Gene profiling of human adipose tissue during evoked inflammation *in vivo*

Running Title: *Evoked adipose inflammation and adipocytokines in humans*

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Objective: Adipose inflammation plays a central role in obesity-related metabolic and cardiovascular complications. However, few human adipose-secreted proteins are known to mediate these processes. We hypothesized that microarray mRNA profiling of human adipose during evoked inflammation could identify novel adipocytokines.

Research Design and Methods: Healthy human volunteers (n=14) were treated with intravenous endotoxin (3 ng/kg lipopolysaccharide, LPS) and underwent subcutaneous adipose biopsies before and after LPS. On Affymetrix U133Plus 2.0 arrays, adipose mRNAs modulated >1.5-fold (with $p<0.00001$) were selected. SignalP 3.0 and SecretomeP 2.0 identified genes predicted to encode secreted proteins. Of these, 86 candidates were chosen for validation in adipose from an independent human endotoxemia protocol (N=7, with 0.6 ng/kg LPS) and for exploration of cellular origin in primary human adipocytes and macrophages *in vitro*.

Results: Microarray identified 776 adipose genes modulated by LPS; 298 were predicted to be secreted. Of detectable prioritized genes, 82/85(96%, 95% CI: 90-99%) were upregulated (fold-changes >1.0) during the lower-dose (LPS 0.6 ng/kg) validation study and 51/85(59%, 95% CI 49-70%) were induced greater than 1.5-fold. Treatment of primary adipocytes with LPS and macrophage polarization to M1 pro-inflammatory phenotype increased expression by 1.5-fold for 58% and 73% of detectable genes, respectively.

Conclusions: We demonstrate that evoked inflammation of human adipose *in vivo* modulated expression of multiple genes likely secreted by adipocytes and monocytes. These included established adipocytokines and chemokines implicated in recruitment and activation of lymphocytes, adhesion molecules, anti-oxidants, and several novel genes with unknown function. induced multiple genes likely secreted by adipocytes and monocytes. These included established adipocytokines and chemokines implicated in recruitment and activation of lymphocytes, adhesion molecules, anti-oxidants and several novel genes with unknown function. Such candidates may represent biomarkers and therapeutic targets for obesity-related complications.

Activation of innate and adaptive immunity is a crucial link between adiposity and its metabolic complications(1-4). In rodents, modulation of toll-like receptor-4 (TLR4)(5), tumor necrosis factor(TNF) receptors(6), chemokines and downstream kinases(7) attenuate diet-induced obesity and insulin resistance. Further, cross-talk between immune cells and adipocytes promotes an inflammatory, insulin-resistant state in obesity. A key initiating event in adipose inflammation is recruitment of T-lymphocytes(8; 9) and monocyte/macrophages(10; 11) with elaboration of inflammatory adipocytokines that modulate metabolic signaling(12-15). Despite experimental evidence in rodent models, most evidence supporting these concepts in humans derives from observational and correlative studies(16-18). Indeed, validated adipokines that mediate, or serve as biomarkers for, complications of human adiposity remain limited.

Expression of inflammatory, insulin-signaling, and lipid genes are perturbed in adipose of obese humans(19-21). Recently, the *in vitro* secretome of subcutaneous and visceral primary human adipocytes was described and includes many unexplored proteins modulated during adipogenesis(1; 22). Remarkably, less than half of genes found in the human subcutaneous adipocyte secretome were previously found in the murine 3T3-L1 preadipocyte secretome(22), underscoring the importance of studies in human tissue.

Experimental human endotoxemia can provide unique insights into the relationship of inflammation to metabolic disturbance in man(23; 24). We and others have shown that endotoxemia induces acute metabolic, lipoprotein, and oxidant responses that resemble the chronic changes in insulin resistance and metabolic syndrome(25; 26).

Notably, endotoxemia induces adipose inflammation(27) with activation of several adipose inflammatory cascades, including cytokines, chemokines and suppressor of cytokine signaling (SOCS) molecules(26) that attenuate insulin signaling and are implicated in obesity and type 2 diabetes(28).

We applied microarray mRNA profiling of human adipose during endotoxemia to identify novel inflammation-induced adipose genes. We focused on genes predicted to be secreted and validated our findings *in vivo* through independent experiments of low-grade human inflammation. Finally, we identified *in vitro* the likely human adipose cellular source of these top candidates.

METHODS

An overview of our design is provided in Figure 1. All clinical studies were performed with approval of University of Pennsylvania Institutional Review Board after written informed consent from all subjects.

Discovery Human Endotoxemia Protocol: As previously described(26), healthy volunteers, age 18-40, with BMI 18-30 kg/m^2 were recruited from the Delaware Valley. Exclusions included past medical history including inflammatory diseases, medication or supplement use, or pregnancy. Participants underwent three Clinical and Translational Research Center visits: visit 1 for screening; visit 2 for dietary run-in; and visit 3 for inpatient stay comprising an acclimatization phase and a post-LPS study phase. Serial whole blood samples were collected before and 2, 4, 6, 8, 12, 16, and 24 hours following IV bolus of 3 ng/kg U.S. standard reference endotoxin (LPS; lot No. CC-RE-LOT-1 + 2; Clinical Center, National Institutes of Health, Bethesda, MD). Serial subcutaneous adipose samples were collected by core needle aspiration through a 4mm gluteal incision from distinct sites, 30 min

before and 4, 12, and 24 h after LPS; samples were treated with RNA Later® and stored at -70C. This report focuses on the subset that underwent microarray mRNA profiling of adipose (N=14).

