THE IMPACT OF SIMIAN IMMUNODEFICIENCY VIRUS ON
SUBCUTANEOUS ADIPOSE TISSUE OF Rhesus Macaques

APPROVED BY SUPERVISORY COMMITTEE

[Signatures and dates]
THE IMPACT OF SIMIAN IMMUNODEFICIENCY VIRUS ON SUBCUTANEOUS ADIPOSE TISSUE OF RHESUS MACAQUES

by

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DISSERTATION

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DOCTOR OF PHILOSOPHY

TULANE UNIVERSITY

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Abstract

Background: Individuals with human immunodeficiency virus (HIV) and undergoing antiretroviral therapy (ART) exhibit high levels of circulating inflammatory cytokines and proteins, which are strongly correlated with shortened time to death and disease. To target damaging inflammation at the source, the drivers of inflammation must be identified. Adipose tissue is a massive organ that contains adipocytes and immune cells capable of producing pro-inflammatory mediators. Dysregulated adipose tissue is implicated in the pathogenesis of obesity and related diseases, such as type 2 diabetes, that are likewise reported in persons with chronic HIV infection. Adipose tissue was therefore explored as a contributor to circulating inflammation in patients with HIV using the rhesus macaque model. Simian immunodeficiency virus (SIV) closely models HIV regarding pathogenesis, including CD4+ T cell depletion, induction of a viral reservoir, and development of opportunistic infections before succumbing to Acquired Immunodeficiency Syndrome (AIDS) and death.

Methods: Subcutaneous adipose tissue (SQAT) from SIV-infected rhesus macaques was characterized using confocal microscopy to describe the major immune cell subsets. Adipose tissue homogenates and plasma were analyzed for expression of genes and proteins related to inflammatory processes using antibody and RNA-based fluorescent multiplex bead technology for protein and gene quantitation, respectively. The functions of adipose tissue immune cells during SIV infection were measured with stimulation and phagocytosis assays.
Results: SQAT from rhesus macaques reflects that from humans by quantity and phenotype of immune cells and became strongly inflamed after SIV±ART. This did not correspond with circulating inflammatory parameters. Immune cells in adipose tissue from SIV±ART had reduced constitutive expression of inflammatory factors, but phagocytosis and response to stimulation were unchanged.

Conclusions: Inflammation in SQAT of rhesus macaques, which closely models that from humans, is greatly pronounced during SIV±ART. This suggests that adipose tissue could be integral to pathogenesis during HIV±ART and an important target for intervention.
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral Therapy</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>LGCI</td>
<td>Low-grade Chronic Inflammation</td>
</tr>
<tr>
<td>ATM</td>
<td>Adipose Tissue Macrophage</td>
</tr>
<tr>
<td>CLS</td>
<td>Crown-like Structure</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-Human Primate</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
</tr>
<tr>
<td>T-SNE</td>
<td>t-stochastic Neighbor Embedding</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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</table>
Chapter 1

INTRODUCTION

Despite the availability and efficacy of antiretroviral therapy (ART), inflammation remains highly problematic in HIV+ individuals. ART first became available in the early 1990s, and it is only now (30 years later) that clinicians have begun to observe the effects of long-term ART administration. Long-term ART has been associated with accelerated and accentuated aging\(^1\). This specifically refers to an increase in the rate or frequency of age-associated diseases, including cardiovascular disease (CVD), atherosclerosis, kidney disease, type 2 diabetes, frailty, neurodegenerative diseases, and non-AIDS associated cancers. The link between long-term ART and the development of accelerated aging is elevated levels of circulating inflammatory biomarkers compared to age-matched HIV-negative individuals\(^2\). It is becoming clear that inflammation is integral to pathogenesis, as high levels of inflammatory cytokines have been linked to poor prognosis in HIV+ patients with and without ART\(^3,^4\). Until a cure and a vaccine are found, targeting inflammation in HIV+ patients could provide significant improvements in lifespan and healthspan\(^5\). We therefore investigated tissue inflammatory mediators, particularly in adipose, that could be contributing to the systemic inflammation observed during HIV infection. The long-term goal of the project is to inhibit or eliminate the contributors of harmful inflammation to improve prognosis for HIV+ individuals, as well as to identify an easily accessible tissue
site that could be a better biomarker for monitoring health and assessing outcome prognosis.
Chapter 2 – Literature Review

CURRENT UNDERSTANDING OF INFLAMMATION, ADIPOSE TISSUE, AND HIV

2. Low-Grade Chronic Inflammation

2.1.1 Overview of Inflammation

Inflammation is the process of healing an injured tissue or clearing infectious organisms from an infected tissue site\(^6\). Classically, inflammation has been characterized as a response to injury or pathogen invasion that comprises clinical signs of heat, fever, pain, swelling, and redness\(^6\). It is a normal process that is critical for the resolution of the numerous insults inflicted upon an organism over the course of its life, and the injury or infection is usually resolved within a period of one to two weeks\(^7\). If the injury is severe enough, fibrosis can occur at the end of the inflammatory process. Inflammation which is resolved in a short period of time is called acute inflammation. When inflammation progresses beyond this time frame, it can become a harmful process whereby the tissue is damaged more from the inflammatory process itself than by the injury or infection which initiated the reaction\(^7\). This long-term, damaging process is called chronic inflammation, and is often reflected by increased levels of inflammatory factors in the circulation\(^8\text{--}10\).
This increase of inflammatory factors in the blood is generally indicative of poor prognosis for the development of large variety of chronic diseases, and several of the most common include CVD, neurodegenerative diseases, type 2 diabetes, and others.\textsuperscript{11-13}

2.1.2 Chronic Inflammation

In contrast to acute inflammation, chronic inflammation takes a very long of time, if ever, to resolve. Chronic inflammation is a slow process that occurs in response to the lack of resolution of an infectious disease (e.g. tuberculosis)\textsuperscript{14}, persistent tissue damage, a consequence of autoimmune diseases (e.g. rheumatoid arthritis)\textsuperscript{15}, or from continuous exposure to a toxic agent (e.g. silica)\textsuperscript{16}. It is exemplified by the ever-presence of macrophages, lymphocytes, and plasma cells, which continually exert inflammatory functions. This can be severely damaging to the tissue and is regularly accompanied by the creation of new connective tissue or fibrosis (scarring of the tissue)\textsuperscript{17}. Scarring reduces the ability for tissue to function normally.

During the chronic inflammatory process, the infiltrating immune cells secrete a variety of factors to communicate with each other; these can often be detected by various immunoassays in the tissue and in the circulatory system. There are often symptoms associated with the presence of chronic inflammation, such as difficulty breathing in the lung when it is inflamed, full of immune cells, and fibrotic. A persistent infiltration of inflammatory cells in the tissue in combination with elevated inflammatory factors in the circulation are strongly indicative of an ongoing chronic inflammatory process.

2.1.3 Low-Grade Chronic Inflammation
Distinct from chronic inflammation, low-grade chronic inflammation (LGCI; also called subclinical inflammation) describes an apparently asymptomatic state of delayed, long-term increases in many of the same inflammatory factors which characterize chronic inflammation. Low-grade chronic inflammation is commonly observed in people who are obese. A key difference between chronic inflammation and LGCI is the source of the disturbance which is causing elevated inflammatory factors. In chronic inflammation, inflammatory factors are secreted in response to an ongoing and unresolved injury or infection in several possible tissues. In LGCI, it is metabolic alterations, particularly in the adipose tissue, which correspond with increased levels of a more specific, though overlapping (with chronic inflammation), group of inflammatory factors. Similar to chronic inflammation, LGCI involves an infiltration of mononuclear cells to the adipose tissue, with a corresponding excess of pro-inflammatory factors. Further, it is common to observe adipocyte cell death followed by macrophage infiltration around this dead or dying cell.

Identification of adipose tissue as the site of LGCI and metabolic inflammation was made possible by the discovery of the relationship between high adiposity (obesity) and circulating chronic inflammation, which did not correspond with any overt pathology or clinical disease. Though the mechanism of metabolic inflammation due to excess weight is not entirely clear, substantial research has been undertaken in the past decade to elucidate this. A more in-depth review of the mechanism of metabolic inflammation in adipose tissue causing LGCI is presented below in Section 3 entitled “Adipose Tissue.”

2.1.4 Biomarkers of Low-Grade Chronic Inflammation
Low-grade chronic inflammation is derived primarily from perturbed adipose tissue. However, it is thought that this inflammation is reflected in the circulation, as exemplified by an increase of corresponding inflammatory factors in the blood, thus allowing for clinicians to diagnose low-grade chronic inflammation using a sample of blood\textsuperscript{23}. Several specific cytokines, chemokines, and other inflammatory factors are secreted during the inflammatory process in the tissue and are widely described as being “pro-inflammatory” because they are most often secreted by inflammatory cells at a site of injury or infection, and function in the resolution of the infection or injury.

As aforementioned, LGCI is generally explained by metabolic inflammation in the adipose tissue. Since adipose tissue is primarily involved in this process, biomarkers in the blood are more specific to inflammatory factors that would be secreted in excess in the adipose tissue when it is inflamed, rather than those specific to other organs. Several notable biomarkers of LGCI (as defined by metabolic inflammation in the adipose tissue) circulate in the blood, and these are described in Table 1.
Detecting the changes in levels of circulating cytokines and chemokines during both chronic inflammation and LGCI is challenging. As opposed to greatly elevated levels of biomarkers during acute phase inflammation, the levels of biomarkers during chronic inflammation and LGCI are subtle and can require large sample sizes to detect a significant change. Since the sample size in this study is limited, biomarkers of chronic inflammation in the research undertaken here are considered both individually and in combination with each other to create a signature of disease status. A deeper review of the specific functions of these cytokines as they relate to adipose tissue and metabolic inflammation is discussed in Section 3 below entitled “Adipose Tissue”.

### Table 1. Biomarkers of metabolic chronic inflammation in the blood.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Major cell types that produce</th>
<th>Function</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>Immune, muscle, bone, epithelial, and endothelial</td>
<td>Pyrogen, stimulates release of amino acids from muscle and fatty acids by adipocytes, helps activate neutrophils and lymphocytes, TNF and IL-1 inhibition, IL-1 and IL-10 activation</td>
</tr>
<tr>
<td>TNFa</td>
<td>Immune, muscle, epithelial, endothelial, adipocytes</td>
<td>Pyrogen, promotes coagulation, CD4+ T cell differentiation, elicits antitumor activity, prevents viral replication, involved in cachexia</td>
</tr>
<tr>
<td>IL-1b</td>
<td>Immune</td>
<td>Pyrogen, attracts and activates neutrophils, stimulates release of amino acids in muscle, supports collagen production, helps activate B and T cells</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>Immune, epithelial, adipocytes</td>
<td>IL-1a and IL-1b inhibition</td>
</tr>
<tr>
<td>IL-8</td>
<td>Immune, epithelial, endothelial</td>
<td>Attracts and activates neutrophils, supports angiogenesis</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Immune</td>
<td>Promotes secretion of inflammatory chemokines and cytokines, protects skin against bacteria and fungus, pertakes in allergic response</td>
</tr>
<tr>
<td>IL-18</td>
<td>Immune, epithelial</td>
<td>Promotes IFNγ, TNF, and IL-1b production. Reduces IgE and IgG1 secretion</td>
</tr>
<tr>
<td>CCL2</td>
<td>Immune, epithelial, endothelial</td>
<td>Strong monocyte recruitment, but also DC and memory cells</td>
</tr>
<tr>
<td>CCL3</td>
<td>Immune</td>
<td>Attracts monocytes, B cells, T cells, and eosinophils to site of inflammation</td>
</tr>
<tr>
<td>CCL5</td>
<td>Immune, epithelial, endothelial</td>
<td>Attracts T cells, basophils, and eosinophils to site of inflammation. Helps to activate NK cells</td>
</tr>
</tbody>
</table>
2.2. HIV, Inflammation, and Disease

2.2.1 HIV+ART and Accelerated Aging

ART was first introduced in the mid-1990s and is currently utilized by 40% of people infected with HIV worldwide\(^24\). The advent of ART was life-changing because people with HIV could expect to live for nearly as long as people without HIV. That is, individuals with HIV could now live to approximately age 70 regardless of age at infection, compared to the short 6-10 years of life expectancy after infection in individuals with HIV\(^25-27\). However, it is only now, approximately 30 years after the first patients with HIV started to receive ART, that clinicians have noted an increase of diseases among long-term treated patients that reflect diseases commonly found during the normal process of aging\(^28\). The acceleration and accentuation of what are normally age-related diseases is called ‘accelerated aging’. These include CVD, renal disease, dementia, Alzheimer’s disease, non-AIDS associated cancers, type 2 diabetes, and frailty\(^29-36\). Furthermore, individuals with HIV+ART also experience metabolic irregularities including dyslipidemia, metabolic syndrome, and lipodystrophy, a condition where adipose tissue degenerates from some regions and hypertrophies in other regions\(^37-42\).

These diseases and metabolic irregularities in people with HIV+ART also occur more frequently and earlier in people who are obese\(^43-47\). In fact, dyslipidemia and metabolic syndrome are hallmark disorders found in people with obesity\(^48-49\). Obesity is
also one of the largest risk factors for development of CVD and type 2 diabetes\textsuperscript{50,51}. Due to the relationship between obesity and age-related diseases and risk factors, obesity is also considered to be a risk factor for accelerated aging.

The parallels between obesity and accelerated aging during HIV infection are more striking when considering that they share a common prognostic indicator for development of disease: inflammation\textsuperscript{11,36,52}. In both obesity and HIV+ART, inflammation is the best prognostic indicator for accelerated aging\textsuperscript{31,36}. Both conditions share overlapping elevated markers of inflammation in the bloodstream, including the cytokines TNF\textalpha and IL-6, though the levels of these are not as high as are typical during an acute inflammatory process\textsuperscript{53-55}. In individuals with HIV+ART, like people who are obese, the inflammation does not correspond with pathology distinctly related to a particular organ system, thus meeting the criteria for LGCI. In both cases, higher levels of LGCI correspond with the development of age-related diseases, morbidity, and mortality.

In people who are obese, LGCI is primarily due to disturbances in the adipose tissue. Whether this same mechanism is responsible for the LGCI seen in HIV+ART is not clear. However, research in subjects who have HIV+ART and a condition called lipodystrophy also exhibit signs of perturbed tissue function in the adipose tissue, including signs of hypoxia and an increase of crown-like structures (covered in the Section 3 entitled “Adipose Tissue”).

A third risk factor for inflammation and developing age-related diseases is “healthy” aging, itself. In “healthy” aging, the process of becoming chronologically older without any ongoing disease processes, inflammation is prognostic of the development of
the appropriately termed ‘age-related diseases’\textsuperscript{56,57}. Thus, it is unclear which comes first: inflammation or aging. In fact, inflammation is so intimately linked with aging that immunologists use the term ‘inflamma-aging’ to describe the process of aging as one of developing inflammation, or vice-versa\textsuperscript{58}. Like in obesity, there is overlap between the inflammatory cytokines which increase during aging, and accelerated aging from obesity and HIV+ART, including elevated plasma levels of IL-6 and TNFα\textsuperscript{59}. The numerous parallels between obesity, aging, and accelerated aging due to HIV+ART, underscores the potential for a shared mechanism.

2.2.2. Overview of Inflammation during HIV Infection

To fully understand the contributions of various mechanisms of inflammation during HIV+ART, understanding the cause of inflammation during HIV infection without ART must be considered. This is because HIV may persist in tissues during HIV+ART and elicit similar pathways of inflammation as during HIV infection without ART\textsuperscript{60}. Moreover, the virus may affect immune cells during active viral replication in a way which is not reversible after the initiation and ongoing use of ART. Inflammation that occurs after HIV infection without ART is akin to chronic inflammation, rather than LGCI.

To understand the source of inflammation during HIV, analyzing circulating levels of inflammatory factors in the blood can provide important information. This is because it is thought that inflammation in the circulation is reflective of inflammation in the tissue, and the specific inflammatory molecules that are measured could give a hint as to which organ is unwell. The blood is also easily accessible. For these reasons, in-depth research has been conducted to measure inflammatory cytokines in blood which
correspond with poor outcome during HIV infection. Poor outcomes during HIV describe endpoints such as time to death, and time to development of a specific AIDS-related disease.

2.2.3. Mechanisms of Inflammation during HIV infection without ART

Several possible mechanisms can explain the inflammation observed during HIV, and these are critical to understand so that scientists and clinicians can accurately target intervention sites. Mechanisms of inflammation during HIV infection without ART have been well-described and include both direct and indirect effects of viral infection. For example, killing of CD4+ T cells in the lymph node occurs through the process of pyroptosis, likely causing the release of inflammatory cytokines and chemokines into the blood. Pyroptosis is a mechanism of programmed cell death, but differs from apoptosis because it is highly inflammatory, and apoptosis is not. During pyroptosis, the dying cell secretes inflammatory cytokines such as IL-1β and IL-18, and responding cells also rush to the area to clean up the dead and dying cells, releasing their own unique milieu of inflammatory cytokines and chemokines in the process. The slow depletion and death of CD4+ T cells by pyroptosis leads to the constant release of inflammatory factors into the blood throughout the course of HIV infection.

A second mechanism of inflammation during HIV infection could derive from monocytes in the blood and macrophages in the tissue which are either directly or indirectly affected by the virus (i.e. via infection or in response to pathogen recognition). For example, it has been reported that the frequency of CD16+ monocytes increases during HIV infection and that these monocytes are preferentially infected by the virus compared to other monocyte subsets. CD16 is a type III Fcγ receptor that internalizes...
antibodies (presumably which are bound to viral particles), and monocytes which express this surface antigen are considered pro-inflammatory because they conventionally secrete high levels of TNFα, IL-1, and IL-12, and low levels of the anti-inflammatory cytokine IL-10. It is unclear whether monocytes become CD16+ after HIV infection because they are responding to the virus or whether they become CD16+ because the virus infects the monocyte and induces upregulation of the protein. However, it is believed that the polarization of monocytes by HIV to become the pro-inflammatory subset that expresses CD16 promotes inflammation. Macrophages in tissue, particularly the microglia of the brain and alveolar macrophages in the lung are also known to be infected by HIV and SIV. HIV infection of microglia, for example, causes rapid inflammasome activation and the release of IL-1β and IL-18, which may be reflected in the circulation.

There could be indirect reasons for inflammation due to HIV as well. For example, the infection and depletion of CD4+ T cells after infection followed by only a partial recovery of this cell type could create an imbalanced immune cell repertoire because it is missing fully-functional CD4+ T cells. Cells which rely on CD4+ T cells to become activated or polarized in a certain direction might function differently without this interaction, and perhaps become more inflammatory in response. It is also conceivable that the gradual loss of CD4+ T cells over time would exacerbate the role of the innate immune system to contain the infection, because the loss of such a key component of the adaptive immune system may disable the entire adaptive arm. In support of this, only 50-70% of gut CD4+ T lymphocytes are reconstituted after HIV infection with prolonged ART, even though the CD4+ T cell numbers in the blood are restored. This could shift the responsibility of the immune system response to the virus to Juan C Pizarro 3/27/2018 10:45 PM

Comment [1]: It is possible that this is also due to the loss of a CD4+ population in a particular compartment, like the GALT?
favor the innate immune response, and potentially cause overexpression of pro-inflammatory cytokines from monocytes and macrophages that must hyper-react to the virus.

2.2.4 Mechanisms of Inflammation During HIV+ART

The mechanism(s) for inflammation during active HIV infection (ART-naïve) are better understood than the reasons behind inflammation that occurs in patients on long-term ART. This is because active monitoring of HIV+ART patients was less relevant until clinical signs of CVD, type 2 diabetes, dementia, and other chronic inflammatory disease began to surface at a much earlier time than was expected. Information regarding inflammation and these aging disease outcomes, therefore, has only become available in recent years, and the distinctions between accelerated aging during HIV±ART versus natural aging, and the relationship to inflammation, have not been delineated. However, there may be overlap between the source of inflammation during HIV infection and during HIV+ART because HIV persists in cellular reservoirs even during long-term ART, highlighting a potential role for the virus to directly affect immune cells long-term.

Patients with HIV infection and administered ART for many years exhibit a reduced level of inflammation compared to those without treatment, however, the levels of inflammatory biomarkers remain higher than in uninfected people and are remarkably similar to the levels of LGCI seen during obesity. Furthermore, some patients on long-term ART develop a condition known as “lipodystrophy” which is the atrophy of subcutaneous adipose tissue beneath the skin (particularly in the limbs and face) and hypertrophy of adipose tissue behind the neck, such that a ‘hump’-like fatty mass forms. Many of these patients also suffer from dyslipidemia and metabolic syndrome even
though they are not obese. The presence of LGCI, lipodystrophy, dyslipidemia, and metabolic syndrome, highlights a potential role for adipose tissue in the mechanism of 

Table 2. Biomarkers which overlap between LGCI (as explained by metabolic chronic inflammation) and HIV+ART

<table>
<thead>
<tr>
<th>Biomarkers of Metabolic Chronic Inflammation</th>
</tr>
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<tbody>
<tr>
<td><strong>Factor</strong></td>
</tr>
<tr>
<td>IL-6</td>
</tr>
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<td>TNFα</td>
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Increased inflammation seen during HIV+ART. Indeed, several biomarkers of HIV+ART overlap with those observed during metabolic chronic inflammation, highlighted in green in Table 2.

