Deficiency of lymphotxin-α does not exacerbate high-fat diet-induced obesity but does enhance inflammation in mice

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Pamir N, McMillen TS, Edgel KA, Kim F, LeBoeuf RC. Deficiency of lymphotxin-α does not exacerbate high-fat diet-induced obesity but does enhance inflammation in mice. Am J Physiol Endocrinol Metab 302: E961–E971, 2012. First published February 7, 2012; doi:10.1152/ajpendo.00447.2011.—Lymphotxin-α (LTα) is secreted by lymphocytes and acts through tumor necrosis factor-α receptors and the LTβ receptor. Our goals were to determine whether LT has a role in obesity and investigate whether LT contributes to the link between obesity and adipose tissue lymphocyte accumulation. LT deficient (LT−/−) and wild-type (WT) mice were fed standard pelleted rodent chow or a high-fat/high-sucrose diet (HFHS) for 13 wk. Body weight, body composition, and food intake were measured. Glucose tolerance was assessed. Systemic and adipose tissue inflammatory statuses were evaluated by quantifying plasma adipokine levels and tissue macrophage and T cell-specific gene expression in abdominal fat. LT−/− mice were smaller (20%) and leaner (25%) than WT controls after 13 wk of HFHS diet feeding. LT−/− mice showed improved glucose tolerance, suggesting that, in WT mice, LT may impair glucose metabolism. Surprisingly, adipose tissue from rodent chow- and HFHS-fed LT−/− mice exhibited increased T lymphocyte and macrophage infiltration compared with WT mice. Despite the fact that LT−/− mice exhibited an enhanced inflammatory status at the systemic and tissue level even when fed rodent chow, they were protected from enhanced diet-induced obesity and insulin resistance. Thus, LT contributes to body weight and adiposity and is required to modulate the accumulation of immune cells in adipose tissue.

macrophages; lymphocytes; cytokines; diabetes
However, LT<sup>−/−</sup> mice showed an enhanced inflammatory status at systemic and adipose tissue levels, suggesting that macrophage and T cell accumulation per se are not requirements for insulin resistance. Overall, LT joins the growing list of inflammatory mediators now known to modulate body composition and glucose homeostasis likely via alterations of T cell and macrophage phenotype profiles (34, 52).

**MATERIALS AND METHODS**

**Mice.** Male mice deficient in LTα (LT<sup>−/−</sup>) (B6.129S2-Lta<sup>tm1Dch</sup>−/J) and their wild-type (WT) C57BL/6J (B6) controls were obtained from The Jackson Laboratory (Bar Harbor ME; strains nos. 002258 and 000664) and housed four per cage unless otherwise noted. At 4 wk of age, mice were randomly assigned to two diet groups and fed either pelleted rodent chow or a high-fat/high-sucrose (HFHS) diet. Body weights were determined weekly for these animals. At necropsy, mice were fasted for 4 h in the morning, bled from the retroorbital sinus into tubes containing 1 mM EDTA, and euthanized by cervical dislocation, and tissues were collected and stored at −80°C until analyses. Mice were maintained in a specific pathogen-free animal facility at the University of Washington at 25°C with a fixed 12:12-h light-dark cycle. All procedures were done in accordance with current National Institutes of Health (NIH) guidelines and approved by the Animal Care and Use Committee of the University of Washington. Results for the WT mice were reported previously in a separate analysis of obesity and TNFR-deficient mice (53). Results for our LT<sup>−/−</sup> mice were obtained in simultaneous feeding studies with the WT and TNFR<sup>−/−</sup> mouse studies, but results for the test strains are being published separately due to the large data of mice and separate types of findings. Thus, these data for WT mice are the simultaneous control group for the current analyses of the role of LT in obesity.

**Glucose tolerance test.** Following a 16-h overnight fast, mice were gavaged orally or injected intraperitoneally (ip) with 1 mg glucose/g body wt or ip with 1 µl/kg body wt insulin (Humulin R; Eli Lilly, Indianapolis, IN). Blood glucose was monitored (OneTouch Ultra, LifeScan) before and serially after glucose or insulin administration. Hyperinsulinemic-euglycemic clamp assessment of insulin resistance (HOMA-IR) values were calculated as HOMA-IR = [insulin (mg/dl) × 22.5]/[glucose (mg/dl)/22.5].

**Body composition.** Body composition was performed on conscious immobilized mice by using quantitative magnetic resonance (EchoMRI whole body composition analyzer; Echo Medical Systems, Houston, TX) (43). Because of the availability of equipment, body composition was performed at one time point (5 wk of diet feeding, 9 wk of age) for a second cohort of mice developed expressly for this purpose.

