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T Cell–Mediated Inflammation in Adipose Tissue Does Not Cause Insulin Resistance in Hyperlipidemic Mice

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Abstract—Obesity is associated with chronic inflammation in adipose tissue. Proinflammatory cytokines including tumor necrosis factor-α and interleukin-6 secreted by adipose tissue during the metabolic syndrome are proposed to cause local and general insulin resistance and promote development of type 2 diabetes. We have used a compound mutant mouse, $Apoe^{-/-} \times$CD4dnTGFbR, with dysregulation of T-cell activation, excessive production of proinflammatory cytokines, hyperlipidemia, and atherosclerosis, to dissect the role of inflammation in adipose tissue metabolism. These mice are lean, which avoids confounding effects of concomitant obesity. Expression and secretion of a set of proinflammatory factors including tumor necrosis factor-α, interferon-γ, and monocyte chemoattractant protein-1 was increased in adipose tissue of $Apoe^{-/-} \times$CD4dnTGFbR mice, as was the enzyme 11β-hydroxysteroid dehydrogenase type 1, which converts cortisone to bioactive cortisol. Interleukin-6, which has an inhibitory glucocorticoid response element in its promoter, was not upregulated. In spite of intense local inflammation, insulin sensitivity was not impaired in adipose tissue of $Apoe^{-/-} \times$CD4dnTGFbR mice unless exogenous interleukin-6 was administered. In conclusion, T-cell activation causes inflammation in adipose tissue but does not lead to insulin resistance in this tissue in the absence of interleukin-6. **(*Circ Res*. 2009;104:961-968.)**

Key Words: adipose tissue ■ cytokines ■ inflammation ■ insulin resistance ■ interleukin-6 ■ T cells

Obesity is associated with a chronic low-grade inflammatory condition in adipose tissue.[1] In the metabolic syndrome, which is characterized by abdominal obesity, insulin resistance, hypertension, and hyperlipidemia, adipose tissue is infiltrated by macrophages and displays secretion of proinflammatory cytokines.[1–4] Very recently, T cells have also been detected in adipose tissue, with increased infiltration in obesity, although their role remains unclear.[5,6] Chemokines expressed in obese adipose tissue likely mediate the recruitment of these cells.[6]

A range of proinflammatory cytokines have been identified in adipose tissue of obese individuals, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6.[7–10] TNF-α and IL-1 induce local expression and secretion of large amounts of IL-6, leading to measurable levels of the latter in the systemic circulation. By acting on hepatocytes, IL-6 induces the acute phase response with expression of C-reactive protein, fibrinogen, and other proteins.

Blood levels of C-reactive protein, fibrinogen, and IL-6 are elevated in obese individuals and correlate with the fat mass,

insulin resistance, and hypertension.[11,12] Obesity may lead to type 2 diabetes and insulin resistance, and it can aggravate atherosclerosis by impairing macrophage function.[13,14] It has been proposed that proinflammatory cytokines are causally linked to the development of insulin resistance.[15,16] Islet inflammation is involved in the regulation of β-cell function in type 2 diabetes, a condition in which IL-1 receptor antagonism has beneficial effects.[17] Both TNF-α and IL-6 have been reported to interfere with insulin signaling, which would lead to impaired glucose metabolism in adipose tissue and skeletal muscle.[18,19] However, administration of blocking anti–TNF-α antibodies does not improve insulin sensitivity in humans,[20] and IL-6 deficiency in mice leads to obesity by acting on the central nervous system.[21] These data illustrate the complexity of cytokine signaling in obesity and demonstrate a need for experimental models of metabolic syndrome and adipose inflammation that are not confounded by obesity.

On the other hand, recent data suggest an increased prevalence of metabolic syndrome during inflammatory chronic diseases, independently of any obesity. Patients with

psoriasis,[22] Crohn's disease,[23] and rheumatoid arthritis[24] appear to be at higher risk for type 2 diabetes. Furthermore, anti-TNF therapy of rheumatoid arthritis has led to improved insulin resistance,[25] arguing for a role of inflammation per se in the regulation of insulin sensitivity in humans.

By crossing 2 mutant mice, we have developed a mouse model characterized by aggravated inflammation, hyperlipidemia, and atherosclerosis. The CD4dnTbR mouse carries a dominant-negative transforming growth factor (TGF)-β receptor II construct under the CD4 promoter, leading to loss of TGF-β–dependent inhibition of T-cell activation and, as a consequence, aggravated T cell–dependent inflammation.[26] The $Apoe^{-/-}$ mouse has defective lipoprotein elimination from circulation, causing hyperlipidemia with excessive levels of very low-density lipoprotein and chylomicron remnants.[27] By crossing CD4dnTbR and $Apoe^{-/-}$ mice, an offspring is obtained ($Apoe^{-/-} \times$CD4dnTbR) that displays hyperlipidemia, excessive vascular inflammation, and dramatically accelerated atherosclerosis.[28]

We speculated that the combination of immune inflammation, hyperlipidemia, and atherosclerosis may cause adipose inflammation and lead to insulin resistance. By using the lean but inflamed CD4dnTbR$\times Apoe^{-/-}$ mouse, it should be possible to determine the effects of inflammation per se in the absence of obesity. Our analysis of white adipose tissue (WAT) in $Apoe^{-/-}$ and $Apoe^{-/-} \times$CD4dnTbR demonstrated that it shares a large set of expressed inflammatory genes with WAT of obese *ob/ob* mice. However, IL-6 expression was not increased, and T cell–driven inflammation did not per se impair insulin sensitivity in adipose tissue. Therefore, our findings show that T cell–driven immune inflammation and obesity-associated inflammation differ in important aspects and that IL-6 may be a critical component in the development of insulin resistance.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Mouse Models

$Apoe^{-/-}$ mice[27] were crossed with transgenic CD4-dnTGFβRIITg$^+$ (CD4dnTbR) mice.[26] Female compound mutant, $Apoe^{-/-}$, *ob/ob*, and C57BL/6 mice were fed standard mouse chow. All studies were approved by the regional ethical committee for animal experiments.

mRNA Analysis

Gonadal WAT cDNA from C57BL/6, $Apoe^{-/-} \times$CD4dnTbR, and $Apoe^{-/-}$ mice (6 mice per group) was hybridized to Affymetrix global microarrays.[29] Signals were normalized across samples. Transcript profiles were compared with published data, as described in the online data supplement. Standard methods were used for real-time RT-PCR.

Immunofluorescence Microscopy

Sections of gonadal WAT were stained with fluorescent antibodies and analyzed using confocal microscopy.

Western Blot

WAT protein extracts were separated by SDS-PAGE, subjected to immunoblot analysis, stained with primary antibodies to 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) or to phosphorylated proteins of the insulin signaling pathway, followed by peroxidase-labeled secondary antibodies, and visualized by chemiluminescence.

Glucose Tolerance Test and Homeostasis Model Assessment Index

After 4 hours of starvation, 1.5 g/kg glucose was injected intraperitoneally into conscious mice. Baseline blood glucose values were at 0, 5, 15, 30, 60, 90, and 120 minutes after injection; homeostasis model assessment (HOMA) index was used to calculate relative insulin resistance ($G_0 \times I_0/22.5$), where I_0 is the fasting plasma insulin (μU/mL) and G_0 the fasting blood glucose (mmol/L).[30]

Lipogenesis, Lipolysis, and Secretion Studies

Fat cells were isolated from gonadal WAT.[31] Glucose transport, lipolysis, and secretion studies were performed as described.[32] Incorporation of radioactive glucose into lipid was used to measure lipogenesis and release of glycerol for lipolysis.

