

The development of antiretroviral therapies (ART) is one of the greatest achievements against the morbidity and mortality associated with HIV infection. However, ART efficacy is limited by factors such as short-term activity (daily ingestion of pills is required), non-specific toxicities, and impaired distribution in certain anatomic regions such as the brain due to the blood-brain barrier. In tissues of virally-suppressed patients, such as lymph nodes where latently infected CD4 T cells persist, intracellular concentrations of ART drugs are reduced in lymph node cells compared to peripheral blood leukocytes [82]. ART penetration and circulation may be reduced in other tissues as well. Adipocytes can also rapidly sequester ART drugs, possibly reducing the exposure of infected CD4 T cells to ART [158].

The antiretroviral compounds Efavirenz (a NNRTI class drug), Emtricitabine (NRTI class), and Tenofovir (NRTI class) are currently administered in combination as Truvada® or Atripla® pills. To determine if adipocytes affect the antiviral function of ART compounds, infected memory CD4 T cells were cocultured with adipocytes (direct contact) and IL2, or medium alone, in the presence of Emtricitabine, Efavirenz, or Tenofovir for 3 days, then extracellular p24 measured. Figure 2.10 shows a p24 ELISA representative of 3 separate experiments indicating that ~1-10µg/ml Emtricitabine, Efavirenz, Tenofovir, or the combination of these drugs reduced HIV replication by memory CD4 T cells in medium alone at least 50%. However, the antiviral efficacy of ~1µg/ml Tenofovir was partially mitigated during coculture with adipocytes. Thus, adipocytes may interfere with the viral suppressive mechanism of some ART drugs.

**Figure 2.10. Mitigation of Tenofovir antiviral efficacy by adipocytes.**

Memory CD4 T cells were purified from peripheral blood and infected with HIV (strain SF162) for 24 hrs in IL2 medium. Cells were then washed and  $5 \times 10^5$  infected cells cocultured with  $5 \times 10^5$  adipocytes (direct contact) or in medium alone (+IL2) in 24-well plates (2ml). Emtricitabine, Efavirenz, or Tenofovir were added at indicated concentrations immediately after starting the coculture. Cells were cultured for 3 days, then supernatants harvested for extracellular p24 measurements (shown is one p24 ELISA representative of 3 separate experiments).



## ***2.7. Suppression of adipogenesis by memory CD4 T cells, and lack influence by hallmark adipokines leptin and adiponectin for HIV replication***

We lastly studied if HIV-infected memory CD4 T cells conversely affect adipose homeostasis such as adipogenesis, a critical function of adipocytes that becomes defective during HIV infection and obesity. Preadipocytes were first cocultured with uninfected or HIV-infected memory CD4 T cells for up to 6 days in IL2 medium, then preadipocyte cell cycle and viability examined. Preadipocyte cell cycle and apoptosis were not affected after 6 days coculture with uninfected or HIV-infected memory CD4 T cells (n=4, Fig. 2.11A). Preadipocytes remained mostly viable after 6 days coculture with CD4 T cells (<2% apoptotic during culture with uninfected or HIV-infected memory CD4 T cells compared to ~0.5% in medium alone), and visual inspection of preadipocytes by bright-field microscopy also showed that preadipocytes cocultured with CD4 T cells remained adherent and morphologically healthy (data not shown).

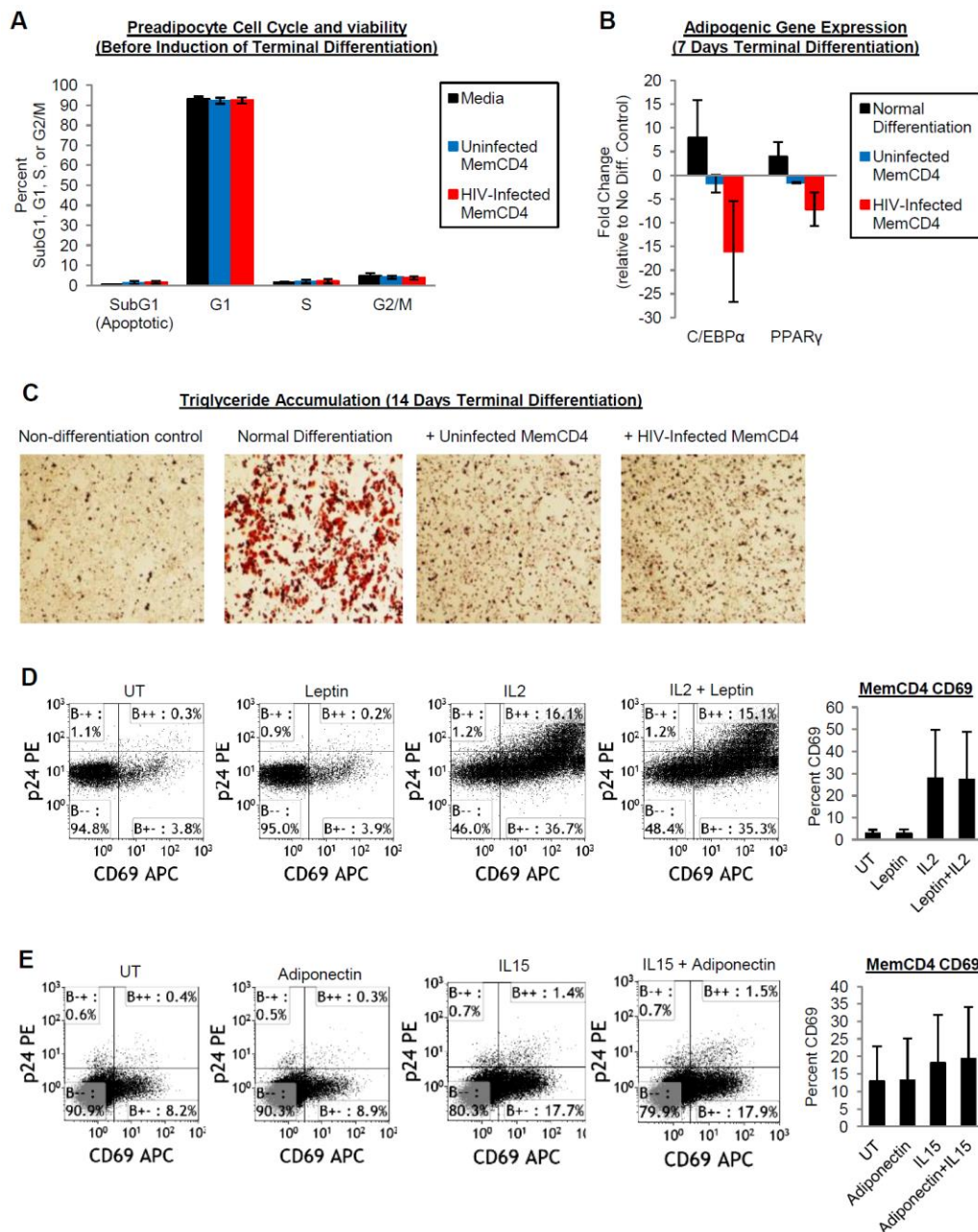
Following coculture of preadipocytes with memory CD4 T cells, the coculture was continued by changing the medium to Adipocyte Differentiation Medium and induction of terminal differentiation to mature adipocytes for 2 weeks. Expression of critical adipogenic genes C/EBP $\alpha$  and PPAR $\gamma$ 2 was measured by real-time PCR after 7 days differentiation, and endpoint lipid accumulation was measured by Oil Red O staining after 14 days of differentiation. Expression of C/EBP $\alpha$  and PPAR $\gamma$ 2 by early differentiated adipocytes after 7 days of differentiation was downregulated by both uninfected and HIV-infected memory CD4 T cells (Fig. 2.11B). Relative to the non-differentiation control, C/EBP $\alpha$  and PPAR $\gamma$ 2 expression were upregulated ~8.0- and 3.9-fold, respectively, in medium alone, whereas C/EBP $\alpha$  expression was reduced ~1.6-1.8-fold, and PPAR $\gamma$ 2 expression reduced ~7.2-16.1-fold by both uninfected and infected CD4 T cells. However, this downregulation of C/EBP $\alpha$  and PPAR $\gamma$ 2 was comparable between uninfected and infected CD4 T cells. Corroborating the downregulation of adipogenic gene expression, both uninfected and infected memory CD4 T cells completely blocked adipocyte lipid accumulation as measured by Oil Red O-staining (Fig. 2.11D shows representative images of 5 separate experiments). Additionally, uninfected or

infected CD4 T cells did not grossly alter adipocyte viability or morphology after 2 weeks of coculture (data not shown). Consistent with other studies, activated CD4 T cells impaired adipocyte development, but whether HIV-infected CD4 T cells impact adipocyte functions more so than uninfected CD4 T cells requires more detailed investigation.

We further explored if leptin and adiponectin, two major adipokines that exert immunomodulatory effects (T cell proliferation, cytokine production, or viability), also affect CD4 T cell activation and HIV replication. Additionally, expression of leptin and adiponectin receptors is upregulated by activated CD4 T cells [122-124]. Memory CD4 T cell activation and HIV production were unaffected by direct treatment with these adipokines, either alone or in conjunction with IL2 or IL15 (Fig. 2.11D and E), nor did leptin or adiponectin synergize with IL6 or TNF $\alpha$  to affect T cell activation (data not shown). Additionally, mRNA expression of leptin and adiponectin by adipocytes was not affected after 5 days coculture with uninfected or HIV-infected memory CD4 T cells (data not shown). Thus, leptin and adiponectin do not appear to have important roles in these short-term in vitro coculture experiments.

**Figure 2.11. Suppression of adipogenesis by memory CD4 T cells.**

(A) Preadipocyte cell cycle and proliferation after 6 days coculture with memory CD4 T cells prior to induction of terminal differentiation. Cell cycle graph shows mean $\pm$ sem SubG1 (apoptotic), G1, S, or G2/M percentages after staining preadipocytes for DNA content with propidium iodide (n=4). (B) Expression of C/EBP $\alpha$  and PPAR $\gamma$ 2 during preadipocyte differentiation and coculture with memory CD4 T cells. Shown are mean $\pm$ sem C/EBP $\alpha$  and PPAR $\gamma$ 2 mRNA fold changes relative to no differentiation negative control after 7 days differentiation (n=3). (C) Adipocyte triglyceride development during coculture with memory CD4 T cells. Shown are representative brightfield microscopy images (40x) of Oil Red O-stained adipocytes after 14 days of differentiation (representative of 5 separate experiments). (D and E) Lack of influence by leptin or adiponectin for T cell activation and HIV production. HIV-infected memory CD4 T cells were treated with 20ng/ml recombinant leptin or adiponectin, as well as IL2 or IL15 for 5 days. Shown are representative CD69/p24 flow cytometry dotplots and mean $\pm$ sem CD69 expression (n=2-3).



## **Adipose tissue of humans and rhesus macaques as viral reservoirs**

### ***2.8. Adipose-resident T cells of HIV-infected humans are primarily activated memory T cells with the capacity for Th1 cytokine production, and experience inversion of CD4/CD8 ratios***

The in vitro coculture results between HIV-infected CD4 T cells and adipose cells demonstrate a possible role for adipose tissue in promoting HIV persistence by enhancing CD4 T cell activation, viability, and HIV replication. Additionally, other human and murine studies clearly demonstrate the infiltration and functional regulation of adipose memory CD4 T cells in vivo, suggesting that infected memory CD4 T cells are also likely to reside in adipose tissue where viral replication or latency establishment may occur. To determine if adipose tissue is a reservoir for HIV-infected memory CD4 T cells, adipose tissue of 5 infected patients (4 of which were cART-treated) were acquired from live or recently deceased donors (Fig. 2.12A shows patient characteristics). Adipose tissue samples were harvested from subcutaneous or visceral depots, and AT-SVF cells isolated by collagenase digestion, which typically yielded  $\sim 0.3\text{-}1 \times 10^6$  AT-SVF cells per gram of adipose tissue (Fig. 2.12B shows the general AT-SVF isolation method). The memory T cell distribution in AT-SVF cells was then examined by flow cytometry, and HIV DNA examined by nested PCR. Peripheral blood and other tissues (lymph nodes or thymus) of infected patients (depending on donor availability) were also studied for comparison of viral detection, and adipose tissue and peripheral blood of uninfected healthy control donors were examined for comparisons of immune cell phenotypes.

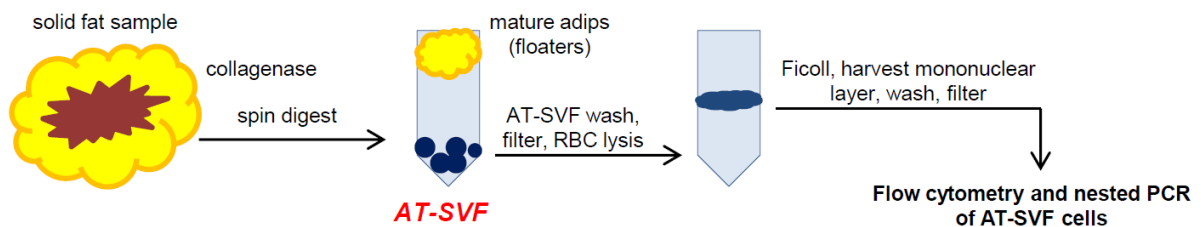
**Figure 2.12. HIV patient characteristics and general method of AT-SVF isolation from adipose tissue samples.**

(A) AT-SVF of 5 HIV-infected persons (from surgical donors or recently deceased patients) were studied (4 were on ART treatment). (B) AT-SVF cells were isolated by collagenase digestion from solid adipose tissue samples, followed by flow cytometry and nested PCR analyses.

