Blocking IL-6 trans-Signaling Prevents High-Fat Diet-Induced Adipose Tissue Macrophage Recruitment but Does Not Improve Insulin Resistance

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SUMMARY

Interleukin-6 (IL-6) plays a paradoxical role in inflammation and metabolism. The pro-inflammatory effects of IL-6 are mediated via IL-6 “trans-signaling,” a process where the soluble form of the IL-6 receptor (sIL-6R) binds IL-6 and activates signaling in inflammatory cells that express the gp130 but not the IL-6 receptor. Here we show that trans-signaling recruits macrophages into adipose tissue (ATM). Moreover, blocking trans-signaling with soluble gp130Fc protein prevents high-fat diet (HFD)-induced ATM accumulation, but does not improve insulin action. Importantly, however, blockade of IL-6 trans-signaling, unlike complete ablation of IL-6 signaling, does not exacerbate obesity-induced weight gain, liver steatosis, or insulin resistance. Our data identify the sIL-6R as a critical chemotactic signal for ATM recruitment and suggest that selectively blocking IL-6 trans-signaling may be a more favorable treatment option for inflammatory diseases, compared with current treatments that completely block the action of IL-6 and negatively impact upon metabolic homeostasis.

INTRODUCTION

The cytokine interleukin 6 (IL-6) is a therapeutic target for many pro-inflammatory diseases, and tocilizumab, a humanized IL-6 receptor inhibiting monoclonal antibody, is approved for clinical use in >100 countries for the treatment of rheumatoid arthritis (RA) (Nishimoto et al., 2005) and other pro-inflammatory diseases (Febbraio et al., 2010). Since the seminal discovery 20 years ago by Hotamisligil and colleagues that tumor necrosis factor alpha (TNF-α) was highly expressed within adipose tissue in several rodent models of obesity (Hotamisligil et al., 1993), inflammation has become a major mechanism linking obesity to type 2 diabetes (T2D). Given that IL-6 is pro-inflammatory, it is widely accepted that, like TNF-α, IL-6 negatively impacts on obesity-induced insulin resistance (Lazar, 2005). In some circumstances, administration of an IL-6-neutralizing antibody to genetic mouse models of obesity improves metabolic homeostasis (Cai et al., 2005; Sabio et al., 2008). However, evidence gathered over the last decade has challenged the concept that IL-6 has a negative impact on metabolic homeostasis. IL-6-deficient (IL-6−/−) mice develop mature onset obesity (Wallenius et al., 2002), glucose intolerance, insulin resistance, and increased liver inflammation in response to a high-fat diet (HFD) (Matthews et al., 2010). In addition, complete blockade of IL-6 signaling in hepatocytes (Wunderlich et al., 2010) or macrophages (Mauer et al., 2014), by tissue-specific deletion of the IL-6 receptor (IL-6R), produces a similar negative metabolic phenotype. It is important to note that IL-6 activates the fuel-sensing kinase AMPK, a known insulin sensitizer, and infusion of recombinant IL-6 into healthy humans improves insulin action during a hyperinsulinemic-euglycemic clamp (Carey et al., 2006). Consistent with these results, tocilizumab, while very effective in treating RA, results in weight gain and hyperlipidemia (Nishimoto et al., 2005).

The controversy regarding the role of IL-6 in metabolic homeostasis may be due to its complex signaling biology. The pro-inflammatory effects of IL-6 signaling appear to be mediated via so-called IL-6 “trans-signaling” (Kraakman et al., 2013; Rose-John et al., 2007). IL-6 trans-signaling is a process by which the IL-6R can be cleaved by the metalloproteinases ADAM10 (Matthews et al., 2003) and/or ADAM17 (Chalaris et al., 2010), resulting in a soluble form of the IL-6R (sIL-6R). IL-6 can bind to the sIL-6R, giving this circulating ligand/receptor complex the capacity to signal in cells that express the gp130, but not IL-6R, on
their plasma membrane. Importantly, specifically blocking IL-6 trans-signaling by genetic overexpression of or pharmacological treatment with soluble glycoprotein 130 protein (sgp130Fc) can prevent inflammation in both an air-pouch model of local inflammation (Rabe et al., 2008) and in atherosclerosis (Schuett et al., 2012). Notably, sgp130Fc does not block the activity of IL-6-mediated signaling via the membrane-bound IL-6R (Jostock et al., 2001). These findings led us to hypothesize that blocking IL-6 trans-signaling is a therapeutic strategy for preventing adipose tissue macrophage (ATM) accumulation and subsequent insulin resistance in obesity, since ablation of macrophages using genetic (Patsouris et al., 2008) or pharmacological (Feng et al., 2011) approaches leads to a reduction in local and systemic markers of inflammation and a concomitant improvement in insulin sensitivity in mice fed a HFD. Such a scenario could represent a major clinical breakthrough in the treatment of T2D, since sgp130Fc has recently entered human clinical trials for the treatment of auto-immune diseases. Here, we show that IL-6 trans-signaling is a major chemotactic signal in ATM recruitment. Moreover, blocking IL-6 trans-signaling with sgp130Fc can prevent HFD-induced ATM recruitment. While this alone is insufficient to improve HFD-induced insulin resistance, blocking IL-6 trans-signaling does not result in exacerbated weight gain, liver steatosis, or insulin resistance, as we previously observed with complete IL-6 blockade (Matthews et al., 2010).