**Food intake.** Food intake was measured over 10 days for HFHS-fed mice and over 4 days for chow-fed mice. Briefly, weighed food was provided daily, and each available food excess was collected, and the difference of food provided vs. food not consumed was used to calculate food intake.

Plasma insulin and adipokine measurements. Plasma insulin levels were measured using the Linco insulin ELISA (catalog EZRMI-13K; Millipore, St. Charles, MO). Plasma levels of TNFα, interleukin (IL)-6, leptin, tissue plasminogen activator inhibitor-1 (PAI-1), resistin, and monocyte chemoattractant protein-1 (MCP-1) were determined using the Lincoplex adipokine panel (catalogue MADPK-71K; Millipore), with plate reading done on a Lumex100 (Lumexin, Austin, TX).

**Cell culture.** The evaluation of insulin receptor signaling was done as described previously (9). Briefly, mouse liver (AML12; ATCC CRL-2254) and myoblast (C2C12; CRL-1772) cell lines were cultured in Dulbecco’s modified Eagle’s medium (Gibco-Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, nonessential amino acid, and penicillin-streptomycin and maintained at 37°C in 5% CO₂. Cells were treated with LT (R & D Systems, Minneapolis, MN) at 5, 10, and 50 ng/ml for 1 h and then stimulated with either 100 nM insulin (Eli Lilly) or vehicle for 15 min before the cell lysates were prepared. Protein concentrations were determined using BCA, and the same amount of protein was used in each lane for Western blotting. SDS-polyacrylamide gel electrophoresis was performed using 4–20% gradient gels (Lonza, Rockland, ME). After transfer, the membrane was incubated with p-Akt antibody (1:1,000; Cell Signaling Technology, Beverly, MA), total Akt antibody (1:1,000; Cell Signaling Technology), or GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The protein bands were analyzed using densitometry and ImageJ image analysis-normalizing phosphorylated protein to total protein bands.

**Real-time quantitative RT-PCR.** Total RNA samples were isolated from epididymal adipose tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified by spectrophotometry. First-strand cDNAs were synthesized from 2.0 to 4.0 µg of total RNA with Moloney murine leukemia virus reverse transcriptase. The primers and probes were purchased from IDT (Coralville, IA), and sequences of primers and probes are given in Table 1. Sequences for Va14/Ja18 are based on those from Nyamuyar et al. (49). Results were analyzed using the ΔΔC<sub>t</sub> method (31).

**Statistical analysis.** Data are presented as means ± SE, and statistical significance was established at α < 0.05 (two-tailed). The t-tests were done using the Prism (La Jolla, CA) statistical programming package, and a two-way ANOVA was used to evaluate main effects and interactions involving genotype and diet status.

**RESULTS**

**LT deficiency results in reduced obesity.** Body weights for LT<sup>−/−</sup> and WT mice fed pelleted rodent chow were comparable over the 13-wk study (Fig. 1A). In contrast, HFHS diet feeding induced greater weight gain in WT than LT<sup>−/−</sup> mice starting at 11 wk, and by 17 wk of age LT<sup>−/−</sup> mice were 16% lighter than WT mice (29.7 ± 0.7 vs. 33.1 ± 1.9 g, P < 0.05, n = 7–10). To test whether reduced weights were due to reduced fat mass, body composition and selected tissue weights were obtained across groups. Brown adipose depots were smaller for LT<sup>−/−</sup> compared with WT mice after 13 wk of diet feeding (Table 1). Direct measurements of body composition performed on a second set of mice (at 9 wk of age following 5 wk of HFHS diet feeding) showed that whereas lean mass was comparable between LT<sup>−/−</sup> and WT mice (Fig. 1B), fat mass was reduced by 30% (8.4 ± 2.3 g for WT vs. 5.7 ± 0.9 g for LT<sup>−/−</sup>, P < 0.01). The proportion of fat mass relative to body weight was reduced for LT<sup>−/−</sup> mice (29.3 ± 5.8% for WT and 23 ± 3.6% for LT<sup>−/−</sup>, P < 0.05). We were unable to detect differences in food intake between the genotypes (data not shown). Overall, LT<sup>−/−</sup> mice did not show significant body weight or body fat gain over WT mice when fed a high-fat diet, and results are comparable with other mouse strains deficient in inflammatory mediators (8, 46).

Plasma total cholesterol levels were increased ~40% for HFHS-fed mice (120 mg/dl) compared with mice fed rodent chow (80 mg/dl), but no significant differences were seen between the genotypes. For plasma triglyceride levels, no differences were seen between the genotypes (38 mg/dl), and plasma triglyceride levels did not change with diet.

