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Depot-specific differences in inflammatory mediators and a role for NK cells and IFN- γ in inflammation in human adipose tissue

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Abstract

Background—Adipose tissue is a primary *in vivo* site of inflammation in obesity. Excess visceral adipose tissue (VAT), when compared to subcutaneous adipose tissue (SAT), imparts an increased risk of obesity-related comorbidities and mortality, and exhibits differences in inflammation. Defining depot-specific differences in inflammatory function may reveal underlying mechanisms of adipose-tissue-based inflammation.

Methods—Stromovascular cell fractions (SVFs) from VAT and SAT from obese humans undergoing bariatric surgery were studied in an *in vitro* culture system with transcriptional profiling, flow cytometric phenotyping, enzyme-linked immunosorbent assay and intracellular cytokine staining.

Results—Transcriptional profiling of SVF revealed differences in inflammatory transcript levels in VAT relative to SAT, including elevated interferon- γ (IFN- γ) transcript levels. VAT demonstrated a broad leukocytosis relative to SAT that included macrophages, T cells and natural killer (NK) cells. IFN- γ induced a proinflammatory cytokine expression pattern in SVF and adipose tissue macrophages (ATM). NK cells, which constitutively expressed IFN- γ , were present at higher frequency in VAT relative to SAT. Both T and NK cells from SVF expressed IFN- γ on activation, which was associated with tumor necrosis factor- α expression in macrophages.

Conclusion—These data suggest involvement of NK cells and IFN- γ in regulating ATM phenotype and function in human obesity and a potential mechanism for the adverse physiologic effects of VAT.

Keywords

adipose tissue; inflammation; IFN- γ ; NK cells

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Conflict of interest

The authors declare no conflict of interest.

Introduction

Human obesity is associated with aberrations in inflammatory function that underlie many obesity-related disease processes. Adipose tissue is a primary *in vivo* site of inflammation in obesity, and adipose tissue macrophages (ATM) have a central role in these processes.^{1,2} Emphasizing the clinical relevance of these observations, macrophages and their inflammatory products are primary causative agents in the pathogenesis of insulin resistance and diabetes.^{3,4} Macrophages may be categorized as M1, an inflammatory phenotype, or M2, a scavenging/remodeling phenotype. ATM with an inflammatory M1-type phenotype have been identified in murine and human obesity,^{2,5} but phenotypic and functional properties of human ATM remain poorly defined.

Depot-specific differences in metabolism and inflammatory function impact on the study of adipose-tissue-based inflammation in obesity. Strong epidemiologic data demonstrate that excess visceral adipose tissue (VAT), when compared to subcutaneous adipose tissue (SAT), is associated with an increased risk of numerous comorbidities of obesity as well as overall mortality.^{6,7} At the cellular level, VAT exhibits increased expression of inflammatory markers and increased ATM infiltration compared to SAT.^{1,8,9} These observations suggest that characterization of depot-specific differences in tissue inflammatory function may serve as a model to identify mechanisms of adipose-tissue-based inflammation.

The goal of this study was to define depot-specific differences in inflammatory mediator gene expression and lymphocyte subset frequencies within adipose tissue from obese humans in order to identify potential mechanisms of adipose-tissue inflammation. We demonstrate depot-specific differences in adipose tissue inflammatory transcript levels and ATM, natural killer (NK) cell and T-cell frequencies. In addition, we identify a population of NK cells that constitutively express interferon- γ (IFN- γ) which is present at much higher frequency in VAT relative to SAT, and a potential role for IFN- γ in regulating inflammatory cytokine expression in adipose tissue. To the best of our knowledge, these data are the first that implicate NK cells in adipose-tissue-based inflammation in humans. These data suggest a model by which NK cells regulate ATM phenotype and a potential mechanism for the adverse physiologic effects of VAT.

Materials and methods

Subjects

Obese women undergoing laparoscopic bariatric surgery recruited from the OHSU Bariatric Surgery Clinic were enrolled and consented to institutional review board approval. All applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed. All subjects met National Institutes of Health (NIH) criteria for surgery (NIH Consensus Conference 1991) and had a body mass index (BMI) ≥ 40 . VAT (10–15 g) was harvested from the greater omentum and SAT (1–3 g) was harvested from the abdominal wall at the beginning of the operation and both were processed immediately.

Four cohorts of subjects provided tissues for separate experiments. Tissue from nine subjects was used for the initial transcriptional analysis (Figure 1; for this cohort mean age 43, mean BMI 53, 22% diabetic as defined by use of oral hypoglycemic agents or insulin, 78% had diagnosis of sleep apnea as defined by a positive polysomnography test, 56% had diagnosis of hypertension as defined by use of antihypertensive medication). A second cohort of 25 subjects was used for flow cytometric phenotyping and magnetic bead sorting, culture and enzyme-linked immunosorbent assay (ELISA) analysis (Figures 2, 3, 4 and 6; mean age 45, mean BMI 49, 36% diabetic, 44% sleep apnea, 64% hypertension). Of these 25 subjects, 25 were used for CD45 and CD14 phenotyping, 9 were used for T-cell phenotyping, 7 were

used for NK cell (CD56) phenotyping and 10 were used for magnetic bead sorting, culture and ELISA analysis. A third cohort of 10 subjects was used for stromovascular cell fraction (SVF) culture and qRT-PCR analysis (Figure 5; mean age 45, mean BMI 45, 20% diabetic, 60% sleep apnea, 60% hypertension). A fourth cohort of eight subjects was used for intracellular cytokine staining (Figure 7; mean age 43, mean BMI 48, 13% diabetic, 13% sleep apnea, 44% hypertension). The four cohorts were similar with respect to the clinical characteristics studied ($P>0.158$ for all variables tested), with the exception of sleep apnea, for which the fourth cohort had a lower prevalence ($P=0.031$). There were no significant differences among subject groups with respect to medication use, including angiotensin-converting enzyme inhibitors (19% of entire group), β -blockers (17% of entire group), statins (19% of entire group) and aspirin or nonsteroidal anti-inflammatory drugs (17% of entire group) ($P>0.500$ in all the cases).

