Characterization of regulatory T cells in obese omental adipose tissue in humans

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Abbreviations
T2D: type 2 diabetes
AT: adipose tissue
Tconv: conventional T cell
Treg: regulatory T cell
Th2: T helper type 2 cell
SVF: stromal vascular fraction
HOMA-β: homeostatic model assessment of β-cell function

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Abstract

Obesity-associated visceral adipose tissue (AT) inflammation promotes insulin resistance and type 2 diabetes (T2D). In mice, lean visceral AT is populated with anti-inflammatory cells, notably regulatory T cells (Tregs) expressing the IL-33 receptor ST2. Conversely, obese AT contains fewer Tregs and more pro-inflammatory cells. In humans, however, there is limited evidence for a similar pattern of obesity-associated immunomodulation. We used flow cytometry and mRNA quantification to characterize human omental AT in 29 obese, 18 of whom had T2D. Patients with T2D had increased proportions of inflammatory cells, including M1 macrophages, with positive correlations to body mass index. In contrast, Treg frequencies negatively correlated to BMI but were comparable between T2D and non-T2D individuals. Compared to human thymic Tregs, omental AT Tregs expressed similar levels of FOXP3, CD25, IKZF2, and CTLA4, but higher levels of PPARG, CCR4, PRDM1, and CXCL2. ST2, however was not detectable on omental AT Tregs from lean or obese subjects. This is the first comprehensive investigation into how omental AT immunity changes with obesity and T2D in humans, revealing important similarities and differences to paradigms in mice. These data increase our understanding of how pathways of immune regulation could be targeted to ameliorate AT inflammation in humans.
Introduction

Obesity-associated visceral adipose tissue (AT) inflammation is thought to promote insulin resistance and contribute to the development of type 2 diabetes (T2D). In lean mice, immune cells resident in visceral AT are typically associated with type 2 immunity, and include regulatory T cells (Tregs), T helper type 2 (Th2) cells, M2 macrophages, group 2 innate lymphoid cells, and eosinophils. Conversely, visceral AT in mice fed with a high fat diet is characterized by a reduction in type 2 immune cells and a parallel accumulation of pro-inflammatory M1 macrophages and cytokines, such as IL-6, TNF-α, CCL2, IFN-γ, and leptin. These inflammatory cytokines are thought to directly contribute to insulin resistance and ultimately the development of T2D [1-4].

Of particular interest is the role of Tregs in maintaining metabolic homeostasis. Evidence that Treg depletion results in AT inflammation and insulin resistance in mice [5, 6] suggests that these cells may be central to maintaining the normal complement of type 2 immune cells. Notably, in mice, visceral AT Tregs acquire a Th2-like phenotype distinct from their peripheral counterparts, with heightened expression of Gata3, Ccr4, and Il10 [7]. This Th2-like phenotype seems to be driven by the IL-33/ST2 pathway, since the majority of visceral AT Tregs in lean mice express the IL-33 receptor ST2, and in vitro or in vivo administration of IL-33 promotes their Th2-like, anti-inflammatory function that protects mice from diet-induced insulin resistance [8, 9]. Consistent with this concept, ST2- or IL-33-deficient mice have reduced visceral AT Tregs and impaired glucose tolerance even when fed a normal chow diet [9]. In vitro, ST2-deficient Tregs are as suppressive as their wildtype counterparts in the presence of IL-33; in vivo, however, ST2-deficient Tregs are unable to prevent adoptive T cell-induced colitis [10], suggesting that the IL-33/ST2 axis is critical for Treg proliferation and maintenance in mice.
In contrast to these well-established paradigms in mice, the immune landscape of human omental AT is unclear. Limited evidence suggests there is a positive correlation between obesity and immune cell counts in omental but not subcutaneous AT [11]. Focussing on macrophages, the lack of definitive M1 versus M2 markers in human AT macrophages has yielded inconsistent reports on macrophage polarization in obesity. Flow cytometry has been used to show that CD11c⁺ monocytes accumulate in obese omental AT [11], but CD11c itself is insufficient to identify M1 macrophages in human AT because most macrophages in the crown-like structures of human omental AT also express high levels of CD206 [12]. Notably, CD206 is traditionally considered an M2 marker in mice; in humans, however, it also marks pro-inflammatory macrophages in omental AT [12-14]. Accordingly, the number of CD11c⁺CD206⁺ pro-inflammatory macrophages in omental AT has been positively correlated to insulin resistance [12]. On the other hand, another study used immunohistochemistry to show that, compared to normal-weight subjects, obese individuals had increased numbers of CD40⁺ cells in omental AT, whereas the number of CD206⁺ cells were unchanged [15]. Overall, more studies are needed to investigate how AT macrophages change in obese humans.