Due to the obvious signs of lipodystrophy, the vast majority of research undertaken to explain the mechanisms of inflammation in HIV-infected patients on long-term ART has been in subjects with lipodystrophy. However, lipodystrophy may be on the extreme end of possible metabolic outcomes due to HIV+ART, and there is a gap in the knowledge of the effects of HIV+ART on individuals without lipodystrophy. It is possible that subjects with less obvious pathology remain at higher risk for disease. Lipodystrophy in humans is primarily associated with the use of protease inhibitors in the ART regimen, a class of drugs which blocks HIV proteases from cleaving polypeptides into functional proteins\textsuperscript{73–75}. Protease inhibitors, however, are extremely effective at controlling virus replication as well as reducing the incidence of resistance of HIV to the
drugs, and thus remains popularly prescribed. It is unclear to what extent other ART drugs could also cause metabolic dysregulations.

The data linking protease inhibitors to LGCI underscores the side effects from the drugs alone. However, inflammation during HIV+ART could also be due to persistent viral infection, or even from permanent changes induced by HIV during productive infection that were not reversed after viral suppression and absence of the virus. The brain is a compelling inflammatory site during virally-suppressed HIV infection for several reasons and serves as a useful example for these LGCI-related phenomena. Macrophages of the brain called microglia become infected and inflamed after HIV infection. However, it is believed that the virus isn’t always cleared from the microglia because it is difficult for ART drugs to cross the blood-brain barrier. The microglia can become a reservoir for infection, constantly secreting low numbers of virus that functionally alter the reservoir cell and surrounding cells. Alternatively, reports on the analysis of brain sections taken during autopsy of HIV-infected patients who are virally suppressed describe signs of neuroinflammation mediated by macrophages in the complete absence of detectable virus. This highlights a potential irreversible change to microglia caused by active viral infection prior to when the individual became virally suppressed after ART.

2.3. Adipose Tissue and Disease

2.3.1. HIV and Adipose Tissue Inflammation

In the setting of obesity (and underweight, though this is less researched), adipose is widely recognized for its contribution to circulating inflammation and downstream
consequences\textsuperscript{30,31}, predominantly driven by inflammation in the adipose tissue compartment. Adipose tissue inflammation in obesity is defined as an increase in number of immune cells and a change in their activation state\textsuperscript{32}. Specifically, adipose tissue macrophages (ATMs), which are the most abundant immune cells in adipose, increase from 4% to 12% of all nucleated cells in human visceral fat and become pro-inflammatory\textsuperscript{30}. Few studies have examined the inflammatory effects of HIV infection on adipose tissue inflammation. One key study in 2015 reported that adipose from humans and cynomolgus macaques harbors HIV and SIV during active infection as well as during ART, thus making adipose a viral reservoir\textsuperscript{83}. Additionally, the report suggested that adipose tissue becomes inflamed during SIV infection based on adipose-tissue macrophages assuming a pro-inflammatory phenotype and increasing in number\textsuperscript{83}. Besides this report, an in-depth characterization of adipose tissue inflammation due to HIV/SIV has not been conducted.

2.3.2. Overview of Adipose Tissue

Adipose is a massive organ by body mass percentage; it accounts for approximately 15-30% of body mass in average men and women, respectively\textsuperscript{84}. It is located between abdominal organs (visceral), under the skin (subcutaneous), surrounding blood vessels (perivascular), above the heart (epicardial), in the bone marrow, and in the axillary and cervical regions. Adipose can also accumulate ectopically in the liver, and in cases of severe obesity, around other organs as well. Large, fat-filled cells called adipocytes constitute the majority of adipose tissue by volume. In addition, immune cells such as T cells and macrophages reside between adipocytes and surrounding other structures in the tissue, including blood vessels and nerves (Figure 1)\textsuperscript{85,86}. 
Figure 1. Model of white adipose tissue. Adipocytes constitute most of adipose tissue by volume, and groups of adipocytes are separated into lobules via connective tissue. Each lobule of adipose is supported by a blood supply and sympathetic nerves. Macrophages and T cells are the predominant immune cells found scattered throughout the tissue. Fibroblasts and mesenchymal stem cells are also commonly dispersed throughout the adipose tissue.

Groups of adipocytes form lobules that are delimited by connective tissue called septa, and many lobules join together to form ‘depots’, such as the subcutaneous adipose tissue depot. Each lobule is supplied by capillaries as well as innervation by sympathetic nerve fibers. The presence of multiple cell types, a blood supply, and a nerve supply, promotes adipose tissue crosstalk with the organism on a system-wide level.
Once considered an organ primarily used for energy-storage and cushion for other organs, adipose is now appreciated as a dynamic tissue with a capacity to regulate endocrine, metabolic, and immunological processes.\textsuperscript{86,91,92} Hormones secreted exclusively by adipocytes are called adipokines and exert effects throughout the body.\textsuperscript{93} For example, the adipokines leptin and ghrelin, are integral to the feelings of hunger and satiety.\textsuperscript{88} Over- or under-expression of these hormones is commonly implicated in the pathological process of obesity, whereby those who are obese tend to feel hungry more often due to decreased expression of leptin and increased expression of ghrelin. Adipokines are also thought to play a role in insulin resistance\textsuperscript{94} and blood pressure regulation\textsuperscript{95}, thus rendering them extremely important to overall health and metabolism.

2.3.3. Subcutaneous Adipose Tissue

Subcutaneous adipose tissue is a type of white adipose tissue found deep to the dermis of the skin (Figure 2). Excess energy intake leading to obesity causes massive
growth of the subcutaneous adipose tissue depot relative to other adipose tissue compartments, whereby new adipocytes form (via hyperplasia) from adipocyte precursors called preadipocytes, and existing adipocytes grow in size (via hypertrophy)\(^9^6\). In humans, subcutaneous adipose tissue is less cellular than other types of adipose tissue, but this may be due to the greater size of adipocytes per unit area compared to other depots rather than a decrease of non-adipocyte cells in relation to the number of adipocytes.

A further distinction between subcutaneous adipose tissue and other sites of adipose tissue, is that subcutaneous adipose tissue is not closely associated with any organ besides the skin. By contrast, visceral adipose tissue in the abdominal area lies in between and surrounding the intestines and peritoneal space, and epicardial adipose tissue lies directly on top of the heart. The anatomical locations distinguish the tissues in different regions by function. For example, subsets of visceral adipose tissue in mice are closely associated with the peritoneum and support lymphocyte-rich clusters of immune cells called Milky Spots in the omental adipose and Fat-Associated Lymphoid Clusters (FALC) in the mesenteric adipose in response to peritoneal inflammation\(^9^7,^9^8\). Conversely, this phenomenon has not been reported in subcutaneous adipose tissue in response to disturbances in the skin.

Another key distinction between subcutaneous adipose tissue and other adipose tissue compartments is the sheer size of the organ. Subcutaneous adipose tissue generally occupies a far greater volume than any other fat depot in the body because it is found underneath the skin throughout the entire body and accumulates in massive amounts in obese individuals. Other fat pads are restricted in their growth, especially considering
their anatomical location deep in the body and between critical organs. However, there is still considerable growth of visceral adipose tissue in people who are obese.

When selecting which type of adipose tissue to use for this research, several factors were considered. The first factor was the biological relevance of the tissue in relation to SIV infection. Previous studies had shown that HIV and SIV infected both visceral and subcutaneous adipose tissue of cynomolgus macaques, thus narrowing our selection of tissues to visceral or subcutaneous because of the knowledge that both can be infected and affected by the virus.\textsuperscript{83,99} Secondly, these same studies highlighted the fact that subcutaneous adipose and visceral adipose tissues both exhibited some degree of inflammation after SIV infection as defined by an increased percent of CD14\textsuperscript{+} macrophages out of CD45\textsuperscript{+} immune cells. However, the magnitude of difference was greater in the subcutaneous adipose tissue. Third, human studies of adipose tissue from patients with HIV+ART are regularly conducted in the subcutaneous adipose tissue, and it is known that HIV+ART perturbs this region.\textsuperscript{40,43} Fourth, the massive size and location of subcutaneous adipose tissue throughout the body emphasized the biological relevance of subcutaneous adipose tissue to metabolic inflammation and disease. If there is a generalized disturbance in the subcutaneous adipose, regardless of how mild it is, the effects could be either local or exacerbated over its huge area and develop into a much larger problem, potentially even being reflected in the blood. Last and perhaps most importantly, the location of subcutaneous adipose tissue right beneath the skin makes it ideal for sampling, considering a biopsy from this location is far easier to access than one from adipose tissue found deep inside the body, thereby producing less stress on the Juan C Pizarro 3/29/2018 12:07 AM
Comment [3]: Have people looked in other adipose tissues?

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Comment [4]: Can perturbations just be local?
animals being sampled. For these reasons, subcutaneous adipose tissue was selected for the purposes of this research.

A final consideration into researching adipose tissue in the context of the rhesus macaque model of AIDS is the fact that the adipose from this species of non-human primate (NHP) is rarely used in metabolic research and has not been immunologically validated as an appropriate representation of human subcutaneous adipose tissue. It has, however, been shown to be anatomically structurally similar\textsuperscript{100}. However, the immunological profile of subcutaneous adipose from rhesus macaques has not been independently validated as a model for adipose tissue for humans, and should be so that future studies can use this animal model to study human diseases.

### 2.3.4. Adipose Tissue Immune Cells

Adipose tissue from all fat depots in humans are thought to contain numerous immune cells, including macrophages (10-50/mm\textsuperscript{2}), T cells (<10/mm\textsuperscript{2}), B cells (<5/mm\textsuperscript{2}), and iNKT cells (0-20\% of T cells), with macrophages being the most abundant\textsuperscript{101}. These cells interact with each other and with adipocytes to orchestrate growth and maintenance of the tissue; they also contribute to the pathology found in adipose during aging\textsuperscript{102}. Adipose tissue macrophages (ATMs) are found between adipocytes and in crown-like structures surrounding dead or dying macrophages\textsuperscript{103}. Involved in the process of creating new blood vessels and deposition of collagen to form connective tissue, ATMs are critical for the growth and maintenance of the tissue\textsuperscript{101}. Furthermore, ATMs interact closely with adipocytes and aid in the removal of excess triglycerides via the secretion of TNF\textalpha during obesity\textsuperscript{101}. A new population of macrophages associated with sympathetic nerves in the tissue has been recently described.
and appears vital to degradation of catecholamines, a process which is central to the ability for adipocytes to hydrolyze triglycerides into free fatty acids, the mobile form of stored energy\textsuperscript{102-104}. T cells in the adipose tissue are also important during obesity, when necrotic adipocytes die and are cleared by macrophages. These macrophages then display antigens in the context of MHC II and induce T cells to secrete IFN\textgreek{y} which interferes with insulin-receptor signaling and impair uptake of glucose\textsuperscript{105}. Invariant NKT (iNKT) cells which secrete both Th1 and Th2 cytokines are also highly represented in adipose tissue compared to the blood, and are thought to interact with both adipocytes and B cells via recognition of an as yet undetermined lipid antigen presented by \textbf{CD1d}, an MHC class I-like molecule. They support the expansion of tbet+CD11c+IgG2c+ B cells in adipose tissue, and this correlates with protection from diabetic symptoms\textsuperscript{106,107}.

The research described in this study will predominantly focus on ATMs, because they are so highly abundant in human adipose tissue compared to the other immune cell types and are intimately involved in pathological processes. Resident ATMs in humans express CD68, CD163, and CD206\textsuperscript{108}. CD68 is a marker expressed in cells of the myeloid lineage, including monocytes, macrophages, and dendritic cells. It is found on the surface of lysosomes and is a member of the lysosomal-associated membrane protein (LAMP) family. It is thought to shuffle between the lysosomal and plasma membranes, where it is involved in adhesion of cells to tissues by binding to tissue- and organ-specific lectins\textsuperscript{109}. CD163 is a hemoglobin/haptoglobin scavenger receptor that clears debris and is found on the cell surface of most macrophages and monocytes\textsuperscript{110}. CD206 is the mannose receptor that is thought to play a role in non-specific pathogen recognition and phagocytosis, as well as in the process of resolution of inflammation\textsuperscript{111}. CD163 and
CD206 are both considered to be ‘anti-inflammatory’ markers and are found on anti-inflammatory macrophages, though that classification is usually described in mice and not completely clear in humans. For example, human macrophages, despite expressing ‘anti-inflammatory’ markers, are still capable of secreting pro-inflammatory cytokines. Thus, it is unknown whether ATMs are distinctly ‘pro- or anti-inflammatory’ and whether that classification is relevant to humans. In addition, it is thought that ATMs are tissue resident, with a population of bone-marrow derived macrophages that traffic into the tissue during obesity (described in more detail below).

2.3.5. Adipose Tissue Inflammation

Adipose tissue has only been seriously researched in the past 15 years because of the increasing prevalence of obesity, which is an important risk factor for the development of several chronic diseases. Due to obesity, the topic of adipose tissue inflammation is one of considerable interest, but the mechanism by which it occurs is not entirely clear. In addition, adipose tissue inflammation has not been well-studied in relationship to diseases other than obesity, so the studies presented in the literature may describe only a single mechanism by which adipose becomes inflamed. Moreover, most of what is known about the mechanisms behind inflammation in adipose tissue is from studies in mice, where genes are knocked out and the resulting phenotype is observed. This often provides results which are useful but not necessarily translatable to understand primate pathologies due to species differences.

An additional hindrance towards understanding mechanisms of inflammation in adipose tissue is the difficulty of obtaining adipose tissue samples. Most adipose tissue depots are inaccessible by simple biopsies and obtaining samples from lean individuals is
logistically challenging due to the paucity of lean individuals donating their fat to research. In addition, adipose tissue has low cellularity in general after excluding adipocytes, and it requires large amounts of tissue to perform *in vitro* experiments. Therefore, *in vitro* experiments using samples from lean humans is extremely challenging because the number of cells is so low. Furthermore, adipose tissue is filled with blood vessels which may contaminate or confound the isolation of adipose tissue cells with cells from the blood. In support of this, the ratio of T cells to macrophages in the adipose tissue using flow cytometry is substantially higher than when using confocal microscopy.
With these caveats in mind, a couple of theories have begun to form to explain inflammation during obesity. It is thought that during obesity, adipocytes hypertrophy and undergo hyperplasia. However, there is a limit to adipocyte hypertrophy, and when an adipocyte grows too large, it eventually dies via necrosis and spills its lipid contents into the surrounding tissue environment (Figure 3). The process of necrosis in adipocytes, as in other cell types, is characterized by the release of signals which attract leukocytes to the area to clean up the dying cell and lipids which were released\(^{112}\). Adipocyte necrosis alone is thought to contribute to LGCI, because adipocytes are capable of secreting pro-inflammatory factors such as TNF\(\alpha\) and IL-6 during death. Besides the adipocytes secreting signals, macrophages that normally live in the tissue detect these signals and
traffic to the area to surround the adipocyte. After recognizing the dying cell, macrophages release chemokines such as CCL2 to recruit monocytes into the tissue to help clear the debris. It has been shown that macrophages in mice can increase from 5-50% of all nucleated cells during obesity\textsuperscript{103}. It is believed that monocytes from the blood are recruited to this area of tissue death, differentiate into macrophages, and surround the adipocyte, thus creating a ‘crown-like structure’ which is the hallmark of adipose tissue inflammation (Figure 4). The recruited monocytes do not express all of the same markers as the tissue-resident macrophages, and likely secrete elevated levels of pro-inflammatory cytokines and chemokines to help resolve the tissue damage\textsuperscript{120,118}. The combination of the adipocyte death, the recruitment

Figure 3. Adipose tissue inflammation after obesity. Adipocytes hypertrophy and die via necrosis, initiating an inflammatory process whereby macrophages surround the adipocyte and begin to clean up the dead area. The dead adipocytes, surrounding adipocytes, and macrophages release factors to signal each other and aid in the resolution.\textsuperscript{118}
of monocytes, and the orchestration of resolution of tissue damage by macrophages could explain the elevated levels of inflammatory cytokines and chemokines observed in the plasma. Since adipose tissue is so large, this process could occur over a vast area of tissue and with high frequency. Indeed, it has been reported that in obese humans, as many as six crown-like structures can be found per 1000 adipocytes throughout the body, compared to fewer than one per 1000 adipocytes in lean individuals.113

An additional explanation for the metabolic inflammation seen in adipose tissue during obesity concerns the process of lipolysis, which is regulated by TNFα. Adipocytes that are hypertrophied but do not die via necrosis secrete high levels of CCL2 and TNFα. CCL2 attracts macrophages to the area, which then secrete inflammatory cytokines including TNFα. TNFα induces lipolysis within the adipocyte and triglycerides are expelled in the form of free fatty acids (FFA). FFAs then travel into the bloodstream and are stored as triglycerides in the skeletal muscle. Additionally, FFAs bind to macrophages via the surface receptor CD36, which then signals the secretion of chemokines and TNFα by macrophages.113 This creates a cycle by which hypertrophic adipocytes can maintain
their survival by releasing excess lipid. In consequence, a pro-inflammatory environment is created in the tissue.\textsuperscript{113}

Signs of inflammation in the tissue can be assessed by the close examination of adipose tissue structural features, as well as by undertaking various assays which can determine whether the expression of certain metabolic inflammatory markers is changed. Identification of chronic inflammation via histology (tissue section stained with hematoxylin & eosin) is generally the first step in determining the presence of inflammation in the adipose. A positive identification of inflammation involves the presence of an elevated quantity of inflammatory cells, especially macrophages, as well as evidence of fibrosis. Further, the presence of dead or dying adipocytes surrounded by numerous macrophages is highly indicative of a metabolic disturbance. A general observation of hypertrophy and hyperplasia by a trained pathologist can also indicate metabolic defects. Additionally, levels of gene expression and protein expression of specific inflammatory cytokines can be measured by processing whole tissue to determine if metabolic inflammation is occurring.

\subsection*{2.3.6. Overview of the Intersection between HIV, Adipose, and Inflammation}

Individuals who are infected with HIV exhibit chronic inflammation for the duration of infection, and this does not completely resolve after the initiation and long-term use of ART. In fact, individuals with HIV who have been undergoing ART for 20-30 years exhibit LGCI and are substantially more susceptible to developing diseases that are commonly associated with chronic inflammation such as CVD and type 2 diabetes, but at an age well below the normal age for the development of these diseases (i.e. in
persons not infected with HIV). The incidence of these typically ‘age-related’ diseases is so high, that the term ‘accelerated aging’ has been coined to describe the increased frequency and rate of development of disease. Additionally, the degree of chronic inflammation as expressed by elevated pro-inflammatory cytokines in the blood are the best prognostic determinant of development of morbidity and mortality in HIV-infected individuals, with and without treatment. Thus, there is substantial evidence implicating a relationship between chronic inflammation and poor outcomes in people who are infected with HIV.

During obesity, adipose tissue becomes inflamed (called metabolic inflammation) and induces a specific type of systemic inflammation called ‘low-grade chronic inflammation’, or LGCI. LGCI is also found during the normal process of aging, and individuals who are infected with HIV+ and on long-term ART appear to be prematurely aged. During obesity, aging, and HIV±ART, inflammation is prognostic of morbidity and mortality. The significant overlap between the inflammation seen during accelerated aging during HIV±ART, obesity, and “normal” aging, highlights the potential of a shared mechanism.

