FUNCTION AND CONTROL OF ADIPOSE TISSUE REGULATORY T CELLS:
IMPLICATIONS FOR OBESITY

by

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Abstract

Obesity is associated with chronic low-grade inflammation in visceral adipose tissue (VAT), which promotes the development of insulin resistance. The role of adaptive immunity in VAT inflammation has only recently been investigated. Initial studies suggest that VAT-resident regulatory T cells (Tregs) have a prominent role in suppressing VAT inflammation and correcting metabolic dysfunction in obese mice. I sought to investigate how Tregs in the VAT are regulated.

Obesity is accompanied by a rise in insulin levels, and whether this hyperinsulinemia affects the progression of inflammation is not known. I first found that Tregs express the insulin receptor, and high levels of insulin inhibited IL-10 production and the ability of Tregs to suppress macrophages. In parallel, Tregs from the VAT of obese mice showed a similar decrease in IL-10 production, suggesting that hyperinsulinemia may contribute to the development of obesity-associated inflammation via an effect of insulin on Treg function.

I then found that the majority of IL-10-expressing Tregs in the VAT expressed the ST2 chain of the IL-33 receptor. The proportion of ST2+ Tregs in VAT was severely diminished in obese mice, and this effect could be completely reversed by treatment with IL-33. IL-33 treatment also reversed VAT inflammation in obese mice, and resulted in a reduction of hyperinsulinemia and insulin resistance. These data suggested that IL-33 is critical for the maintenance of ST2+ Tregs in the VAT, and that delivery of IL-33 may be a new therapeutic approach to reverse obesity-associated Treg deficiency, inflammation and insulin resistance.

It is not known whether Tregs and adipocytes can directly interact in the VAT. I found that soluble factors produced by adipocytes significantly increased survival and IL-10 production
from Tregs, and caused a shift towards oxidative metabolism \textit{in vitro}. Similarly, Tregs resident in mouse VAT have substantially increased expression of IL-10 compared to those found in the periphery, indicating that the interaction between Tregs and adipocytes may contribute to the functional specialization of Tregs in the VAT.

Taken together, these data suggest that insulin, IL-33 and adipocyte-produced factors regulate IL-10-expressing Tregs and their ability to control inflammation in the VAT.
Preface

Chapter 1. A version of the introduction chapter has been published. Han, J. M. and Levings, M. K. 2013. Immune regulation in obesity-associated adipose inflammation. *J Immunol* 191(2): 527-532. Copyright 2015. The American Association of Immunologists, Inc. I did the research for this review article, and Megan Levings and I wrote the manuscript for the published review article.

Chapter 2. A version of chapter 2 has been published. Han, J. M., Patterson, S. J., Speck, M., Ehses, J. A., and Levings, M. K. 2014. Insulin inhibits IL-10-mediated regulatory T cell function: implications for obesity. *J Immunol* 192: 623-629. Copyright 2015. The American Association of Immunologists, Inc. Scott Patterson assisted with experiments to analyze AKT phosphorylation. Madeleine Speck and Jan Ehses assisted with setting up the high fat/sucrose diet model of obesity and performed the experiments measuring the metabolic parameters of the mice. I performed all other experiments. Megan Levings and I designed the project and wrote the manuscript for the published paper.

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performed all the experiments in the chapter with the help of the above co-authors. I designed the study and wrote the manuscript for the published paper with Megan Levings.

Chapter 4. Chapter 4 is a version of a manuscript being prepared or publication. Han, J. M., Wu, D. and Levings, M. K. 2015. 3T3-L1 adipocyte-derived factors increase metabolic activity and IL-10 production of regulatory T cells. Dan Wu performed the Seahorse experiments. I performed all other experiments and wrote the manuscript. I designed the study with Megan Levings.

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<th>Description</th>
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<tbody>
<tr>
<td>2-DG</td>
<td>2-Deoxy-D-Glucose</td>
</tr>
<tr>
<td>AA</td>
<td>Antimycin A</td>
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<tr>
<td>Adipo</td>
<td>Adipocyte</td>
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<tr>
<td>Adipo CM</td>
<td>Adipocyte-Conditioned Media</td>
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<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
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<td>AT</td>
<td>Adipose tissue</td>
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<td>BMDC</td>
<td>Bone Marrow-derived Dendritic Cells</td>
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<td>BMDM</td>
<td>Bone Marrow-Derived Macrophages</td>
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<tr>
<td>CCL</td>
<td>CC Chemokine Ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC Chemokine Receptor</td>
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<tr>
<td>CTLA4</td>
<td>Cytotoxic T-Lymphocyte Associated protein 4</td>
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<tr>
<td>CXCL</td>
<td>CXC Chemokine Ligand</td>
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<td>Dendritic Cells</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immuno Assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent-Activated Cell Sorting</td>
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<td>FCCP</td>
<td>Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone</td>
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<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
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<tr>
<td>Glc</td>
<td>Glucose</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage – Colony Stimulating Factor</td>
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<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid</td>
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<tr>
<td>HFD</td>
<td>High fat/sucrose diet</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T cell COStimulator</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>InsR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-Associated Peptide</td>
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<td>Lamina Propria</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>Mitogen-activated protein kinase kinase 1/2</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>mTOR</td>
<td>mammalian Target of Rapamycin</td>
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<tr>
<td>NCD</td>
<td>Normal chow diet</td>
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<tr>
<td>NKT</td>
<td>Natural Killer T cells</td>
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<td>NLRP3</td>
<td>Nod-Like Receptor, Pyrin domain containing 3</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>Oligo</td>
<td>Oligomycin</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>Rot</td>
<td>Rotenone</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>Tconv</td>
<td>Conventional T cells</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>Th1</td>
<td>T helper 1</td>
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<tr>
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<td>T helper 2</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
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</table>
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Dedication

To my parents
Chapter 1: Introduction

Obesity is a risk factor for developing insulin resistance, defined as the inability of cells such as adipocytes, hepatocytes and myocytes to respond normally to insulin and adequately activate pathways leading to glucose uptake. A commonly held view is that in a subset of obese, insulin-resistant individuals, beta cell dysfunction ensues leading to decreased insulin production, poor blood glucose regulation, and ultimately type 2 diabetes (T2D) (1, 2). It is also possible that beta cell dysfunction arises prior to, or in parallel with, insulin resistance, since in some cases it can be detected well before the onset of T2D (3, 4). Investigation into why obesity is a risk factor for developing insulin resistance is an area of intense research, with increasing evidence for a major role of inflammation. Specifically, the development of excess adipose tissue (AT) is strongly associated with the development of chronic inflammation caused by infiltration of activated immune cells and over-production of pro-inflammatory cytokines. Mechanistically, pro-inflammatory cytokines, such as TNF-α, can cause serine phosphorylation and inactivation of insulin receptor substrate-1 (IRS-1), and hence block insulin receptor (InsR) signaling in multiple cell types, including adipocytes and hepatocytes (Figure 1.1) (5, 6). As a result, PI3K cannot be effectively recruited to the InsR, and its downstream activation of Akt and translocation of GLUT4 to the plasma membrane are abrogated. Interestingly, visceral adiposity is more tightly linked with metabolic abnormalities than subcutaneous adiposity (7, 8), possibly because visceral AT (VAT) is more vulnerable to loss of immune regulation and hence inflammation. For example, mast cell infiltration and a proportional decrease in T regulatory cells (Tregs) are more prominent in visceral than subcutaneous AT of obese individuals (9-11). Whether the initiating trigger is adiposity, inflammation, and/or other factors remains unknown. Here I provide a brief review of recent studies that are beginning to reveal a central role for loss
of immune regulation as a major factor contributing to AT inflammation and obesity-associated pathologies.

**Figure 1.1 TNFα signaling interferes with insulin receptor-mediated PI3K/Akt signaling.**

Ligation of the insulin receptor triggers IRS1-mediated recruitment of PI3K, leading to downstream signaling events, including the activating phosphorylation of Akt at Thr308. To fully be activated, Akt requires a second phosphorylation at Ser473 (12). Akt can then phosphorylate AS160 to induce the translocation of GLUT4 (13), and activate mTORC1 to promote protein synthesis (14). TNFα signaling leads to the activation of JNK and IKK, which can both phosphorylate IRS1 at inhibitory serine residues (5, 6), thereby dampening InsR signaling. Green and red dots represent activating and inhibiting phosphorylation, respectively.

### 1.1 Adipose tissue inflammation is driven by innate and adaptive immune cells

Obesity-associated inflammation has long been attributed to the presence of elevated levels of pro-inflammatory cytokines. Only recently, however, have the cellular sources of these cytokines been investigated in detail, with current evidence pointing to roles for both innate and adaptive
immune cells in obese AT. In terms of innate immune cells, one of the defining features of AT inflammation in obesity is a marked increase in the accumulation of macrophages that surround adipocytes in “crown-like structures” (15, 16). In contrast to the M2 macrophages typically found in lean AT, the macrophages in inflamed adipose tissue are predominantly inflammatory M1 macrophages that produce substantial amounts of pro-inflammatory cytokines such as TNF-α (17). In addition to macrophages, there is growing evidence for a role of other innate immune cells. For example, the AT in obese mice is also infiltrated by CD11c$^{\text{high}}$F4/80$^{\text{low}}$ dendritic cells, which have been shown to induce the differentiation of pro-inflammatory Th17 cells (18) and promote further macrophage infiltration (19). Similarly, mast cells, whose numbers are increased in obese AT, have also been shown to promote AT inflammation in obesity (20). Neutrophils also transiently infiltrate AT as early as 3 days after the initiation of high fat diet (HFD) in mice (21), and their production of elastase contributes to AT inflammation and may directly cause insulin resistance (22). Indeed, increased activity of neutrophil elastase has been detected in the serum of obese human subjects, and mice deficient in neutrophil elastase are protected against HFD-induced obesity and insulin resistance (23).

Adaptive immune cells have likewise been implicated in AT inflammation. Both T cells and B cells accumulate in the VAT of obese mice (10, 11, 24-26), and their phenotypes are skewed towards pro-inflammatory subtypes. For example, both CD4$^+$ and CD8$^+$ T cells in obese AT express markers of effector memory cells and produce high amounts of IFN-γ (10, 27), suggesting that an over-active Th1 cell response could play a role in AT inflammation. Infiltrating CD8$^+$ T cells also produce elevated levels of chemokines such as CCL5 and CXCL1, and thus contribute to the further recruitment of macrophages into AT (25, 27). Interestingly, since obese AT-resident T cells exhibit limited TCR diversity (27), specific antigens may drive
these T cell responses. Although the nature of the relevant antigens remains unclear, there is some evidence that absorbed intestinal antigens could have a role (28). Studies using B cell-deficient mice or antibody-mediated B cell depletion have also shown that B cells contribute to obesity-associated inflammation in the VAT (24) and systemically (26). Interestingly, Winer et al (24) found that B cells from obese HFD mice produce elevated levels of pathogenic IgG2c antibodies, and that via an Fc-dependent mechanism, transfer of serum IgG from these mice rapidly induced insulin resistance in recipient mice. DeFuria et al (26) did not, however, observe an increase in anti-nuclear autoimmune antibodies in HFD mice, but rather that follicular B cells promoted cell-mediated inflammation. Collectively these studies demonstrate that the progression of AT inflammation is strongly associated with over-active innate and adaptive immune responses. Which immune cells initiate the process, however, remains controversial. Although it is often argued that macrophages are the early perpetrators in an immune response, some studies suggest that infiltration by adaptive immune cells precedes macrophage accumulation (24, 29).

1.2 Immune regulation is lost in obese adipose tissue

In parallel to the increase in pro-inflammatory cells in obese AT, the steady-state high proportion of regulatory immune cells is also reduced (10, 11, 30). For example, in obese AT, macrophages undergo a phenotypic switch from the IL-10 producing M2 macrophages that normally occupy lean AT, to pro-inflammatory M1 macrophages (17, 31, 32). Normally, the predominant M2 phenotype is maintained by IL-4, with eosinophils and Th2 cells thought to be major sources of this cytokine. Remarkably, ninety percent of IL-4-expressing cells in the AT are eosinophils, and evidence that AT M2 macrophages depend on IL-4 and IL-13-expressing eosinophils, suggests
these cells have an important role in sustaining alternative activation of macrophages in healthy AT (33). In support of a similar protective role for Th2 cells, transfer of wild-type but not STAT6−/− CD4+ T cells, which have impaired Th2 differentiation, into Rag1−/− mice on a HFD normalizes obesity-associated pathologies (10). Although the loss of Th2 cell-derived cytokines likely affects the state of AT macrophage polarization, it should be noted that T cell transfer into immunodeficient mice can also induce colitis, and consequent effects on weight, microbiome and immune homeostasis may confound interpretation of effects on the AT (34).

Another subset of regulatory immune cells which is normally found in AT is the invariant NKT (iNKT) cell. iNKT cells are enriched in lean AT compared to peripheral lymphoid organs, but their numbers are markedly reduced in obese AT (30, 35). Furthermore, mice lacking iNKT cells show increased insulin resistance and weight gain on HFD, correlating with increased infiltration of pro-inflammatory macrophages into the AT (30). As an increasing number of studies report the effects of immune modulation on weight gain/loss, it is important to study if these changes could be due to effects on parameters such as food intake, energy expenditure, and/or changes in anatomical distribution of energy stores rather than direct effects on metabolism.

In addition to a predominance of the M2 variety of macrophages, the VAT in lean mice contains a substantially higher proportion of CD4+FOXP3+ Tregs within the CD4+ T cell population than is found in the peripheral lymphoid organs (11); and this proportion is markedly reduced in obese VAT. Through their broad range of suppressive activities (36), AT-resident Tregs could normally control the activity of many, if not all, of the types of immune cells that contribute to AT inflammation. Illustrating the central role of Treg-mediated regulation in metabolic homeostasis, acute depletion of Tregs by administration of diphtheria toxin to mice
expressing the diphtheria toxin receptor under control of the FOXP3 promoter, results in insulin resistance as indicated by reduced insulin-stimulated phosphorylation of the insulin receptor in VAT and liver (11). Interestingly, the phenotype of AT-resident Tregs is distinct from that of their peripheral counterparts, with a notable increase in IL-10 expression (11, 37). In addition to its classical anti-inflammatory function, IL-10 can also suppress TNF-α-induced insulin resistance in 3T3-L1 cells differentiated into adipocytes (17), suggesting that IL-10-producing Tregs could have relevant direct effects on adipocytes. An outstanding question is whether the Tregs that reside in obese AT are functionally normal or not. Although the proportion of Tregs is reduced, the absolute number of Tregs per gram of lean mouse AT is not significantly different from that in AT of obese HFD-fed mice (10), suggesting that factors in addition to a simple lack of sufficient numbers of Tregs contribute to a lack of immune regulation. In support of the possibility that Tregs in inflamed AT may be functionally defective, we found that Tregs from obese VAT of mice fed a HFD have reduced expression of IL-10 (38). Systemically, follicular B cells from obese mice also have reduced IL-10 production (26). More recently, B cells in the VAT of HFD mice have also been shown to express reduced levels of IL-10 (39). Hence, the overall reduction of IL-10 in obese AT (17) may reflect the combined loss of IL-10 producing M2 macrophages, Tregs and B cells.