Focussing on T cells, there is also no clear evidence that either the proportion or the phenotype of T cells changes with obesity in humans [11, 16, 17]. There are reports of omental AT T cells skewing towards a Th1/Th17 phenotype, with a parallel decrease in Th2-associated cytokines and transcription factors [16, 18-20]. For AT Tregs, there are even fewer studies, with both negative and positive correlations found in small cohorts. Some reports found negative correlations between the amount of FOXP3 mRNA and obesity or insulin resistance [5, 6, 16], whereas others reported positive correlations [17, 21, 22]. To date, only two studies examined human omental AT Tregs using flow cytometry [23, 24]. Moreover, there are no reports on the phenotype of human omental AT Tregs, leading to a gap in
knowledge about whether human AT Tregs possess a unique, tissue-specific phenotype that could lead to AT-specific mechanisms of immune regulation.

Here we aimed to characterize the immune landscape of human omental AT, examining in particular how the phenotype and frequencies of macrophages and CD4^+ T cell subsets change with obesity and in the presence or absence of T2D.

Results

*Obesity and T2D alter metabolic gene expression in human omental AT*

An initial cohort of 29 participants with obesity, 18 diagnosed with T2D and 11 without the disease, were recruited (Table 1). The cohort had a range of BMIs, from ~30 to ~70 kg/m^2^. Participants with or without T2D had a similar sex ratio, age, BMI and blood lipid profiles. As expected, fasting glucose and A1C levels were significantly higher in the T2D group.

Omental AT was collected at the time of bariatric surgery and an unfractionated portion was processed for mRNA expression analysis using a NanoString custom codeset. We first quantified expression of key metabolic genes thought to regulate insulin resistance (PPARγ), adipose browning (UCP1), and immune function (adiponectin and leptin), and assessed their correlation to relevant clinical parameters. We found that *PPARG*, *UCP1*, and *ADIPOQ* (adiponectin) all negatively correlated to clinical parameters indicative of obesity, whereas *LEP* (leptin) exhibited the opposite trend (Fig. 1A, Supporting Table 1). Similarly, omental AT from patients with T2D had lower levels of *PPARG*, *UCP1*, and *ADIPOQ* but higher levels of *LEP* compared to non-T2D controls (Fig. 1B). These data are consistent with the concept that obesity and T2D lead to reduced expression of favourable metabolic genes [1, 25].
Obesity and T2D are associated with increased innate inflammation in human omental AT

Visceral AT inflammation in obesity and T2D has been documented in both humans and mice [1, 3, 4, 25]. In humans, although macrophages are known to accumulate in omental AT in severe obesity, there are contradictory results regarding their M1 versus M2 phenotype [26]. In our cohort, we found that BMI positively correlated with expression of several pro-inflammatory cytokines and chemokines in omental AT, including CCL2 (MCP1), IL1B, IL6, and CXCL8 (Fig. 2A) as well as with the anti-inflammatory cytokines IL10 and IL1RN (IL-1RA) (Fig. 2B). In addition, BMI positively correlated to innate-cell and functional markers such as ITGAX (CD11c), CD1D, TLR2, and CD86 (Fig. 2C). Furthermore, omental AT from patients with T2D had higher expression of CD68, TLR4, CD86, and IL10 than non-T2D individuals (Fig. 2D). Together, these data suggest that obesity and T2D both correlate with increased innate inflammation in human omental AT.

M1 macrophages accumulate in the omental AT of patients with T2D

To more definitively examine how immune cells change with human obesity, we next used flow cytometry to quantify changes in M1 versus M2 macrophages. Using the gating strategy for M1 versus M2 macrophages developed by Wentworth et al. [12], we gated on live CD45⁺CD14⁺ cells in the stromal vascular fraction (SVF), and defined M1 macrophages as CD206⁻CD11c⁺ cells and M2 macrophages as CD206⁺CD11c⁻ cells (Fig. 3A, Supporting Fig. 1A). Consistent with Wentworth et al. [12], the omental AT of patients with T2D exhibited a significantly higher proportion of macrophages, entirely due to an increase in M1 macrophages as other macrophage subpopulations remained unchanged (Fig. 3B). Accordingly, M1 macrophage proportions in omental AT also positively correlated to A1C levels (Fig. 3C). Interestingly, we observed that patient age also affects M1 and M2 macrophage proportions in omental AT, with older age associated with an accumulation of
M1 macrophages and a concomitant reduction of M2 macrophages (Fig. 3D). Overall, individuals with T2D have a significant accumulation of M1 macrophages in their omental AT.