The overarching theme of this dissertation is to examine the interplay between HIV infection, adipose tissue, macrophages, and inflammation. This is based on observations that adipose tissue is infected by HIV and remains infected after long-term ART. It is also vulnerable to dysregulation by the drugs alone. Furthermore, adipose tissue dysfunction is the hallmark of LGCI observed during obesity. Subcutaneous adipose tissue is an easily accessible site for longitudinal sampling and has been understudied in the context of HIV±ART as an inflammatory mediator.
2.4. Non-human Primate Model of HIV Infection

2.4.1. Simian Immunodeficiency Virus (SIV) infection as a Model for HIV infection

AIDS was first recognized in 1981 after a group of young, otherwise healthy, homosexual men in California developed Pneumocystis carinii pneumonia, and a second group of healthy, young, homosexual men in New York developed aggressive forms of Kaposi’s Sarcoma. In 1982, the Centers for Disease Control and Prevention used the term Acquired Immunodeficiency Syndrome for the first time, and the following year, the causative agent, HIV, was identified by groups in France and the United States. By 1984, researchers at the Delta Regional Primate Research Center (now Tulane National Primate Research Center) and at the New England Primate Research Center discovered the existence of two lentiviruses called STLV-IIIDeltaB670 (now SIVDelta) and STLV-IIImac (now SIVmac), respectively, that closely mimicked the pathogenesis of AIDS when inoculated into rhesus macaques. Since then, simian immunodeficiency virus (SIV) was discovered to be the original virus that evolved into HIV in humans and has been characterized and used extensively to model HIV in different species of monkeys. Strains of SIV differ by cellular tropism and length of infection, but almost all are characterized by a depletion in CD4+ T cells and the onset of opportunistic infections before succumbing to death. Furthermore, SIV is susceptible to the same ART used in
humans, including integrase inhibitors, reverse transcriptase inhibitors, and protease inhibitors, and macaques on long-term ART control viral load and maintain CD4+ T cell counts. A key difference between SIV and HIV is the presence of the Vpx gene in SIV and HIV-2, and its absence in HIV-1. Vpx is an accessory gene which enables replication of HIV-2 in myeloid cells by counteracting certain host defense factors (e.g. SAMHD1). However, HIV-1 can still infect myeloid cells, and thus, SIV is considered to adequately model the pathogenesis of HIV120. The research undertaken here utilizes the strains SIVmac239 and SIVmac251, as well as a simian-human immunodeficiency virus (SHIV) chimera called SHIV162p3. SIVmac239 is an aggressive, clonal virus strain that induces death in rhesus macaques after an average of one to two years of infection. SIVmac251 is a swarm of viral clones, but also causes death in rhesus macaques after about one to two years of infection. By contrast, SHIV162p3 is CCR5-tropic and more closely resembles the progression of HIV-1, in that the course of infection is longer (approximately 2-3 years before progression AIDS) compared to the SIVmac strains121. In this research, all three strains of virus are grouped together and referred to as “SIV” for simplicity, and because we expect that the all the virus strains are appropriately reflective of HIV infection.

2.4.2. Rhesus Macaque Model of HIV

NHP closely resemble humans genetically and are susceptible to many diseases that humans experience, including SIV infection. Conversely, there is no other animal model that is susceptible to HIV or related virus that can infect and persist in a different species. Humanized mouse models for HIV have been developed, however, their genetic distance from humans and inbred feature reduces the ability to translate results to
humans. A strength of the rhesus macaque model over other monkey models is that certain species of NHP are natural hosts of SIV and do not succumb to disease when infected with the virus. Such species include African Green Monkeys and Sooty Mangabeys and they are important for comparative studies between species to highlight why some monkeys are susceptible to diseases while others are not. Rhesus macaques are not natural hosts and are susceptible to SIV infection pathogenesis and progression to AIDS, and ultimately death. SIV infection in rhesus macaques exhibits CD4+ T cell depletion and AIDS after infection with HIV, like in humans. Further, after infection with SIV, plasma viral loads in rhesus macaques peak after approximately two weeks, after which time the viral load decreases to a setpoint of approximately $10^4 - 10^6$ particles/mL. Long-term infection typically leads to wasting and diarrheal diseases, followed by the onset of diseases such as pneumonia and meningoencephalitis, and ultimately death. In addition, rhesus macaques are one of the most extensively utilized and characterized species of NHP and thus there is a multitude of reagents and antibodies available for use in immunological and biochemical assays. Considering their short genetic distance from humans and susceptibility to the closely-related SIV, the similar pattern of CD4+ T cell depletion before the onset of AIDS, similar viral replication timing and quantity in the blood, and presence of opportunistic infections during AIDS, rhesus macaques are excellent models for studying HIV.
Chapter 3

HYPOTHESIS, AIMS, AND RESEARCH OBJECTIVES

Individuals infected with HIV exhibit increased levels of inflammation which do not completely subside after long-term ART. Long-term ART is increasingly associated with accelerated aging, the increased rate and incidence of typically age-related diseases. The primary predictor for development of accelerated aging is LGCI in individuals with HIV+ART. LGCI is also strongly associated with progression to disease during obesity and aging. In obesity, LGCI is associated with metabolic inflammation, that is, inflammation of the adipose tissue compartment. Due to the relationship between adipose tissue inflammation and LGCI seen in individuals with HIV+ART, we decided to conduct a comprehensive analysis of the inflammatory effects of SIV±ART on adipose tissue. We also chose to investigate the inflammatory effects of SIV on ATMs of rhesus macaques with and without ART. We then related “local” tissue and cell-specific inflammatory markers with “circulating” markers of inflammation to define associations and better understand the contribution of adipose tissue and ATMs to the overall LGCI during SIV infection. The overall goal of this project is to identify specific inflammatory changes that can be used to identify contributors of systemic inflammation. Therapeutic targeting of these drivers could provide a necessary improvement to current therapy regimens and has the potential to eliminate an important source of systemic inflammation.
during HIV. In addition, identifying a signature of inflammatory factors in the tissue that can identify a specific disease would be useful as a biomarker for disease status during HIV±ART. We hypothesize that adipose tissue becomes inflamed after SIV infection and contributes to the circulating markers of inflammation, and that adipose tissue macrophages are the primary producers of this inflammation.

**Aim 1. Validate subcutaneous adipose tissue in rhesus macaques as immunologically representative of human subcutaneous adipose tissue.**

It is necessary determine the immune cell populations and their abundance in the adipose tissue of rhesus macaques compared to humans. We quantified the number of macrophages, T cells, and B cells, via immunohistochemistry. Further, the phenotype of ATMs in rhesus macaques compared to humans is uncharacterized and this information will be important for translating the results to humans. To achieve this, we phenotyped ATMs using confocal microscopy. Additionally, it is unknown whether ATMs from rhesus macaques are derived from the bone marrow or long-lived. This is important to understand so that future treatments can be targeted appropriately (i.e. treat circulating monocytes or treat macrophages at the tissue level). To address this, we injected dextran which is taken up and retained by long-lived macrophages. Subsequently, confocal microscopy was applied to identify the presence of dextran-labeled long-lived macrophages in relation to recent immigrant macrophages lacking dextran.

**Aim 2. Define the contribution of adipose tissue inflammation to circulating inflammation after SIV infection, with and without ART.**

**Subaim 2a. Define changes in adipose tissue inflammation after SIV±ART.**

Adipose tissue inflammation after HIV/SIV infection is understudied yet has the
potential to be a major contributor to systemic inflammation. We defined adipose tissue inflammation by quantification of major immune cell populations via confocal microscopy and measuring tissue-level gene and protein expression using high-throughput QuantiGene and ProCarta assays, respectively.

**Subaim 2b. Define associations between adipose tissue inflammatory markers and circulating inflammatory markers.**

The relationship between adipose tissue inflammation and circulating inflammatory markers could suggest a contribution of adipose tissue in the pathogenesis of long-term HIV/SIV infection. We will use the Luminex multiplex assay to analyze inflammatory cytokines from the plasma of SIV-uninfected and SIV±ART rhesus macaques and assess the relationship between these and the previously identified inflammatory markers in the adipose tissue.

**Aim 3. Define the contribution of adipose tissue macrophages to inflammation after SIV infection, with and without ART.**

**Subaim 3a. Define changes in adipose tissue macrophages after SIV±ART.**

Adipose tissue macrophage functional changes are appreciated during obesity, but unknown after HIV/SIV infection. We assessed functional changes in macrophages before and after infection, with and without ART, utilizing flow cytometry to conduct a phenotypic analysis of the macrophages, as well as by stimulation and phagocytosis assays to assess functional changes.

**Subaim 3b. Define associations between adipose tissue macrophage functional changes and circulating inflammation markers.**
The relationship between adipose tissue macrophage functional responses and circulating inflammatory markers could suggest a contribution of adipose tissue macrophages in the pathogenesis of long-term HIV/SIV infection. We used the Luminex multiplex assay to analyze inflammatory cytokines from the supernatant of cultured adipose tissue macrophages isolated from the adipose tissue of SIV-uninfected and SIV±ART rhesus macaques. We then assessed the relationship between these and corresponding circulating inflammatory markers from the plasma.
Chapter 4

MATERIALS AND METHODS

Aim 1. Validate subcutaneous adipose tissue in rhesus macaques as immunologically representative of human subcutaneous adipose tissue.

Purpose: a detailed analysis of subcutaneous adipose tissue from rhesus macaques is necessary for relating results to human data. Considering NHP infected with SIV offer the only closely-related animal model to humans infected with HIV, it is critical that the animal model tissue in question be an appropriate immunological representation of human tissue.

Study population and sample collection

Adult Indian-ancestry rhesus macaques (Macaca mulatta) between ages of 4-8 years old of normal weight as assessed by the veterinarian assigned to the study were used. All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Tulane University Institution Animal Care and Use Committee. Subcutaneous adipose tissue was collected either from biopsies or necropsies by veterinarian technicians from experimental macaques that were part of an unrelated SIV study, prior to infection. Samples derived from biopsies were collected opportunistically from either the abdominal or inguinal regions following anesthesia. In brief, each animal was
anaesthetized and the area of surgery was aseptically treated, followed by tissue being collected bluntly using a hemostat from beneath the skin following a 2 cm incision. Samples were then treated as described in each section that follows.

1. Quantification of macrophages (CD68+), T cells (CD3+), and B Cells (CD20+)

The total number of CD68+ macrophages, CD3+ T cells, and CD20cy+ B cells stained by enzyme immunohistochemistry were counted and averaged from a minimum of 10 randomly selected 400x fields per animal. Antibodies used for immunohistochemistry were anti-CD68 (1:100; Clone: KP1; catalog no. M0814; Agilent, Santa Clara, CA), anti-CD3 (1:400; Clone: polyclonal; catalog no. A0452; Agilent, Santa Clara, CA), anti-CD20cy (1:400, Clone: L26; catalog no. M0755; Agilent, Santa Clara, CA), and anti-Ki-67 (1:50; Clone: MIB-1; catalog no. M7240; Agilent, Santa Clara, CA). Samples were fixed overnight in Z-fix (Catalog # 170-175, Anatech LTD, Battle Creek, MI) and embedded in paraffin wax blocks. Sections were cut at 5 µm thickness each and baked overnight at 58-60°C in a dry oven. Slides were deparaffinized, rehydrated, and pretreated with a high pH antigen unmasking solution (Catalog # H-3301; Vector Laboratories, Burlingame, CA) in a microwave for 22 minutes at 400W followed by incubation in low pH antigen unmasking solution (Catalog # H-3300; Vector Laboratories, Burlingame, CA) for one hour starting at boiling and gradually cooling to room temperature. Samples were blocked with Background Punisher for 20 minutes (Catalog # BP974; BioCare Medical, Pacheco, CA) washed in TBS-Tx100 for 5 minutes, then blocked with Pierce Protein-free Blocking Buffer (Catalog # 37584, ThermoFisher Scientific, Waltham, MA) prior to addition of antibody diluted in Pierce Protein-free
Blocking Buffer for 60 minutes. Slides were washed in detergent solution (TBS-Tx100 twice for 5 minutes, then TBS 5 minutes) followed by addition of BioCare Medical Probe (Pacheco, CA) specific to either mouse (MP530L) or rabbit (RP531L) for 40 minutes, another set of washes, and the BioCare Medical Polymer specific to either mouse (MAP532L) or rabbit (RAP533L) for 40 minutes. BCIP/NTB (Catalog #B1911, Sigma-Aldrich, St. Louis, MO) was used for development for 12 minutes, followed by dehydration and mounting with glycerol-based mounting media. Slides were imaged with a light microscope.

2. Phenotypic analysis of key macrophage surface proteins.

Macrophages were stained for CD68, CD163, and CD206, viewed using confocal microscopy, and counted manually based on detecting a nucleus and a fluorescent label in close association with each other. Macrophages from at least 10 400x fields were counted per animal. Nuclei were automatically counted with NIH ImageJ version 2.0 with Fiji. Adobe Photoshop software (version 7.0, Adobe Systems) was used to process and assemble the images. Primary antibodies used for immunofluorescence staining were mouse IgG1 anti-CD68 (1:20; Clone: KP1; catalog no. M0814; Agilent, Santa Clara, CA), mouse IgG1 anti-CD163 (1:50; Clone: 10D6; catalog no. NCL-L-CD163; Leica, Wetzler, Germany), rabbit anti-CD206 (also named anti-MRC1; 1:100; Clone: polyclonal; catalog no. HPA004114; Sigma-Aldrich, St. Louis, MO), anti-Ki67 (1:50; Clone: MIB-1; catalog no. M7240; Agilent, Santa Clara, CA), and chicken anti-dextran (1:200; custom). Secondary antibodies used were Alexa Fluor 488-, 594-, or 647. Samples were fixed, embedded, and pretreated as described for immunohistochemistry. After antigen unmasking, tissue sections were then blocked with 10% normal goat serum.
in phosphate-buffered saline (PBS) for 40 minutes, followed by incubation with primary antibodies for one hour. Samples were then washed for 10 minutes of washing with 2% fish skin gelatin (FSG) and 1% Triton-X100 in PBS twice, followed by 10 minutes with 2% FSG in PBS. Sections were then incubated with secondary antibodies for 40 minutes, followed by the previously described washing protocol. DAPI (1:10,000; 5mg/mL stock solution) was applied for 10 minutes, followed by a repeated series of washes. Slides were dehydrated and mounted in glycerol-based mounting media. Imaging was performed with a Leica TCS SP2 confocal microscope equipped with four lasers (Leica Microsystems) under oil immersion (x400, fluotar/NA 1.0) with a resolution of 512x512 pixels.

3. Quantification of long-lived macrophages

Dextran is a derivative of the sugar dextrose which is incorporated by macrophages and other cells expressing the L-SIGN or DC-SIGN family of surface receptors. Dextran cannot be broken down by mammalian cells which lack the enzyme necessary for degradation. We therefore used dextran to identify and track the lifespan of long-lived macrophages in the adipose tissue. Dextran (D1860, Molecular Probes, Eugene, OR) was prepared at 50mg/mL in saline (pH 5.6, 0.9% Sodium Chloride, NDC 0409-7983-02, Hospira, Lake Forest, IL) and filter sterilized prior to i.v. inoculation at 12.5 mg/kg (n=2), 75 mg/kg (n=6), or 300 mg/kg (n=1). Dextran was injected either 11 days (n=8) or 43 days (n=1) prior to sample collection. Long-lived macrophages were identified by immunostaining and visualization with confocal microscopy for intracellular incorporation of dextran, along with surrounding positive surface staining for macrophage markers CD163 and/or CD206 in relation to closely-associated nuclei.
Aim 2. Define the contribution of adipose tissue inflammation to circulating inflammation after SIV infection, with and without ART.

Purpose: understanding whether adipose tissue becomes inflamed and contributes to circulating inflammation could provide a target for intervention in patients with HIV±ART.

Study Population and Sample Collection

Characteristics of the Indian-ancestry rhesus macaques used in this study are listed in Table 3. The three groups of animals did not statistically differ in respect to age, weight, or the number of standard deviations from normal weight based on the Tulane National Primate Research Center colony data on sex and age. The distribution of the sexes differed between groups, with the uninfected groups being all males compared to the other two groups which were a mix of males and females.

Three different virus strains were used in these studies, including SHIV162p3, SIVmac239, and SIVmac251, and these were mixed between groups, with SIVmac239 being more heavily represented among SIV-infected animals and SIVmac251 being more heavily represented among SIV+ART animals. As described in the literature review, the kinetics of these virus infections exhibit somewhat different average lengths of time before the animals progress and succumb to AIDS, where monkeys infected with SHIV162p3 take approximately one year longer than those infected with SIVmac239 or SIVmac251 to develop AIDS. The lengths of viral infection were recorded at the time of sampling and averaged 258 ±40 days of infection, which was comparable to the length of viral infection prior to onset of ART in the SIV+ART group (284 days ± 164). Samples
from SIV-infected animals were collected when viral loads were at setpoint, $10^4 - 10^5$.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Weight (kg) (std from normal)(^2)</th>
<th>Std from normal</th>
<th>Virus</th>
<th>Length of Infection(^2) (days)</th>
<th>Viral Load(^3)</th>
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<tr>
<td>F</td>
<td>6.6</td>
<td>-1.3</td>
<td>SHIV162p3</td>
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<td>2.16E+04</td>
</tr>
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<td>9.4</td>
<td>0.94</td>
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<td>1.55E+05</td>
</tr>
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<td>SHIV162p3</td>
<td>226</td>
<td>2.00E+05</td>
</tr>
<tr>
<td>M</td>
<td>14.98</td>
<td>1.5</td>
<td>SIVmac239</td>
<td>300</td>
<td>6.03E+04</td>
</tr>
<tr>
<td>M</td>
<td>13.74</td>
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<td>SIVmac239</td>
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<td>4.66E+04</td>
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<tr>
<td>M</td>
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<td>0.63</td>
<td>SIVmac239</td>
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<td>5.00E+05</td>
</tr>
<tr>
<td>M</td>
<td>9.6 (2.2)</td>
<td>-0.26</td>
<td>SHIV162p3 (3): SIVmac239 (3)</td>
<td>258 (40)</td>
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</tr>
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</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th>Weight (std from normal)(^2)</th>
<th>Std from normal</th>
<th>Virus</th>
<th>Length of Infection(^2) (days)</th>
<th>Length of ART(^5) (days)</th>
<th>ART regimen</th>
<th>Viral Load(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>6.8</td>
<td>-1.3</td>
<td>SHIV162p3</td>
<td>288</td>
<td>707</td>
<td>210 days DTG/PMPA/FTC; 497 days DTG/PMPA/FTC/TDF</td>
<td>&lt;83 copies/mL</td>
</tr>
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<td>M</td>
<td>10.25</td>
<td>-0.69</td>
<td>SIVmac239</td>
<td>622</td>
<td>686</td>
<td>189 days DTG/PMPA/FTC; 497 days DTG/PMPA/FTC/TDF</td>
<td>&lt;83 copies/mL</td>
</tr>
<tr>
<td>F</td>
<td>7.0</td>
<td>0.04</td>
<td>SHIV162p3</td>
<td>288</td>
<td>111</td>
<td>DTG/PMPA/FTC</td>
<td>&lt;83 copies/mL</td>
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<td>-0.02</td>
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<td>DTG/PMPA/FTC</td>
<td>&lt;83 copies/mL</td>
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<tr>
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<td>-1.57</td>
<td>SIVmac239</td>
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<td>DTG/PMPA/FTC</td>
<td>&lt;83 copies/mL</td>
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<td>M</td>
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<td>0.44</td>
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<td>DTG/PMPA/FTC</td>
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<td>SIVmac239</td>
<td>174</td>
<td>184</td>
<td>DTG/PMPA/FTC</td>
<td>&lt;83 copies/mL</td>
</tr>
<tr>
<td>M</td>
<td>8.8 (2)</td>
<td>-0.54</td>
<td>SHIV162p3 (2): SIVmac239 (4): SIVmac239 (1)</td>
<td>284 (164)</td>
<td>307 (224)</td>
<td>&lt;83-332 copies/mL</td>
<td></td>
</tr>
</tbody>
</table>

Normal weights were calculated using reference weight per age data from the Tulane National Primate Research Center outdoor-housed colony.

\(^1\) Length of treatment, respectively, prior to sample collection.

\(^2\) Normal weight per age data from the Tulane National Primate Research Center outdoor-housed colony.

\(^3\) Viral load for each animal is the average of two consecutive measurements.
particles/mL, and viral loads in SIV+ART animals were undetectable in all but one animal at the time of sampling. The one animal with detectable viral load appeared to be ‘blipping’, or showing a small resurgence in viremia after a long period of undetectable viral levels. The viral load in this animal was 332 particles/mL.

Adipose tissue biopsy and necropsy samples were collected by on-site veterinarian technicians as described in Aim 1, and were either historical samples available from prior unrelated studies or newly obtained samples available because they were subjects participating in ongoing unrelated studies. After collection, samples were immersed in RNAlater and stored at -20°C for up to three years. Plasma samples were collected in tubes with EDTA to prevent clotting of whole blood after centrifugation for 10 minutes at 3000 RPM, and then stored at -80°C for up to three years.