**LT<sup>−/−</sup> mice are modestly protected from diet-induced glucose intolerance.** We evaluated glucose tolerance for mice after 11 and 12 wk of diet feeding. Our data suggest that LT<sup>−/−</sup> mice had modest improvements in glucose tolerance compared with WT mice. During oral glucose challenge (Fig. 2A), HFHS diet-fed WT but not LT<sup>−/−</sup> mice experienced an extensive glucose...
Table 1. List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Bank ID</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>LTα</td>
<td>7106343a1</td>
<td>CCACTCTTGTAGGCTGTGG</td>
<td>CATGTGAGAAGAGGGGACAT</td>
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<tr>
<td>SDF-1α</td>
<td>7305465a1</td>
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<td>GATAGGCTCTGAGATGACATXT</td>
<td>TTCGCCAATCAGGCTGAGAG</td>
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<tr>
<td>CD3γ</td>
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<td>AGTGGGCCACAGATACATGCT</td>
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<td>GCGATCTTTCTGAGCTTCTC</td>
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<tr>
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<td>GGTGTTGAGATATAGGAGTTC</td>
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<tr>
<td>IFNγ</td>
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Primer sequences and gene bank IDs are provided. LTα, lymphotoxin-α; SDF-1α, stromal cell-derived factor-1α; CXCR4, chemokine (C-X-C motif) receptor 4; MCP-1, monocyte chemoattractant protein-1; MGL1, megalin-1; Arg1, arginase-1; iNOS, inducible nitric oxide synthase; Mhc2, major histocompatibility complex class II.

excursion at \( t = 15 \) min, implying that first-phase insulin secretion, which is required for reducing the initial glucose excursion, was less sufficient for WT mice than for LT\(^{-/-}\) mice. Also, the rate of return to pregavage glucose values by 120 min was significantly slower for WT compared with LT\(^{-/-}\) mice, indicating reduced insulin sensitivity for WT mice. These findings led to a significantly larger area under the curve (AUC) value for the HFHS WT mice (Fig. 2A, inset, \( P < 0.01 \)). For mice fed the HFHS diet, the rate of glucose lowering after ip insulin injection was comparable between genotypes for \( \geq 60 \) min (Fig. 2B). However, the return to initial glucose values was more rapid for WT compared with LT\(^{-/-}\) mice. This suggests that LT\(^{-/-}\) mice experienced a sustained responsiveness to insulin. However, we cannot rule out alternative explanations, such as enhanced insulin secretion or reduced counterregulatory responses, that may have contributed. Also of note was that 16-h fasting blood glucose levels (\( t = 0 \) min; Fig. 2A) were \( \sim 40\% \) lower for LT\(^{-/-}\) vs. WT mice (104 \( \pm 4.8 \) mg/dl for LT\(^{-/-}\) vs. 141 \( \pm 9.2 \) mg/dl for WT, \( P < 0.01 \)), which is also consistent with greater comparative impaired glucose tolerance in WT mice. Although we were not able to evaluate plasma insulin at 16-h fasting times due to lack of plasma, no differences in insulin levels or calculated HOMA-IR values were observed between genotypes for mice fed either diet after 4-h fasting times (Table 1).

Because body fat mass can directly contribute to insulin resistance phenotypes, we repeated the glucose tolerance tests using WT and LT\(^{-/-}\) matched for body weight. Mice were fed the HFHS diet for 5 wk, and animals were selected for comparable body weight and fat mass. Results supported our observations above that WT mice have impaired glucose tolerance, because oral glucose tolerance AUC values were again significantly higher for WT mice compared with LT\(^{-/-}\) mice (Fig. 2C). Overall, LT deficiency modestly protects mice from HFHS diet-induced aberrant glucose homeostasis.

**LT interaction with insulin receptor signaling.** Since LT deficiency protected mice from diet-induced obesity and insulin resistance, we tested the hypothesis that LT may directly influence insulin-signaling events. Protein from harvested cells was used to determine p-Akt protein concentrations by Western blotting, as reported previously (69). LT did not reduce insulin-stimulated p-Akt production in cultured mouse hepatocytes.
cytes and myoblast cells (data not shown), suggesting that the effects of LT are not directed through the insulin-signaling pathway.

Leptin and systemic inflammatory proteins. Plasma leptin levels are positively associated with body weight and body fat mass (15). Feeding the HFHS diet increased body weight and plasma leptin levels significantly within each genotype, as expected (Fig. 3A). Although leptin levels were comparable for HFHS diet-fed mice, chow-fed LT−/− mice had modest but significantly greater leptin levels than WT mice. It may be that the higher levels of adipose tissue inflammation seen for LT−/− compared with WT (see below) resulted in the stimulation of greater leptin production.