**Obesity is associated with decreased Treg proportions in human omental AT**

To examine how obesity and T2D affect Tregs, we used flow cytometry to quantify and characterize Tregs in human omental AT, using omental AT samples from 6 non-obese patients obtained during hernia repair or laparoscopic cholecystectomy as controls (**Supporting Table 2**). As comparators, we used the same panel to quantify Tregs in the blood of healthy controls or patients with T2D (**Supporting Table 3**). Tregs were defined as CD45⁺CD3⁺CD4⁺FOXP3⁺ cells and conventional T cells (Tconvs) as CD45⁺CD3⁺CD4⁺FOXP3⁻ cells (**Fig. 4A, Supporting Fig. 1B**). Proportions of CD3⁺ and CD3⁺CD4⁺ cells were comparable between omental AT and blood, as well as in omental AT among all subjects regardless of obesity or T2D status (**Fig. 4B**, top panels).

Focusing on Tregs, in non-T2D individuals, Treg proportions were significantly higher in omental AT than in blood, while in patients with T2D, AT Treg proportions became comparable to those of blood (**Fig. 4B**, bottom panels). Similarly, non-obese individuals also had higher Treg proportions in omental AT than in the blood of non-T2D individuals (**Fig. 4B**, bottom panels). These data suggest that omental AT Treg proportions may progressively decline as obese individuals acquire T2D. This possibility is supported by evidence that BMI negatively correlated with Treg proportions in omental AT and, conversely, positively correlated to CD3⁺ and Tconv proportions (**Fig. 4C**). While Spearman’s correlation testing did not reveal a significant correlation between A1C levels and Tregs in omental AT, there was a trend towards a negative correlation (**Fig. 4D**).
Similar to our findings with AT macrophages, we observed an aging-related effect in CD4\(^+\) T cell populations, with aging promoting Tconv accumulation and reducing Treg frequencies in omental AT (Fig. 4E). Together these results suggest that increased obesity, especially in conjunction with T2D, is associated with decreased Treg proportions in human omental AT.

**Human omental AT Tregs have a tissue-specific phenotype**

To further characterize human Tregs in omental AT, we sorted CD45\(^+\)CD3\(^+\)CD4\(^+\)CD25\(^{hi}\)CD127\(^{lo}\) cells in omental AT (Fig. 5A; for comparison a typical staining pattern in blood is also shown) Similar to blood, the CD25\(^{hi}\)CD127\(^{lo}\) population contains the majority of the FOXP3\(^+\)CD25\(^{hi}\) Treg cells (Supporting Fig. 1B). We then isolated RNA for analysis with a custom NanoString codeset for Treg-related markers. From 10g of omental AT, we isolated on average ~1000 Tregs per sample, limiting our analysis to mRNA quantification. To compare omental AT Tregs to *bona fide* Tregs free from potential Tconv contamination, we also isolated Tregs from human thymus for parallel NanoString analysis. Hierarchical clustering revealed that the gene signature of omental AT Tregs was closely related to that of thymic Tregs (Fig. 5B), with both expressing high levels of FOXP3, IL2RA (CD25), and IKZF2 (HELIOS) transcripts (Fig. 5C). Human AT Tregs and thymic Tregs also had comparable expression of TBX21 (TBET), GATA3, and RORC (Fig. 5D), indicating that AT Tregs are unlikely to be poised to produce inflammatory cytokines. The expected negligible production of inflammatory cytokines in AT Tregs was confirmed by intracellular staining for IL-2, IFN-\(\gamma\), and IL-17A (Supporting Fig. 2).

In terms of markers of suppressive potential, omental AT Tregs also closely resembled thymic Tregs, with high expression of CTLA4, TNFRSF18 (GITR), and ICOS, and lower expression of GZMB, CCL3, and CCL4 compared to AT Tconvs (Fig. 5E). Distinct from
thymic Tregs, omental AT Tregs had increased expression of ENTPD1 (CD39) but reduced TGFβ transcript levels (Fig. 5E).