RESULTS

The sIL-6R Is a Major Chemotactic Cytokine that Correlates with Type 2 Diabetes

Patients with T2D have elevated plasma sIL-6R (Kado et al., 1999), but the significance of this observation is unclear. To determine whether the sIL-6R plays a role in inflammation, insulin resistance, or T2D, we first studied three age-matched cohorts of patients: a lean group (Lean), obese patients with impaired glucose tolerance (Obese, IGT), and obese patients with type 2 diabetes (Obese, T2D). The patients’ characteristics, as well as confirmation of the categorization of Obese, IGT versus Obese, T2D status in the patients, are described in detail in Table S1. We obtained plasma and abdominal subcutaneous white adipose tissue (WAT) biopsy samples from these patients and analyzed them for markers of inflammation and components of IL-6 signaling. The mRNA expression of the macrophage marker CD68 was higher in the WAT of Obese, T2D compared with Lean (Figure 1A). Both the mRNA expression of ADAM17 in WAT (Figure 1A) and plasma sIL-6R concentration (Figure 1B) were higher in both obese groups compared with Lean, but the plasma concentration of sgp130 did not differ among groups (Figure 1B). To ascertain whether these observations were related, we performed correlation analyses. As expected, the mRNA expression of WAT CD68 positively correlated with both HOMA-IR (Figure 1C) and HbA1c (Figure 1D), indicating that ATMs are, indeed, a marker of insulin resistance and T2D. The concentration of sIL-6R in plasma also positively correlated with HOMA-IR, HbA1c, and body mass (Figures 1E–1G), indicating that the sIL-6R is also a disease marker. We further examined whether the sIL-6R was related to the mRNA expression of CD68 in WAT of these patients and, importantly, observed a significant correlation between these two measures (Figure 1H). These data suggested that the sIL-6R is a major chemotactic signal for ATM recruitment in human obesity. Given the tight association between ATM accumulation and sIL-6R in both humans, we next tested if IL-6 trans-signaling could directly induce bone marrow-derived macrophage chemotaxis in a cell migration assay system. To mimic trans-signaling, we used Hyper-IL-6, a fusion protein that mimics IL-6 trans-signaling (Fischer et al., 1997). As expected, monocyte chemoattractant protein-1 (MCP-1) (positive control) induced marked macrophage chemotaxis in a dose-dependent manner. Importantly, Hyper-IL-6-induced macrophage chemotaxis with efficacy similar to that of MCP-1, providing clear evidence that IL-6 trans-signaling is a potent chemotactic signal (Figure 1I). Moreover, Hyper-IL-6-induced macrophage recruitment was completely blocked in the presence of sgp130Fc (Figure 1J). Sgp130Fc did not affect MCP-1-induced chemotaxis, suggesting that the anti-chemotactic effects of sgp130Fc selectively block the chemotactic signaling mediated by the sIL-6R (Figure 1I). Taken together, these results demonstrate that IL-6 trans-signaling can induce macrophage and that ATM accumulation is a marker of metabolic disease.

Genetic Overexpression of sgp130Fc Increases Adipogenesis in Response to High-Fat Feeding

While IL-6−/− mice are protected from a variety of pro-inflammatory diseases, such as RA (Alonzi et al., 1998), hepatocellular carcinoma (Nagler et al., 2007), and colitis (Gay et al., 2006), they paradoxically display mature-onset obesity, hepatosteatosis, liver inflammation, and insulin resistance (Matthews et al., 2010; Wallenius et al., 2002). Sgp130Fc transgenic mice show high serum levels of sgp130Fc (Figure S1A) resulting in impaired IL-6 trans-signaling but intact, membrane-bound IL-6R signaling and protection from inflammation (Rabe et al., 2008). However, the metabolic phenotype of these mice is unknown. Sgp130Fc mice displayed normal body mass, fat mass and lean mass, glucose tolerance (Figures S1B and S1C), food intake, and energy expenditure (data not shown) compared with wild-type (WT) control mice. Next, we placed the mice on either a standard chow or HFD (42% of calories from fat) for a period of 12 weeks. As expected, the HFD increased both body mass and fat mass compared with chow-fed animals (Figures 2A and 2B; Figures S1D and S1E). Consistent with the phenotype of the IL-6−/− mice (Matthews et al., 2010; Wallenius et al., 2002), sgp130Fc mice fed a HFD displayed a greater body mass and fat mass compared with WT and a decrease in lean mass in response to the HFD (Figures 2A and 2B; Figures S1D and S1E). As expected, energy intake was higher in response to the HFD, but hyperphagia was not responsible for the higher body mass and fat mass in the sgp130Fc mice (Figure 2C). Rather, this phenotype was due to a decrease in energy expenditure as measured by both oxygen consumption (VO2) (Figure 2D) and the change in body weight in response to an overnight fast (Figure S1G). In addition, locomotor activity was decreased by both consumption of HFD and expression of sgp130Fc mice (Figure 2D). This is generally accepted that fewer and larger adipocytes are characteristic of metabolic dysregulation in obesity. Accordingly, we next examined the adipose tissue beds in detail. In line with our in vivo measures, epididymal fat pad mass was higher in the sgp130Fc mice irrespective of diet (Figure 2E), while serum leptin was higher in the sgp130Fc mice on the HFD (Figure S1H). Importantly, however, and in direct contrast
Figure 1. The Soluble IL-6 Receptor Correlates with White Adipose Tissue Macrophage Accumulation and Insulin Action in Obesity and Mediates Macrophage Migration