Obesity in mice and humans is associated with increased circulating levels of inflammatory adipokines (22, 75). Thus, we expected to observe reduced inflammatory adipokine levels in plasma of the somewhat leaner LT−/− mice. Surprisingly, plasma TNFα and IL-6 levels were significantly greater for LT−/− compared with WT mice (Fig. 3B). TNFα levels for rodent chow-fed WT mice were near the assay detection level (12 pg/ml) but elevated for LT−/− mice (38 ± 14 pg/ml), and chow-fed values for IL-6 were more than twofold higher for LT−/− (48 ± 19 pg/ml) than WT (16 ± 6 pg/ml). TNF values for HFHS-fed LT−/− mice were markedly higher (413 ± 117 pg/ml) than for WT mice. Of note, these levels were also greater than seen for mice with severe obesity and diabetes [e.g., ob/ob mice at 80 pg/ml (82)], pointing to a major deregulation in systemic inflammatory potential with loss of LT.

tPAI-1 protein levels trended toward higher values with HFHS diet feeding but did not differ between the genotypes.

Fig. 3. Plasma adipokine protein panel. At 13 wk of rodent chow (open bars) and HFHS diet challenge (light gray, dark gray, and black bars), plasma samples were taken from 4-h-fasted animals and evaluated as described in the text for levels of leptin (A), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) (B), and tissue plasminogen activator inhibitor (tPAI-1) and resistin (C); n = 6/group. *P < 0.05 between genotypes; †P < 0.05 between diets.
and TNF that were not seen for resistin, tPAI-1, or MCP-1, markedly enhanced following feeding of the obesigenic diet. In inflammation profile compared with WT mice, which was for both strains and diets (data not shown).

Plasma MCP-1 levels were below the detection limit of 8 pg/ml for 4 h prior to being euthanized Metabolic and tissue weight traits for WT and LT Table 2.

were not significantly different between strains (Fig. 3 C (Fig. 3 C)). Resistin levels, associated with insulin resistance in mice (26, 66), increased 30% with the HFHS diet being fed but were not significantly different between strains (Fig. 3 C). Plasma MCP-1 levels were below the detection limit of 8 pg/ml for both strains and diets (data not shown).

Overall, LT deficiency resulted in an aberrant systemic inflammation profile compared with WT mice, which was markedly enhanced following feeding of the obesigenic diet. In particular, LT deficiency resulted in elevations in leptin, IL-6, and TNF that were not seen for resistin, tPAI-1, or MCP-1, suggesting specificity in inflammatory regulatory systems modulated by LT.

Increased adipose tissue macrophage accumulation without increased cytokine levels for LT−/− mice. Obesity is accompanied by macrophage infiltration into adipose tissue and increased expression of proinflammatory adipokines and cytokines (22, 34, 63). Since LT−/− mice are leaner than WT mice, we expected to see a reduced adipose tissue cytokine inflammatory profile as well as reduced macrophage accumulation for LT−/− mice. These parameters were evaluated by quantifying molecular markers expressed in epididymal fat depots of mice fed the HFHS or chow diet for 13 wk (Fig. 4 and Table 2).

Surprisingly, rodent chow-fed LT−/− mice expressed sevenfold higher levels of macrophage marker F4/80 transcript than did WT mice, suggesting that LT−/− mice experienced a dramatic accumulation of adipose tissue macrophages in the basal state (Fig. 4A). This was dependent upon LT deficiency and not diet, because HFHS diet-fed LT−/− mice showed no further increase in F4/80 expression. In contrast, WT mice exhibited the commonly seen increase in macrophage marker expression with high-fat diet feeding (34). These results suggest that monocyte recruitment in adipose tissue is stimulated by the loss of LT. This finding was supported by the marked increase in MCP-1 expression for rodent chow-fed LT−/− compared with WT mice (Fig. 4A). WT mice showed an increase in MCP-1 expression with HFHS diet feeding, as seen by others (25, 67). For LT−/− mice fed HFHS, we were surprised to see a decrease in MCP-1 mRNA levels compared with LT−/− mice fed chow and WT mice fed the HFHS diet. However, levels for LT−/− remained significantly higher than those seen for the WT mice fed chow supporting continued induction of monocyte recruitment.

The phenotype of macrophages was examined using markers associated with subtypes of macrophages (34). Overall, it is likely that macrophage polarization within adipose tissue of LT−/− mice favored the M2 subtype since MGL1 and Arg-1 levels (Fig. 4B) were markedly elevated for chow fed LT−/− mice compared with WTs. Inducible nitric oxide synthase transcript levels (Fig. 4C), associated with a more M1 polarization phenotype, were not different between strains or diets.