Extensive work in mice has shown that visceral AT Tregs have a Th2-like phenotype with high expression of Pparg, Gata3, Prdm1 (BLIMP1), Il10, Il1rl1 (ST2), Ccr4, and Cxcl2 [7-9]. Consistent with this phenotype, human omental AT Tregs had elevated expression of PPARG, PRDM1 (BLIMP1), and CXCL2 compared to thymic Tregs, and increased levels of CCR4 transcript compared to AT Tconvs (Fig. 5F). In addition to these well-established mouse visceral AT Treg markers, Cipolletta et al. previously reported a comprehensive list of differentially expressed genes in visceral AT versus splenic Tregs in both lean and obese mice [7]. Consistent with this study, genes that were reduced in mouse visceral AT Tregs such as SATB1 and TCF7 were also reduced in human omental AT Tregs compared to thymic Tregs (Fig. 5G). In contrast, genes highly expressed in mouse visceral AT Tregs, including AREG, ANXA1, CXCR6, KLRG1, PLIN2, PCYT1A, CTSH, and DGAT1, were all either reduced or unchanged compared to omental AT Tconv and/or thymic Tregs (Fig. 5H).

Undetectable expression of ST2 on human omental AT Tregs

Notably, we did not detect any IL10 or IL1RL1 (ST2) expression in purified human omental AT Tregs. IL1RL1 was only detectable in whole AT mRNA, with gene expression not correlating to BMI, A1C (Fig. 6A), or other clinical parameters (data not shown). To further explore the finding that IL1RL1 (ST2) mRNA was undetectable on obese AT Tregs, we used flow cytometry to measure ST2 protein on FOXP3+ T cells. We first validated antibody specificity by confirming its ability to bind to 293T cells transfected with an ST2-encoding expression vector (Fig. 6B). We also confirmed the ability of this antibody to detect endogenous ST2 expressed by a subset of tonsil CD19+ B cells, and that endogenous ST2 was not susceptible to collagenase II digestion using the same protocol used to digest adipose
tissue (Supporting Figure 3). In contrast, no ST2 expression was detected on CD4⁺ T cells in either the FOXP3⁺ or FOXP3⁻ fractions isolated from obese omental AT (Fig. 6C). Since we and others have shown that mouse Treg expression of ST2 wanes with obesity [7-9], we also investigated ST2 expression on CD4⁺ T cells isolated from lean omental AT. Similar to the obese omental AT samples, we were also unable to detect ST2 expression on Tregs or other CD4⁺ T cells from lean omental AT (Fig. 6C).

Discussion

Extensive work in mouse AT has revealed a critical role for immunoregulatory cells at the interface between metabolism and inflammation; however, whether similar pathways are relevant in human AT have remained poorly characterized. Here we report the first comprehensive study of how human omental AT immune cells change with increasing obesity and correlate with the presence or absence of T2D. We found that the proportion of AT M1 macrophages is elevated in patients with obesity and T2D, and that although Treg frequencies negatively correlated to BMI, they did not differ between T2D and non-T2D individuals. Human omental AT Tregs express high levels of canonical Treg-defining markers, including FOXP3, CD25, IKZF2, and CTLA4, and possess a unique AT-specific signature characterized by high expression of PPARG, PRDM1 (BLIMP1), and CXCL2, but notably not ST2, IL10, or AREG.

Humans have two major visceral adipose depots, omental and mesenteric; increased adiposity of either depot is associated with an elevated risk of insulin resistance [27, 28]. Unlike mesenteric AT, omental AT contains “milky spots”, or clusters of leukocytes [29]. Few studies have characterized the immune composition of the milky spots in human omental AT, but overall the majority have been reported to be T cells, followed by B cells and macrophages [30, 31]. In our study, we found that ~70% of the CD45⁺ cells are CD3⁺, whereas ~10% are macrophages, consistent with previous reports. Although milky spots are
also found in mouse omental AT [32], it is important to note that most of the studies examining the role of visceral AT in controlling the animal’s overall metabolism studied male gonadal fat pads which lack milky spots [23, 24]. Therefore, it is difficult to make direct comparisons between mouse and human data due to the disparate features of these two tissues.

Obesity is known to alter adipokine levels, which can contribute to systemic low-grade inflammation. Specifically, the pro-inflammatory adipokine leptin is elevated in the serum and AT of obese individuals, whereas adiponectin, the anti-inflammatory adipokine, is reduced in serum [33]. Indeed, we confirm that obese AT has elevated leptin expression and report the new finding that adiponectin declines in proportion to the degree of obesity and in the presence of T2D. In addition, we found that UCP1 transcript levels in omental AT were lower in patients with T2D, consistent with the idea that in obesity a progressive loss of beige AT and the accumulation of white AT leads to cellular stress in adipocytes, triggering the release of pro-inflammatory cytokines and ultimately a change in the immune cell composition in AT [1, 25].