Stromovascular and adipocyte cell fraction preparation

All cell culture media and reagents were certified and tested by the manufacturer to have endotoxin levels of less than 0.030 EU per ml. Serum and collagenase had endotoxin levels of less than 1 EU per ml. Vessels were carefully dissected from adipose tissue, then washed twice in phosphate-buffered saline (PBS) and 4% bovine serum albumin (BSA), minced, washed twice again and digested with type II collagenase (175Uml^{-1} in PBS and 2% BSA; Gibco Inc., Carlsbad, California, USA) for 60 min at 37°C with gentle agitation followed by centrifugation at 200 g for 10 min. The stromovascular (SVF) cell pellet was retrieved, washed, filtered and used for subsequent analyses. The adipocyte layer was also retrieved between the top lipid layer and the intermediate aqueous phase and RNA was isolated.

SVF culture

One million SVF cells isolated as above were cultured in RPMI and 10% fetal calf serum, penicillin, streptomycin and glutamine for 24 h in 1 ml of media with or without IFN- γ (BioLegend, Inc. San Diego, CA, USA; 100 ng ml^{-1}), LIGHT (R&D Systems, Inc., Minneapolis, MN, USA; 100 ng ml^{-1} , or phorbol 12-myristate 13-acetate (PMA) and ionomycin (50 ng ml^{-1} and 1 mg ml^{-1} , respectively), after which cells were harvested and studied with qRT-PCR, ELISA or intracellular cytokine staining as described below.

qRT-PCR

RNA was prepared from SVF and adipocyte cell fractions using an RNeasy lipid kit (Qiagen Inc., Germantown, MD, USA). A fixed volume ($150\text{ }\mu\text{l}$) of adipocyte cell suspension derived from 5 g of collagenase-digested adipose tissue was used to prepare adipocyte RNA. SVF RNA was prepared from one million cells. Equal amounts of input RNA were used from both fractions for each sample, thus normalizing qRT-PCR results by the amount of input RNA. RNA was reverse transcribed using random hexamer primers. qRT-PCR was performed using SYBR Green I reagent and transcript-specific primers. Inflammatory qRT-PCR arrays were used according to the manufacturer's instructions (SA Biosciences, Inc., Frederick, MD, USA; catalogue no. APHS-PL04; data in Table 1). For all other reverse transcription-PCR (Figure 5), gene-specific primers were used. GAPDH and actin were used as endogenous controls and provided similar results in all cases. Fold changes relative to GAPDH are reported (IFN- γ FOR: 5'-AGCGGATAATGGAAGTCTTTTCTTAG-3', REV: 5'-AAGTTTGAAGTAAAGAAGACAATTTGG-3'; interleukin (IL)-1Ra FOR: 5'-GGAATCCATGGAGGGAAGAT-3', REV: 5'-CCTTCGTCAGGCATATTGGT-3'; IL-6 FOR: 5'-AATTCGGTACATCCTCGACGG-3', REV: 5'-GGTTGTTTTCTGCCAGTGCCT-3'; IL-8 FOR: 5'-CCAAGCTGGCCGTGGCTCTCTTGG-3', REV: 5'-GACATCTAAGTTCTTTAGCACTCC-3'; IL-10 FOR: 5'-GCCTGGTCCTCCTGACTGGG-3', REV: 5'-GCAGGTTGCCTGGGAAGTGG-3'; CCL-2

FOR: 5'-GCAATCAATGCCCCAGTCA-3', REV: 5'-TGCTGCTGGTGAATCTTCTATAGCT-3'; tumor necrosis factor- α (TNF- α) FOR: 5'-TCTCGAACCCCGAGTGACA-3', REV: 5'-GGCCCGGCGGTTCA-3'; GAPDH FOR: 5'-TGCACCACCAACTGCTTAG-3', 5'-GATGCAGGGATGATGTTTC-3'; actin FOR: 5'-CCGCATCGTCACCAACTG-3', REV: 5'-GGCACACGCAGCTCATTG-3'). The $2^{-\Delta\Delta C_t}$ relative quantification method was used to calculate fold difference in transcript levels between samples,¹⁰ and efficiencies of amplification for all primer pairs were verified to be equivalent over a range of template concentrations. All qRT-PCR were performed using an ABI 7900 real-time thermocycler (Applied Biosystems Inc., Foster City, CA, USA).

Flow cytometry

Cells were incubated with appropriate antibodies (CD14-APC-Cy7, CD3-PE, CD11b-PE, CD11c-FITC, CCR5/CD195-PE, CD206-FITC, CD163-Alexa Fluor 647, HLA-DR-Alexa Fluor 647, CD64 PE-Cy7, CD4 PE-Cy7, CD8-APC (BioLegend Inc.), CD45 PE-Cy5.5 (eBiosciences Inc., San Diego, CA, USA), CD56-PE, CCR2/CD192-APC (BD Pharmingen Inc.)) for 30 min, washed with PBS+0.5% BSA+0.1% NaN₃, fixed with Cytotfix/Perm solution (BD Pharmingen Inc.) and analyzed on an LSR II flow cytometer (Becton Dickinson and Co.). All antibodies were tested with fixed and unfixed cells to ensure equivalent results. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Data were compensated after acquisition and isotype controls and Fluorescence Minus One gating¹¹ were used to determine borders between positive and negative groups. All data were analyzed after exclusion of doublets and nonviable cells using viable dye (Invitrogen Inc., Carlsbad, California, USA) staining. A large forward and side scatter gate was used to include all viable cells, within which subsequent analysis was restricted to CD45+ cells.