**A** HIV patient characteristics

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

**B** Adipose tissue stromal-vascular fraction (AT-SVF) isolation

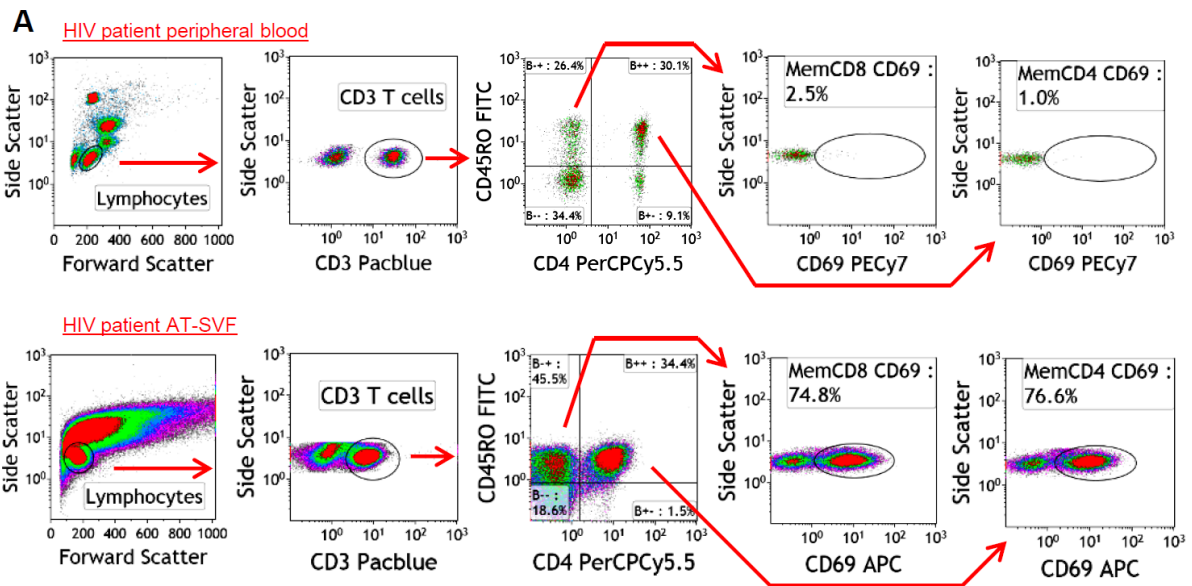


Measurement of the CD3 T cell distribution in AT-SVF cells of uninfected and infected donors showed that most CD4 and CD8 T cells were of a memory CD45RO<sup>+</sup> phenotype (Fig. 2.13A shows flow cytometry gating schemes for examination of activated memory T cells in peripheral blood or AT-SVF cells). Within the CD3 T cell population, uninfected healthy control donor (HC) AT-SVF contained the highest proportion of memory CD4<sup>+</sup>CD45RO<sup>+</sup> cells (~63%), whereas the proportion of memory CD4<sup>+</sup>CD45RO<sup>+</sup> cells of HC PB CD3 T cells was ~40% (n=3-4, p<0.05, Fig. 2.13B). The proportion of HC memory CD4 T cells gated on CD3 T cells was also higher than that of memory CD8 T cells in HC PB (~18%) and AT-SVF (~15%) (p<0.01). However, in AT-SVF of HIV patients, the opposite distribution of CD4/CD8 T cells was observed (Fig. 2.13C shows a representative CD4/CD45RO dotplot comparing HC and HIV patient AT-SVF). Compared to HC AT-SVF, the proportion of CD4<sup>+</sup>CD45RO<sup>+</sup> cells in HIV

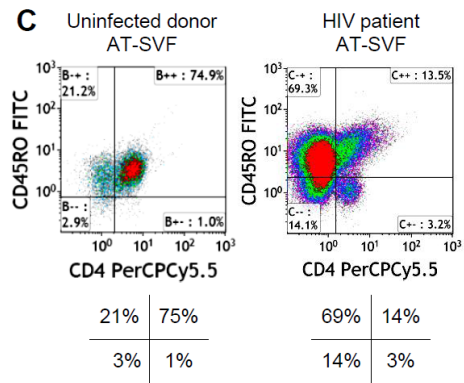
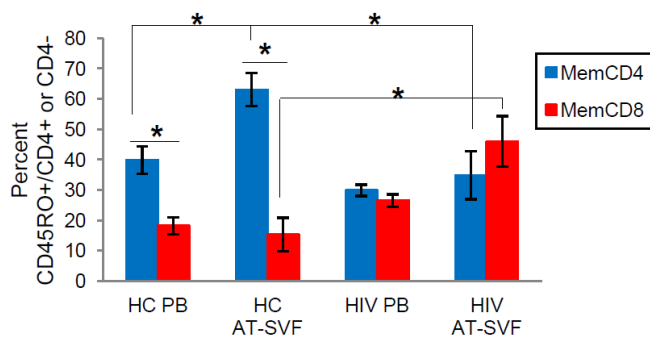
patient AT-SVF was reduced to ~35%, whereas that of CD4-CD45RO<sup>+</sup> cells increased to ~46% (n=4, p<0.05 compared to HC AT-SVF, Fig. 2.13B).

As expected, memory T cells in peripheral blood of both HC and HIV patients expressed little to no CD69 (1-3%, n=3-4), whereas CD69 expression by AT-SVF memory T cells was substantially higher and similar between HC (~61-72%, n=5) and HIV patients (~60-67%, n=4, Fig. 2.13D). These CD69 expression levels by adipose memory T cells are also similar to memory T cell activation levels in lymphoid and intestinal tissues (~60-80%), and even similar to CD69 levels of memory CD4 T cells during coculture with adipose cells in vitro (~50-70%) shown in Figure 2.2 of the present study [192-194]. Lastly, in PBMC and AT-SVF of 3 healthy control donors, Th1 cytokine functionality (IL2 and IFN $\gamma$  production) by memory T cells was also examined since Th1 cells regulate adipose functions and inflammation, as well as serve as productive hosts for HIV. PBMC and AT-SVF cells were stimulated with PMA/IO+GolgiPlug for 5hrs, then stained for CD3, CD4, CD45RO, and either IL2 or IFN $\gamma$ . IL2 and IFN $\gamma$  production by activated AT-SVF memory CD4 and memory CD8 T cells mostly resembled those of PB memory T cells, although AT-SVF CD8 T cells appeared to have a greater capacity for IL2 and IFN $\gamma$  production compared to AT-SVF CD4 T cells (Fig. 2.14). In summary, adipose tissue of HIV-infected persons harbor activated memory CD4 T cells that phenotypically resemble CD4 T cells in other lymphoid and non-lymphoid tissues where virus persists.

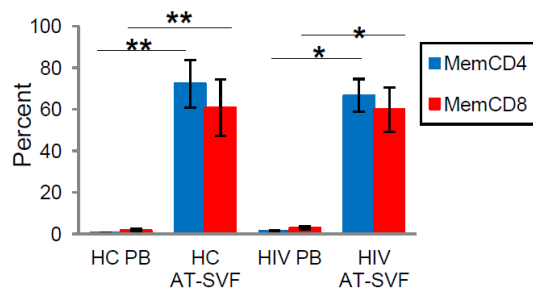
*Figure 2.13. Distribution of activated memory T cells in adipose tissue of HIV-infected persons.* AT-SVF were isolated from adipose tissue of uninfected healthy control (HC) and HIV-infected donors. Peripheral blood was also examined for comparison. (A) Representative flow cytometry gating scheme for examination of memory CD4 (CD3+CD4+CD45RO+) and memory CD8 (CD3+CD4-CD45RO+) T cells, and activation levels (CD69). (B) Mean±sem distribution (gated on CD3 T cells) of memory CD4+CD45RO+ or CD4-CD45RO+ T cells in peripheral blood or AT-SVF of uninfected HC donors (n=4-5) and HIV patients (n=3-4, \*p<0.01). (C) Representative flow cytometry dotplots showing inversion of memory CD4/memory CD8 distribution of HIV patient AT-SVF compared to uninfected HC donor AT-SVF. (D) Mean±sem CD69 expression by peripheral blood and AT-SVF memory T cells (gated on CD3 T cells) of uninfected HC donors (n=4-5) and HIV patients (n=3-4, \*\*p<0.001 and \*p<0.01).



**B** MemCD4 / MemCD8 distribution (gated on CD3 T cells)

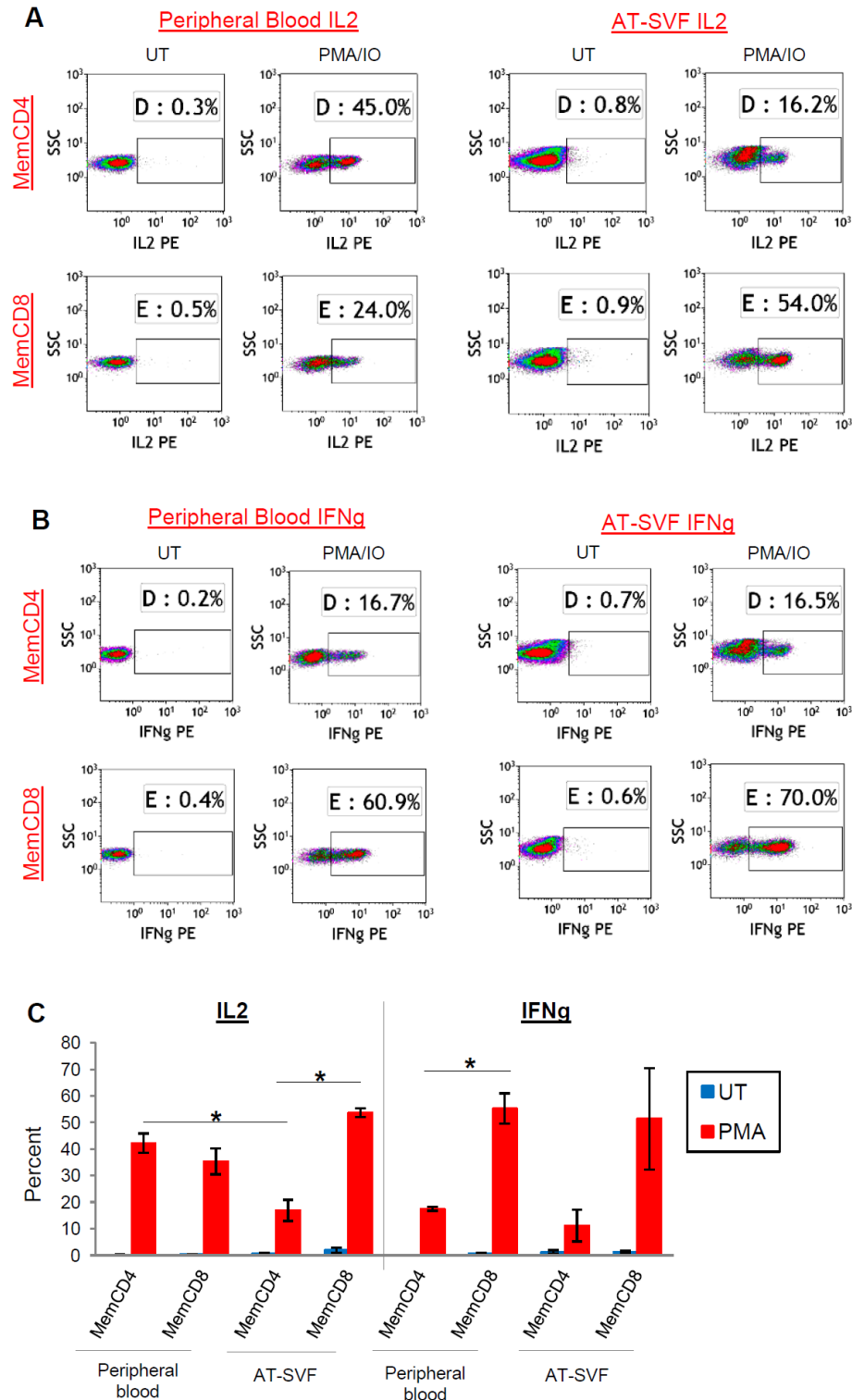


**D** Memory T cell CD69+



**Figure 2.14. Th1 functionality of adipose tissue memory T cells.**

AT-SVF were isolated from adipose tissue, and PBMC were isolated from peripheral blood, of healthy donors and treated with PMA/IO+GolgiPlug for 5hrs, followed by flow cytometry staining for memory T cell IL2 or IFN $\gamma$  production. Shown are representative flow cytometry dotplots (A-B) and mean $\pm$ sem (C) IL2 and IFN $\gamma$  expression by memory CD4 (gated on CD3+CD4+CD45RO+ cells) or memory CD8 (gated on CD3+CD4-CD45RO+ cells) T cells (\* $p$ <0.05, n=3).



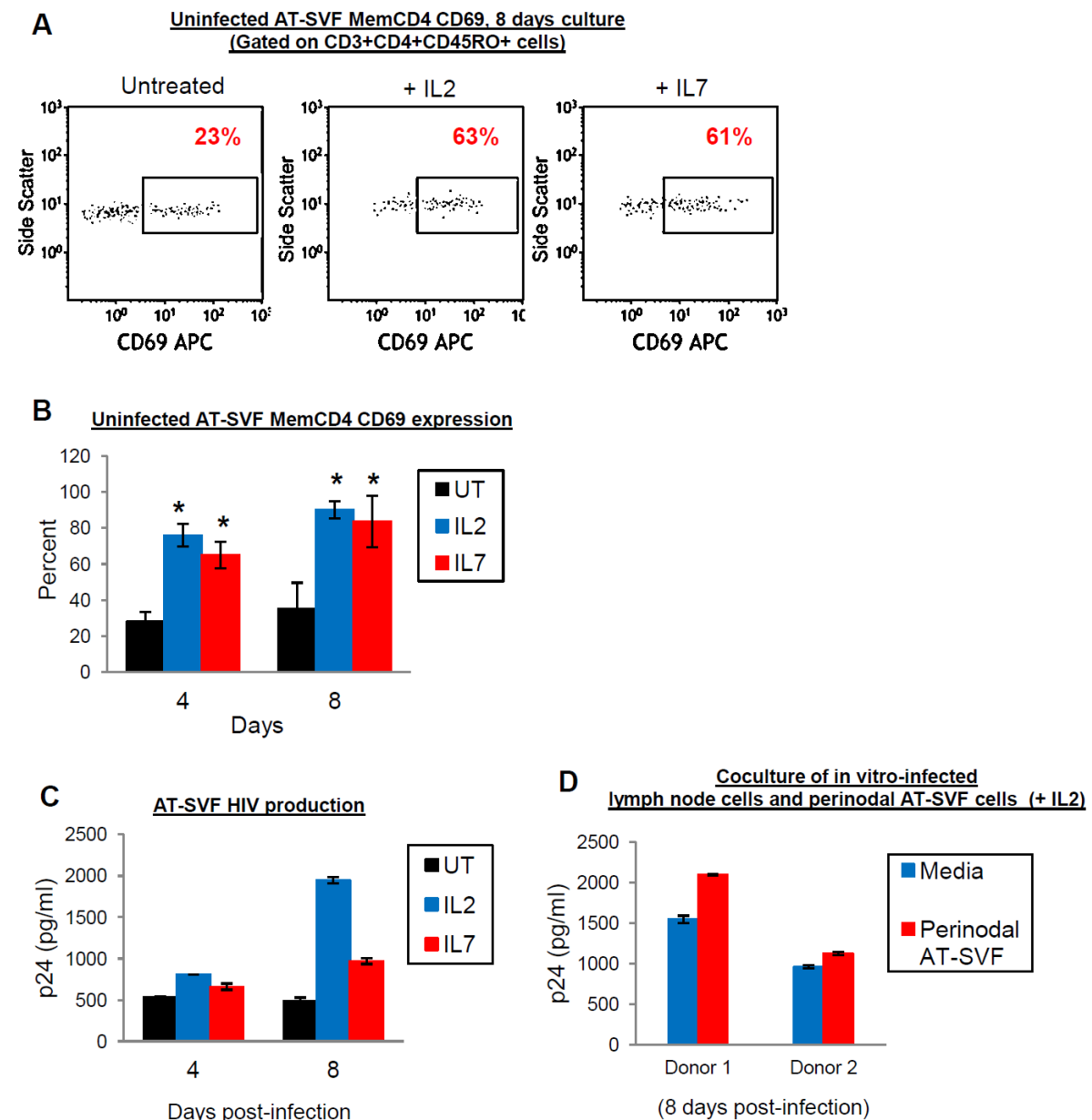
## **2.9. Exposure of AT-SVF cells to HIV ex vivo results in productive viral replication**

We additionally determined if AT-SVF cells could support productive HIV infection ex vivo. AT-SVF cells were isolated from uninfected healthy donor adipose tissue, infected with HIV (R5-tropic) for 24hrs, then cultured for up to 8 days, with or without IL2 or IL7. After 8 days culture, CD69 expression by untreated, uninfected AT-SVF memory CD4 T cells declined to ~28-35% (compared to >60% expression by initially isolated AT-SVF), but remained high at ~65-90% if cultured with IL2 or IL7 (Fig. 2.15A and B, n=2-4). HIV production by infected AT-SVF cells was also observed, even without the addition of IL2 or IL7 (Fig. 2.15C shows one p24 ELISA representative of 2-3 separate experiments).