Overall, the loss of immune regulation clearly contributes to the development and/or exacerbation of AT inflammation in obesity, with combined deficits in the number and/or function of multiple types of regulatory cells leading to the inability to sustain M2 macrophages. Although what causes these immunoregulatory mechanisms to fail is unknown, inappropriate stimulation of pro-inflammatory cells, could be one of the inciting events.
1.3 Multiple factors contribute to the development of adipose tissue inflammation

What initiates the reprogramming of immune cells in obese AT towards pro-inflammatory subtypes? The answer is likely a combination of different endogenous and exogenous danger signals. In terms of endogenous signals, saturated fatty acids directly activate TLR4 and TLR2 in macrophages, and even in adipocytes themselves, resulting in pro-inflammatory cytokine production (40, 41). Saturated fatty acids, specifically palmitate, can also activate the NLRP3 inflammasome, causing maturation and release of IL-1β by macrophages (42). In addition to dietary sources, fatty acids are also released from adipocytes during lipolysis, a process that occurs at an increased rate in obese AT (43). Since macrophages surround adipocytes (15, 16), they would be one of the first immune cell types to encounter fatty acids and other endogenous danger signals, such as ATP, that may be released by dying adipocytes. Notably, TNF-α secreted by stimulated macrophages can further stimulate lipolysis (44), resulting in a positive feedback loop that exacerbates AT inflammation. Interestingly, fatty acids inhibit differentiation of IFN-γ-producing Th1 cells (45), a finding that seems to contradict the observed accumulation of Th1 cells in obese AT, which has high free fatty acid levels. This apparent paradox might be explained by the dominant contribution of other factors, such as leptin (see below), which favour Th1 cell accumulation.

In addition to endogenous danger signals, pathogen associated molecular patterns (PAMPs), such as LPS, are also found at chronically elevated levels in the plasma of obese mice (46). In humans, high fat, high carbohydrate meals induce an increase in plasma LPS in as little as 2 hours (47). Since chronic low levels of LPS do not induce endotoxin tolerance in macrophages (48), a high-fat high-carbohydrate diet could result in chronic innate-immune driven inflammation. Moreover, obesity reduces adiponectin levels (49), and since adiponectin
can promote endotoxin tolerance (50), obesity may exacerbate the endotoxin-mediated effect by removing a potentially protective factor. Interestingly, a major source of PAMPs in obesity may be the intestinal microbiota: in mice, a HFD induces adherence and translocation of commensal bacteria from the intestine into the blood and AT, correlating with an increase in inflammatory cytokines (51). Moreover, a HFD causes a robust change in the composition of gut microbiota (52, 53), which could have a major influence on immune cell function (54). Thus a HFD could disrupt the intestinal epithelial cell barrier, and initiate inflammation by providing a source of microbial PAMPs and antigens that stimulate innate and adaptive immune cells in the VAT (28). A consideration is that inflammation itself may affect intestinal microbiota and barrier integrity; it is thus difficult to conclude with certainty that changes in intestinal microbiota precede and cause inflammation. Regardless, it is likely that changes in intestinal permeability contribute to a vicious cycle that exacerbates obesity-associated inflammation.

Corresponding with the aforementioned increase in endogenous and foreign danger signals, several chemokines are up-regulated in obese AT compared to lean AT. The expression of CCL2, 3, 5, 7, 8, 11 and 20, as well as CXCL14 is elevated in obese AT (55-57), creating a strong chemotactic gradient for innate and adaptive immune cells. Many of the studies on chemokines in AT have focused on CCL2 (MCP-1), and systemic deletion of CCL2 prevents macrophage accumulation in AT and ameliorates insulin resistance (58). These data suggest that chemokines produced by AT-resident immune cells or adipocytes themselves, which can also produce chemokines (59), significantly contribute to the development of AT inflammation.

An additional mechanism that could lead to obesity-related inflammation is a change in hormonal balance that affects regulatory immune cells. For example, leptin is an adipocyte-derived hormone that is produced at elevated levels in obesity (60), and acts directly on T cells to
stimulate IFN-γ production, thereby promoting Th1 cell differentiation while suppressing Th2 (61) and the proliferation of Tregs (62). Leptin-induced IFN-γ production can stimulate adipocytes to increase MHCII expression, causing a positive feedback pathway of T cell stimulation (29). Notably, leptin-deficient (ob/ob) and leptin-receptor-deficient (db/db) mice are often used as a model of obesity, and although these mice do become obese, leptin-mediated exacerbation of obesity-associated inflammation is absent. Hence the mechanisms driving AT inflammation in ob/ob and db/db mice may be distinct, especially in the T cell compartment, and likely cannot be directly extrapolated to wild-type mice. Counteracting the effects of leptin, is adiponectin, another adipokine, which promotes M2 macrophage polarization (63) and inhibits T cell activation (64). Thus a combination of increased leptin and reduced adiponectin production by adipocytes in obese AT worsens AT inflammation.

1.4 Restoring immune regulation in obesity improves metabolic parameters

Since AT inflammation plays a key role in the development of obesity-associated pathologies, it seems logical that restoration of immune regulation in targeted tissues could be therapeutic in insulin resistance and T2D. Interestingly, some of the currently used therapeutic strategies for T2D have previously unknown effects on inflammation and may actually restore immune regulation. For example, metformin, the most commonly used drug for T2D, enhances the proportion and numbers of Tregs by activating AMPK and promoting fatty acid oxidation, which is the primary biochemical pathway used by Tregs for cellular metabolism (45). Recently, it has also been shown that metformin therapy inhibits caspase-1 activation and IL-1β maturation in peripheral monocyte-derived macrophages human obese T2D subjects (65). PPARγ agonists, such as pioglitazone, are also commonly used in T2D and seem to have anti-inflammatory
effects. Compared to peripheral Tregs, AT-resident Tregs express high levels of PPARγ, and pioglitazone enhances the interaction between PPARγ and FOXP3 (11). Remarkably, treating obese mice with pioglitazone prevents the obesity-driven reduction of the proportion of Tregs in AT (11), induces apoptosis of AT macrophages (66), and favours the restoration of M2 macrophages (31). However, whether or not the effects of these drugs on AT inflammation contributes to their mechanism of action is unknown.

In addition to these pharmacological therapies, cell-based therapies that were originally designed to restore immune regulation in autoimmunity can also ameliorate obesity-associated pathologies in mouse models. For example, therapies that enrich Tregs, such as IL-2/anti-IL-2 complexes or anti-CD3 antibodies, can alleviate both inflammation and insulin resistance in HFD models (10, 11, 67). Cell therapy with regulatory CD4+LAP+ T cells has a similar effect (67), as does increasing iNKT cell numbers by adoptive cell transfer, delivery of α-galactosylceramide to expand iNKT cells and stimulate production of IL-4 and IL-10 (30), or depletion of B cells using anti-CD20 antibodies (24). In humans, it seems unlikely that anti-inflammatory therapy alone will be effective in T2D, and it will be important to test combination therapies that target both immune and metabolic parameters.

1.5 Summary

Obesity-associated AT inflammation appears to be caused by infiltration of inflammatory immune cells and a parallel loss, or functional re-programming, of immunoregulatory cells (Figure 1.2). Together, these changes lead to a variety of positive feedback pathways that not only sustain chronic inflammation, but also contribute to the development of insulin resistance. Now that the essential roles of innate and adaptive immunity in metabolic dysregulation are
recognized, a future area of focus will be to determine whether strategies that are designed to restore immune regulation can prevent and/or reverse this process. Many of the therapies that could be tested are already known to work in the setting of “traditional” immune-mediated diseases, and it will be of great interest to investigate whether these approaches will be similarly effective in metabolic diseases.

Figure 1.2 The loss of immune regulation in obesity-associated AT inflammation.

Lean AT contains regulatory immune cells (blue) that suppress pro-inflammatory immune cells (red) and sustain alternative activation of macrophages via Th2-associated cytokines. Adipocytes in lean AT are of normal size and produce adiponectin, which has anti-inflammatory properties. In contrast, obese AT is infiltrated with pro-inflammatory immune cells that produce high amounts of inflammatory cytokines and chemokines. M1 macrophages accumulate in crown-like structures around hypertrophic adipocytes that have increased rate of lipolysis, secreting free fatty acids (FFA) that can serve as endogenous danger signals to stimulate production of...
inflammatory cytokines, such as TNF-α. Adipocytes in obese AT also have increased leptin production, which promotes Th1 cells and inhibits Treg expansion. The gut barrier is disrupted in obesity, causing gut antigens and pathogen-associated molecular patterns such as LPS to enter the AT and stimulate inflammation. Furthermore, immune cells in the blood migrate into the AT in response to heightened chemokine production.

1.6 Objectives

Among immunoregulatory mechanisms in the AT, Tregs have a major role in the regulation of AT inflammation in obesity. As discussed above, the population of Tregs in the AT dwindles in obesity, and the restoration of normal AT Tregs could be beneficial for controlling AT inflammation and insulin resistance. Curiously, these AT-resident Tregs are phenotypically distinct from those in the periphery. These findings led us to ask the following research questions:

- What causes the inhibition of Tregs in obese AT?
- How can we therapeutically reverse the Treg deficit in obese AT?
- Why do Tregs residing in the AT have a distinct phenotype from those in the periphery?

My overall objective was to find mechanisms that regulate AT-resident Tregs and their ability to control obesity-associated AT inflammation. To answer the above research questions, I devised three independent objectives:

1. To determine whether insulin-mediated activation of the AKT pathway inhibits Treg function (Chapter 2)
2. To determine whether IL-33 can be used to expand AT Tregs, reverse AT inflammation and ameliorate insulin resistance in obesity (Chapter 3)
3. To determine whether soluble factors made by adipocytes contribute to the unique phenotype of AT-resident Tregs (Chapter 4)
Chapter 2: Insulin inhibits IL-10 mediated Treg function: implications for obesity

2.1 Introduction

One of the hallmarks of obesity is a marked rise in plasma insulin (68), with recent evidence suggesting that hyperinsulinemia may even be required for obesity (4). The canonical role of insulin is to promote glucose uptake in cells such as adipocytes, hepatocytes and myocytes, a function that is primarily mediated via insulin receptor-mediated stimulation of the AKT pathway (5, 69). Notably, the AKT signaling pathway also has a major role in regulating the development and function of Tregs, and hence immune tolerance and inflammation. For example, relatively low activity of the AKT pathway is essential for the normal development and function of Tregs (12). In addition, fully developed Tregs normally have diminished AKT activity in response to T cell receptor (TCR) or IL-2 stimulation (70, 71), and forced AKT activation inhibits their function (12, 70, 72). Downstream of AKT, mTOR activation is also linked to reduced Treg differentiation and function (73-75), providing a molecular mechanism for why the mTOR inhibitor rapamycin promotes tolerance (76, 77).

Because obesity-associated hyperinsulinemia is correlated with a reduction in Treg proportion (10, 11), we hypothesized that insulin may have biological effects on Tregs via the AKT/mTOR pathway. Here, we show that in contrast to TCR stimulation, insulin strongly activates AKT signaling in Tregs. Activation of AKT results in specific inhibition of IL-10 production and reverses the ability of Tregs to suppress TNF-α production from macrophages. Similarly, Tregs isolated from the VAT of obese, hyperinsulinemic mice were specifically
impaired in IL-10 production and produced significantly higher amounts of IFN-γ. Together these data reveal a previously unknown link between metabolism and immunity and suggest that insulin-stimulated activation of the AKT pathway in Tregs is important for regulation of IL-10 production and may contribute to the perpetuation of chronic inflammation in obesity.

2.2 Materials and methods

Mice. Mice were C57Bl/6 (B6), B6 FOXP3-EGFP (78) or B6 FOXP3-mRFP (79) x IL-10-EGFP (80) mice (6-12 weeks old; the Jackson Laboratory or bred in-house). Where indicated, 3-4 week old male mice were placed on normal chow diet or 58 kcal% fat with sucrose diet (Research Diets Inc.) for 13 or 16 weeks.

Glucose tolerance tests and determination of homeostatic model assessment of insulin resistance (HOMA-IR). For glucose-tolerance testing, eight hour fasted mice were orally gavaged with 2 g/kg body weight glucose. Glycemia was measured using a OneTouch Ultra 2 glucometer, and plasma insulin was determined using Luminex technology (Millipore, USA). HOMA-IR was calculated as fasting blood glucose (mmol/l) x fasting insulin (mU/ml) / 22.5 as described (81).

Cell isolation. Spleens and lymph nodes were mashed. Epididymal fat pads (visceral adipose tissue, VAT) were minced and digested with collagenase type II (Worthington) for 1h at 37°C with shaking. The stromal-vascular fraction was obtained after centrifugation, and passed through a cell strainer. CD4+ T cells were isolated with EasySep CD4+ selection kit (StemCell Technologies), and CD25+ selection was performed with CD25 microbeads (Miltenyi Biotec). For gene expression analysis, CD4+ T cells were sorted into CD4+FOXP3-EGFP+ Tregs and CD4+FOXP3-EGFP- T conventional (Tconv) cells to a >98% purity on a FACSARia.
Cell culture and signaling. RPMI-1640 was supplemented with 5 or 10% FBS, HEPES, and penicillin G and streptomycin. FBS contains a relatively small amount of endogenous insulin (0.002 ng/ml) (6), whereas serum insulin concentrations in obese hyperinsulinemic humans can rise to as high as 38 ng/ml upon glucose challenge (82). To mimic these conditions, where indicated, T cells were cultured with 0, 10 or 100 ng/ml insulin (Sigma-Aldrich) in the presence of 25 U/ml IL-2 for 24h, then stimulated with plate-bound α-CD3 (10 μg/ml), soluble α-CD28 (2 μg/ml) and 100 U/ml IL-2 in continuous insulin (0, 10 or 100 ng/ml). After 4 days, cell free conditioned media was collected. In some cases 25 nM rapamycin, 2 μM Akti1/2 or 2 μM UO126 (Sigma-Aldrich) was present throughout the culture. For suppression assays, irradiated APCs and soluble α-CD3 (0.7 μg/ml) were used to stimulate CFSE- or cell proliferation dye eFluor670 (eBioscience)-stained Tconvs that were co-cultured without or with Tregs (4:1 ratio) for 3 days. For cell signaling, CD4+ T cells were rested in serum free RPMI for 4h, or overnight in RPMI 1% FBS then serum free RPMI for 4h for VAT-derived cells, then stimulated with 10 μg/ml insulin, or 10 μg/ml αCD3 and 4 μg/ml αCD28 for the indicated time points. Activation was arrested by fixation in fix/perm buffer (eBioscience), and methanol in the case of pS6, then phosphorylation was detected with α-pAKT (Ser473) and α-pS6 (Ser235/236) Abs (Cell Signaling Technology) using flow cytometry by measuring the geometric mean fluorescence intensity (MFI).

