T-Cell Profile in Adipose Tissue Is Associated With Insulin Resistance and Systemic Inflammation in Humans

Tracey McLaughlin, Li-Fen Liu, Cindy Lamendola, Lei Shen, John Morton, Homero Rivas, Daniel Winer, Lorna Tolentino, Okmi Choi, Hong Zhang, Melissa Hui Yen Chng, Edgar Engleman

Objective—The biological mechanisms linking obesity to insulin resistance have not been fully elucidated. We have shown that insulin resistance or glucose intolerance in diet-induced obese mice is related to a shift in the ratio of pro- and anti-inflammatory T cells in adipose tissue. We sought to test the hypothesis that the balance of T-cell phenotypes would be similarly related to insulin resistance in human obesity.

Approach and Results—Healthy overweight or obese human subjects underwent adipose-tissue biopsies and quantification of insulin-mediated glucose disposal by the modified insulin suppression test. T-cell subsets were quantified by flow cytometry in visceral (VAT) and subcutaneous adipose tissue (SAT). Results showed that CD4 and CD8 T cells infiltrate both depots, with proinflammatory T-helper (Th)-1, Th17, and CD8 T cells, significantly more frequent in VAT as compared with SAT. T-cell profiles in SAT and VAT correlated significantly with one another and with peripheral blood. Th1 frequency in SAT and VAT correlated directly, whereas Th2 frequency in VAT correlated inversely, with plasma high-sensitivity C-reactive protein concentrations. Th2 in both depots and peripheral blood was inversely associated with systemic insulin resistance. Furthermore, Th1 in SAT correlated with plasma interleukin-6. Relative expression of associated cytokines, measured by real-time polymerase chain reaction, reflected flow cytometry results. Most notably, adipose tissue expression of anti-inflammatory interleukin-10 was inversely associated with insulin resistance.

Conclusions—CD4 and CD8 T cells populate human adipose tissue and the relative frequency of Th1 and Th2 are highly associated with systemic inflammation and insulin resistance. These findings point to the adaptive immune system as a potential mediator between obesity and insulin resistance or inflammation. Identification of antigenic stimuli in adipose tissue may yield novel targets for treatment of obesity-associated metabolic disease. (Arterioscler Thromb Vasc Biol. 2014;34:2637-2643.)

Key Words: adaptive immunity ■ C-reactive protein ■ human adipose tissue-specific secretory factor ■ inflammation ■ insulin resistance ■ interleukin-6 ■ interleukin-10 ■ obesity ■ T helper lymphocytes ■ T lymphocytes ■ visceral fat

Although obesity has been clearly linked to insulin resistance and type 2 diabetes mellitus, the underlying mechanisms have not been fully elucidated. Accumulating evidence implicates adipose tissue inflammation as a contributor to insulin resistance via increased systemic inflammation and direct impairment of insulin-mediated glucose uptake. Obese adipose tissue is associated with increases in serum and tissue proinflammatory molecules, such as interleukin-6 (IL-6) and tumor necrosis factor-α, which can induce insulin resistance in vitro.1,2 Macrophages accumulate in adipose tissue in proportion to body mass index (BMI) and adipose cell size,3,4 and adopt a proinflammatory phenotype in response to diet-induced obesity.5 It is thought that changes in hypertrophic adipose cells, such as hypoxia, necrosis, chemokine secretion, and fatty acids, attract and activate macrophages, but the specific stimuli and pathways by which this occurs are unclear. Also unclear is the potential role of other immune cells in adipose tissue, and their relationship to localized and systemic inflammation and insulin resistance. Studies in mice indicate that with diet-induced obesity, proinflammatory T-helper 1 (Th1) and CD8 lymphocytes infiltrate visceral adipose tissue (VAT), where they overcome the anti-inflammatory effects of Th2 and T-regulatory (Treg) cells, promoting classical activation of macrophages and systemic insulin resistance or glucose intolerance.6–13 T cells have been identified in human adipose tissue,14,15 but there are scant data on T-cell phenotypes or their relationship to systemic inflammation and insulin resistance. To test the hypothesis that numerical dominance of proinflammatory CD8 and Th1 over anti-inflammatory Th2 and T-reg cells would be related to systemic inflammation and insulin resistance in humans, we used flow cytometry.
to quantify the relative frequency of these T-cell subsets in subcutaneous adipose tissue (SAT) and VAT of healthy overweight or obese subjects who underwent quantitative insulin-sensitivity testing.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Forty-seven prescreened, nondiabetic, healthy subjects were studied: 20 Roux-en-Y gastric bypass patients with sampling of both SAT and VAT for flow cytometric analysis of T-cell subsets (n=10) and real-time polymerase chain reaction of T-cell–associated cytokines (n=10), and 27 nonsurgical overweight/obese subjects with sampling of SAT alone for similar analyses (Table I in the online-only Data Supplement). Thirteen nonsurgical subjects underwent collagenase digestion of fresh adipose tissue for flow cytometric analysis of T-cell subsets: 6 of these subjects and an additional 14 frozen tissue specimens from subjects meeting study criteria and harvested in an identical manner were included in the real-time polymerase chain reaction analyses of T-cell–associated cytokine expression. Same day peripheral blood for flow cytometry was obtained from all subjects who underwent flow cytometric analysis of adipose tissue. Using the staining and gating strategies depicted in Figure I in the online-only Data Supplement, we determined relative frequencies of T-cell subsets in SAT, VAT, and peripheral blood. Primary analyses were of 2 types: (1) comparison of T-cell subsets and relevant cytokines in SAT versus VAT depots from gastric bypass patients (Tables 1 and 2), including relationships between fat depots and peripheral blood T cells (Figure 1) and plasma high-sensitivity C-reactive protein (hsCRP), a marker of systemic inflammation (Figure 2); (2) measurement of the association between T-cell subsets or cytokines and systemic insulin resistance, which was quantified in all nonsurgical subjects and approximately half of the bariatric surgery subjects and harvested in an identical manner were included in the real-time polymerase chain reaction analyses of T-cell–associated cytokine expression. Same day peripheral blood for flow cytometry was obtained from all subjects who underwent flow cytometric analysis of adipose tissue. Using the staining and gating strategies depicted in Figure I in the online-only Data Supplement, we determined relative frequencies of T-cell subsets in SAT, VAT, and peripheral blood. Primary analyses were of 2 types: (1) comparison of T-cell subsets and relevant cytokines in SAT versus VAT depots from gastric bypass patients (Tables 1 and 2), including relationships between fat depots and peripheral blood T cells (Figure 1) and plasma high-sensitivity C-reactive protein (hsCRP), a marker of systemic inflammation (Figure 2); (2) measurement of the association between T-cell subsets or cytokines and systemic insulin resistance, which was quantified in all nonsurgical subjects and approximately half of the bariatric surgery subjects, who were willing to undergo time-intensive metabolic testing before their surgery (Figure 3).