(A and B) The mRNA expression of the macrophage marker cluster of differentiation 68 (CD68) and the metalloproteinase ADAM17 (A) in white adipose tissue (WAT), and the plasma concentration of the soluble IL-6 receptor (sIL-6R) and the soluble gp130 (B) in lean (Lean); obese, impaired glucose tolerance (IGT); and type 2 diabetes (T2D) patients. *p < 0.05 denotes difference from lean (n = 6 per group). Data are expressed as mean ± SEM.

(C–H) Correlations between HOMA-IR and WAT CD68 mRNA (C), HbaA1c and WAT CD68 mRNA (D), HOMA-IR and sIL-6R (E), and HbaA1c and sIL-6R (F) body mass and sIL-6R (G) and WAT CD68 mRNA and sIL-6R (H) in all patients. Correlative analyses (Pearson) were performed using a two-tailed p value (n = 18).

(I) Bone marrow-derived macrophages from C57BL/6 mice were cultured, and their migration was measured in response to increasing doses of MCP-1, Hyper-IL-6 (the designer cytokine consisting of IL-6 and the sIL-6R joined by a peptide linker), and/or sgp130Fc. Three technical replicates performed in three independent experiments are shown. Data are expressed as mean ± SEM. * denotes p < 0.05 compared with non-treated control; † denotes p < 0.05 compared with Hyper-IL-6 (10 or 25 ng/ml).
Figure 2. Metabolic Phenotyping of sgp130Fc Mice

(A and B) Total body mass progression during 12-week dietary period (A) and pre- and post-total body mass, fat mass, and lean mass (B), n = 33–52 animals. (C and D) Average daily food (energy) (C) and oxygen (VO₂) consumption (D). In (D), the shaded area denotes dark cycle and the solid line denotes a.m. n = 5–11 cages per group (C); n = 5–6 cages per group (D).

(E and F) Epididymal fat pad mass (E) and adipocyte area and number (F), n = 24–39 (E); n = 14–27 (F).

(G and H) Histogram of adipocyte cross-section area averaged from 20 x magnified fields per section (G) and the mRNA expression of CCAAT/enhancer-binding protein beta (C/EBPβ), fatty acid-binding protein 4 (FABP4), vascular endothelial factor alpha (VEGFα), cluster of differentiation 31 (CD31), and hypoxia-inducible factor alpha (HIF1α) (H) in sgp130Fc and WT mice fed a chow diet or HFD for 12 weeks. * denotes main effect (p < 0.05) for diet. † denotes main effect (p < 0.05) for genotype. Data are expressed as mean ± SEM. n = 14–27 (G); n = 5–19 (H).
with previous observation in IL-6−/− mice (Matthews et al., 2010), the increase in adiposity was not due to adipocyte hypertrophy, since mean adipocyte area was lower in the sgp130Fc compared with WT mice (Figure 2F). Although we did not detect a significant increase in total adipocyte number per cross-sectional image (Figure 2F), when we stratified the cross-sectional area, we observed many smaller, but fewer larger, adipocytes in the sgp130Fc compared with WT mice (Figure 2G). Together, these data indicate that the increase in adipose tissue mass in the sgp130Fc mice is due to increased adipogenesis rather than adipocyte hypertrophy, as previously seen in IL-6−/− mice (Matthews et al., 2010). To determine a possible mechanism for the current observation, we first examined serum adiponectin, a peptide hormone known to induce adipose tissue expansion in a manner similar to our observed phenotype (Kim et al., 2007), but this was not different when comparing mice (Figure S1H). We next examined the mRNA expression of several genes involved in adipogenesis and differentiation. CCAAT1/enhancer-binding protein beta (C/EBPβ) is known to regulate adipogenesis and was higher in sgp130Fc compared with WT mice, as was the mRNA expression of fatty acid-binding protein 4 (FABP4), known to regulate adipocyte differentiation (Figure 2H). In addition, since angiogenesis modulates adipogenesis (Cao, 2007), we also examined the mRNA expression of vascular endothelial factor alpha (VEGFα), cluster of differentiation 31 (CD31), and hypoxia-inducible factor alpha (HIF1α), all implicated in the process of angiogenesis. The mRNA expression of all three genes was higher in the sgp130Fc compared with WT mice (Figure 2H). It was clear from these data that the etiology of the increase in adiposity when comparing the IL-6−/− with the sgp130Fc mice was different, as the former displayed adipocyte hypertrophy (Matthews et al., 2010) while in this study the sgp130Fc mice displayed an increase in adiposity consistent with adipogenesis. Accordingly, we next performed a detailed comparison of adipose tissue gene expression between the two strains of mice. First, we retrospectively examined the mRNA expression of the genes presented in Figure 2H in adipose tissue samples from the IL-6−/− mice. In contrast with the observations in the sgp130Fc mice, deletion of IL-6 had no effect on the expression of any of these genes (Figure 3A). Next we performed gene array analyses on adipose tissue samples from WT, IL-6−/− mice and sgp130Fc mice. The pattern of gene expression was markedly different when comparing the IL-6−/− with the sgp130Fc mice (Figures 3B–3D). Importantly, gene ontology analyses revealed that genes involved in angiogenesis produced the second-highest enrichment score (Figure 3B). Taken together, these data demonstrate that genetic overexpression of sgp130Fc, but not deletion of IL-6, results in increased adipogenesis leading to increased fat pad mass due to an increased number of smaller adipocytes in response to a HFD.