Flow cytometry and ELISA. All flow cytometry and ELISA antibodies were commercially obtained from BD Pharmingen or eBioscience. For insulin receptor staining, cells were fixed and permeabilized with a Transcription Factor Staining Buffer Set (eBioscience), and stained with anti-FOXP3 (eBioscience) and insulin receptor β (4B8) rabbit mAb (Cell Signaling Technology) antibodies, followed by subsequent staining with Alexa Fluor 647 labeled-anti rabbit IgG (H+L)
antibody (Life Technologies). Isotype control was rabbit (DA1E) mAb IgG XP isotype control (Cell Signaling Technology). Flow cytometry was performed on BD FACSCanto or LSR II, and analysis was performed using FlowJo 8.7.

**Bone marrow derived macrophages (BMDM).** BMDMs were differentiated with GM-CSF for 6 days then incubated with 10 ng/ml IL-10, or media containing 12.5% of medium conditioned by Tregs or Tconvs (prepared as above with or without insulin) for 4 hours, then 10 ng/ml of LPS was added for 20 hours.

**RT-PCR analysis.** Gene expression was measured on Applied Biosystems 7500 Fast Real-Time PCR System. Primer sequences were: 18s, CAAGACGGACCAGAGCGAAA (5’) and GGCGGGTCATGGGAATAAC (3’); IL-10, TTGCCAAGCCTTATCGGAAA (5’) and TGCTCCACTGCCTTGCTCTT (3’). Data were normalized to 18s.

**Statistical analysis.** Student’s T tests or Mann-Whitney tests were used to analyze significance. The p values are indicated as follows: * p < 0.05 and ** p < 0.01.

### 2.3 Results

**2.3.1 Insulin activates the AKT pathway in Tregs.**

Insulin is arguably the best-characterized activator of AKT signaling. Since Tregs have diminished activation of the AKT pathway in response to TCR (12, 72) or IL-2 stimulation (71), we investigated if Tregs would be similarly hyporesponsive to insulin. To test this, CD4⁺ T cells were purified from B6 mice, stimulated with insulin for various times, then analyzed by flow cytometry to measure the amounts of AKT Ser473 phosphorylation in FOXP3⁺ Tregs versus
FOXP3+ Tconv cells. As shown in Fig. 2.1A, surprisingly insulin-induced AKT phosphorylation was significantly higher in Tregs than Tconv cells, which is the opposite of how these cells respond to TCR stimulation (Fig. 2.2). To test if signaling downstream of AKT was also higher in Tregs, we measured insulin-induced phosphorylation of ribosomal protein S6, which is dependent on the activation of mTORC1 (14) (Fig. 2.1B). Similar to AKT, insulin-stimulated S6 Ser235/236 phosphorylation was significantly higher in Tregs compared to Tconv cells. This greater response to insulin in Tregs compared to Tconv cells was not related to differences in expression of the insulin receptor, since Tregs and Tconvs express equivalent levels of the receptor ex vivo or after 72 h of TCR activation (Fig. 2.1C). These data indicate that despite the poor ability of TCR or IL-2 to stimulate the AKT pathway in Tregs (71, 72), they are fully competent to activate this pathway upon exposure to insulin.
Figure 2.1 Insulin activates AKT in Tregs.

(A-B) CD4+ T cells were purified from spleens and stimulated with 10 μg/ml insulin for the indicated times. Phosphorylation of (A) AKT Ser473 (n = 8) or (B) S6 Ser235/236 (n = 12) relative to unstimulated controls was measured by flow cytometry in Tregs and Tconvs cells gated as CD4+FOX3+ or CD4+FOX3- cells, respectively. MFIs relative to time 0 and representative histograms (with absolute MFIs indicated in brackets) are shown. Amounts of phosphorylation in unstimulated (t=0) Tregs and Tconv cells were equivalent. (C) Insulin receptor expression was measured by flow cytometry on FOX3- Tconvs and FOX3+ Tregs ex vivo, or after 72 hours of stimulation with α-CD3 and α-CD28 (n = 3-5). The MFI of the isotype control is shown. Error bars represent SD.
CD4+ T cells were purified from spleens and stimulated with 10 μg/ml insulin or 10 μg/ml αCD3 + 4 μg/ml αCD28 for the indicated times. Phosphorylation of AKT Ser473 (n = 2) relative to unstimulated controls was measured by flow cytometry in Tregs and Tconv cells gated as CD4+FOXP3+ or CD4+FOXP3- cells, respectively. Error bars represent SD.

**2.3.2 Insulin selectively inhibits IL-10 production by Tregs via AKT/mTOR activation.**

Tregs suppress immune cells via a number of different mechanisms including production of inhibitory cytokines (83). Since high AKT activity is associated with loss of Treg function (12, 70, 72), we asked whether exposure to insulin might affect one or more of these suppressive mechanisms. Tregs and Tconv cells were isolated, cultured overnight in 0, 10 or 100 ng/ml of insulin to stimulate the AKT pathway, and then stimulated via the TCR. Neither cell proliferation nor survival was affected by insulin (Fig. 2.3A&B), and consistent with our previous studies of forced AKT activation in Tregs (72), insulin-induced AKT activation did not reduce FOXP3 expression in Tregs (Fig. 2.4A). We then measured expression of surface proteins associated with contact-dependent Treg suppression, including CTLA-4, CD39, CD25 and LAP, but did not find
any significant effect of insulin (Fig. 2.4B). To test if insulin might affect cytokine-mediated suppression, Tregs and Tconv cells were stimulated for 4 days, and the amounts of secreted IL-10 and TGF-β were measured by ELISA (Fig. 2.4C). In contrast to the lack of effect on TGF-β production, 10 and 100 ng/ml of insulin significantly inhibited the production of IL-10 by 40.9 ± 22.0% and 39.9 ± 13.2%, respectively.

**Figure 2.3 Insulin does not affect apoptosis and proliferation of Tregs and Tconvs.**

Tregs and Tconvs were cultured with 0, 10 or 100 ng/ml insulin for 24h, then stimulated with plate bound α-CD3 and soluble α-CD28 in the continuous presence of 0, 10 or 100 ng/ml insulin for 3 days. (A) Apoptosis was measured with Annexin V and 7AAD, and (B) proliferation was measured via CFSE dilution (representative data from n = 2). Error bars represent SD.
Figure 2.4 Insulin specifically inhibits IL-10 production by Tregs.

(A-C) CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconv cells were cultured with 0, 10 or 100 ng/ml insulin for 1 day, then stimulated with α-CD3, α-CD28 mAbs and IL-2 for 4 days in the continual presence of 0, 10 or 100 ng/ml insulin. (A) The proportion of cells expressing FOXP3, and (B) the expression of CTLA-4, CD39, LAP and CD25 were measured in FOXP3⁺ Tregs and FOXP3⁻ Tconvs via flow cytometry (n = 3-12). (C) Supernatants were collected, and IL-10 (n = 12) and TGF-β (n = 5) were measured via ELISA. Error bars represent SD.
To determine whether the specific effect of insulin on IL-10 production was dependent on insulin-induced activation of AKT signaling, we used inhibitors of AKT (Akti1/2), mTOR (rapamycin) or the AKT pathway-unrelated kinase MEK1/2 (UO126), which is also activated by insulin (84). Tregs or Tconv cells were cultured overnight in 0, 10 or 100 ng/ml insulin in the presence or absence of the indicated inhibitors, then stimulated via the TCR for 4 days. As shown in Fig. 2.5A&B, inhibitors of the AKT pathway (Akti1/2 and rapamycin), but not MEK1/2 pathway (UO126), completely reversed the inhibitory effect of insulin on IL-10 production. Fig. 3A displays a representative experiment, and the cumulative data in Fig 3B were normalized to account for the fact that, as expected, Akti1/2 and rapamycin reduced cell proliferation and caused an overall reduction in IL-10. Thus, insulin-mediated suppression of IL-10 production was dependent on AKT and mTOR, but not the MEK/ERK signaling pathway.

Figure 2.5 Insulin inhibits IL-10 production by Tregs via the AKT/mTOR pathway.
(A-B) CD4*CD25+ Tregs and CD4*CD25- Tconvs were cultured with 0, 10 or 100 ng/ml insulin for 1 day, then stimulated with α-CD3, α-CD28 mAbs and IL-2 for 4 days in the continual presence of 0, 10 or 100 ng/ml insulin, in the presence or absence of 2 μM Akti1/2, 25 nM rapamycin or 2 μM UO126. Supernatants were collected and IL-10 was measured via ELISA. In (A) the representative absolute amounts of IL-10 and in (B) the average fold change in IL-10 compared to the without insulin condition is shown (n = 5-12). Error bars represent SD.

### 2.3.3 Insulin impairs the ability of Tregs to exert cytokine-mediated suppression.

Since IL-10 is one of the major mechanisms used by Tregs to suppress target cells (83, 85, 86), we next tested whether insulin alters Treg suppressive function. Tregs were cultured with or without insulin for 24 hours, then tested for their ability to suppress the proliferation of Tconv cells (Fig. 2.6A) in an assay that is known to be cytokine independent (87). Consistent with our finding that insulin did not affect molecules associated with contact-dependent mechanisms of suppression, insulin had no effect on Treg-mediated suppression of Tconv cell proliferation. Similar results were obtained with co-culture suppression assays in the presence or absence of insulin using Tregs that had not been pre-treated with insulin (data not shown). Moreover, insulin did not affect the proliferation of Tconv cells in this assay (Fig. 2.2B).
Figure 2.6 Insulin inhibits the ability of Tregs to suppress TNF-α production by macrophages via soluble factors.

(A) CD4+CD25+ Tregs were cultured with 0, 10 or 100 ng/ml insulin for 24h, then added in increasing numbers to CFSE-labeled CD4+CD25- Tconvs cells. The co-cultures were stimulated with anti-CD3 mAbs and irradiated APCs for 3 days in the absence of insulin. Tconv cell proliferation was measured by CFSE dilution and division index (DI). (A) Representative data and average percent suppression from multiple experiments at a 4:1 ratio of Tconvs:Tregs are shown (n = 3). (B) Bone marrow derived macrophages (BMDMs) were incubated with 10 ng/ml rIL-10, or media with 12.5% of media that had been conditioned by CD4+CD25+ Tregs or CD4+CD25- Tconvs in
the presence or absence of α-IL-10 blocking mAbs (n = 2-5). (C) BMDMs were incubated with media containing 12.5% of medium conditioned by CD4+CD25+ Tregs that had been stimulated with α-CD3, α-CD28 and IL-2 with 0, 10 or 100 ng/ml insulin (n = 3) for 4 hours. BMDMs were then stimulated with 10 ng/ml LPS and cultured for an additional 20 hours and TNF-α production was measured. The % suppression of TNF-α release compared to cultures without Treg conditioned medium is shown. Error bars represent SD.

To test if insulin affects contact-independent Treg suppression, we tested the effects of insulin on the ability of Tregs to reduce TNF-α production by macrophages (Fig. 2.6B&C). Bone marrow derived macrophages (BMDMs) were incubated for 4 hours in media that was conditioned by Tregs activated in the absence or presence of insulin, then stimulated with LPS. After 20 hours, TNF-α production was measured. As expected, addition of recombinant IL-10 effectively inhibited TNF-α production. Similarly Treg conditioned media suppressed TNF-α production by an average of 70.5 ± 7.5%. Addition of neutralizing α-IL-10 mAbs blocked the suppression of TNF-α production by the Treg-conditioned media, and further potentiated its production, demonstrating that Treg-mediated suppression of TNF-α is IL-10-dependent. In contrast to the effects of Tregs, media conditioned by Tconv cells significantly enhanced TNF-α production by an average of 464 ± 112 %. Tconv cells alone produced an average of 2.3 ± 0.3 ng/ml TNF-α (n = 3) (data not shown), and since only 12.5% of the total BMDM media is conditioned by Tconv cells, they only made a minor contribution to these elevated levels of TNF-α.

Next we asked how exposure to insulin affected Treg-mediated suppression of macrophages. BMDMs were stimulated with LPS with or without supernatants from Tregs that had been cultured in the absence or presence of insulin. These supernatants had reduced concentrations of IL-10 due to the effect of insulin on Tregs as shown in Fig. 2.3C. Exposure of
Tregs to 10 and 100 ng/ml insulin reduced their ability to suppress TNF-α by 46.8 ± 23.7% and 58.1 ± 12.1%, respectively. Addition of insulin itself did not affect TNF-α production by BMDMs (Fig. 2.7). These data show that exposure of Tregs to high levels of insulin impairs their ability to exert cytokine-mediated suppression of macrophages via IL-10.

Figure 2.7 Insulin does not affect TNF-α production by macrophages.
Bone marrow-derived macrophages were cultured with 0, 10 or 100 ng/ml insulin for 4 hours, then 10 ng/ml LPS was added and the macrophages were cultured for a further 20 hours. TNF-α in the culture media was measured via ELISA (n = 3). Error bars represent SD.

2.3.4 Tregs from obese, hyperinsulinemic mice have reduced IL-10 and increased IFN-γ expression.
We next examined the phenotype of Tregs residing in the VAT in a hyperinsulinemic environment in vivo. As expected in a model of diet-induced obesity, serum insulin levels were substantially increased to 9.48 ± 3.43 ng/ml in B6 FOXP-EGFP mice fed a high fat/sucrose diet (HFD) for 16 weeks compared to 3.17 ± 1.41 ng/ml in mice fed a normal chow diet mice (NCD) (Fig. 2.8A). Furthermore, mice fed a HFD had increased body weight (Fig. 2.9A), and impaired glucose tolerance and insulin sensitivity, as measured by an oral glucose tolerance test and
HOMA-IR calculation (Fig. 2.9B-C). These data are consistent with a previous report using the same diet (88) and confirm the suitability of the model to ask how changes in insulin sensitivity affect immune cells.

Figure 2.8 Tregs in the VAT of obese hyperinsulinemic mice are present in reduced proportions, but express normal levels of proteins that mediate contact-dependent suppression.

(A) B6 FOXP3-EGFP mice were fed a high fat/sucrose diet (HFD) for 16 weeks and their serum insulin levels were measured (n = 8). (B) The percentage of FOXP3+ Tregs of CD4+ cells was measured in spleen and VAT of NCD and HFD mice (n = 5). Representative plots of VAT CD4+ cells and their FOXP3+ Treg gates are shown. (C) The absolute numbers of CD4+FOXP3- Tconv cells and CD4+FOXP3+ Tregs per grams of tissue were measured (n = 3-4). (D) Expression of CTLA-4, CD39, CD25 and LAP on Tregs and Tconvs from spleen and VAT of HFD and NCD mice were measured by flow cytometry (n = 2-4). Error bars represent SD.
C57Bl/6 mice were fed a HFD or normal diet for 16 (A) or 13 (B-C) weeks. (A) Their body weights were measured ($n = 24$). Error bars represent SD. (B) Oral glucose tolerance test was performed on fasted mice. Blood glucose and plasma insulin levels are shown ($n = 4$). (C) HOMA-IR is calculated using fasting glucose and fasting insulin concentrations to measure insulin resistance ($n = 4$). Error bars represent SEM.