Differences in T-Cell Frequency

According to Regional Fat Depot

Proinflammatory Th1, Th17, and interferon (IFN)-γ+ CD8 cells, expressed as percentage of total CD4 or CD8 T cells, were markedly increased in VAT relative to SAT, and in both adipose compartments relative to peripheral blood mononuclear cells (PBMC; Table 1). This finding can also be seen in Figure 4, depicting intense staining for both CD4 and CD8 in the VAT of an insulin-resistant subject as compared with SAT. Interestingly, CD4 and CD8 density in a comparable VAT sample of an IS control with similar BMI was much less dense. Although there was no significant difference in anti-inflammatory Treg% between SAT, VAT, and PBMC, the relative frequency of anti-inflammatory Th2 cells was significantly higher in SAT, but not VAT, as compared with PBMC. T-cell profiles in SAT and VAT were highly correlated with each other with respect to Th1, Th17, CD4, and CD8 cell frequency (Figure 1A–1D), but not Th2 (r=0.46; P=0.19) or Treg (r=0.13; P=0.76). Of note, the frequency of Th1 in both adipose tissue depots was 10- to 20-fold greater than that of Th2, Treg, and Th17. Given the robust correlations between T-cell subsets in SAT and VAT, we considered the possibility that T-cell profiles might be reflected in peripheral blood. In support of this idea, strong

### Table 1. Flow Cytometric Analysis of T-Cell Subsets in Peripheral Blood Mononuclear Cells, Subcutaneous, and Visceral Adipose Tissues in Bariatric Surgery Subjects

<table>
<thead>
<tr>
<th>T-Cell Subset, %</th>
<th>PBMC (n=10)</th>
<th>SAT (n=10)</th>
<th>VAT (n=10)</th>
<th>SAT vs PBMC</th>
<th>VAT vs PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T cells (total T)</td>
<td>61.9±15</td>
<td>59.6±14.1</td>
<td>53.6±12.2</td>
<td>0.008</td>
<td>§</td>
</tr>
<tr>
<td>Th1 (CD4 T)</td>
<td>18.4±13</td>
<td>47±19</td>
<td>63.0±5</td>
<td>0.008</td>
<td>§</td>
</tr>
<tr>
<td>Th2 (CD4 T)</td>
<td>0.63±0.6</td>
<td>1.9±1.1</td>
<td>1.4±0.8</td>
<td>0.15</td>
<td>†</td>
</tr>
<tr>
<td>Th17 (CD4 T)</td>
<td>0.53±0.5</td>
<td>3.5±1.9</td>
<td>5.6±3.2</td>
<td>0.013</td>
<td>§</td>
</tr>
<tr>
<td>Treg (CD4 T)</td>
<td>1.4±1.0</td>
<td>2.2±2.7</td>
<td>3.8±4.3</td>
<td>0.42</td>
<td>NS</td>
</tr>
<tr>
<td>CD8 T cells (total T)</td>
<td>32.9±13.8</td>
<td>30.7±9.6</td>
<td>38.4±6.8</td>
<td>0.87</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ+ CD8 cells (CD8 T)</td>
<td>30.9±19.3</td>
<td>69.2±15.6</td>
<td>81.3±7.0</td>
<td>0.04</td>
<td>§</td>
</tr>
</tbody>
</table>