A major finding here was that despite the striking increase in circulating IL-6 and TNF levels seen for LT−/− mice, this was not repeated within adipose tissue for levels of mRNA (Fig. 4C). TNF mRNA levels were reduced twofold for LT−/− mice compared with WT for mice fed either the chow or HFHS diet. Furthermore, IL-6 levels were comparable between the

![Fig. 4. Adipose tissue mRNA levels related to macrophage infiltration. Total RNA was isolated from epididymal fat pads taken from WT and LT−/− mice after 13 wk of being fed rodent chow and HFHS diets. Data are presented as normalized to chow-fed WT mice. F4/80 and monocyte chemoattractant protein-1 (MCP-1; A), megalin-1 (MGL1) and argenase-1 (Arg1) (B), and TNFα, IL-6, and inducible nitric oxide synthase (iNOS) (C); n = 5–8/group. *P < 0.05 between genotypes; †P < 0.05 between diets.](http://ajpendo.physiology.org/Downloadedfrom)

### Table 2. Metabolic and tissue weight traits for WT and LT−/− mice fed rodent chow and HFHS diets for 13 wk and fasted for 4 h prior to being euthanized

<table>
<thead>
<tr>
<th>Trait</th>
<th>WT Chow</th>
<th>WT HFHS</th>
<th>LT−/− Chow</th>
<th>LT−/− HFHS</th>
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<tr>
<td>Insulin, ng/ml</td>
<td>0.7 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>133 ± 11</td>
<td>167 ± 13</td>
<td>118 ± 12</td>
<td>199 ± 15</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.1 ± 1.4</td>
<td>7.0 ± 2.1*</td>
<td>4.5 ± 1.2</td>
<td>8.4 ± 1.8*</td>
</tr>
<tr>
<td>Liver, g</td>
<td>1.26 ± 0.06</td>
<td>1.28 ± 0.09</td>
<td>1.13 ± 0.03</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>Liver, %</td>
<td>4.68 ± 0.17</td>
<td>4.08 ± 0.23</td>
<td>4.34 ± 0.10</td>
<td>3.67 ± 0.14</td>
</tr>
<tr>
<td>BAT, g</td>
<td>0.10 ± 0.01</td>
<td>0.13 ± 0.01*</td>
<td>0.06 ± 0.01†</td>
<td>0.08 ± 0.01†</td>
</tr>
<tr>
<td>BAT, %</td>
<td>0.37 ± 0.03</td>
<td>0.39 ± 0.03</td>
<td>0.25 ± 0.02†</td>
<td>0.31 ± 0.04†</td>
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</table>

Values are means ± SE; n = 7–10. WT, wild type; HFHS, high-fat/high sucrose; HOMA-IR, homeostatic model assessment of insulin resistance; BAT, brown adipose tissue. *P < 0.05 between diets; †P < 0.05 between genotypes.
two strains for both diets. Since TNF and IL-6 are primarily expressed by the nonadipocyte constituent of adipose tissue (13), these results also support a macrophage component that is polarized toward the M2 subtype. However, a caveat exists in that reduced mRNA TNF levels in this particular strain of LT<sup>−/−</sup> mice may be indirectly affected due to the nature of targeting vector used in generating the LT<sup>−/−</sup> mice (28). Overall, loss of LT resulted in significant adipose tissue macrophage recruitment and accumulation that was characterized predominantly by M2-associated phenotypic markers without concomitant increases in tissue proinflammatory cytokines, TNF, or IL-6.

LT<sup>−/−</sup> mice exhibit enhanced adipose tissue lymphocyte content. Because of the dramatic macrophage accumulation phenotype that occurred in LT<sup>−/−</sup> mice even without an obesigenic stimulus, we tested the hypothesis that rodent chow-fed LT<sup>−/−</sup> mice also showed aberrant T cell infiltration. It is now clear that T lymphocytes accumulate in adipose tissue in the early stages of adipose tissue expansion (5, 27) and may in part be responsible for subsequent monocyte infiltration and macrophage differentiation. We tested this idea using a panel of T cell gene transcripts indicative of lymphocyte subclasses (Fig. 5).