How obesity affects the expression of pro-inflammatory molecules in omental AT has previously been investigated using histology and/or RT-PCR [19, 33, 34]. Consistent with these reports, we found that expression of several pro-inflammatory cytokines (CCL2, CXCL8, IL1B, and IL6) had a weak positive correlation with BMI. Interestingly, BMI also positively correlated with two anti-inflammatory cytokines, IL10 and IL1RN (IL-1RA) [33], implying the presence of counter-regulatory mechanisms.

Several groups have examined the phenotype of omental AT macrophages in humans [12-14]. Although there is a clear consensus that macrophages accumulate in obesity, their phenotype is less well-defined. CD14+CD206+ macrophages, traditionally considered M2-like, are reported to produce pro-inflammatory molecules and form crown-like structures
around inflamed adipocytes [12, 14]. Using a similar gating strategy, we found that patients with T2D exhibited elevated frequencies of M1 (CD14^+CD11c^+CD206^+) macrophages in their omental AT. Our findings further support the notion that these M1 macrophages are important contributors to human omental AT inflammation.

In mice, ST2^+ Tregs represent a dominant component of the immune cell composition in visceral AT. Lean visceral AT is highly enriched in ST2^+ Tregs, with up to 40% CD4^+ T cells expressing Foxp3 and more than half of visceral AT Tregs expressing ST2 [8, 9, 35]. Treg proportions are reduced but not eliminated by diet-induced obesity in mice, decreasing to ~20% of CD4^+ T cells in obese visceral AT [8]. Our data in human omental AT contrast with these findings in mice: the average proportion of FOXP3^+ cells within human omental AT CD4^+ T cells was 10% (range of 2–20%). To date, two other studies have examined human omental AT Tregs by flow cytometry, finding ~5% FOXP3^+ cells or CD25^hiCD127^lo cells within the CD4^+ gate [23, 24].

As in mice, Treg frequencies in human omental AT negatively correlated with BMI. These data are consistent with Gyllenhammer et al. who found that lower omental AT Treg numbers were associated with worsened homeostatic model assessment of β-cell function (HOMA-β), a measure of β-cell function [23]. Interestingly, non-T2D individuals had significantly higher Treg frequencies in omental AT than in blood, while they were similar between AT and blood in patients with T2D, suggesting a trend towards loss of omental AT Tregs in T2D. This observation is in line with the previous finding that metabolic unhealthy obese individuals have reduced omental AT Treg frequencies compared to lean individuals [24]. Together our results further support the notion that, despite differences in AT Treg phenotype and proportions between mice and humans, AT Tregs may nevertheless be involved in suppressing inflammation-induced insulin resistance.
Our observations also indicate an age-related effect on the innate and adaptive immune cell compartments. Older individuals exhibit an overall reduction in the proportion of CD14\(^+\) macrophages in their omental AT, and the composition of macrophage subsets additionally alters with age: M1 macrophages increased, while M2 macrophages decreased, with older age. In contrast, in the few studies carried out in mice, older mice exhibited an accumulation of M2 macrophages in the spleen, lymph nodes, and bone marrow [36, 37].

On the other hand, we found that omental AT Treg frequencies decrease with age. In mice, visceral AT Tregs initially accumulate over time, peaking at \(~40\%\) of CD4\(^+\) T cells at 25 weeks, but then gradually decline to \(~10\%\) by 40 weeks of age [7]. Thus, our findings in humans suggest that there is a similar aging-associated reduction in omental AT Tregs and a concomitant shift in macrophage polarization. Further work is needed to explore their potential contribution to aging-associated inflammation.

Finally, we report the first data on the phenotype of human omental AT Tregs. We find that they are similar to thymic Tregs in their expression of lineage-defining and functional molecules, including FOXP3, CD25, IKZF2 (HELIOS), ENTPD1 (CD39), and CTLA4. Distinct from thymic Tregs, and similar to mouse visceral AT Tregs [38], they also had high expression of PPAR\(G\) and PRDM1 (BLIMP1). However, expression of IL1RL1 (ST2) or IL10 was undetectable. This finding is consistent with a previous report that ST2 is exclusively expressed by endothelial cells and not by any immune cells in human omental AT [39]. In support of this conclusion, we also detected abundant levels of IL1RL1 transcript in total omental AT lysate, suggesting that ST2 is expressed outside of the CD4\(^+\) T cell compartment. Overall, while omental AT Treg frequencies are reduced in obesity, they appear to be lineage-committed cells that have adopted a tissue-specific profile.