Genetic Overexpression of sgp130Fc Does Not Exacerbate Liver Steatosis or Glucose Intolerance

Complete ablation of IL-6 signaling in the liver either via genetic deletion of IL-6 (Matthews et al., 2010) or via liver-specific deletion of the IL-6 receptor (Wunderlich et al., 2010) results in
hyperlipidemia, increased hepatosteatosis, and liver inflammation and damage when mice are fed a HFD. Accordingly, we next examined whether this was the case when only IL-6 trans-signaling is blocked. Selective blockade of IL-6 trans-signaling did not exacerbate HFD-induced increases in circulating non-esterified fatty acid concentration (data not shown), hepatosteatosis, liver inflammation, or liver function (Figures S2 A–S2F). Furthermore, and in contrast with IL-6−/−/C0/C0 mice (Matthews et al., 2010), sgp130Fc mice did not display glucose intolerance relative to WT mice (Figure S2 G).

**Genetic Overexpression of sgp130Fc Ameliorates Adipose Tissue Macrophage Accumulation in Response to High-Fat Feeding**

As we demonstrated in Figure 1, the sIL-6R appeared to be a major chemotactic signal in humans, and IL-6 trans-signaling-induced chemotaxis was blocked by treatment with sgp130Fc in vitro. Accordingly, we next examined whether this link persisted in the sgp130Fc mice. Consistent with our data in humans, the HFD induced an increase in serum sIL-6R (Figure S3A). The mRNA expression of the IL-6R decreased, while ADAM10 increased in WAT (Figure S3 B). The systemic overexpression of sgp130Fc resulted in a marked accumulation of sgp130Fc protein in the WAT (Figure 4 A), but this did not affect serum sIL-6R concentration, nor the mRNA expression of either the IL-6R or ADAM10 in WAT (Figure S3 B). Strikingly, and consistent with our hypothesis, overexpression of sgp130Fc ameliorated the ATM accumulation. While the HFD induced marked increases in the mRNA abundance of *Emr1*, the gene encoding F4/80, and *Itgax*, the gene encoding CD11c, these increases were abrogated in the sgp130Fc mice (Figure 4 B). We next examined the protein expression of F4/80 within WAT by two independent methods, immunohistochemistry and flow cytometry. While the HFD increased the appearance of crown-like structures ~4-fold in the WAT of WT mice, this increase was abrogated in sgp130Fc mice (Figures 4C and 4D). In addition, the percentage of cells positively stained for both F4/80 and CD11c in the WAT of mice fed the HFD was markedly lower in the sgp130Fc compared with WT mice (Figures 4 E and 4F). Fibrosis is associated with the appearance of crown-like structures and inflammation in WAT and systemic insulin resistance (Halberg et al., 2009). Accordingly, we also measured collagen content using picro-sirius red staining in WAT. Collagen staining
in WAT was increased in WT but not in sgp130Fc mice in response to HFD (Figures S3D and S3E). These data suggest that transgenic expression of sgp130Fc can prevent macrophage accumulation and fibrosis in WAT of mice fed a HFD. It is well known that macrophages release pro-inflammatory cytokines that can signal in neighboring adipocytes to activate serine threonine kinases such as inhibitor of kappa B kinase (IKK) and c-Jun NH2-terminal kinase (JNK) to impair insulin action (Wellen and Hotamisligil, 2003). Accordingly, we next examined these pathways. We first measured MCP-1 and observed an increase in the mRNA expression of this gene in WAT of mice fed a HFD but, critically, no difference when comparing genotypes (Figure 4G), indicating that the expression of MCP-1 was not responsible for the lack of ATM in sgp130Fc mice. As expected, the mRNA expression of TNF-α was increased in mice fed HFD, but—somewhat unexpectedly—this increase was not decreased in sgp130Fc mice (Figure 4G), despite the fact that the increase in HFD-induced ATM accumulation was prevented. Although the HFD led to mild increases in the phosphorylation of IKK and JNK (p = 0.057), this was unaffected by genotype (Figures S3F and S3G). Somewhat unexpectedly, the mRNA expression of IL-6, IL-1β, and Nod-like receptor protein 3 (NLRP3) in WAT were all elevated in the sgp130Fc mice (Figure 4G), despite the near absence of ATM. These increases appeared not to affect circulating cytokine expression, as we observed neither a diet or genotype effect on circulating IL-6, IL-1β, IL-10, or TNF-α measured in serum by multiplex ELISA (data not shown).