Microarray: Adipose tissue RNA was extracted using RNeasy Lipid Tissue Mini kit (QIAGEN Inc., Valencia, CA) and underwent extensive QC (including spectrometry, picogreen quantitation and electrophoresis for RNA quality) before first-strand cDNA synthesis (100ng), cRNA synthesis, and hybridization to Affymetrix GeneChip Human Genome U133Plus 2.0 arrays (Santa Clara, CA) in the Penn Microarray Facility(www.me.upenn.edu/microarr/facility). Microarrays were washed, stained with amplified fluorescence markers and signals collected on a confocal scanner at 3µm resolution (excitation at 570nm). Affymetrix Microarray Suite 5.0 was used to quantitate expression levels of average signal from two sequential scans. Array signal intensities passed a 2-stage QC process including affyQCReport package. All arrays had similar background values and distributions of intensity.

Adipose Microarray Statistical and Informatic Methods: *Identification of Candidate LPS-modulated Genes:* Arrays were normalized, adjusted for background signal using robust multi-array analysis(RMA)(29) and filtered by removing probesets with little evidence of expression using the PM/MM probes(63) or expression levels consistently less than three-fold background. For the remaining 13,646 genes, mixed effects models using shrinkage estimates of the variance components were fit, with the goal of identifying genes with highly significant fold-changes in expression between baseline and each timepoint (4, 12, and 24h). Genes were selected for further study if the permutation-based P-value for the null hypothesis test of a fold-change of unity

at any timepoint was less than 1×10^{-5}(30). Permutation-based false discovery rates were assessed at each timepoint(31). To increase the chance of identifying genes with detectable changes at the protein level, we required a minimum cut-off of at least 1.5-fold change in intensities per gene. Analyses were performed using Bioconductor (www.bioconductor.org) run under R 2.6(R Foundation for Statistical Computing, Vienna, Austria) with libraries including *affy, maanova*, and *panp*.

Secreted protein identification: We interrogated *in silico* our candidate LPS-modulated genes using the validated protein informatic software SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/)(32; 33) and SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/)(34) to identify the subset of human genes with highest probability of encoding either signal peptides (SignalP) or protein sequences predictive of non-classical secretion (SecretomeP). For SignalP, we used a cutoff D-score of 0.7, as the score above which frequency of containing a signal peptide is greatly increased in known eukaryotic proteins(32). A SecretomeP cutoff of 0.7 was chosen as the cut-off above which Bendsten et al. demonstrated that all positive training examples, and no negative training examples were classified as non-classically secreted(34). Genes encoding sequences that met the cut-off value for either classical or non-classical secretion comprise our preliminary candidate "human adipose tissue inflammatory secretome".

Prioritization for validation: For initial prioritization, we focused on upregulated genes, as more likely to have detectable and increased protein levels in adiposity-related disease. Thus, candidate inflammatory, adipose-secreted proteins were prioritized as follows; (1) novelty – genes with unknown functions, (2) >3 fold upregulation at any timepoint, (3) >1.5 fold

upregulation at two or more timepoints, (4) prior publication as secreted proteins *in vitro* or *in vivo*, (5) evidence for functional roles in inflammation, insulin signaling, or adipose biology, and (6) known association with diabetes, obesity, metabolic syndrome, or cardiovascular disease. Genes that did not meet any of the criteria were not studied further. This resulted in a list of 86 candidates (including positive controls IL6, TNF, MCP-1) that were selected for validation and identification of adipose tissue cell of origin.

Functional Classification: Prioritized genes were uploaded to DAVID (Database for Annotation, Visualization, and Integrated Discovery) Bioinformatics Resource and the Gene Functional Classification tool was utilized to generate clusters of related, and over-represented genes(35; 36). The enrichment score (geometric mean of –log p values) is presented.

Validation Human Endotoxemia Protocol: Healthy volunteers (n=10) were recruited from the same population as Discovery Study, using similar criteria. Eligible participants underwent dietary counseling and were admitted for inpatient stay including acclimatization and post-LPS phases. Serial whole blood (at 0, 2, 4, 6, 12, 16, and 24 hours) and subcutaneous gluteal adipose tissue samples (at 0, 4, 12, and 24 hours) were collected before and after IV infusion of 0.6 ng/kg endotoxin and processed as above. We focused on the subset (n=7) in whom adipose tissue samples were assayed by quantitative PCR.

Cell Studies: *Primary Human Adipocyte Studies:* Fresh human adipose tissue obtained from abdominal surgery specimens via the Penn Human Adipose Resource was the source of primary human adipocytes. Briefly, the specimen was digested with collagenase (Roche Applied Science, IN), filtered, centrifuged, and cellular layer resuspended in OF medium [DMEM/F12 plus penicillin/streptomycin,

biotin (4mg/L), and pantothenate (8mg/L)] with 20% fetal bovine serum and plated at 30,000cells/cm^2. Once confluent, cells were differentiated in serum-free differentiation medium [OF media, insulin (20nM), hydrocortisone (1μM), dexamethasone (250nM), human transferrin (10mg/L), T3 (0.2nM), isobutylmethylxanthine (500μM), and PPARγ agonist GW347845 (2μM), (gift from GlaxoSmithKline, King of Prussia, PA)] until 80% mature(7-10 days). Cells were washed, and treated±LPS 100 ng/ml in DMEM/F12 with 0.1% FBS plus penicillin/streptomycin for 2, 4, 12, and 24 hours for two independent experiments, in triplicate. Unless noted, reagents were purchased from Sigma-Aldrich, St Louis, MO.