**Subaim 2a. Define changes in adipose tissue inflammation after SIV±ART.**

1. Adipose Tissue Gene Expression

Samples stored in RNAlater were removed from the tube and excess RNAlater was dabbed off with a KimWipe, followed by weighing the tissue and cutting sections until samples of 20mg were obtained. Each tissue sample was added to a 5mL round-bottom polystyrene Falcon FACS tubes (Becton Dickinson, Catalog #14-959-1A) and homogenized using the Qiagen TissueRuptor II (Catalog #9002755) in Homogenizing Solution mixed with Proteinase K (ThermoFisher Scientific, Catalog # QS0106, Waltham, MA) until each of the samples were completely homogenized, approximately 30-60 seconds. Samples were then twice clarified by centrifuging for 5 minutes at 1700
RPM and retaining only the middle layer; the top layer comprised fat and the bottom layer contained debris. Clarified samples were used as the input for the QuantiGene Plex assay (ThermoFisher, custom panel). RNA transcripts encoded from 40 genes were detected using the QuantiGenePlex 6.0 Reagent System according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA). In brief, magnetic fluorescent beads conjugated with Capture Probes bind to short strands of RNA specific to the selected genes. The RNA is then bound to sequential molecules which serve to amplify the signal, culminating with the binding of streptavidin-PE to the complex which is detectable using the Luminex 100xMAP system and interpreted with Bio-Plex software (version 5.0; Bio-Rad Laboratories, Hercules, CA). The data generated is reported as Mean Fluorescent Intensity (MFI) for each transcript. Negative controls to detect background signal were run in triplicate. Background fluorescence was subtracted from each sample, followed by elimination of values that were below the limit of detection, which is equal to the background fluorescence + three standard deviations above the background signals. Values were normalized using beta-actin, and fold-changes were the relative ratios between the normalized values of the eight SIV±ART animals and the animals which were uninfected.

3. Adipose Tissue Protein Expression

Tissues stored in RNAlater for analyzing gene expression can and were also used for quantification of proteins. The process of weighing, homogenizing, and clarifying the samples were the same as the preparation for gene expression except for the use of a different homogenization buffer (Cell Lysis Buffer, ThermoFisher Scientific, Catalog #
EPX-99999-000). Protein levels from 30 pro- or anti-inflammatory cytokines and chemokines were detected using the NHP ProCartaPlex System according to the manufacturer's protocol (Catalog # EPX300-40044-901, ThermoFisher Scientific, Waltham, MA). In brief, magnetic fluorescent beads conjugated with antibodies bind to specific analytes (i.e. cytokines and chemokines). Detection antibodies conjugated with biotin then bind to the analytes, which can be detected by the addition of streptavidin-PE. The intensity of the streptavidin-PE signal and recognition of the specific beads is detectable using the Luminex 100xMAP system and interpreted with Bio-Plex software (version 5.0; Bio-Rad Laboratories, Hercules, CA). The data generated was reported in Mean Fluorescent Intensity (MFI) for each analyte which was compared to a standard curve for quantification of the protein concentration in pg/mL.

Subaim 2b. Define associations between adipose tissue inflammation and circulating inflammation.

1. Plasma protein levels

Plasma samples stored at -80°C for up to three years were thawed on ice and used for detection of cytokines and chemokines; the same volume of plasma was used for all samples. Protein levels from 30 pro- or anti-inflammatory cytokines and chemokines (the same proteins that were analyzed in the tissue) were detected using the NHP ProCartaPlex System according to the manufacturer's protocol (Catalog # EPX300-40044-901, ThermoFisher Scientific, Waltham, MA) and as described in the methods for protein expression in adipose tissue. The data generated is reported in Mean Fluorescent
Intensity (MFI) for each analyte which is compared to a standard curve for quantification of the protein concentration in pg/mL.

**Data analysis**

Based on a power analysis using cross-sectional and preliminary results, a minimum of six monkeys per group was calculated to support scientific rigor and provide statistical significance at $P < 0.05$ by nonparametric Mann-Whitney test. We calculated interquartile ranges, mean and standard deviation values for each data set. Since our cohorts were independent and not matched, independent comparisons of monkey groups at specific time points were conducted using Kruskal-Wallis one-way analysis of variance, and associated pairwise comparisons were performed using Dunn’s post-test. Linear correlations between adipose tissue inflammation and circulating inflammation were quantified using Spearman’s correlation coefficient and its associated hypothesis test. The statistical significance threshold for all of the analyses was set at $P < 0.05$. Graphs were created using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, California, USA and R. Packages used for this effort were “ggplot2”, “ggpubr”, “ggsci”, “xlsx”, and “cowplot”. Correlations between genes and proteins to each other, including scatterplots, distribution plots, and the calculation of correlation coefficients were performed using R packages “xlsx”, “ggplot2”, and “GGally”. The heatplot was generated using the R packages “xlsx” and “pheatmap”. Clustering using tSNE was conducted using R packages “xlsx”, “ggplot2”, “Rtsne”, “ggpubr”, and “ggsci”. Default settings were used except for the perplexity, which was set to 6, and PCA_scale=TRUE. tSNE default settings were as follows: dims = 2, initial_dims = 50, theta=0.5, PCA=true, max_iter=1000.
Aim 3. Define the contribution of adipose tissue macrophages to inflammation after SIV infection, with and without ART.

Purpose: understanding which cell subset(s) contributes to the adipose tissue inflammation could help target intervention specifically at the source in individuals with HIV±ART.

Study Population and Sample Collection

Characteristics of the Indian-ancestry rhesus macaques used in this study are listed in Table 4. Monkeys did not differ between groups by age, weight, or the number of standard deviations in variation from normal weight based on the Tulane National Primate Research Center colony data considering sex and age. Males were overrepresented in the SIV-infected group due to the nature of opportunistic sample collection from studies that were either analyzing all males or all females, but not both. Three different virus strains were used in these studies, including SHIV162p3, SIVmac239, and SIVmac251, and these were mixed between groups, with SIVmac239 exclusively represented among SIV-infected animals and a mix of the three strains more heavily represented among SIV+ART animals. Several of the macaques in the SIV-infected group were given ART for three months beginning 40 days after viral
inoculation and then allowed to proceed to infection for at least 450 days after removal of ART; one animal received ART for 30 days prior to necropsy but was not virally
suppressed. Animals were categorized for SIV infection status based on their viral load at the time of sampling. The lengths of viral infection were recorded at the time of sampling.
and averaged 360 ± 198 days, which was comparable to the length of viral infection prior
to onset of ART in the SIV+ART group (366 ± 203 days). SIV-infected animal samples

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Std from normal</th>
<th>Virus</th>
<th>Length of Infection (days)</th>
<th>Viral Load</th>
<th>ART regimen</th>
</tr>
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<tbody>
<tr>
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<tr>
<td>Male 4</td>
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<td>SIVmac239</td>
<td>132</td>
<td>1.01E+04</td>
<td></td>
</tr>
<tr>
<td>Female 1</td>
<td>F</td>
<td>14.98</td>
<td>1.3</td>
<td>SIVmac239</td>
<td>300</td>
<td>4.66E+04</td>
<td></td>
</tr>
<tr>
<td>Female 2</td>
<td>F</td>
<td>13.74</td>
<td>0.78</td>
<td>SIVmac239</td>
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<tr>
<td>Female 3</td>
<td>F</td>
<td>12.71</td>
<td>0.63</td>
<td>SIVmac239</td>
<td>300</td>
<td>2.00E+06</td>
<td></td>
</tr>
</tbody>
</table>

SIV-infected animal samples were calculated using reference weight per age data from the Tulane National Primate Research Center outdoor-housed colony. `n` or duration of treatment, respectively, prior to sample collection.
were collected when viral loads were at setpoint, $10^4$-$10^6$ particles/mL, and viral loads in SIV+ART animals were undetectable in all animals at the time of sampling.

Subcutaneous adipose tissue samples were collected at necropsy by on-site veterinarian technicians as described in Aim 1, and were either historical samples available from prior unrelated studies or newly obtained samples available because they were from subjects participating in ongoing unrelated studies. After collection, adipose tissue was finely minced and mixed with collagenase solution and then incubated at 37°C for 45 minutes in a shaker. The collagenase solution consisted of R10 media (1% Pen/Strep, 1% L-glutamine, 1% 1M HEPES, and 10% FBS) mixed with Type II collagenase (0.75mg/mL). After shaking, samples were centrifuged at 1700 RPM for 5 minutes, at which time the tissue mixture separated into three clear layers (Figure X). All the top layers which included the oil, adipocytes, and media, were discarded. Only cells of the stromal vascular fraction (SVF) were stored in Bambanker serum-free freezing media (Wako Chemicals, Catalog # 302-14681, Richmond, VA) at a concentration of $1\times10^6$ cells/mL in liquid nitrogen for up to four years. Plasma samples were collected in tubes containing EDTA to prevent clotting of whole blood followed by centrifugation for 10 minutes at 3000 RPM, and storage at -80°C for up to three years.

Subaim 3a. Define changes in adipose tissue macrophages after SIV±ART.

1. Analysis of Immune Cell Populations
Flow cytometry using a 3-laser LSRII (Becton Dickinson, San Jose, CA) was applied to analyze the immune cell populations present in the subcutaneous adipose tissue. Table 5 shows the antigens, fluorochromes, and antibody clones used. Data was analyzed using FlowJo (version 10.1, TreeStar).

2. Macrophage Stimulation Assay

Frozen stromal vascular fractions from adipose tissue were thawed and plated at a concentration of \(10^5\) cells per well in a 24 well plate. R10 media was added to each well (1mL/well) and cells were incubated for one hour at 37°C in 5% CO\(_2\) to allow adherence and acclimation to the new environment. After one hour, lipopolysaccharide (LPS) (final concentration 100ng/mL) or R10 was added to the wells. Cells were incubated overnight and supernatants were collected 16 hours later. Supernatant samples were analyzed using the Cytokine NHP 29-plex Monkey Panel (ThermoFisher, Catalog # LPC0005M, Waltham, MA) according to the manufacturer’s instructions. In brief, magnetic fluorescent beads conjugated with antibodies bind to specific analytes (i.e. cytokines and chemokines). Secondary antibodies conjugated with biotin then bind to the analytes, which can be detected by the addition of streptavidin-PE. The intensity of the streptavidin-PE signal and recognition of the specific beads is detectable using the Luminex 100xMAP system and interpreted with Bio-Plex software (version 6.2; Bio-Rad Laboratories, Hercules, CA). The data generated was reported in Mean Fluorescent

<table>
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<th>Fluorochrome/Channel</th>
<th>Marker</th>
<th>Antibody Clone</th>
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<td>PE-CF594</td>
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<td>GHI/61</td>
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<tr>
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<td>CD4</td>
<td>L200</td>
</tr>
<tr>
<td>BV510</td>
<td>Live/Dead</td>
<td>Dye</td>
</tr>
<tr>
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<td>CD3</td>
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</tr>
<tr>
<td>APC-Cy7</td>
<td>CD206</td>
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</table>
Intensity (MFI) which was compared to a standard curve for quantification of the protein concentration in pg/mL.

3. Macrophage Phagocytosis Assay

Frozen stromal vascular fractions from adipose tissue were thawed and plated at a concentration of $10^5$ cells per well in a 24 well plate. R10 media was added to each well (1mL/well) and cells were incubated for one hour at 37°C in 5% CO$_2$ to allow adherence and acclimation to the new environment. After one hour, melamine-resin FITC-marked microparticles 3um in diameter (Sigma-Aldrich, Catalog # 72439, St. Louis, MO) were added to achieve a final concentration of .005% by volume and cultures were incubated at 37°C in 5% CO$_2$ for 45 minutes. Adherent cells were then collected using warm (37°C) trypsin-EDTA (0.25%) for 5 minutes and gentle pipetting up and down, and then stained for flow cytometry. Anti-FITC antibodies were also added to quench surface-bound beads (clone F4/1). Macrophages incorporating beads were detected by positive expression for FITC, and the average number of beads incorporated per macrophage (phagocytic index) was calculated by multiplying the percent of macrophages incorporating a certain number beads out of the total population of macrophages which ingested beads, by that corresponding number of beads:

$$\sum_{i=1}^{n} \left(\frac{q_i}{p} \times n\right)$$

where

$n = \text{number of beads,}$

$p = \text{percent of macrophages incorporating beads,}$ and

$q = \text{percent of macrophages incorporating } n \text{ beads}$
Subaim 3b. Define associations between adipose tissue macrophage functional changes and circulating inflammation.

1. Plasma protein levels

Plasma samples stored at -80°C for up to three years were thawed on ice and used for quantification of cytokines and chemokines. Protein levels from 30 pro- or anti-inflammatory cytokines and chemokines (the same proteins that were analyzed in the tissue) were detected using the Cytokine NHP 29Plex System according to the manufacturer's protocol (Catalog # LPC0005M, ThermoFisher, Waltham, MA). In brief, magnetic fluorescent beads conjugated with antibodies bind to specific analytes (i.e. cytokines and chemokines). Secondary antibodies conjugated with biotin then bind to the analytes, which can be detected by the addition of streptavidin-PE. The intensity of the streptavidin-PE signal and recognition of the specific beads is detectable using the Luminex 100xMAP system and interpreted with Bio-Plex software (version 6.2; Bio-Rad Laboratories, Hercules, CA). The data generated is reported in Mean Fluorescent Intensity (MFI) of each analyte which is compared to a standard curve for quantification of the protein concentration in pg/mL.

Data analysis

Based on a power analysis using cross-sectional and preliminary results, a minimum of six monkeys per group were calculated to support scientific rigor and provide statistical significance at \( P < 0.05 \) by nonparametric Mann-Whitney test. We calculated interquartile ranges, mean and standard deviation values for each data set.
Since our cohorts were independent and not matched, independent comparisons of monkey groups at specific time points were conducted using Kruskal-Wallis one-way analysis of variance, and associated pairwise comparisons were performed using Dunn’s post-test. Linear correlations between ATM inflammation and systemic inflammation were quantified using Spearman’s correlation coefficient and its associated hypothesis test. The statistical significance threshold for all of the analyses was set at \( P < 0.05 \).

Graphs were created using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, California, USA and R\(^\text{122}\). Packages used for this effort were “ggplot2”, “ggpubr”, “ggsci”, “xlsx”, and “cowplot”. Correlations between genes and proteins to each other, including scatterplots, distribution plots, and the calculation of correlation coefficients were performed using R packages “xlsx”, “ggplot2”, and “GGally”. The heatplot was generated using the R packages “xlsx” and “pheatmap”. tSNE was conducted using R packages “xlsx”, “Rtsne”, “ggpubr”, and “ggsci”. Default settings were used except for the perplexity, which was set to 6, and \texttt{PCA\_scale=TRUE}. tSNE default settings were as follows: \texttt{dims = 2}, \texttt{initial\_dims = 50}, \texttt{theta=0.5}, \texttt{PCA=true}, \texttt{max\_iter=1000}, \texttt{pca\_scale=FALSE}, \texttt{pca\_center=TRUE}, \texttt{momentum=0.5}, \texttt{final\_momentum=0.8}, \texttt{eta=200}, and \texttt{exaggeration\_factor = 12}. Scatterplots were created using the R packages “xlsx”, “ggplot2”, “ggpubr”, “ggsci”, and “cowplot”; correlation coefficients and p-values were also calculated using R and the package “ggpplot2”.

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Chapter 5

VALIDATION OF SUBCUTANEOUS ADIPOSE TISSUE FROM Rhesus Macaques AS AN IMMUNOLOGICAL MODEL FOR HUMAN SUBCUTANEOUS ADIPOSE TISSUE

Introduction

Subcutaneous adipose tissue from rhesus macaques has not been validated as a model for human subcutaneous adipose tissue from an immunological perspective. In this chapter of the dissertation, the abundance of major immune cell populations, as well as the phenotype and tissue residence/infiltration of adipose tissue macrophages from rhesus macaques is described in relation to human subcutaneous adipose tissue.

Macrophages comprise the major immune cell population in subcutaneous adipose tissue from rhesus macaques

In human subcutaneous adipose tissue, macrophages are the most abundant cell type (10-50/mm² area) followed by T cells (<10/mm² area) and B cells (<5/mm² area). The proportion of different immune cell populations in subcutaneous adipose tissue from rhesus macaques is unknown, and the cells that are present could dictate the possible
biology and mechanisms which regulate the tissue functions. Healthy, non-obese rhesus macaques exhibiting no signs of short- or long-term weight loss were used to generate baseline data. We used CD68, CD3, and CD20cy to identify macrophages, T cells, and B cells using enzyme immunohistochemistry. CD68 (also known as macrosialin or LAMP-4), is preferentially expressed in late endosomes of cells of the monocyte/macrophage lineage, and is commonly used to quantify macrophages in subcutaneous adipose tissue of human specimens. CD3 is the T cell co-receptor required for activation, and the transmembrane protein CD20cy identifies precursors and mature B cells.

All three cell types were identified in our samples. Macrophages (Figure 5) were

![Figure 5. Identification of major immune cell types in subcutaneous adipose tissue. Enzyme immunohistochemistry shows CD68+ macrophages, CD3+ T cells, and CD20cy+ B cells. Sections shown are representative of nine monkeys and were imaged using a light microscope at x100 original magnification.]

Figure 6. Immune cells per unit area in rhesus macaque subcutaneous adipose tissue. Immune cells per field were counted and averaged from a minimum of 10 fields; each point represents one individual animal.
replete throughout the tissue, whereas T cells were rarer and typically found between adipocytes. B cells were the rarest cell type and were also found interstitially. On average (mean), 22 ± 16.5 CD68+ macrophages were found per mm$^2$, 3.6 ± 5.2 CD3+ T cells were found per mm$^2$, and 2.2 ± 2.3 CD20+ B cells were found per mm$^2$ (Figure 6). Based on immunohistochemistry and confocal microscopic imaging of subcutaneous adipose tissue in rhesus macaques, macrophages represented 79% of the immune cells, while T cells and B cells each accounted for 13% and 8%, respectively (Figure 7).

**Spatial distribution of macrophages in adipose tissue**

The abundance of macrophages found throughout the adipose tissue raised questions about histological location relative to phenotype and function, considering organs like the lung\(^{122}\) and spleen\(^{123,124}\) contain macrophage populations which differ by location and function. It is also expected that macrophages with different tissue localizations come in physical contact with different cell types, thus influencing their
functional potential. Detailed descriptions of macrophage populations surrounding dead or dying adipocytes called crown-like structures (CLS) and in interstitial spaces between adipocytes have been reported for both mice and humans, but the same information has not been investigated for rhesus macaques.
To characterize locations of macrophages in subcutaneous adipose tissue of rhesus macaques, enzyme immunohistochemistry was applied. CD68+ macrophages were detected in at least six different compartments: interstitially between adipocytes (Figure 8A), within the connective tissue (Figure 8B), perivascularly (Figure 8C), in association with neurons (Figure 8D), within crown-like structures (Figure 8E), and as aggregates between adipocytes (Figure 8F). Notably, the layer of macrophages around the crown-like structures was thin and generally one-cell deep, but the cells tended to be in contact with each other. Interstitial macrophages and perivascular macrophages were scattered within their respective regions, found individually, and were not often seen touching each other. Conversely, macrophages in the clusters were aggregated very closely together. In
the connective tissue regions of the subcutaneous adipose tissue, macrophages were often extended longitudinally and found in close association with each other, such that their elongated pseudopodia were touching end to end. Nerve-associated macrophages in the adipose lied in close proximity to each other, but unlike those from the connective tissue, they generally were not touching. Crown-like structures and interstitial macrophage clusters were exceedingly rare and detected in only 2 out of 9 individuals, respectively. On the other hand, interstitial macrophages, macrophages in connective tissue, and those surrounding blood vessels and nerves were present in nearly every sample from all animals.

Figure 8. Distribution of macrophage subsets throughout the subcutaneous adipose tissue. Enzyme immunohistochemistry was used to detect CD68+ macrophages interstitially between adipocytes (A), within the connective tissue or septa (B), perivascularly (C), in association with neurons (D), within crown-like structures (E), and forming interstitial clusters between adipocytes (F). Sections shown are representative of nine monkeys and were imaged using a light microscope at x100 final magnification, except for E and F, which are shown at 200x final magnification.
The majority of adipose tissue macrophages in rhesus macaques express CD206 and CD163

Most ATMs from humans express both CD163 and CD206 on their cell surface. CD163 is a hemoglobin/haptoglobin scavenger receptor exclusively found on the cell surface of monocytes/macrophages, and CD206 is a mannose receptor commonly found on the surface of macrophages which is thought to be involved in a variety of processes including phagocytosis and antigen presentation. CD206 can also be expressed by a variety of other cell types, including endothelial cells and fibroblasts, but can distinguish macrophages based on location and morphology, or more specifically, by the co-expression of other macrophage-defining molecules such as CD68 or CD163. The expression of either CD163 or CD206 on the surface of macrophages generally defines an ‘anti-inflammatory’ phenotype in mice, though the distinction between pro- and anti-inflammatory macrophages in humans and rhesus macaques is less clear.