A “macrophage-centric” view of obesity-associated inflammation has reigned for several years, despite the fact that many other immune cell types play an important role in obesity-associated WAT inflammation (Mathis, 2013). As this is an important consideration, we next examined the immune cell milieu in both the WAT and circulation. As previously shown (Feuerer et al., 2009; Wu et al., 2011), we observed decreases in the percentage of leukocytes staining positive for eosinophils (Figures 5A and 5E). In addition, as previously demonstrated (Nishimura et al., 2009), the HFD increased the percentage of lymphocytes stained positively for CD8, but not CD4, T cells (Figures 5B and 5E). It has been suggested that a HFD increases both neutrophils and B cells within WAT, the former of which releases the enzyme elastase to induce insulin resistance (Talukdar et al., 2012). In contrast with these findings, we observed a HFD-induced decrease in both the percentage of leukocytes staining positively for neutrophils (Figures 5A and 5E) and B cells (Figures 5C and 5E) and a similar decrease in the mRNA expression of ELANE, the gene encoding elastase (Figure S3H). Importantly, however, unlike ATM, the overexpression of sgp130Fc had no effect on any of these measures in WAT of mice irrespective of the diet (Figures 5A–5C and 5E; Figure S3H). Regulatory T cells (Tregs) have emerged as important negative regulators of WAT inflammation and metabolic homeostasis (Cipolletta et al., 2012; Feuerer et al., 2009). Accordingly, we next examined the accumulation of Tregs in the adipose tissue of the mice. Within the time frame of our experiment, we did not observe a HFD-induced decrease in Tregs in WT mice, but did so in sgp130Fc mice (Figures 5D and 5E). In addition, we observed a decrease in Foxp3 mRNA in WAT of sgp130Fc mice (Figure S3H). We next examined a set of immune cells in the blood of the mice (Figures S4A–S4C). Although not statistically significant, overexpression of sgp130Fc tended to increase the percentage of macrophages in the blood (Figure S4A), a result consistent with the blocking of migration of macrophages from the blood to the WAT. The percentages of monocytes, natural killer T cells, and B cells as well as hemoglobin, hematocrit, and red blood cell numbers were affected by neither diet nor genotype (Figures S4A–S4C). The HFD decreased the percentage of CD8 cells (Figure S4A) and increased the platelet count (Figure S4B) irrespective of genotype. In general terms, the immune status of the sgp130Fc mice was normal.

**sgp130Fc Drug Therapy Regresses High-Fat Diet-Induced ATM Accumulation but Does Not Affect Insulin Resistance**

Our data in the sgp130Fc mice provided a strong rationale for testing whether sgp130Fc could be used as a drug therapy to treat ATM accumulation and whether this influenced insulin resistance. We deemed the transgenic mouse a less than ideal model to test the efficacy of sgp130Fc therapy. Since the transgene was expressed shortly after birth, the accumulation of ATM would have been prevented from the onset of obesity, which does not occur in human treatment interventions for metabolic disease. Accordingly, we next performed a drug intervention trial in C57BL/6 mice (protocol summarized in Figure S5A). Briefly, four groups of mice were studied from 8 weeks of age. Three groups were administered a HFD, while the fourth received a regular chow diet. After 12 weeks of diet, the chow-fed animals (Chow Control) and one HFD group (HFD Basal) were killed. The remaining groups were treated with either 0.5 mg/kg sgp130Fc protein (HFD Sgp130Fc) or sham control (HFD Saline) twice weekly for a further 6 weeks. The dosing regime was based on pharmacokinetics of sgp130 (Figure 6A). Unlike the transgenic model reported above, while the HFD increased total body mass and fat mass, no differences were observed between the HFD saline, basal, or sgp130Fc in these measures (Figure 6B) either during the dietary intervention or during the drug treatment (Figures S5B and S5C). Lean mass was not different across groups (Figure 6B; Figure S5D). In contrast to the study in sgp130Fc transgenic mice, we observed no differences in epididymal fat pad mass (Figure 6C) or adipocyte number (Figure 6E; Figure S5E) across groups. We also examined a number of inflammatory genes in the adipose tissue and liver from the mice and circulating cytokines. In general, the HFD increased or tended to increase the expression of all measured genes in the adipose tissue, but notably, sgp130Fc treatment reduced or tended to reduce HFD-induced increases in these genes, indicating that sgp130Fc treatment was indeed anti-inflammatory in adipose tissue (Figure S5F). We observed no such effects in liver or circulating cytokines (Figures S6A and S6B). Consistent with our study in the sgp130Fc transgenic mice, the HFD increased serum sIL-6R concentration (Figure S5G), and importantly, we observed a marked regression of ATM in response to 6 weeks of sgp130Fc treatment as measured by the mRNA expression of *Emr1* and *Itgax* (Figure 6D) and the appearance of F4/80 measured by immunohistochemical detection in adipose tissue cross-sections (Figures 6E and 6F). Given the efficacy in regression of ATM without affecting adipose tissue morphology with sgp130Fc treatment, we next performed hyperinsulinemic-euglycemic clamps in the mice. While the glucose infusion rate
Figure 5. sgp130 Overexpression Has Minimal Effects on High-Fat Diet-Induced Modification of Immune Cells in White Adipose Tissue