The proportion of Tregs amongst total CD4$^+$ T cells was normal in the spleen of HFD mice, but reduced from 32.8 ± 3.9% in NCD to 16.4 ± 1.1% in the VAT of HFD mice (Fig. 2.8B). However, NCD and HFD mice had equivalent numbers of Tregs per gram of VAT tissue (Fig. 2.8C) (10, 11, 89) due to the increase in immune cell infiltration in this setting. Since diet-induced obesity causes insulin resistance in adipocytes residing in VAT (90), we examined the ability of VAT Tregs from HFD mice to respond to insulin and found that they remained sensitive to insulin as judged by induction of AKT phosphorylation (Fig. 2.10A). Furthermore, Tregs remained sensitive to insulin in the presence of TNF-α (Fig. 2.10B), which has been shown to promote a loss of insulin signaling in adipocytes (91).
Figure 2.10 Tregs from HFD mice remain sensitive to insulin.

(A) B6 mice were fed a HFD or normal diet for 16 weeks, then CD4+ T cells were isolated from spleen and VAT (n=2), and stimulated with 10 μg/ml insulin. AKT Ser473 phosphorylation was measured in FOXP3+ Tregs. Relative data and histograms are shown. (B) CD4+ T cells were isolated from spleens of B6 mice (n=2), and stimulated with 10 μg/ml insulin with or without 100ng/ml TNF-α. AKT Ser473 phosphorylation was measured in FOXP3+ Tconvs and FOXP3+ Tregs. Relative data are shown. Error bars represent SD.

Whether or not Tregs in the VAT of HFD mice have an altered suppressive function has never been examined. To investigate whether Tregs in this environment are dysfunctional we measured expression of proteins associated with Treg suppressive capacity. Consistent with our in vitro data, in a setting of hyperinsulinemia in vivo, Tregs from the spleen or VAT of HFD mice had no significant change in expression of CTLA-4, CD39, CD25 or LAP in comparison to cells from NCD mice (Fig. 2.8D).

We next examined IL-10 production in Tregs from NCD and HFD mice. Tregs were sorted as GFP+ cells from the spleen and VAT of FOXP3-GFP-reporter mice fed a NCD or HFD and expression of IL-10 mRNA was measured. We found that the Tregs from both the spleen and the
VAT of HFD mice had a significant reduction in the amount of IL-10 mRNA (Fig. 2.11A). To confirm these data at the protein level, we fed FOXP3-RFP x IL-10-EGFP mice a NCD or HFD, then analyzed spleen and VAT Tregs by flow cytometry (gating shown in Fig. 2.8B). Both the MFI of IL-10 and percentage of IL-10\textsuperscript{hi} cells indicate that VAT Tregs produce markedly higher levels of IL-10 compared to splenic Tregs (Fig. 2.11B). In VAT Tregs from HFD mice however, production of IL-10 was significantly reduced compared to Tregs from NCD mice.

Figure 2.11 Tregs in hyperinsulinemic obese mice have reduced expression of IL-10 and increased expression of IFN-\(\gamma\).
(A) CD4+FOXP3+ Tconv cells and CD4+FOXP3+ Tregs were FACS sorted from spleen or VAT of FOXP3-GFP mice fed a NCD or HFD, and expression of IL-10 mRNA was measured by quantitative RT-PCR (n = 2-3). (B) B6 FOXP3-RFP x IL-10-EGFP mice were fed a HFD or NCD, then IL-10-EGFP expression was measured in FOXP3-RFP+ Tregs from the spleen and VAT. The average MFI, % of IL-10hi cells and representative histograms are shown (n = 8-9). (C) IFN-γ production of Tregs and Tconv cells in spleen and VAT of NCD and HFD mice was measured via flow cytometry. Representative and average data are shown (n = 3). Error bars represent SD.

Treg dysfunction is associated with induction of expression of effector cytokines such as IFN-γ and IL-17 (92). Therefore, in parallel to IL-10, we measured production of IFN-γ in HFD versus NCD mice. As expected, HFD mice had an enhanced T\textsubscript{H}1 response in the VAT (10), as evidenced by an increased proportion of IFN-γ+ Tconv cells. VAT, but not splenic Tregs in HFD mice also had a significant increase in IFN-γ expression: 15.1 ± 0.4 % of Tregs from HFD mice expressed IFN-γ compared to only 6.9 ± 1.8 % in NCD mice (Fig. 2.11C). Expression of IL-17 was not detectable under any conditions (data not shown). These data suggest that in HFD mice, VAT Tregs may lose their lineage stability and take on a T\textsubscript{H}1-like phenotype.

2.4 Discussion

Insulin has long been known as a metabolic hormone that regulates glucose homeostasis, and its physiological effects on myocytes, hepatocytes and adipocytes in health and disease are well established. Little is known, however, about the effects of insulin on immune cells, and its effects on Tregs have never been investigated. Here, we show that Tregs express the insulin receptor, and we reveal a novel physiological role for insulin: regulation of IL-10 production and the suppressive function of Tregs. Tregs resident in the VAT of hyperinsulinemic mice produced
significantly less IL-10 than lean mice fed a normal diet, suggesting that changes in metabolism may specifically affect this mechanism of Treg suppression.

Tregs normally have dampened TCR- or IL-2-induced AKT phosphorylation at Ser473, at least partly due to high expression of the protein phosphatase PHLPP (70, 71). This low AKT signaling is functionally relevant for Tregs, as forced activation of the pathway by overexpression of active forms of AKT or deletion of inhibitory phosphatases blocks their normal development and function (12, 70, 72). We show here, however, that unlike TCR or IL-2 stimulation, insulin-induced activation of AKT was significantly higher in Tregs compared to Tconv cells, suggesting that Tregs may be uniquely responsive to this hormone. Insulin-induced AKT activation also resulted in the activation of mTORC1, as judged by phosphorylation of ribosomal protein S6, a downstream target of mTORC1 (14). This finding provides the first evidence that Tregs are not universally hyporesponsive to stimulation of the AKT pathway.

The effect of insulin on Tregs was remarkably specific: inhibition of IL-10 production without any effect on other proteins associated with Treg function, including CTLA-4, CD39, LAP, or TGF-β. To the best of our knowledge, such a specific cytokine/growth factor-stimulated effect on IL-10 production by Tregs has not been previously reported. The insulin-mediated suppression of IL-10 production was dependent on AKT and mTOR, but not the MEK/ERK signaling pathway, which is also activated by insulin (84). Of the few studies that have investigated the relationship between AKT signaling and IL-10 production in Tregs, some have reported an inverse association between AKT activation and IL-10 production in Tregs (93, 94) much like our data; while other studies suggest the opposite (95-97). Thus, the role of AKT signaling in IL-10 production by Tregs may be context dependent. Our data suggest that of the major known mechanisms of Treg action, IL-10 production may be particularly sensitive to
modulation of the AKT signaling pathway, and that, in the context of insulin stimulation, AKT activation negatively regulates this aspect of Treg function.

A well-established feature of obesity associated with hyperinsulinemia is chronic inflammation, characterized by a prominent infiltration of macrophages into adipose tissue, and an over-production of TNF-α (16, 98, 99). We found that insulin significantly inhibited the ability of Tregs to exert IL-10-mediated suppression of TNF-α production by macrophages. Tregs in HFD mice also produced lower amounts of IL-10, in accordance with a previous report that IL-10 expression in the total stromal-vascular fraction of VAT from HFD mice is reduced (17). Since IL-10 inhibits TNF-α, which is over-produced by macrophages in obese VAT (16, 98), and can directly alleviate TNF-α-induced insulin resistance in adipocytes (17), reduced IL-10 production by Tregs may directly contribute to both inflammation by macrophages and insulin resistance in non-immune cells.

As a mouse model of obesity, HFD mice are known to be hyperinsulinemic, and have a reduced proportion of Tregs specifically in VAT, which is a key site for insulin resistance and chronic inflammation (100, 101). However, whether these Tregs are functionally altered, was hitherto unknown. Consistent with a previous study (10, 89), we found that, on a per gram of tissue basis, the absolute number of adipose Tregs was unchanged in HFD mice, indicating that a simple reduction in Treg numbers likely does not contribute to obesity-associated inflammation. Our data are the first to suggest that there is major functional impairment of Tregs in HFD mice, specifically their cytokine-dependent function. Furthermore, the proportion of Tregs making IFN-γ in HFD mice is significantly increased, possibly indicating a loss of Treg lineage stability in the HFD environment.
There is striking resemblance between insulin-treated Tregs \textit{in vitro} and Tregs from the VAT of hyperinsulinemic obese mice. In both settings, Tregs encountered high levels of insulin, and in both, Tregs acquired a specific defect in IL-10 production, while retaining normal expression of other proteins associated with Treg function. Thus, our data suggest a possible pathological effect of insulin on Tregs in the setting of obesity, similar to an observation previously made in myeloid cells (102), where insulin receptor expression has been shown to contribute to the development of obesity-associated inflammation. In conclusion, Tregs become phenotypically altered in conditions of hyperinsulinemia, and the loss of IL-10 production and gain of IFN-\(\gamma\) could contribute to the immune dysregulation that ensues in VAT during obesity. Our data establish a new molecular and cellular paradigm for how hyperinsulinemia might affect inflammation in obesity.
Chapter 3: IL-33 reverses an obesity-induced deficit in visceral adipose tissue

ST2⁺ Tregs and ameliorates adipose tissue inflammation and insulin resistance

3.1 Introduction

Obesity-associated visceral adipose tissue (VAT) inflammation is thought to result in insulin resistance and contribute to the development of type 2 diabetes (103, 104). Diet-induced obesity (DIO) results in infiltration of many pro-inflammatory immune cells into the VAT, with strong evidence that accumulation of macrophages producing pro-inflammatory cytokines, such as TNF-α and IL-6, have a major causal role in driving inflammation (17, 105). In parallel to expansion of inflammatory macrophages, obese VAT has a reduced proportion and function of anti-inflammatory FOXP3⁺ regulatory T cells (Tregs), which normally comprise a significant proportion of CD4⁺ T cells in lean VAT (10, 11, 38). In addition, mice in which Tregs are ablated have worsened VAT inflammation and insulin resistance (11). These findings have led to the hypothesis that poor Treg function in obese VAT is a major factor contributing to chronic VAT inflammation. In support of this possibility, treatments that enhance Tregs, such as administration of anti-CD3 (10), or IL-2 anti-IL-2 complex (11), or adoptive transfer of Tregs themselves (106), can improve insulin sensitivity.

Tregs resident in lean VAT have a unique phenotype that is at least partly due to the fact they express PPARγ, a transcription factor associated with adipocyte differentiation and function, which co-operates with FOXP3 to drive a distinct VAT Treg gene signature (37). In addition, we previously showed that, in comparison to splenic Tregs, lean VAT-resident Tregs
express high levels of IL-10 (38). This high expression of IL-10 by VAT Tregs is specifically altered in diet-induce obesity: Tregs from obese VAT have a significant reduction in IL-10 production, with unchanged expression of CTLA-4, CD39, CD25 or LAP (38). Since IL-10 is necessary for Treg-mediated suppression of TNF-α production from macrophages (38), this obesity-induced change in VAT Treg function likely contributes to inflammation and insulin resistance.

Here we further investigated the phenotype of Tregs in lean VAT and found that, in comparison to Tregs in the spleen, or conventional T cells (Tconv) in lean VAT or spleen, they also express proportionately higher levels of the ST2 chain of the IL-33 receptor. The proportion of this pool of ST2\(^+\) Tregs is reduced in obese VAT, and can be completely restored by treatment with IL-33. Treatment with IL-33 also reduced VAT inflammation and ameliorated insulin resistance. Hence therapeutic maintenance of ST2\(^+\) Tregs in VAT by IL-33 may be a new approach to control obesity-associated inflammation and metabolic disorders.

### 3.2 Materials and methods

**Mice and IL-33 injections.** 4 week old male C57Bl/6 FOXP3-eGFP or C57Bl/6 FOXP3-RFP x IL10-GFP mice (bred in-house) were fed a normal chow diet (NCD, Lab Diets Cat #5053) or 58 kcal\% fat with sucrose diet (Research Diets Inc. Cat # D12331) for 14-16 additional weeks. Diets were not matched for micronutrients. Where indicated, mice were then injected I.P. for 10 consecutive days with 0.5µg/day of recombinant IL-33 or PBS (eBioscience), while maintained on their respective diets, and were sacrificed 10 days after the start of treatment. To determine metabolic effects of IL-33, 10 consecutive days of injections were followed by one injection every 2-3 days of 0.5µg IL-33 or PBS. Fasting blood glucose and plasma insulin levels were
measured at 14 and 17 days after the start of treatment. All animal studies were approved by the Canadian Council on Animal Care.

**Cell isolation and in vitro generation of ST2+ Tregs.** Spleens and epididymal fat pads (VAT) were processed as described (38). The stromal-vascular fraction (SVF) was obtained after centrifugation, and passed through a cell strainer. Intestines were minced and digested with collagenase, and live cells were isolated using Percoll density centrifugation. Bone marrow-derived dendritic cells (BMDCs) were differentiated with GM-CSF and 10ng/ml IL-4 in RPMI and 10% FCS for 7 days, then purified as CD11c+ cells (STEMCELL Technologies), and rested overnight in complete media, with or without 10ng/ml of IL-33. CD4+ T cells were magnetically sorted (STEMCELL Technologies) from spleens. A ratio of 10 T cells to 1 DC were cultured with 0.01-0.1µg/ml anti-CD3 (2C11, BD) in the presence or absence of 10ng/ml IL-33 for 5 days.

**Flow cytometry, RT-PCR and cytokine analysis.** All flow cytometry and ELISA antibodies were commercially obtained from BD Pharmingen or eBioscience. A portion of VAT was immediately immersed in lysis buffer and dissociated using gentleMACS (Miltenyi Biotec) for analysis of gene expression. Data were normalized to 18s. The SVF from digested VAT was cultured overnight at 2x10^6 cells/ml, and amounts of secreted IL-6 were measured in the culture media by cytometric bead array (BD).

**Determination of blood glucose, insulin and homeostatic model assessment of insulin resistance (HOMA-IR).** Mice were fasted for 4-6 hours at day 14 and 17 after the start of
treatment, respectively. Blood glucose was measured using a OneTouch Ultra 2 glucometer, and plasma insulin was measured by ELISA (ALPCO). HOMA-IR was calculated as fasting blood glucose (mmol/l) x fasting insulin (mU/l) / 22.5 as described (81).

**Statistical analysis.** Unpaired Student’s T tests were used to analyze significance. The $p$ values are indicated as follows: * $p < 0.05$ and ** $p < 0.01$.

### 3.3 Results

#### 3.3.1 A majority of Tregs in lean VAT express ST2 and Th2 cell associated markers.

We previously found that Tregs resident in VAT express high levels of IL-10 which is likely involved in restraining the production of pro-inflammatory cytokines from macrophages (38). To further characterize the phenotype and function of VAT Tregs, we asked whether these cells also express ST2, one of chains of the IL-33 receptor, since expression of this protein has recently been reported on Tregs resident in other tissues (107, 108). Single cell suspensions from the VAT of 20-week-old mice maintained on NCD were analyzed by flow cytometry. We found that $54.5 \pm 14.3\%$ ($n = 14$) of VAT Tregs expressed ST2, compared to just $2.9 \pm 0.9\%$ ($n = 14$) of splenic Tregs (Fig. 3.1A). In addition, only $6.8 \pm 4.2\%$ ($n = 14$) of CD4$^+$FOXP3$^-$ conventional T cells (Tconvs) in the VAT expressed ST2. CD4$^+$FOXP3$^+$ Tregs in the VAT also had high expression of CD25, CTLA-4 and CD39 regardless of whether or not they express ST2 (Fig. 3.2A). VAT Tconvs expressed significantly higher levels of CD25, CTLA-4 and CD39 compared to splenic Tconvs, albeit at lower levels than on Tregs, and interestingly, ST2$^+$ Tconvs in the VAT express increased levels of CD25 compared to ST2$^-$ Tconvs (Fig. 3.2A). The proportion of Tregs expressing ST2 in lean VAT was significantly higher than in the intestinal lamina propria, in which an average of $17.7 \pm 0.4\%$ ($n = 3$) of Tregs expressed ST2 (Fig. 3.2B).
Figure 3.1 Phenotypically distinct ST2+ Tregs populate lean VAT.