Perinodal fat can stimulate lymph node T cells via paracrine interactions during inflammation [102]. To determine if perinodal adipose cells influence HIV production by lymph node cells, lymph node cells from healthy donors were first isolated and infected in vitro with R5-tropic HIV, and the surrounding perinodal AT-SVF cells were also isolated. Infected cells were then placed into transwells and cocultured with the autologous perinodal AT-SVF cells in the lower wells. After 8 days coculture in IL2 medium, extracellular p24 from infected lymph node cells was modestly higher during coculture with perinodal AT-SVF cells compared to medium alone (Fig. 2.15D). Thus, direct exposure of AT-SVF cells to HIV results in productive infection in the presence of common gamma-chain cytokines, and perinodal AT-SVF cells may further enhance HIV production by lymph node CD4 T cells during HIV infection.

**Figure 2.15. Direct exposure of AT-SVF cells to gamma-chain cytokines and HIV ex vivo results in memory CD4 T cell activation and HIV production.**

(A and B) CD69 expression (representative flow cytometry dotplots and mean $\pm$ sem CD69%) by healthy donor AT-SVF memory CD4 T cells (gated on CD3+CD4+CD45RO+ cells) after 8 days culture with IL2 or IL7 (n=2-4, \*p<0.05). (C) HIV production by healthy donor AT-SVF cells after in vitro infection. AT-SVF cells were infected with HIV (R5-tropic, 0.1 MOI) for 24hrs, washed, then cultured 4-8 days with IL2 or IL7 (shown is a p24 ELISA representative of 2-3 separate experiments). (D) HIV production by ex vivo-infected lymph node cells during coculture with autologous perinodal AT-SVF cells. Surrounding adipose tissue of lymph nodes was first removed and AT-SVF cells isolated and cultured. Lymph nodes were digested, then directly infected with HIV (R5-tropic, 0.1 MOI). Infected lymph node cells were then washed, seeded into transwells ( $\sim 5 \times 10^5$ ), and cocultured with perinodal AT-SVF cells ( $\sim 5 \times 10^5$  in lower wells) for 8 days with IL2 (shown are p24 ELISA's of 2 experiments).



### ***2.10. HIV provirus is readily detectable in the adipose tissue stromal-vascular-fraction cells of ART-treated patients***

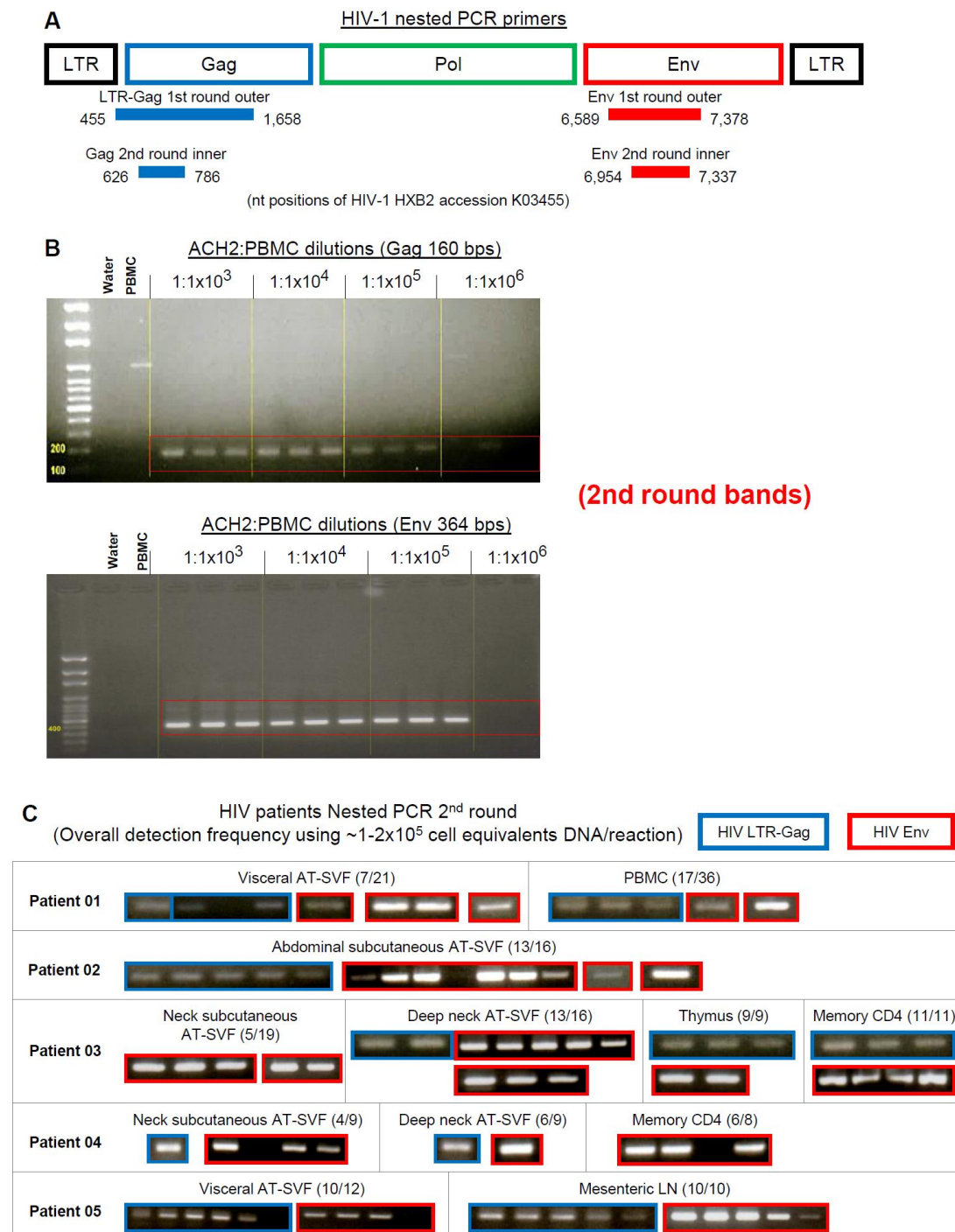
Following the observation of activated memory CD4 T cells in adipose tissue of HIV-infected patients, the presence of virus was next determined. We first determined the qualitative presence of HIV DNA in AT-SVF of 5 patients (4 ART-treated and 1 untreated) by nested PCR using primer-pairs for LTR-Gag (a conserved region) and Envelope (a highly variable region) (Fig. 2.16A). Assessment of primer sensitivity using ACH2 cells (latently infected T cell line harboring one proviral copy) diluted in healthy donor PBMC (irrelevant cell DNA) indicated detection limits of ~1 HIV copy per  $1 \times 10^5$  PBMC (Fig. 2.16B shows 2nd round products for LTR-Gag-160bps and Env-364bps). Using these PCR conditions, HIV DNA was detected in AT-SVF from different adipose depots (subcutaneous, deep neck, visceral abdominal) of all 5 patients with both Gag and Env primers (Fig. 2.16C indicates the overall replicates tested and detection frequency). Other tissues such as PBMC, memory CD4 T cells purified from peripheral blood, thymus, or lymph nodes were also examined and yielded positive bands as expected. Bands were observed mostly after the 2nd round of PCR (35-40 cycles), and typically not after the 1st round (35-40 cycles). More quantitative detection was initially attempted by real-time PCR, but yielded inaccurate results below ~100 viral DNA copies per  $1 \times 10^5$  AT-SVF cell equivalents DNA. Additionally, sorting of AT-SVF cells to determine whether the virus is present mainly in adipose CD4 T cells or macrophages would be a next logical step, but sample limitations make this method challenging.

The second round nested PCR products were then gel-purified and sequenced to assess primer specificity, cross-contamination, and viral diversity. Sequences were then examined and compared amongst patient tissues using multiple sequence alignment and phylogenetic methods. A global phylogenetic analyses comparing all patient Env sequences simultaneously resulted in unique patient and reference strain clustering, indicating lack of cross-contamination (Fig. 2.17). Phylogenetic analyses of Env sequences via maximum likelihood and Bayesian methods recovered monophyly of sequences from individual patients

with strong support, and very similar intra-individual relationships using unpartitioned or partitioned analyses. For patients with sequences from multiple tissues (patients 1 and 3-5), there were no obvious phylogenetic patterns according to tissue. Viral genetic diversity was apparent in sequences from patients 1, 3, and 4, whereas all sequences from patient 5 were similar (only one sequence was available from patient 2). Relationships between patient samples and reference sequences differed between the unpartitioned and partitioned analyses, although support for the relevant bipartitions was low in both cases. Additionally, the diversity and phylogenetic patterns of LTR-Gag nucleotide sequences were generally similar to these Env results (data not shown). Thus, HIV DNA is present in the stromal-vascular-fraction of diverse adipose tissue depots despite “viral suppressive” therapy, but whether HIV evolves in adipose tissue and forms a unique viral species requires further study.

**Figure 2.16. Detection of HIV DNA in adipose tissue of infected patients.**

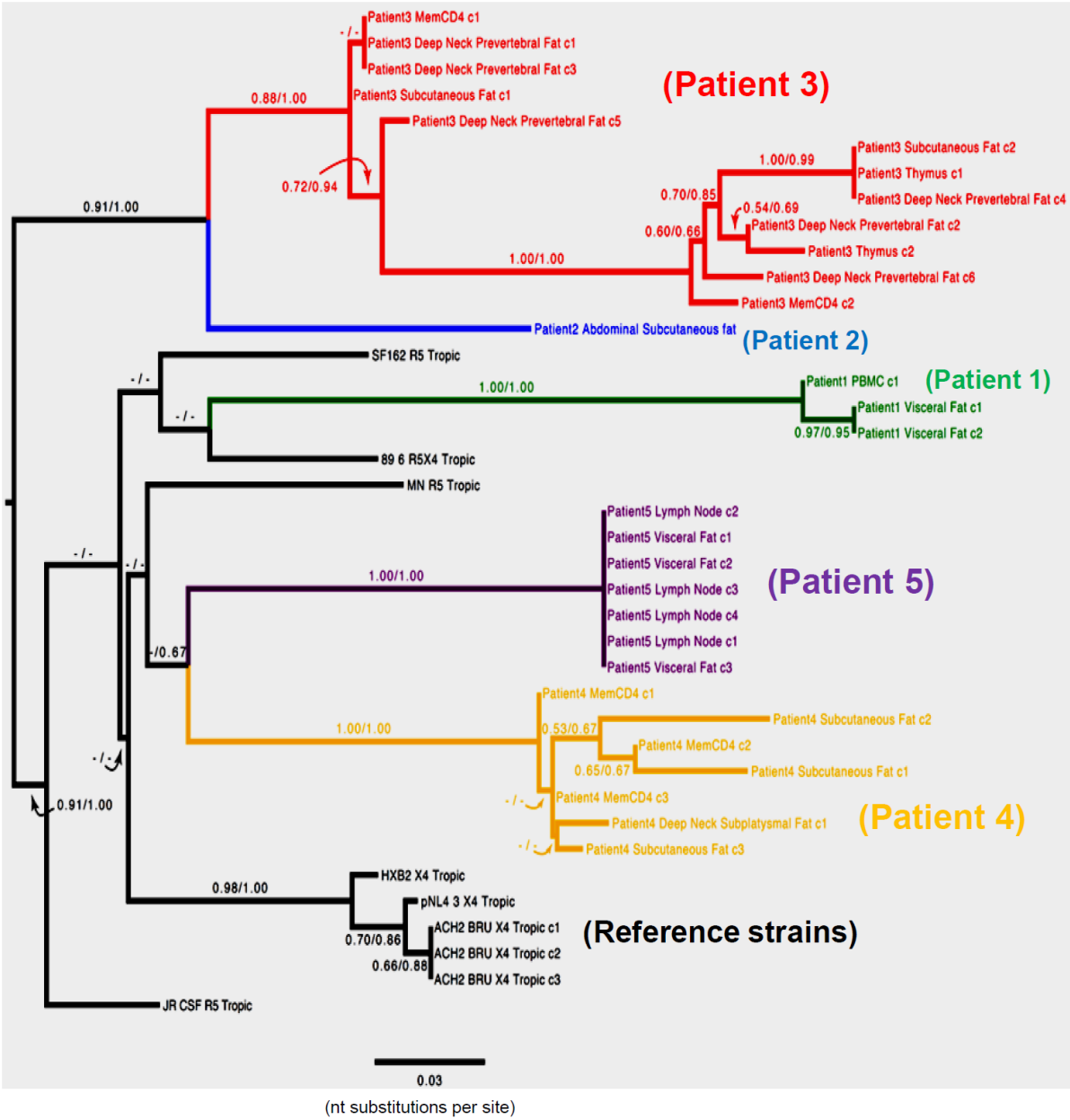
AT-SVF cells were isolated from patient adipose tissue samples (visceral, subcutaneous, or neck regions), followed by DNA extraction from  $\sim 1\text{-}2 \times 10^5$  AT-SVF cells. DNA was also extracted from other tissues (PBMC, memory CD4 T cells purified from peripheral blood, thymus, or mesenteric lymph node) for nested PCR comparisons. (A) Nucleotide positions of HIV LTR/Gag and Env genes targeted for PCR reactions. (B) Sensitivity of nested PCR reactions indicating detection limits of approximately one proviral copy (ACH-2 cell lines harboring one genomic HIV copy per cell) out of  $1 \times 10^5$  irrelevant cells (healthy donor PBMC). (C) Shown are representative 2nd round bands of nested PCR reactions, and overall detection frequencies indicated in parentheses (total positive bands observed/total reactions tested).



*Figure 2.17. Inter- and intra-patient sequence diversity of adipose tissue HIV.*

Nested PCR 2nd round products of patient tissues were gel-purified and sequenced. Nucleotide sequences of patient Env (C2V3C3 region) were analyzed globally by phylogenetics. Samples included AT-SVF cells (from various fat depots), PBMC, memory CD4 T cells (purified from peripheral blood), thymus, or lymph nodes. Shown is a maximum-likelihood phylogenetic tree inferred using an unpartitioned model. Sequences were aligned with MAFFT, and models of sequence evolution tested via jModelTest 2.1.1 and PhyML 3.0. Maximum-likelihood (ML) inference was performed in Garli 2.0 using 50 independent searches and Bayesian inference was performed in MrBayes 3.2.2 using 4 independent Markov chains. 200 non-parametric bootstrap pseudo-replicates with 5 independent searches each were used to assess branch support for ML estimates. Bootstrap proportions and posterior probabilities were calculated from the output of Garli and MrBayes, respectively, using SumTrees. The tree is arbitrarily rooted at the midpoint for ease of display. Branches are labeled with ML bootstrap proportions and Bayesian posterior probabilities, in that order. Branch lengths correspond to the expected number of substitutions per site. [Figure prepared by Dr. Jeremy Brown of Louisiana State University].