IFN indicates interferon; PBMC, peripheral blood mononuclear cells; NS, nonsignificant value; SAT, subcutaneous adipose tissue; Th, T-helper cells; Treg, T-regulatory cells; and VAT, visceral adipose tissue.

*Paired Student t test.
†P<0.05 for PBMC vs SAT; ‡P<0.05 for PBMC vs VAT; and §P<0.05 for PBMC vs both SAT and VAT.

### Table 2. Relative Expression of T-Cell–Associated Cytokines Quantified by Real-Time Polymerase Chain Reaction in Subcutaneous and Visceral Adipose Tissues From Bariatric Surgery Subjects

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>SAT (n=10)</th>
<th>VAT (n=10)</th>
<th>PValue*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>1.3±1.2</td>
<td>2.7±1.6</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.3±0.9</td>
<td>0.3±0.32</td>
<td>0.08</td>
</tr>
<tr>
<td>IL-17</td>
<td>1.09±0.54</td>
<td>3.63±3.62</td>
<td>0.04</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.65±1.9</td>
<td>2.2±1.6</td>
<td>0.57</td>
</tr>
</tbody>
</table>

IFN indicates interferon; IL, interleukin; SAT, subcutaneous adipose tissue; and VAT, visceral adipose tissue.

*Paired Student t test.
correlations were seen between PBMC and SAT for Th1, CD4, and CD8 T-cell frequency and, despite the small number, between PBMC and VAT for CD4 (Figure 1E–1H). The correlation between CD8 frequency in VAT and PBMC was of borderline statistical significance ($r=0.82; P=0.09$; not shown). Neither Th2 and Treg nor Th17 in PBMC was correlated with frequency in SAT or VAT, confirming that observed correlations were not an artifact from blood contamination of tissue. To assess expression of Th cytokine genes in adipose tissue, we performed real-time polymerase...
chain reaction on VAT and SAT from 10 healthy bariatric subjects (Table 2). Expression of IL-6, a marker of Th1 and M1 macrophage activation, was 2-fold greater in VAT than SAT ($P=0.02$), and IL-17, a marker of Th17 activation (also proinflammatory), was 3-fold greater in VAT than SAT ($P=0.04$). IL-10, a marker of Th2 and M2 activation and potential mediator of insulin sensitivity, was 4-fold lower in VAT versus SAT ($P=0.08$).

**T-Cell Phenotype: Relationship to Circulating Inflammatory Markers**

To ascertain whether Th profiles in adipose tissue were associated with systemic inflammation, correlations between Th1 and Th2 in SAT and VAT were performed with respect to both plasma hsCRP and IL-6 concentrations in our cohort. Th1 in VAT was robustly associated with plasma hsCRP ($r=0.91; P=0.002$), whereas Th2 in VAT was inversely

Figure 4. Representative histology of CD4 and CD8 T cells in human subcutaneous (SAT) and visceral (VAT) adipose tissues in insulin-sensitive (IS) and insulin-resistant (IR) subjects. Brown indicates CD4-positive cells, whereas blue indicates CD8-positive cells. Images are shown at a ×200 magnification. VAT from the IR subject has more CD4 and CD8 cells compared with SAT (left) and VAT from the IS subject (top right). The 60-year old, white female IS subject has body mass index (BMI) 41 kg/m², steady-state plasma glucose (SSPG) 129 mg/dL, plasma interleukin-6 (IL6) 8.8 pg/mL; and the 56-year old, white female IR subject has BMI 47.3 kg/m², SSPG 346 mg/dL, and plasma IL6 28.3 pg/mL.
associated with plasma hsCRP ($r = -0.75; P = 0.03$; Figure 2). Th1, but not Th2, in SAT was also strongly associated with plasma hsCRP ($r=0.64; P=0.035$). Frequencies of the other CD4 and CD8 cells were not associated with hsCRP. Results were unchanged after adjustment for sex. These results point to VAT as having a particularly strong association with systemic inflammation, with bidirecional relationships between Th1 and Th2 and plasma hsCRP. Th1, but not Th2, frequency in SAT also correlated with IL-6 concentrations in peripheral blood (Figure II in the online-only Data Supplement; $r=0.65; P=0.03; n=15$). A similar trend was also demonstrated for Th1 in VAT, although sample size was too limited to draw firm conclusions ($r=0.86; P=0.38; n=4$).