The present study fills a knowledge gap in the immune landscape of human omental AT, particularly how the phenotype and proportion of macrophages and CD4\(^+\) T cells change.
with increasing obesity and the presence of T2D. We provide the first phenotypic
descriptions of human omental AT Tregs and discover important similarities and differences
to the paradigms developed in mice. For instance, high expression of PPARG, which is
essential for Treg function in mouse visceral AT [40] suggests that the function of this
transcription factor in AT Tregs is highly conserved. On the other hand, the lack of detectable
ILIRL1 (ST2) and AREG expression indicates that more research is needed to define the
relevance of these proteins in human AT Tregs. Our data increase our understanding of how
pathways of immune regulation could be targeted to ameliorate AT inflammation in humans.

Materials and Methods

Patients. Human samples were collected according to protocols approved by the University
of British Columbia Clinical Research Ethics Board, including written informed consent. For
omental AT, 29 adults undergoing bariatric surgery between 2014 and 2016 were recruited
from the Bariatric Surgery Program at Richmond Hospital. Demographic data including age,
sex, weight, height, calculated BMI, waist circumference, fasting glucose, and A1C levels
were recorded at the time of surgery. A lipid profile (triglycerides, total cholesterol, low-
density lipoprotein (LDL) and high-density lipoprotein (HDL)) was obtained prior to surgery
(Table 1). Omental AT from 6 non-obese controls was collected during hernia repair or
laparoscopic cholecystectomy (Supporting Table 2). For comparisons between omental AT
Tregs and blood Tregs, peripheral blood mononuclear cells (PBMCs) were collected from 22
patients with T2D and 10 non-diabetic age-matched controls (Supporting Table 3). T2D
PBMCs were from participants consented under protocols approved by the Benaroya
Research Institute Institutional Review Board. For comparing omental AT Tregs and thymic
Tregs, thymus tissue discarded from pediatric cardiac surgery was dissociated as described
[41], and CD25^+CD8^- Tregs were isolated by sequential CD25 positive selection and CD8
negative selection using custom EasySep reagents (STEMCELL Technologies) according to
the manufacturer’s protocols. CD25⁺CD8⁻ thymic Tconvs were isolated from the CD25⁺ fraction by CD8 negative selection.

**Omental AT processing.** Approximately 10g of omental AT obtained during bariatric surgery or hernia repair or laparoscopic cholecystectomy was minced and digested in 0.5mg/ml collagenase II (Worthington Biochemical) at 37°C for 45 min, passed through a 100µm cell strainer, and centrifuged to obtain SVF cells. SVF cells were resuspended in 1ml red blood cell lysis buffer (0.8% NH₄Cl, STEMCELL Technologies), incubated for 5 min at room temperature, washed with FACS buffer (PBS, 0.5% BSA, 2mM EDTA), and stained for flow cytometry using the antibodies listed in Supporting Table 4. A portion of omental AT was homogenized and lysed in gentleMACS M tubes using the gentleMACS Dissociator (Miltenyi Biotec). RNA was isolated using an EZNA Total RNA kit (OMEGA Bio-tek).

**Flow cytometry and cell sorting.** For phenotypic analysis, cells were acquired on an LSRFortessa X-20 (BD Biosciences) and analyzed using FlowJo V10 (Tree Star) following the guidelines of Cossarizza et al [42]. Each AT sample was collected and analyzed on a different day. Replicate aliquots of blood and/or tonsil mononuclear cell samples run in parallel each time served as staining and instrument calibration controls. AT macrophages were gated as CD45⁺CD14⁺ cells, then subgated as M1 (CD206⁺CD11c⁺) or M2 (CD206⁺CD11c⁻, Supporting Fig. 1A) [12, 43]. To isolate Tregs and Tconvs, flow cytometric sorting was performed on a FACSARia II (BD Biosciences). Tregs were sorted as CD45⁺CD3⁺CD4⁺CD25hiCD127lo cells and Tconvs as CD45⁺CD3⁺CD4⁺CD25loCD127hi cells (Supporting Fig. 1B) directly into RLT lysis buffer (Qiagen). For intracellular cytokine staining, SVF cells treated with 10µg/ml brefeldin A were either left unstimulated or stimulated with 10ng/ml PMA and 500ng/ml ionomycin for 6 hours, then fixed/permeabilized
with the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) and stained with the antibodies listed in Supporting Table 4.

**Gene expression analysis.** mRNA expression was measured using a custom NanoString nCounter codeset (49 genes including 5 reference genes). 100ng of adipose tissue RNA or 4μl of unpurified lysate from sorted Tregs was analyzed as previously described [44]. Background subtraction, sum normalization, and log₂ transformation of mRNA counts were performed prior to analysis.