(A–D) Stromal vascular cells were isolated from epididymal white adipose tissue (WAT) and assessed by flow cytometry. Levels of eosinophils and neutrophils as a percentage of total leukocytes (A), levels of cytotoxic CD8\(^+\) and CD4\(^+\) T cells as a percentage of total lymphocytes (B), levels of B cells as a percentage of total leukocytes (C), and levels of regulatory T cells (Tregs) as a percentage of CD4\(^+\) T cells (D) are shown. All cells were first gated of CD45\(^+\) cells. Eosinophils were sorted as Siglec F\(^+\) and neutrophils as Gr1\(^{hi}\). T cells were sorted as TCR\(\beta\)^+, CD4\(^+\), or TCR\(\beta\)^+. CD8\(^+\) B cells were sorted as TCR\(\beta\)/CD0^+ or CD19\(^+\). Tregs were sorted as TCR\(\beta\)^+, CD4\(^+\), or FoxP3\(^+\).

(E) Quantification of all cell populations as % parent cell populations. * denotes main effect (p < 0.05) for diet; † denotes difference (p < 0.05) between WT and sgp130Fc on HFD.

Data are expressed as mean ± SEM. n = 6–9 (A–E).
(GIR) was reduced -2-fold by the HFD, the sgp130Fc treatment had no effect in rescuing insulin sensitivity despite the marked ATM regression (Figure 6G; Figures S5H, S5I, and S6C–S6E). Moreover, this pattern was observed for both glucose disposal rate (GDR; Figure 6G) and the percent suppression of hepatic glucose production (HGP) (G) and the rates of 2-deoxyglucose uptake (Rg') in visceral white, subcutaneous white, and brown (BAT) adipose tissue (H) during a hyperinsulinemic-euglycemic clamp in C57BL/6 mice fed a chow diet or HFD during the dietary and during the drug intervention period, which involved twice weekly injections of sgp130Fc (0.5 mg/kg) or saline. n = 9–11. (G and H) The glucose infusion rate (GIR), glucose disposal rate (GDR), and percent suppression of hepatic glucose production (HGP) (G) and the rates of 2-deoxyglucose uptake (Rg') in visceral white, subcutaneous white, and brown (BAT) adipose tissue (H) during a hyperinsulinemic-euglycemic clamp in C57BL/6 mice fed a chow diet or HFD during the entire intervention period. * denotes difference (p < 0.05) from chow control. † denotes difference (p < 0.05) from HFD saline. n = 10–13. Data expressed as mean ± SEM.

Prevention of ATM Accumulation in sgp130Fc Transgenic Mice Does Not Improve Insulin Sensitivity

The results from our drug intervention study clearly demonstrated that more than a 2-fold regression in ATM after the onset of obesity had no effect on HFD-induced insulin resistance or glucose intolerance. However, it is possible that only prevention of ATM accumulation, rather than regression of ATM once accumulated, may protect against insulin resistance. In addition, in the treatment study the reduction in macrophage accumulation with treatment was not as marked as was observed in the sgp130Fc mouse study. Accordingly, we next studied a subset of sgp130Fc mice, carefully matched to WT mice for total body mass and fat mass, to remove differences in these parameters as confounding measures (Figure 7A). Although this cohort of mice was matched for adiposity, the HFD-induced increase in can largely ameliorate HFD-induced ATM accumulation, but this does not affect metabolic homeostasis.
ATM, as measured by the mRNA abundance of EmR1 and Itgax (Figure 7B), and the appearance of crown-like structures in adipose tissue cross-sections (Figure 7C) was prevented in the sgp130Fc mice. Despite this prevention, the HFD-induced intolerance (Figure 7D) was not improved. Moreover, GIR, GDR, and HGP (Figure 7E) during a hyperinsulinemic-euglycemic clamp were not different when comparing the sgp130Fc with WT mice. In addition, the HFD-induced decrease in WAT and BAT glucose uptake was not improved in sgp130Fc mice (Figure 7F), despite the complete prevention of ATM accumulation.

**DISCUSSION**

There are several important findings in our study. These include the metabolic phenotype of the sgp130Fc mice, the identification of the sIL-6R as a chemotactic signal in regulating ATM accumulation, and the observation that pharmacological therapies that target ATM accumulation appear ineffective in protection against insulin resistance.