IL-6 Infusion

Alzet osmotic pumps loaded with 16 μg/mL recombinant human IL-6 or saline only were implanted subcutaneously in 11-week-old mice.[33] After 7 days, mice were euthanized, the vasculature was perfused, and gonadal WAT was removed for analysis. For insulin signaling studies, Actrapid insulin (0.375 mU/g body weight) was injected IP after 4 hour of starvation, 10 minutes before euthanasia.

Results

Body Weight and Fat Cell Size of Transgenic Mice

There was no difference in average weight or fat cell diameter between $Apoe^{-/-}$ single-mutant and $Apoe^{-/-} \times$CD4dnTbR mice (Online Table I). None of these mice was overtly obese, and their weight and mean fat cell diameter were similar to those of wild-type C57BL/6 (B6) mice of the same age. $Apoe^{-/-} \times$CD4dnTbR, as well as $Apoe^{-/-}$, mice displayed severe hyperlipidemia, with dramatically increased very-low-density and low-density lipoproteins.[28]

Immunolocalization of Immune Cells in WAT

Immunofluorescent microscopy was used to identify immune cells in WAT. In *ob/ob* mice, CD68$^+$ macrophages were typically present in crown-like structures surrounding adipocytes (Figure 1). Significant numbers of macrophages were also found in $Apoe^{-/-}$ and $Apoe^{-/-} \times$CD4dnTbR mice (Figure 1). Only occasional CD68$^+$ macrophages were observed in wild-type B6 mice (Figure 1). CD3$^+$ T cells were found in gonadal WAT of $Apoe^{-/-} \times$CD4dnTbR but also *ob/ob* mice (Figure 2). Interestingly, many T cells were present in aggregates, suggestive of clonal activation. Very few T cells could be detected in B6 mice (Figure 2). Most WAT T cells belonged to the CD4$^+$ subset (compare CD4 staining in Figure 2E with CD8 in Figure 2H).

CD4 T cells are activated by antigens presented through the endocytic pathway involving major histocompatibility complex (MHC) class II molecules in antigen-presenting cells. The MHC class II protein I-A^b, as well as CD4, was abundantly expressed in WAT of *ob/ob* and $Apoe^{-/-} \times$CD4dnTbR mice (Figure 2J and 2K). Therefore, WAT contains the molecular machinery involved in CD4$^+$ T-cell activation.
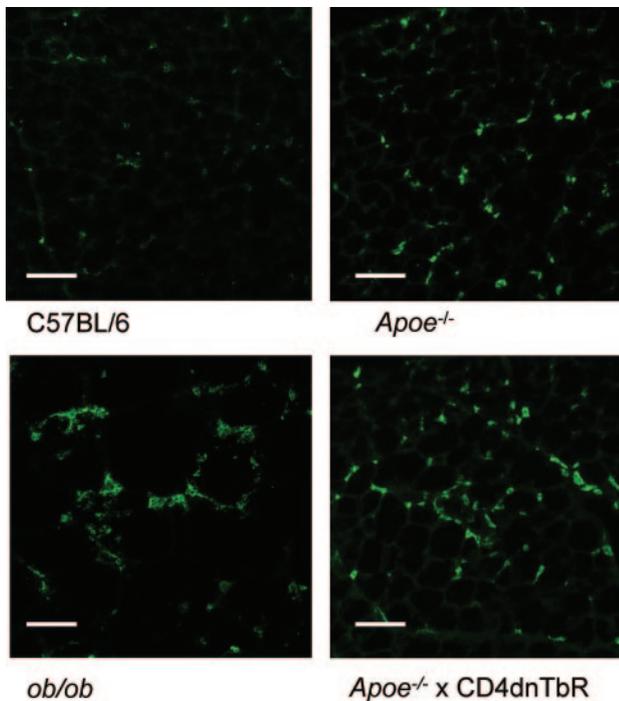
Figure 1. Confocal immunofluorescent microscopy of macrophages in adipose tissue. Macrophages were visualized in adipose tissue from the indicated mouse strains by immunostaining for CD68. Scale bars=20 μm. Representative micrographs from 1 of 3 experiments.

Cytokine Secretion by Adipose Tissue

Incubation experiments with isolated gonadal fat pads were performed to identify cytokine proteins in WAT (Figure I in the online data supplement). Incubation for 2 hours at 37°C led to significant release of TNF-α and monocyte chemoattractant protein (MCP)-1 from $Apoe^{-/-}\times$CD4dnTbR mice, as compared to $Apoe^{-/-}$ and B6 mice. This confirms that WAT from mice with T cell–driven inflammation produces excessive amounts of these cytokines and suggests a paracrine cascade in which T cells activate macrophages (and possibly other cells) to produce TNF-α and MCP-1.

Glucose Metabolism, Adipokine Secretion, and Lipogenesis in Inflamed Adipose Tissue

To test whether T cell mediated inflammation affects glucose metabolism, we performed intraperitoneal glucose tolerance tests. Whereas B6 mice displayed a normal response to glucose injection, $Apoe^{-/-}$ mice developed a significantly higher glycemic response (Figure 3A). This is in line with the previous notion that $Apoe^{-/-}$ mice have a relative insulin resistance. Because $Apoe^{-/-}\times$CD4dnTbR mice have adipose inflammation as well as hyperlipidemia, we expected them to be even more resistant to insulin action. However, their response during glucose tolerance tests was not significantly different from that of $Apoe^{-/-}$ mice (Figure 3A), implying that inflammation did not cause any additional impairment of glucose tolerance in these mice.

Serum levels of insulin and adiponectin did not differ significantly between $Apoe^{-/-}\times$CD4dnTbR and $Apoe^{-/-}$ mice (Figure 3B and Online Table I). Because the levels of adiponectin are inversely correlated to the degree of insulin

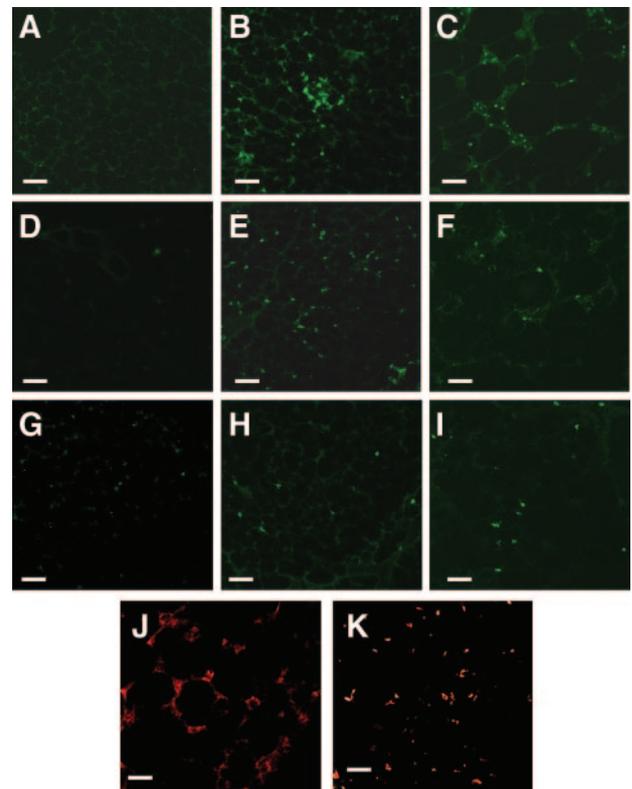**Figure 2.** Confocal immunofluorescent microscopy of T cells and MHC class II protein in adipose tissue. A through C, CD3$^+$ T cells in B6 (A), $Apoe^{-/-}\times$CD4dnTbR (B), and ob/ob mice (C). D through F, CD4$^+$ T cells (MHC II–restricted T cells) in B6 (D), $Apoe^{-/-}\times$CD4dnTbR (E), and ob/ob mice (F). G through I, CD8$^+$ T cells (MHC I–restricted T cells) in B6 (G), $Apoe^{-/}$$^-\times$CD4dnTbR (H), and ob/ob mice (I). J and K, I-A^b (MHC class II antigen) expression in adipose tissue of $Apoe^{-/-}\times$CD4dnTbR (J) and ob/ob mice (K). Scale bars=20 μm. Representative micrographs from 1 of 6 experiments.