Transcript levels for the pan T cell marker CD3 required for membrane expression and function of T cell receptors (18, 64) were more than sixfold greater for LT<sup>−/−</sup> than for WT mice fed rodent chow, and CD3 levels for LT<sup>−/−</sup> mice were not additionally modified by HFHS diet feeding (Fig. 5A). For WT mice, CD3 levels were increased to those of LT<sup>−/−</sup> following challenge with the obesigenic diet. This pattern of gene expression mirrored tightly that for the macrophage marker F4/80 (Fig. 4A) and suggests that LT<sup>−/−</sup> may be involved in both T cell and monocyte recruitment/macrophage retention events.

Infiltration of lymphocytes into human adipose tissue is due primarily to CD4<sup>+</sup> cells (27), and thus, we surveyed our mice for markers associated with CD4 subclasses. Following appropriate antigen stimulation, naïve CD4<sup>+</sup> cells can differentiate into at least four distinct T helper (Th) subsets, which are Th1, Th2, Th17, and iTreg cells (44, 84, 85). These subclasses are characterized in part by the expression of specific cytokines (41) that in turn can contribute to Th subset differentiation.

Th17 cells have been implicated in inflammatory and autoimmune diseases and are characterized by expression of IL-17 (20) and require Ror<sup>γt</sup> expression for differentiation (24). Ror<sup>γt</sup> expression was fourfold lower for chow-fed LT<sup>−/−</sup> compared with WT mice (Fig. 5B), suggesting a loss of Th17 differentiation potential with loss of LT<sup>−/−</sup>. The HFHS diet reduced Ror<sup>γt</sup> expression in WT mice, although no changes were seen for LT<sup>−/−</sup> mice. Thus, this T cell subclass is...
sensitive to nutrient environment, and LT is likely involved in its appropriate expression.

Foxp3 is required for the development of specific sets of Tregs, including natural regulatory T cells primarily in the thymus and inducible Tregs present in the periphery, which are often associated with limiting proinflammatory events in tissues (6, 85). In contrast to results for Rorγt, Foxp3 expression was dramatically elevated in chow fed LT−/− mice compared with WT mice (9-fold; Fig. 5C), consistent with the presence of an important complement of iTreg cells accumulating in LT−/− mouse adipose tissue. Again, the diet or obese state served to alter the relative abundance of this marker with opposing consequences in LT−/− (2-fold decrease) vs. WT mice (3-fold increase). IFNγ, a marker for Th1 cells, was fivefold elevated in LT−/− mice fed chow compared with WT (Fig. 5D). Levels of this marker were unchanged with HFHS diet feeding for LT−/− but markedly increased for WT mice. Thox expression, strongly induced in differentiating Th1 cells and CD8+ cytotoxic effector cells, was elevated somewhat by the HFHS diet for WT mice but overall was comparable between genotypes (data not shown). Significantly, the expression of major histocompatibility complex class II (Mhc2) was two- to fourfold elevated for LT−/− compared with WT (Fig. 5E). Since MHC2 expression occurs primarily on antigen-presenting cells such as macrophages, dendritic cells, and B lymphocytes, these data are somewhat consistent with results for F4/80 expression and signify elevated levels of antigen-presentation potential in LT−/− adipose tissue. Finally, natural killer T cells have recently been shown to be important for the development of adipose tissue inflammation and glucose intolerance in mice (51). mRNA levels for Vct14/Jx18, a specific marker for natural killer T cells, were comparable between genotypes and diets, although we observed a modest but significant increase for LT−/− fed the HFHS diet (Fig. 5F). Overall, outcomes from the deficiency in LT−/− included marked changes in immune markers related to T cell development. Because most changes were not further altered by diet, T cell modulation was likely independent of the status of body fat.

The chemokine stromal cell-derived factor-1 (SDF-1) has been implicated in the recruitment of T cells to peripheral tissues, and its transcription is regulated in part by LT (32, 37). Therefore, we expected the level of SDF-1 to be reduced in LT−/− mice, and this was confirmed by mRNA analyses of mouse epididymal fat tissue. Figure 6, A and B, shows lack of expression for LT−/− and SDF-1 in LT−/− adipose tissue. In contrast, chemokine (C-X-C motif) receptor 4 (CXCR4), a receptor for SDF-1, showed expression in both genotypes (Fig. 6C). For both SDF-1 and CXCR4, significant (2.5-fold) elevations were seen for WT mice with HFHS diet feeding. Thus, SDF-1 expression is not a requirement for T cell accumulation into adipose tissue since CD3 cells were more abundant in the genotype (LT−/−) lacking SDF-1 expression.

Overall, LT plays a key role in the accumulation and phenotypic expression of adipose tissue T cells in basal and obese states.