We previously showed that very high concentrations of sgp130Fc, in the presence of murine sIL-6R, can block classic IL-6 signaling (Garbers et al., 2011). However, in this previous study, mice were also injected with IL-6 and sgp130Fc at doses comparable to the present study. In this circumstance, IL-6 classical signaling through membrane-bound IL-6R was intact, as proven by the conserved phosphorylation of STAT3 in hepatocytes, indicating that sgp130Fc in vivo only blocks IL-6 trans-signaling and not classical IL-6 signaling. This notion is strengthened by the comparison between IL-6−/− and sgp130Fc mice. Apart from the reduced ATM observed in the WAT of the sgp130Fc mice, they displayed no hallmarks of adverse metabolic homeostasis, in direct contrast with IL-6−/− mice that have liver inflammation and insulin resistance (Matthews et al., 2010). The differences when comparing the sgp130Fc with the IL-6−/− mice have major ramifications for the development of therapeutics to treat inflammatory disease. While tocilizumab is a very effective treatment for RA (Nishimoto et al., 2005; Smolen et al., 2008), weight gain, hypercholesterolemia, hyperlipidemia, and bacterial infection are noted side effects (Febbraio et al., 2010). In contrast, in the current study, selective blockade of IL-6 trans-signaling prevented ATM accumulation, but critically did not result in these adverse metabolic side effects. In contrast with the IL-6−/− mice (Matthews et al., 2010),
sgp130Fc mice displayed an increase in smaller adipocytes and a corresponding decrease in larger adipocytes, which is a hallmark of adipogenesis and improved metabolic health (Figures 3 and 4). Microarray analyses revealed a very different pattern of adipose tissue gene expression with gene ontology analyses, showing that angiogenesis produced the second-highest enrichment score (Figure 3B). It should also be noted that the sgp130Fc mice display reduced locomotor activity on the HFD, which may also contribute to the slightly greater adiposity (Figure S1F), whereas the IL-6−/− mice did not show any such differences (Matthews et al., 2010). In addition, unlike IL-6−/− mice, which display hepatosteatosis and liver inflammation even when fed a chow diet (Matthews et al., 2010), the sgp130Fc mice do not display hepatosteatosis and liver inflammation even when fed a chow diet or HFD. Given these results, we suggest that sgp130Fc mice may present a viable therapeutic treatment option for inflammatory diseases where IL-6 blockade is effective, because the unwanted metabolic side effects of complete ablation of IL-6 signaling may be prevented. This is particularly important since patients treated for inflammatory diseases must be medicated for several years, as current medications do not cure the disease. Even in diseases such as RA, where initial weight gain may not pose a problem, weight gain over several years inevitably leads to adverse changes in metabolic homeostasis. A drug that will have efficacy but not result in weight gain would be particularly advantageous for such diseases.

MCP-1, primarily responsible for monocyte/macrophage chemotaxis in inflammatory diseases and atherosclerosis (Charo et al., 1994), is also thought to mediate monocyte/macrophage migration into WAT in obesity (Kamei et al., 2006; Kanda et al., 2006). Accordingly, MCP-1 inhibitors have been the subject of much interest, resulting in the development of mNOX-E36, a so-called Spiegelmer that binds MCP-1 with high affinity and neutralizes its action (Kulkarni et al., 2007). While mNOX-E36 has shown efficacy in mouse models of lupus nephritis (Kulkarni et al., 2007), liver fibrosis (Ehling et al., 2014), and diabetic kidney disease (Darisipudi et al., 2011), there are no reports of efficacy using mNOX-E36 in preventing ATM accumulation. Based on previous findings in MCP-1-deficient mice (Inouye et al., 2007; Kirk et al., 2008) and our current findings, this is not surprising. In the current study, we identify IL-6 trans-signaling as a major mediator of ATM accumulation. Importantly, Hyper-IL-6 induced marked macrophage migration in a dose-dependent manner, which was completely blocked in the presence of sgp130Fc. These data, in conjunction with the reduction of ATM accumulation in sgp130Fc mice, demonstrate that IL-6 trans-signaling is directly responsible for mediating ATM recruitment. Thus, together with our in vivo data, we have identified IL-6 trans-signaling as a major mechanism of ATM accumulation.

Despite the prevailing dogma that HFD-induced ATM accumulation contributes to insulin resistance, our data clearly demonstrate that prevention of ATM accumulation both genetically and pharmacologically appears not to be a viable strategy for the treatment of insulin resistance and/or T2D. While these results appear at odds with previous studies that have adopted different strategies to ablate macrophages, there are several important differences between our study and those performed previously (Feng et al., 2011; Patsouris et al., 2008). First and foremost, previous studies used strategies to completely ablate either all (Feng et al., 2011) or selectively “M1-like” (Patsouris et al., 2008) macrophages. Our study employed a drug intervention strategy to prevent ATM accumulation, rather than ablation of macrophages. From a therapeutic point of view, ablation of macrophages to treat metabolic disease is not a viable strategy, as it would severely compromise immunity. Hence, our data are of major significance for the development of drugs to treat T2D. Based on our data, it is likely that in previous studies where macrophages have been ablated (Feng et al., 2011; Patsouris et al., 2008), improved insulin action was mediated by effects of macrophage independent of the adipose tissue.