To evaluate whether ATMs from rhesus macaques are phenotypically and potentially functionally similar to humans, as well as to elucidate differences in the macrophage subsets depending on their location in the adipose, we first examined the expression of CD206 on CD68+ ATMs using confocal microscopy, and then investigated the expression of CD163 on CD206+ macrophages. Macrophages from the parenchymal space between adipocytes, the connective tissue, crown-like structures, and perivascular space were analyzed henceforth, because these regions are the most thoroughly described in humans and thus useful for comparing whether subcutaneous adipose tissue from humans and macaques are immunologically similar.
The majority of CD68+ cells from the parenchyma (98%), connective tissue (86%), and perivascular areas (83%) were positive for expression of CD206 (Figures 9A-D and 10A-D), and CD206 expression was very bright among these macrophages. Macrophages in crown-like structures (CLS) dimly stained for CD206 and were

Figure 9. Phenotypic characterization of macrophages from different regions of subcutaneous adipose tissue in rhesus macaques. Regions shown are A. Between adipocytes (parenchyma), B. Connective Tissue, C. Crown-like structures, D. Perivascular. CD68 is shown in green, and CD206 is shown in red. DAPI-stained nuclei are shown in grey. Tissue samples were imaged under x400 original magnification under oil (x40 objective, fluotar/NA 1.0) using a Leica TCS SP2 confocal microscope equipped with four lasers (Leica Microsystems). Results shown are representative of studies from nine monkeys.
classified as negative for the purposes of this analysis (Figure 9C). In contrast to macrophages from other regions, only 11% of macrophages from the CLS expressed CD206 brightly (Figure 10C).

We next investigated the expression of the scavenger receptor CD163 on CD206+

macrophages, because in other tissues such as the lung, CD163 can be expressed alone or in combination with CD206 to define different macrophage subsets. CD163 was expressed on nearly all cells which expressed CD206, and this was the same among all of the regions (Figure 11A-D). Of note, CD206 expression was commonly observed on endothelial cells surrounding the blood vessels (Figure 11D), but these were distinguished from macrophages based on location and morphology and were excluded.

![Figure 10. Representation of expression of CD68 and CD206 on macrophages by region.](image)

Macrophages expressing CD68+CD206+ or CD68+CD206- were counted and shown relative to each other in the different histological areas, including A. the parenchymal area between adipocytes, B. the connective tissue, C. in the perivascular space, and D. within crown-like structures.
from counting. Although CD163 was co-expressed on all macrophages which expressed CD206 macrophages within the crown-like structures dimly expressed CD206 but had no discernable expression of CD163 (Figure 11C).

**Figure 11.** Expression of CD163 on CD206+ macrophages from different regions of subcutaneous adipose tissue in rhesus macaques. Regions shown are A. Between adipocytes (parenchyma), B. Connective Tissue, C. Crown-like structures, D. Perivascularly. CD163 is shown in green, and CD206 is shown in red. DAPI-stained nuclei are shown in grey. Tissue samples were imaged under x400 original magnification under oil (x40 objective, fluotar/NA 1.0) using a Leica TCS SP2 confocal microscope equipped with four lasers (Leica Microsystems). Results shown are representative of studies from nine monkeys.
Adipose tissue macrophages from rhesus macaques are predominantly tissue resident and longer-living

Reports have indicated that among ATMs in humans, CD163+CD206+ macrophages are predominantly tissue resident and not recruited into the tissue from circulating monocytes. To investigate whether the same is true for rhesus macaques, we used dextran to quantify long-lived tissue-resident macrophages in the adipose tissue. Dextran is a derivative of the sugar dextrose which cannot be broken down by mammalian cells and thus it is able to be traced in cells over time. It is also endocytosed by cells with the L-SIGN or DC-SIGN family of receptors, which expressed on the surface of macrophages. It should therefore be taken up and retained by macrophages for the duration of their lifespan, and macrophages incorporating dextran can be identified by using labeled antibodies specific to dextran.

Dextran was injected i.v. either 11 days (n=5) or 44 days (n=1) prior to sampling, and subcutaneous adipose tissue was imaged and quantified for dextran-labeled macrophages using confocal microscopy. We first confirmed that CD163+ macrophages readily incorporate dextran intracellularly (Figure 12). We then counted the number of CD163+CD206+ macrophages that incorporated dextran from all regions of the tissue and found that >80% of CD163+206+ ATMs throughout the tissue incorporated dextran after either 11 days (grey bars) or 44 days (blue bar) (Figure 13A-B). Of the macrophages that did not incorporate dextran, CD163+CD206+ macrophages accounted for 68%, CD163+CD206- macrophages accounted for 23%, and CD163-CD206+ macrophages accounted for 9% (Figure 13C).
We then analyzed whether the incorporation of dextran among ATM differed by histological region. Long-lived dextran+ cells were found in all regions of subcutaneous

Figure 12. Identification of rhesus macaque ATM incorporating dextran. Immunofluorescence and confocal microscopy imaging showed that dextran was intracellularly incorporated by CD163+ macrophages and readily detected in the adipose tissue. Tissue samples were imaged under x630 original magnification under oil (x63 objective, fluotar/NA 1.0) using a Leica TCS SP2 confocal microscope equipped with four lasers (Leica Microsystems). Results shown are representative of
adipose tissue that we investigated, including the parenchyma between adipocytes (Figure 14A), in the connective tissue (Figure 14B), in the perivascular space (Figure 14C), and rarely around crown-like structures (Figure 14D). Although crown-like structures were small and rare in our samples, it was notable that most of the cells in the immediate circular region of cells in the CLS did not incorporate dextran, but rather, it was the cells directly adjacent to these structures which were long-lived (Figure 14C). In addition, ATMs that did not incorporate dextran were found scattered throughout the tissue and not localized to one specific region.

Locally proliferating cells are scattered throughout the subcutaneous adipose tissue
Previous studies in mice have indicated that ATMs proliferate locally in response to a pathogen or injury, prior to infiltration by circulating monocytes, but also in small amounts (~2%) during steady state. The existence of locally proliferating macrophages in humans during steady state is not clear\textsuperscript{129}. To first measure whether we could observe local proliferation of any cell type in the adipose tissue, we used Ki67 as a marker for cell

Figure 14. Expression of CD206 on CD163+dextran+ macrophages in subcutaneous adipose tissue of rhesus macaques. Regions shown are A. Between adipocytes (parenchyma), B. Connective Tissue, C. Crown-like structures, D. Perivascular. Dextran is shown in green, CD163 is shown in red, and CD206 is shown in blue. DAPI-stained nuclei are shown in grey. Tissue samples were imaged under x400 original magnification under oil (x40 objective, fluotar/NA 1.0) using a Leica TCS SP2 confocal microscope equipped with four lasers (Leica Microsystems). Results shown are representative of studies from nine monkeys.
division to identify whether any cells in the adipose tissue were dividing at the time of sampling. Only one out of nine animals exhibited any positivity for Ki67. In this animal, enzyme immunohistochemistry confirmed the presence of numerous cells proliferating locally within the subcutaneous adipose tissue. These cells were primarily found in aggregates near blood vessels and peripheral nerve bundles, but were occasionally found between adipocytes and in connective tissue (Figure 15A-E). We also observed Ki67+

cells in clusters of cells around the connective tissue (Figure 15C).

To distinguish whether the locally proliferating cells we found in the subcutaneous adipose tissue were macrophages, we triple-stained for CD206, dextran, and Ki67+ expression of cells and imaged and analyzed tissues using immunofluorescence and confocal microscopy. In our samples, we did not observe any

Figure 15. Identification of Ki67+ cells in subcutaneous adipose tissue of rhesus macaques. Regions shown are A. Parenchyma between adipocytes, B. in connective tissue, C. in cell cluster, D. around nerves, and E. around blood vessels. Ki67-staining cells are indicated by arrowheads. Tissue samples were imaged under x100 final magnification.
Discussion

Considered a metabolically inert organ for decades, adipose tissue has recently become accepted as a dynamic metabolic, endocrine, and immunological organ, replete with stromal and immune cells whose functions are critical for daily maintenance and homeostasis. Epidemic levels of obesity and metabolic diseases have underscored the importance of understanding adipose tissue biology. Adipose tissue is the primary storage compartment for excess fat, and dysregulation is thought to cause systemic low-grade inflammation that exacerbates the risk of developing a wide breadth of chronic diseases. Additionally, adipose tissue can harbor infectious agents, including Epstein-Barr virus, cytomegalovirus, and SIV/HIV. It is therefore imperative to understand adipose tissue biology under homeostatic conditions, so that baseline data can be available to study disease states. Studies using mice have moved the field forward tremendously, however, mice cannot adequately recapitulate many human diseases, including HIV, as well as do non-human primates. It was for these reasons that we decided to characterize immune cells from rhesus macaques, which are phylogenetically similar to humans and simulate HIV/AIDS in humans when infected with SIV.
Previous reports have shown that ATMs constitute a major portion of the immune cell population in adipose tissue\(^{138}\). Results from our study confirmed that ATMs from subcutaneous adipose tissue in rhesus macaques, like humans, express CD68 and were also the predominant immune population compared to T cells and B cells, thus highlighting their critical role under healthy, uninfected conditions. ATMs of RM were, like humans, found in areas such as the crown-like structures and scattered between adipocytes, however, the presence of CLS was exceedingly rare and found in only 2 of 9 animals used in this study. Besides the CLS and interstitial spaces, ATMs were also identified in four other areas rarely described elsewhere in humans: namely perivascularly, around neurons, within connective tissue, and in interstitial clusters between adipocytes. These distinct localizations could highlight functional differences; for example, perivascular macrophages could be sampling antigens from the blood, or they might also be helping in the generation of new blood vessels, which is required prior to the creation of new adipocytes\(^{150}\). Similarly, macrophages in the connective tissue could have a very specific function in helping to create the septa, fibrous tissue that separates groups of adipocytes into lobules, which then form larger units that together comprise adipose tissue depots\(^{151,152}\). Additionally, important roles for neuron-associated ATMs in catecholamine degradation and lipolysis have recently been recently reported\(^{102}\).

Macrophages from different locations in other organs (e.g. lung and spleen) can be distinguished by the expression of CD68, CD163, or CD206, and these same markers are expressed in the majority of ATMs from humans. Most CD68\(^+\) macrophages in rhesus macaques expressed CD206, in accordance with human studies. In addition, the expression of CD163 on all of the cells expressing CD206 with the exception of those
from the crown-like structures is also what we would expect to find when comparing results from rhesus macaques with human data. The preponderance of CD163+CD206+ macrophages was suggestive that ATMs in rhesus macaques, like humans, are mostly ‘anti-inflammatory’ in phenotype. CD163+CD206- macrophages were less frequent and were scattered throughout the tissue. Since CD163 expression is also found in circulating monocytes, it is possible that this subset of macrophages may correspond with a circulating repertoire of macrophages that constantly surveil the tissue and return to the blood.

Macrophages within the crown-like structures appeared completely different than macrophages from the rest of the subcutaneous adipose tissue in terms of the expression of surface markers. CLS macrophages largely expressed CD68 but not CD163 and had only dim expression of CD206, suggesting that these macrophages may be the ‘pro-inflammatory’ macrophages found in excess during obesity in mice and humans. However, it was surprising that there was more expression of CD206 than CD163, considering CD163 is found on circulating monocytes and therefore likely to be expressed on these cells as they exit the blood and traffic into the tissue. Additionally, CD206 is associated mainly with tissue-resident cells, and not those trafficking from the circulation. Thus, the dim expression of CD206 and lack of expression of CD163 could suggest not only a change in phenotype of blood-derived cells after infiltration, but might also be evidence for the polarization of tissue-resident cells changing in phenotype after trafficking to the area of damage. In concordance with our observations, a report from humans analyzing the expression of CD40, CD163, and CD206 on crown-like structures
found that CD206 was capable of identifying CLS macrophages in their samples, but not CD163$^{154}$.

Reports using the cell membrane-binding dye, PKH26, to label macrophages but not monocytes in mice found that there exists a population of ATMs in the interstitial spaces that live in the tissue for at least 28 days$^{126-128}$, and other studies have confirmed that these are maintained via self-renewal$^{108}$. It is also thought that ATMs from humans are tissue-resident, though their lifespan in the tissue is unknown. To investigate this phenomenon in rhesus macaques, we injected them with dextran, an inert sugar-derivative that labels ATMs for the duration of their lifespan, and detected them using immunofluorescence. Our results confirmed that the vast majority of subcutaneous ATMs in rhesus macaques were longer-living and survived for at least 11 days in five macaques and at least 44 days in one. These longer-living ATMs co-expressed CD163 and CD206, and were detected in all regions of the tissue.

Interestingly, dextran+ macrophages did not comprise the immediate areas surrounding the dying adipocytes of CLSs, but were directly outside of the region of the CLS, suggesting that they may be required for orchestrating the CLS but not specifically involved in clearing the debris. However, the rarity of CLS in our samples does not preclude the possibility that dextran+ cells could be more closely partaking in the debris-clearing process, particularly during obesity when crown-like structures are far more common and more taxing on the tissue environment.

We also examined the phenotype of macrophages which did not incorporate dextran, and were surprised to find that most of them were CD163+CD206+ macrophages, followed by CD163+CD206- macrophages and then CD163-CD206+...
macrophages. Since the expression of CD206 is typically indicative of tissue-resident macrophages, we expected that the majority of macrophages that were not long-living would be CD163+CD206- macrophages derived from monocytes in constant circulation through the tissue. The lack of dextran in these cells could be due to several reasons which we did not test here, but may include that the dose of dextran was not sufficiently high to label all of the macrophages in every animal, or that they are, in fact, being constantly replenished from the blood. An alternative possibility is that these macrophages are a distinct subset based on other features, such as potentially lacking expression of L-SIGN or DC-SIGN, that they have proliferated and lost their dextran particles, or that they have the ability to eject particles of dextran better than other macrophages.

Although locally proliferating macrophages have been identified in mice, particularly during obesity, we did not find evidence of local proliferation of macrophages in our samples. However, our monkeys were all of a healthy weight and so we did not expect to find a huge number of self-renewing cells, and, confocal microscopy is less sensitive that other assays such as flow cytometry. Thus, steady-state locally proliferating macrophages may have been missed in our study. Alternatively, macrophages in adipose tissue from humans may self-maintain by simply surviving for a very long time and not being easily susceptible to apoptosis after injury. Considering macrophages in other tissues such as microglia in the brain are known for long-term survival, it is possible that ATMs are similar and very rarely need replacing, and that when this replacement is necessary, it can be accomplished by either circulating monocytes or self-renewal. In spite of the lack of self-renewing macrophages in the
adipose tissue, we did find a large number of Ki67+ cells other than macrophages in one animal and they were generally aggregated around blood vessels and peripheral nerves. These dividing cells could be indicative of adipocyte growth around a blood and nerve supply, as it is well-documented that adipose tissue growth follows the formation of new blood vessels.

Non-human primates are exceptional animal models for the study of human diseases because of their phylogenetic proximity and social relationships, as well as their susceptibility to many human diseases. Our characterization of ATMs from the subcutaneous adipose tissue of rhesus macaques provides evidence that adipose tissue contains many immune cell types that are also found in human adipose tissue, with macrophages being the most abundant. Like in humans, ATMs localize to a variety of regions, are predominantly CD163+CD206+, and are tissue-resident and long-lived. This study is also the first to highlight the minimum lifespan of ATMs from areas beyond the CLS and interstitial space, including the connective tissue and perivascular area. Experimental limitations in humans and species-specific differences in disease pathogenesis reflects the need to answer questions related to immunometabolism and chronic diseases in a non-human primate model. Furthermore, recent studies point to infection of adipose tissue by SIV/HIV, and wasting is a clear symptom of AIDS, suggestive of adipose tissue involvement in disease. The extent to which ATMs are affected and contribute to pathology in adipose tissue after obesity or infection in humans can be greatly enhanced by research utilizing rhesus macaques. This study provides the baseline data and validation necessary to study the effects of a variety of diseases using adipose tissue from rhesus macaques.
Chapter 6

CONTRIBUTION OF ADIPOSE TISSUE INFLAMMATION TO CIRCULATING INFLAMMATION AFTER SIV INFECTION, WITH AND WITHOUT ART

Introduction

Chronic inflammation has been reported in individuals with HIV infection, and low-grade chronic inflammation is observed in those with HIV+ART. This inflammation is prognostic of morbidity and mortality among HIV±ART individuals, including the development of accelerated aging. Adipose tissue is a major contributor to low-grade chronic inflammation due to metabolic inflammation among people who are obese. Since HIV infects adipose, and ART itself may exert metabolic consequences on adipose tissue, we studied the inflammatory effects of SIV±ART on adipose tissue and assessed the relationship between adipose tissue inflammatory factors and those in the blood.
Expression of inflammatory genes in subcutaneous adipose tissue from rhesus macaques is upregulated after SIV±ART

Elevated levels of circulating inflammatory factors, especially cytokines and chemokines, are indicative of developing a variety of diseases during HIV±ART and is also prognostic of mortality, but the tissue source of this inflammation is unclear. Adipose tissue is one source of inflammation during obesity. To test whether adipose could be a source of the circulating inflammation during HIV±ART, we measured gene and protein expression of a variety of pro- and anti-inflammatory factors in the subcutaneous adipose tissue of fifteen young adult rhesus macaques infected with SIV±ART. We used historical samples from Indian ancestry rhesus macaques which had been inoculated with SIVmac239, SIVmac251, or SHIV163p3 (all referred to as SIV for the purposes of this study) for approximately eight months, at which time seven macaques were euthanized and blood and subcutaneous adipose tissue was collected. The remaining macaques received ART (dolutegravir (DTG)/tenofovir (PMPA)/emtricitabine (FTC) or dolutegravir (DTG)/emtricitabine (FTC) /tenofovir disoproxil (TDF) for approximately nine months. The ART regimen included integrase inhibitors (DTG) and reverse transcriptase inhibitors (PMPA, FTC, TDF) but not protease inhibitors. At the time of sampling, all rhesus macaques without ART were actively replicating virus at 10^4-10^6 copies/mL in the blood, and those on ART all had undetectable viremia except for one which appeared to blip at the time of sampling (332 copies/mL) (Table 3, Methods).
We first measured the expression of 40 genes related to infection, inflammation, and tissue growth in subcutaneous adipose tissue samples stored in RNAlater (Figure 16) and subjected to the QuantiGene Plex Assay protocol procedures (Thermo Fisher Scientific). Overall, the vast majority of these genes in subcutaneous adipose tissue were upregulated in SIV-infected compared to uninfected animals, and a large number of these genes remained upregulated even after ART reduced virus levels to below detection. Several chemokines and cytokines related to inflammation were increased upon infection.
and remained high or increased after ART, including CCL2, IL-10, CX3CR1, CXCL10, IL-18, and CCR1. In spite of the higher inflammatory profile, we detected SIV Pol gene in only 2 out of 7 SIV-infected macaques, and 0 out of 7 SIV+ART macaques. Surprisingly, one animal in the SIV-infected group which exhibited the highest levels of multiple inflammatory genes did not have any detectable SIV.

We more closely analyzed several inflammatory mediators in subcutaneous adipose tissue that are frequently elevated in the circulation of HIV-infected individuals. These included IL-1b, TNFα, IL-1RN, CCL2 (MCP-1), IL-6, and IL-18, all of which are considered to be genes that encode ‘pro-inflammatory’ cytokines or chemokines except for IL-1RN, the gene which encodes IL-1RA. The mean fold-change expression of each of these factors relative to beta-actin rose after SIV infection (Figure 17) and both IL-1RN and CCL2 trended towards significance (p=.109 and p=.098, respectively) (Table 6). IL-1RN and CCL2 increased expression by 3.14-fold and 2.31-fold, respectively.
Likewise in the SIV+ART group, the mean expression of CCL2, IL-6, and IL-18

Figure 17. Gene expression of inflammatory mediators in subcutaneous adipose tissue of rhesus macaques. Box and whiskers plots show the minimum to maximum points and detail the interquartile ranges and medians. P-values <.05 are denoted with an asterisk.
Table 6. Inflammatory cytokines and chemokines shifts in subcutaneous adipose tissue of rhesus macaques before and after SIV, with and without ART.

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P-values were calculated using the means of each group and in comparison to results from uninfected rhesus macaques.

Percent change was calculated using the means of each group and in reference to uninfected rhesus macaques.

P-values <0.1 are highlighted in light blue, and those <.05 are highlighted in dark blue. Percent change >100% is highlighted in coral.

were 2.08-fold, 1.16-fold, and 1.5-fold, respectively, higher relative to the SIV-negative animals. IL-10 was significantly upregulated during SIV+ART (p=.023), in addition to the chemokine CCL2 (p=.090) and cytokine IL-18 (p=.061) which trended towards significance (Figure 17, Table 6).

We also more closely examined the gene expression of several other cytokines and chemokines which were detected in the adipose tissue and found that CXCL10 was significantly increased in expression after SIV infection (p=.041), and IL-10 had significantly higher expression in SIV+ART compared to SIV-uninfected (p=.023), and trended towards significance in the SIV-infected group compared to SIV-uninfected.
animals (p=.071) (Table 6). Furthermore, the mean expression of CCL3, IFNa13, CXCL10, and CCL4L1 all increased more than 3-fold in the SIV-infected group compared to the SIV-uninfected animals. The mean expression of CCL3, IL-5, IL-12rb2, and CCL4L1 also increased more than 3-fold in the SIV+ART group compared to SIV-uninfected animals.