DISCUSSION

This report focuses on a key cytokine responsible in part for T cell differentiation, maturation, and activation. Because obesity research has now incorporated knowledge showing that T cells contribute to adipose tissue inflammation (14, 34, 51, 76, 79), it is important to begin to understand the role of individual T cell modulators in obesity and glucose homeostasis. Toward this end, we show for the first time that LT contributes to body fat accumulation and glucose homeostasis.

There are several main findings in this study. First, mice lacking LT expression are protected from the marked diet-induced obesity seen in several other cases of inflammatory gene-deficient mice (8, 46, 55, 68, 86). Body weight and fat mass were significantly reduced for LT−/− compared with WT mice after 13 wk of HFHS diet feeding. Also, LT−/− mice showed modest improvement in glucose homeostasis. Second, although LT−/− and WT mice showed comparable body weights when maintained on rodent chow, significant disturbances in inflammatory status were seen for LT−/− mice. Adipose tissue macrophage and total T cell content were markedly greater for LT−/− than WT mice even in this “lean” environment. Yet, all mice maintained on rodent chow showed normal glucose homeostasis, suggesting that the increased accumulation of immune cells does not itself predict insulin resistance. Third, feeding of the HFHS diet to LT−/− mice selectively enhanced proinflammatory markers in plasma (IL-6, TNF), but this was not reflected within adipose tissue.
Most importantly, HFHS diet feeding of LT$^{−/−}$ mice did not alter the phenotype classes of adipose tissue macrophages or T cells, except in one case (reduction in Foxp3$^{+}$ T cells). It is clear that loss of LT$^{−/−}$ resulted in adipose tissue accumulation of macrophages and T cells independent of test diet feeding. Overall, LT plays a role in body weight maintenance likely through influencing the repertoire and accumulation of specific macrophage and T cell subtypes. How these subtypes influence energy balance and nutrient storage is as yet unclear.

It is now supported by several studies that T lymphocytes infiltrate into adipose tissue during early stages of diet-induced adipose tissue expansion (5, 27). Evidence that T cells offer proinflammatory consequences is that long-term depletion of T cells in whole animals is an effective means of reversing insulin resistance in obesity (77). Their early presence in adipose tissue is followed later by macrophage infiltration and accumulation that has been associated with insulin resistance (27). Our data are supportive of some of these observations because elevations in adipose tissue T cells were as well as an increased accumulation of adipose tissue macrophages in WT mice with HFHS feeding. We observed dramatically increased levels of CD3+$^{+}$, MCP-1, F4/80, and two M2 type macrophage markers for LT$^{−/−}$ mice (MGL1 and Arg1) compared with WT for mice in the basal state. With high-fat diet feeding, adipose tissue macrophages (ATM) and T cell markers for LT$^{−/−}$ mice were largely unchanged, arguing that the T cell and macrophage climate was set by LT and not by the diet feeding or resultant body fat gain. Thus, LT plays a role in control of both T cell and macrophage subtype determinations and contributes to their accumulation.

Others have reported that it is not only the accumulation of immune cells but their phenotype that contributes to body weight and glucose homeostasis (17, 33). This is likely occurring with LT deficiency since the LT$^{−/−}$ mice showed improved glucose tolerance despite the increased immune cell accumulation. The character of the accumulated macrophages in LT$^{−/−}$ mice was likely to be weighted toward an M2 transcript profile due to elevated levels of markers MGL1 and Arg1 for chow-fed mice and decreased levels of TNF for both diets in LT$^{−/−}$ as opposed to WT adipose tissue (34). The M2 phenotype is thought to be anti-inflammatory in nature (69) and not associated with insulin resistance, although we did see a preponderance of Arg1 mRNA for WT HFHS diet-fed mice in our study. This may be due to experimental design since macrophage phenotype marker expression is dynamic and influenced by diet duration (62). Our feeding studies were relatively short (13 wk) compared with other reports, and we utilized a different type of obeseogenic diet (33). The T cell profile in LT$^{−/−}$ mice showed contradictory elements. IFN$γ^{−/−}$ mice are protected from obesity-induced adipose tissue inflammation and insulin resistance (57), yet the LT$^{−/−}$ mice showed sevenfold higher expression levels than those seen for WT mice. Rorγt levels were reduced in LT$^{−/−}$ mice, but Foxp3 was markedly higher. These results indicate important changes in lymphocyte biology associated with LT. Contradictory findings have also been seen in obesity and ATM phenotypes for a variety of genetically engineered mouse strains. Because obesity is associated with low-grade systemic inflammation, the expectation is that genetic ablation of pro-inflammatory cytokines would prevent the sequelae of obesity and/or insulin resistance. In contrast to this concept are mice lacking IL-1R1, IL-1Ra, IL-6, IL-18, and granulocyte macrophage colony-stimulating factor (8, 16, 55, 73, 86) that show maturity onset increases of fat mass and aberrant glucose homeostasis. Unfortunately, ATM and lymphocyte phenotyping was not performed, making it difficult to compare immune phenotypes. Overall, LT deficiency is associated with markedly altered inflammatory profiles compared with WT mice.