**T-Cell Subsets: Relationship to Insulin Resistance**

To ascertain the relationship between Th profiles and systemic insulin resistance, we quantified insulin-mediated glucose uptake using the modified insulin suppression test (see Methods in the online-only Data Supplement), denoted by the steady-state plasma glucose (SSPG). Although studies in obese mice have shown that proinflammatory Th1 and CD8 cells play important roles in the adipose tissue inflammation that underlies insulin resistance,6,8 we found no correlation between insulin resistance and the percentages of Th1, CD8, or Th17 cells in SAT, VAT or PBMC. Also in contrast to mice studies,13 anti-inflammatory Treg frequency in SAT and VAT was not significantly associated with SSPG (data not shown). On the contrary, despite lower overall frequency in both SAT and VAT than proinflammatory Th1, the percentage of anti-inflammatory Th2 was inversely correlated with insulin resistance (Figure 3A and 3B). Th2 in PBMC was also significantly inversely associated with SSPG (Figure 3C). Thus, the results indicate that Th2 bias is independently inversely associated with insulin resistance.

To further explore the relationship between Th2 and insulin resistance, we measured the relative expression of IL-10, a Th2-secreted cytokine that potently inhibits proinflammatory cytokines and has also been shown to enhance whole body insulin sensitivity in mice.11 Relative expression of this cytokine in the SAT of 20 moderately obese, BMI-matched individuals, and confirms prior studies demonstrating Th17 cells are present in human fat. Importantly, results demonstrate that subpopulations of T cells differ according to regional fat depot and are associated with both systemic inflammation and insulin resistance. In our healthy overweight/moderately-obese humans, proinflammatory Th1, Th17, and IFN-$\gamma$+ CD8 cells were markedly increased in VAT relative to SAT and peripheral blood. Despite these differences, proinflammatory T-cell frequencies in VAT correlated highly with SAT, and with peripheral blood, indicating that triggers to the adaptive immune response occur in multiple adipose tissue depots, and may even be systemic. Th1 and Th2 cells in VAT bore robust bidirectional relationships to plasma hsCRP, a systemic inflammatory marker that indicates increased risk for both cardiovascular disease and type 2 diabetes mellitus. Furthermore, Th1 frequency in SAT was associated with both plasma hsCRP and IL-6 concentrations. Finally, and perhaps most importantly, Th2 frequency in adipose tissue and peripheral blood correlated inversely with insulin resistance, indicating a protective effect. Adipose tissue expression of IL-10, a Th2-related cytokine, was also highly inversely associated with insulin resistance. These relationships were independent of obesity per se, and thus the relative dominance of Th1 versus Th2 responses in adipose tissue may account in part for the metabolic heterogeneity that is now well described in overweight and obese humans.18

The present findings represent an important extension to currently published data on the potential role of inflammatory cells as mediators of insulin resistance. Although the majority of studies investigating inflammation and insulin resistance have focused on macrophages, 3 recent studies in mice6,8,13 demonstrated an important role for CD4 and CD8 lymphocytes in the development of insulin resistance. We previously demonstrated that CD4 T cells in adipose tissue promote insulin resistance in diet-induced obese mice via dramatic increases in proinflammatory Th1 over static numbers of anti-inflammatory Treg and Th2. CD4+ T cell transfer, predominantly through Th2 cells, reversed insulin resistance, supporting a causal role. In addition, treatment of obese WT and ob/ob (leptin-deficient) mice with CD3-specific antibody or its F(ab’)2 fragment, reduced the predominance of Th1 cells over Treg cells, reversing insulin resistance for months.6 Two other studies in mice have shown that Treg and CD8 T cells play opposing roles in adipose tissue inflammation and insulin resistance,6,13 and another showed that that obesity was associated with selective expansion of the proinflammatory Th17 sublineage.14 Importantly, 2 of these studies6,13 demonstrated little heterogeneity of the T-cell receptor repertoire in VAT, implying localized antigenic stimulation and expansion of antigen-specific T cells. This is plausible because it is known that hypertrophic or hypoxic adipose cells become necrotic, releasing cellular contents and secreted molecules into the extracellular space which serve as antigens to T cells.7,4