Intracellular cytokine staining

Stromovascular cell fractions (one million cells per ml) were cultured with or without PMA and ionomycin (50 ng ml⁻¹ and 1 mg ml⁻¹, respectively) for 2 h, then Brefeldin A (BioLegend Inc.) was added and stimulation continued for 12 h, after which SVF was harvested, washed and stained with surface antibodies, followed by fixation/permeabilization and intracellular staining with antibody specific for IFN- γ or TNF- α or IFN- γ -Pacific Blue, TNF-FITC (eBiosciences Inc.). Both surface and intracellular stains were preceded by preincubation with murine IgG. SVF were washed and resuspended in flow buffer and data were acquired on an LSR II flow cytometer. Intracellular isotype control staining was used to determine positive staining for intracellular IFN- γ and TNF- α .

Cytokine ELISA

ELISA of cell culture supernatants was performed using standard cytokine-specific ELISA kits (BioLegend Inc.) following the manufacturer's instructions.

Cell sorting

Cell sorting was performed using antibody-coated magnetic beads (Miltenyi Inc., Bergisch Gladbach, Germany) per manufacturer's instructions. CD14-, CD3- and CD16-coated beads were used. SVF underwent two rounds of sorting with CD3 beads (20 μ l beads per 10⁷ cells). Unbound cells then underwent two rounds of sorting with CD14 beads followed by two rounds of sorting with CD16 beads. Flow cytometry was used to assess enrichment of bead-enriched populations. Twenty million SVF cells were used as the input number of cells for all bead sorting for all conditions. Once sorted, cells were counted again, and one million sorted cells were cultured in 1ml of media in all the cases.

Statistical analysis

For comparison of subject groups, analysis of variance was used to compare continuous variables between groups (age, BMI) and χ^2 -test was used to compare dichotomous variables between groups (comorbidities, medications). Owing to limitations in SVF cell yields from adipose tissue depots, different experiments used cells from different groups of patients. All data were tested for normality and parametric or nonparametric tests were used for normally distributed data, whereas nonparametric tests were used for data that were not normally distributed. ΔC_t values were compared for statistical analysis of qRT-PCR data. Cell subset frequencies as a percent of the immediate proximal parent population were compared for analysis of flow cytometry data. Paired *t*-test was used to compare flow cytometry, qRT-PCR and ELISA data between paired VAT and SAT specimens within obese subject cohorts and between media and IFN- γ or LIGHT (homologous to Lymphotoxin, Inducible expression, competes with herpes simplex virus Glycoprotein D for HVEM on T cells)-stimulated arms of *in vitro* cultures.

Results

SVF is the dominant source of inflammatory cytokines in human AT, and demonstrates an M1 transcriptome shift in VAT relative to SAT

Adipose tissue is comprised of adipocytes and an SVF that is rich in lymphocytes. We initially compared SVF and adipocyte cell fractions to determine if SVF was a major source of inflammatory cytokines in adipose tissue. RNA from SVF and adipocyte cell fractions isolated from VAT and SAT from nine obese female subjects was studied with qRT-PCR. SVF was a dominant source of IFN- γ , TNF- α , IL-10, IL-8, CCL-2 and IL-1Ra transcripts, whereas adipocytes were the primary source of leptin transcript. IL-6 transcript was present at similar levels in both fractions (Figure 1). These data demonstrate that SVF is the major source of inflammatory cytokines within adipose tissue.

We next studied depot-specific differences in 15 inflammatory transcript levels in SVF by applying SVF RNA isolated from VAT and SAT from 9 obese female subjects to inflammatory qRT-PCR arrays (Superarray Inc.; catalogue no. APHS-025C) (Table 1). IFN- γ and the TNF superfamily member LIGHT¹² were the most elevated transcripts in VAT relative to SAT (8.2 and 9.9 fold difference, respectively). Other macrophage-related genes were also implicated: GM-CSF transcripts were elevated, whereas HOX1, IL-10 and bcl-3 transcripts were decreased in VAT.

Increased ATM, NK cells and T cells in VAT relative to SAT

Others have shown that ATM increased in VAT relative to SAT.¹ We used the pan-leukocyte marker CD45 to study all leukocytes within SVF and observed an increase in CD45⁺ cells in VAT relative to SAT in these 25 obese subjects (2.1×10^5 and 8.2×10^4 cells per gram of tissue for VAT and SAT respectively, $P=0.036$) (Figure 2a). We next studied T and NK cells, as these latter cell types are potential sources of increased IFN- γ in VAT. Subgroups of the 25 subjects used to study CD45⁺ cells were studied with antibodies directed toward the macrophage marker CD14 ($n=25$), T-cell markers CD3, CD4 and CD8 ($n=9$), and the NK cell marker CD56 ($n=7$). The absolute number of CD14⁺ ATM was increased in VAT relative to SAT (2.7×10^4 and 1.2×10^4 cells per gram of tissue for VAT and SAT respectively, $P=0.018$). The absolute number of CD3⁺ T cells was increased in VAT relative to SAT (9.1×10^4 and 3.0×10^4 cells per gram of tissue for VAT and SAT respectively, $P=0.008$), and involved both CD4⁺ and CD8⁺ compartments (5.2×10^4 and 2.4×10^4 cells per gram of tissue, $P=0.021$ for CD3⁺ CD4⁺ cells and 4.2×10^4 and 7.4×10^4 cells per gram of tissue, $P=0.007$ for CD3⁺ CD8⁺ cells in VAT and SAT, respectively). The

absolute number of CD56+ cells was increased in VAT relative to SAT (1.9×10^4 and 8.1×10^3 cells per gram of tissue for VAT and SAT respectively, $P=0.018$) (Figure 2b).

