Interleukin-33 Induces Protective Effects in Adipose Tissue Inflammation During Obesity in Mice

Ashley M. Miller, Darren L. Asquith, Axel J. Hueber, Lesley A. Anderson, William M. Holmes, Andrew N. McKenzie, Damo Xu, Naveed Sattar, Iain B. McInnes, Foo Y. Liew

Rationale: Chronic low-grade inflammation involving adipose tissue likely contributes to the metabolic consequences of obesity. The cytokine interleukin (IL)-33 and its receptor ST2 are expressed in adipose tissue, but their role in adipose tissue inflammation during obesity is unclear.

Objective: To examine the functional role of IL-33 in adipose tissues and investigate the effects on adipose tissue inflammation and obesity in vivo.

Methods and Results: We demonstrate that treatment of adipose tissue cultures in vitro with IL-33 induced production of Th2 cytokines (IL-5, IL-13, IL-10) and reduced expression of adipogenic and metabolic genes. Administration of recombinant IL-33 to genetically obese diabetic (ob/ob) mice led to reduced adiposity, reduced fasting glucose and improved glucose and insulin tolerance. IL-33 also induced accumulation of Th2 cells in adipose tissue and polarization of adipose tissue macrophages toward an M2 alternatively activated phenotype (CD206+), a lineage associated with protection against obesity-related metabolic events. Furthermore, mice lacking endogenous ST2 fed high-fat diet had increased body weight and fat mass and impaired insulin secretion and glucose regulation compared to WT controls fed high-fat diet.

Conclusions: In conclusion, IL-33 may play a protective role in the development of adipose tissue inflammation during obesity. (Circ Res. 2010;107:650-658.)

Key Words: obesity ■ inflammation ■ diabetes mellitus ■ interleukins

Obesity is a consequence of many complex factors, including increased calorie consumption and reduced physical activity. Many studies also implicate chronic low-grade inflammation in the interplay between obesity and metabolic complications (reviewed elsewhere1). During obesity, white adipose tissue (WAT) is infiltrated by immune cells, such as macrophages and T cells2–4 which, along with the adipocytes themselves, secrete a variety of proinflammatory cytokines and chemokines (reviewed elsewhere5). Recent studies demonstrate that the infiltrating T cells display a Th1 pattern of activation with enhanced interferon-γ production,6 and that an imbalance may exist in obese adipose between dominant Th1 responses and reduced Treg or Th2 responses.7–9 This, in turn, leads to macrophage recruitment and a switch from a protective alternatively activated (M2) macrophage to a classically activated (M1) proinflammatory phenotype.7–10 Several observations indicate a role for the interleukin (IL)-1 family of cytokines in obesity. IL-1 inhibits adipocyte differentiation, stimulation of lipolysis and induces the development of an insulin resistant phenotype.11 Adipocytes also produce IL-1 receptor antagonist (IL-1Ra), and levels of IL-1Ra are strongly elevated in the serum of obese patients and highly correlated with insulin resistance.12 IL-18 concentrations are increased in serum of individuals with obesity and type 2 diabetes,13 but absence of IL-18 or IL-18R or the overexpression of IL-18 binding protein in genetically modified mice, leads to abnormalities characteristic of the metabolic syndrome.14 Furthermore, inhibition of IL-1–associated pathways in patients with diabetes improved glycemia and β-cell secretory function and reduced markers of systemic inflammation.15,16 These findings highlight the therapeutic potential of targeting IL-1 family of cytokines in type 2 diabetes.

IL-33 is a newly identified member of the IL-1 cytokine family that induces production of Th2 cytokines,17 by interacting with a heterodimeric receptor comprising ST2 and IL-1 receptor accessory protein (IL-1Racp).18 The ST2 gene encodes 2 protein isoforms: ST2L, a transmembrane receptor; and a secreted soluble (sST)2 form, which can serve as a decoy receptor for IL-33. Several lines of evidence suggest a role for the IL-33/ST2 pathway in cardiovascular biology.
Serum elevations of sST2 predict mortality and heart failure in patients with acute myocardial infarction.\textsuperscript{19,20} Furthermore, we have demonstrated that the IL-33/ST2 pathway has a protective role in the progression of atherosclerosis via the induction of Th2 cytokines and anti-oxLDL antibodies.\textsuperscript{21} More recently, IL-33 and ST2 were shown to be expressed in human adipose tissue.\textsuperscript{22}

In this study, we show that IL-33 has clear protective effects against obesity and type 2 diabetes. IL-33 induced Th2 cytokines in WAT, and the polarization of WAT macrophages toward an M2 alternatively activated phenotype with reduced adipose mass and fasting glucose. Taken together, these results demonstrate a novel protective role for IL-33/ST2 during obesity and suggest that induction of Th2 cytokines and alternative macrophage polarization by manipulating IL-33 expression may be a useful therapeutic strategy for treating or preventing type 2 diabetes in obese patients.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animals