Despite the impressive findings in rodent studies, human data on the role of T cells in adipose tissue are scant and there are no studies addressing their potential relationship to insulin resistance. To our knowledge, there are only 3 published studies demonstrating CD4 T cell subsets in human adipose tissue using flow cytometry. The first, which examined SAT only, demonstrated increased prevalence of Th17 and Th22 cells in metabolically abnormal versus metabolically normal obese.15 In this study, CD4 T cells staining for IL13 (Th2) and IFN-$\gamma$ (Th1) were not associated with metabolic profile. It is important to note that the BMI of the metabolically abnormal versus metabolically normal obese subjects in this study was 43.8 versus 34.9 kg/m², which does not rule out the possibility of
that obesity per se contributed to the findings. Furthermore, T cells were expanded in culture for 2 weeks before flow cytometric analysis, which leaves open the possibility that T-cell phenotypes were altered by the culture process. The second study, which compared SAT and VAT of morbidly obese diabetic and nonobese patients with SAT of nonobese controls, also showed increased frequency of IL17 and IL22-secreting T cells in obese subjects with or without diabetes mellitus as compared with the nonobese controls. Furthermore, exposure of CD4 T cells to macrophage-derived or recombinant IL1B increased production of IL17 and IL22, suggesting cross-talk between immune cells in adipose tissue. The third study demonstrated increased Th17 in VAT of overweight or obese as compared with lean women. As in the first study, these results cannot exclude the possibility that alterations in T-cell phenotypes were caused by obesity per se because the comparisons were made between largely different BMI groups. Beyond these reports, there is 1 published study documenting the presence of CD4 cells in human fat via immunohistochemistry, and one showing a relative increase in gene expression of FOXP3, a marker for anti-inflammatory Treg cells, in SAT as compared with VAT.

Indirect support for our finding that adipose tissue T sub- sets are associated with insulin human resistance is found in 1 published study, demonstrating the presence of both classically activated proinflammatory macrophages and alternatively activated anti-inflammatory macrophages in human adipose tissue, with associations between the ratio of pro- to anti-inflammatory macrophages and insulin resistance as measured by the homeostasis model assessment of insulin resistance. Although the signals responsible for macrophage activation in adipose tissue have not been clearly elucidated, we have shown in mice that proinflammatory Th1 cells stimulate macrophage activation via secretion of IFN-γ. Thus, it is conceivable that localized T-cell activation in adipose tissue is a primary event, which results in macrophage recruitment and activation. This is consistent with findings in mice fed a high-fat diet, in which T-cell infiltration into VAT and insulin resistance was observed at 5 weeks but macrophage recruitment was not observed until 10 weeks of feeding. T-cell secreted cytokines may also directly impair insulin action in target tissues. Inflammatory cytokines (eg, tumor necrosis factor-α) elaborated by activated T-cells directly impair insulin-mediated glucose uptake via stimulation of IKKB (inhibitor of nuclear factor kappa-B kinase subunit beta) and JNK (c-Jun amino-terminal kinase). Other cytokines, such as IL-10, secreted by anti-inflammatory T cells, are associated with enhanced insulin sensitivity and protection from inflammation in mice. Our results support a protective role for IL-10 and are the first to demonstrate an association between expression of this cytokine in human adipose tissue and systemic insulin sensitivity.

In the context of accumulating data implicating inflammation as causal in the development of atherosclerotic vascular disease and type 2 diabetes mellitus, the potential role of T cells in determining systemic inflammation is of great interest. The present results show that T cells in VAT, both CD8 and CD4, adopt a proinflammatory phenotype as compared with SAT. Furthermore, the frequencies of Th1 and Th2 in VAT are bidirectionally and robustly associated with circulating hsCRP, an inflammatory marker predictive of both type 2 diabetes mellitus and atherosclerotic heart disease. hsCRP is secreted by the liver in response to stimulation by IL-6, a classic proinflammatory cytokine. Given the proximity of VAT to the liver, which is the main source of hsCRP, it is plausible that inflammatory immune cells in VAT provide a particularly potent stimulus to hsCRP secretion, via direct cytokine release into the portal vein. In the present study, expression of IL-6, secreted by Th1 and proinflammatory macrophages, was significantly higher in VAT than in SAT, supporting the hypothesis that proinflammatory immune cells in VAT play a particularly important role in determining hsCRP release by the liver. Whether inflammation in VAT is a stronger determinant of systemic inflammation than SAT is an important question, and although prior clinical studies show that abdominal and visceral fat mass bear modestly stronger independent associations with plasma hsCRP than total adiposity, this is the first study to characterize immune cell phenotypes in VAT with respect to systemic inflammation. Additional data supporting a role of T cells in determining systemic inflammation are found in an increasing number of studies showing that the balance of pro- to anti-inflammatory T cells is associated with development of atherosclerotic plaque.

Our study is limited by the relatively small number of presurgical subjects willing to undergo comprehensive insulin-resistance testing (SSPG) and the inability to perform comprehensive flow cytometry for all T-cell subsets on every tissue sample. Furthermore, as in most human studies, it is difficult to prove causality. In particular, proving that Th2 cells protect humans from insulin resistance would require selective manipulation of Th2 cells, in vivo, which is not feasible. Considering the present findings together with those from our prior diet-induced obese mouse studies, in which transfer of Th2 cells protected against the development of insulin resistance, the present results are highly suggestive of a similar relationship in humans.