**Hierarchical clustering.** The hierarchical clustering module (public GenePattern server, Broad Institute) was used to generate heat maps and cluster trees by pairwise average linkage according to Spearman’s correlation coefficient.

**Statistical analysis.** For all correlation analyses between gene expression and clinical parameters, Spearman’s nonparametric correlation was used. Comparison of gene expression between two groups were performed with the Mann-Whitney test, and for more than two groups, one-way ANOVA with a Bonferroni post hoc test was performed. * represents P < 0.05; ** represents P < 0.01; *** represents P < 0.001. Error bars in figures represent the SEM.

**Validation of ST2 mAb specificity.** A monoclonal anti-human ST2 antibody (R&D MAB523) was conjugated to PE by the University of British Columbia Antibody Lab. 293T cells were plated in a 24-well plate at 40,000 cells/ml in media (DMEM, 10% fetal bovine serum, 1% penicillin-streptomycin, 2mM L-glutamine) and transfected with a lentiviral vector encoding human ST2 (pLVX-EF1a-IL1RL1-IRES-emGFP, a gift from Drs. Guy
Charron and John Rioux, University of Montreal) using jetPRIME reagent (Polyplus Transfections) according to the manufacturer’s protocol. Cells were collected 48 hours later and stained with the PE conjugated anti-human ST2 antibody. Mononuclear cells from human tonsil samples were stained with the same anti-ST2 antibody. In some cases tonsil samples were exposed to 0.5mg/ml collagenase II (Worthington Biochemical) at 37°C for 45 min.

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Conflict of Interest Disclosure

The authors declare no commercial or financial conflict of interest.
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associated with higher fasting glucose and lower beta-cell function in adults with obesity. *Obesity (Silver Spring)* 2016. 24: 1274-1282.


Table 1. Characteristics of cohort undergoing bariatric surgery.