(A) The percentages of ST2+ cells ($n = 14$), and (B) mean fluorescence intensities of CCR4 ($n = 14$) and GATA3 ($n = 6$) on gated CD4+FOXP3+ Tregs and CD4+FOXP3- Tconvs from the spleen and VAT of 20 weeks old NCD mice were analyzed by flow cytometry. The mean fluorescence intensities of (C) CCR4 ($n = 14$) and GATA3 ($n = 6$), (D) percentages of IL-10hi ($n = 6$), (E) mean fluorescence intensities of Nrp1 ($n = 7$), and (F) percentages of Helios+ cells ($n = 7$) in ST2+ versus ST2- FOXP3+ Tregs in the VAT of 20 weeks old NCD mice are shown. Plots and histograms shown are representative data. Graphs show averages (A, D, F) or paired biological replicates (B, C, E) from the
number of mice indicated analyzed in 3-4 (A-C) or 2 (D-F) independent experiments. Error bars represent SD. * p < 0.05 and ** p < 0.01.

ST2 expression has also been previously been linked to Th2 cells (109, 110), thus, we measured expression of Th2-associated proteins CCR4 and GATA3 in VAT Tregs. We found that VAT Tregs expressed significantly higher levels of CCR4 and GATA3 than splenic Tregs (Fig. 3.1B), but did not express Th2-related cytokines such as IL-13 and IL-5 (data not shown). VAT Tconvs expressed slightly higher levels of GATA3, but not of CCR4, compared to splenic Tconvs (Fig. 3.1B). By comparison, VAT Tregs did not express increased levels of Th1- (TBET, CXCR3) or Th17- (RORγt) associated proteins (Fig. 3.2C). However, VAT Tregs expressed significantly more CCR6 than splenic Tregs (Fig. 3.2C), but so did VAT Tconvs, indicating that this change is not Treg specific.
Figure 3.2 Characterization of VAT CD4^+FOXP3^+ Tregs.

(A) The expression of CD25, CTLA-4 and CD39 amongst CD4^+ FOXP3^+ and CD4^+ FOXP3^-, and their ST2^+ and ST2^- subpopulations in the spleen and VAT of 20 weeks old NCD mice were analyzed by flow cytometry (n = 3-5).

(B) The percentage of ST2^+ cells on gated CD4^+FOXP3^+ Tregs and CD4^+FOXP3^- Tconvs from the spleen, VAT and intestinal lamina propria of 20 weeks old NCD mice were analyzed by flow cytometry (n = 3-12). (C) Mice were fed a NCD or HFD for 16 weeks and the expression of CXCR3, TBET, CCR6 and RORγt on Tregs and Tconvs in spleen and VAT was measured by flow cytometry (n = 4). Graphs show averaged data. Plots and histograms show representative data. Error bars represent SD. * p < 0.05 and ** p < 0.01.
To test if the expression of Th2 markers is associated with expression of ST2, we examined expression of CCR4 and GATA3 in ST2+ vs ST2− VAT Tregs. As shown in Fig. 3.1C, VAT-specific expression of GATA3 and CCR4 was restricted to ST2+ Tregs, indicating that ST2 appears to specifically mark Tregs with a Th2-like phenotype. Of note, ST2+ Tconvs also expressed significantly more CCR4 than ST2− Tconvs (Fig. 3.1C). Since GATA3 enhances the expression of IL-10 in CD4+ T cells (111), and VAT Tregs express high levels of IL-10 (38) we used FOXP3-RFP x IL10-GFP mice to determine the correlation between ST2+ and IL-10. Indeed, the vast majority (90.8 ± 6.7%, n = 6) of IL-10-expressing Tregs in VAT also expressed ST2, and by corollary, ST2+ Tregs in the VAT expressed significantly more IL-10 than did ST2− Tregs (Fig. 3.1D): 68.1 ± 13.1% of ST2+ Tregs expressed IL-10, compared to only 17.9 ± 9.1% of ST2− Tregs.

To determine whether the Th2-like phenotype and IL-10 production by ST2+ Tregs was related to IL-33 signaling, we used BMDCs and IL-33 to differentiate ST2+ Tregs in vitro from splenic CD4+ T cells, which had very low levels of ST2 expression prior to stimulation (112). This culture system with IL-33 resulted in the induction of ST2+ Tregs which had increased expression of both CCR4 and IL-10 (Fig. 3.3A and B).
Figure 3.3 *In vitro* differentiated ST2+ Tregs have increased expression of CCR4 and IL-10.

CD4+ T cells were co-cultured with bone marrow-derived DCs in the presence of 10ng/ml IL-33 and 0.01-0.1µg/ml anti-CD3 at a ratio of 10 T cells to 1 DC. *(A)* Representative plot depicting ST2 expression in Tregs is shown, and *(B)* the expression of CCR4 and IL-10 were measured in ST2+ and ST2- Tregs by flow cytometry. Representative and averaged data from *n* = 6 are shown. Error bars represent SD. * *p* < 0.05 and ** *p* < 0.01.

Interestingly, ST2+ Tregs also had increased expression of neuropilin-1 (Nrp1) (Fig. 3.1E), which is known to promote Treg survival and function (113). In contrast, analysis of Helios revealed no significant differences in expression between ST2+ and ST2- Tregs (Fig. 3.1F), suggesting that these cells are comprised of similar proportions of thymically- and peripherally-derived cells.

**3.3.2 Obesity diminishes VAT-resident ST2+ Tregs.**

DIO results in a reduced proportion of VAT Tregs due to an expansion of pro-inflammatory Tconvs (10, 11). In addition, Tregs from obese VAT have diminished expression of IL-10, while
other suppressive mechanisms, including CD39, CTLA-4 and LAP, remain unchanged compared to cells in lean VAT (38). To investigate the effect of DIO on ST2+ Tregs, littermates to the lean NCD mice shown in Figure 3.1 were fed with a 58 kcal% fat with sucrose diet (HFD). We found that obese VAT had a substantially reduced proportion of ST2+ Tregs (Fig. 3.4A): 54.5 ± 14.3% (n = 14) of VAT Tregs expressed ST2 in lean VAT, compared to just 16.3 ± 7.1% (n = 14) in obese VAT from HFD mice. There was a similar HFD-induced reduction in the minor population of VAT Tconv cells that also expressed ST2.

Figure 3.4 ST2+ Tregs are diminished in obese VAT.

(A) The percentage of ST2+ cells (n = 14) and the mean fluorescence intensities of (B) CCR4 (n = 14), (C) GATA3 (n = 6) and (D) Nrp1 (n = 7) in gated CD4+FOXP3+ Tregs versus CD4+FOXP3- Tconvs in the spleen and VAT of
mice that had been fed the indicated diet for 16 weeks were analyzed by flow cytometry. The mean fluorescence intensities of (E) CCR4 \( (n = 14) \), (F) GATA3 \( (n = 6) \) and (G) Nrp1 \( (n = 7) \) in ST2\(^+\) and ST2\(^-\) Tregs from the VAT of NCD versus HFD mice were analyzed by flow cytometry. Representative data for Tregs gated as CD4\(^+\)FOXP3\(^+\) cells are shown. Graphs show averages from the number of mice indicated analyzed in 4 (A-B, E), 3 (C, F) or 2 (D, G) independent experiments. Error bars represent SD. * p < 0.05 and ** p < 0.01.

In parallel, VAT Tregs from HFD mice also had reduced expression of multiple proteins that were associated with ST2 expression, including CCR4, GATA3 and Nrp1 (Fig. 3.4B-D). VAT Tregs in HFD mice had a 56.9 ± 19.7% \( (n = 12) \) reduction in CCR4, and a 43.6 ± 12.9% \( (n = 6) \) reduction in the expression of GATA3. VAT Tconvs had a similar percentage reduction of GATA3, but no reduction in the expression of CCR4. Interestingly, HFD induced a large 84 ± 11.6% \( (n = 3) \) reduction of Nrp1 in VAT Tconvs, compared to 53.4 ± 34.8% \( (n = 3) \) in VAT Tregs. The expression of Th1- (TBET, CXCR3) or Th17- (ROR\(\gamma\)t, CCR6) associated proteins on VAT Tregs was not affected by HFD (Fig. 3.2C).

Within the populations of ST2\(^+\) Treg and ST2\(^+\) Tconvs in the VAT, DIO significantly reduced the expression of CCR4 (Fig. 3.4E) and Nrp1 (Fig. 3.4G), but not GATA3 (Fig. 3.4F). The expression of Nrp1 in ST2\(^-\) Tregs and ST2\(^-\) Tconvs was also down-regulated in HFD mice (Fig. 3.4G), indicating that both ST2\(^+\) and ST2\(^-\) cells were affected by DIO. In addition, although the overall expression of CCR4, GATA3 and Nrp1 in total VAT Tregs and/or Tconvs was decreased in HFD mice, ST2\(^+\) Tregs and Tconvs retained higher expression of these proteins compared to ST2\(^-\) cells (Fig. 3.5).
Figure 3.5 VAT ST2+ Tregs have increased expression of CCR4, GATA3 and Nrp1 in HFD mice.

Mean fluorescence intensities of CCR4 (n = 14), GATA3 (n = 6) and Nrp1 (n = 7) in ST2+ versus ST2- Tregs in the VAT of 20 weeks old HFD mice were analyzed by flow cytometry. Graphs show paired biological replicates. * p < 0.05 and ** p < 0.01.

3.3.3 IL-33 reverses the reduction of ST2+ Tregs in obese VAT.

Injection of IL-33 is known to expand peripheral ST2+ Tregs in a model of cardiac transplantation (114), so we asked whether in vivo administration of IL-33 could reverse the diet-induced reduction of ST2+ Tregs in VAT. Mice were fed a NCD or HFD for 16 weeks, then injected intraperitoneally with IL-33, or PBS as a control, for 10 consecutive days. Notably, 10 days of IL-33 treatment did not change the body weights of NCD or HFD mice (Fig. 3.6A and B). Mice fed a HFD had a significant reduction in the proportion of Tregs, and injection of IL-33 completely reversed this effect, resulting in restoration of the normal proportions of Tregs (Fig.
IL-33 also significantly increased the absolute number of Tregs per gram of VAT. However, IL-33 did not increase the numbers of Tconvs, and, contrary to a previous report (114), IL-33 injections did not expand Tregs in the spleen.

**Figure 3.6** 10 days of IL-33 administration does not affect body weights.

Mice were fed a NCD (n = 2-3) or HFD (n = 8) for 16 weeks, and then injected with PBS or IL-33. Weights were measured before (d0) and after treatment (d10), and shown as (A) averaged data. Error bars represent SD. (B) Paired analysis comparing the weights of mice before and after treatment of each replicate. Each line represents one mouse.

* p < 0.05 and ** p < 0.01.

IL-33 administration also increased the proportion of ST2-expressing Tregs in the VAT by three fold in HFD mice, resulting in restoration of the normal population balance of VAT ST2+ Tregs found in lean NCD control mice (Fig. 3.7B). The absolute number of ST2+ Tregs per gram of VAT increased by eight fold, whereas the number of ST2+ Tconvs was not significantly
affected. GATA3 expression and Ki67 staining were also increased in VAT Tregs of HFD mice receiving IL-33 treatments (Fig. 3.7C and D). Injection of IL-33 also upregulated ST2 expression on VAT Tconvs by four fold, and on splenic Tregs by two fold, although the overall proportion of ST2+ cells in these populations always remained significantly lower than that of VAT Tregs.

**Figure 3.7 IL-33 treatment reverses the deficit of ST2+ Tregs in obese VAT.**

(A-D) Mice were fed a HFD for 16 weeks, then injected with PBS or IL-33 for 10 days. (A) The percentage of FOXP3+ Tregs of CD4+ cells in the spleen and VAT (n = 9), and the absolute number of cells per gram of VAT (n = 7) was determined by flow cytometry. (B) The percentage (n = 9) and absolute number (n = 7) of ST2+ cells, (C) mean fluorescence intensity of GATA3 (n = 4) and (D) the percentage of Ki67+ cells (n = 5-6) in gated CD4+FOXP3+ Tregs and CD4+FOXP3- Tconvs in the spleen and VAT were measured. Dot plots and histograms shown are representative data from VAT. Representative dot plots of NCD mice injected with PBS for 10 days are also shown for (A-B). Percentages and MFI shown are averages from the number of mice indicated analyzed in 3 (A-B) or 2 (C-D) independent experiments. Absolute numbers in (A-B) are representative data from 1 of 2 independent experiments. Error bars represent SD. * p < 0.05 and ** p < 0.01.
3.3.4 IL-33 treatment reduces VAT inflammation.

We next asked whether the IL-33-induced restoration of ST2\(^+\) Tregs in the VAT of HFD mice was accompanied by a reduction in inflammation. As expected, compared to NCD mice, HFD mice had an increase in F4/80\(^+\) macrophages infiltrating the VAT, and this was almost completely reversed by IL-33 administration (Fig. 3.8A). By comparison IL-33 did not significantly alter the proportion of CD4\(^+\), CD8\(^+\) or CD11c\(^+\) cells in the VAT (Fig. 3.9C). RT-PCR analysis of the VAT revealed that IL-33 treatment of HFD mice also reduced the expression of Tnfa mRNA in the VAT (Fig. 3.8B), but did not significantly affect levels of Ccl2 or Il10 mRNA (Fig. 3.9D). We also isolated the SVF from the VAT, which contains all VAT-resident immune cells, and measured the amount of IL-6 released in the culture media. SVF cells isolated from the VAT of HFD mice that had been injected with IL-33 produced significantly less IL-6 than did SVF cells isolated from HFD mice receiving PBS (Fig. 3.8C). Indeed, IL-33 administration was able to reduce TNF\(\alpha\) and IL-6 in the VAT to the level of expression found in lean NCD control mice, as indicated by the dashed lines (Fig. 3.8B and C).
Figure 3.8 IL-33 administration reduces VAT inflammation.