Patient HIV Envelope nucleotide phylogeny

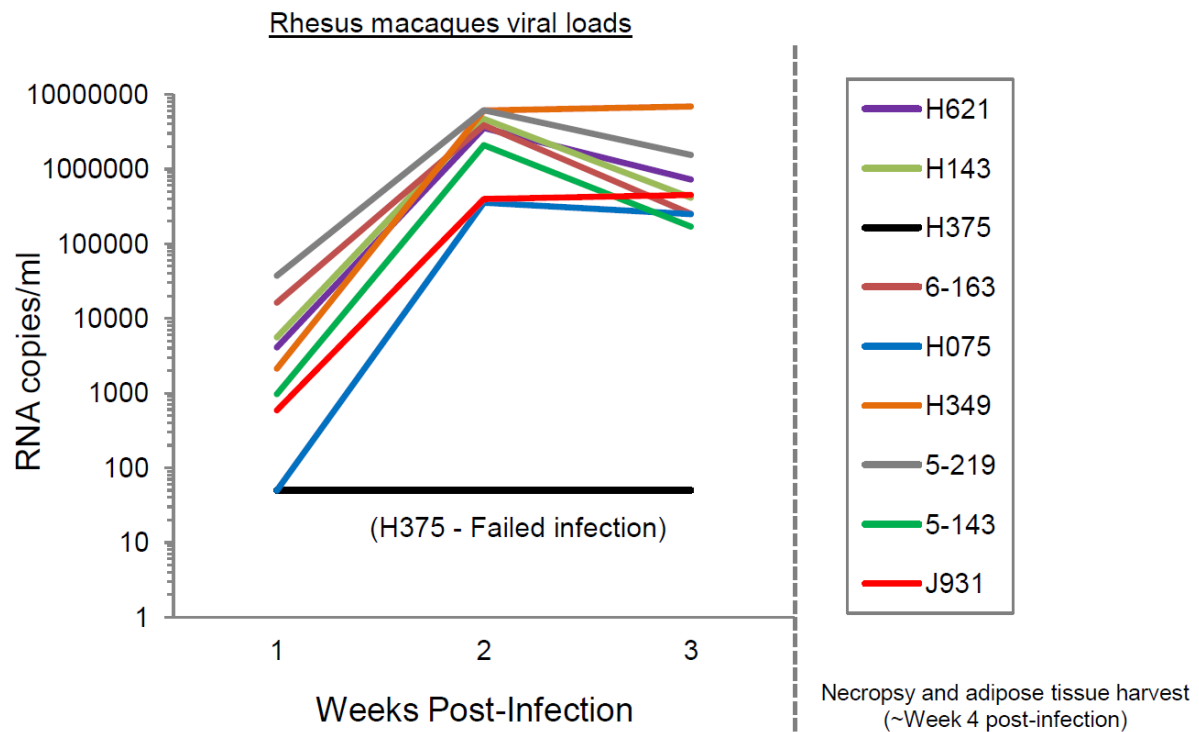


### ***2.11. Adipose tissue stromal-vascular-fraction cells of SHIV-infected rhesus macaques harbor activated memory T cells and provirus***

Rhesus macaques represent the best animal of HIV infection. However, very few studies of immunometabolism and adipose tissue inflammation have been conducted with non-human primates. As part of a larger study that involved SHIV-infected rhesus macaques, we obtained adipose tissue samples following necropsies. 9 rhesus macaques were intra-rectally infected with SHIV strain SF162p3 (SIV mac239 backbone with HIV envelope insertion). 8 out of the 9 monkeys were productively infected as determined by measurement of plasma RNA viral load 1-3 weeks post-infection (Fig. 2.18). Monkeys were necropsied approximately 4 weeks post-infection, and ~10-20grams solid adipose tissue samples harvested from abdominal subcutaneous and visceral regions. Using protocols similar to the human studies, AT-SVF cells were then isolated and examined for activated memory T cells by flow cytometry and SHIV DNA by nested PCR. Peripheral blood and AT-SVF of uninfected rhesus macaques were also examined for immune cell characteristics.

**Figure 2.18. Infection of rhesus macaques prior to isolation of AT-SVF cells.**

Plasma viral loads of 9 rhesus macaques after intra-rectal infection with SHIV.SF162p3. Immediately following necropsy, solid adipose tissue samples (~10-20g) were harvested from abdominal subcutaneous and visceral depots, and AT-SVF cells isolated for flow cytometry and nested PCR measurements.

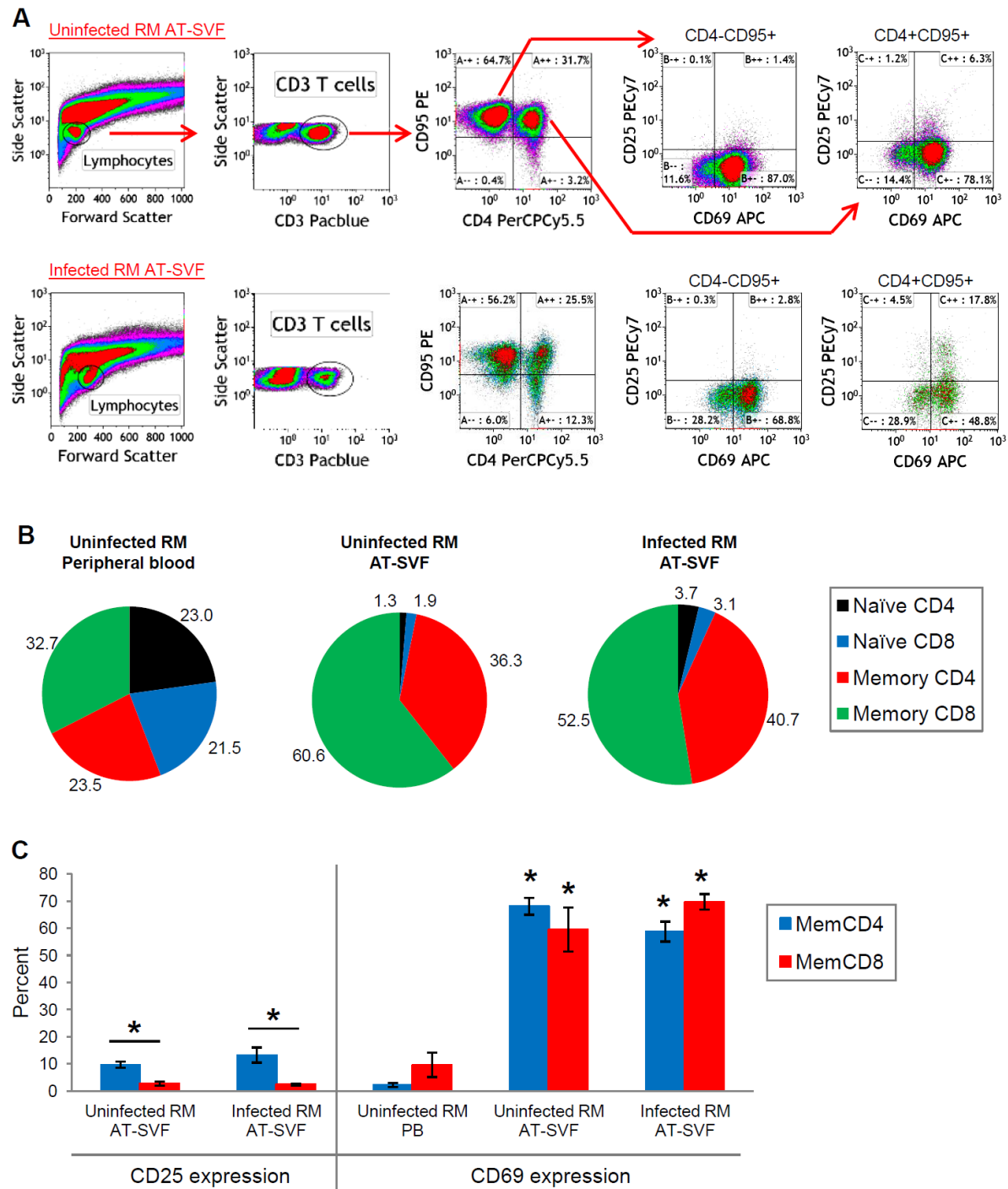


To first determine the content of activated memory T cells in RM AT-SVF, cells were stained for CD3, CD4, CD95 (a pan-memory marker for non-human primate T cells), CD25, and CD69 (Fig. 2.19A shows flow cytometry gating schemes). Peripheral blood was not collected from infected RM's, but was collected from uninfected RM's and examined for comparison to uninfected RM AT-SVF immune cells. Similar to human AT-SVF, the majority of CD4 and CD8 T cells in RM AT-SVF were memory T cells (Fig. 2.19B shows the distribution of naïve and memory T cells gated on CD3 T cells). But by contrast to human AT-SVF, the proportion of memory CD8 (CD3+CD4-CD95+) T cells was higher (~61% of uninfected, and ~53% of infected) than memory CD4 (CD3+CD4+CD95+) T cells (~36% of uninfected, and ~41% of infected) in both uninfected and infected RM AT-SVF (Fig. 2.19B). Additionally, little

change of the CD4:CD8 ratio was observed in infected RM's, likely due to the short time period of infection (~1 month). As with human AT-SVF memory T cells, CD69 expression by RM AT-SVF memory T cells was also high (~60-70%), and similar between uninfected and infected monkeys (Fig. 2.19C). AT-SVF memory CD4 T cells also expressed ~10% CD25, whereas memory CD8 T cells expressed little to no CD25 (<1-2%), possibly indicating the presence of Tregs as extensively described in human and mouse studies of obesity and adipose inflammation.

**Figure 2.19.** Distribution of activated memory T cells in AT-SVF of uninfected or infected rhesus macaques.

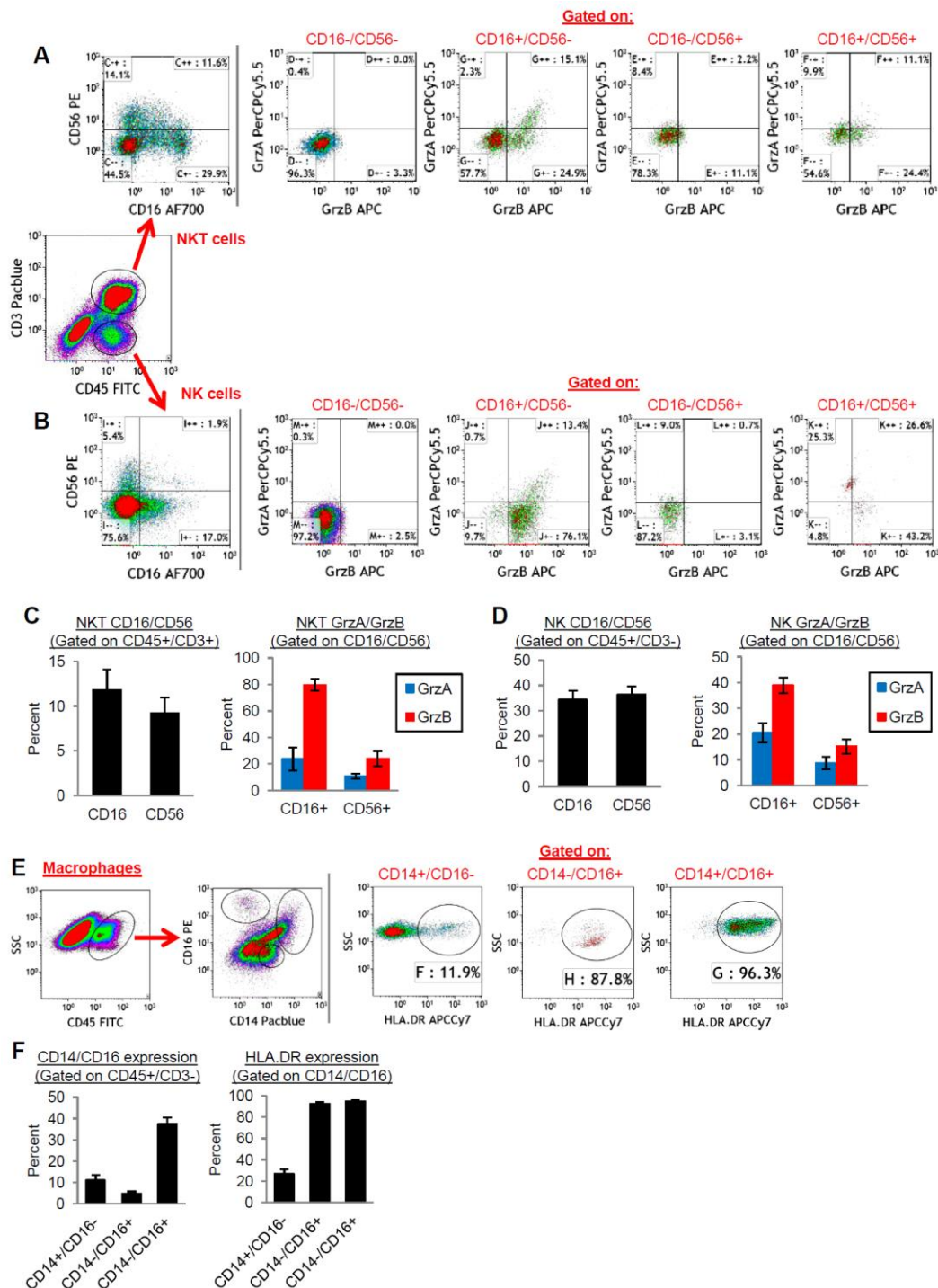
(A) Gating scheme for examination of activation levels (CD25/CD69) by memory CD4+CD95+ or CD8+CD95+. (B) Mean distribution of naïve (CD4+CD95- or CD8+CD95-) or memory (CD4+CD95+ or CD8+CD95+) T cells gated on CD3 T cells of peripheral blood of uninfected RM (n=8), or of AT-SVF of uninfected (n=5) or infected (n=7) RM. (C) Mean±sem CD25 (n=5, \*p<0.05) and CD69 (n=5, \*p<0.05 compared to PB of uninfected RM) expression by memory T cells of RM AT-SVF or peripheral blood.