In summary, the present findings extend previous studies in mice and add to the small but growing body of literature in humans implicating T lymphocytes in obesity-related insulin resistance and type 2 diabetes mellitus. Not only Th1 and Th2 CD4 cells, as well as IFN-γ+ CD8 T cells, are abundantly present in the VAT and SAT of healthy overweight and obese humans but also the balance of pro- to anti-inflammatory subtypes is associated with systemic inflammation and insulin resistance. The high frequencies of Th1, Th2, and Th17 cells in 2 separate adipose tissue depots are consistent with antigenic stimulation in these tissues and indicate a widespread effect. Thus, these results provide compelling evidence for a role of the adaptive immune system in systemic inflammation and insulin resistance in overweight or obese humans. Identification of antigens and other key components of this process may ultimately prove useful in the prevention and treatment of obesity-associated metabolic diseases.

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The biological mechanisms linking obesity to insulin resistance are not fully elucidated. It has been suggested that inflammation in adipose tissue may play a role as follows: proinflammatory cytokines and macrophages have been identified in human adipose tissue, and overfeeding mice shifts macrophage phenotype in adipose tissue from anti- to proinflammatory. Other types of immune cells may also potentiate localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005;46:2347–2355.


Significance

The biological mechanisms linking obesity to insulin resistance are not fully elucidated. It has been suggested that inflammation in adipose tissue may play a role as follows: proinflammatory cytokines and macrophages have been identified in human adipose tissue, and overfeeding mice shifts macrophage phenotype in adipose tissue from anti- to proinflammatory. Other types of immune cells may also potentiate localized inflammation and insulin resistance. In mice, the ratio of pro- to anti-inflammatory T-helper cells in adipose tissue increases with weight gain, and blocking their activity prevents obesity-induced insulin resistance. Here, we show that T-helper 1 (proinflammatory) and T-helper 2 (anti-inflammatory) cell frequency in human subcutaneous and visceral adipose tissues correlate with systemic inflammation and insulin resistance. These results extend findings in mice to humans and provide strong support for a role of the adaptive immune system in mediating obesity-induced inflammation and insulin resistance.


References

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Material and Methods

Subjects: Healthy women and men 35-65 years of age and BMI 25-35 kg/m² were recruited via local newspaper advertisements “seeking overweight and moderately-obese healthy volunteers.” Eligibility required that subjects be nondiabetic, defined as fasting plasma glucose <126 mg/dL, and free of major organ disease, chronic inflammatory conditions, cancer, active psychiatric disease, history of liposuction or gastric bypass procedures, pregnancy and lactation. In addition, subjects were required to be weight-stable for a minimum of three months and not taking medications known to alter glucose metabolism, immune function, or body weight. In order to evaluate T-cell subsets (flow cytometry) and their respective cytokines (RT-PCR) from visceral adipose tissue, subjects were also recruited from the Stanford Bariatric Surgery Clinic. These subjects, like the others, were required to be healthy and free of diabetes and major organ disease, although there was no upper limit on BMI (maximum 49.5 kg/m²). Due to limited tissue sample size and high number of cells required for flow cytometry, in tissue from subjects with smaller biopsies, flow cytometry was prioritized and thus comparable subjects with banked frozen tissue, harvested in an identical manner, were used to augment the sample size for real-time-polymerase chain reaction (RT-PCR) analyses. All subjects gave written, informed consent, and the protocol was approved by the Stanford University IRB.

Quantification of insulin-mediated glucose uptake: Subjects were admitted to the Stanford Clinical and Translational Research Unit (CTRU) after a 12 hour overnight fast for measurement of fasting body weight and height, on standardized scale and stadiometer. Blood was drawn for measurement of plasma glucose (oximetric method), lipid/lipoproteins (ultracentrifugation), and hsCRP (bariatric patients). Insulin-mediated glucose uptake was quantified by the modified insulin suppression test as originally described¹, which has been validated with the euglycemic hyperinsulinemic clamp². Briefly, after an overnight 12 hour fast, subjects were infused for 180
min with octreotide (0.27 µg/m2•min) to suppress endogenous insulin secretion, insulin (25 mU/m2 min), and glucose (240 mg/m2 •min). Blood was drawn at 10-min intervals from 150 to 180 min of the infusion for measurement of plasma glucose and insulin concentrations: the mean of these values was used as the steady-state plasma insulin (SSPI) and glucose (SSPG) concentration for each individual. As SSPI concentrations are, by design, similar in all subjects, the SSPG concentration provides a direct and quantitative measure of the relative ability of insulin to mediate disposal of an infused glucose load; the higher the SSPG concentration, the more insulin resistant the individual. Based on a prior study of the distribution of SSPG concentrations in 449 healthy nondiabetic adults³, we classified volunteers as being insulin resistant (IR) if their SSPG concentration was in the top tertile, and insulin sensitive (IS) if their SSPG concentration was in the lowest tertile of the SSPG distribution as previously described. The use of SSPG tertiles for the purpose of classification is based on prospective studies showing that individuals with SSPG in the top tertile suffer adverse health effects such as DM2, cardiovascular disease, and hypertension, whereas those in the lowest SSPG tertile do not⁴,⁵.