As outlined above, our pre-clinical data suggest that developing drugs that target ATM chemotaxis is an ineffective therapeutic strategy to treat insulin resistance and T2D. There are, however, some important considerations to these findings. First, although macrophages have been recognized for some time as being a hallmark of adipose tissue inflammation (Weisberg et al., 2003; Xu et al., 2003), it is now acknowledged that many other cells of the innate and adaptive immune system are also an important part of the immunological milieu of adipose tissue in obesity (Mathis, 2013). Thus, it is the balance of pro- and anti-inflammatory immune cells that is important for the regulation of obesity-induced adipose tissue inflammation and insulin resistance (Mathis, 2013). Therefore, one possibility is that marked reduction of all adipose tissue macrophages, as in our study, is ineffective, as metabolic parameters may be determined by the balance of M1 to M2 macrophages. Furthermore, while sgp130Fc was strikingly effective in preventing ATM accumulation, it had little effect on other immune cell changes seen with HFD-induced weight gain (Figure 5). Our data highlight the fact that adipose tissue inflammation is far more complex than simply a function of ATM accumulation. Furthermore, it is important to consider that the results from our intervention studies in mice may not be readily transferred to human obesity. This is an especially important point, as we observed in our patient group convincing correlations between insulin resistance (as measured by the HOMA-IR), sIL-6R plasma concentrations, and ATM accumulation (as measured by CD68 transcripts). Therefore, it remains possible that blocking IL-6 trans-signaling in humans may have clinical utility that goes beyond the prevention of ATM accumulation. This is a particularly important consideration because sgp130Fc Phase I clinical trials have been completed, with Phase II trials about to commence.

In conclusion, we demonstrate that IL-6 trans-signaling is a major chemotactic signal in ATM recruitment. Although ATM accumulation is blocked by sgp130Fc treatment, it does not improve insulin resistance. However, given the fact that sgp130Fc mice do not present with a negative metabolic phenotype, while nonetheless possessing anti-inflammatory properties, our data suggest that sgp130Fc treatment may be a more favorable treatment option for inflammatory diseases such as RA compared with current treatments that negatively impact upon metabolic homeostasis.

**EXPERIMENTAL PROCEDURES**

**Mouse Models**

Mice that overexpress human sgp130Fc were generated as previously described (Rabe et al., 2003). Heterozygous animals were bred together and litters were genotyped. Animals negative for the transgene were designated WT, and animals expressing the transgene were bred for ELISA analysis, as
genotyping cannot distinguish between homozygous and heterozygous mice. Mice exhibiting a human sgp130Fc circulating concentration of >10 μg/ml were considered homozygous, while animals testing positive, but below that threshold, were considered heterozygous. Littermate WT and heterozygous mice were born in parallel, and their litters were used for the study. Sgp130Fc transgenic and C57BL/6 mice were bred and housed at the Alfred Medical Research and Education Precinct Animal Centre (AMREP PAC) in a pathogen-free facility under controlled environmental conditions and exposed to a 12:12 hr light:dark cycle. IL-6−/− mice were generated as previously described (Matthews et al., 2010). Animal experiments were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee and conducted in accordance with the National Health and Medical Research Council (NHMRC) of Australia Guidelines for Animal Experimentation. All experiments commenced when mice were 6–8 weeks of age. Mice were fed either a chow diet (5% of total energy from fat) or a HFD (43% of total energy from fat) for 12–18 weeks depending on the experiment. Animals were given their prescribed diet and water ad libitum.

Metabolic Measurements

Plasma Measurements

ELISAs were performed for the soluble IL-6 receptor (Duoset mouse IL-6 sR ELISA Kit, DY1830), total adiponectin (Quantikine Mouse Adiponectin/Acrp30, MRP300), and leptin (Quantikine Mouse/Rat Leptin, MOB00) (all from R&D Systems) according to the manufacturer’s specifications. Insulin concentrations were measured using a Mouse Ultrasensitive Insulin ELISA kit (ALPCO) according to the manufacturer’s instructions.

EchoMRI

Mouse body composition (fat mass [FM] and lean body mass [LBM]) was measured weekly with a 4-in-1 EchoMRI body composition analyzer (Columbus Instruments) and standard laboratory scales.

GTT

Intraperitoneal glucose tolerance tests (pGTTs) (1 g/kg LBM) were performed in 5 hr-fasted mice as previously described (Matthews et al., 2010). Oral GTTs (2 g/kg LBM) were performed in 5 hr-fasted mice.

Hyperinsulinenic-Euglycemic Clamps

Hyperinsulenic-euglycemic clamp studies were performed in mice 4 days after catheterization. Details of the surgical procedures, insulin clamp, and the determination of plasma and tissue radioactivity are described elsewhere (Bruce et al., 2012).

Indirect Calorimetry

Oxygen consumption (VO2) and activity (beam breaks) were measured using a 12-chamber indirect calorimeter (Oxymax series) with an airflow of 0.6 l/min.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was used to detect main effects for diet (chow versus HFD) and genotype (WT versus sgp130Fc). Post-hoc analyses (Holm-Sidak) were performed when a significant interaction effect occurred. When not using a two-way ANOVA, one-way ANOVA or a Student’s t test (Holm-Sidak) were performed when a significant interaction effect occurred.

Results

Oxgen consumption (VO2) and activity (beam breaks) were measured using a 12-chamber indirect calorimeter (Oxymax series) with an airflow of 0.6 l/min.

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S.R.-J. is an inventor of patents owned by CONARIS Research Institute, which develops the sgp130Fc protein together with Ferring Pharmaceuticals, and he has stock ownership in CONARIS.

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