<table>
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<th>Characteristics</th>
<th>No T2D (n=11)</th>
<th>T2D (n=18)</th>
<th>P-value</th>
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<td>Sex – female n (%)</td>
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<td>15 (83.3)</td>
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<td>Duration of diabetes to surgery – mean in years (SD)</td>
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<td>48.4 (9.7)</td>
<td>0.99</td>
</tr>
<tr>
<td>Waist circumference – mean in cm (SD)</td>
<td>129.9 (12.8)</td>
<td>139.3 (15.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>A1C – mean in percentage (SD)</td>
<td>5.4 (0.5)</td>
<td>7.4 (1.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting glucose – mean in mmol/L (SD)</td>
<td>5.7 (0.7)</td>
<td>8.3 (2.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lipid Profiles – mean in mmol/L (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>4.78 (0.71)</td>
<td>4.39 (0.76)</td>
<td>0.24</td>
</tr>
<tr>
<td>LDL</td>
<td>2.69 (0.69)</td>
<td>2.34 (0.66)</td>
<td>0.19</td>
</tr>
<tr>
<td>HDL</td>
<td>1.29 (0.19)</td>
<td>1.23 (0.49)</td>
<td>0.16</td>
</tr>
<tr>
<td>Non-HDL</td>
<td>3.48 (0.87)</td>
<td>3.11 (0.75)</td>
<td>0.15</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.75 (0.73)</td>
<td>1.82 (0.90)</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. *Obesity and T2D alter metabolic gene expression in human omental AT.* Omental AT was collected from 29 obese patients undergoing bariatric surgery. RNA was isolated from unfractionated tissue and expression of the indicated genes was measured by NanoString nCounter Gene Expression. Each circle represents one subject; tissue from each subject was collected and processed on a different day. NanoString was run with batches of 12 samples per run over several days. (A) Gene expression data were correlated to A1C, waist circumference, and BMI using Spearman’s rank correlation. (B) Gene expression in subjects with (n=11) or without T2D (n=18). Comparisons of gene expression between two groups were performed with the Mann-Whitney test. Correlations between gene expression and clinical variables were performed with Spearman’s rank correlation. * represents P < 0.05; ** represents P < 0.01; *** represents P < 0.001. Error bars represent the SEM.
Figure 2. Obesity and T2D are associated with increased innate inflammation in human omental adipose tissue. Omental AT was collected from 29 obese patients undergoing bariatric surgery. RNA was isolated from unfractionated tissue and expression of the indicated genes was measured by NanoString nCounter Gene Expression. Each circle represents one subject; tissue from each subject was collected and processed on a different day. NanoString was run with batches of 12 samples per run over several days. Gene expression of (A) pro-inflammatory cytokines, (B) anti-inflammatory cytokines, and (C) innate immune cell markers were correlated to BMI. (D) Gene expression in subjects with (n=11) or without T2D (n=18). Comparisons of gene expression between two groups were performed with the Mann-Whitney test. Correlations between gene expressions and clinical variables were performed with Spearman’s rank correlation. * represents P < 0.05; ** represents P < 0.01; *** represents P < 0.001. Error bars represent the SEM.
Figure 3. Flow cytometry analysis of ex vivo macrophage populations from omental AT reveals increased M1 macrophages in T2D. In a subset of subjects (no T2D, n=4; T2D, n=8), a portion of the SVF was analyzed by flow cytometry for monocyte/macrophage populations. Each circle represents one subject; tissue from each subject was collected and analyzed on a different day. (A) Representative gating strategies for the human AT macrophages, pre-gated on live CD45$^+$ cells. (B) Proportions of the indicated macrophage subpopulations. (C) Proportions of M1 macrophages were correlated to A1C levels. (D) Age-associated changes in macrophage proportions. Comparison of population frequencies between two groups were performed with the Mann-Whitney test. Correlations between population frequencies and clinical variables were performed with Spearman’s rank correlation. * represents P < 0.05; ** represents P < 0.01; *** represents P < 0.001. Error bars represent the SEM.
Figure 4. Flow cytometric analysis of ex vivo Tregs from human omental AT and peripheral blood. In a subset of obese subjects (no T2D, n=9; T2D, n=13) and non-obese controls (n=6), a portion of the SVF was analyzed by flow cytometry for Treg and Tconv frequencies. Each SVF sample was analyzed on a different day. Data were compared to cryopreserved blood from aged-matched controls with (n=10) or without T2D (n=22). Cryopreserved blood samples were analyzed in 5 separate batches on 5 days. (A) Representative flow cytometry data for Tregs, pre-gated on CD45+CD3+CD4+ cells. (B) Quantification of the indicated T cell populations. (C-D) Obesity-associated changes in T cell populations in the omental fat. (E) Aging-associated changes in CD4+ T cells in omental AT. Comparison of population frequencies between multiple groups were performed with one-way ANOVA with a Bonferroni post hoc test. Correlations between population frequencies and clinical variables were performed with Spearman’s rank correlation. * represents P < 0.05; ** represents P < 0.01; *** represents P < 0.001. Error bars represent the SEM.
Figure 5. Gene expression analysis of human omental AT Tregs. Ex vivo Tregs from omental AT (n=3) or thymus (n=5) were isolated and mRNA levels measured by NanoString nCounter Gene Expression. Tissue from each subject was collected and processed on a different day. NanoString was run with batches of 12 samples per run over several days. (A) Representative staining pattern of human omental AT Tregs, as determined by flow cytometry. For comparison, a typical staining pattern for Tregs in blood is shown. (B) Hierarchical clustering of Tconv and Tregs isolated from omental AT or thymus by pairwise average linkage according to Spearman’s correlation coefficient. Each column represents one individual. (C-H) Comparison of gene expression by omental AT Tregs and thymic Tregs: (C) Treg markers, (D) Th1/2/17 transcription factors, (E) Treg functional markers, and (F, G, H) signature genes of mouse visceral AT Tregs. Comparison of gene expression between multiple groups were performed with one-way ANOVA with a Bonferroni post hoc test. Correlations between gene expression and clinical variables were performed with Spearman’s rank correlation. * represents P < 0.05; ** represents P < 0.01; *** represents P < 0.001. Error bars represent the SEM.
Figure 6. Human omental AT Tregs do not express detectable ST2. (A) *IL1RL1* (ST2) expression in whole omental AT correlated to BMI or A1C (n=29). Samples were collected and analyzed as in Figure 5. (B) Validation of the anti-human ST2 flow antibody. Representative flow plots of surface ST2 expression on ST2-overexpressing 293T cells analyzed by flow cytometry (n=2 independent experiments). (C) Representative flow plots of ST2 expression in human omental AT CD4⁺ T cells (non-obese, n=6; obese, n=3). Tissue from each subject was collected and processed on a different day and analyzed by flow cytometry.
The proportion of regulatory T cells (Tregs) in human omental adipose tissue decreases in obesity. These human omental adipose Tregs exhibit a tissue-specific phenotype but lack ST2 expression, in contrast to Tregs resident in mouse epididymal adipose tissue.

Word count: 35/50