The CD14-enriched cell population has a macrophage phenotype and is a dominant source of inflammatory cytokines in SVF

Detailed phenotyping of the CD14+ subpopulation in VAT and SAT was performed by gating on CD14+ cells and studying expression of macrophage-related markers. Subgroups of the subjects above were studied with antibodies directed toward CD11b and CD11c ($n=9$), CD56 and HLA-DR ($n=7$), CD64, CCR2 and CCR5 ($n=10$), CD163 ($n=5$), and CD206 ($n=7$). The phenotype of CD14+ cells was consistent with a tissue macrophage lineage, including expression of the myeloid/macrophage integrins CD11b and CD11c, the macrophage chemokine receptors CCR2 and CCR5, and tissue macrophage-specific markers CD64, CD163 and CD206 (Figure 3). The CD14+ population expressed uniform levels of CD64, CD11c and CCR2, and a relatively broad but overall high distribution of HLA-DR expression. CD14+ cells were heterogeneous with respect to CCR5, CD11b, CD163 and CD206. Approximately 40% of all CD14+ cells in VAT and SAT expressed CCR5, whereas the remainder were CCR5- (mean percent of all CD14+ cells: VAT, 42% CCR5+ vs SAT, 38% CCR5+; $P=0.637$ for paired comparison between VAT and SAT). Depot-specific differences in CD11b, CD163 and CD206 subpopulations were observed: VAT demonstrated a lower frequency of CD11b^{bright} cells and a higher frequency of CD11b^{dim} cells compared to SAT (mean percent of CD14+ cells: VAT, 35% 11b^{bright} vs SAT, 51% 11b^{bright}, $P=0.024$), and a higher frequency of CD163^{bright} and CD206^{bright} cells with a decrease in respective dim subpopulations (mean percent of CD14+ cells: VAT, 32% CD163^{bright} vs SAT, 16% CD163^{bright}, $P=0.016$; VAT, 42% CD206^{bright} vs SAT, 19% CD206^{bright}, $P=0.006$).

To define functional differences between CD14+, CD3+ and CD16+ subpopulations, *in vitro* 24 h basal cytokine expression was compared in antibody-coated magnetic bead CD14-, CD3- and CD16-enriched SVF. Serial antibody-coated magnetic bead enrichment provided populations that were enriched to a purity of 83% of all viable cells for CD3+ cells and 45% for CD14+ cells. CD14+ cells were the dominant source of inflammatory cytokines compared to CD3+ and CD16+ cells, expressing cytokines at levels similar to bulk SVF. These data also demonstrate basal expression levels of IL-6 and IL-8 in CD14-enriched SVF an order of magnitude higher than basal expression levels of IL-10 and IL-1Ra. No differences were noted in basal IFN- γ expression levels between bulk, CD14-enriched, CD3-enriched and CD16-enriched cells (Figure 4).

IFN- γ induces M1 cytokine expression in bulk and CD14-enriched primary human VAT SVF, and LIGHT induces a modest upregulation of IFN- γ transcription in bulk SVF

Since IFN- γ and LIGHT were the most differentially expressed transcripts between VAT and SAT, and have been implicated in the regulation of macrophage function,¹³⁻¹⁶ we next studied their involvement in regulating cytokine expression in ATM from primary human VAT SVF. VAT SVF cells from 16 obese female subjects were cultured for 24 h in the presence or absence of IFN- γ or LIGHT, after which cytokine transcript levels were studied with qRT-PCR. IFN- γ induced increased transcription of TNF- α and CCL-2, and decreased transcription of IL-10 in bulk SVF, consistent with an M1 cytokine expression profile. IFN- γ also induced its own transcription in primary human SVF, suggesting an autocrine positive feedback regulatory mechanism. LIGHT induced a modest but statistically significant ($P=0.043$) upregulation of IFN- γ transcription (30% increase) in primary human SVF, but had no effect on transcription of other cytokines tested (Figure 5).

We next studied the function of IFN- γ and LIGHT in regulating cytokine expression at the protein level in ATM and T cells within VAT SVF. Bulk SVF from VAT and SVF from VAT that was enriched for either CD3+ or CD14+ cells using antibody-coated magnetic beads from a subgroup of 11 subjects from the 25 subjects used for flow cytometry phenotyping were cultured with or without IFN- γ , and cytokine levels in culture supernatants were studied using ELISA. IFN- γ induced expression of TNF- α and CCL-2, typically considered M1 cytokines, and downregulated expression of IL-10, typically considered an M2 cytokine, in bulk SVF and CD14-enriched SVF (Figure 6). IFN- γ did not regulate cytokine expression in CD3-enriched SVF, and LIGHT did not regulate cytokine expression in bulk, CD3-enriched or CD14-enriched SVF for any cytokines tested (data not shown).