resistance,[34] these data supported the conclusion that insulin resistance was not proportional to the extent of inflammation in adipose tissue. Furthermore, the HOMA index did not differ significantly between groups (Figure 3C), confirming that increased inflammation was not associated with aggravated insulin resistance in $Apoe^{-/-}\times$CD4dnTbR mice.

Because circulating levels may not reflect the local situation in WAT, we analyzed adipokine secretion from fat pads. No differences between $Apoe^{-/-}$ and $Apoe^{-/-}\times$CD4dnTbR mice were detected in the secretion of leptin or adiponectin during in vitro incubation (Online Figure I, C and D). As direct tests of insulin-dependent responses, we analyzed lipogenesis (ie, incorporation of glucose-derived radioactivity into lipids), as well as lipolysis in isolated adipocytes. Basal lipogenesis was elevated under fasting conditions, both in $Apoe^{-/-}$ and $Apoe^{-/-}\times$CD4dnTbR mice, and a further increase in lipogenesis was detected in response to insulin stimulation (Figure 3D). Of note, insulin-induced lipogenesis was significantly more efficient in fat cells of $Apoe^{-/-}\times$CD4dnTbR than in adipocytes from $Apoe^{-/-}$ mice, suggesting that the former mice responded more vividly to insulin in spite of their adipose inflammation. No difference in basal (spontaneous), catecholamine-stimulated, or insulin-inhibited lipolysis in adipo-
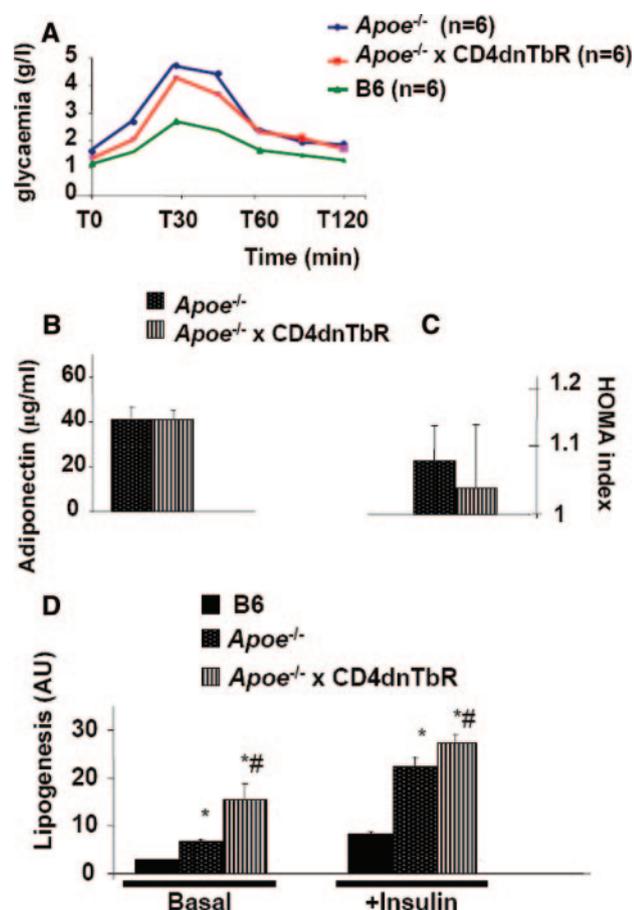
Figure 3. Metabolic status of the $Apoe^{-/-} \times$CD4dnTbR mouse. A, Intraperitoneal glucose tolerance test showed a similar response to glucose in $Apoe^{-/-} \times$CD4dnTbR as in $ApoE^{-/-}$ mice. B and C, Plasma adiponectin (B) and the HOMA Index (C) did not differ between the 2 strains. Data show means±SE (n=6 mice per group). D, Lipogenesis in gonadal adipose tissue showed significant incorporation of ^{3}H-glucose into lipid and a vivid response to insulin in $Apoe^{-/-} \times$CD4dnTbR mice. Data show arbitrary units of radioactivity per gram lipid divided by average radioactivity per gram lipid in B6 adipose tissue not exposed to insulin, means±SEM. *$P<0.05$ vs B6, #$P<0.05$ vs $Apoe^{-/-}$.

cytes was observed between the different types of mice (data not shown).

Gene Expression in Adipose Tissue

The global transcriptome was assessed in gonadal WAT from $Apoe^{-/-} \times$CD4dnTbR, single-knockout $Apoe^{-/-}$, and wild-type C57BL/6 mice. Affymetrix expression array analysis showed increased expression of 490 transcripts in $Apoe^{-/-} \times$CD4dnTbR mice when compared with $Apoe^{-/-}$ mice. These transcripts were then compared with those reported to be upregulated in obese mice (leptin-deficient *ob/ob* mice and wild-type mice exposed to a high fat diet)[2]: 25 transcripts were elevated both in $Apoe^{-/-} \times$CD4dnTbR and obese mice (Table). These transcripts were mainly derived from inflammatory genes and several of them are known to be induced by interferon (IFN)-γ, a cytokine of activated T cells. Of note, the adipose tissue cytokine IL-6 was not among the upregulated genes. The global expression analysis thus suggested that T cell–derived cyto-

Table. Transcripts With Elevated Adipose Tissue Expression Both in $Apoe^{-/-} \times$CD4dnTbR and *ob/ob* Mice

Gene Family and Affymetrix Reference	Gene Name	Fold Change
Immune cell genes		
1415983_at	Lymphocyte cytosolic protein-1	3.97
1422903_at	Lymphocyte antigen 86	3.1
1427076_at	Macrophage expressed gene 1	3.89
Ig family		
1417876_at	Fc receptor IgG high affinity	3.06
1420464_s_at	Paired-Ig-like receptor A6	2.16
1428018_a_at	Immunoglobulin superfamily member 7	2.04
Cytokines		
1438855_x_at	Tumor necrosis factor α-induced protein 2	2.48
1422476_at	Interferon γ inducible protein 30	2.12
MHC family		
1425137_a_at	Histocompatibility 2 Q region locus 10	1.65
1417063_at	Complement component 1q subcomponent β polypeptide	3.01
Chemokines		
1417936_at	Chemokine (C-C motif) ligand 9	2.38
1450678_at	Integrin β2	4.81
1419128_at	Integrin αX	4.50
Other genes		
1454268_a_at	Cytochrome b-245α polypeptide	2.98
1450165_at	Schlafen 2	2.91
1419394_s_at	S100 calcium binding protein A8 (calgranulin A)	1.62
1417647_at	Sorting nexin 5	1.58
1456424_s_at	Phospholipid transfer protein	1.55
1417620_at	RAS-related C3 botulinum substrate 2	3.31
1426454_at	Rho GDP dissociation inhibitor β	3.26
1418296_at	FYXD domain–containing ion transport regulator 5	1.76
1448561_at	Neutrophil cytosolic factor 2	1.74

The fold change in cDNA signal shown is for $Apoe^{-/-} \times$CD4dnTbR vs $Apoe^{-/-}$ mice. Data represent means fold change from 2 experiments with adipose tissue mRNA pooled from 6 mice per group. This data set was compared with a published data set of the transcriptome in WAT of ob/ob mice.[2]

kine(s) operate in adipose tissue but do not induce IL-6 expression.