Primary Human Macrophage Studies: Human monocytes (peripheral blood mononuclear cells, >95% expression of CD14 and HLA-DR), were isolated from donor blood following apheresis and elutriation in the Penn Center for AIDS Research. As described(37), cells were plated at 3x10^5 cells/cm^2 and cultured in RPMI with 20% FBS, and penicillin/streptomycin, then supplemented with 100 ng/ml MCSF (Sigma, St Louis, MS) to promote differentiation to macrophages over 7 days. In two independent experiments, each in triplicate, macrophages were washed and treated for 16 hours with: RPMI + 5% FBS alone, 100 ng/ml LPS and 20 ng/ml Interferon Gamma (R&D Systems, Minneapolis, MN) for differentiation to the M1 phenotype, or 20 ng/mL recombinant human IL4 (R&D Systems, Minneapolis, MN) for differentiation to the M2 phenotype(38).

General Laboratory Methods: *RNA extraction, cDNA synthesis, and Quantitative PCR:* RNA was isolated from adipose tissue, adipocytes and macrophages using Trizol reagent (Invitrogen, Carlsbad, CA) and was reverse-transcribed (500ng) to cDNA (High Capacity cDNA Archive Kit, ABI; Foster

City, CA). Expression of genes was determined by Real-Time PCR (ABI7900 system) using TaqMan Universal PCR MasterMix, primers and probes. To control for between-sample differences, mRNA levels were normalized to ß-actin for each sample by subtracting the Ct for ß-actin from the Ct for gene of interest, producing a ΔCt value. The ΔCt for each post-treatment sample was compared with the mean ΔCt for all pre-treatment samples using the relative quantitation $2^{-\Delta\Delta Ct}$ method to determine fold change from baseline(39).

Metabolic and Inflammatory Markers: As previously described(26), plasma levels of tumor necrosis factor alpha (TNF), interleukin-6 (IL-6) (Linco Multiplex ELISAs on Luminex IS100; Austin TX), and C-reactive protein (high-sensitivity Latex turbidimetric immunoassay; Wako Ltd., Osaka Japan) were measured in duplicate per manufacturers' guidelines. Intra- and inter-assay CVs for TNF ELISA were 8**.**66% and 20.4% and for IL-6, 8.7% and 10.9%. Plasma lipids and glucose were measured enzymatically on Hitachi 912 automated chemistry (Wako Diagnostics, Richmond, VA)(26). Subject data are reported as mean±SD for continuous variables, and as proportions for categorical variables. Effects of endotoxemia on clinical parameters were tested by mixed effects modeling or repeated measures analysis of variance (ANOVA)(26).

Statistical Analysis of Quantitative PCR Data: For validation study adipose tissue, fold-changes at each time point were estimated using the method of generalized estimating equations(GEE) to account for within-subject correlations. For adipocytes and macrophages, analysis of mRNA fold-change utilized geometric means of the independent replicated samples. For the timepoint with peak fold-change, the mean and 95% confidence intervals are reported for all studies. Correlation analyses between studies were performed by Spearman's rank correlation of the log10 peak fold-change. The proportions of upregulated genes for validation, adipocyte, and macrophage studies were reported and their 95% exact binomial confidence intervals were calculated using Clopper and Pearson(40). Analyses were performed using the R 2.6 and Stata 10 (StataCorp LP, College Station, TX).

RESULTS

Baseline characteristics of participants and clinical responses to endotoxemia: Participants in the discovery and validation studies had similar baseline characteristics with normal blood pressure, plasma lipoproteins and BMI, although the validation study enrolled more females (Table 1). In the discovery study, as reported(26), there was a transient clinical and biochemical inflammatory response to LPS 3 ng/kg, whereas the lower LPS dose of 0.6 ng/kg in the validation study produced a sub-clinical response with modest increases in plasma cytokines but no significant symptoms or fever (Table 2).

Endotoxemia modulates expression of many human adipose tissue genes. We identified 696 genes at 4h, 130 genes at 12h, and 24 genes at 24h that met criterion for selection. The false discovery rates for the three timepoints were 3×10^{-5}, 4×10^{-4} and .001 respectively suggesting strong evidence of differential expression among these genes. Many genes were modulated at multiple time points, resulting in 776 unique genes modulated at any timepoint (696 at 4h, 68 additional at 12h, and 12 additional at 24h). Approximately half of the genes were upregulated (fold change >1.5), the remaining were downregulated (Table 3).

A subset of modulated adipose tissue genes is predicted to encode secreted proteins. Based on the SignalP 3.0 and SecretomeP 2.0 analysis, a total of 266 genes at 4h, 72 at 12h, and 15 at 24h were predicted to encode secreted proteins (Table 3). This

corresponds to 298 unique genes and represents 38% of all modulated genes, suggesting an over-representation of secreted proteins relative to the entire Affymetrix U133Plus 2.0 array (~24%) and the human genome (15-20%). The majority of these (75%) were predicted to be classically secreted. More were upregulated (63% at 4h and 12h, 54% at 24h) than downregulated.

For the current report, only upregulated genes predicted to encode secreted proteins were chosen for further validation (n=86). These inflammatory, human adipose tissue-secreted factors included cytokines (e.g., IL6, TNF), chemokines (e.g., CXCL9, CCL4), growth factors (e.g., BMP-2), acute phase reactants (e.g., PTX3, HP), adhesion molecules (e.g., ICAM1, VCAM), cell surface markers (e.g., CD14, CD274), anti-oxidants (e.g., metallothioneins), and novel genes with unknown functions (e.g., C19orf59, KIAA0247). In addition, several prioritized genes were identified that have been implicated in inflammatory processes in human or animal models, but not described in adipose function (e.g., CEACAM, ISG15, CX3CL1). The heatmap in Supplemental Figure 1 (available in the online appendix at http://diabetes.diabetesjournals.org) depicts fold changes (ranked from highest to lowest fold-change) in the discovery study adipose, as determined by microarray. Greatest mRNA modulation occurred at 4h but some genes had later induction or sustained modulation over time.