A unique population of NK cells that constitutively express IFN- γ is present at higher frequency in human VAT compared to SAT

We next studied potential sources of IFN- γ in adipose tissue. Primary human SVF from VAT was studied with intracellular cytokine staining and antibodies specific for T-cell (CD3, CD4 and CD8) and NK cell markers (CD56) and intracellular IFN- γ . A small but distinct population of NK cells that expressed low levels of IFN- γ constitutively was present at much higher frequency in VAT compared to SAT (mean 1.39 and 0.13% of all CD56+ cells for VAT and SAT respectively, $P=0.001$, paired t -test). CD3+ cells expressed IFN- γ constitutively at very low levels in both depots (Figure 7). We also studied IFN- γ expression in NKT cells, as defined by CD3 expression, within the NK cell population. CD56+CD3+ (NKT) cells that expressed low levels of IFN- γ constitutively were present at similar frequencies in VAT and SAT (mean 0.32 and 0.10% of all CD56+ cells for VAT and SAT respectively, $P=0.315$, paired t -test). CD56+CD3- (classic NK) cells that expressed low levels of IFN- γ constitutively were present at higher frequency in VAT compared to SAT (mean 1.41 and 0.42% of all CD56+ cells for VAT and SAT respectively, $P=0.019$, paired t -test), consistent with data presented in Figure 7b for CD56+ cells independent of CD3 expression. These data suggest that classic NK cells rather than NKT cells are the primary source of constitutive IFN- γ expression in VAT. Finally, separate intracellular cytokine staining experiments did not demonstrate IFN- γ expression by CD14+ cells within SVF.

T and NK cells do not express IFN- γ in response to LIGHT, but have the capacity to express IFN- γ on activation, which is associated with TNF- α expression in CD14+ ATM

We next sought to determine if LIGHT was an initiating *in vivo* stimulus for increased IFN- γ expression in VAT. We used intracellular cytokine staining to study IFN- γ expression in response to LIGHT stimulation in T and NK cells within SVF. SVFs from VAT were cultured for 12 h in the presence or absence of LIGHT, after which cells were stained with antibodies directed toward CD3, CD4, CD8, CD56, CD14 and intracellular IFN- γ . LIGHT did not induce IFN- γ expression in NK or T cells, or TNF- α expression in CD14+ cells as determined by intracellular cytokine staining (data not shown).

Finally, we studied the capacity of T and NK cells within SVF to express IFN- γ on activation with PMA and ionomycin. SVF from VAT was cultured with PMA and ionomycin followed by intracellular cytokine staining using antibodies directed toward CD3, CD56 and intracellular IFN- γ . Both T cells (CD3+) and NK cells (CD56+) markedly upregulated IFN- γ expression in response to PMA and ionomycin (mean difference between media and PMA/ionomycin-stimulated cells: 35.8%, $P=0.001$ for CD56+ cells and 52%, $P<0.001$ for CD3+ cells), demonstrating that these cells have the capacity to express IFN- γ in response to nonspecific activation.

Discussion

The increased clinical morbidity and differences in metabolism in VAT relative to SAT suggest that comparison of inflammatory processes within these anatomic tissue depots may identify underlying mechanisms of adipose-tissue-based inflammation. We found a marked increase in the number of ATM, NK cells and T cells and differences in inflammatory transcript levels in VAT relative to SAT. Furthermore, we demonstrate a unique population of IFN- γ -expressing NK cells in human VAT as well as a function for NK-cell-derived IFN- γ in regulating cytokine expression in ATM in human obesity. To the best of our knowledge these data are the first that implicate NK cells and IFN- γ in the regulation of inflammation within human adipose tissue.

Differences in inflammatory transcript levels and increased ATM in human VAT relative to SAT

We observed an increase in CD14⁺ ATM in VAT relative to SAT in obese humans, consistent with data from others.¹ In addition, transcriptional profiling demonstrated differences in inflammatory transcript levels in VAT relative to SAT, including lower levels of transcripts encoding the M2 macrophage products IL-10, bcl-3 and HMOX1,^{9,17-19} and increased levels of IFN- γ and GM-CSF transcripts, cytokines that induce an M1 phenotype.²⁰⁻²³ Furthermore, we demonstrate that CD14⁺ ATM have the capacity to express cytokines that, in general, both promote (for example, TNF- α , IL-6 and IL-8) and inhibit (for example, IL-10 and IL-1Ra) inflammation, consistent with data from others.⁵ Two pieces of evidence, however, suggest that, despite these opposing capacities, CD14⁺ ATM in VAT may be predisposed to an M1 phenotype. First, *in vitro* 24 h culture basal expression levels of IL-6 and IL-8, cytokines that generally promote inflammation, by CD14⁺ ATM were approximately 10-fold higher than IL-10 and IL-1Ra, cytokines that in general suppress inflammatory responses (Figure 4). Second, IFN- γ , transcript levels of which were present at approximately 10-fold higher levels in VAT relative to SAT, induced expression of TNF- α while decreasing expression of IL-10. Taken together, these observations suggest an increase in ATM predisposed to an M1 phenotype in VAT relative to SAT in human obesity, at least in part mediated by increased levels of IFN- γ , and suggest a mechanism by which VAT may exert its detrimental physiologic effects.

Aspects of our transcriptional profiling data conflict with previously published data. For example, others have shown higher levels of IL-6^{9,24} and bcl-3⁹ in VAT compared to SAT, whereas our data demonstrate only modestly higher levels of IL-6 in VAT, a difference that did not reach statistical significance, and lower levels of bcl-3 in VAT. In contrast to our data that are derived from omental adipose tissue, one earlier study⁹ examined epiploic rather than omental adipose tissue, suggesting unique transcriptomes among different intraabdominal adipose tissue depots (for example, omental, epiploic, mesenteric and retroperitoneal). In addition to depot-specific differences, variability in published tissue transcriptional profiles in human obesity^{8,9,25} reflects the heterogeneity of the patient populations studied. Finally, no significant depot-specific difference in TNF- α transcript levels was observed, contrary to the hypothesis that inflammation is greater in VAT. Others have also demonstrated higher levels of TNF- α transcript in human SAT relative to VAT,²⁶ consistent with our data and suggesting that depot-specific differences in transcripts are complex. TNF- α activity may be regulated at the post-transcriptional level, or may require *in vivo* stimuli not present *in vitro*.