To validate these findings, key mRNA species were quantified by real-time RT-PCR (Figure 4). The T cell–specific gene, CD3γ was highly expressed in adipose tissue of $Apoe^{-/-} \times$CD4dnTbR mice. Substantial CD3γ mRNA levels were also found in adipose tissue of *ob/ob* mice, confirming that T cells infiltrate the adipose tissue in this model of obesity. IFN-γ was profoundly increased in $Apoe^{-/-} \times$CD4dnTbR mice, suggesting T-cell activation in WAT of these mice. Interestingly, obese mice also displayed elevated WAT IFN-γ mRNA, pointing to the
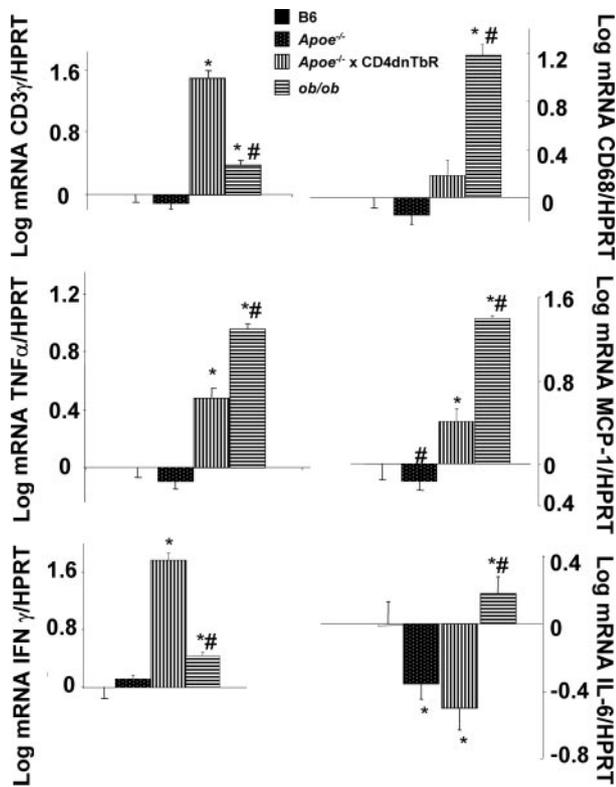
Figure 4. Inflammatory gene expression in adipose tissue of different mouse strains. Data show relative mRNA levels measured by real-time RT-PCR of total adipose tissue RNA. Note similar trends of change vs B6 in $Apoe^{-/-} \times CD4dnTbR$ and ob/ob mice, although absolute levels of expression differ. Data represent means±SEM (n=6 mice per group). *$P<0.05$ vs B6, #$P<0.05$ vs $Apoe^{-/-} \times CD4dnTbR$.

possibility that obesity is associated with T-cell activation and IFN-γ secretion in WAT.

As expected, the macrophage-specific gene, CD68 (macrosialin) was highly expressed in adipose tissue of ob/ob but also in $Apoe^{-/-} \times CD4dnTbR$ mice (Figure 4). TNF-α mRNA was increased, as was the chemokine MCP-1 (Figure 4). In contrast, mRNA was not elevated for any of these cytokines in $Apoe^{-/-}$ mice as compared to wild-type mice (Figure 4). In fact, MCP-1 mRNA was significantly reduced in $Apoe^{-/-}$ mice.

Reduced IL-6 Expression in Inflamed WAT of $Apoe^{-/-} \times CD4dnTbR$ Mice

IL-6 has been proposed to play a key role in the development of insulin resistance but was not among the genes overexpressed in both models (see above). RT-PCR analysis of IL-6 mRNA in WAT showed significantly increased levels in ob/ob as compared to wild-type mice (Figure 4). In contrast, IL-6 mRNA was substantially reduced in $Apoe^{-/-}$ and particularly in $Apoe^{-/-} \times CD4dnTbR$ mice. IL-6 protein levels were not detectable in the systemic circulation of these mice, nor in $Apoe^{-/-}$ mice (data not shown). The lack of IL-6 induction was surprising in view of the excessive inflammatory status of the $Apoe^{-/-} \times CD4dnTbR$ mice and the fact that IL-6 protein is detectable at ≈150 pg/mL in sera of ob/ob mice on the same B6 genetic background.[35]

Upregulated 11β-HSD I in WAT

We speculated that IL-6 expression could be actively suppressed in inflamed WAT of $Apoe^{-/-} \times CD4dnTbR$ mice. IL-6 is largely regulated on the transcriptional level, with promoter elements including nuclear factor κB, activator protein-1, and serum response element–mediating IL-6 transcription in response to IL-1, TNF-α, and several other stimuli[36] and with several glucocorticoid response elements inhibiting IL-6 transcription. Glucocorticoids can be produced by several cell types including CD4$^+$ T cells, which express 11β-HSD1.[37] This enzyme regenerates bioactive cortisol from inactive 11-keto metabolites.[38] We found significantly increased 11β-HSD1 mRNA in the adipose tissue of $Apoe^{-/-} \times CD4dnTbR$ mice when compared to ob/ob, as well as wild-type B6 mice (Figure 5A). Ob/ob mice did not differ from wild-type B6 mice with regard to 11β-HSD1 mRNA (Figure 5A). Western blot analysis of WAT extracts confirmed the presence of 11β-HSD1 protein and showed increased amounts in $Apoe^{-/-} \times CD4dnTbR$ mice (Figure 5B). The relative amount of 11β-HSD1 protein was 3.11±0.33 in $Apoe^{-/-} \times CD4dnTbR$ versus 1.04±0.56 U in $Apoe^{-/-}$ WAT (densitometric units of 11β-HSD1/β-actin; means±SEM, n=4, $P<0.05$).