The majority of prioritized genes fall into six functional categories defined by DAVID. Figure 2 depicts these over-represented categories which include cell adhesion, chemokine and cytokine, acute phase response, T-cell activation, metal-binding and lipid metabolism. Remarkably 28 genes, including many unknowns, remained uncategorized. Supplemental Table 1 gives the full lists of genes in each category.

Inflammatory modulation of many prioritized genes replicates in human adipose tissue samples during an independent validation endotoxemia study. We replicated endotoxemia-induced adipose tissue genes in an independent study of lower-grade endotoxemia (0.6 ng/kg). This dose resulted in modest cytokine induction without clinical significant responses and therefore may more closely reflect the modest inflammation observed in human obesity. Of the 86 prioritized genes, 85 (98% 95% CI: 94-100%) were confirmed to be expressed (CT value <37). During 0.6 ng/kg endotoxemia, 82/85 (96%, 95% CI: 90-99%) were increased (fold-changes >1.0) and 51/85 (60%, 95% CI 49-70%) were upregulated by greater than 1.5-fold. The heatmap in Supplemental Figure 2 depicts mRNA expression changes over time. Compared to the discovery study, the lower dose LPS validation study induced a more modest but similar pattern of expression (Figure 3A). Remarkably, there was a relatively high correlation (r=0.64, p<0.0001) of peak mRNA fold-change between the discovery and validation studies (Figure 3B).

The majority of the putative adipose tissue inflammatory secretome is expressed in human adipocytes and induced by LPS *in vitro*. Analysis of primary human adipocytes revealed that 77/86 (90%) prioritized genes had detectable expression (CT value <37) in resting adipocytes, 68 of these 77 (88%, 95% CI: 79-94%) were increased (>1.0-fold) and 50/77 (65%, 95% CI 53-75%) were upregulated by greater than 1.5-fold after 100 ng/mL LPS. There was a remarkable correlation of peak mRNA fold-change between discovery human adipose and adipocytes (Figure 4A) as well as between validation adipose and adipocytes (Figure 4B) suggesting that adipocytes are a major source of inflammatory modulated genes in human adipose tissue *in vivo*.

Many candidate genes are expressed in human macrophages and upregulated during polarization to the M1-phenotype. Next, expression was tested in classically-activated pro-inflammatory (M1) and alternatively-activated anti-inflammatory (M2) macrophages(41; 42) relative to undifferentiated macrophages. In resting macrophages, 72 of 86 mRNAs (84%) were detectable (CT < 37) while 66 of these 72 (92%, 95% CI: 83-97%) were increased (>1.0-fold), and 63/72 (88%, 95% CI 78-94%) were upregulated by >1.5-fold during polarization to the M1 phenotype, supporting their potential role in inflammatory macrophage signaling in human adipose tissue. In contrast, genes upregulated in adipose tissue tended to be unchanged or downregulated in M2 macrophages; only 22/72 (31%, 95% CI 21-43%) were increased >1.0-fold, and 14/72 (20%, 95% CI 11-31%) were upregulated >1.5-fold.

A summary of findings in the discovery and validation studies as well as in primary human adipocytes and human macrophages is presented in Supplemental Table 2. Overall, of the 51 genes confirmed to be upregulated >1.5-fold in human adipose tissue in our validation study, the majority (46/51, 90%) was induced by LPS in adipocytes and/or M1 macrophages (Table 4).

DISCUSSION

This mRNA microarray analysis of human adipose tissue during experimental endotoxemia is the first attempt to identify novel adipocytokines using an *in vivo* evoked adipose-inflammatory model. Our strategy identified genes encoding established adipokines implicated in obesity-related insulin resistance as well as emerging adipose tissue-secreted factors(1) and multiple secreted proteins with unknown adipose functions. These experimental human studies are internally consistent, provide independent validation and also define the likely cellular origin. Such candidates may represent new biomarkers and therapeutic targets for the treatment of obesity-related complications.

Microarray analysis of gene expression is established for discovery of disease-related genes(43). Indeed, mRNA profiling of adipose has identified novel genes involved in obesity and type 2 diabetes(44). Several reports identified genes involved in inflammation, insulin signaling, and lipid metabolism as differentially expressed in adipose tissue of obese humans(19-21). We also identified many of these genes suggesting that our model may be useful in human adipokine discovery. For example, nine of 89 genes differentially expressed in omental adipose of obese subjects(20) were also upregulated in our study [ADRB2, IFIT2, IL10RA, IL6, ISG20, JAK2, LDL4, MAP3K4, NGFR]. In adipocytes cultured *ex vivo* from subcutaneous adipose of obese vs. non-obese Pima Indians, 38 upregulated genes were also increased in our study(21), including 13 likely secreted proteins that we prioritized for validation [APOL1, APOL3, CCL2, CCL4, CD14, CXCL1, CXCL2, CXCL3, ICAM2, IER3, MT2A, SERPINE1, TNFRSF1A].

The dose of LPS (3 ng/kg) used in the discovery study induced an acute and more marked inflammatory state than that found in obesity. This super-physiologic activation of innate immunity allowed us to identify *in vivo* a broad range of adipose inflammatory genes, with the caveat that the degree of modulation may be greater than in human adiposity. To explore our findings in a setting more relevant to the low-grade inflammation in central obesity, we validated adipose tissue upregulation in an independent human study of very-low-dose endotoxemia (0.6 ng/kg) which results in modest cytokine induction without significant clinical inflammatory responses. Notably, the majority of adipose tissue upregulated genes identified in the discovery study were also induced in the

8

validation study, suggesting that they may be modulated during the more modest adipose inflammation of obesity.