A role for NK cells and IFN- γ in regulating cytokine expression in VAT ATM

Few previous data show depot-specific adipose tissue T-cell and NK cell frequency in obesity. We observed a lymphocytosis in VAT relative to SAT that involves not only ATM,

but T and NK cells as well. These data confirm reports of increased adipose tissue T-cell infiltration in murine and human obesity,^{27,28} and are the first of which we are aware of, demonstrating depot-specific differences in human adipose tissue NK cell frequencies. These observations are particularly relevant given the observed increase in IFN- γ in VAT and its involvement in regulating ATM cytokine expression. As known sources of IFN- γ , we demonstrate that adipose-derived NK and T cells have the capacity to upregulate IFN- γ expression on activation. Furthermore, we demonstrate a unique population of NK cells that constitutively express IFN- γ which is present at much higher frequency in VAT relative to SAT, suggesting that NK cells may be a potential source of increased IFN- γ in VAT.

IFN- γ regulates macrophage function and induces an M1 phenotype in monocytes and macrophages in numerous systems.^{20–23} Others have demonstrated a role for IFN- γ in regulating inflammation and insulin resistance in murine obesity.²⁹ Our data suggest a role for IFN- γ in the regulation of macrophage phenotype within human VAT, in which IFN- γ induced expression of TNF- α , a paradigmatic M1 cytokine, while downregulating expression of IL-10, an M2 cytokine. Despite these observations, IFN- γ downregulated expression of IL-6, typically considered an M1-cytokine. We suspect that high basal levels of IL-6 expression may make ATM unresponsive to further upregulation by IFN- γ . These observations suggest a model in which increased IFN- γ expression by NK cells drives an M1 shift in CD14⁺ ATM in VAT, inducing increased adipose tissue inflammation and its sequelae, for example, local-tissue-based insulin resistance.^{30,31} Such a model provides a possible explanation for the detrimental physiologic effects of VAT. These data also suggest an important and novel function for NK cells and IFN- γ in regulating tissue macrophage-mediated inflammatory responses in human obesity.

LIGHT

The *in vivo* stimuli that regulate IFN- γ expression within adipose tissue in obese humans remain unknown. LIGHT has been implicated in the regulation of both macrophage and T-cell function,^{13,14,32–35} and induces IFN- γ expression in T cells in a number of systems,^{14,32–35} whereas a single report demonstrates that LIGHT induces activation of and IFN- γ expression by NK cells in a murine tumor model.³⁶ We were therefore surprised that LIGHT induced only modest upregulation of IFN- γ transcription in bulk SVF, but did not induce cytokine expression in bulk SVF, NK cells or T cells when studied with ELISA or intracellular cytokine staining. SVF lymphocytes may be resistant to further stimulation with LIGHT secondary to chronic elevated levels within VAT. Alternatively, other adjunctive stimuli may be necessary, or LIGHT may regulate ATM independent of IFN- γ . LIGHT induces IL-8 expression in synovial fluid macrophages from patients with rheumatoid arthritis,¹³ and TNF- α and IL-8 expression in a human macrophage cell line.³⁷ Despite these data, however, we observed no effect of LIGHT on cytokine expression in adipose tissue SVF using qRT-PCR, ELISA or intracellular cytokine staining. Further research is necessary to define the role of LIGHT in regulating macrophage and T-cell function within adipose tissue.

CD14⁺ cells demonstrate heterogeneous expression of a number of macrophage markers (Figure 3), and are likely composed of multiple macrophage phenotypes. Depot-specific differences were noted with respect to expression of CD11b, CD163 and CD206, and to a lesser extent, HLADR. Variable expression of HLA-DR may represent differences in activation states among ATM, whereas differences in expression of CD11b, CD206 and CD163 may define M1 and M2 subpopulations. The functional significance of these differences will require further study. Nonetheless, the majority of these cells express CD64, CD11b, CD206 and CD163, markers associated with a tissue macrophage phenotype.^{5,38,39} Despite this heterogeneity, the CD14⁺ population is functionally discrete and therefore provides a useful tool for study, as evidenced by the observation that these cells were

responsible for the majority of *in vitro* basal inflammatory cytokine expression within SVF compared to CD3+ and CD16+ cells (Figure 4). Although CD3+ cells expressed similar basal levels of TNF- α compared to CD14+ cells, CD3+ cells did not upregulate TNF- α in response to IFN- γ , unlike CD14+ cells (data not shown).

Study limitations

Lymphocyte numbers were normalized by tissue weight, but might alternatively be normalized to adipocyte number, as data from other studies suggest that adipocyte size may differ between depots.⁴⁰ Future research will study adipocyte size and its relation to lymphocyte number in adipose tissue. To compare our results to previously published data using similar techniques,^{5,41} we removed vessels from adipose tissue before collagenase digestion. Others have shown important role for vascular endothelial cells in mediating inflammation,⁴² effects which would not be detected in these experiments. Future research will focus on adipose tissue endothelial cells as potential mediators of inflammation. Tissue culture was performed in standard plastic tissue culture ware, which may activate macrophages. Nonetheless, virtually all previously published data studying ATM^{2,5,41,43} use similar techniques, and comparisons between VAT and SAT and unstimulated and IFN- γ -stimulated experimental arms were internally controlled for this variable. Future experiments will study this and other potential confounders of this *in vitro* system. Other technical limitations include possible effects of antibody-coated magnetic beads on cell activation and cytokine expression. All IFN- γ -stimulated bead-sorted cells were compared to bead-sorted unstimulated cells to internally control for such effects, and separate experiments by our laboratory demonstrate no effects of antibody-coated magnetic beads on SVF cytokine expression (data not shown). Bead-sorted populations were not completely purified, likely because of relatively high levels of nonspecific antibody binding. Despite the relatively lower purity of CD14-enriched cells, a purification of more than twofold over bulk SVF was accomplished. Flow cytometry sorting of cells and intracellular cytokine staining may provide more purified cell populations for future experiments. The SVF isolation procedure, including collagenase digestion, low levels of endotoxin exposure and other variables, is a concern for all *in vitro* systems and may independently induce inflammation. Nonetheless, all specimens underwent identical processing to internally control for any artifact from isolation procedures, thus validating the paired comparisons made from these data.