IL-6 Administration Reduces Insulin Sensitivity in Inflamed Adipose Tissue

We next tested the hypothesis that lack of IL-6 accounts for the maintained insulin sensitivity in $Apoe^{-/-} \times CD4dnTbR$ mice. Recombinant human IL-6, which is recognized by
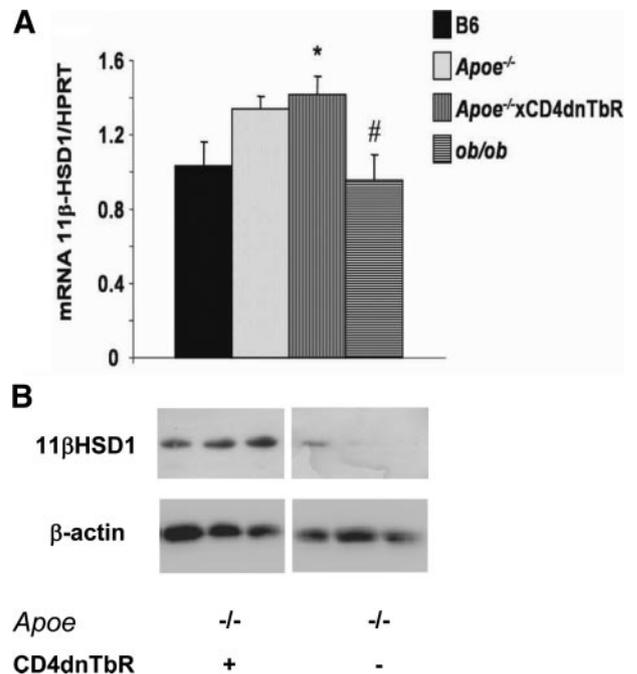

Figure 5. 11β-HSD1 in adipose tissue. A, Real-time RT-PCR analysis of 11β-HSD1 mRNA levels in gonadal adipose tissue from different mouse strains (means±SEM, n=6 per group). *$P<0.05$ vs B6, #$P<0.05$ vs $Apoe^{-/-} \times CD4dnTbR$. B, Western blot analysis of 11β-HSD1 protein in gonadal adipose tissue from $Apoe^{-/-} \times CD4dnTbR$ and $Apoe^{-/-}$ mice.
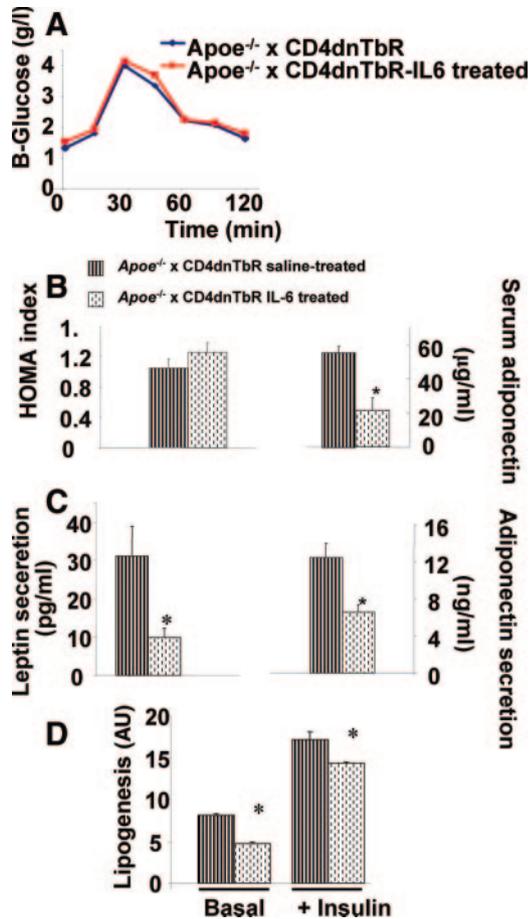
Figure 6. Metabolic status of *Apoe*$^{-/-}$×CD4dnTbR IL-6–treated mouse. A, Glucose tolerance test showed no difference between groups (n=5 per group). B, HOMA index did not differ between groups, whereas serum adiponectin was reduced in IL-6–treated mice (n=6). C, In vitro secretion of leptin and adiponectin tissue was significantly reduced in adipose tissue from IL-6–treated mice (n=6). D, IL-6–treated mice displayed significantly reduced basal, as well as insulin-stimulated, lipogenesis. Data show arbitrary units as described in the legend of Figure 3.

mouse IL-6 receptors, was infused for 1 week via osmotic minipumps. This treatment resulted in significant circulating levels of human IL-6, reaching concentrations of nearly the same magnitude as those observed in obese individuals and patients with type 2 diabetes (93.9±4.9 pg/mL versus undetectable levels in saline-treated mice). No interference with endogenous mouse IL-6 took place as no IL-6 was detected by immunoassay in saline-treated mice.

IL-6 treatment *Apoe*$^{-/-}$×CD4dnTbR mice led to significant reduction of serum adiponectin levels (Figure 6), suggesting that IL-6 causes insulin resistance in WAT. In line with this, adiponectin and leptin secretion was significantly reduced in gonadal fat pads from IL-6–treated *Apoe*$^{-/-}$×CD4dnTbR mice (Figure 6). In contrast, IL-6 treatment did not significantly affect glucose tolerance test results or HOMA index (Figure 6), implying that systemic effects of IL-6 may differ from those on WAT.

To directly test whether WAT insulin sensitivity was affected by IL-6 treatment, lipogenesis was analyzed in isolated adipocytes from IL-6–treated and untreated *Apoe*$^{-/-}$×CD4dnTbR

mice (Figure 6). Basal and insulin-stimulated lipogenesis from radiolabeled glucose was significantly reduced after IL-6 treatment. This confirms that IL-6 administration reduced WAT insulin sensitivity in these mice.

Finally, we determined whether IL-6 directly affects the insulin receptor signaling complex in WAT. Insulin-induced phosphorylation of Akt and extracellular signal-regulated kinase 1/2, key enzymes along metabolic and mitogenic signaling pathways, was unaltered by IL-6 treatment (Online Figure II). Therefore, IL-6 is likely to modulate insulin-dependent responses in WAT through indirect mechanisms.

Discussion

This study demonstrates several novel features of inflammation in adipose tissue. (1) Inflammation can occur in WAT in the absence of obesity and does not necessarily lead to obesity. (2) It involves T cells, the orchestrators of adaptive immunity, in addition to macrophages and stromal cells. (3) WAT inflammation is associated with expression of a host of "proximal" proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1β, irrespective of whether it is caused by obesity or caused by T cell–driven inflammation and hyperlipidemia. (4) The 2 forms of WAT inflammation differ in IL-6 expression, which is present in obesity-associated but not in immune inflammation. (5) Immune inflammation and hyperlipidemia does not per se lead to insulin resistance, either at the local (WAT) or systemic level.

Our findings confirm the recent observation by Rocha et al that IFN-γ is expressed by infiltrating T cells in adipose tissue.[39] They concluded that Th1 cell–derived IFN-γ regulates fat inflammation and glucose homeostasis. Our findings in the CD4dnTR model, which has uncontrolled Th1-cell activation and excessive IFN-γ expression, argues against a decisive role for the Th1 cell in regulating glucose metabolism in WAT. Instead, our findings show that marked Th1-type inflammation caused by loss of TGF-β control of T cells is not sufficient to cause WAT insulin resistance. Instead, local modulation of IL-6 expression may control glucose metabolism in inflamed WAT and may depend on additional stimuli and modulators in addition to proinflammatory Th1 cytokines including IFN-γ (Figure 7). These findings support the notion that IL-6 plays a key role in the
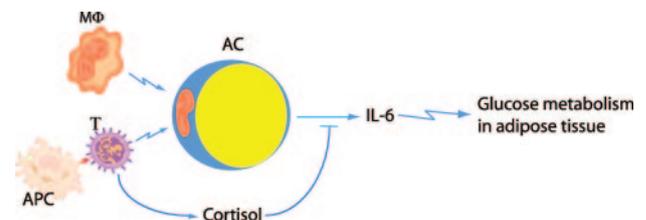

Figure 7. Hypothetical scheme depicting immune activation and metabolic regulation in adipose tissue. Activation of innate immunity (macrophage) (upper left), as well as adaptive immunity (T cell–activated by antigen-presenting cell) (lower left), generates mediators that can induce IL-6 production in adipocytes. This, in turn, modulates glucose metabolism in adipose tissue. However, T-cell activation also leads to local production of cortisol that inhibits IL-6 transcription. Therefore, T-cell activation during a local adaptive immune response may not impair glucose metabolism to the same extent as innate immune activation.

development of insulin resistance.[19,33,40] However, they also show that IL-6 is not the proximal modulator of insulin receptor signaling but likely depends on secondary mediators.