Of note is that the chemokine SDF-1 has been implicated in the recruitment of T cells to peripheral tissues, and its transcription is regulated in part by LT (32, 37). Our data suggest that SDF-1 expression is not a requirement for T cell accumulation into adipose tissue since CD3+$^{+}$ cells were more abundant in the LT$^{−/−}$ mice, for which we saw no SDF-1 signal.

We also found that transcript levels of several adipokines (e.g., TNF, IL-6) did not reflect overall plasma protein levels. One possible explanation is dissociation between transcript and protein levels. In addition, differences in cytokine clearance and/or utilization may be occurring between adipose and blood compartments. The mechanisms by which LT participates in modulating adipokine expression remain unknown.

Both LT$^{−/−}$ and TNF$^{−/−}$ strains are protected from significant diet-induced obesity. However, it is important to keep in mind that deficiency in these and other immune regulators can lead to alterations in immune organ differentiation and loss of specific leukocyte populations (e.g., see Refs. 39, 50, and 71). Thus, modulation of metabolic phenotypes may be directly related to loss of the cytokine or due to a collection of changes related to tissue defects. In addition, design of targeting vectors may influence phenotypes especially for closely linked genes such as LT, TNF, and LTβ (28). For instance, “conventional” LT$^{−/−}$ mice exhibit markedly reduced TNF responses to systemic LPS treatment. Our LT$^{−/−}$ mice showed disparate TNF changes with markedly high TNF levels seen in plasma, especially following HFHS feeding, and reduced levels seen in adipose tissue compared with WT mice. Our data support the concept that reduced weight gain in LT$^{−/−}$ mice is due to factors related to LT and not TNF since TNF was present in these critical compartments.

TNF triggers TNF receptors, and this activity is implicated in the TNF-mediated interference of insulin receptor signaling reported for obese animals (70). For LT, the story may be more complex since we were unable to show that LT directly inhibited insulin receptor signaling as monitored by p-Akt quantification. In addition to TNF receptors, LT binds to membrane-bound LTβ (LTα1β2), and this heterotrimer (membrane LT) is the ligand for the membrane-bound LTβ receptor (LTβR). Membrane LT is expressed by activated T, B, and NK cells, and the LTβR is expressed in nonlymphoid tissues and dendritic cells (40, 80). Thus, lack of LT may influence the ability of the LTβR to direct inflammatory cell responses in adipose tissue. LT is secreted as a soluble cytokine primarily from leukocytes and in this form could modulate obeseogenic phenotypes through binding to TNF receptors that exist on most cell types as well as the LTβR. Since T cells are potentially the initiators of monocyte recruitment, it is possible that LT is the initial ligand responsible for early events in macrophage maturation and stimulation. Because of the impressive alterations in T cell and macrophage markers seen for LT$^{−/−}$ mice in the basal state (fed rodent chow), we suggest that LT contributes to diet-induced obesity by modulation of adipose tissue inflammatory architecture possibly through the
maturation and/or activation of specific T cell subtypes that are responsible for monocyte recruitment and macrophage activation states.

Our report is the first to demonstrate that LT contributes to modulating obesity. Overall, we report that LT plays a physiological role in body weight and adiposity and is required to regulate the accumulation of T cells and myeloid cells in adipose tissue. LT also modulates the activation phenotype of both types of immune cells, the character of which leads to protection from diet-induced obesity. Markers associated with activation are not themselves predictive of an improved body weight and glucose homeostasis state. Further work is needed using these mice to identify in more detail the identities and functions of adipose tissue immune cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.P., K.A.E., and R.C.L. did the conception and design of the research; N.P., T.S.M., K.A.E., F.K., and R.C.L. drafted the manuscript; N.P., T.S.M., K.A.E., F.K., and R.C.L. acquired the results of the experiments; N.P. and T.S.M. prepared the figures; N.P. performed the experiments; N.P., T.S.M., F.K., and R.C.L. analyzed the data; N.P., T.S.M., K.A.E., F.K., and R.C.L. interpreted the results of the experiments; N.P. and T.S.M. prepared the figures; N.P. and R.C.L. drafted the manuscript; N.P., T.S.M., K.A.E., F.K., and R.C.L. edited and revised the manuscript; N.P., T.S.M., K.A.E., F.K., and R.C.L. approved the final version of the manuscript.

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