To eliminate gender as a potential confounder, we limited the study to women, who comprise the majority of the bariatric surgical population. This study also lacks a lean control subject comparator group, limiting the conclusions that can be drawn regarding pathologic implications of these data. Access to VAT from healthy lean subjects in a general surgical population is rare, as most patients undergoing abdominal surgery are either overweight or obese. Future research will study men and lean control subjects to address these issues.

Although we demonstrate a unique population of IFN- γ -expressing NK cells that is present at higher frequency in VAT relative to SAT, these data do not confirm that these cells are the *in vivo* source of elevated IFN- γ in VAT. This cell population is small in number, and other cell types also likely contribute to adipose tissue inflammation. Macrophages have also been described as a potential source of IFN- γ ,⁴⁴ although our intracellular cytokine staining data do not support ATM as a source of IFN- γ in human VAT. Further experiments will be necessary to definitively ascribe a pathogenic role for NK cells within VAT.

Conclusions

We demonstrate differences in inflammatory transcript levels in SVF from VAT relative to SAT in obese humans, as well as increased numbers of ATM, NK cells and T cells in VAT relative to SAT. Our data also implicate IFN- γ in the regulation of ATM-mediated inflammation within human adipose tissue and identify a unique population of IFN- γ -expressing NK cells that is increased in frequency in VAT relative to SAT. Taken together, these observations suggest a model in which NK-cell-derived IFN- γ induces ATM cytokine expression in VAT. Important avenues for future research include identification of the initiating stimuli and precise sources of IFN- γ expression within VAT, the cause of increased ATM, NK and T-cell homing to adipose tissue, and the specific role of these cell subsets in regulating inflammation within adipose tissue. Such research will provide potential explanations for the detrimental physiologic effects of VAT and elucidate underlying mechanisms of inflammation within human adipose tissue.

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Figure 1.

Transcript levels in stromovascular cell fraction (SVF) compared to matched adipocyte cell fraction in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). Mean fold differences comparing transcript levels in SVF to its matched (from the same subject and same depot) adipocyte cell fraction within VAT (left) or SAT (right) from nine obese female subjects; *P*-values from paired *t*-test comparing transcript levels between paired SVF and adipocyte fractions for VAT and SAT respectively were: leptin: <0.001, <0.001, interferon- γ (IFN- γ): <0.001, <0.001; tumor necrosis factor- α (TNF- α): <0.001, 0.001; interleukin (IL)-6: 0.775, 0.438; IL-8: <0.001, <0.001; IL-10: <0.001, 0.002; IL-1Ra: <0.001, 0.001; CCL-2: 0.046, 0.488. These data demonstrate that the SVF fraction is the dominant source of inflammatory cytokines relative to the adipocyte fraction in both VAT and SAT. Fold differences are presented in numeric form above each data bar. Bars represent middle quartiles, whiskers represent standard deviation (s.d.). Asterisks represent *P*<0.05 for paired comparison between matched SVF and adipocyte fractions. Abscissa is logarithmic scale.

Figure 2.

A pan-leukocytosis in visceral adipose tissue (VAT) relative to subcutaneous adipose tissue (SAT). **(a)** Leukocyte infiltration is increased in stromovascular cell fraction (SVF) from VAT relative to SAT. Flow cytometric analysis demonstrating the absolute number of CD45+ cells within the entire SVF cell fraction from VAT and SAT from 25 obese subjects (mean 207 240 and 82 437 cells per gram of tissue for VAT and SAT respectively, $P=0.036$, paired t -test). **(b)** Adipose tissue macrophages (ATM), T-cell and natural killer (NK) subpopulation cell numbers are increased in SVF from VAT relative to SAT. Flow cytometric analysis of SVF demonstrating the absolute number of CD45+CD14+, CD45+CD3+, CD45+CD3+CD4+ and CD45+CD3+CD8+ cells per gram of adipose tissue in SVF from nine obese subjects (T-cell markers) or seven obese subjects (NK cell marker CD56) that were subsets of the 25 subjects included in the analysis in **(a)**; CD14+ cell numbers were derived from the entire cohort of 25 subjects; P -values represent comparisons between VAT and SAT for each cell subset and are derived from paired t -tests between matched VAT and SAT specimens. Error bars: s.d.

Figure 3.

CD14+ population phenotype. Representative dot-plots of (a) CD45 and CD14 gating strategy (top two dot-plots). Left: CD45+ cells gated within all stromovascular cell fraction (SVF) cells; right: all CD14+ cells within the CD45+ gated cells from the left plot, and (b) CD14+ visceral adipose tissue (VAT) adipose tissue macrophages (ATM) phenotype studied with antibodies directed toward macrophage markers CD14, CD64, CD11b, CD11c, CCR2, CCR5, CD163 and CD206. CD14+ cells express markers characteristic of adipose-tissue-associated macrophages. Representative phenotype data for VAT SVF are shown. Although subcutaneous adipose tissue (SAT) SVF ATM phenotype was similar, depot-specific differences in the frequencies of bright and dim subpopulations of CD11b, CD163 and CD206 are described in the Results section.