(A-C) Mice were fed a HFD for 16 weeks, then injected with PBS or IL-33 for 10 days. (A) The percentages of F4/80+ macrophages of total live cells in the spleen and VAT were analyzed by flow cytometry (n = 6-7). (B) The relative amounts of Tnfα mRNA in the VAT were detected by quantitative RT-PCR (n = 6-7). (C) The average amounts of IL-6 produced by SVF cells during overnight culture were measured by cytometric bead array (n = 4). The dashed lines indicate the average values from the VAT of NCD mice injected with PBS for 10 days. Graphs show averages from the number of mice indicated analyzed in 2 independent experiments. Error bars represent SD. * p < 0.05 and ** p < 0.01.
Figure 3.9 IL-33 administration does not affect the expression of CCL2 and IL10 mRNAs in the VAT.
Mice were fed a NCD ($n = 2-3$) or HFD ($n = 8$) for 16 weeks, and then injected with PBS or IL-33. (A) The percentages of CD4+ , CD8+ and CD11c+ cells of total live cells in the spleen and VAT were determined by flow cytometry ($n = 4-8$). (B) The relative amounts of CCL2 and IL10 mRNA in the VAT were detected by quantitative RT-PCR ($n = 4-6$). The dashed lines indicate the average values from the VAT of NCD mice injected with PBS for 10 days. All graphs show averaged data. Error bars represent SD. * $p < 0.05$ and ** $p < 0.01$.

3.3.5 IL-33 treatment ameliorates insulin resistance in diet-induced obese mice
To determine whether IL-33-induced resolution of VAT inflammation could also lead to an improvement of metabolism, mice fed a HFD for 16 weeks were injected with IL-33 for 10 consecutive days to expand VAT Tregs and reduce VAT inflammation, followed by one injection every 2-3 days. At days 14 and 17, mice were fasted for 4-6 hours, and their fasting blood glucose and serum insulin were measured and the HOMA-IR, (homeostatic model assessment-insulin resistance), a mathematical equation used to estimate insulin resistance using fasting blood glucose and insulin levels (115), was calculated. There was no significant
difference in body weights between PBS and IL-33 treated mice before and after treatment (Fig. 3.10A); however, paired analysis revealed IL-33 injections resulted in a reduction of body weight after 14 and 17 days of treatment, whereas PBS injections did not (Fig. 3.10B). IL-33 significantly reduced fasting glycemia and amounts of plasma insulin (Fig. 3.10C and D). Consequently, the HOMA-IR, was reduced to normal levels in mice receiving IL-33 treatment (Fig. 3.10E), indicating a significant improvement in insulin resistance.

Figure 3.10 IL-33 treatment reverses insulin resistance in diet-induced obese mice.

(A-E) Mice were fed a HFD for 14-15 weeks, then injected with PBS or IL-33 for 10 consecutive days followed by one IL-33 injection every 2-3 days. (A) Averaged body weights were measured before treatment (d0) and after 14 and 17 days (n = 6). (B) Paired analysis of body weights before and after PBS or IL-33 treatment with each line representing one mouse (n = 6). (C) Fasting plasma insulin (n = 4) and (D) fasting blood glucose (n = 4) were measured at day 14 and 17 after initial treatment. (E) HOMA-IR (n = 4) was determined as an indicator of insulin resistance. Dashed lines indicate normal levels found in control NCD mice. Graphs show averaged data from the indicated number of mice per group analyzed in 2 independent experiments. Error bars represent SD. * p < 0.05 and ** p < 0.01.
3.4 Discussion

A deficit in FOXP3+ Tregs in the VAT is thought to be a key factor in the development of adipose inflammation, resulting in the accumulation of pro-inflammatory macrophages and cytokines which promote insulin resistance (5, 6, 11, 89, 103, 105, 116). Here, we show that the VAT contains a large proportion of ST2+ Tregs which have a phenotype that is distinct from ST2- Tregs and consistent with cells likely to have superior stability and IL-10-dependent suppressive function. IL-33 can be used therapeutically to completely reverse the diet induced obesity-associated reduction in the proportion of ST2+ Tregs, resulting in reduced VAT inflammation and improved metabolic health of obese mice. Thus manipulation of ST2+ Tregs may be a new approach to control obesity-associated inflammation and insulin resistance.

Previous studies have shown that the VAT of lean mice is populated with a high proportion of Tregs that are phenotypically unique compared to peripheral Tregs due to high expression of PPARγ (11, 37) and IL-10 (38). Our study adds to this knowledge by showing that the majority of Tregs in lean VAT express ST2, and that they can be therapeutically expanded with IL-33. Thus VAT Tregs may be uniquely sensitive to IL-33, possibly requiring continual IL-33 stimulation for their survival, stability and/or function (107, 112, 114). A previous study reported that Tregs in the colon also express ST2 (107). Interestingly, we found that the proportion of ST2+ Tregs was significantly higher in lean VAT compared to Tregs isolated from the whole intestinal lamina propria, suggesting that the IL-33:ST2 axis could be relatively more influential in VAT compared to the intestine.

ST2+ Tregs as well as ST2+ Tconvs in lean VAT expressed increased levels of the Th2-related proteins GATA3 and CCR4 (117, 118). Similar to VAT Tregs, ST2 expression on colonic Tregs is also associated with GATA3 expression (107). As GATA3 promotes Treg
stability and function (119, 120), VAT resident ST2+ Tregs may be more stable and potent than their ST2− counterparts. In support of this hypothesis, ST2+ Tregs also have enhanced expression of IL-10 compared to ST2− Tregs. Since IL-10 produced by Tregs inhibits TNF-α production by macrophages (38), and IL-10 is well known to limit obesity-associated inflammation (16, 98) and TNF-α-induced insulin resistance (17), ST2+IL-10+ Tregs are likely key regulators of immune responses in the VAT. Evidence that ST2+ Tregs also express increased levels of Nrp1, which is critical for the maintenance and function of Tregs (113), further supports the hypothesis that ST2+ Tregs possess superior stability and function. Although ST2+ Tregs are not more suppressive than ST2− Tregs in in vitro suppression assays (112, 114), evidence that ST2-deficient Tregs have attenuated suppressive function in vivo (107), supports the notion that this subset may have unique functional properties.

In obese VAT, not only is the frequency of Tregs reduced, but also the proportion of the remaining Tregs expressing ST2 is severely diminished. As a result, there was an overall reduction in the expression of CCR4, GATA3 and Nrp1 in VAT Tregs. It remains unclear whether this reduction is due to the loss of ST2+ Tregs, selective expansion of ST2− Tregs, and/or an intrinsic change in the phenotype of VAT resident ST2+ Tregs. Since, as discussed above, the expression of ST2, GATA3 and Nrp1 have all been associated with Treg expansion, stability and function, the phenotype of Tregs in obese VAT suggests these cells may be less potent. This possibility is supported by our previous finding that Tregs from obese VAT have diminished expression of IL-10 (38). Since the HFD and NCD were not micronutrient matched, we cannot exclude the possibility that there may also be obesity-independent, but diet-related effects.

Therapeutic IL-33 administration corrected the obesity-associated deficit in VAT ST2+ Tregs, suggesting that IL-33 is sufficient to maintain a pool of functionally-specialized VAT-
resident ST2+ Tregs. IL-33 increased both the proportion and absolute numbers of ST2+ Tregs, indicating that IL-33 induced the active expansion of ST2+ Tregs. In contrast, although IL-33 treatment increased the proportion of ST2+ Tconvs, their absolute number did not significantly change. Evidence that IL-33 reprograms DCs to selectively expand ST2+ Tregs (112), and the paucity of ST2+ Tregs which could respond to IL-33 in HFD mice raises the possibility that, at least initially, the effect of IL-33 on the expansion of ST2+ Tregs may be indirect. Contrary to a previous report (114), we found that IL-33 injections did not expand Tregs in the spleen, possibly because the 20 week old mice used in these experiments had a higher age-related (121) proportion of Tregs in the spleen than the younger mice reported by Turnquist et al. (114).

IL-33 therapy also reversed VAT inflammation in obese mice, reducing expression of TNF-α and IL-6 to the levels found in the VAT of healthy lean mice. Since macrophages express almost all of the TNF-α, and a significant amount of IL-6, in the VAT (16), changes in these cytokines likely originate from these cells. Although the reversal of VAT inflammation was correlated with the expansion of ST2+ Tregs in the VAT, IL-33 could also have many other effects in the adipose tissue including the activation of eosinophils (122) and ILC2-mediated beiging of VAT (123). IL-33 also affects macrophages; however, it is unclear whether IL-33 promotes the polarization of alternatively-activated macrophages (124), or enhances inflammatory cytokine production (125), and studies of the effects of IL-33 specifically on VAT-resident macrophages remain to be done. Thus the IL-33-mediated reduction of inflammation is likely due to a combination of anti-inflammatory effects of this cytokine on multiple cell types.

Both TNF-α and IL-6 have previously been shown to induce insulin resistance in adipocytes (126, 127), leading us to the hypothesis that IL-33 may also improve the metabolic parameters of obese mice. One hallmark of obesity is an over-production of insulin, which at
high levels can inhibit IL-10-dependent Treg function (38). IL-33-induced reduction of hyperinsulinemia may thus dampen the inhibitory effect that insulin exerts on Tregs in obesity. Using HOMA-IR to estimate the severity of insulin resistance (115), we found that IL-33 decreased the severity of insulin resistance in obese mice. Notably, we observed the metabolic effects of IL-33 at day 14, but not at day 10 (data not shown). Since IL-33 had already reversed VAT inflammation at day 10, the delayed improvement in insulin sensitivity may thus be subsequent to the effect of reduced inflammation. Longitudinal analysis of individual mice revealed that IL-33 treatment reduced body weight at day 14, coincident with improved metabolic parameters. Although the difference between the body weights of PBS versus IL-33-treated mice was not significant, alleviation of insulin resistance may be partly related to the reduction of body weight.

Our data is consistent with a recent report that the majority of VAT-resident Tregs express ST2, and IL-33 treatment promoted the increase of VAT Tregs, reduced VAT inflammation and improved glucose tolerance of HFD mice (128). In addition, we show that DIO diminishes ST2+ Tregs and the expression of ST2-associated proteins such as CCR4, GATA3 and Nrp1. IL-33-mediated correction of the Treg deficit and VAT inflammation lasted as long as 25 days after initial treatment (data not shown), suggesting a prolonged therapeutic effect. IL-33 has also been reported to reduce genetically-driven insulin resistance in leptin deficient (ob/ob) mice (129). However, since ob/ob mice have an abnormal T cell compartment (130) and leptin has direct effects on T cells, including Treg proliferation (62), it is difficult to interpret these findings as they relate to T cells.

In summary, we show that more than half of the Tregs in normal VAT have a unique Th2-like and ST2+ phenotype, and express markers associated with potent and activated Tregs
(i.e. IL-10, Nrp-1). Diet induced obesity severely diminishes the proportion of VAT Tregs with this phenotype, and this effect can be completely reversed by treatment with IL-33. Treatment with IL-33 also reverses the infiltration of macrophages into the VAT and the accumulation of pro-inflammatory cytokines. Subsequent to the reduction in inflammation, IL-33 reduces body weight, hyperinsulinemia and insulin resistance, resulting in an anti-obesity effect that targets multiple aspects of DIO. Thus IL-33-induced therapeutic expansion of ST2\(^+\) Tregs may be a new approach to control obesity-associated inflammation and insulin resistance.
Chapter 4: Adipocyte-derived factors increase IL-10 production by regulatory T cells and modulate their metabolic activity

4.1 Introduction

The adipose tissue (AT) is now recognized as an immunologically active compartment. Immune cells residing in the AT influence the metabolic parameters of the AT, and conversely, adipocytes can have effects on immune cells. Since macrophages were the first cells thought to contribute to adipose tissue inflammation, several groups have investigated the interaction between macrophages and adipocytes. The over-production of pro-inflammatory cytokines, such as TNF-α by macrophages is thought to interfere with insulin signaling in adipocytes and promote insulin resistance in the AT of obese mice and humans (105, 131). The influence of adipocytes on macrophages has been studied, but the results remain inconclusive. For example, some studies suggest that adipocytes or adipocyte-derived soluble factors promote the production of pro-inflammatory cytokines such as IL-6 (132-134) and TNF-α (132) by macrophages. On the contrary, others have suggested anti-inflammatory effects of adipocytes such as the inhibition of IL-12p40 secretion (135) and polarization towards M2 macrophages (136). Adipocyte-derived factors have also been described to increase IFN-γ production and proliferation of CD4+ T cells (137), indicating that a wide range of immune cells may directly interact with adipocytes.

More recently, regulatory T cells (Tregs) have been shown to partake in controlling the inflammatory milieu of the AT and preventing insulin resistance in obesity (10, 11). AT-resident Tregs are phenotypically distinct than those found in the periphery or other tissues due to their high expression of PPARγ (34, 37). These data suggest that their unique phenotype may be a result of modulation by their neighboring adipocytes, but whether or not adipocyte-derived
factors can affect Tregs is not known. Notably, AT-resident Tregs have substantially increased expression of the anti-inflammatory cytokine IL-10 (38), indicating that they may have superior suppressive function compared to peripheral Tregs. Since Tregs residing in the AT are distinct and can be found in close proximity to adipocytes, we asked whether adipocytes-derived factors can influence their phenotype and function.

Here, we show that 3T3-L1 adipocyte-derived factors enhance the production of IL-10 by Tregs, thereby increasing the ability of Tregs to suppress macrophages. Interestingly, 3T3-L1 adipocyte-derived factors increase the rate of cellular metabolism of Tregs, and that this modulation of cellular metabolism is required for the effects of adipocytes on IL-10 production.

4.2 Materials and methods

Mice and cell isolation. 8-12 week old male C57Bl/6 or C57Bl/6 FOXP3-RFP x IL10-GFP mice (bred in-house) were sacrificed, and spleens and lymph nodes were collected and mashed. Bone marrows were obtained from the femur and tibia of C57Bl/6 or C57Bl/6 IL-10R2+/− mice. CD4+ T cells were isolated with EasySep CD4+ selection kit (StemCell Technologies), and CD25+ selection was performed with CD25 microbeads (Miltenyi Biotec).

Cell lines maintenance and differentiation. 3T3-L1 cells were maintained in DMEM supplemented with 10% newborn bovine serum, and were differentiated into mature adipocyte-like cells in DMEM supplemented with 10% FBS. Briefly, 3T3-L1 cells were cultured past confluence then incubated in media containing insulin, dexamethasone and 3-isobutyl-1-methylxanthine for 2-3 days, followed by insulin containing media for 2-3 days (138). The cells were then cultured for an additional 3 days in DMEM 10% FBS to complete the differentiation.
Fully differentiated 3T3-L1 were cultured in fresh DMEM 10% FBS for 3 days, then the cell-free media was collected and used as adipocyte-conditioned media in subsequent experiments.

**T cell culture.** RPMI-1640 was supplemented with 10% FBS, GlutaMAX, HEPES, and penicillin G and streptomycin. T cells were stimulated with plate-bound α-CD3 (10 μg/ml), soluble α-CD28 (2 μg/ml) and 100 U/ml IL-2, and 50% of the media consisting of control (Ctrl: DMEM 10% FBS) media or adipocyte-conditioned media (CM: DMEM 10% FBS conditioned by differentiated 3T3-L1 adipocytes) for 2 or 4 days. For suppression assays, Tregs were pre-incubated with control or conditioned media for 24 hours in the presence of IL-2, then co-cultured with proliferation dye eFluor670 (eBioscience)-stained Tconvs (1 Treg:4 Tconvs ratio) in the presence of α-CD3/28 coated beads for 4 days. Where indicated, 2.5nM oligomycin, 7nM rotenone + 45nM Antimycin A, 50µM etomoxir, 0.5mM 2-Deoxy-D-glucose (2-DG), 20nM rapamycin or 2µM Akti-1/2 were added to the culture media.