Our *in-silico* predicted adipose inflammatory secretome is remarkable because it included (1) many established adipocytokines including TNFα, IL-6, MCP-1, PAI-1, and SELE, (2) secreted inflammatory molecules with known immune functions but no known role in adipose or metabolic signaling (e.g., CX3CL1, ISG15, EBI3), (3) a number of proteins recently identified in the human visceral adipose secretome(1) (e.g. CSF-1, PAI-1, ADAMSTS, PTX3), and (4) multiple genes encoding secreted proteins without known functions. Functional classification of the 86 genes revealed several expected inflammatory categories including acute phase response, chemokines and cytokines, as well as cell adhesion molecules. The over-representation of T-cell activating genes is of particular interest, given the recent implication of T-cell recruitment in obesity-related adipose tissue inflammation and insulin resistance(2; 8; 45). An inflammatory impact on adipocyte metabolic and redox activities is suggested by excess representation of lipid and metal-binding gene categories. Notably 28 genes, many with completely unknown functions, remained uncategorized highlighting the potential for this model to provide novel insights into human adipose biology.

As macrophage infiltration is a key event in adipose tissue inflammation and insulin resistance(10; 11), we expected that the evoked inflammatory state would induce factors involved in recruitment and differentiation of monocytes. Chemokines such as MCP1, CSF1, CXCL3, and CX3CL1 were upregulated at early time points. Macrophage cell markers, such as CD14 and CD64, and macrophage-secreted proteins such as MIP-1(CCL3), IL8, and IL18BP were also induced by LPS. In fact, many of the overexpressed genes are known to be macrophage-secreted, and our validation studies in human M1 macrophages confirmed that ~75% of genes induced in adipose tissue by LPS were also upregulated during *in vitro* polarization to inflammatory macrophages.

Macrophages are polarized from a resting state to "classically activated" (M1) or "alternatively activated" (M2) phenotypes with pro-inflammatory or anti-inflammatory activities, respectively(41; 42). In mouse models, obesity induces a phenotypic switch in adipose tissue macrophages (ATMs) from a predominantly M2 phenotype that protect against insulin resistance to an M1 inflammatory state that confer sensitivity to TNFα-induced insulin resistance(46). In humans, ATMs possess a complex phenotype with some M2 markers but M1-like pro-inflammatory cytokine production(47). Of note, our predicted adipose inflammatory secretome contained 22 genes found to be upregulated in the M1 phenotype compared to resting macrophages(48) and our human macrophage studies confirmed M1-macrophages as a potential source of many inflammatory molecules.

T-cell recruitment to adipose tissue has been recently identified in obesity-induced adipose inflammation. In fact, some findings suggest T-cells are found in adipose and can induce insulin resistance prior to macrophage recruitment(2; 8; 45). These inflammatory T-cells secrete cytokines/chemokines that switch resident ATMs to the M1 phenotype while recruiting additional circulating monocytes. Several potent T-cell chemokines, including CCL4, CXCL9, CXCL10, CXCL11, and IL15 were highly induced by endotoxin. Additionally, overexpression of T-cell induced genes (ISGs, ICOSLG, IRFs) was detected, suggesting increased T-cell activity.

We confirmed *in vitro* that both human adipocytes and M1-pro-inflammatory macrophages are likely cellular sources for the vast majority of our adipose inflammatory

secretome. By focusing on these cells, we have excluded other adipose components (e.g., stromal cells). Of genes validated as upregulated in human adipose inflammation, however, only five were not induced by LPS in either adipocytes or macrophages. This finding suggests these cells are the principle source of inflammatory modulation.

Our study used subcutaneous gluteal adipose tissue from healthy humans. Although visceral adipose tissue is commonly held to be more active in obesity-related inflammation, it appears that the evoked-inflammatory response in subcutaneous adipose tissue approaches a similar phenotype. Many genes previously found to be over-expressed in visceral adipose were upregulated in subcutaneous adipose during *in vivo* endotoxemia. Several of the proteins identified in a recently published human visceral adipose secretome(1) were also upregulated in our discovery microarray [NETO1, RBP, PAI-1, THSD1, CD14, PTX3, LUM, SPON2]. These findings support our use of subcutaneous adipose tissue. In fact, a recent analysis revealed that both subcutaneous and visceral depots displayed a pro-inflammatory phenotype with obesity(49).

We acknowledge that our acute evoked inflammatory model does not reproduce the chronic low-grade changes in diet and obesity-related adipose inflammation. However, our prioritized inflammatory adipose molecules are cogent biomarkers for obesity-related diseases. First, the transient inflammatory and metabolic responses to endotoxemia resemble those observed in insulin resistance and metabolic syndrome (23; 24). Second, rodent models have proven that adipose inflammatory pathways play important roles in diet and obesity-related metabolic disturbance(5-7). Finally, mRNA microarray and secretome profiling of human adipose tissue and adipocytes identify inflammatory pathways in obesity similar to those classically activated by endotoxin(1; 20; 21). Additional studies are required to confirm changes at the protein level and mechanistic studies are required to determine whether our prioritized genes are truly involved in adipose metabolic functions.

CONCLUSIONS

Human endotoxemia induces adipose inflammation and upregulates many adipose genes and proteins implicated in the complications of obesity. Microarray analysis of human adipose tissue during endotoxemia also identified novel genes that may serve as potential biomarkers of and therapeutic targets for obesity-related diseases.