Figure 4.

CD14+ cells are a dominant source of inflammatory cytokines in stromovascular cell fraction (SVF). Bulk SVF and antibody-coated magnetic bead CD14-enriched, CD3-enriched and CD16-enriched SVF were cultured in media alone for 24 h, and supernatants were harvested and studied with enzyme-linked immunosorbent assay (ELISA). CD14-enriched SVF was the dominant source of interleukin (IL)-6, IL-8, IL-10, IL-1Ra and CCL-2 (P -values <0.03 in all cases, paired t -tests comparing CD14-enriched data to CD3- or CD16-enriched data). CD3 and CD14 cells expressed similar basal levels of tumor necrosis factor- α (TNF- α) ($P=0.097$, paired t -test). No differences in basal interferon- γ (IFN- γ) expression levels were noted between bulk, CD14-enriched, CD3-enriched and CD16-enriched cells ($P>0.05$ in all cases, independent t -test). Bars represent middle quartiles, whiskers represent standard deviation (s.d.). Note different scales on abscissas, all represented in picograms per milliliter of culture supernatant.

Figure 5.

Response of bulk visceral adipose tissue (VAT) stromovascular cell fraction (SVF) to stimulation with interferon- γ (IFN- γ) or LIGHT. Transcript levels in bulk SVF from VAT stimulated with IFN- γ or LIGHT for 24 h relative to unstimulated SVF referent are shown. Fold change in transcript levels in response to IFN- γ stimulation are shown above data bars. *P*-values for paired analysis between media and IFN- γ -stimulated arms were: IFN- γ , 0.028; tumor necrosis factor- α (TNF- α), 0.028; interleukin (IL)-6, 0.490; IL-8, 0.142; IL-10, <0.001; IL-1Ra, 0.585; CCL-2, 0.022. *P*-values for paired analysis between media and LIGHT-stimulated arms were: IFN- γ , 0.043; TNF- α , 0.293; IL-6, 0.065; IL-8, 0.137; IL-10, 0.698; IL-1Ra, 0.286; CCL-2, 0.938. Fold differences are presented in numeric form above each data bar. Bars represent middle quartiles, whiskers represent standard deviation (s.d.). Asterisks represent *P*<0.05. Abscissa is logarithmic scale.

Figure 6.

Interferon- γ (IFN- γ) induces an M1 cytokine expression pattern in human stromovascular cell fraction (SVF) enriched for CD14 cells. Cytokine levels (pg ml^{-1}) measured by enzyme-linked immunosorbent assay (ELISA) in supernatants from bulk and CD14-enriched visceral adipose tissue (VAT) SVF using antibody-coated magnetic bead sorting and cultured for 24 h with or without IFN- γ . IFN- γ did not regulate cytokine expression in CD3-enriched SVF, and LIGHT did not regulate cytokine expression in bulk, CD3-enriched or CD14-enriched SVF for any cytokines tested (data not shown). *P*-values were derived from paired *t*-test comparing media and IFN- γ -stimulated cells. Bars represent middle quartiles, whiskers represent standard deviation (s.d.). Note different scales on abscissas, all represented in pg ml^{-1} of culture supernatant.

Figure 7.

(a) Constitutive expression of interferon- γ (IFN- γ) by natural killer (NK) cells is increased in visceral adipose tissue (VAT) relative to subcutaneous adipose tissue (SAT). Representative dot-plots of SVF rested for 12 h in culture followed by intracellular staining for IFN- γ and surface staining for CD45, CD56 and CD3. (b) Depot-specific frequencies of constitutive IFN- γ expressing NK and T-cell subsets. Percent of total parent population (CD3+, CD3+CD4+ or CD3+CD8+ T cells or CD56+ NK cells) that constitutively expressed IFN- γ within VAT and SAT SVF. The percentage of CD56+ cells that constitutively expressed IFN- γ was increased in VAT compared to SAT. CD3+ cells demonstrated similar low levels of constitutive IFN- γ expression with no difference between depots. Values reported are percent of CD56+ or CD3+ populations positive for intracellular IFN- γ minus percent of populations (CD56+ or CD3+ populations) positive for intracellular isotype-matched control antibody. *P*-values were derived from paired *t*-test comparing VAT and SAT for each matched culture. Note that multiple points overlap at zero for NK cell SAT arm and T-cell VAT and SAT arms. A total of eight subjects were included for each arm of these experiments, as described in the Materials and methods section. Error bars: s.d.

Table 1

Transcriptional profiling of matched human SVF from VAT and SAT depots

Gene	Fold diff	P-value
<i>bcl-3</i>	0.33	0.001
<i>CCL-2</i>	0.88	0.640
<i>G-CSF</i>	0.57	0.402
<i>GM-CSF</i>	2.86	0.030
<i>HMOX1</i>	0.21	0.003
<i>IFN-γ</i>	8.2	0.000
<i>IFN-α1</i>	2.09	0.164
<i>IFN-β1</i>	2.65	0.043
<i>IL-1α</i>	0.64	0.049
<i>IL-1β</i>	0.66	0.068
<i>IL-6</i>	1.56	0.156
<i>IL-8</i>	0.50	0.018
<i>IL-10</i>	0.58	0.027
LIGHT	9.82	0.000
<i>TNF-α</i>	0.61	0.062

Fold-difference in levels of 15 transcripts related to inflammation and macrophage biology in VAT SVF relative to SAT SVF referent from 9 obese female subjects is displayed. These data demonstrate differences in inflammatory cytokine transcript levels in SVF between VAT and SAT depots within subjects. Paired *t*-test was used to determine the significance of differences in ΔC_t values between matched VAT and SAT SVF specimens.