It is surprising that IL-6 was not increased in the inflamed WAT of $Apoe^{-/-} \times$CD4dnTbR mice, particularly because its transcription is induced by IL-1 and TNF-α, both of which were significantly upregulated. The paradoxical absence of IL-6 may be attributable to transcriptional inhibition. A glucocorticoid response element in the IL-6 promoter inhibits transcription when cortisol occupies the glucocorticoid receptor.[36] Cortisol can be produced in WAT by bioconversion of inactive steroids.[38] This reaction is catalyzed by 11β-HSD1, an enzyme found in adipocytes, macrophages, T cells, and several other cell types. It is upregulated on activation of T cells,[37] and the high expression levels of 11β-HSD1 in adipose tissue of $Apoe^{-/-} \times$CD4dnTbR mice could at least partly be attributable to abundant expression in activated T cells (Figure 7). It is also plausible that T cell–dependent activation of other cells led to upregulation of their 11β-HSD1. Because cortisol efficiently silences IL-6 expression,[36] our data suggest that T-cell activation–dependent, 11β-HSD1–mediated cortisol production may inhibit IL-6 expression in WAT (Figure 7).

Administration of recombinant IL-6 reduced insulin-dependent responses in WAT of $Apoe^{-/-} \times$CD4dnTbR mice. However, it did not impair the insulin-dependent phosphorylation cascade. Therefore, IL-6 likely modulates WAT insulin sensitivity through indirect pathways. The effects of IL-6 on insulin responses in WAT were not mirrored on the integrative level. Blood glucose, insulin, HOMA index, and glucose tolerance test results remained unchanged after IL-6 administration. This difference likely reflects that whole-body insulin sensitivity is determined by several different tissues including skeletal muscle and liver.[41,42] It is possible that insulin sensitivity is regulated differentially in these tissues and that IL-6 is particularly important in WAT. Alternatively, WAT may respond more rapidly to IL-6 than other insulin-responsive tissues.

Several studies have suggested that TNF-α is the instigator of insulin resistance in the inflammatory state.[1,7] Although it remains possible that TNF-α may have such an effect in skeletal muscle, our data suggest that this may not be the case in adipose tissue. Infiltration of activated T cells initiated expression of a set of proinflammatory cytokines, mRNA for which was 10- to 50-fold increased in WAT of $Apoe^{-/-} \times$CD4dnTbR mice when compared to wild-type or $Apoe^{-/-}$ mice. In spite of this, insulin sensitivity remained on the same level as in the $Apoe^{-/-}$ single-knockout mouse, which has modestly reduced insulin sensitivity as compared to wild-type mice. Therefore, dramatically elevated local TNF-α expression per se did not reduce insulin sensitivity any further in the mouse with hyperlipidemia and severe immune inflammation. Similarly, drastically elevated expression of IFN-γ, a major macrophage-activating T-cell cytokine, was not sufficient to cause WAT insulin resistance. It is possible that obesity-associated changes such as enlargement of adipocytes and enhancement of lipolysis are required for TNF-α induced insulin resistance; our inflamed mice had normal fat cell size and normal rates of adipocyte lipolysis. However, recent data suggest that

TNF-α may not cause insulin resistance even when obesity is present.[43]

Ob/ob mice expressed higher levels of TNF-α and MCP-1 and lower levels of IFN-γ in adipose tissue as compared with $Apoe^{-/-} \times$CD4dnTbR mice. The inflammatory infiltrate also differed, with a relatively higher proportion of macrophages and lower of T cells in *ob/ob* mice. These differences may reflect a more important non–T cell–driven inflammation under conditions of leptin deficiency. We speculate that this might be attributable to innate immune activation of macrophages in *ob/ob* mice, for instance by metabolically modified molecules eliciting pattern recognition receptor-dependent activation of macrophages (Figure 7). Interestingly, Toll-like receptor-4 ligation has been implicated in vascular inflammation, IL-6 expression, and insulin resistance.[14,16,44] However, T cell infiltrates were also identified in *ob/ob* mice in our study, suggesting that T cell–dependent macrophage activation is involved in adipose tissue inflammation also in this model.

Our data and those of several other investigators were derived from mutant mouse models that represent extreme cases of metabolic dysregulation and immune activation. Adipose tissue inflammation under conditions more similar to human pathophysiology could conceivably display different patterns of glucose metabolism. It is also possible that compensatory mechanisms may operate in mutants with congenital defects in metabolism and immunity. In future studies, it will be therefore important to use alternative strategies to analyze the effects of inflammation on WAT metabolism.

To summarize, using a mutant mouse model of systemic inflammation and hyperlipidemia, we demonstrate that this combination is not sufficient to elicit a complete metabolic syndrome. Proximal cytokines such as TNF-α and IFN-γ do not cause insulin resistance per se when IL-6 is downregulated in WAT. These findings point to factors inducing and regulating local inflammation as critical for determining insulin sensitivity under pathological conditions.

Disclosures
None.

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Supplement Material

A Sultan, D Strodthoff et al.
T cell mediated inflammation in adipose tissue does not cause insulin resistance in hyperlipidemic mice

Material and Methods

Mouse models:

Apoe^{-/-} mice [1](Taconic) were crossed with transgenic CD4-dnTGFßRIITg[+] (CD4dnTbR) mice [2]. Both strains had been backcrossed seven times to C57BL/6 (B6). Female littermates were fed standard mouse chow and sacrificed under anesthesia. B6 mice were obtained from Taconic and *ob/ob* mice on the B6 background from Charles River. All studies were approved by the regional ethical committee for animal experiments.

Tissue collection, total RNA preparation:

12-week old mice were sacrificed by CO_2 inhalation, tissues collected on dry ice, rapidly frozen, and stored at -80^0C. Total gonadal WAT RNA was isolated from 12-week-old mice after homogenization in FastRNA® Pro Green (Q-Biogene) with RLT lysis buffer (Qiagen) and phenol-CHISAM (Sigma-Aldrich). Total RNA was purified using RNeasy (Qiagen) with a DNase step. RNA concentration and quality were assessed by capillary electrophoresis (Agilent Technologies). cDNA was synthesized from total RNA (1μg) using Superscript-II (Invitrogen) and random hexamers.