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Table 1: Baseline Characteristics of the Discovery and Validation Study Samples.

	Discovery Study 3 ng/kg endotoxin (n=14) Mean (SD)	Validation Study 0.6 ng/kg endotoxin (n=7) Mean (SD)
Age (years)	25.0 (6.5)	24.4 (3.1)
Female Sex, N (%)	4 (28%)	5 (71%)
Race		
Caucasian (%)	12 (86%)	6 (86%)
African American (%)	2 (14%)	1 (14%)
Systolic Blood Pressure (mg Hg)	121 (9)	115 (7.9)
BMI (kg/m^2)	24.0 (4.0)	24.4 (2.6)
Fasting Glucose (mmol/L)	4.68 (0.28)	3.89 (0.77)
Total Cholesterol (mmol/L)	4.53 (0.52)	4.65 (0.60)
LDL Cholesterol (mmol/L)	2.46 (0.67)	2.21 (0.56)
HDL Cholesterol (mmol/L)		
Males	1.33 (0.26)	1.24 (0.05)
Females	1.69 (0.34)	1.81 (0.19)
Triglycerides (mmol/L)	0.86 (0.39)	1.0 (0.3)

Table 2: Clinical and Inflammatory Responses to Endotoxemia.

	Discovery Study (N=14)			Validation Study (N=7)		
	Pre-LPS Mean (SD)	Post-LPS Mean (SD)	Time of Peak	Pre-LPS Mean (SD)	Post-LPS Mean (SD)	Time of Peak
TNF (pg/mL)	3.0 (1.1)	*507 (251)	2 hours	1.0 (0.6)	†22 (13)	2 hours
IL6 (pg/mL)	3.2 (17.1)	*1607 (650)	2 hours	1.7 (1.8)	*107 (31)	2 hours
HR (bpm)	76 (11)	*107 (13.6)	4 hours	64 (11)	‡80 (15)	4 hours
Temp (^{o}C)	36.5 (0.3)	*37.8 (0.9)	4 hours	36.4 (0.4)	36.7 (0.4)	4 hours
WBC count	6.5 (1.2)	*13.8 (3.2)	12 hours	6.1 (1.4)	‡8.4 (1.9)	4 hours
hsCRP (mg/L)	0.49 (0.21)	†42.4 (8.4)	24 hours	1.5 (1.5)	†16.7 (7.6)	24 hours

All data presented as mean (SD). LPS=lipopolysacharide; TNF=tumor necrosis factor alpha; IL6=interleukin-6; HR=heart rate; bpm=beats per minute; WBC=white blood cell; hsCRP=high sensitivity C-reactive protein. For each study, within-subject pre vs. post LPS comparisons are reported. *p<0.0001; † p<0.005: ‡ p<.05.

Table 3: Summary of Data for Modulated Genes and Secreted Proteins in Adipose during Human Endotoxemia.

Time Post LPS	Modulated Genes	Secreted	Classically Secreted
4 hour			
Total Number	696	266	198 (75%)
Upregulated (%)	414 (61%)	168 (63%)	
12 hour			
Total Number	130	72	57 (80%)
Upregulated (%)	75 (58%)	45 (64%)	
24 hour			
Total Number	24	15	11 (72%)
Upregulated (%)	11 (46%)	8 (46%)	

* Number of genes up- or downregulated >1.5 fold are shown.

Table 4: Induction of Validated Adipose Genes (N=51) by LPS in Human Adipocytes and during Polarization to M1 Macrophages.

	Up in Adipocytes	Not up in Adipocytes
Up in M1 Macrophages	32 (63%) *CCL8, CXCL10, MT2A*	9 (18%) *CXCL9, IL2RA, SLAMF1*
Not up in M1 Macrophages	5 (10%) *TNFSF14, ICOSLG, ANGPT2*	5 (10%) *LCN6, DIO3, CEACAM1*

*Number of genes upregulated >1.5 fold in respective cell-type are shown, along with examples of genes in each category.

Figure Legends

Figure 1: **Overview of study design and results.** The Discovery Study utilized microarray analysis of human adipose tissue isolated during *in vivo* endotoxemia (3 ng/kg) to identify genes modulated by inflammation (n=776). Bioinformatics tools (SignalP 3.0 or SecretomeP 2.0 score >0.7) to establish which of these were likely to encode secreted proteins (n=298). This list was prioritized to create a subset of genes (n=86) based upon (1) novelty, (2) degree of induction (3) sustained upregulation, (4) prior publication as secreted proteins *in vitro* or *in vivo*, (5) evidence for functional roles in inflammation, insulin signaling, or adipose biology, and (6) known association with diseases of interest. The Validation Study involved RT-PCR analysis of these 86 genes in human adipose isolated during *in vivo* endotoxemia (0.6 ng/kg) to confirm microarray findings and to identify genes modulated during lower-grade, more physiologic inflammation. Cell studies, in primary human adipocytes after treatment with LPS and macrophages after polarization to inflammatory (M1) phenotype were also performed to determine likely cellular origin of these 86 genes.

Figure 2: Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional categories with enrichment scores. Numbers of genes in each category are listed above each bar. The majority of genes are categorized into inflammatory pathways but some cluster into lipid and metal-binding categories. Many genes (28/86), often with unknown functions, did not classify into any known categories.