Adipose tissue inflammatory signature using gene analysis

Numerous cytokines, chemokines, and other tissue-related factors work in concert to resolve tissue damage. Thus, it is helpful to examine multiple factors at once to determine whether numerous subtle changes could globally define if a tissue is undergoing an inflammatory process. New methods to reduce high-dimensional data are currently available that provide a mathematical basis for combining many parameters to create a molecular ‘signature’. One such method, called t-Distributed Stochastic Neighbor Embedding (t-SNE), was developed in 2008 with the purpose of mapping samples with numerous parameters on one two-dimensional plot. This is achieved by calculating the probability that two points are similar based on their distance from a t-distribution of a specific parameter. Based on these values, the points are attracted and repelled from each other through time such that they move through a two-dimensional space until points which are most similar to each other are grouped together, and those that are most dissimilar are spread apart. The axes represent relative distances between points and are shown on a ‘map’ (scatterplot) which allows visualization of not only which points are most similar to each other, but also their relative similarity. There are several advantages to using t-SNE over other clustering methods, including that it is non-linear (e.g. PCA), unsupervised, and uses a t-distribution instead of a normal distribution.
to calculate probabilities that similar points will fall near each other on the map. This allows points to aggregate further apart and not all cluster in the center of the map. Using this method, we clustered the gene expression data for all 40 genes on a single map to determine whether animals from each group would cluster together and provide a unique signature that would define a global inflammatory status after SIV infection (Figure 18A). Although there were clear differences between groups, the confidence intervals overlapped (Figure 18B). We followed this first analysis with a second that included only
the six genes that were the best prognostic indicators in plasma for progression to disease and which were more closely analyzed previously (IL-1b, TNFα, IL-1RN, CCL2 (MCP-1), IL-6, and IL-18) for re-plotting the t-SNE map (Figure 18C). Confidence intervals for these groups dramatically overlapped and the groups failed to separate distinctly (Figure 18D).

**Relationship between inflammatory genes and SIV+ART in adipose tissue**

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**Figure 18. Gene expression signatures in subcutaneous adipose tissue of rhesus macaques with or without SIV infection and ART**

- **A.** t-SNE mapping of all genes in the data set.
- **B.** t-SNE of all genes with 80% confidence ellipses.
- **C.** t-SNE mapping of six selected genes from the dataset.
- **D.** t-SNE of six selected genes with 80% confidence ellipses.
The subset of six genes most commonly elevated in the plasma of individuals with HIV±ART did not create a unique inflammatory signature which distinguished between groups. However, the complete set of 40 inflammatory genes together was able to highlight distinct differences in the inflammatory genes expressed in each group. Thus, there could be unique biological pathways mediating inflammatory processes during steady state, SIV-infection, and SIV+ART, which may confer distinct targets for intervention. To determine whether inflammatory pathways were unique to each disease state, we assessed the relationship between all 40 of the inflammatory cytokines shown to be expressed from our original gene set and separated these relationships out by group (Figure 19).

Correlations were plotted with and without hierarchical clustering to allow comparisons between pathways (no clustering) and easy visualization of cytokines relationships (with clustering). Since several inflammatory chemokines and cytokines were expressed at very low levels in the SIV-negative group, most factors were not significantly correlated with each other often due to a low level of expression. In the SIV-negative group, the clear majority of genes with detectable levels of expression were in positive association with each other (Figure 19A-B). Chemokines and chemokine receptors tended to lie mostly
Figure 19. Correlation plots of subcutaneous adipose tissue gene expression in rhesus macaques. Negative correlations are denoted in red, and positive correlations are denoted in blue. The size of the circles indicates the strength of the correlation. Samples were from: A. SIV-negative animals without hierarchical clustering. B. SIV-negative with hierarchical clustering. C. SIV-infected animals without hierarchical clustering. D. SIV-infected animals with hierarchical clustering. E. SIV+ART animals without hierarchical clustering. F. SIV+ART animals with hierarchical clustering.
in positive association with each other. However, groups of specific chemokines and hemokine ligands were distinct from other groups of chemokines and their ligands. In addition, there were only three factors significantly associated with any negative correlations, most notably COX2 which had a negative correlation with over 10 inflammatory cytokines and chemokines. IL-18 correlated inversely with IL-3 and IL-6, and CCL3 inversely correlated with IL1R1.

By contrast, numerous adipose tissue genes were upregulated during SIV infection and this underscored both positive and negative correlations with other cytokines and chemokines (Figure 19C-D). These associations were not only due to increased expression, but because the relationships between them changed. For example, COX2 correlated positively with several chemokines and cytokines, as in SIV-negative animals, but was also strongly negatively correlated with several others, which was not seen in SIV-negative animals. Like in SIV-uninfected animals, groups of chemokines and chemokine receptors tended to be organized in several clusters which were distinct from each other, including CCL2 and CCR1, CXCL10 and CCL5, and CCL3, CCR4, CCL4L1, CCR5, CCR6, and CXCR3. In addition, the gene for SIV most strongly correlated with IRF1, CXCL10, CCL5, TNF, and IL-18, and showed no significant correlations with any other cytokines, indicating that the presence of the virus itself exerts a very specific inflammatory reaction in the adipose tissue.

During SIV+ART, expression was increased for several adipose genes compared to those in SIV-negative controls, but the relationships between the genes were very similar to those in SIV-animals (Figure 19E-F). The primary difference was in the magnitude of the expression of the genes, rather than in the relationships between them.
In SIV+ART, COX2 negatively correlated with most cytokines and chemokines in a similar fashion as in SIV-negative, and there was also a negative association between IL-18 with IL-3 and IL-6. Unique to SIV+ART, CCL2 significantly correlated with over 10 inflammatory factors, which was more than any other chemokine. CXCL10 also positively correlated with many inflammatory factors during SIV+ART.

**Inflammatory proteins are elevated in subcutaneous adipose tissue from rhesus macaques**

Proteins are the endpoint of transcription and translation and are the biological molecules which exert an actual function, however, the magnitude of RNA expression does not always reflect the concentration of protein in the tissue. To investigate whether increased expression of inflammatory genes in the tissue reflected an increase of corresponding proteins, and to assess the biological relevance of the detected genes, we measured protein expression from the same adipose tissue samples used for measuring gene expression. The proteins selected largely corresponded with the genes selected for the gene expression assay, and results of all of the proteins measured are shown in Figure 20. There was a general trend towards increased expression of inflammatory cytokines and chemokines in the SIV+ group (green bars) and several of these factors were higher, remained high, or did not completely return to baseline levels after ART, including sCD40L, IL-6, and IL-23.
We more closely analyzed the same subset of factors analyzed via gene

Figure 20. Protein expression of inflammatory mediators from subcutaneous adipose tissue of rhesus macaques. The same volumes of subcutaneous adipose tissue used in the QuantiGene Plex assay for measuring gene expression were also applied here for protein analysis using the ProCarta Plex System. Box plots show the interquartile ranges and medians.
expression which are best known to be elevated during HIV±ART and prognostic of disease (Figure 21). All the measured chemokines and cytokines within this subset increased after SIV infection, and several trended towards significance, including IL-18 (p=.062) and IL-1RA (p=.072). Further, the mean expression of TNFα increased by 57%, the mean expression of IL-1RA increased by 134%, and that of IL-18 increased by 620% compared to adipose from uninfected animals. Interestingly, the only cytokine of the subset which returned to baseline levels in the SIV+ART animals was IL-1RA, the cytokine which counteracts the action of pro-inflammatory cytokines IL-1a and IL-1b. IL-6 (+190%, p=.088), and IL-18 (+81% p=.031) were particularly elevated in comparison to both baseline and after SIV-infection levels. There was also a distinct increase of CCL2 and TNFα among nearly all animals in the SIV±ART group compared
with SIV-negative animals, though statistically this was not significant because of the
tremendous variability in the SIV- animals.

We analyzed the statistical difference and percent change of several other proteins
which are less commonly associated with HIV (Table 7). Of these, CXCL13 stood out as
having a 923% increase in the SIV-infected group compared to the SIV-uninfected group
and trended closely towards significance (p=.077). Additionally, IL-13 (+98%, p=.036),
IL-23 (+278%, p=.002), sCD40L (+420%, p=.080), and G-CSF (+92%, p=.091) were
elevated in the SIV+ART group.

*Adipose tissue inflammatory signature using protein analysis*
In the previous section, using all of the genes in concert allowed for clustering of adipose tissue between the three groups, but some overlap remained. Using the same t-SNE method, we clustered the protein expression data for all 30 proteins on a single map.

Table 7. Inflammatory cytokines and chemokines in subcutaneous adipose tissue of rhesus macaques before and after SIV, with and without ART.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>SIV- Mean</th>
<th>SIV- SD</th>
<th>SIV+ Mean</th>
<th>SIV+ SD</th>
<th>Percent Change</th>
<th>p-value</th>
<th>SIV+ART Mean</th>
<th>SIV+ART SD</th>
<th>Percent Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>0.23</td>
<td>0.00</td>
<td>0.23</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.23</td>
<td>0.00</td>
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<td>CCL3</td>
<td>3.02</td>
<td>0.00</td>
<td>3.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>2.99</td>
<td>0.07</td>
<td>0.334</td>
<td>-0.81</td>
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<td>CXCL11</td>
<td>2.82</td>
<td>0.00</td>
<td>2.95</td>
<td>0.23</td>
<td>0.122</td>
<td>4.72</td>
<td>2.82</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>sCD40L</td>
<td>64.70</td>
<td>167.52</td>
<td>236.63</td>
<td>221.32</td>
<td>0.111</td>
<td>265.72</td>
<td>336.12</td>
<td>369.99</td>
<td>0.080</td>
<td>419.90</td>
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<td>IL-10</td>
<td>0.05</td>
<td>0.78</td>
<td>3.65</td>
<td>5.57</td>
<td>0.156</td>
<td>458.58</td>
<td>0.38</td>
<td>0.00</td>
<td>0.334</td>
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<td>IL-17A</td>
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<td>4.12</td>
<td>1.67</td>
<td>0.302</td>
<td>18.13</td>
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<td>CCL9</td>
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<td>0.00</td>
<td>8.62</td>
<td>12.42</td>
<td>0.225</td>
<td>181.61</td>
<td>3.06</td>
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<td>IL-4</td>
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<td>3.62</td>
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<td>IL-5</td>
<td>4.60</td>
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<td>4.75</td>
<td>1.17</td>
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<td>4.75</td>
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<td>IL-12</td>
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<td>45.25</td>
<td>56.20</td>
<td>0.638</td>
<td>30.23</td>
<td>18.90</td>
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<td>43.02</td>
<td>52.29</td>
<td>0.532</td>
<td>57.06</td>
<td>14.40</td>
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<td>GM-CSF</td>
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<td>16.97</td>
<td>5.02</td>
<td>4.84</td>
<td>0.495</td>
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<td>3.19</td>
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<td>0.296</td>
<td>-67.16</td>
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<td>G-CSF</td>
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<td>1991.16</td>
<td>5372.84</td>
<td>5713.97</td>
<td>0.118</td>
<td>201.21</td>
<td>3430.90</td>
<td>1613.15</td>
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<td>IL-1 beta</td>
<td>3.61</td>
<td>8.88</td>
<td>13.52</td>
<td>30.89</td>
<td>0.400</td>
<td>274.10</td>
<td>7.28</td>
<td>8.63</td>
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<td>IL-18</td>
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<td>21.44</td>
<td>249.68</td>
<td>298.43</td>
<td>0.063</td>
<td>630.42</td>
<td>62.78</td>
<td>25.32</td>
<td>0.031</td>
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<td>41.58</td>
<td>672.29</td>
<td>1323.59</td>
<td>0.223</td>
<td>784.33</td>
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<td>146.70</td>
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<td>18.88</td>
<td>442.99</td>
<td>870.83</td>
<td>0.190</td>
<td>2385.97</td>
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<td>21.30</td>
<td>0.696</td>
<td>21.32</td>
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<td>144.44</td>
<td>184.64</td>
<td>0.148</td>
<td>267.34</td>
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<td>75.04</td>
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<td>38.71</td>
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<td>156.55</td>
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<td>31.47</td>
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<td>7713.55</td>
<td>9564.98</td>
<td>0.077</td>
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<td>77.70</td>
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<td>343.80</td>
<td>1006.60</td>
<td>753.06</td>
<td>0.072</td>
<td>134.54</td>
<td>465.16</td>
<td>208.84</td>
<td>0.804</td>
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<td>278.37</td>
<td>485.89</td>
<td>519.37</td>
<td>0.470</td>
<td>47.69</td>
<td>304.53</td>
<td>157.71</td>
<td>0.832</td>
<td>-7.43</td>
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<td>116.15</td>
<td>0.440</td>
<td>-41.96</td>
<td>38.73</td>
<td>21.17</td>
<td>0.099</td>
<td>-79.37</td>
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</table>

P-values were calculated using the means of each group and in reference to SIV-uninfected animals. Percent change was calculated using the means of each group and in reference to SIV-uninfected animals.

P-values <0.1 are highlighted in light blue, and those <.05 are highlighted in dark blue. Percent change >100% is highlighted in coral.
to determine whether these would confer a clustering advantage over the gene expression clustering and provide a unique signature that could define a global inflammatory status after SIV infection (Figure 22A-D). We followed this by clustering the samples according to a subset of six proteins which corresponded with the subset of six genes
used for the secondary t-SNE analysis previously, namely, IL-1b, TNFα, IL-1RA, CCL2, IL-6, and IL-18.

Figure 22. Protein expression signature in subcutaneous adipose tissue of rhesus macaques. A. t-SNE mapping of all proteins in the data set. B. t-SNE of all proteins with 80% confidence ellipses. C. t-SNE mapping of six selected proteins from the dataset. D. t-SNE of six selected proteins with 80% confidence ellipses.

In contrast with the gene expression data, when analyzing all of the proteins from the data set at once, the samples within each group aggregated near each other within groups, but also were closer together between groups, and the 80% confidence intervals
drastically overlapped (Figure 22A-B). Interestingly, there was more overlap between SIV-uninfected and SIV-infected groups than between SIV+ART and either of the other groups. Re-plotting the t-SNE map using the subset of six proteins resulted in somewhat more distinct clustering between groups (Figure 22C-D), which contrasted with the less distinct clustering using the corresponding genes. Interestingly, in the subset of six proteins, the SIV-infected and SIV+ART animal groups were clustered more closely together than SIV-uninfected. Additionally, the groups were much closer (relatively) to each other using the subset of six proteins compared to the entire data set when considering the scales of both sets of plots.

Relationship between inflammatory proteins and SIV±ART in adipose tissue

Although the subset of six proteins conferred a more robust separation between groups than all of the proteins together, we nonetheless investigated the relationship between our entire panel of proteins to determine whether the proteins in this subset were related to each other and working in concert, and whether these relationships differed between groups (Figure 23). Consistent with the results from the gene expression data, adipose tissue protein relationships differed with infection status. Uninfected animals expressed several more proteins which were inversely correlated with each other in comparison with SIV-infected and SIV+ART groups. These relationships were consistent with the gene expression data, including IL-18 negatively correlating with the expression of several cytokines. Interestingly, IL-18 showed only positive associations between cytokines and chemokines in the SIV+ART group, and not in the SIV-uninfected or SIV-infected groups. Additionally, IL-12 and IL-13 negatively correlated with multiple
Figure 23. Correlation plots of adipose tissue proteins from subcutaneous adipose tissue from rhesus macaques. Negative correlations are denoted in red, and positive correlations are denoted in blue. The size of the circles indicates the strength of the correlation. Samples were from groups of animals as follows: A. SIV-uninfected without hierarchical clustering. B. SIV-uninfected with hierarchical clustering. C. SIV-infected without hierarchical clustering. D. SIV-infected with hierarchical clustering. E. SIV+ART without hierarchical clustering. F. SIV+ART with hierarchical clustering.
cytokines and chemokines, a pattern which was either not present (SIV-infected group) or reversed (SIV+ART group). In general, the transition from SIV-uninfected to SIV-infected only and then to SIV+ART status constituted a continually increasing concentration of inflammatory proteins which correlated positively with each other.

**Relationship between adipose tissue inflammation and circulating inflammation**

Having established the existence of adipose tissue inflammation in rhesus macaques with SIV+ART using both gene and protein analysis, we wanted to investigate whether this inflammation in the tissue was contributing to that found in the circulating inflammation. We first analyzed in the plasma the same 30 proteins that we investigated

![Figure 24. Protein expression of inflammatory mediators from plasma from rhesus macaques.](image)

The same volumes of subcutaneous adipose tissue used in the QuantiGene Plex assay for measuring gene expression were also applied here for protein analysis using the ProCarta Plex System. Box plots show the interquartile ranges and medians.
in the adipose tissue, and found that overall, there was a trend towards the elevated expression of numerous inflammatory cytokines and chemokines during SIV infection (Figure 24). Several of these were increased or remained high after ART, including IL-6, IL-18, and IL-23. Notably, robust expression of sCD40L which was seen in the adipose tissue from all groups was not correspondingly elevated in the plasma.

We then closely analyzed the initial subset of six inflammatory cytokines and chemokines that were analyzed in the adipose tissue, which are prognostic of disease in individuals with HIV±ART (Figure 25). In contrast to the adipose tissue, the mean levels of IL-1b, IL-1RA, IL-6, and IL-18 were not increased in plasma after SIV infection, although all except for CCL2 were higher after SIV+ART. CCL2 was nearly significantly increased in SIV+ART compared to SIV-uninfected (+904%, p=.057). Additionally, IL-1RA showed a significant increase in expression in plasma of SIV+ART animals compared to that in SIV-uninfected (+130%, p=.028) (Table 8).
After analyzing the remaining proteins, no additional factors approached

**Figure 25.** Protein expression of selected inflammatory mediators in plasma from rhesus macaques. Box and whiskers plots show the minimum to maximum points and detail the interquartile ranges and medians. P-values <.05 are denoted with an asterisk.
significance in the SIV-infected group as compared to SIV-uninfected, and only GM-CSF
approached significance in the SIV+ART group compared to SIV-negative group
(±118%, p=.088). Considering that low-grade chronic inflammation in the plasma is
defined by very small increases in proteins, this result was expected given our sample
Table 8. Inflammatory cytokines and chemokines in plasma before and after SIV, with and without ART in rhesus macaques.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>SIV- Mean (pg/ml)</th>
<th>SD</th>
<th>SIV+ Mean (pg/ml)</th>
<th>SD</th>
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P-values were calculated using the means of each group and in reference to SIV-uninfected animals. Percent change was calculated using the means of each group and in reference to SIV-uninfected animals.

P-values <0.1 are highlighted in light blue, and those <0.05 are highlighted in dark blue. Percent change >100% is highlighted in coral.

In spite of the relatively small changes in inflammatory mediators in the plasma from size.
our samples, there was a consistent trend towards the increase of several cytokines and chemokines after SIV±ART in both adipose tissue and plasma. We therefore compared levels of gene and protein expression in the adipose tissue with the expression of proteins in plasma to
determine whether inflammation from the adipose tissue was contributing to that in the circulation. Unexpectedly, there were no significant correlations between the expression of adipose tissue genes or proteins and plasma protein levels after considering all of the animals.

Figure 26. Relationship between subcutaneous adipose tissue and plasma inflammatory mediators in rhesus macaques.

A. Plasma protein expression compared to subcutaneous adipose tissue gene expression and B. Plasma protein expression compared to subcutaneous adipose tissue protein expression.
together and separated by group. In fact, the only cytokine which even moderately related with plasma proteins was IL-1RA (p=.227), and the remainder failed to show any statistically significant relationships between plasma inflammatory factors and those in the adipose.

**Discussion**

Subcutaneous adipose tissue was identified as an infection and reservoir site for HIV as recently as 2015. Only about fifteen years earlier was adipose tissue acknowledged to function as an endocrine and metabolic organ, but also as an immunological organ. The finding that adipose tissue becomes inflamed during obesity by Weisberg, et al., 2003, set into motion the investigation of adipose tissue serving multi-functional roles related to metabolic injury and overall systemic inflammation. The presence of numerous immune cell types, including T cells and macrophages, supports the fact that adipose can be infected by the CD4-tropic HIV virus, a receptor expressed on the surface of both T cells and, occasionally, macrophages. Regardless of whether they become infected, CD4+ T cells and macrophages play pivotal roles in immune defense and are likely to encounter or be affected by viral particles in relation to other cells which are actively fighting the infection. It stands to reason that the immune cell-rich environment of the adipose tissue could therefore change in response to HIV infection.