Other leukocytes that regulate adipose functions and inflammation in humans and mice, such as NK, NKT, macrophages, and B cells, were also examined in adipose tissue of 3 uninfected rhesus macaques since the immune cell composition in adipose tissue of non-human primates has not been studied. NKT and NK cells were identified based on expression of CD16, CD56, GrzA, and GrzB (gated on CD45+CD3+ for NKT or CD45+CD3- for NK cells) (Fig. 2.20A-D shows flow cytometry gating schemes and mean $\pm$ sem NKT/NK marker expression). Expression of NKT cell surface markers was ~12% CD16+ and ~9% CD56+ (gated on CD45+CD3+ cells), and expression of granzymes were ~24% GrzA and ~80% GrzB (gated on CD45+CD3+CD16+), and ~11% GrzA and ~24% GrzB (gated on CD45+CD3+CD56+ cells). Expression of NK cell surface markers was ~34% CD16+ and ~36% CD56+ (gated on CD45+CD3- cells), which was higher than NKT cell CD16 and CD56 expression, and expression of granzymes were ~21% GrzA and ~39% GrzB (gated on CD45+CD3-CD16+), and ~9% GrzA and ~15% GrzB (gated on CD45+CD3-CD56+ cells). Macrophages were identified by CD14, CD16, and HLA.DR expression (gated on CD45+ cells in the high FSC/SSC region of light scatter dotplots which were distinctly above the low FSC/SSC lymphocyte cells). Surface expression was ~11% CD14+CD16- (~27% HLA.DR+), ~5% CD14-CD16+ (~93% HLA.DR+), and ~37% CD14+CD16+ (~95% HLA.DR+) (Fig. 2.20E and F). The pan-B cell marker CD19 was not detected, but ~43% CD27 (a memory B cell marker) was observed (gated on CD45+CD3- cells) (data not shown). Thus, adipose tissue of rhesus macaques harbors various innate immune cells which may regulate adipose homeostasis and interact with adipose T cells during SIV infection and chronic inflammation.

**Figure 2.20. Distribution of innate immune cells in adipose tissue of uninfected rhesus macaques.**

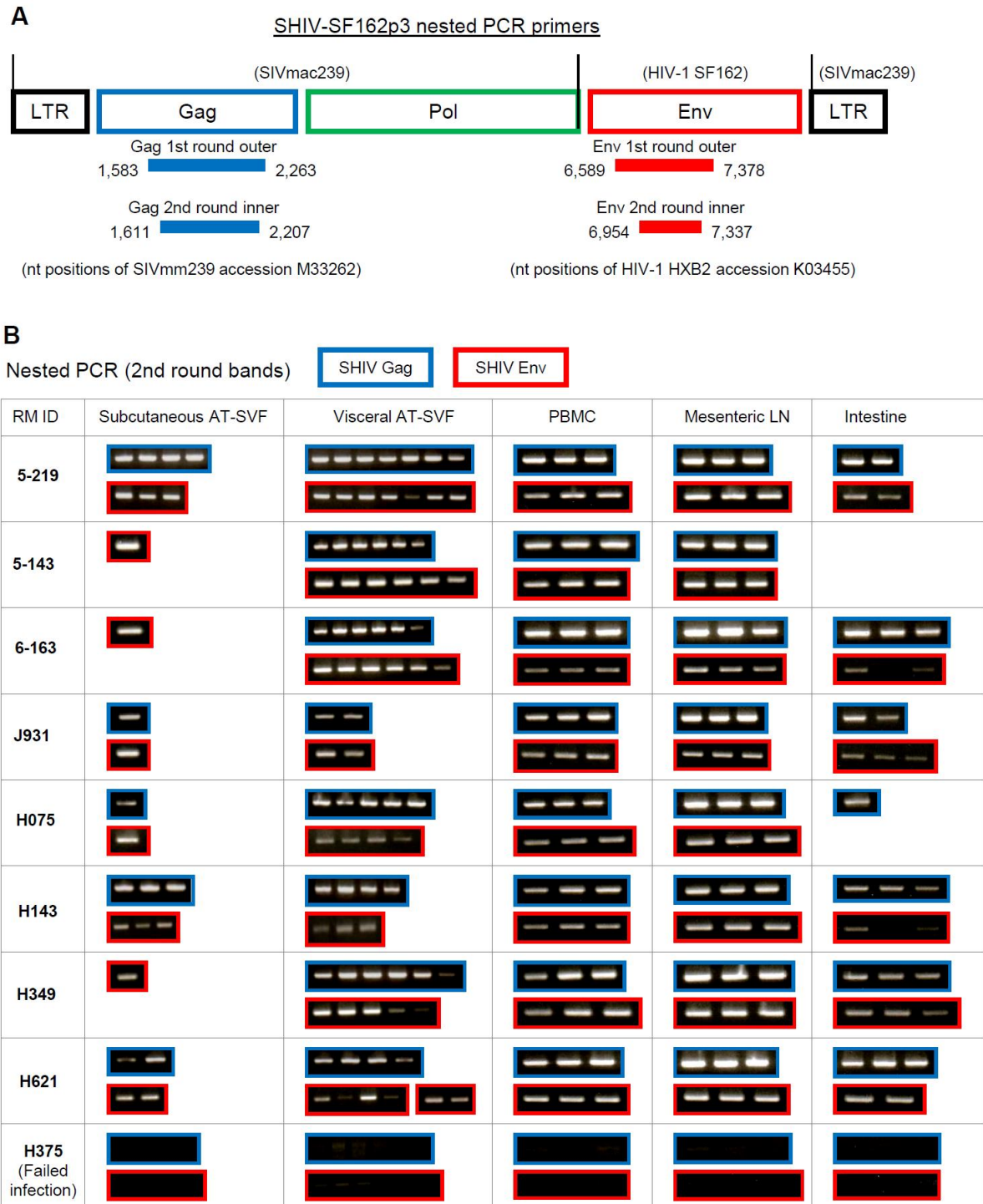
AT-SVF were isolated from adipose tissue of uninfected RM's and flow cytometry stained for NK, NKT, and macrophage markers. (A and B) Gating scheme for examination of AT-SVF NKT (CD16, CD56, GrzA, and GrzB gated on CD45+CD3+ cells) and NK (CD16, CD56, GrzA, and GrzB gated on CD45+CD3- cells) cells. (C and D) Mean±sem expression of CD16/CD56 and GrzA/GrzB (gated on CD16/CD56 cells) by AT-SVF NKT and NK cells. (E and F) Examination of AT-SVF CD14/CD16 macrophages (gated on CD45+ cells) and HLA.DR expression.



To examine AT-SVF cells for the presence of SHIV DNA, DNA was extracted and nested PCR performed in similar fashion to human AT-SVF cells. SIVmac239 Gag and human Env genes were targeted for PCR amplification (Fig. 2.21A). DNA was also extracted from PBMC, mesenteric lymph nodes, and intestinal tissues and examined for comparison. Replicates for 6-9 were tested for SHIV detection in subcutaneous and visceral AT-SVF, and replicates of 3 were tested for detection in PBMC, MLN, and intestinal tissues. SHIV Gag and Env DNA were detected in virtually all tissues of all 8 infected monkeys (Fig. 2.21B). The detection frequency in visceral AT-SVF was also higher than subcutaneous AT-SVF, likely due to the higher AT-SVF vascularization and yield. In corroboration of the plasma viral load of RM H375 indicating a failed infection (Fig. 2.18), SHIV DNA was also not detected in any tissues. PCR bands were then sequenced and compared by multiple sequence alignment to assess evolution and diversity. However, Env and Gag sequences were nearly identical amongst all monkeys (Fig. 2.22 shows Env nucleotide sequence alignments), consistent with other reports of high sequence homology and minimal viral evolution during this early infection period of rhesus macaques [205-208]. These data collectively show that activated memory CD4 T cells and virus become compartmentalized within adipose tissue of rhesus macaques during acute infection.

**Figure 2.21. Detection of SHIV provirus in adipose tissue of infected rhesus macaques.**

(A) Nucleotide positions of SHIV Gag and Env genes targeted for nested PCR reactions. (B) Nested PCR 2nd round gel bands of AT-SVF (~5x10<sup>4</sup>-2x10<sup>5</sup> cell equivalents of DNA), PBMC, mesenteric lymph nodes, or intestinal tissues of 8 infected RM's. Replicates of 6-9 were tested for subcutaneous and visceral AT-SVF, and replicates of 3 were tested for PBMC, MLN, and intestinal tissues.



*Figure 2.22. Near sequence identities of SHIV Env genes derived from adipose tissue of infected rhesus macaques.*

Products from the SHIV Env 2nd round nested PCR reactions of AT-SVF DNA of 8 infected monkeys (subcutaneous and visceral) were gel-purified, sequenced, and aligned with Clustal-Omega software. Yellow-highlighted nucleotides indicate a nucleotide difference compared to other nucleotides in the alignment column (sequences include nucleotide positions G:7042 to C:7329 relative to HIV HXB2).

[illegible]

## Chapter 3

### Discussion and Future Directions

#### ***3.1. Immunometabolism and HIV pathogenesis***

In the past several years, a substantial amount of new information has been learned about adipose tissue physiology and metabolism that has greatly enhanced our understanding of metabolic disorders such as HIV Lipodystrophy and metabolic syndrome, diabetes, obesity and chronic inflammation. A new field of “Immunometabolism” research has emerged describing the intricate relationship between the immune system and adipose tissue homeostasis [98]. Amongst the more novel findings include the following; the majority of adipose CD4 T cells are memory effector subsets such as Th1, Tregs, or Th17 that express high levels of activation markers (CD69, CD25, and HLA.DR); adipocytes express not only adipokines, but proinflammatory and immune-regulatory common gamma-chain cytokines (IL2, IL7, and IL15); and adipose CD4 T cells are activated by adipose-resident macrophages and dendritic cells, and by adipocytes via direct contact interactions [106-110]. In addition to the ubiquity of adipose tissue in subcutaneous and visceral regions, adipocytes are also abundant in lymphoid organs such as lymph nodes and bone marrow, tissues known for HIV persistence in antiretroviral-treated patients. These findings suggest that adipose depots may regulate HIV infection, and may promote rather than suppress most steps of HIV replication due to regulation and stimulation of T cells in adipose tissue.

The goal of the present study was to address the hypothesis that adipose tissue and adipocytes indeed directly influence HIV pathogenesis to contribute to viral persistence. Through in vitro and in vivo studies, the present study demonstrates that adipose cells stimulate infected memory CD4 T cells to enhance HIV replication in a physiologically relevant extracellular milieu, and that the adipose tissue stromal-vascular-fraction of infected humans and rhesus macaques harbors activated memory CD4 T cells and viral DNA. Although the

presence of HIV-infected CD4 T cells or macrophages has been reported in virtually every tissue and organ of an infected person, the present findings are the first report of adipose tissue, and suggest that viral distribution is substantially more widespread than currently believed.

Although in vitro models do not recapitulate the complexities of in vivo biology, the coculture experiments conducted between primary adipose cells and HIV-infected memory CD4 T cells yielded important new information that point to additional mechanisms of HIV induction specific for the adipose tissue milieu. Although adipocytes alone were incapable of stimulating T cells, the inclusion of either IL2, IL7, or IL15 (cytokines that are ubiquitous in vivo) in the milieu allowed adipocytes to increase the level of T cell activation and HIV replication by approximately 2-fold compared to these cytokines alone with adipocytes. Additionally, these cytokines can be expressed not only by adipose-resident immune cells or other accessory cells such as MSC's, but also by adipocytes [133-141]. These in vitro data imply that adipose depots may be important, self-sufficient reservoirs wherein adipocytes, SVF cells, specific common gamma-chain cytokines and proinflammatory cytokines cooperate to promote CD4 T cell activation and HIV replication, while the activated T cells conversely exert inimical effects on adipocyte turnover that contribute to adipose dysfunction and metabolic defects. Furthermore, less appreciated is the fact that adipocytes and these stimulatory cytokines are present in lymphoid tissues where latently infected CD4 T cells persist such as lymph nodes and bone marrow, suggesting that adipocytes at these sites could exert supporting effects for HIV reactivation or viral rebound.

### ***3.2. Role of adipose IL6 and soluble extracellular matrix proteins for CD4 T cell stimulation and HIV replication***

A clear role for adipose IL6 in enhancing T cell activation and HIV replication was observed, which is not be surprising for several reasons. IL6 is multifunctional cytokine and adipocytes are major sources of systemic IL6 [195-196]. Expression of adipose IL6 significantly increases

during HIV infection, and in the present study, IL6 was upregulated in preadipocytes by HIV-infected memory CD4 T cells (Fig. 2.3) [126-131]. This increase in adipose IL6 was further associated with upregulation of T cell activation and HIV production in concert with IL2 as shown in Fig. 2.4, consistent with a previously reported synergy amongst IL2 and proinflammatory cytokines such as IL6 and TNF $\alpha$  that induce HIV replication [38-39]. Additionally, the IL6 receptor (CD126) was highly expressed (>90%) by both uninfected and infected memory CD4 T cells during coculture with preadipocytes (Fig. 2.5), highlighting the importance of IL6 signaling for activation of T cell functions. Lastly, macrophages or microbial products, which are also increased in adipose tissue of infected persons, could further contribute to IL6-mediated effects on HIV replication as suggested by the upregulation of adipocyte IL6 during culture with macrophages and LPS (Fig. 2.9) [173]. Thus, if HIV replication does occur in adipose-resident CD4 T cells in vivo, IL6 is likely to be a key mediator.

A more unexpected result was the role of integrin signaling for adipose enhancement of T cell activation and HIV replication. Integrins are ubiquitous cellular transmembrane receptors composed of a heterodimeric alpha chain (1-11 or D, E, L, M, V, X, 2B) and beta chain (1-8) that can activate T cell motility and other functions. The extracellular ligands that bind integrin receptors are also diverse and include mainly ECM proteins such as collagens, fibronectin, and laminins. In conjunction with blocking IL6, blockade specifically of memory CD4 T cell integrin  $\alpha$ 1 and  $\beta$ 1 chains (VLA-1) completely mitigated the adipose enhancement of HIV replication (Fig. 2.4). Similar to the proinflammatory cytokines IL6 and TNF $\alpha$ , collagens and fibronectin also enhance T cell activation or HIV replication in concert with polyclonal stimulants [47-48]. Additionally, VLA-1 is a unique marker for a subset of activated memory CD4 T cells in inflamed tissues that regulates their activation, proliferation, and phenotype. In the present study, memory CD4 T cells expressed moderate levels of integrin  $\alpha$ 1 (~30%) and  $\beta$ 1 (~70%) during coculture with preadipocytes (Fig. 2.5) [198-200]. The breakdown and reorganization of adipose tissue is a frequent event in obese and HIV-infected persons that can lead to fibrosis

and increased deposition of collagens and fibronectin [142-143]. These results emphasize the diversity of agents in adipose tissue that increase HIV replication from CD4 T cells.