**Bone marrow derived macrophages (BMDM).** BMDMs were differentiated with GM-CSF for 6 days then incubated with 10 ng/ml IL-10, or media containing 12.5% of medium conditioned by Tregs or Tconvs (cultured as above with control or conditioned media) for 4 hours, then 10 ng/ml of LPS was added for 20 hours.

**Flow cytometry, ELISA, cytometric bead array and RT-PCR.** All flow cytometry and ELISA antibodies were commercially obtained from BD Pharmingen or eBioscience. Fixable viability dye eFluor780 was purchased from eBioscience. Cytometric bead array was performed
using BD CBA mouse Th1/Th2/Th17 kit (BD Biosciences). RT-PCR data were normalized to 18s ribosomal RNA.

**Metabolic assays.** T cells were stimulated for 4 days in control or conditioned media, then were adhered onto 96 well plates using Cell Tak (BD) in unbuffered media in a non-CO₂ incubator for 1 hour. Glycolysis results in the release of protons from cells, and thus the extracellular acidification rate (ECAR) can be used as an indication of glycolytic rate. Oxygen is consumed in the mitochondria during OXPHOS, and the oxygen consumption rate (OCR) is used as a measure of the rate of OXPHOS. ECAR was measured using a Glycolysis Stress Test Kit (Seahorse Bioscience), and OCR was measured using a Mito Stress Test Kit (Seahorse Bioscience). ECAR and OCR was measured using an XF⁹6 Extracellular Flux Analyzer (Seahorse Bioscience) (139).

**Statistical analysis.** Unpaired Student’s T tests were used to analyze significance. The $p$ values are indicated as follows: * $p < 0.05$ and ** $p < 0.01$.

4.3 **Results**

4.3.1 **Adipocyte-derived factors delay T cell activation and enhance Treg viability.** To obtain adipocyte-derived factors, we differentiated 3T3-L1 cells, an immortalized cell line which has been widely used to study adipocyte biology, into mature adipocytes. Differentiated 3T3-L1 cells were cultured for 3 days to obtain adipocyte-conditioned media (adipo CM), and to investigate whether adipocytes can affect Tregs and Tconvs, we subsequently used this adipocyte-conditioned media to culture Tregs and Tconvs *in vitro.*
To determine whether adipo CM affected T cell activation, we stimulated Tregs and Tconvs with plate bound anti-CD3 and soluble anti-CD28 in the presence of IL-2, and measured the expression of activation marker CD44. After 24 hours of activation, adipo CM reduced the induction of CD44 expression on Tregs by 33.5 ± 0.7%, and on Tconvs by 57.2 ± 15.2% compared to control (Fig. 4.1A). After 48 hours of activation, adipo CM continued to attenuate CD44 expression by 38.7 ± 12.3% on Tregs, but the effect of adipo CM on CD44 expression by Tconvs were no longer evident. Adipo CM also mildly, but significantly, attenuated the up-regulation of CD25 expression, another hallmark of T cell activation, by 22.8 ± 2.2% on Tregs after 24 hours of activation (Fig. 4.2A), but this effect was not apparent 48 hours post-activation. Adipo CM did not affect expression of CD25 by Tconvs. These data suggest that adipocyte-derived soluble factors may have attenuated or delayed the activation of Tregs and Tconvs.
Figure 4.1 Adipocytes dampen CD44 up-regulation and enhances Treg viability.

CD4^+^CD25^+^ Tregs and CD4^+^CD25^- Tconvs were activated with plate bound α-CD3, soluble α-CD28 and IL-2 in the presence of control or adipo CM. (A) CD44 MFI was measured by flow cytometry after 24 or 48h (n = 3). (B) Cell viability was measured using eBioscience Fixable Viability Dye eFluor780, and (C) FOXP3 expression was measured by flow cytometry after 4 days of culture (n = 12). Dot plots and histograms show representative data. Graphs show averaged data. Error bars represent SD. * p < 0.05 and ** p < 0.01.
To ask whether adipo CM affects the viability of Tregs and the expression of Treg-associated proteins, we cultured Tregs and Tconvs in control media or adipo CM for 4 days. Tregs that were cultured in adipo CM had a greater proportion of viable cells (51.0 ± 8.8%) compared to Tregs in control media (33.1 ± 7.4%) (Fig. 4.1B), whereas FOXP3 expression remained equivalent between Tregs cultured in adipo CM and control media (Fig. 4.1C). On the other hand, the percentage of viable cells and FOXP3 expression of Tconvs were not affected by adipocyte-derived factors. Exposure to adipo CM had no effect on the proliferation (Fig. 4.2B), and the expression of Treg-associated markers CTLA-4, CD25, ICOS, CD39 and LAP (Fig. 4.2C). These data indicate that adipocyte-derived factors enhance the survival of Tregs in vitro.
Figure 4.2 Adipocytes do not affect proliferation, and the expression of CTLA-4, ICOS, CD39 and LAP by Tregs.

CD4+CD25+ Tregs and CD4+CD25- Tconvs were activated with plate bound α-CD3, soluble α-CD28 and IL-2 in the presence of control or adipo CM. (A) The percentage of cells expressing CD25 was measured by flow cytometry after 24 or 48h (n = 3). (B) Proliferation was assessed by division index measured by the dilution of cell proliferation dye after 4 days of culture (n = 3). (C) The expression of levels of CTLA-4, CD25, ICOS, CD39 and LAP were measured by flow cytometry after 4 days of culture (n = 2-6). Graphs show averaged data. Error bars represent SD. * p < 0.05 and ** p < 0.01.
4.3.2 Adipocyte-derived factors enhance the production of IL-10 by T cells

We next determined whether adipo CM affected the expression of proteins commonly associated with the suppressive function of Tregs. Tregs or Tconvs were stimulated in vitro in the presence of adipo CM or control media, and the release of IL-10 in the media was analyzed by ELISA. Adipo CM significantly enhanced Treg-produced IL-10 by 62 ± 20% (Fig. 4.3A). Using cells isolated from FOXP3-RFP x IL-10-GFP mice, flow cytometry analysis also showed that adipo CM significantly increased the expression of IL-10-GFP by 37 ± 12% in Tregs (Fig. 4.3B). Although not statistically significant, trends from these experiments suggested that adipo CM also increased IL-10 production by Tconvs.

![Graph A](image1)

![Graph B](image2)

**Figure 4.3 Adipocytes enhance IL-10 production by Tregs.**

(A) CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconvs were activated in the presence of control or adipo CM for 4 days, and the release of IL-10 into the media was measured vs ELISA \( (n = 6) \). (B) CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconvs from FOXP3-RFP x IL-10-GFP mice were activated in the presence of control or adipo CM for 48h, and the expression of IL-10 was detected by flow cytometry \( (n = 8-11) \). Histograms show representative data. Graphs show averaged data. Error bars represent SD. * \( p < 0.05 \) and ** \( p < 0.01 \).
To determine whether the up-regulation of IL-10 is specifically due to 3T3-L1 adipocytes rather than a general effect of media conditioned by any cell types, we performed the same experiment using 3T3 fibroblast-conditioned media. Contrary to adipo CM, 3T3-conditioned media inhibited the production of IL-10 by Tregs (Fig. 4.4), indicating that the effect on IL-10 was not a general consequence of being cultured in CM. Using cytometric bead array, we found that adipo CM had no effect on the secretion of IFN-γ, IL-17A, TNF-α or IL-2 by Tregs (Fig. 4.5). In agreement with the trends observed in Fig. 4.3, our cytometric bead array analysis showed that adipo CM significantly increased the production of IL-10 by Tconvs (Fig. 4.5). Furthermore, adipo CM also enhanced the secretion of TNF-α by Tconvs. Since adipo CM did not affect the expression of proteins important for Treg function, such as CTLA-4, CD39 and LAP (140) (Fig. 4.2C), these data suggest that adipocyte-derived factors were able to enhance IL-10 production by both Tregs and Tconvs.

**Figure 4.4 3T3 fibroblast-conditioned media reduce IL-10 production by Tregs.**

CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconvs were stimulated in the presence of control or 3T3 fibroblasts-conditioned media for 4 days. The release of IL-10 into the media was measured by ELISA (n = 3). Graphs show averaged data. Error bars represent SD. * p < 0.05 and ** p < 0.01.
Adipocytes enhance the production of IL-10, but not IFN-γ, IL-17A, TNF-α and IL-2 by Tregs. CD4^+CD25^+ Tregs and CD4^+CD25^- Tconvs were activated in the presence of control or adipo CM for 4 days, and the release of IFNγ, IL-10, IL-17A, TNFα and IL-2 were measured by cytometric bead array (n = 6). Graphs show averaged data. Error bars represent SD. * p < 0.05 and ** p < 0.01.

4.3.3 Adipocyte-derived factors enhance the IL-10-dependent suppressive function of Tregs

IL-10 is a key anti-inflammatory cytokine produced by Tregs that is critical for controlling inflammation in vivo (85, 86), and adipose tissue-resident Tregs have been shown to express elevated levels of IL-10, indicating the importance of IL-10 in maintaining immune homeostasis in the adipose tissue (11, 38). Since adipo CM enhanced IL-10 production by Tregs, we investigated whether adipo CM has any effects on the ability of Tregs to suppress target cells.
Figure 4.6 Adipocytes promote the ability of Tregs to suppress macrophages.

(A) CD4+CD25+ Tregs were cultured with control or adipo CM for 24h, then added in increasing numbers to CFSE-labeled CD4+CD25- Tconvs cells. The co-cultures were stimulated with anti-CD3/28 beads for 4 days. Tconv cell proliferation was measured by CFSE dilution and division index (DI). Representative data of the suppression assay at a 4:1 ratio of Tconvs:Tregs are shown (n = 3). (B) Bone marrow derived macrophages (BMDMs) were incubated with media, or media containing 12.5% of medium conditioned by CD4+CD25+ Tregs that had been stimulated in the presence of control or adipo CM, for 4 hours. BMDMs were then stimulated with 10 ng/ml LPS and cultured for an additional 20 hours and TNF-α production was measured (n = 4-5). The % suppression of TNF-α release compared to cultures without Treg conditioned medium is shown. The left panel show representative data, and the right panel show averaged data. Error bars represent SD. * p < 0.05 and ** p < 0.01.

As expected, prior exposure to adipo CM did not alter the ability of Tregs to suppress Tconvs in an assay which is known to be IL-10-independent (Fig. 4.6A). To test IL-10-dependent

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suppression, we detected TNF-α production from macrophages cultured in media conditioned by Tregs that had been cultured in the presence of adipo CM or control media. We observed that exposure to adipo CM enhanced the ability of Tregs to suppress bone marrow-derived macrophages in a contact-independent manner in this assay (Fig. 4.6B). Tregs that had been exposed to adipo CM suppressed TNF-α production from macrophages by 75.8 ± 5.8%, whereas Tregs in control media suppressed TNF-α production by 60.4 ± 7.0% \((P = 0.017)\). Notably, adipo CM did not affect TNF-α production by macrophages (data not shown). To confirm that the effects on TNF-α secretion by macrophages were IL-10 dependent, we performed the assay using macrophages derived from the bone marrow of IL-10R2−/− mice, and found that Tregs were unable to suppress TNF-α production from macrophages that could not respond to IL-10 (Fig. 4.7). Together, these data indicate that adipocyte-derived factors enhanced the ability of Tregs to exert their suppressive function in an IL-10-dependent manner.

**Figure 4.7 Suppression of TNF-α production by macrophages is dependent on IL-10.**

WT or IL-10R2−/− bone marrow derived macrophages (BMDMs) were incubated with media, or media containing 12.5% of medium conditioned by CD4+CD25+ Tregs that had been stimulated in the presence of control or adipo CM, for 4 hours. BMDMs were then stimulated with 10 ng/ml LPS and cultured for an additional 20 hours and TNF-α production was measured \((n = 3-5)\). The % suppression of TNF-α release compared to cultures without Treg conditioned medium is shown. Graph shows averaged data. Error bars represent SD. * \(p < 0.05\) and ** \(p < 0.01\).
4.3.4 Adipocyte-derived factors alters Treg metabolism

Tregs are thought to use lipid oxidation as a primary means for cellular metabolism, and the addition of exogenous fatty acids can promote generation of Tregs *in vitro* (45). In addition, increased glycolytic rate has been linked to reduced Treg function (141), and the inhibition of OXPHOS by rotenone decreased Treg survival (142). Since adipo CM enhanced Treg function and survival, we next investigated whether adipocyte-derived factors might alter Treg metabolism. We first used an extracellular metabolic flux analyzer to measure extracellular acidification rate (ECAR) by measuring the release of protons, which are produced during glycolysis. Exposure to adipo CM did not affect ECAR in Tregs and Tconvs (Fig. 4A). However, we found that adipo CM significantly increased the oxygen consumption rate (OCR) of both Tregs and Tconvs, indicating increased rate of OXPHOS (Fig. 4B). However, the maximal respiratory capacity of Tregs and Tconvs upon the addition of FCCP was not affected by adipo CM. These data suggest that adipo CM preferentially increased the mitochondrial OXPHOS of Tregs and Tconvs.
Figure 4.8 Adipocyte-derived factors increases OXPHOS.

CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconvs were activated in the presence of control or adipo CM for 4 days, and
(A) a glycolytic stress test was performed to measure ECAR with the additions of glucose (glc), oligomycin (oligo) and 2-deoxy-D-glucose (2-DG) (n = 4). (B) OCR was measured in a mito stress test in the presence of glucose, and the additions of metabolic inhibitors oligomycin (oligo), FCCP, and rotenone + antimycin A (Rot/AA) (n = 2).

Representative plots are shown. * p < 0.05 and ** p < 0.01.

4.3.5 Alteration of Treg metabolism is required for the effect of adipocyte-derived factors on IL-10 production

To investigate whether the adipo CM-induced changes in cellular metabolism were required for the effect of adipo CM on IL-10, we cultured Tregs and Tconvs in the presence of control media or adipo CM with the addition of several metabolic inhibitors. We first used inhibitors of OXPHOS: oligomycin, which inhibits ATP synthase (143), and a combination of rotenone and antimycin A (Rot/AA), which inhibit complex I and complex III of the electron transport chain,
respectively (144). Both oligomycin and Rot/AA inhibited the effect of adipo CM on IL-10 (Fig. 4.9A), indicating that the increased rate of OXPHOS was required for the enhancement of IL-10 expression by adipo CM. On the other hand, the inhibition of fatty acid oxidation using etomoxir (145) did not interfere with the effect of adipo CM (Fig. 4.9B), suggesting that fatty acid oxidation was not involved in the response to adipo CM.