Male obese (ob/ob) mice (B6c-Lep ob/J) were purchased from The Jackson Laboratory at 5 weeks old and allowed to acclimatize for 1 week. These mice were fed normal diet and at 6 weeks randomly grouped and injected intraperitoneally 3 times per week for 3 weeks with control PBS or recombinant IL-33 (Biolegend, 2 µg/injection). Male BALB/c wild-type (WT) mice and BALB/c ST2\textsuperscript{−/−} mice were bred in-house in a pathogen-free facility and fed either normal diet or a high fat diet (HFD) (0.15% cholesterol and 21% lard, Special Diet Services) ad libitum from 6 weeks of age. All animal experiments complied with United Kingdom Home Office guidelines.

Statistical Analysis

All data are means±SEM. Statistics were performed using unpaired Student’s t tests or ANOVA with GraphPad Prism Software.

Results

IL-33 Induces Th2 Cytokines and Chemokines in WAT Cultures In Vitro

To establish the cellular localization of ST2 expression in WAT, we carried out flow cytometric double immunostaining of collagenase digested murine epididymal stromal vascular fraction (SVF) cells. Both F4/80\textsuperscript{+} macrophages and F4/80\textsuperscript{−} cells expressed ST2 (Figure 1A), indicating the potential for multiple cell types in WAT to respond to IL-33. We then examined the effect of IL-33 treatment on cytokine and chemokine secretion from murine SVF cells cultured from epididymal WAT (eWAT) of WT and ST2\textsuperscript{−/−} mice in vitro. Addition of IL-33 to SVF cultures from WT but not ST2\textsuperscript{−/−} mice induced secretion of the cytokines IL-5, IL-6, IL-13 (Figure 1B), and chemokines MCP-1, MIP-1\textalpha, and KC (Figure 1C). Macrophage depletion markedly reduced, but did not abolish, IL-33–induced expression of IL-6, MCP-1 and KC (Figure 1D). However, IL-33–induced expression of IL-13 and MIP-1\textalpha were completely abolished by macrophage depletion. In contrast, IL-5 expression was unaffected by macrophage depletion. Intracellular staining in IL-33–treated SVF cultures demonstrated IL-6 expression in both F4/80\textsuperscript{+} and F4/80\textsuperscript{−} cells (Figure 1E). These studies indicate that IL-33 can act on both macrophages and other F4/80\textsuperscript{−} cells in the SVF (eg, preadipocytes) to induce cytokine and chemokine production.

IL-33 Induces Changes in Lipid Storage and Expression of Metabolic Genes in WAT Cultures In Vitro

To further investigate the mechanisms of action of IL-33 in WAT, we examined the effect of IL-33 on adipocyte differentiation in vitro. Addition of IL-33 for 7 days during the differentiation process of murine SVF cells significantly inhibited lipid accumulation (oil red O staining) (Figure 2A), and this effect was absent in cells from ST2\textsuperscript{−/−} mice (Figure 2B). IL-33 treatment also reduced the mRNA expression of C/EBP\textalpha (CAAT enhancer–binding protein–\alpha), SREBP-1\textepsilon (sterol regulatory element-binding protein 1c), liver X receptor (LXR)\alpha, LXR\beta, and PPAR\gamma, all associated with lipid metabolism and adipogenesis (Figure 2C). Furthermore, we used a mouse proteome array to simultaneously examine the expression of 38 obesity-related proteins in the supernatants of IL-33–treated (100 ng/mL) SVF cultured cells (Figure 2D and 2E). The expression of molecules shown to be protective against obesity, such as FGF-acidic, FGF-21, IGFBP-1, IL-10, IL-11, and RAGE,\textsuperscript{23–26} were >2-fold increased by IL-33, whereas expression of Resistin, which induces insulin resistance,\textsuperscript{27} was <2-fold decreased by IL-33. Together, these results indicate that IL-33 has potent effects on adipocyte differentiation and expression of metabolic genes and adipokine proteins in SVF cultures and support a role for IL-33 in the metabolic regulation of WAT.

Effect of Exogenous IL-33 on Metabolic Parameters In Vivo

To directly investigate the effect of IL-33 on obesity in vivo, we treated spontaneously obese (ob/ob) mice with PBS or recombinant IL-33. IL-33 did not affect the final body weight of ob/ob mice (Figure 3A; PBS-treated, 41.4±0.7 g versus IL-33–treated, 40.1±0.7 g) or food intake (Figure 3B), but eWAT weight was decreased by 9.9% (Figure 3C and 3D). Furthermore, IL-33–treated mice displayed a 13% decrease in body fat (AUClipid) and a 17% increase in fat-free mass.