Microarray analysis of transcriptome

Pooled total RNA from C57BL/6, *Apoe*^{-/-} x CD4dnTbR, and *Apoe*^{-/-} mice (6 mice per group) was converted into biotinylated, fragmented cRNA and hybridized to microarrays (Murine Genome Array MOE430A, Affymetrix) at 45°C for 16 hours[3]. The samples were stained and washed on a Fluidics Station 400 (Affymetrix) and scanned on a GeneArray Scanner (Affymetrix). The analysis was repeated twice for each strain. Primary data extraction was performed with Microarray Suite 5.0 (Affymetrix) and signal normalization across samples was carried out using all probe sets with a mean expression value of 500. To compare transcript profiles obtained in this study with those published

by Weisberg et al [4], we downloaded a file comparing the MOE430A and MG_u74av2 arrays from the Affymetrix website, parsed the data files and used custom Perl scripts for analysis.

Real-time RT-PCR analysis of mRNA:

cDNA was PCR amplified in an ABI 7700 Sequence Detector (Applied Biosystems) using primers and probes obtained from Affymetrix as assay-on-demand and with hypoxanthine guanidine ribonucleosyltransferase (HPRT) as a "housekeeping gene". Data were expressed as arbitrary units obtained by comparing the threshold cycle value of the test sample with that of HPRT (ΔC_T). We analyzed the data using the $\Delta\Delta C_T$ method, normalizing the *Apoe$^{-/-}$* x CD4dnTbR, *Apoe$^{-/-}$* and *ob/ob* samples to the B6 samples. Results are expressed as $2^{-\Delta\Delta CT}$.

Immunofluorescence microscopy:

Portions of gonadal WAT were flash frozen in OCT compound or embedded in paraffin. 10μm cryosections were fixed with ice-cold acetone, pretreated with 10% normal goat serum in PBS followed by a biotin-avidin blocking kit (Vector Lab), and incubated with monoclonal rat-anti-mouse-CD68, rat-anti-mouse CD4, rat-anti-mouse CD8, or rat-anti-mouse CD68 (PharMingen) in PBS with 10% goat serum. Sections were stained with Oregon green-488 conjugated goat-anti-rat IgG (Molecular Probes). Rat-anti-mouse-CD3 antibodies were used on paraffin sections and visualized with Oregon Green-goat-anti-rat-IgG. All antibodies were used at optimal concentrations determined by titration on mouse spleen. Images were obtained with a BioRad MRC 1024 confocal unit attached to a Nikon Diaphot 200 inverted microscope. Focus was set at the height where the cell diameter was maximal.

Western blot

WAT specimens were crushed and lysed at 4ºC in 1% Triton-X 100, Tris-HCL pH 7.6 and 150 mmol/L NaCl supplemented with protease inhibitors[5], homogenized, centrifuged at 14,000 rpm for 30 min, and the infranatant collected and assayed for protein contentusing BCA Protein Assay (Pierce). 50-100 μg protein was loaded on SDS-polyacrylamide gels and transferred to polyvinylidine fluoride membranes (Amersham Pharmacia Biotech). Blots were blocked for 1 h at room temperature in Tris-buffered

saline with 0.1% Tween-20 and 5% non-fat dried milk. This was followed by overnight incubation at 4°C with antibodies against 11β-HSD1 (Santa Cruz, Inc.), phospho-Akt-Ser(473), or phospho-Erk1/2 (Cell Signaling Technology)[5]. Secondary anti-rabbit-IgG antibodies conjugated to horseradish peroxidase were from Sigma. Antigen-antibody complexes were detected by chemiluminescence (Supersignal from Pierce) and specific bands identified using a Chemidoc XRS system (BioRad). Images were analyzed using BioRad software. β-actin and glyceraldehyde-3-phosphate dehydrogenase antibodies (Sigma) was used as loading controls (arbitrary units).

Glucose tolerance test:
After 4 hours starvation, 1.5g/kg glucose was injected intraperitoneally into conscious mice. Baseline blood glucose values were obtained from tail vein by using an AccuCheck Go (Roche) glucometer. Additional measurements were made at 5, 15, 30, 60, and 90 minutes after injection, animals sacrificed after 120 min and blood collected after heart punction. Serum insulin was measured by ELISA (Mercodia).

HOMA Index:
HOMA index was used to calculate relative insulin resistance (G_0 x I_0 / 22.5), where I_0 is the fasting plasma insulin (μU/ml) and G_0 the fasting blood glucose (mmol/l) [6].

WAT preparation for lipogenesis, lipolysis and secretion studies:
Fat cells were isolated from gonadal WAT [7]. Mean adipocyte diameter was calculated from 100 cells. Isolated cells were used to study glucose transport and lipolysis, and additional WAT pieces used for secretion studies.

Lipogenesis:
Fat cells were incubated for 2h at 37°C with (3-^{3}H) glucose (5×10^6 dpm/ml), unlabelled glucose (1 μmol/l) and human insulin (10^{-15} -10^{-6} M)[8]. Incorporation of radio labeled glucose into adipocyte lipids, which reflects lipogenesis, was normalized to gram lipid.

Lipolysis:
Glycerol release was used as an index of lipolysis. Adipocytes were incubated for 2 hours at 37°C in Krebs-Ringer-phosphate buffer containing 2% BSA, 1 mg/ml glucose; insulin at 10^{-12} to 10^{-9} M and norepinephrine at 10^{-12}-10^{-5} M. Total lipid content of cells was measured gravimetrically after heptane extraction. Glycerol was analyzed in a cell-free

aliquot by bioluminescence. Lipolysis was expressed as glycerol per mg lipid.

Secretion studies:

WAT was incubated for 2h at 37°C in KRP buffer, endotoxin-free 2% BSA and 1mg/ml glucose, and aliquots of the medium analyzed by ELISA (IL-6, TNF-α and IFN-γ with kits from R&D Systems, adiponectin and leptin with Biovendor kits). Secretion was expressed per gram lipid.

IL-6 infusion:

Alzet osmotic pumps (model no. 2001) were filled with 16 µg/ml recombinant human IL-6 (RDI Systems) or saline only[9]. Pumps were implanted into the intrascapular subcutaneous space of anesthetized 11-week-old mice. After 7 days, mice were sacrificed, the vasculature perfused through the heart with sterile RNase-free PBS, and gonadal WAT removed for analysis. For insulin signaling studies, Actrapid® insulin (0.375 mU/g body weight) was injected IP 10 minutes before sacrifice and after 4h starvation.

Statistical analysis:

All results are expressed as mean ± S.E.M. Statistically significant differences were determined by two-way analysis of variances (ANOVA) followed by Mann Whitney test. *p* values <0.05 were considered significant. For RT-PCR data, relative mRNA levels are expressed as log values.

References to Online Data supplement:

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Supplemental Table I: Body weight and fat cell diameter of 12-week old mice.