Laboratory analysts were blinded as to insulin resistance status of the subjects. High-sensitivity C-reactive protein (hsCRP) was quantified on fasting plasma samples by immunoassay.

**Human IL6 measurement**

Human inflammatory cytokine kit (BD bioscience Cytometric Bead Array, CBA) was used to measure IL6 levels in EDTA-treated plasma from human subjects. 10uL samples were run in duplicate. CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence. Fluorescence was measured using flow cytometry.

**Adipose tissue biopsies:** After an overnight fast, under sterile conditions and local anesthesia, nonbariatric surgery subjects underwent superficial (just under the skin) subcutaneous periumbilical adipose tissue biopsies as previously described⁶. For bariatric surgery patients,
superficial SAT from the periumbilical area and VAT from the greater omentum were obtained in a standardized manner at the outset of the bariatric surgery procedure and immediately processed by laboratory personnel. For both surgical and nonsurgical patients, a portion of adipose tissue was flash frozen on liquid nitrogen for preparation of cDNA. Isolation of adipocytes and stromal-vascular cells from remaining tissue was performed via collagenase digestion using a modification of the original protocol of Rodbell\textsuperscript{7}. Sterile harvested tissue was immediately placed in sterile PBS with 2% bovine serum albumin until separation was initiated. Mechanically minced adipose tissue was digested for 30 min at 37°C in a shaking water bath with collagenase I, 1mg/mL in 0.1M HEPES, 0.12M NaCl, 0.05M KCl, 0.005M glucose, 1.5% wt/vol BSA, 1mM CaCl\textsubscript{2}, 2H2O, pH7.4. The cell suspension was then filtered through a 500-um nylon mesh and centrifuged for 5 min at 500RPM at room temperature. Isolated cells were washed and mature fat cells removed by flotation. The resulting cell pellet was collected after centrifugation at 1200 RPM for 5 minutes and then incubated in erythrocyte lysis buffer (Invitrogen) for 10 min at 37°C. PBS with 2% BSA was added to the cell suspension following erythrocyte lysis procedure.

**Flow cytometry:** Single cell suspensions of mononuclear cells from fresh adipose tissue and heparinized whole blood were analyzed by multicolor flow cytometry for the frequency and activation state of CD4 T cell subsets as previously described\textsuperscript{8}. These experiments utilized fluorescently labeled monoclonal antibodies (mAbs) specific for CD2, CD4, CD8, CD14, CD16, CD19, CD56, CD45, IL-13, IL-17, and IFN-\gamma, along with the appropriate isotype-matched control Abs. Cells were analyzed directly or, for intracellular cytokine detection, stimulated with PMA (10 ng/ml) and Ionomycin (1\mu g/ml) for 4 hours with addition of Brefeldin A (10 \mu g/ml) 1-hour post stimulation. Subsets of interest included Th1 (IFN\textsuperscript{+}), Th2 (IL-13\textsuperscript{+}), Th17 (IL-17\textsuperscript{+}) and Treg (FoxP3\textsuperscript{+}), which were expressed as % CD4 cells, and total CD4 and CD8 T cells, expressed as % total T cells. Details of the gating procedure are shown in **Supplemental Figure 1.**
Stimulated cells were subsequently permeabilized with Cytofix/Cytoperm and analyzed by flow cytometry with BD LSRII and FACSDiva software. To identify Tregs, cells surface-labeled cells were resuspended with 1 ml of Fix/perm buffer (1x) and incubated at room temperature for 20 min. After washing twice with MACS buffer, cells were incubated with FoxP3 perm buffer at room temperature for 15 minutes. The supernatant was discarded and FoxP3 Perm buffer (100 ul) was added to the cells prior to staining with intracellular FoxP3 Pacific Blue antibody (Biolegend), for an additional 30 min. Cells were washed and resuspended with MACS buffer prior to flow cytometry analysis. The cells were kept in the dark during the process. Although in the current study identification of Th2 cells was based on detection of intracellular IL-13 in CD4 T cells, under the assay conditions used there is >95% overlap between IL-13 and IL-4 producing cells. All fluorochrome-conjugated antibodies and reagents were purchased from either BD Biosciences or Biolegend.