In keeping with the stimulatory potential of adipose tissue for HIV induction, IL7 or IL15 could also substitute for IL2, which are gamma-chain cytokines that may be more abundantly produced than IL2 in adipose tissue. All the common gamma-chain cytokines (IL2, IL4, IL7, IL9, IL15, and IL21) are expressed by mammalian adipocytes or preadipocytes, as well as by adipose SVF cells such as T cells, monocytes/macrophages, dendritic cells, and mesenchymal stem cells [133-141]. However, IL7 and IL15 are well known for positive effects on T cell activation and HIV replication, with impact on adipose tissue homeostasis and metabolic pathways. For example, IL7 expression by adipocytes is upregulated in obese humans, and IL7 or IL15 regulate fat loss, insulin sensitivity, and lipolysis in mice and humans [209-213]. In the present study, adipose cells expressed IL7 and IL15/IL15R $\alpha$  mRNA more than IL2, and IL15/IL15R $\alpha$  was further upregulated during coculture with infected CD4 T cells (Fig. 2.6A), suggesting that IL15 expression may be an indicator of adipose inflammation. IL15 is a unique cytokine which is primarily not secreted but “trans-presented” bound to IL15R $\alpha$  by IL15-presenting cells such as monocytes/macrophages, dendritic cells, and epithelial cells [201-204]. Trans-presentation of IL15 occurs via direct cell-to-cell contact to IL15-recipient (IL15R $\beta\gamma$ C-expressing) CD4 and CD8 T cells and NK cells, and promotes their maturation, proliferation and survival. Additionally, IL15-expressing adipocytes promote maturation of NK cells in adipose tissue in a mouse model [140]. At the protein level, preadipocytes expressed IL15 and little to no IL15R $\alpha$  intracellularly (Fig. 2.6B and C), whereas mature adipocytes expressed both surface and intracellular IL15 and IL15R $\alpha$  (in the absence of soluble IL15 secretion), suggesting that IL15 trans-presentation may be a function of mature adipocytes. Further direct contact experiments between IL15/IL15R $\alpha$ -expressing adipocytes and CD4 T cells are required to better understand this interaction. However, expression of IL15 and IL15R $\alpha$  is upregulated during infections and inflammation, and trans-presentation activates signaling pathways such as MAPK's, PI3K, and STAT's in IL15-recipient T cells that could

induce HIV transcription. These results suggest that IL7 or IL15 may be more abundant, and more potent, mediators of T cell activation in adipose tissue than IL2.

### **3.3. Adipose tissue as compartments of high immune cell activity during viral infections**

As summarized in Table 1, bacteria, viruses, and parasites can localize within adipose tissue during infection, and based on Figures 2.16 and 2.21 of the present study, this microbial repertoire of adipose tissue includes HIV and SIV as well. HIV DNA was detected in the stromal-vascular-fraction of adipose tissue, implicating infected CD4 T cells or macrophages as the likely source of virus, but this requires confirmation. Other studies of adipose microbes have focused on their compartmentalization within the mature adipocyte fraction and in lipid droplets. For example, human studies and mouse models of *M. tuberculosis* infection demonstrate that adipocytes express scavenger receptors for *Mtb*. binding, and that bacteria accumulate within lipid droplets and persists in a dormant state [180-181]. Bacterial uptake by adipocytes has also been shown for *T. cruzi* in mice and humans, and *E. faecalis* in human patients with ulcerative colitis or Crohn's disease [179,185-186]. The mode of adipose localization for HIV in the present study appears to be via infected CD4 T cells or macrophages, but whether virus persists mostly in a dormant latent state, or if some level of active replication occurs, is still to be determined. Also to be investigated is the impact of infected immune cells on adipocyte metabolism and homeostasis, and how their infiltration into adipose tissues is associated with adipose dysfunction during HIV infection and lipodystrophies in the absence of cART therapy.

The T cells in adipose tissue of humans and rhesus macaques were primarily memory T cells that expressed high levels of CD69 (Fig. 2.13 and 2.19), similar to T cell phenotypes in other tissues where HIV replication occurs such as lymph nodes and GALT. Additionally, the memory T cells in human adipose tissue demonstrated Th1 functionality as demonstrated by inducible expression of IL2 and IFN $\gamma$  (Fig 2.14), but other subsets such as Tregs and Th17 may also be present. Intriguingly, CD69 expression levels by human AT-SVF memory CD4 T cells

in vivo (Fig. 2.13) were comparable to those of peripheral blood memory CD4 T cells during in vitro coculture with adipocytes and IL2 (Fig. 2.2). In vivo, CD4 T cells in adipose tissue can be activated by macrophages, dendritic cells, mesenchymal stem cells, or cytokines such as IL2, IL7, IL15, IL6, or TNF $\alpha$  [111-116]. A decline of memory CD4 and increase of CD8 T cell proportions in adipose tissue of HIV patients also occurred (Fig. 2.13), consistent with inversion of the peripheral blood CD4/CD8 ratio observed in HIV patients. Although CD4 T cells were decreased in adipose tissues of HIV patients, sustained viability in the adipose milieu may occur as indicated in Fig. 2.8, a process that could be mediated by adipose factors such as IL7, leptin, or free fatty acids. Additionally, during acute infection, SIV-infected rhesus macaques also experience substantial depletion of CD4 T cells in tissues such as GALT and lymph nodes that correlates to viral peak replication. But in the present study, the proportion of CD8 T cells compared to CD4 T cells in adipose tissue of rhesus macaques remained similar between uninfected and infected monkeys, suggesting that infected CD4 T cells may survive longer within adipose tissues compared to other reservoirs such as intestine, bone marrow, or lymph nodes.

#### ***3.4. Significance of finding HIV-1 and SHIV in adipose tissue of every human or monkey examined***

HIV and SHIV were detected in AT-SVF cells of all 5 infected humans and all 8 infected rhesus macaques examined, and in both subcutaneous and visceral depots, suggesting widespread distribution of infected CD4 T cells or macrophages in adipose tissue. The finding of SHIV in adipose tissue of rhesus macaques strongly complements the human results as the monkeys and humans that were studied differed in some important aspects of infection. By contrast to the infected humans who were chronically infected and ART-treated, the monkeys were studied during acute infection in the absence of antiretroviral treatment, representing a more natural scenario of viral reservoir establishment in adipose tissue. From a quantitative perspective, the size of the latent reservoir in memory CD4 T cells is potentially underestimated by ~60-fold if

“non-inducible proviruses” (integrated proviruses that mostly remain quiescent during conventional stimuli, but still possess the potential for reactivation under optimal conditions in vivo) are considered [61]. But due to the prevalence of adipose tissue, as well as the abundance of adipose-resident CD4 T cells and macrophages, the viral reservoir anatomically is likely to be substantially underestimated further. HIV DNA was detected in different fat depots (subcutaneous, visceral, and neck regions) of all 5 patients studied, and each nested PCR replicate contained  $\sim 1 \times 10^5$  AT-SVF cell equivalents of DNA, of which  $\sim 1$ -10% were memory CD4 T cells based on flow cytometry analyses. Assuming the presence of at least one HIV copy per positive PCR product (and that AT-SVF viral DNA is harbored mostly in CD4 T cells), the frequency of infected CD4 T cells in adipose tissue could be one per  $1 \times 10^4$  CD4 T cells, which is comparable to infected CD4 T cell frequencies in other reservoirs such as lymph nodes and GALT [194]. Additionally, lean to overweight adult humans possess  $\sim 30$ -50kg of fat tissue, and if each gram of fat in an HIV-infected person contains  $\sim 2$ - $5 \times 10^5$  T cells (of which  $\sim 1$ -2% of the T cells are infected), it is possible that up to  $10^8$  copies of HIV could be harbored within adipose tissue, with significantly more in obese patients. Furthermore, much of this virus in adipose tissue could be sequestered away from ART drugs if some compounds such as tenofovir are less effective in adipose tissue as suggested in Fig. 2.10.

Lastly, sequencing and phylogenetic analyses of HIV Gag and Env PCR products from human AT-SVF cells demonstrated unique inter-patient differences as expected, but intra-patient differences and whether viral evolution occurs in adipose tissue that manifests viral species distinct from virus in other tissues requires further samples. However, most of the patients were on ART treatment, which may render these genomic analyses more difficult since replication and diversity have been suppressed. HIV evolution is driven by the high mutation rate of reverse transcriptase enzyme and genetic recombination, antiretroviral compounds, and immune selection pressures such as CD8 T cell responses. Intra-patient differences of HIV sequences such as Gag, Nef, or Env have been described amongst tissues such as peripheral blood, brain, intestine, and kidney [214-216]. Patients 3 and 4 in Fig. 2.17 indicated some

modest differences of Env DNA sequences between adipose tissue and other tissues, but patient 5 indicated little to no differences (although patients 3 and 4 were live donors treated with cART that provided neck subcutaneous fat, whereas patient 5 was a cadaver without ART the last 6 months of life and provided visceral fat, factors that influence viral diversity). The phylogeny of patient 5 Env sequences indicates that the virus is monoclonal, or may be the survivor HIV species in the donor. The near-identity of adipose tissue SHIV Env and Gag sequences amongst the rhesus macaques (Fig. 2.22) is likely due to the short time period of infection, consistent with other reports demonstrating lack of SIV evolution during acute infection [205-208]. It is also possible that viral evolution in adipose tissue may be limited due to lack of replication (ie. virus may remain mostly quiescent and latent), or due to antiviral control by adipose CD8 T cells, but more patients and tissue-derived sequences are required to better assess viral evolution in adipose tissue.

### ***3.5. Resemblance of adipose tissue T cells to tissue-resident memory T cells ( $T_{RM}$ )***

Although the functions of adipose tissue CD8 and CD4 T cell subsets, such as Treg, Th1, Th17, and NKT cells, have been extensively characterized, the existence of a distinct adipose-resident memory T cell subset has not been investigated. Memory T cells are generally categorized as either central or effector memory T cells based on expression of CCR7 and CD62L, which direct their migratory and homing patterns to lymphoid (CCR7+CD62L+ central memory) or non-lymphoid (CCR7-CD62L- effector memory) tissues. More recently, additional subsets of memory T cells, such as memory stem T cells ( $T_{SCM}$ ) and tissue-resident memory T cells ( $T_{RM}$ ) have been described, and some adipose T cells may fall under  $T_{RM}$  classification [217]. Adipose tissue T cells are predominantly memory CD69+ T cells which produce IL-2, IFN $\gamma$ , and granzyme B, which resemble  $T_{RM}$  phenotypes in skin and intestinal tissue.  $T_{RM}$  cells are non-circulating memory CD8 and CD4 T cells that primarily reside in non-lymphoid tissues, such as skin, intestine, brain, and lung, and are believed to be derived from effector memory T cells of previous immune responses for the purpose of mediating more immediate immune

reaction upon rechallenge. The epidermal layer of skin, for example, harbors CD8 and CD4 T<sub>RM</sub> cells that upon antigenic exposure, are rapidly activated and secrete mediators such as IFN $\gamma$ , IL-2, TNF $\alpha$ , and granzyme B, activities that precede the recruitment of additional memory T cells from the circulation [218]. T<sub>RM</sub> cells lack expression of CCR7 and CD62L, and instead express high levels of CD69 and CD103 (integrin  $\alpha$ E) in conjunction with low levels of the transcription factor KLF2, markers which may be important for the retention of T<sub>RM</sub> cells within non-lymphoid tissues [219]. Additionally, the proliferation and homeostatic maintenance of T<sub>RM</sub> cells relies in part on IL-7 and IL-15 in non-lymphoid tissues, cytokines produced by adipocytes as well. Based on the intimate anatomical proximity between the dermis and subcutaneous fat, and also between the intestinal mucosa and visceral fat, it is possible that migratory surveillance of skin and intestinal T<sub>RM</sub> cells include the adipose depots associated with skin and intestinal tissue. However, more extensive phenotyping of adipose-resident T cells (including surface markers, cytokine production, and transcription factors) is required to better profile these T cells within the T<sub>RM</sub> category.

### ***3.6. Associations of adiposity or obesity with virological parameters in HIV patients***

The impact of HIV infection and antiretroviral treatments for adipose tissue homeostasis and metabolism is well-studied, but the converse aspects, such as the influence of obesity levels or BMI for HIV replication and pathogenesis have been less studied. As the present dissertation suggests that adipose tissue may promote HIV infection and replication, the implications of these findings may be extended to additionally propose that obese HIV-infected persons, who experience more inflammation and chronic immune activation compared to infected lean persons, may have higher viral loads and worse metabolic pathologies as well. Interestingly, several studies correlating obesity and BMI with viral loads and immunological status of HIV patients appear to suggest the contrary, reporting that higher BMI levels are associated with lower viral loads and improvement of CD4 T cell counts and suggesting that obesity or weight gain may instead be protective against HIV disease progression. For example, a study of 875

infected women (HIV Epidemiology Research study - HER cohort) showed that women with higher BMI's also had increased CD4 T cell counts and slower progression of AIDS-related disease and death compared to normal weight or underweight women [220]. Additionally, in a study of 125 infected male and female drug users (Miami HIV-1-infected drug abusers - MIDAS cohort), higher BMI and obesity were associated with higher CD4 T cell counts and lower mortality [221]. Another study of 711 infected males and females in the US (HIV Outpatient Study - HOPS study) also observed that obese patients had lower baseline viral loads compared to normal weight patients [222]. These findings also appear to be consistent with a study of 83 HIV-infected South African women demonstrating an inverse correlation between serum leptin (a principal adipokine directly related to BMI) and plasma RNA viral load [223]. However, another study of 158 infected men and women on virally-suppressive ART treatment showed no difference of viral load and CD4 T cell counts between lean and obese patients [224]. These studies suggest that increased adiposity may moderate HIV pathogenesis, but the influences amongst adiposity, metabolism, and HIV pathogenesis is a complex relationship that is likely influenced by many factors such as demographics, antiretroviral treatments, and immunological and disease status.

### ***3.7. Adipose tissue effects on antiretroviral function***

Despite extensive research associating HAART with lipodystrophy and dysfunctional adipose physiology and metabolism in vivo and in vitro, the actual distribution and concentrations of ART drugs have not been studied in adipose tissue of infected persons. The circulation and pharmacodynamics of ART drugs are highly complex and influenced by factors such as drug half-life, binding of systemic proteins such as albumin, tissue distribution, and drug-drug interactions and synergies [225-227]. Whereas antiviral drug efficacy is studied mainly by direct treatment of infected cells, the influence of cells and tissues proximal to infected CD4 T cells and macrophages are less understood. The partial neutralization of Tenofovir by adipocytes during coculture with infected CD4 T cells shown in Fig. 2.10 is intriguing as it

suggests that mechanisms of antiretroviral compounds may differ in adipose tissue. Several reasons may underlie the reduction of Tenofovir efficacy in an adipose tissue milieu. Tenofovir and Emtricitabine are both NRTI class antiviral compounds which inhibit the viral reverse transcriptase enzyme, but differ with respect to chemical and metabolic properties. Both compounds have short half-lives (~10-17hrs) and bind plasma and serum proteins at low levels (~4-7%) [228]. By contrast to Emtricitabine, which has rapid absorption rates and bioavailability (>75%) unaffected by food, the bioavailability of Tenofovir is much lower (~25%) and increased by food, particularly by high-fat diets. Efavirenz is an NNRTI class drug that is metabolized by the cytochrome p450 system, whereas Emtricitabine and Tenofovir are unaffected by these enzymes [228]. Additionally, Efavirenz is highly bound by plasma and serum proteins (nearly 100%), and has a substantially longer half-life of at least 40hrs [228]. Adipocytes also express drug transporters such as ABC transporters that could influence ART efficacy, and the rapid uptake of some protease inhibitors by adipocytes has been demonstrated using fluorescently-labeled compounds such as lopinavir and ritonavir [158]. Furthermore, drugs such as Efavirenz and Abacavir are relatively more lipophilic and may be readily sequestered by adipocyte lipid droplets [229]. The blood-brain barrier is a well-known impediment for antiretroviral drug penetration into the CNS. Another recent study describes an association of viral replication in lymph nodes with lower drug concentrations (Efavirenz, Emtricitabine, and Tenofovir) in lymph node cells (compared to circulating leukocytes), suggesting that insufficient distribution of ART drugs within tissues can further contribute to viral persistence in tissues [82]. Thus, different tissues possess unique obstacles for antiviral drug distribution and function, and it will be important to determine if adipose tissue present additional novel barriers.