<table>
<thead>
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<th>Non-standard Abbreviations and Acronyms</th>
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<tr>
<td>eWAT epididymal white adipose tissue</td>
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<tr>
<td>FFM fat-free mass</td>
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<tr>
<td>HFD high-fat diet</td>
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<tr>
<td>IL interleukin</td>
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<tr>
<td>LXR liver X receptor</td>
</tr>
<tr>
<td>PPAR peroxisome proliferator–activated receptor</td>
</tr>
<tr>
<td>retla resistin-like molecule/found in inflammatory zone</td>
</tr>
<tr>
<td>sST2 soluble ST2</td>
</tr>
<tr>
<td>SVF stromal vascular fraction</td>
</tr>
<tr>
<td>WAT white adipose tissue</td>
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(FFM) (AUCH2O), compared to PBS-treated controls (Figure 3E and 3F) by magnetic resonance spectroscopy (MRS) analysis. Mean adipocyte size in eWAT was reduced in IL-33–treated ob/ob mice (Figure 3G; PBS-treated, 1935/11006/110090/110090/110062 m2 versus IL-33–treated, 1335/11006/110068/110090/110062 m2; P<0.001). In addition, IL-33 treatment significantly reduced total serum cholesterol (4.0/11006/110060.1 versus 3.3/11006/110060.1 mmol/L), but did not affect high-density lipoprotein cholesterol (1.4/11006/110060.1 versus 1.1/11006/110060.1 mmol/L), or triglyceride (0.6/11006/110060.1 versus 0.7/11006/110060.1 mmol/L) concentrations (Figure 3H).

Weekly analysis of fasting blood glucose levels in IL-33–treated ob/ob mice demonstrated lower levels than the PBS-treated controls (Figure 3I). Surprisingly though, no significant difference in serum insulin levels was found (Figure 3J), suggesting that insulin secretion was not altered by IL-33, nor responsible for the lowered fasting glucose levels. IL-33–treated mice also showed significantly lower glucose levels after a glucose load (Figure 3K). To investigate whether altered insulin sensitivity explains the changes in glucose levels and tolerance we carried out an insulin tolerance test in the mice at week 7 and 9. Improved insulin sensitivity was found in IL-33–treated mice at week 7 but not at week 9 (Figure 3L), when perhaps some tolerance to the effects of IL-33 were evident.

**IL-33 Induces Accumulation of Th2 Cytokines in Serum and Th2 Cells in WAT**

We analyzed changes in circulating cytokines and adipokines following IL-33 treatment in serum of ob/ob mice. IL-33–

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**Figure 1. IL-33 induces Th2 cytokines and chemokines in both macrophages and adipocytes in WAT cultures.** Epididymal fat pads from 10 age-matched male BALB/c WT (filled bars) and BALB/c/ST2–/– (open bars) mice were pooled, dissected, and separated into mature adipocyte and SVF populations. A, Expression of ST2 by FACS in SVF cells from BALB/c mice stained with anti-F4/80 and either isotype control or anti-ST2 mAbs. SVF cells were then cultured for 7 days with or without IL-33 (10 to 100 ng/mL). Cytokines (B) and chemokines (C) in cell supernatants (all pg/mL) collected on day 7 were measured by Luminex assay. Data shown are means±SEM, n=4 to 5 independent experiments. *P<0.05, **P<0.01 (1-way ANOVA with Dunnett’s post test comparing all values to respective control column or Student’s unpaired t test). Cells with or without macrophages depleted (by F4/80+ cell sorting) were then cultured for 7 days with or without IL-33 (10 ng/mL), and cytokines and chemokines measured on day 7 were measured by Luminex assay (D) or intracellular cytokine staining (E) for IL-6 in day 7 SVF cells restimulated with PMA/Ionomycin/GolgiPlug. Numbers indicate mean percentage of CD45+ cells. Data are means±SEM; n=2 to 3 independent experiments. *P<0.05 (Student’s unpaired t test).
treated mice produced markedly more IL-5, IL-6, IL-10 and IL-13 (Figure 4A) than PBS-treated mice, but no significant change in TNFα, MCP-1, Resistin or tPAI-1 (data not shown). Furthermore, given the large increase in the chemokines MCP-1, MIP1α and KC released by IL-33–treated SVF cells we assessed inflammatory cell infiltration into eWAT from PBS versus IL-33–treated cultures we assessed inflammatory cell infiltration into eWAT from PBS versus IL-33–treated cultures. Consistent with this, eWAT from IL-33–treated ob/ob mice contained more IL-5–producing CD45⁺ macrophages but lower percent of TLR2⁺ macrophages. Taken together, these results suggest that IL-33 treatment increases the accumulation of macrophages in WAT and has substantial influence over their polarization toward an M2 phenotype.