**Gene expression:** Total RNA was extracted from frozen adipose tissue using TriZol (Invitrogen, Carlsbad, CA) and the Adipose Tissue RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized from total RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA). A threshold cycle (Ct value) was obtained from each amplification curve, and a Δ Ct value calculated by subtracting the Ct value for 18S ribosomal RNA from the Ct value for each sample. A ΔΔCt value was then calculated by subtracting the Δ Ct value of a SAT adipocyte sample (control). Gene expression of cytokines reflective of pro-inflammatory Th1 and Th17 activation (IL-6, IFNγ and IL-17) as well as anti-inflammatory IL-10 were quantified, using Taqman primers/probes (Applied Biosystems). Fold changes vs control were determined using the comparative threshold method ($2^{-\Delta\Delta Ct}$).

**Immunohistochemistry:** Fresh SAT and VAT tissue samples were fixed in 10% formalin then paraffin embedded by Histo-Tec laboratory (Histo-Tec, Hayward CA). Sections were
deparaffinized in 3 changes of xylene followed by graded alcohols. Antigen retrieval was performed using pH 6.0 Diva universal retrieval solution (Biocare Medical) inside a pressure cooker (Biocare Medical). Endogenous peroxidase activity was quenched using Peroxidazed 1 (Biocare Medical). Sections were blocked with serum-free protein blocker (Dako). The primary antibodies used were: mouse monoclonal anti-CD4 (clone: 4B12, 1:10, Vector), rabbit monoclonal anti-CD8 (clone: EP1150Y, 1:100, Abcam). For detection, MACH2 Double Stain 2 (Biocare Medical) was used. Color development was performed with freshly prepared 3,3’ Diaminobenzidine (DAB) solution and Ferangi Blue solution (Biocare Medical) before mounting with Faramount Mounting Medium (Dako).

**Statistical analysis:** Data are expressed as mean ± standard deviation. Normality of data was tested via quantile plots. Non-normally distributed data (PBMC Th2 and hsCRP) were log-transformed for statistical tests. Comparison of gene expression and T-cell data between adipose tissue depots and peripheral blood was done utilizing paired Student’s t-test. Pearson’s correlations were used to determine univariate associations between T-cell variables in tissue and blood with insulin resistance (SSPG). Linear regression with adjustment for BMI was used to measure associations between T-cell frequency and IL-10 expression and insulin resistance (SSPG), as well as between Th frequency and plasma cytokines. P< 0.05 was considered statistically significant.

**References**


**Supplementary Table I.** Subject characteristics (mean ± SD)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nonbariatric Subjects for T Cells (n=13)</th>
<th>Bariatric Subjects for T Cells (n=10)</th>
<th>Nonbariatric Subjects for rtPCR (n=20*)</th>
<th>Bariatric Subjects for rtPCR (n=10)</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
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<td>56 ± 9.3</td>
<td>45.3 ± 12.6</td>
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<td>7/3 update</td>
<td>19/1</td>
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<tr>
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<td>5/4/1/0</td>
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<tr>
<td>BMI (kg/m2)</td>
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<td>45.3 ± 3.4</td>
<td>31.7 ± 4.6</td>
<td>46.2 ± 8.6</td>
</tr>
<tr>
<td>SSPG (mg/dL)</td>
<td>127 ± 52</td>
<td>231 ± 88</td>
<td>166 ± 80</td>
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</tbody>
</table>

*includes 6 subjects from the T cell cohort and 14 separate subjects for a total of 27 nonbariatric subjects

rtPCR: real time polymerase chain reaction

C: Caucasian; H: Hispanic; B: Black; A: Asian
**Supplementary Figure I.** Gating strategy to separate T cell subsets via flow cytometry performed on human adipose tissue and blood. **Panel A:** T helper cells: After gating out doublet cells, the CD45+ lymphocyte population is selected and non-viable cells are excluded. CD2+ T cells are separated from other lineages. Side scatter and CD8 expression are then used to identify CD4 and CD8 populations. Positive selection of CD4 T cells is avoided due to down modulation of the CD4 molecule upon activation with PMA/Ionomycin. IFNγ, IL13, and IL17 are plotted against CD4 to determine TH1, TH2, TH17 expression, respectively.

**Panel B:** Plots displaying IFNγ, IL13 and IL17 expression of CD4 cells before and after stimulation with PMA/Ionomycin.

**Panel C:** Regulatory T cells: After gating out doublets, the CD45+ lymphocyte population is selected and non-viable cells are excluded. CD2+ cells are excluded from other lineages. Side scatter and CD8 expression are then used to identify CD4 and CD8 population. CD4+ cells expressing FoxP3 are then identified as regulatory T cells.

![Graph showing the relationship between plasma interleukin-6 concentrations in association with human subcutaneous adipose tissue (SAT) Th1 frequency.](image)

**Supplementary Figure II.** Relationship between plasma interleukin-6 concentrations in association with human subcutaneous adipose tissue (SAT) Th1 frequency.