### ***3.8. Sizing up the adipose tissue HIV reservoir in comparison to other reservoirs***

During primary infection with HIV, cellular and anatomic reservoirs of virus are established which become resistant to eradication by immune responses and antiretroviral drugs. Although ART drugs suppress viral replication to force the virus into dormancy, viral replication resumes

soon after treatment interruption. The induction and reactivation of latently infected CD4 T cells in lymphoid tissues such as lymph nodes and GALT are believed to mostly account for this viral rebound, whereas other non-lymphoid tissues harboring infected immune cells, such as skin, lungs, or brain, may contribute to the systemic viral load at lesser extents. The findings of the present dissertation demonstrate adipose tissue to be a non-lymphoid reservoir in humans and monkeys, as well as the potential for adipocytes to upregulate HIV replication, but are too preliminary to define the general contribution of adipose tissue HIV for systemic persistence, viral rebound, and AIDS disease progression. Key studies that can help to assess the broader impact of adipose HIV for viral pathogenesis are described in the following “future studies” section. For example, detection of high viral RNA loads, and the demonstration of replication-competence of adipose HIV via Q-VOA assays, would provide strong evidence that adipose HIV could be highly impactful for viral loads and disease progression, especially considering the anatomic prevalence of adipocytes. However, if viral RNA were to be undetectable in adipose tissue, or if adipose HIV is mainly non-infectious, this may suggest that adipose tissues are simply regions of viral compartmentalization with little impact for HIV pathogenesis. The present dissertation also proposes that a latently infected memory CD4 T cell may experience viral induction upon migration into adipose tissue via mechanisms that also reactivate latent HIV in lymphoid tissues, such as by APC-mediated CD3/TCR activation or by cytokines. However, the converse scenario may be just as possible, in which the enforcement of latency by anti-inflammatory cytokines or adipokines, or by mechanisms that counteract immune activation and prevent viral replication, predominates in adipose tissue. Furthermore, the penetration and efficacy of ART drugs in adipose tissue may adequately prevent viral replication, or antiviral immunity mediated by adipose-resident CD8 CTL’s or CD4 Tregs may mostly eliminate infected CD4 T cells or macrophages. Like any other tissue or organ, the homeostasis of adipose tissue is complex and highly regulated by multiple mechanisms and signals, and it will be important to determine how these regulatory pathways influence HIV infection and pathogenesis in adipose depots.

### **3.9. Future studies**

The present study suggests that adipose tissue may promote HIV infection and persistence, but a number of additional in vitro and in vivo studies could better clarify the broader influence of adipose tissue.

With respect to the in vitro coculture results in Fig. 2.2 showing that adipose cells enhance T cell activation and HIV replication in the presence of IL2, IL7, or IL15, additional experiments would better characterize these interactions. Based on their previously described effects of enhancing T cell activation or HIV replication in other scenarios, as well as being abundantly expressed in adipose tissue, the focus on adipose-derived IL6 and integrin ligands and the use of neutralizing abs in coculture experiments in Fig. 2.4 confirmed their involvement as enhancers of HIV replication. However, more robust methods for exploration of candidate factors, such as genomic and protein arrays of the adipose and immune cells, could reveal additional mediators of crosstalk during cocultures. The coculture experiments may also be biased by the exogenous addition of factors such as IL2, IL7, and IL15, while inadequately examining other mechanisms that suppress T cell activation and HIV replication. For example, the adipose milieu in vivo could also include IL4, IL10, or adiponectin that may predominate over stimulatory agents and limit T cell activation and HIV replication. The inclusion of antiviral CD8 T cells in cocultures would also be relevant as adipose infiltration by CD8 T cells increases during obesity and inflammation, as well as during HIV infection as shown in the present study (Fig. 2.13). Additionally, pan-memory CD4<sup>+</sup>CD45RO<sup>+</sup> T cells were infected and used in coculture experiments, but more specific subsets such as Th1/Th2 or Th17/Tregs may differ as productive hosts for HIV, and likely interact differently with adipose cells. The specific signals that attract T cells into adipose tissue are still mostly unclear, but in vitro chemotaxis assays could be utilized to determine if adipocytes affect migration of HIV-infected CD4 T cells differently than uninfected T cells.

Fig. 2.11 indicates that both uninfected and HIV-infected CD4 T cells suppress adipocyte differentiation similarly, but specific viral proteins such as Vpr, Nef, and Tat have

unique, and mostly detrimental, effects on adipose functions such as adipogenesis. Thus, more refined experimental conditions may better reveal differences between uninfected and infected CD4 T cells upon adipose physiology. Although macrophages are considered a much smaller cellular reservoir for HIV compared to CD4 T cells, macrophage infiltration and regulation in adipose tissue can be substantial during obesity and HIV infection, and it would be important to study by coculture experiments whether adipocytes affect HIV replication in monocytes and macrophages as well. Lastly, the coculture experiments in Fig. 2.10 suggest that adipocytes impair the antiretroviral efficacy of Tenofovir. This could be due to adipocyte sequestration of ART compounds or to drug degradation. Future studies should include measurement of intracellular adipocyte concentrations of ART drugs during coculture and correlation with antiviral efficacy.

Studies of tissues from infected humans and rhesus macaques clearly show that adipose tissue is a reservoir for memory CD4 T cells and viral DNA, but this adipose-localized virus requires much more characterization to better understand the broader impact for HIV pathogenesis and persistence. Mainly due to technical and sample limitations, the majority of adipose tissue samples were prioritized to first confirm the presence of memory CD4 T cells and provirus. For a few infected humans and monkeys that provided more tissues, conventional PBMC coculture viral outgrowth assays were used to examine inducibility and infectiousness of AT-SVF virus, but this assay yielded inconsistent and unclear results, and future attempts are needed that will utilize more recently developed and improved methods of quantitative viral outgrowth assays (Q-VOA). Additionally, measurement of viral RNA by real-time PCR or in situ hybridization would better indicate the replication status of adipose virus. PCR assays such as the *Alu*-LTR real-time PCR method could also better define the nature of AT-SVF virus by determining if virus is present as stably integrated forms in the genomes of adipose immune cells (and thus more likely to be inducible and a source of rebound viremia), as opposed to being mostly unintegrated and more labile. Sorting of AT-SVF cells to determine if CD4 T cells or macrophages represent the main host for adipose virus is another important

question to be addressed, as well as determining if virions or viral proteins can further be sequestered by mature adipocytes. Lastly, SIV-infected rhesus macaques are the best animal model of HIV infection, but it would be important to determine if adipose tissue of infected humanized mice can be viral reservoirs as well. Although a limitation of humanized mouse models may be restricted immune cell migration within adipose tissue due to the lack of chemokine receptors, humanized mouse models are constantly being improved (for example, a transgenic mouse model expressing human CCR5 and Cyclin T1, two host factors essential for HIV replication, is recently reported) [230]. The labeling of infected CD4 T cells and in vivo tracking in rhesus macaques to examine their migration into adipose tissue is also a possibility. In summary, the findings of the present study may be just “the tip of the iceberg” of a potentially larger issue regarding HIV hideouts and sanctuaries, presenting yet more complications for HIV cure research.

## Chapter 4

### Materials and Methods

#### Cells and culture

The majority of in vitro experiments utilized human memory CD4 T cells and primary adipose cells (preadipocytes and mature adipocytes). PBMC were first isolated from peripheral blood of healthy donors (Gulf Coast Regional Blood Center, Houston, TX) with Ficoll-Paque (GE Healthcare). Memory CD4<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> T cells were then purified from PBMC using EasySep magnetic bead negative selection kits (STEMCELL Technologies), and purities were 90-95%. Cells were maintained in complete RPMI-1640 medium (Gibco) containing 10% heat-inactivated FBS, 2mM L-glutamine, 0.1mM MEM non-essential amino acids, 2mM sodium pyruvate, 25mM HEPES, and 1X antibiotic/antimycotic at 37°C+5% CO<sub>2</sub> until used for experiments.

Primary subcutaneous preadipocytes and adipocytes were commercially acquired from Zen-Bio (Research Triangle Park, NC). Each vial of cryopreserved preadipocytes consisted of 2x10<sup>6</sup> preadipocytes pooled from 5-7 healthy female, non-obese donors (BMI ranges of ~25-30), and were tested free of HIV-1, HIV-2, HTLV-1, HTLV-2, HBV, HCV, and endothelial cells. Preadipocytes were cultured in DMEM Preadipocyte Maintenance Medium (Zen-Bio) before experimentation. To obtain mature adipocytes, preadipocytes were differentiated to adipocytes in 6-well plates in accordance with manufacturer's instructions. Terminal differentiation was induced by culturing preadipocytes for 7-10 days in DMEM Adipocyte Differentiation Medium (Zen-Bio) containing insulin, dexamethasone, isobutylmethylxanthine and PPAR $\gamma$  agonist. Medium was then changed to DMEM Adipocyte Maintenance Medium (Zen-Bio) containing insulin and dexamethasone (Zen-Bio), and cultured an additional 7-10 days, during which each plate well became ~50% confluent with lipid droplets. Mature adipocytes were then maintained in adipocyte maintenance medium until used for experiments.

## **Viruses and infections**

HIV-1 stocks were generated by the Baylor College of Medicine-University of Texas Health Science Center at Houston Center for AIDS Research (CFAR) Virology Core. HIV-1 strains utilized include R5-tropic SF162 and NSN-SX, X4-tropic 93BR019 and NL4.3, and R5X4-dual tropic 89.6. For infections, purified memory CD4 T cells were either first pre-activated by CD3/CD28 costimulation or directly infected as resting cells. For infection of pre-activated memory CD4 T cells, cells were costimulated with 1µg/ml coated CD3 (clone UCHT-1) + 1µg/ml soluble CD28 (clone CD28.2) mabs (BD Biosciences) for 2 days in RPMI medium. Cells were then washed and cultured with viral stocks at 0.01-0.1 MOI for 24-48 hrs in medium containing 20ng/ml recombinant IL2 (Biolegend). Cells were then washed 2x and used in experiments. For infection of resting memory CD4 T cells, cells were cultured with viral stocks at 0.01-0.1 MOI for 24-48 hrs in IL2 medium, washed 2x, then used for experiments. Uninfected cells were cultured in medium alone in parallel to infected cells.

## **Coculture experiments between human memory CD4 T cells and adipose cells**

Most coculture experiments utilized transwells in which memory CD4 T cells were cultured in transwells (0.4µm pore size, Costar), and preadipocytes or mature adipocytes were cultured in lower wells of 6-well plates (Costar), and the addition of appropriate reagents (cytokines or antibodies). For coculture of memory CD4 T cells with preadipocytes,  $1-2 \times 10^5$  uninfected or HIV-infected memory CD4 T cells were seeded into transwells in 2ml Preadipocyte Maintenance Medium and placed in 6-well plates with  $1-2 \times 10^5$  preadipocytes (seeded 1-2 days earlier in the lower wells) in 3ml medium. Cells were then cocultured for indicated time periods (usually 3-6 days) and appropriate T cell or adipose measurements conducted as described below. For coculture of memory CD4 T cells with mature adipocytes,  $1-2 \times 10^5$  preadipocytes were first differentiated for 14 days in 6-well plates as described above.  $2 \times 10^5$  uninfected or HIV-infected memory CD4+ T cells were seeded into transwells in 2ml Adipocyte Maintenance Medium, then placed in wells with adipocytes in 3ml medium.

For coculture experiments that involved addition of recombinant cytokines (IL2, IL7, IL15, IL4, IL6, IL8 and TNF $\alpha$ ), 10-20ng/ml cytokine (Biolegend and R&D Systems) was added to coculture medium at the start of the coculture experiment. For coculture experiments utilizing neutralizing antibodies, memory CD4 T cells or preadipocytes and adipocytes were separately pre-incubated in medium with 2-10 $\mu$ g/ml blocking abs for 1hr prior to the start of coculture. Blocking antibodies tested include goat polyclonal antibodies against IL2, IL6, IL8, IL10, IL15, GP130, TNF $\alpha$ , TNF $\beta$ , collagens I and IV, fibronectin, RANTES, CCR5, CXCR4, SDF1 $\alpha$ , MIP1/2/3, MCP1/3, BAFF/BAFF receptor, TRAIL, and TWEAK, and monoclonal antibodies against CD126 (IL6 receptor), CD49a (integrin  $\alpha$ 1), and CD29 (integrin  $\beta$ 1) (R&D Systems and Biolegend). For experiments involving direct treatment of memory CD4 T cells alone with recombinant cytokines, 1-2x10<sup>5</sup> uninfected or HIV-infected memory CD4 T cells were cultured in 48-well plates in 1ml complete RPMI medium with 10-20ng/ml cytokines IL2, IL7, IL15, IL6, IL8, TNF $\alpha$ , Leptin (Santa Cruz Biotechnology), or Adiponectin (BioVision).

### **Measurement of T cell activation and HIV replication**

Activation of memory CD4 T cells (mostly CD69 and CD25) was measured by flow cytometry. At appropriate time points, memory CD4 T cells were harvested, washed with 2%FBS/PBS, then incubated with 1 $\mu$ g/ml directly conjugated mabs against CD69 (APC, PE, PerCPCy5.5, or APCCy7), CD25 (PE or PECy7), or isotype controls (Biolegend and BD Biosciences) for 30mins at 4°C. Cells were then washed and analyzed with a Gallios Flow Cytometer and Kaluza1.2 software (Beckman-Coulter). For experiments examining surface expression of IL6 receptor (CD126), and integrins  $\alpha$ 1 (CD49a) and  $\beta$ 1 (CD29) by memory CD4 T cells, cells were stained with CD126-APC, CD49a-FITC, and CD29-FITC mabs (Biolegend).

For measurement of HIV replication in memory CD4 T cells, intracellular and extracellular p24 was measured. For intracellular p24 measurement, memory CD4 T cells were washed with 2%FBS/PBS, then fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 30mins at 4°C. Cells were then washed with Perm/Wash buffer, and

incubated with 1µg/ml p24-PE mabs (clone KC57, Beckman-Coulter) for 30mins at 4°C. Cells were then washed and analyzed with flow cytometer. Extracellular p24 was measured in cell-free culture supernatants by ELISA with a detection limit of 10pg/ml (Advanced BioScience Laboratories).