We next used the glucose analog 2-deoxy-D-glucose (2-DG) to inhibit glycolysis (146), and found that 2-DG was also able to block the effect of adipo CM. Since 2-DG inhibits both glycolysis and glycolysis-dependent OXPHOS (147), these data suggest that glycolysis is also required for adipo CM-mediated increase in IL-10 (Fig. 4.9C). Furthermore, glycolysis provides metabolic intermediates for the synthesis of proteins (148), and 2-DG treatment may thus limit the capacity of Tregs to synthesize proteins more IL-10. The Akt/mTOR pathway plays a major role in promoting glucose metabolism in T cells (149-151), thus we asked whether Akt or mTOR are involved in mediating the effect of adipo CM. Inhibition of mTOR by rapamycin interfered with the effect of adipo CM; however, adipo CM still significantly increased IL-10 production by Tregs in the presence of Akt inhibitor Akti1/2, although this effect is reduced (Fig. 4.9D). These data indicate that OXPHOS, glycolysis and the Akt/mTOR pathway are all required for the effect of adipo CM on IL-10 production.
Figure 4.9 OXPHOS and glycolysis are both required for the effect of adipocytes on IL-10.

CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconv from FOXP3-RFP x IL-10-GFP mice were activated in the presence of control or adipo CM for 2 days, with the addition of (A) oligomycin, rotenone + antimycin A, (B) etomoxir, (C) 2-deoxy-D-glucose, and (D) rapamycin or Akti-1/2 (n = 3-6). The percentage of cells expressing IL-10 was measured by flow cytometry. Graphs show averaged data. Error bars represent SD. * p < 0.05 and ** p < 0.01.

4.4 Discussion

Tregs resident in the adipose tissue are known to have a unique phenotype compared to those found in the periphery (10, 11); however, which environmental factors regulate their phenotype is unclear. Here, we show that adipocytes themselves may play a role in driving the phenotypic specialization of adipose tissue-resident Tregs.

Our finding that adipocyte-derived factors dampened up-regulation of CD44 following T cell receptor activation suggests that adipocytes may delay T cell activation. We plan to investigate whether this is true using additional activation markers such as CD69 and Nur77. Since T cell activation is associated with an increased ratio of glycolysis to OXPHOS (152), we
can speculate that the delayed T cell activation may be related to the adipo CM-induced increase in OXPHOS.

Most interestingly, adipocyte-derived factors increased the expression of IL-10, but not other cytokines or other proteins associated with Treg function. The phenotype of adipo CM-exposed Tregs resembles the phenotype of adipose tissue-resident Tregs, which also distinctively express increased levels of IL-10 (37, 38). The adipo CM-induced increase in percentage of viable cells \textit{in vitro} likely contributed to the overall increase of IL-10 secretion as detected by ELISA; however, using the FOXP3-RFP x IL-10-GFP mice, we were able to determine that adipo CM also increased IL-10 expression on a per cell basis. Notably, this increase in IL-10 production resulted in increased IL-10-dependent Treg function, as shown in the suppression of TNF-\(\alpha\) production by macrophages. Similarly, the enhanced IL-10 expression by adipose tissue-resident Tregs may be critical to control TNF-\(\alpha\) by macrophages, which could promote insulin resistance if left unchecked (105, 131). Our data suggest that adipocyte-derived factors enhance IL-10 production by Tregs, and thus increase the capacity of Tregs to suppress inflammatory responses by macrophages. Our data indicate that adipocyte-derived factors also increased IL-10 production by Tconvs, although not as substantial as the increase of IL-10 observed in Tregs, suggesting that Tregs may be more sensitive to modulation by adipocytes in the adipose tissue. Additionally, adipocyte-derived factors enhanced TNF-\(\alpha\) secretion by Tconvs; however, almost all of the TNF-\(\alpha\) produced in the adipose tissue \textit{in vivo} is from macrophages (16), and thus Tconvs may not be as significant source of TNF-\(\alpha\).

Recent studies have shown that Tregs primarily use OXPHOS, and on the contrary, effector Tconvs utilize heightened glycolytic metabolism (45). Adipocyte-derived factors substantially increased the rate of OXPHOS in Tregs and Tconvs. Our metabolic data thus
suggest that adipocyte-derived factors skewed the metabolism of T cells towards mitochondrial respiration. To our knowledge, no studies thus far have reported a link between cellular metabolism and IL-10 production. Here, we show that adipo CM-mediated increase in IL-10 expression is dependent on the modulation of OXPHOS. Despite the reported reliance of Tregs on lipid oxidation (45) for cellular metabolism, the inhibition of lipid oxidation using etomoxir did not inhibit the effect of adipo CM on Tregs. These data suggest that the oxidation of glucose, rather than lipids, is used to fuel IL-10 production. Even though adipo CM did not affect glycolysis, the inhibition of glycolysis using 2-DG also dampened the effect of adipo CM. This finding suggests that high rates of glycolysis is necessary for enhanced IL-10 production, perhaps by providing key building blocks for the synthesis of new proteins (153). In parallel, others have suggested that optimal cytokine production by effector T cells also requires glycolysis (152). Furthermore, we found that adipo CM-mediated increase in IL-10 production is dependent on mTOR activity. This finding was expected since mTOR is a key signaling molecule that orchestrates T cell metabolism (150, 154, 155), and the inhibition of mTOR diminishes both glycolysis and mitochondrial OXPHOS (156, 157). In the presence of Akt inhibition, adipo CM still enhanced IL-10 production by Tregs, although to a lesser extent, suggesting that an Akt-dependent and -independent modulation of mTOR activity may be involved in mediating the effect of adipo CM. Alternatively, since mTOR activity is critical for protein translation (158), rapamycin-mediated mTOR blockade may simply inhibit the translation of new IL-10.

It remains unclear what factor(s) specifically mediate the effect of adipocytes on Tregs and Tconvs. Adipocytes secrete factors such as free fatty acids and adipokines, which can affect immune cells. Free fatty acids for example, have been shown to activate TLR2 and TLR4 on
macrophages (159, 160). Furthermore, saturated fatty acids activate the inflammasome and promote IL-1β production in macrophages (42). Adipokines such as leptin and adiponectin also have direct effects on immune cells. Adiponectin has been reported for both pro- and anti-inflammatory effects. For example, adiponectin can promote Th1 cell differentiation in human CD4+ T cells (161), and yet has also been shown to inhibit CD8+ T cell proliferation and IFN-γ production (64). Interestingly, adiponectin can increase IL-10 expression in human macrophages (162), suggesting that the effect of adipocyte-derived factors on IL-10 production by Tregs may be partially mediated by adiponectin. Leptin promotes Th1 (61) and Th17 cells (163), and inhibits the proliferation of Tregs (62). In addition, the leptin receptor is required for glycolysis and mitochondrial respiration (164), suggesting that the production of leptin by adipocytes may also in part mediate the effect of adipocyte-derived factors on cellular metabolism. Hence it is likely a combination of soluble factors produced by adipocytes that together promote the unique phenotype and function of VAT Tregs.

In summary, we report that adipocyte-derived factors modulate T cell activation, and enhance IL-10 dependent Treg function. We show that the effect of adipocyte-derived factors on IL-10 expression is dependent on an increased rate of mitochondrial respiration, and an optimal rate of glycolysis. Since Tregs residing in the adipose tissue are important in controlling obesity-associated inflammation and metabolic disorders, our study fills a missing gap of knowledge on the interaction between Tregs and adipocytes. Overall, our study suggests that adipocyte-derived factors may contribute to the unique phenotypic and functional specialization of adipose tissue-resident Tregs in controlling macrophage-driven adipose tissue inflammation via IL-10.
Chapter 5: Conclusion

To summarize, my findings suggest that insulin inhibits IL-10-dependent Treg function, IL-33 expands AT-resident Tregs and inhibits AT inflammation, and adipocyte-derived factors promote IL-10 secretion by Tregs (Fig. 5.1A-C). The three chapters above elucidate three novel and independent mechanisms for the regulation of Tregs that contribute to the control of AT inflammation in obesity.

Figure 5.1 Tregs are regulated by adipocyte-derived factors, insulin and IL-33.

(A) Adipocyte-derived soluble factors promote IL-10-producing Tregs that are able to effectively suppress inflammation. (B) High levels of insulin, such as those found in DIO, inhibit IL-10-dependent Treg function. (C) IL-33 therapy expands Tregs in the AT, reduces AT inflammation and ameliorates insulin resistance.

5.1 Future directions

The significance of these studies have been highlighted above in their respective chapters. In this section, I will briefly evaluate the limitations of the above data, and how they can be improved with further experimentations.
5.1.1 Chapter 2: Insulin inhibits IL-10-dependent Treg function

In this chapter, I showed that insulin inhibits IL-10 production by Tregs in an AKT dependent manner in vitro, and then generated parallel data showing reduced expression of IL-10 by VAT Tregs in hyperinsulinemic HFD mice. As a future direction, it will be important to investigate the biochemical basis for how insulin activated AKT in Tregs and to formally demonstrate a functional role for insulin on Tregs in vivo.

First, I showed that despite equivalent expression of insulin receptors, insulin more strongly activates AKT in Tregs than Tconvs. To investigate the mechanism of this differential activation, we can measure whether Tregs have increased expression or phosphorylation of the adaptor proteins IRS-1/2 by Western blot. A number of biochemical experiments could be performed to dissect this pathway.

IGF-1 receptor can also bind insulin at a low affinity (165). In the phospho flow experiments, I used a high dose of insulin that could possibly activate the IGF-1 receptor. Further studies are thus required to rule out the possible effect of insulin on IGF-1R-mediated AKT phosphorylation. However, in our functional assays, I used a much lower dose of insulin that should not activate the IGF-1R. Interestingly, despite similar structure and downstream signaling activity to the insulin receptor, IGF-1R signaling in Tregs has been suggested to be beneficial to Tregs (166, 167).

Using HFD mice, I showed that hyperinsulinemia is correlated with reduced expression of IL-10 in VAT-resident Tregs. However, further experiments are needed to prove that insulin has a causal role in the inhibition of IL-10 expression in Tregs in vivo. I have now crossed FOXP3-Cre mice with insulin receptor floxed (InsR fl/fl) mice to obtain mice with insulin receptor deletion specifically in Tregs. We plan to feed these mice a HFD to induce hyperinsulinemia. I
hypothesize that FOXP3-Cre x InsR fl/fl mice will be resistant to the effect of insulin on Tregs, and thus VAT Tregs will retain their normal level of IL-10 expression, and consequently these mice will have reduced inflammation in the VAT. A fellow graduate student is currently performing experiments with the FOXP3-Cre x InsR fl/fl mice to answer whether insulin receptor signaling affects Tregs in vivo.

5.1.2 Chapter 3: IL-33 reverses obesity-induced deficit in ST2+ Tregs, and ameliorates adipose tissue inflammation and insulin resistance.

In this chapter, I showed that IL-33 expanded ST2+ Tregs in the VAT. Further studies are needed to show that this restoration of ST2+ Tregs is dependent on a direct effect of IL-33 on VAT Tregs. Our attempts to culture VAT Tregs in vitro in the presence or absence of IL-33 have been unsuccessful due to poor cell viability. However, Vasanthakumar et al was able to demonstrate that IL-33 exposure can directly promote the expansion of VAT Tregs via in vitro and mixed bone marrow chimera experiments (128).

I next showed that IL-33 also reversed VAT inflammation and insulin resistance, but whether this reversal is dependent on the IL-33-induced expansion of VAT ST2+ Tregs is not known. To answer this question, ST2(fl/fl) mice, which are not currently commercially available, could be crossed with FOXP3-Cre mice to obtain ST2 deletion specifically in Tregs. Another possible approach would be to administer IL-33 to FOXP3-DTR mice after the ablation of Tregs; however, the development of inflammation resulting from Treg ablation is a major caveat to this approach. I attempted to perform adoptive Treg transfer into HFD mice, with the goal to transfer Tregs isolated from WT vs ST2+/ mice. Unfortunately, I could not successfully detect the donor
Tregs in the VAT of recipient mice, and could not detect a reduction of VAT inflammation after the adoptive transfer.

A major outstanding question is whether human VAT Tregs also have increased expression of ST2 and ST2-associated proteins (CCR4, GATA3, Nrp1 and IL-10), and whether the expression levels of these proteins are related to the metabolic health of patients. I have established a collaboration with clinicians at Richmond General Hospital to obtain omental adipose tissue samples from bariatric surgery patients and lean controls. These studies will also answer whether Tregs in obese human VAT have reduced IL-10 expression, akin to what we have observed in obese mice in chapter 3. Studies are ongoing to answer this question and further characterize human VAT Tregs. Another graduate student will continue this work after my completion in the lab.

5.1.3 Chapter 4: Adipocyte-derived factors modulate the cellular metabolism and IL-10 production of Tregs

In this study, I showed that 3T3-L1 adipocyte-derived factors modulate Tregs. To prove that the effect of 3T3-L1 adipocytes on Tregs are relevant, I would first need to investigate whether the effect of 3T3-L1 adipocyte-conditioned media can be recapitulated using primary adipocyte conditioned media.

I have shown that exposure to adipo CM has beneficial effects on IL-10-dependent Treg function, but we have not been able to pinpoint what specific factors in the conditioned media have effects on Tregs and Tconvs. I attempted using denaturation and size exclusion columns to answer this question, but no one method could consistently reverse the effect of adipo CM, suggesting that multiple factors are involved in mediating this effect. I found that fatty acids
alone could not recapitulate the effects of adipo CM on Tregs; however, these preliminary experiments showed that fatty acids could improve the viability of Tregs cultured at low cell density. Thus, further studies are ongoing on whether fatty acids could be used to improve human Treg expansion protocols. In addition, it would be intriguing to investigate whether human adipocyte-derived factors also have similar effects on human Tregs and Tconvs.

5.2 Overall significance

Overall, my research has significantly contributed to the lab, and to the fields of immunology and metabolism. My research has initiated and established a new line of research for our lab at the interface between these two disciplines. My project has led to a collaboration with the Bariatric Surgery Program at Richmond General Hospital. My research has equipped the lab with techniques, funding and collaborations to continue in the field of immunometabolism.

Immunometabolism is a relatively new field of investigation, and the role of adaptive immunity in obesity has only emerged in the past 5 years. Chapter 2 shows for the first time that excessive levels of insulin has a negative effect on the function of Tregs, suggesting that hyperinsulinemia inhibits Tregs and thus promotes inflammation in obesity. I then showed that IL-33 can therapeutically reverse AT Treg deficit and AT inflammation in obesity, and subsequently ameliorates insulin resistance. Although inflammation is known to promote insulin resistance, current treatments for obesity-associated disorders may not effectively target inflammation. Our study in chapter 3 suggests that the incorporation of IL-33 into a therapeutic regimen may improve its efficacy to target both insulin resistance and inflammation in obesity. In chapter 4, I reported a previously unknown interaction between Tregs and adipocytes, and showed how an alteration in Treg metabolism can modulate its function.
Put together, these studies suggest that insulin, IL-33 and adipocyte-derived factors regulate IL-10-producing Tregs in the VAT. Furthermore, these three novel mechanisms of Treg regulation may be important for the functional specialization of VAT-resident Tregs to effectively control obesity-associated VAT inflammation. Our research has presented the possibilities that these pathways can be exploited for beneficial effects in therapeutic strategies for obesity-associated disorders. Finally, chapters 2, 3 and 4 above have broadened our understandings of the cellular biology of Tregs in the contexts of cell signaling, cytokine regulation and cellular metabolism, respectively.
Bibliography