Studies in human adipose tissue samples are often limited to examining those individuals who experience lipodystrophy, which is the atrophy of the subcutaneous fat and hypertrophy of fat behind the neck. Further, these studies focused on the effects on
adipocytes themselves, in particular mitochondrial function and oxygen levels of the environment. Human adipose tissue studies have also been confounded by not always knowing the length of infection prior to ART initiation, a wide variety of drugs combined with the ART treatments which differ by type and dose, compliance, and difficulty obtaining samples from ‘healthy’ control subjects, especially for matching age, gender, and risk behaviors often observed in HIV-infected individuals. Thus, studies using rhesus macaques may be better suited for controlling against confounding risk factors. Our previous set of results indicated that the subcutaneous adipose tissue from rhesus macaques is immunologically representative of that from humans, and thus comprises an ideal model with which to study the effects of viral infection on adipose tissue.

In this study, we showed that the adipose tissue becomes inflamed during SIV infection after measuring numerous inflammatory genes and proteins from homogenized parallel adipose tissue samples. This inflammation was defined by the increased mean expression of genes and proteins from inflammatory cytokines and chemokines, including IL-1β, IL-6, TNFα, IL-1RA, IL-18, and CCL2, several of which are known to be increased in the plasma of HIV-infected individuals. Of note, however, only a few pro-inflammatory cytokines and chemokines were statistically significantly increased after viral infection. This was a somewhat expected result, considering that individuals with HIV and HIV+ART normally exhibit chronic inflammation and LGCI, respectively, in the plasma of infected individuals. Correspondingly, these low levels of inflammation are only statistically significant when utilizing very large sample sizes, as required in human studies. Still, the percent change in expression of a large breadth of cytokines and
chemokines did increase substantially in both SIV-infected and SIV+ART groups compared to SIV-uninfected animals.

We were surprised to find that adipose tissue from animals in the SIV+ART group exhibited higher levels of inflammation considering that they did not show any signs of lipodystrophy and had undetectable viral loads in both the plasma and the adipose. In addition, the fact that our monkeys experienced metabolic inflammation after <12 months on ART suggested that the onset of ART-related inflammation occurs much more quickly than has been previously regarded. This could demonstrate that more overt signs (e.g. lipodystrophy) may only be apparent after a longer progression of metabolic disturbances, or could indicate species differences. Interestingly, our SIV+ART monkeys were not receiving protease inhibitors as part of their treatment regimen, which are commonly associated with metabolic changes. This data suggests that reverse transcriptase inhibitors and/or integrase inhibitors may cause metabolic inflammation in the adipose via a pathway that is not currently understood. However, this cannot be confirmed without an analysis of an ART-only group of rhesus macaques.

We expected that evaluating the large groups of adipose tissue genes and proteins together and utilizing an unsupervised clustering technique would confer a robust separation between groups. Instead, however, we found that while the groups did separate, their confidence intervals remained overlapping. When comparing the clusters, the group of 40 genes and the subset of 6 proteins clustered better than the corresponding subset of 6 genes and 30 proteins that are closely related to inflammation. This was not expected, and could be explained by the distinct mechanisms which regulate post-transcriptional and post-translational regulations and modifications. Further testing of
different combinations of genes and proteins in concert may confer better separation than
the subset used here, which was chosen based on the frequent association of these genes
and proteins with inflammation in the plasma of HIV-infected individuals.

The difference in gene and protein expression suggests that while the adipose
undergoes large changes on the entire transcriptional level in response to viral infection
and/or medication, it is only certain proteins that remain stably elevated. Furthermore,
mRNA transcripts are exclusively localized to the cell which produces them. Conversely,
proteins are initially localized to the cell where they are created, but then can quickly be
secreted to move away from the cell and into the bloodstream, where they would not be
as easily detected by assays examining the tissue protein levels. Both genes transcripts
and proteins are susceptible to rapid degradation, and the rate of degradation for each
individual gene mRNA and protein could not be quantified within the scope of this study.
Proteins are also engaged in binding to cell receptors for initiating signaling processes,
and thus may be undetectable in tissue lysates (i.e. containing only soluble proteins).
Regardless, we expect the cell receptor binding or degradation rates to be biased in the
same direction between groups, allowing us to compare between groups the genes and
proteins which do remain detectable.

When analyzing in combination genes mRNA and proteins levels that were
expressed at slightly higher levels of many cytokines and chemokines in SIV±ART
compared to SIV-uninfected, these differences were too small to result in clearly distinct
groupings or associations. However, the groupings still sufficiently distinct that it was
clear that the tissues were undergoing a generalized inflammatory process which varied
in magnitude by each individual monkey and was broadly similar between groups. Due to
these different states of inflammation corresponding with SIV±ART, we analyzed the relationships between the genes and proteins to each other to try to elucidate mechanistic pathways and a potential target for intervention. This was investigated to determine whether different pathways were occurring during steady-state, SIV-infection alone, and during SIV+ART. Pathways were more similar between SIV-uninfected and SIV+ART animals when compared to SIV infected animals without treatment. Results from the SIV+ART group could be distinguished primarily by the increased expression of genes and proteins involved in these pathways. This suggests that there is probably some degree of inflammatory processes underway in ‘healthy’ animals which may be normal or homeostatic, but that these processes are exacerbated in the SIV+ART animals. Thus, a target for intervention during SIV+ART may require reduction of the inflammatory processes to a level closer to ‘normal’. On the other hand, the relationships between the transcripts and protein levels in adipose tissue from SIV-infected animals differed from each other, suggesting that active viremia exerted a strong and distinct influence on the expression of the inflammatory factors and their relationships to each other.

We hypothesized that adipose tissue would become inflamed after SIV infection, and that this inflammation would contribute to the inflammation exhibited in the circulation and serve as a biomarker of disease pathogenesis (e.g. in adipose). It was surprising that there were little to no relationships between the inflammatory factors in the adipose tissue locally compared to those in the circulating plasma, regardless of infection/disease status. There could be multiple explanations for this observation. Adipose tissue inflammation could be highly localized and not require leukocytes from the bloodstream to enter because it is already replete with innate immune cells which can
respond to injury. Additionally, as new adipocytes form near blood vessels, older adipocytes grow farther and farther away from a blood source, rendering the tissue hypoxic but also making it more difficult for inflammatory cytokines to travel into the bloodstream and reach the circulation. Alternatively, expression of some of the cytokines and chemokines were expressed at levels below the limit of detection in our assays, particularly in the plasma. Therefore, our methods may not have been sufficiently sensitive enough to detect a correlation. Finally, it is possible that the tissue environment of adipose tissue is not reflective of that in the circulation.

This is the first study focusing on the inflammatory environment of subcutaneous adipose tissue in rhesus macaques after SIV infection, with and without ART. We showed that adipose tissue becomes inflamed, but that this inflammation occurs at a lesser magnitude than that typically seen during acute infections. The relationships between inflammatory factors differed in the group of animals which received SIV+ART, so targeted intervention should be different depending on infection status. Additionally, adipose tissue inflammation did not correspond with circulating inflammation, suggesting that adipose tissue inflammation is highly localized and that circulating inflammation may be derived from a different stimulus source. Future studies are warranted to tease apart these differences in inflammatory stimuli to identify specific targets for intervention.
Chapter 7

CONTRIBUTION OF ADIPOSE TISSUE MACROPHAGE INFLAMMATION TO CIRCULATING INFLAMMATION AFTER SIV INFECTION, WITH AND WITHOUT ART

Introduction

Subcutaneous adipose tissue becomes inflamed in rhesus macaques with SIV+ART. This fat depot is comprised of numerous immune cell subsets, including macrophages, which are known to be key mediators of inflammatory processes. The contribution of adipose tissue macrophages to the inflammation found in the adipose tissue and in the circulation is investigated here.

Subcutaneous Immune Cell Populations after SIV±ART

Previous reports indicate that the percent of macrophages in the subcutaneous adipose tissue increases as a proportion of CD45+ immune cells after SIV infection, and that these macrophages lose expression of CD163 and CD206 to become pro-inflammatory. Whether the same is true in animals infected with SIV and administered ART is currently unknown. To examine this, we isolated the stromal vascular fraction (all cells except adipocytes) from the adipose tissue of SIV-uninfected, SIV-infected, and
SIV+ART rhesus macaques and used flow cytometry to determine the proportion of macrophages, T cells, and B cells out of all CD45+ leukocytes (Figures 27A-B and 28A-

Figure 27. Gating strategy for analyzing major immune cell populations in the stromal vascular fraction of rhesus macaque subcutaneous adipose tissue. A. Gating of CD45+ leukocytes from singlets. B. Gating of B cells, CD14+ macrophages, CD3+ T cells, followed by phenotypic analysis of CD14+ macrophages by CD163 and CD206, and CD3+ T cells by CD4 and CD8.

C).

Consistent with previous findings, the percent of CD14+ macrophages significantly increased after SIV infection, but were not different when comparing stromal vascular fraction from SIV-uninfected animals and from the SIV+ART group.
(Figure 28A). The percent of CD3+ T cells decreased after SIV infection, and returned to close to baseline levels after ART. CD20+ B cells comprised a very low percent of the CD45+ leukocyte population, and did not change after SIV±ART. Of the CD3+ T cells, the percent of CD8+ T cells was higher as a fraction of CD3+ T cells after SIV infection compared to SIV-uninfected, but was unchanged after SIV+ART (Figure 28B),
suggesting that the drop in total CD3+ T cell percentage could be due to a depletion of CD4+ T cells in the adipose after SIV infection rather than from an increase of CD14+ macrophages during SIV infection. In contrast to previous reports, the phenotype of CD14+ macrophages in terms of CD163 and CD206 expression did not change.

Figure 28. Immune cell populations in subcutaneous adipose tissue of rhesus macaques. A. Percent of CD14+ macrophages, CD3+ T cells, and CD20+ B cells out of CD45+ leukocytes. B. Percent of CD8+ T cells out of CD3+ T cells. C. Expression of CD163 and CD206 out of CD14+ macrophages.
Subcutaneous Adipose Tissue Macrophage Function after SIV±ART

The flow cytometry data suggested that macrophages may infiltrate the tissue during SIV infection, but the increased percentage could also reflect a depletion of CD4+ T cells, among other possibilities. Additionally, there was no evidence of macrophage infiltration during SIV+ART, and macrophages did not lose their ‘anti-inflammatory’ phenotype after infection. However, our previous data indicated that the tissue does undergo inflammatory processes during SIV±ART. To determine whether functional changes in the macrophages, rather than an infiltration or phenotype change, contribute to a pro-inflammatory environment, we investigated the function and pro-inflammatory potential of ATMs after SIV±ART.

One of the most important roles of macrophages is to phagocytose pathogens and debris, and several reports indicate that phagocytosis is inhibited in macrophages from individuals with HIV. To investigate whether macrophage phagocytosis ability was compromised or changed after SIV infection, we cultured ATMs from SIV- uninfected and SIV±ART rhesus macaques for 45 minutes and compared their capacity to ingest fluorescent beads that were 300 nm in diameter (Figure 29A-C). The number of fluorescent beads ingested by live CD14+ macrophages corresponded with the number of peaks in the CD14+ population (Figure 29B). Phagocytic macrophages were phenotyped for expression of CD163 and CD206, and back gated to determine their forward- and side-scatter and confirm CD14+ positivity (Figure 29C). The percent of macrophages which ingested any number of beads out of all CD14+ macrophages did not differ between groups (Figure 30A) and was approximately 43% of all macrophages regardless...
of the group. Furthermore, the phagocytic index (PI), or the average number of beads
ingested per macrophage, was also equivalent between groups, with a PI of 2.2 beads per
macrophage for the SIV-uninfected and SIV+ART groups, and 2.4 beads per macrophage

Figure 29. Rhesus macaque adipose macrophage gating strategy for detecting phagocytosis of fluorescent beads ex vivo. A. CD45+ leukocytes were gated on singlets, and then identified using CD14+ followed by live/dead discrimination. B. FITC-positive fluorescent peaks corresponded with the number of beads ingested. C. Phenotyping and back gating of CD14+ macrophages that ingested beads.
in the SIV-infected group (Figure 30B). Of all CD14+ macrophages which ingested

Figure 30. Phagocytosis ability by rhesus macaque subcutaneous ATMs ex vivo. A. Percent of CD14+ macrophages which ingested any number of beads. B. Average number of beads ingested per CD14+ macrophage (phagocytic index). C. Percent of macrophages which ingested 1, 2, 3, 4, or 5+ beads out of all macrophages which ingested beads. D. Phenotype of macrophages which ingested beads. E. Percent of CD14+CD163+CD206+ macrophages which ingested any number of beads. F. Average number of beads ingested per CD14+CD163+CD206+ macrophage (phagocytic index).
beads, the same percent of macrophages in all three groups ingested 1, 2, 3, 4, or 5+ beads (Figure 30C). The phenotype of phagocytic macrophages was also the same between groups, and >95% of phagocytic macrophages were CD163+CD206+. (Figure 30D). We then gated exclusively on CD14+CD163+CD206+ to measure whether phagocytosis in this particular group of cells was different between groups of macrophages, and both the percent of phagocytic cells and the phagocytic index did not change between groups (Figure 30E-F).

The ability to phagocytose fluorescent particles did not differ between macrophages from the three groups of monkeys and could not explain the differences in inflammation we previously found between groups. However, macrophages have several other functions which could alter after infection and promote a pro-inflammatory environment. Macrophages secrete several cytokines and chemokines that recruit other cell types to the tissue and communicate with responding cells to exert specific functions during cell death, injury, or pathogen invasion. Many cytokines and chemokines are secreted at steady state without requiring any outside stimulation. To investigate whether ATMs became ‘pro-inflammatory’ and constitutively secrete higher levels of cytokines and chemokines, we cultured the adipose tissue stromal vascular fraction overnight (16 hours) and analyzed their supernatants for 29 cytokines and chemokines related to inflammation in general, followed by a detailed analysis of the cytokines secreted predominantly by macrophages.

Overall, the levels of inflammatory cytokines and chemokines were higher in cultures from the SIV-uninfected and SIV-infected groups compared to cells from the SIV+ART animals, which had a significantly lower expression of several cytokines
(Figure 31, Table 9). There was a high amount of variation between individuals in the SIV-infected group, where approximately half were highly expressive of many inflammatory factors and the other half had very low levels of expression, akin to the SIV+ART group. When looking at cytokines and chemokines predominantly secreted by macrophages, a consistent trend was observed. Constitutive secretion of macrophage-derived factors tended to be observed at a similar level or slightly lower level in SIV-infected macaques compared to SIV-uninfected macaques, however, the expression was
consistently reduced in SIV+ART group (Figure 32).

The constitutive expression of macrophage-derived inflammatory cytokines and chemokines secreted constitutively in culture supernatants from adipose tissue stromal vascular fractions.

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<th>SIV+</th>
<th>SIV+ART</th>
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<th>% Change</th>
<th>Mean</th>
<th>SD</th>
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p-values <0.1 are highlighted in light blue.
p-values <.05 are highlighted in dark blue.
Percent change is relative to SIV-uninfected. Positive percent change is colored in red. Negative percent change is colored in blue.
chemokines was slightly decreased after SIV infection, but this was exacerbated in combination with ART. However, the degree of response to exogenous stimuli could also differ between groups, such that macrophages from SIV-infected animals are either more or less “inflammatory” after being exposed to a macrophage-stimulating molecule. To investigate their responsiveness, we cultured the adipose tissue stromal vascular fractions overnight with lipopolysaccharide (LPS), a bacterial-derived antigen which specifically stimulates macrophages by interacting with Toll-Like Receptor 4 on their cell surface. We then analyzed the secretion of cytokines and chemokines predominantly derived from
macrophages.

Interestingly, supernatant analysis of the stromal vascular fraction from all groups of macaques showed that these cells could respond to LPS stimulation, and the magnitude of response was similar between groups (Table 10). Likewise, the percent change of

![Figure 32](image.png)

Expression of selected macrophage-derived cytokines and chemokines in supernatants of cultures of stromal vascular fraction from subcutaneous adipose tissue of rhesus macaques in response to LPS stimulation. Stromal vascular fractions were cultured overnight and supernatants and proteins from supernatants were analyzed using the ProCartaPlex System (ThermoFischer). SIV-uninfected is shown in red, SIV+ is shown in green, and SIV+ART animals is shown in blue. P-values.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Mean ± SD</th>
<th>p-value</th>
<th>% Change</th>
<th>Mean ± SD</th>
<th>p-value</th>
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<th>p-value</th>
<th>% Change</th>
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<td>0.66 ± 0.53</td>
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<td>911.17 ± 1045.36</td>
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p-values <.01 are highlighted in blue
p values <.05 are highlighted in dark blue

Percent change is calculated relative to baseline levels within groups. Positive percent change is colored in red. Negative percent change is colored in blue.
primarily macrophage-derived cytokines and chemokines was also similar between groups (Figure 33).

*Relationship between adipose tissue macrophage inflammation and circulating inflammation*
Our analysis of the cytokines and chemokines derived from the stromal vascular fraction and macrophages specifically demonstrated decreased constitutive expression of macrophage-derived inflammatory factors after SIV infection, particularly in monkeys on long-term ART. However, macrophages responded equally to LPS stimulation between

Figure 33. Percent change in selected cytokine secretion in adipose tissue stromal vascular fraction cell culture supernatants after LPS stimulation ex vivo. of selected macrophage-derived cytokines and chemokines after LPS stimulation. Cytokine secretion from cells of SIV-uninfected is shown in red, SIV-infected animals is shown in green, and SIV+ART animals is shown in blue.
groups. We therefore investigated whether the diminished constitutive expression of several pro-inflammatory cytokines and chemokines from the stromal vascular fraction correlated with the levels of these factors in the circulation, because a decreased basal expression of cytokines and chemokines could demand an increase of these same factors from the circulation for the tissue to function optimally. However, we were unable to detect any relationship between supernatant cytokines and those in the plasma, including
those which were predominantly derived from macrophages (Figure 34).

**Discussion**

Inflammatory cytokines and chemokines are elevated in the plasma of HIV±ART individuals, and this is considered prognostic of accelerated aging, morbidity, and mortality. Our previous results confirmed that plasma from rhesus macaques infected

![Figure 34. Scatterplots relating plasma cytokines and chemokines with those constitutively expressed from the stromal vascular fraction culture supernatant from subcutaneous adipose tissue of rhesus macaques.](image)

SIV-uninfected is shown in red, SIV-infected animals are shown in green, and SIV+ART animals are shown in blue.
with SIV±ART also show increases in inflammatory factors. We also demonstrated that
the subcutaneous adipose tissue becomes inflamed during SIV infection and remains
inflamed, but to somewhat lesser levels, after ART. Adipose tissue is replete with
immune cells, especially macrophages, which are key mediators of inflammation.
Macrophages in other tissues, such as the microglia in the brain and alveolar
macrophages in the lung are susceptible to HIV/SIV infection, but may also be indirectly
affected by several mechanisms, such as a change in cell-to-cell function with other cells
that are infected (*i.e.* CD4+ T cells), by a shift in the environmental milieu of cytokines
due to active viremia, or by the recognition and processing of circulating viral particles
themselves. During ART, macrophages could also experience negative effects from the
drugs alone. We therefore investigated whether inflammation derived from ATMs
contributed to inflammation in the circulation, which would provide a target for
intervention and reduce the negative side effects of long-term ART.

In our study, we confirmed other reports that the proportion of CD14+
macrophages out of CD45+ leukocytes in the adipose tissue increases after infection. We
also found that this proportion did not increase after ART. However, flow cytometry data
must be interpreted very carefully: an increase of one subset of cells could mean an
increase in absolute number, or it could reflect a depletion of a different cell type. In
addition, flow cytometry data incorporates a much greater volume of tissue/cells
compared to other methods such as confocal microscopy. Tissue-digested cell lysates
evaluated by flow cytometry also contains numerous blood vessels, allowing cells from
the blood to contaminate the samples and prevent analysis of tissue cells exclusive of
blood cells. With this in mind, the proportional increase of CD14+ cells out of CD45+
leukocytes found during SIV infection does not necessarily reflect a true infiltration of macrophages into the tissue, especially because there was a corresponding decreased ratio of CD3+ T cells, of which CD8+ T cells increased in percent out of CD3+ T cells. This suggests either an infiltration of CD8+ T cells and/or a depletion of CD4+ T cells, as is known to happen during SIV infection, and a depletion of CD4+ T cells would result in false interpretation about the ‘infiltration’ of CD14+ macrophages. The flow cytometry data also did not correspond with data from our previous study regarding the proportion of macrophages, T cells, and B cells in relation to each other. In this study using flow cytometry analysis on tissue digests, macrophages represented about 45% of these immune cells in SIV-uninfected animals, and T cells represented 35%, whereas confocal data suggested that nearly 80% of these cells are macrophages and 13% are T cells. We also found here that approximately 38% of CD14+ macrophages in the adipose tissue expressed CD163 and CD206, whereas the confocal data suggests that over 80% of macrophages express CD163 and CD206. This discrepancy also could be due to blood contamination in the flow cytometry studies. The second largest subset of macrophages we found were CD163+CD206- (34%), which could correspond with monocyte contamination from the blood because macrophages express both CD163 and CD14, but not CD206. In spite of this, it was notable that we found no phenotypic changes in the macrophage subsets between groups and thus no indication that they become ‘pro-inflammatory’ by losing expression of CD163 and CD206, assuming that the blood contamination bias is equivalent between groups.