For measurement of memory CD4 T cell proliferation, uninfected or HIV-infected cells were first pre-labeled with CellTrace CFSE Cell Proliferation Kit (Life Technologies), then cocultured with preadipocytes in IL2 medium for 7 days. Memory CD4 T cells were then washed, and CFSE dilution and proliferation analyzed with flow cytometer. For measurement of memory CD4 T cell viability, cells were washed, then stained with LIVE/DEAD Cell Viability Kit (Life Technologies) and analyzed with flow cytometer.

### **Real-time PCR and flow cytometry of adipose cells**

mRNA levels of IL6, IL2, IL7, IL15, and IL15Rα in preadipocytes or adipocytes were measured by real-time PCR (primers were acquired from Sigma Genosys and are listed in Table 2). At appropriate coculture time points, adipose cells were lysed, and mRNA purified with RNeasy kit (Qiagen). mRNA was then reverse-transcribed to cDNA using High Capacity RNA-to-cDNA Kit (ABI). SYBR green real-time PCR reactions were conducted with ABI Prism 7000 using reaction conditions of 1 cycle at 95°C for 10mins (polymerase activation), then 40 cycles at 95°C for 15secs (denaturation) and 60°C for 60secs (annealing/extension). TBP housekeeping gene was used as internal control, and fold change comparisons were calculated by  $2^{-\Delta\Delta CT}$ . Extracellular IL6 was measured by ELISA (eBioscience).

Surface and intracellular expression of IL15/IL15Rα by preadipocytes or adipocytes were measured by flow cytometry after 5 days coculture. For measurement of surface IL15/IL15Rα, transwells were removed, and adipose cells rinsed with PBS. Cells were then detached with TrypLE cell dissociation solution (Life Technologies), washed, and incubated with 1µg/ml IL15-APC and IL15Rα-PE mabs (R&D Systems and Biolegend) for 30mins at 4°C. Cells were then washed and analyzed with flow cytometer. For measurement of intracellular

IL15/IL15R $\alpha$ , cells were detached, washed, and fixed/permeabilized with Cytofix/Cytoperm solutions. Cells were incubated with IL15-APC and IL15R $\alpha$ -PE mabs for 30mins at 4°C, washed, then analyzed with flow cytometer. Extracellular IL15 was measured by ELISA (eBioscience).

*Table 2. Primers used for real-time PCR of adipose cells.*

Gene	Forward	Reverse
TBP	5'-CCACTCACAGACTCTCACAAC-3'	5'-CTGCGGTACAATCCCAGAACT-3'
PGK1	5'-TGGACGTAAAGGGAAGCGG-3'	5'-GCTCATAAGGACTACCGACTTGG-3'
GAPDH	5'-GGAGCGAGATCCCTCCAAAAT-3'	5'-GGCTGTTGTCATACTTCTCATGG-3'
IL6	5'-ACTCACCTCTTCAGAACGAATTG-3'	5'-CCATCTTTGGAAGGTTTCAGGTTG-3'
IL2	5'-TACAAGAACCCGAACTGACTCG-3'	5'-ACATGAAGGTAGTCTCACTGCC-3'
IL7	5'-TTCCTCCCCTGATCCTTGTTG-3'	5'-CTTGCGAGCAGCACGGAATA-3'
IL15	5'-TTTCAGTGCAGGGCTTCCTAA-3'	5'-GGGTGAACATCACTTCCGTAT-3'
IL15R $\alpha$	5'-ACAACAGCAGCTATTGTCCCG-3'	5'-GTGGCCCTGTGGATACACAC-3'
Adiponectin	5'-TGCTGGGAGCTGTTCTACTG-3'	5'-TACTCCGGTTTCACCGATGTC-3'
Leptin	5'-TGCCTCCAGAAACGTGATCC-3'	5'-CTCTGTGGAGTAGCCTGAAGC-3'

### **Assessment of adipogenesis during coculture with memory CD4 T cells**

Adipogenic capacity of adipose cells was examined during coculture of differentiating preadipocytes with uninfected or HIV-infected memory CD4 T cells. For measurement of preadipocyte cell cycle and proliferation prior to induction of terminal differentiation, preadipocytes were cocultured with memory CD4 T cells (in transwells) for 3-6 days in Preadipocyte Maintenance Medium (Zen-Bio) and IL2. Preadipocytes were then trypsinized, washed with 2%FBS/PBS, and fixed and permeabilized with 70% ethanol for 1hr at -20°C. Cells were then washed and incubated for 1hr at 4°C with 50 $\mu$ g/ml DNA dye propidium iodide and RNase (Sigma). Cells were then analyzed with flow cytometer.

For examination of preadipocyte differentiation and lipid accumulation during coculture with memory CD4 T cells, cells were cocultured in Adipocyte Differentiation Medium (Zen-Bio) to induce terminal differentiation. After 7 days differentiation, mRNA expression of C/EBP $\alpha$  and PPAR $\gamma$  was measured by Taqman real-time PCR. Adipocyte mRNA was harvested with

RNeasy kit, reverse-transcribed to cDNA, and real-time PCR performed using ABI TaqMan Gene Expression Assays for C/EBP $\alpha$  and PPAR $\gamma$ 2. GAPDH was used as internal control and relative fold change comparisons were calculated by  $2^{-\Delta\Delta CT}$ . After 14 days differentiation, triglyceride accumulation was measured by staining lipid droplets with Oil Red O dye (Sigma). Differentiated adipocytes were gently rinsed with PBS, then fixed with 10% formalin for 10mins at room temp. Wells were rinsed with 60% isopropanol, then incubated with Oil Red O for 15mins. Wells were rinsed with water, then multiple representative brightfield images (40X) of each well recorded.

### **Treatment of HIV-infected memory CD4 T cells with conditioned media of macrophage and adipocyte cocultures**

Conditioned media (CM) of activated adipocytes was prepared by culturing mature adipocytes with LPS or human monocyte-derived macrophages (MDM). For treatment with LPS, differentiated adipocytes were cultured with 500ng/ml LPS (Sigma) for 5 days in 5ml media. LPS in media alone was also cultured. For culture with MDM, MDM were prepared by purifying human monocytes from healthy buffy coat donors with monocyte negative selection EasySep kits (Stemcell Technologies). Monocytes were then differentiated into macrophages by culturing in 6-well plates with 100ng/ml recombinant M-CSF (R&D Systems) in complete RPMI medium for 7 days.  $2 \times 10^5$  MDM were then placed in transwells (0.4 $\mu$ m pore size) with adipocytes in lower wells of 6-well plates and cultured for 5 days in Adipocyte Maintenance Medium (Zen-Bio). MDM were also cultured with medium alone or with 500ng/ml LPS in parallel with Adipocyte+MDM and Adipocyte+MDM+LPS cultures. After 5 days of culture, extracellular IL6 in conditioned media was measured by ELISA. Conditioned media was harvested, spun to pellet MDM or adipocytes, and 4ml cell-free conditioned media transferred directly to  $2 \times 10^5$  uninfected or HIV-infected (strain NSN-SX) memory CD4 T cells and cultured for 5 days with 20ng/ml IL2. T cell activation (CD69) and HIV production (intracellular and extracellular p24) were measured by flow cytometry and ELISA.

### **Assessment of antiretroviral drug efficacy during cocultures between HIV-infected memory CD4 T cells and adipocytes**

Memory CD4 T cells were purified from peripheral blood and infected with R5-tropic HIV (SF162 at 0.1 MOI) for 24-48 hrs in IL2 medium. Cells were washed, then added ( $1 \times 10^5$ ) directly to mature adipocytes ( $5 \times 10^4$ ) in 2ml Adipocyte Maintenance Medium (Zen-Bio) with 20ng/ml IL2 in 24-well plates. Immediately after cell additions, ART drugs - Emtricitabine-FTC, Efavirenz, or Tenofovir (NIH AIDS Reagent Program) were added at indicated concentrations, and cells cultured for 3 days. After 3 days culture, cell-free supernatants were harvested and extracellular p24 measured by ELISA.

### **Isolation of adipose tissue stromal-vascular-fraction (AT-SVF) cells**

Solid adipose tissue samples from subcutaneous and visceral depots were obtained from humans and rhesus macaques. For human samples, protocols were approved by Institutional Review Boards of Baylor College of Medicine and University of Texas Health Science Center at Houston. Adipose tissue samples (5-50g) of uninfected and HIV-infected persons were procured either from recently deceased cadavers (National Disease Research Interchange (NDRI, Philadelphia, PA), or from live donors undergoing elective surgical procedures. Other tissues (blood, lymph nodes, or thymus) were also obtained depending on donor availability. For rhesus macaques samples, adipose tissue (10-20g) were harvested immediately after necropsy and transported for AT-SVF processing.

For isolation of AT-SVF cells from humans and monkeys, 2-4g of whole adipose tissue was minced with scissors and digested with 100 $\mu$ g/ml collagenase (Sigma) for 30-60mins at 37°C with rotation. Cells were then filtered through mesh (100 $\mu$ M), and spun at 1,500rpm for 5mins to pellet the SVF cells. Adipocyte floaters were collected and stored at -80°C. AT-SVF cells were washed, then RBC's lysed with lysis buffer. Cells were washed, filtered (40 $\mu$ M), then centrifuged by density-gradient sedimentation with Ficoll-Paque to improve the leukocyte

yield. Cells were then cultured in complete RPMI medium until used for experimentation (mainly flow cytometry or PCR measurements).

### **Flow cytometry of AT-SVF cells**

Flow cytometry analyses of human AT-SVF cells and peripheral blood leukocytes mainly examined activated memory T cells. AT-SVF cells were stained with CD3-Pacific blue, CD4-PerCPCy5.5, CD45RO-FITC, CD25 (PECy7), and CD69 (APC or PECy7) mabs, and isotype controls (Biolegend and BD Biosciences). Cytokine functionality of AT-SVF memory T cells from some uninfected donors was also examined by Intracellular Cytokine Assay (ICS). AT-SVF cells were stimulated with PMA/IO+GolgiPlug (BD Biosciences) for 5hrs, then stained for CD3, CD4, CD45RO, and either IL2-PE or IFN $\gamma$ -PE using Cytofix/Cytoperm solutions.

For direct infection of AT-SVF cells isolated from adipose tissues of uninfected human donors, cells were infected with HIV (R5-tropic at 0.1 MOI) for 24hrs, washed, then cultured with IL2 or IL7 for up to 8 days. AT-SVF memory CD4 T cells were stained by flow cytometry (CD3, CD4, CD45RO, and CD69), and HIV production measured by p24 ELISA. For coculture of lymph node cells and perinodal AT-SVF cells, uninfected donor lymph nodes were isolated by collagenase digestion, then infected with HIV for 24hrs. Perinodal AT-SVF cells from the same donor were isolated and cultured in the lower wells ( $2 \times 10^5$ ) of 24-well plates. Infected lymph node cells were washed, then placed in transwells ( $5 \times 10^5$ ) and cocultured with perinodal AT-SVF cells and IL2 for 8 days.

AT-SVF and peripheral blood of rhesus macaques was examined by flow cytometry for T cells, macrophages, NK and NKT cells. T cells were stained with CD3-Pacific blue, CD4-PerCPCy5.5, CD8-FITC, CD95-PE, CD25-PECy7, and CD69-APC mabs (Biolegend or BD Biosciences). NK and NKT cells were stained with CD45-FITC, CD3-Pacific blue, CD16-PE, CD56-AF700, and Granzyme A-PerCPCy5.5 and Granzyme B-APC mabs. Macrophages were stained with CD45-FITC, CD14-PE, CD16-Pacific blue, and HLA.DR-APCCy7 mabs.

## Nested PCR and DNA sequence analyses

Proviral DNA in AT-SVF and other tissues (PBMC, lymph nodes, or thymus) of HIV-infected humans and SHIV-infected (strain SF162p3) rhesus macaques was examined by nested PCR. DNA was extracted from tissues with QIAamp Microkit (Qiagen) and stored at -80°C. Gag and Env genes of HIV for humans and SHIV for rhesus macaques were targeted for amplification (primers used are listed in Table 3). First round reactions included 40µl PCR SuperMix (Life Technologies), 200-300nM primers, 0.1µg DNA, and 10µl water, and PCR conditions were 1 cycle at 94°C (2mins), then 35-40 cycles at 94°C (30secs), 55°C (30secs), and 72°C (55secs), then final extension at 72°C (10mins). Second round reactions included 40µl PCR SuperMix, 200-300nM primers, 2-5µl of the first round product, and 10µl water, and PCR conditions were 1 cycle at 94°C (2mins), then 35-40 cycles at 94°C (30secs), 55°C (30secs), and 72°C (30secs), then final extension at 72°C (10mins). PCR products were gel-purified with QIAquick kits (Qiagen), and sequenced by the Baylor College of Medicine DNA Sequencing Core using ABI 3130XL Genetic Analyzer and BigDye Terminator methods. Multiple sequence alignments were conducted with Clustal Omega, MAFFT, or PhyML-3.0 software, and phylogenetic analyses performed with MrBayes-3.2.2 and Garli-2.0.

*Table 3. Nested PCR primers used for detection of HIV-1 in humans or SHIV in rhesus macaques.*

Gene	(Primer ID) Forward sequence	(Primer ID) Reverse sequence	Reference
HIV-1 LTR-Gag (Outer)	(RU5) 5'-GGTCTCTCTGGTTAGACCAGAT-3'	(SK39) 5'-TTTGGTCCTTGCTTATGTCCAGAAT-3'	[231]
HIV-1 LTR-Gag (Inner)	(US5) 5'-TCTCTAGCAGTGGCGCCGAACA-3'	(US3) 5'-TCTCCTCTAGCCTCCGCTAGTC-3'	
HIV-1 Env (Outer)	(PCR5) 5'-TAACCCCACTCTGTGTTA-3'	(R344) 5'-CAGTAGAAAAATCCCCTC-3'	[232]
HIV-1 Env (Inner)	(EN1) 5'-ACAGTACAATGTACACATGG-3'	(EN4B) 5'-AATTTCTGGGTCCCCCTCCTG-3'	
SHIV-SF162p3 Gag (Outer)	(GO1) 5'-AGAAAGTGAAACACACTGAGGAAGC-3'	(GO2) 5'-TCATCCAATTCTTACTGCTGCAT-3'	[233]
SHIV-SF162p3 Gag (Inner)	(GO3) 5'-ACAGATAGTGACAGACACCTAGTGG-3'	(GO4) 5'-CTGTCTACATAGCTCTGAAATGGCTC-3'	

(HIV-1 Env primers also used for SHIV Env detection)

**Statistics**

Analyses were performed using MS Excel or GraphPad. Comparisons between groups or conditions utilized unpaired or paired t-tests, or Wilcoxon signed-rank tests as appropriate.  $P < 0.05$  was considered significant.

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## **Vita**

Jacob Couturier was born in Seoul, Korea, the son of Thomas and Roberta Couturier, on March 15, 1976. He graduated from Greenville High School in 1995, and received a Bachelor of Science in Physiology from Michigan State University in 2005. He then joined the lab of Dr. Dorothy Lewis as a research assistant at Baylor College of Medicine in 2006. He entered the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences Virology and Gene Therapy program in 2010.