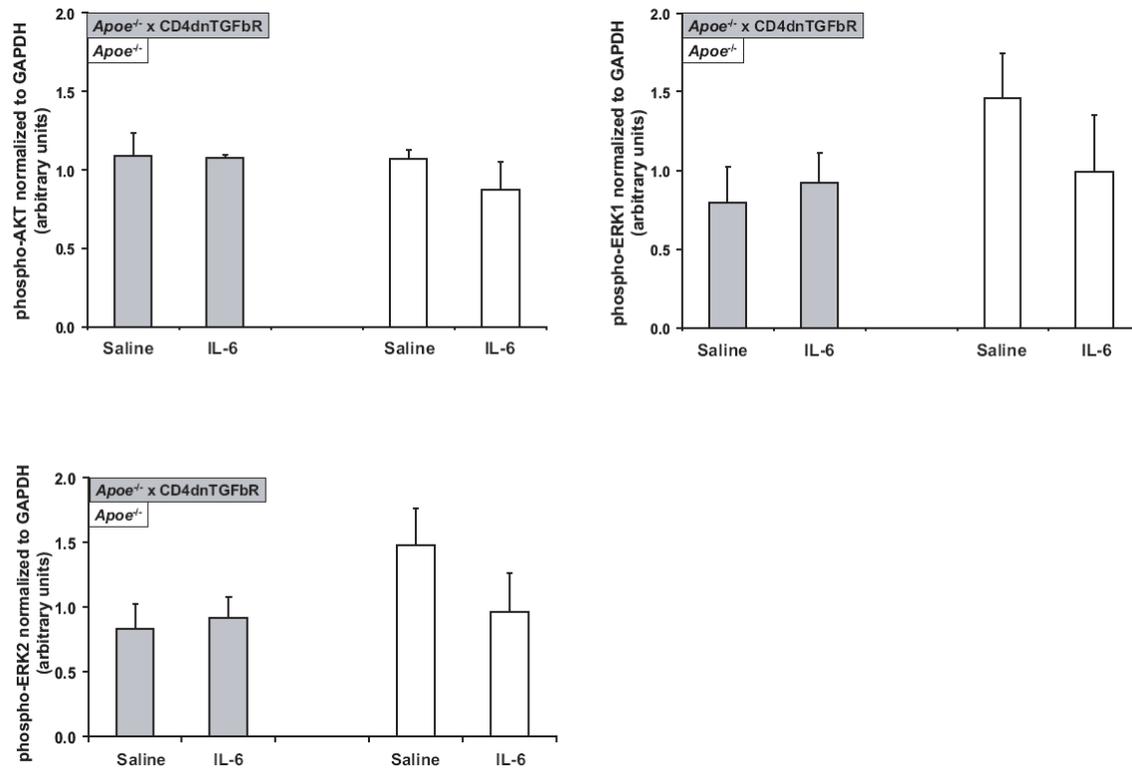| Strain	*Apoe*^{-/-}	*Apoe*^{-/-}x CD4dnTbR
Weight, gram (mean ± SEM)	20.13 ± 0.48	20.75 ± 0.45
Fat cell diameter, μm (mean ± SEM)	46.1 ± 7.55	44.9 ± 6.83
Serum insulin (ng/mL)	1.33 ± 0.17	1.67 ± 0.17

Data shown are mean±S.E.M. (n=10 per group). No significant differences were observed.
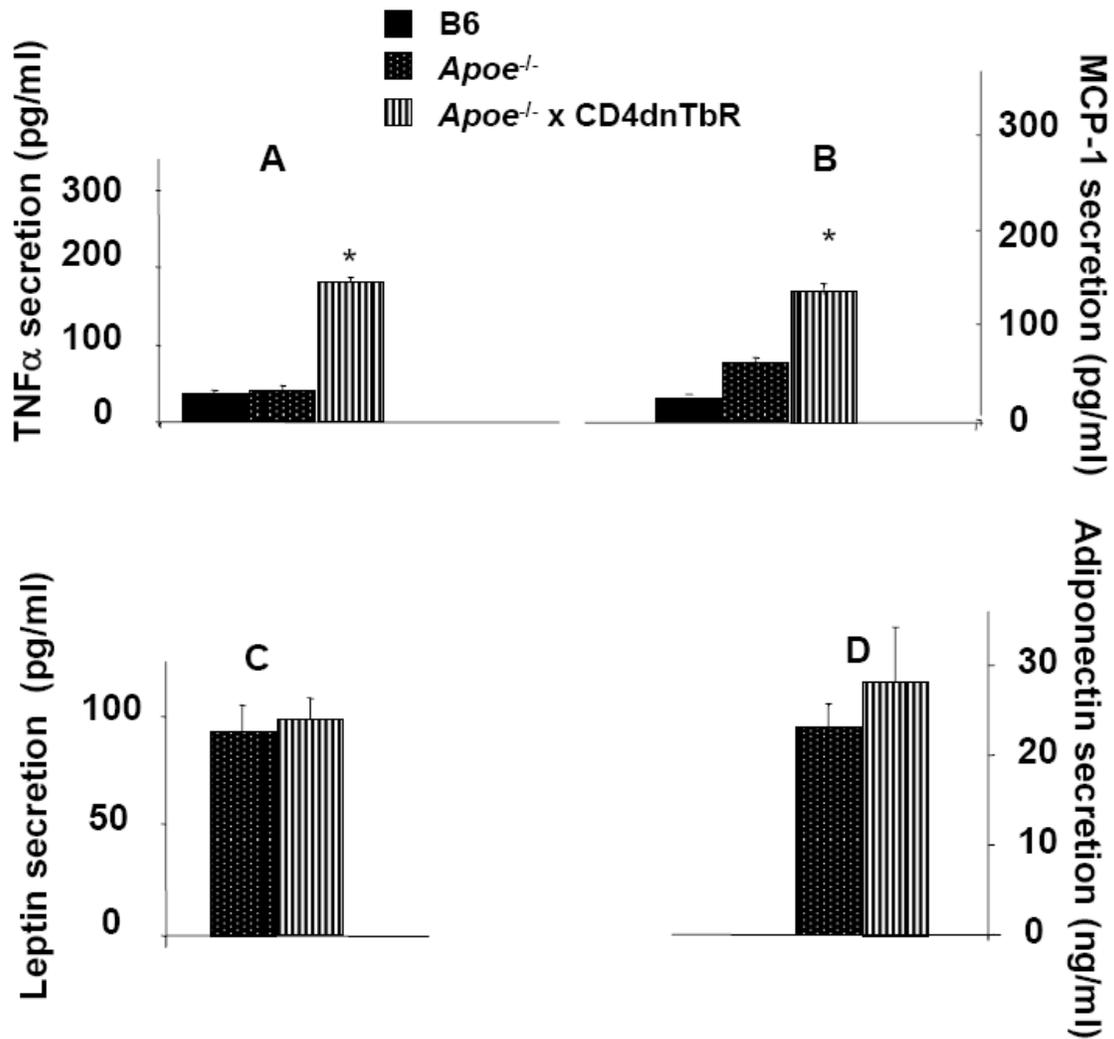
Supplemental Figure I. **TNFα (A), MCP-1 (B), leptin (C) and adiponectin (D) secretion by gonadal adipose tissue.**

Note the higher TNFα and MCP-1 secretion by adipose tissue of *Apoe*^{-/-}x CD4dnTbR mice. The secretion of leptin and adiponectin by adipose tissue did not differ between strains. Results show concentration in medium after a 2h incubation; mean ± SEM (n=6).
*) p<0.05 *vs* B6.

Supplemental Figure II. Insulin signalling in adipose tissue.

Western blot analysis of phosphorylated proteins in the insulin signalling cascade in adipose tissue. *Apoe*^{-/-} x CD4dnTbR and *Apoe*^{-/-} mice were treated for 7 days with recombinant IL-6 or saline and received insulin intraperitoneally 10 min before sacrificing. pAKT, phospho-protein kinase B; and pERK1/2, phospho-extracellular signal-regulated kinase were analysed for comparison and normalized to GAPDH. 6 to 9 mice were analyzed per group. Results are shown as mean ± SEM and did not reveal significant differences between groups.