To investigate the function of ATMs after SIV infection, we measured their capacity to phagocytose fluorescent particles, their constitutive expression of several pro-
and anti-inflammatory cytokines, and their ability to respond to a TLR-4 agonist (LPS). In organs like the lung, impairment of phagocytosis during HIV is thought to reduce the ability for macrophages to respond adequately to opportunistic pathogens, thus being an important part of disease pathogenesis. In adipose tissue, an inhibition of phagocytosis by HIV could reduce the ability for macrophages to clear viral particles from the tissue, allowing the virus to infect the macrophages or surrounding T cells. Additionally, inhibition of phagocytosis could impair the clearance of dead or dying adipocytes, delaying the resolution process and creating an environment of long-term metabolic damage.

In our study, no loss of phagocytic function was detectable among ATMs in SIV+ART groups compared to SIV-uninfected animals. The percent of macrophages which ingested beads, in addition to the phagocytic index, was the same between groups. Additionally, the clear majority of phagocytic macrophages co-expressed CD163 and CD206, suggesting that these were, indeed, ATMs and not circulating monocytes. When gating on these cells alone using flow cytometry, the phagocytic ability, again, did not change. In this study, we only tested phagocytosis using fluorescent beads that were 300 nm in diameter. The size chosen for this assay was based upon studies finding that particles approximately 200-300 nm in diameter were the most readily phagocytosed by macrophages. However, the material, shape, size, and charge of the particles all affect phagocytosis, and our study does not preclude that phagocytosis may still be impaired under different conditions.

An alternative explanation for the inflammatory environment of the adipose tissue is that macrophages become polarized to exhibit pro-inflammatory functions after
infection. If macrophages were to become pro-inflammatory, we would expect to find an increased number of macrophages and/or reduced expression of the anti-inflammatory surface markers CD163 and CD206. However, we could not conclusively show that macrophages were infiltrating after SIV±ART, and levels of CD163 and CD206 did not differ between groups in our study. After measuring the constitutive expression of secreted cytokines from macrophages cultured overnight, we were surprised to find a trend towards decreased expression of macrophage-specific pro-inflammatory cytokines and chemokines after SIV infection, and this expression was even lower in the cultures from SIV+ART animals. Intriguingly, these macrophages retained the ability to respond normally to LPS stimulation.

One interpretation is that ATMs do not change in number during SIV±ART, but are affected by SIV and/or ART in such a way that they constitutively express lower quantities of ‘steady-state’ cytokines. For example, a defective macrophage could receive a signal to produce and secrete the particular protein, but in the wrong amounts. In this scenario, exposure to an exogenous stimulant such as LPS would result in an appropriate increase of magnitude relative to the original level of secretion, but it would still be at levels considerably lower than normal. Thus, a defect in the ‘inner machinery’ could render the cells incapable of producing normal levels of these factors. Alternatively, our data could miss a depletion of ATMs after infection because of a change in other cell types. For example, a decrease in numbers of both T cells and macrophages at the same time would show no change in the proportion of either of these cell types, because the denominator changed. A third explanation is that the absolute number of macrophages are unchanged, but that the types of macrophages within our population is changing, and
this change isn’t reflected by a differential expression of CD14, CD163, or CD206. A polarization or replacement of the normal macrophages for a more ‘anti-inflammatory’ subset could explain the reduced capacity for macrophages to secrete inflammatory cytokines after SIV±ART.

These data do not explain the increase of inflammation in the adipose tissue environment found in our previous study. Instead, our data suggests that ATMs exhibit a reduction of constitutive expression of inflammatory mediators, and that this lack of expression may be modifying the overall tissue environment to its detriment. Additionally, our data suggests that inflammation during SIV±ART may be derived from a different cellular source in the adipose tissue, such as adipocytes, which are excluded from the stromal vascular fraction that was tested here. In this scenario, macrophages which constitutively secrete fewer cytokines may not provide the signals necessary for optimal adipocyte function, resulting in hypersecretion from adipocytes of inflammatory factors as a compensatory mechanism.

Constitutive expression of inflammatory cytokines from the stromal vascular fraction, and specifically those derived from macrophages, did not show any relationship (either positive or negative) with the circulating inflammation. Therefore, although adipose tissue inflammation likely underscores a metabolic impairment in the individual, this is likely not the source of circulating inflammation found in patients with HIV±ART, and further research to define this source is still needed. In spite of this, the metabolic inflammation found in our previous study in conjunction with the reduced expression of inflammatory cytokines from ATMs underscores a need to understand the metabolic impact of HIV and ART on individuals, even when lipodystrophy is not present, as is the
case with our animals. Adipose tissue is the primary storage unit of energy, and also mobilizes energy when supplies are scarce. It also is intimately involved with insulin sensitivity, in addition to playing a role in blood pressure regulation. During obesity, metabolic inflammation corresponds with accelerated aging and the development of several chronic diseases in which the mechanism is still poorly understood, including cardiovascular disease, renal disease, and neurodegenerative diseases. Thus, although the effects of metabolic inflammation may not be reflected in the circulation, the impact of the inflamed adipose tissue during HIV on the system as a whole remains a critical point for investigation.
Chapter 8

DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

Inflammation is a severe consequence of HIV/AIDS with and without treatment, and is an indicator of poor prognosis, including accelerated aging, morbidity, and mortality. Adipose tissue is an important source of circulating inflammation in people with obesity and is also a key risk factor for development of disease in this group of people. Defining whether inflammation is derived from adipose tissue during HIV could elucidate a target for intervention, particularly in people with HIV+ART who are undergoing accelerated aging. Therefore, adipose tissue was investigated as a potential source of circulating inflammation in rhesus macaques with SIV+ART.

In Aim 1, we validated subcutaneous adipose tissue from rhesus macaques as being immunologically similar to subcutaneous adipose tissue from humans. The numbers of macrophages, T cells, and B cells per unit area was similar between our monkeys compared to humans, with macrophages being the most predominant subset, followed by T cells and then B cells. Furthermore, the phenotype of ATMs represented that from humans and was predominantly 'anti-inflammatory' as shown by the co-expression of CD163 and CD206. This was true regardless of where in the tissue the cells were located, except for those found in crown-like structures, which were largely negative for both CD163 and CD206. Using a cell tracing technique, we also found that
ATMs were mostly long-lived, survived in the tissue for over a month, and were not actively being replaced by circulating monocytes. There was scant evidence of self-renewal of the macrophages in our samples using expression of Ki67, confirming the low turnover of these cells in the tissue.

The validation of rhesus macaque adipose tissue in comparison with humans is essential for future research regarding obesity, type 2 diabetes, and infectious diseases which may affect the adipose tissue. Our data provides strong evidence that rhesus macaques can and should be more often used to study chronic diseases, because NHP are more phylogenetically similar to humans compared to other model organisms like mice, and research is more translatable. Additionally, our evidence suggests that ATMs are long-lived, and presents implications that these cells, like other immune system cells, may be vulnerable to exhaustion or senescence over time. This exhaustion or senescence could have a wide impact among many tissues, because macrophages are essential for both infection control as well as normal tissue homeostasis. Thus, processes that provide constant stimulation over time (i.e. obesity) or normal aging might impact long-lived macrophages such that their functions are severely compromised. Additionally, long-lived macrophages could be susceptible to viruses and bacteria (i.e. HIV, TB) that change the function of the cells without killing them, thus altering the normal tissue environment and being a potential reservoir for continual viral/bacterial production.

In **Aim 2**, we confirmed that adipose tissue becomes inflamed after SIV+ART, but that this inflammation does not correspond with circulating inflammation. The magnitude of the inflammation varied widely between individual animals, especially in the SIV+ART-naïve group, suggesting that different stages of viral progression could
have different severities of metabolic consequences. The monkeys which exhibited the strong levels of inflammation varied by both virus strain and sex, suggesting that host factors may play a role in these differences. Additionally, although inflammatory factors were increased broadly during SIV±ART, most were not statistically significantly different, underscoring inflammation probably being akin to 'low-grade chronic inflammation' that requires much larger sample sizes to detect statistically significant differences. Regardless, the generalized increase of inflammatory factors suggested a biologically significant alteration in the tissue. An interesting feature of our study is that none of our SIV+ART rhesus macaques had observable lipodystrophy, which may be because of species differences, lipodystrophy could require a longer amount of time to develop, or the ART regimen in rhesus macaques with SIV did not include protease inhibitors. It is possible that by the time lipodystrophy is noticed in humans, the metabolic inflammation has become so severe that it is better reflected in the circulation, as well. Importantly, the fact that we identified inflammation in the subcutaneous adipose tissue after eight months of infection and nine months of ART suggests that the onset of metabolic inflammation occurs rapidly after initiating ART and may worsen over time.

The fact that adipose tissue inflammation did not correspond with circulating inflammation suggests that even though ART suppresses viremia and extends the lifespan of those infected with HIV, it may not adequately address pathogenesis or disease occurring in adipose tissue and in other tissues as well. This pathogenesis could be due to persistent viremia or an effect from the drug itself and should be studied using ART-only NHP models to determine the extent of drug-related disease in multiple organ systems. In addition, the inflammation seen in the circulation could be derived from a different tissue,
such as the lymph node, but it also may represent bits of inflammatory processes occurring in multiple tissues at the same time, thus rendering it not a particularly useful indicator of how to intervene, but rather a general measure of the health of the system as a whole. However, our results from adipose tissue biopsies could be an extremely useful gauge of the metabolic status of an individual rather than a reflection of the system. For example, we achieved some separation between groups by using gene and protein analyses that reflected their increased inflammatory status. Although a subset of six cytokines and chemokines most often utilized in humans could not distinguish the groups, research into the development of a key panel of genes or proteins in adipose tissue biopsy samples that can more clearly distinguish between groups could be extremely useful as an indication of metabolic disease status.

In **Aim 3**, we investigated whether adipose tissue stromal vascular fraction and ATMs in particular were responsible for the inflammatory profile seen in the tissue samples from Aim 2. We found that the ability for macrophages to phagocytose fluorescent beads did not change after SIV-infection, and that impairment in phagocytosis could not explain the inflammatory changes found after SIV. We did, however, find evidence that ATMs after SIV±ART secrete inflammatory factors constitutively at a lower level than our control macaques, and that these macrophages are still able to respond equally (by percent change) to a TLR-4 stimulant (*i.e.* LPS). This suggests that ATMs are changed after infection in such a way that their baseline capacity to secrete molecules is highly diminished, even though they are still able to respond to stimuli. Counterintuitively, this decreased baseline capacity to secrete inflammatory cytokines could, in fact, be a major reason why the adipose as a whole becomes inflamed.
Adipocytes are the primary sources of TNFα in adipose tissue during obesity. They can also secrete several other factors, including IL-6 and IL-10. Macrophages and adipocytes closely associate with each other, and during obesity, this communication is central to inflammation and pathogenesis, whereby adipocytes that are overly hypertrophied secrete excess free fatty acids, which then bind to CD36 on the surface of macrophages that in turn secrete TNFα and act on the adipocytes to expel more free fatty acids. During SIV±ART, this is probably not occurring in the same way because macrophages are not secreting more TNFα compared to macrophages in SIV-uninfected macaques. However, the lack of macrophage secretion of inflammatory factors suggests that there may be a reduction of cell-to-cell communication between macrophages and adipocytes that could incite adipocytes to secrete excess inflammatory cytokines as a compensatory mechanism. In other words, if an adipocyte senses that there is a problem within itself or an outside threat, but the macrophages are not responding accordingly, they may hypersecrete inflammatory factors to induce the macrophages to exert their functions.

It is intriguing that macrophages may become less functional after SIV±ART, which could be explained by the information gathered from Aim 1 demonstrating that macrophages in the adipose tissue are long-lived. These macrophages may be directly or indirectly affected by SIV (through infection or a bystander effect) and over time lose their function because of constant stimulation by the virus. Furthermore, the drugs in the ART regimen may induce damage to adipocytes that requires constant help from macrophages, also 'exhausting' them. Alternatively, direct or indirect effects of the virus could change the macrophages to where they no longer function the way that they
normally should, such as polarizing them towards one phenotype, or by directly infecting
them and altering their capacity to produce inflammatory factors. Regardless of the
mechanism by which the macrophages change their function, our data has important
implications about targets for intervention. For example, if the effects on macrophage
function were reversible, we could treat them by using a drug which ‘restores’ their
potential. Conversely, if the macrophage effects are irreversible, we could target them
specifically for deletion and allow them to be replaced with “fresh” monocytes from the
blood stream, or by the addition of hematopoietic stem cells. A more general solution
could be the addition of anti-inflammatories to the cocktail of ART drugs administered to
individuals.

Our study had several important limitations and confounders. We were unable to
obtain body mass index (BMI) data on our rhesus macaques because the historical
samples that we used did not include the crown-to-rump length, which is required for
calculating the BMI of rhesus macaques. Knowing the BMI of individuals is very
important when studying adipose tissue, because adipose is the primary storage sight of
excess energy and can become inflamed during obesity. Thus, if an animal is obese, it
may be ‘inflamed’ to begin with. Conversely, it is unclear what the inflammatory effects
would be in adipose from an underweight animal. To compensate, we compared weights
of our animals to what their ‘healthy’ weight should be according to their age and gender,
using data from our colony. However, this is an indirect measure, and our macaques may
not have truly been matched for BMI.

Our cohorts were also poorly matched for gender and virus strain. Data in the
literature indicates that adipose tissue exhibits sexual dimorphism, whereby females tend
to gain more weight subcutaneously, and males tend to gain more weight viscerally. This distinction could be a result of sex-specific differences in each compartment that render one depot more resistant to weight gain than the other, and could possibly reflect differences in inflammatory profiles. Additionally, immunity itself is sexually dimorphic, and overall, females tend to exhibit immune responses of higher magnitudes than in males. Thus, although our monkeys did not appear to have differential responses to the virus based on sex, the possibility still exists and could have been missed based on the endpoints we measured.

The virus strains that we used were also often not matched between groups, which probably impacted the reliability of the data and increased the variability. This could lead to a bias in either direction: if a virus which is more pathogenic is highly represented in the SIV-infected group compared to the SIV+ART group, then the inflammation from the SIV-infected group may be much higher than the SIV+ART group simply due to the virus strain and not because of the viral reservoir and/or drug. Conversely, if a less pathogenic virus strain is highly represented in the SIV-infection group compared to the SIV+ART group, then inflammation from SIV+ART would look inflated compared to SIV-infection, making it seem as if SIV+ART was worse compared to SIV-infection than it really was. Thus, it is difficult to truly compare the differences between SIV-infection and SIV+ART, but what remains clear is the fact that in both scenarios, viral infection had a detrimental and inflammatory effect.

A key missing component was confocal microscopy data detailing whether macrophage numbers increase after SIV infection, which would have been highly relevant towards interpreting why the macrophages appeared to be less functional after
SIV±ART. Understanding whether differences were just due to a change in absolute count would have explained the changes very easily. However, the available historical samples were taken so closely to lymph nodes that it was impossible to discern whether the immune cell populations present were an ‘extension’ of the lymph node, or whether the process in the adipose was occurring independently from surrounding organs.

In addition, several of our ‘control’ monkeys were animals from the outdoor-housed colony, whereas all of the animals with SIV infection were on projects and housed inside for a long period of time prior to sampling. This could have strong effects on the status of the immune system because of the reduced exposure to pathogens among the indoor-housed macaques, as well as a change in commensal gut microbiota because of exposure to unique pathogens.

The length of storage of our samples varied widely. Some samples were processed within a few days after collection, and several had been in storage for over three years. This could affect the preservation of the proteins, RNA, and cells. However, all of our samples were stored the same way and also in the most ideal way for each sample type to preserve their use, *i.e.* RNAlater samples were stored at -20°C, samples blocked in paraffin were safely stored at room temperature for years, and our isolated cells were kept in freezing media in liquid nitrogen. Interestingly, two of our oldest samples actually expressed the highest levels of inflammatory factors, which lends the possibility that the results were either accurate or an underestimate of their true inflammation compared to those with a shorter length of storage time. The biggest concern for storage in this study would be proteins in RNAlater. RNAlater is not truly suitable for preservation of proteins in tissue, however, the samples being frozen helped
prevent them from being degraded. Different rates of protein degradation could have played a factor in our results, however, the trends were still clear, in that the groups with SIV±ART expressed overall higher levels of inflammatory factors. Essentially, a lack of detecting a protein doesn’t necessarily mean that it wasn’t expressed; rather, it could have been degraded anywhere in the process. It is thus fairer to compare proteins that were expressed in all groups, rather than make any conclusions about those which were not. A study whereby samples were either processed immediately or fresh frozen would be an ideal follow-up for making stronger conclusions about the expression of inflammatory proteins.

Our study was also limited by sample size in addition to the fact that few cells by weight are recovered from subcutaneous adipose in NHP and humans. Due to its history as an organ of little importance outside of energy storage, adipose tissue is not regularly banked among primate centers. The samples which were available were opportunistically collected from a mix of several studies, and our study could have benefited from a couple of additional animals per group, especially because of the high variability that was observed after SIV infection. Also, adipose tissue yields a lower number of cells per gram compared to other organs, and so it was difficult to run samples in duplicates because of limited tissues and cells. Interestingly, the larger the size of the tissue received (typically from an obese individual), the lower the cellularity, so bigger samples do not always yield more cells. By volume, adipocytes constitute a huge portion of space, so the larger they individuals are, the fewer additional cells are returned after processing for the same unit volume. Therefore, the confidence per sample measurement was not as high as it
would have been had we been able to perform more replicates, and the reliability suffers as a result.

Lastly, the length of SIV infection and ART were both substantially shorter than what is typical for humans, but the pathogenesis of SIV is still considered to be very similar to what occurs in humans. However, it is common for people today to be diagnosed earlier and earlier after infection, and irreversible changes due to the virus alone may be ameliorated if the time to diagnosis is shortened in humans. For example, metabolic inflammation after one year of HIV infection in humans followed by one year of ART may look different than one year of SIV infection in macaques followed by one year of ART. A major difference between ART usage in humans and our population of monkeys is that ART is used for decades in most people before they see side effects, but was only administered for under a year in our macaques, which exhibit noticeable inflammation in the adipose tissue. This could be because the virus used in rhesus is more aggressive and persistence may be more problematic in spite of ART. It could also be that the drugs affect rhesus macaques more strongly than humans. More likely, ART is affecting adipose from humans much more quickly than was previously known, but the lack of signs renders it difficult to diagnose and was not suggestive of undertaking research to investigate it.

The limitations and confounders of this study are numerous, but despite this, the results provided the baseline and experimental data necessary to move the field forward. NHP research is expensive, time-consuming, and ethical constraints limits the type and amount of work that can be done. So, using historical data is imperfect but critical for testing ideas which are not currently described or tried in the literature. Ideally, a
longitudinal study using one strain of SIV, both sexes divided equally between groups, same length of infection and ART, and updated methods for storing tissue for proteins would follow to better understand the exact magnitude of changes to be expected from SIV±ART. Testing a variety of drugs would be instrumental from a clinical perspective.

It is very important to mention that circulating inflammation corresponds with poor outcomes in humans, but we did not test whether metabolic inflammation alone could relate to poor outcomes in rhesus macaques. In follow-up studies, it would be very important to assess whether the inflammation in the adipose tissue has a negative clinical impact. This is because it is possible (though unlikely) that this inflammation is innocuous, and that a different inflammatory process altogether predicts poor outcome during SIV±ART. In other words, although we clearly showed that adipose tissue becomes inflamed after SIV±ART, we did not show that it related to circulating inflammation or poor outcomes, and this is an important next step. Relevant clinical endpoints to analyze in follow-up studies could be examining lipodystrophy (i.e. DEXA scans), dyslipidemia, glucose tolerance, cognitive testing, blood vessel thickness, and measures of kidney function, which may end up being more relevant endpoints than the cytokines especially as they pertain to age-related diseases. Furthermore, measuring the change in adipokine levels such as leptin and adiponectin would be key to any follow-up study, because these interact with the brain and could be consequential for the whole organism.

If it can be sufficiently shown that metabolic inflammation relates with poor outcome using an endpoint other than a relationship to circulating inflammation, then improvement of macrophage function in the adipose tissue should be considered. One
idea would be to use an anti-inflammatory agent (e.g. THC) to reverse macrophage function. A second would be to utilize macrophage depletion (e.g. liposome-encapsulated bisphosphonates) to remove faulty macrophages and replace them with ‘fresh’ monocytes from the blood. If it is the case that adipose tissue inflammation relates to accelerated aging, then treatments such as these could be clinically relevant into updating the care of individuals with HIV+ART. Until a cure is found, studies such as these are vital for helping these individuals extend not only their lifespan, but also their healthspan, and may simultaneously help individuals suffering from obesity as well as natural aging.


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