Figure 3: Comparison between endotoxin-induced adipose mRNA fold-change in Discovery vs. Validation Study. (A) Heatmap depicting direct comparison of 4h log10 expression changes of 86 high-priority genes in Discovery Study vs. Validation Study, ranked in order of log10 fold-change in Discovery Study. NCBI Gene ID names are listed on the right. Time 0 values are set to 1 (log10 changes = 0). Color coding: 0 is set to orange as seen at baseline, yellow indicates upregulation, red indicated downregulation. **(B)** Correlation (Spearman) between log10 peak fold-change of 85 detectable genes in Discovery vs. Validation study.

Figure 4: Comparison between endotoxin-induced mRNA fold-change in human adipose vs. primary adipocytes. (A) Spearman correlation between log10 peak fold-change of 77 detectable genes in primary human adipocytes (after 100 ng/mL LPS) and human adipose in Discovery Study (3 ng/kg endotoxin). **(B)** Correlation between log10 peak fold-change of 77 detectable genes in primary human adipocytes (after 100 ng/mL LPS) and human adipose in Validation Study (0.6 ng/kg endotoxin).
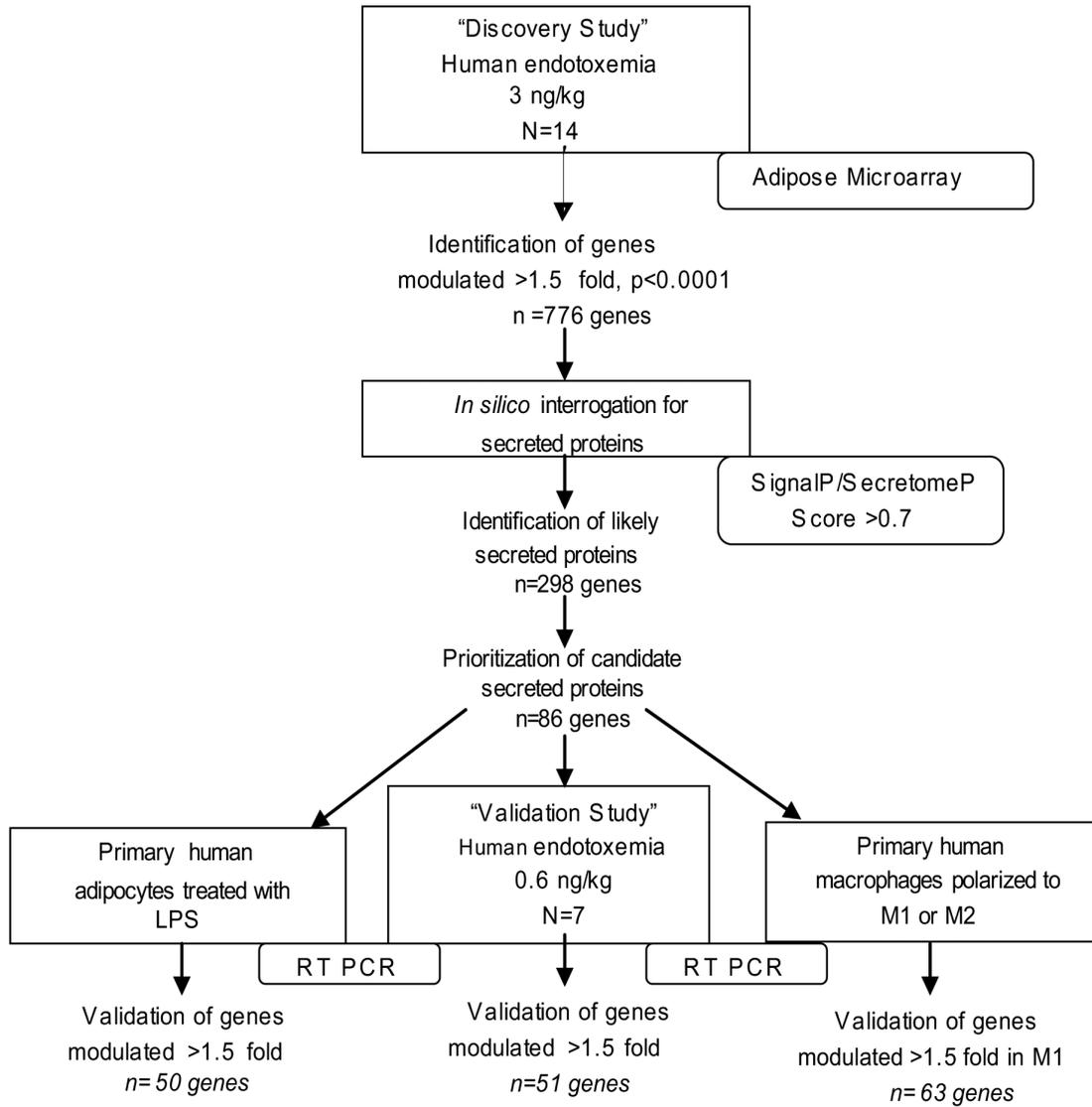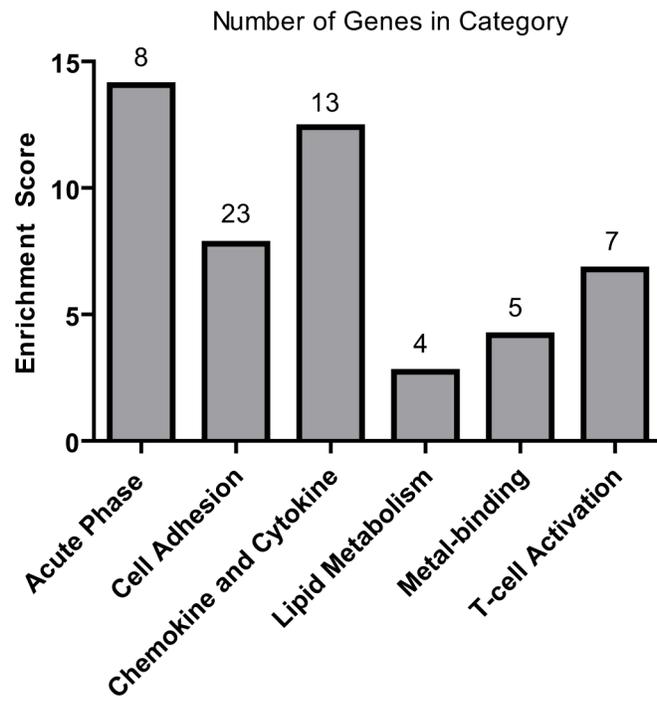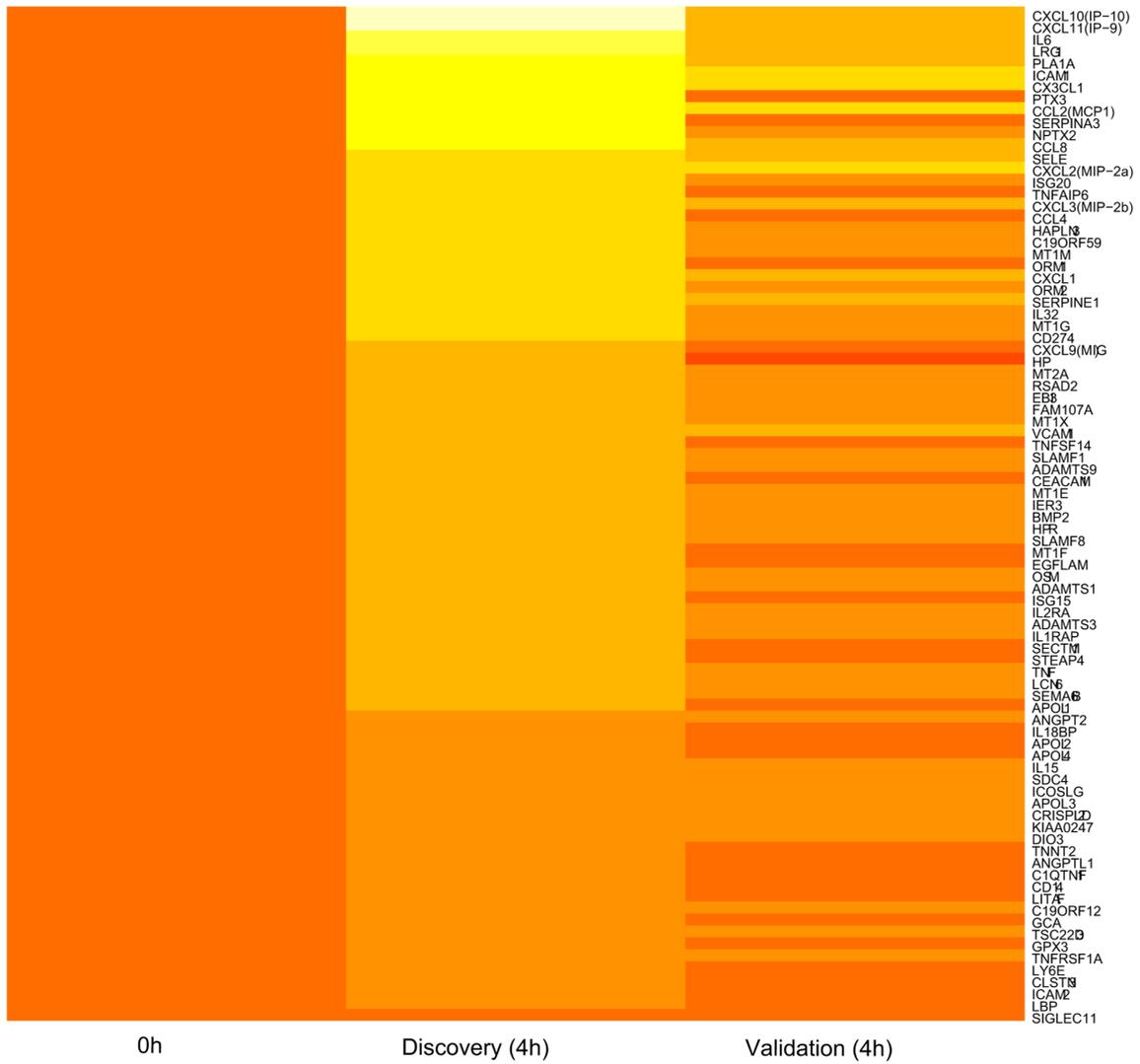
Figure 1

"Discovery Study"
Human endotoxemia
3 ng/kg
N=14

Adipose Microarray

Identification of genes
modulated >1.5 fold, p<0.0001
n =776 genes

In silico interrogation for
secreted proteins

SignalP/SecretomeP
Score >0.7

Identification of likely
secreted proteins
n=298 genes

Prioritization of candidate
secreted proteins
n=86 genes

Primary human
adipocytes treated with
LPS

"Validation Study"
Human endotoxemia
0.6 ng/kg
N=7

Primary human
macrophages polarized to
M1 or M2

RT PCR

RT PCR

Validation of genes
modulated >1.5 fold
n= 50 genes

Validation of genes
modulated >1.5 fold
n=51 genes

Validation of genes
modulated >1.5 fold in M1
n= 63 genes

Figure 2

Number of Genes in Category

Figure 3A

Figure 3B

r=0.64, p<0.0001

Figure 4A

r=0.49, p<0.0001

Figure 4B

r=0.42 p<0.0001