Expression of Hepatic M2 Macrophage Genes in Mice Treated With IL-33

We examined the metabolic changes and gene expression in the liver of obese mice treated with IL-33. IL-33 treatment did not affect liver weight (3.6±0.2 g versus 3.3±0.2 g), serum levels of the aminotransferase enzymes AST (285.8±33.4 versus 279.6±33.6 U/L) or ALT (27.6±12.0 versus 28.2±6.4 U/L), or liver histology (data not shown). Additionally, expression of the phosphoenolpyruvate carboxykinase (PCK-1) gene, involved in gluconeogenesis in liver, was not altered by IL-33 treatment (mean expression, 1.22±0.08 versus 1.25±0.09), indicating that downregulation of PCK-1 is not likely responsible for the glucose lowering actions of IL-33. However, we did not investigate the expression of other genes involved in gluconeogenesis (eg, glucose-6-
Endogenous ST2 Regulates Body Weight, Adipose Tissue, and Glucose Homeostasis

To investigate the physiological significance of basal endogenous IL-33/ST2 signaling in metabolism we measured metabolic parameters, body weight/composition of WT and ST2−/− mice over 24 weeks of feeding either normal diet or HFD. On normal diet body weight of ST2−/− mice was similar to that of the WT mice (Figure 7A). Mice on the BALB/c background are generally resistant to diet-induced obesity; however, at 24 weeks of age body weight of ST2−/− mice fed HFD was increased by on average 11.5% compared to WT controls (Figure 7A; P < 0.05). Furthermore, weight of eWAT was increased in ST2−/− mice fed either HFD (2.0 g vs. 1.3 g, P < 0.01) or normal diet (0.62 g vs. 0.31 g, P < 0.0001) when compared to WT controls (Figure 7B). ST2−/− mice displayed a 17% increase in percentage body fat (AUClipid) and a 9% decrease in FFM (AUCH2O), compared to WT controls (Figure 7C and 7D) fed HFD at 24 weeks of age. However, serum total cholesterol, triglycerides, and high-density lipoprotein cholesterol were not different between WT and ST2−/− mice (data not shown).

To investigate whether the obese phenotype in ST2−/− mice fed HFD is mediated through effects on glucose homeostasis, we measured fasting serum insulin concentration was significantly lower at 24 weeks compared to WT controls (Figure 7E), but insulin responsiveness was not different (Figure 7H). These results suggest that ST2−/− mice are not insulin resistant but may have impaired pancreatic insulin production in response to glucose overload. Taken together, these data demonstrate that ST2 signaling appears to play some role in controlling body weight gain and the regulation of WAT and glucose homeostasis.
Discussion

In this study, we show for the first time that IL-33 treatment can modulate cytokine production and macrophage phenotype in WAT and liver and induce protective effects on body composition and glucose homeostasis.

Although IL-33 has already been shown to be expressed in adipose tissue, the cellular source of IL-33 in adipose tissue is still unclear. However, recent studies have shown endothelial cells express IL-33 as a vascular alarmin, and macrophages and fibroblasts in other tissues express IL-33. Therefore, multiple cell types in adipose tissue have the potential to produce IL-33. Treatment of ob/ob mice with IL-33 led to production of strong Th2 responses in WAT. Our findings agree with Kang et al, who have previously demonstrated that adipocytes can produce the Th2 cytokines IL-4 and IL-13, which led to alternative activation of macrophages. Furthermore, recent work suggests that during obesity a relatively constant pool of Th2 fat-associated T cells fail to limit an expanding Th1 pool of proinflammatory cells and that Th2 cells exert protective functions in insulin resistance. In addition, a recent study has documented the existence of a new population of cells, termed “natural helper cells,” in fat, which are ST2 positive, respond to IL-33 and produce large amounts of Th2 cytokines, though the role of these cells in obesity is not yet known. Therefore, IL-33 may exert its protective effects via an expansion of a Th2 pool of fat-associated T cells or natural helper cells and increased production of Th2 cytokines.

The significance of raised systemic levels of IL-6 in obesity is controversial. In humans, blood levels of IL-6 are elevated in obesity and correlate with adiposity and insulin resistance. However, our results show that IL-33 has beneficial effects on metabolism despite an increase in circulating IL-6. It has previously been shown in mice that absence of IL-6 led to maturity onset obesity though a central action. Furthermore, infusion of IL-6 in mice affected adipose insulin sensitivity but did not affect systemic glucose tolerance test or HOMA index. Therefore, the effects of IL-6 in the context of metabolism may be species-dependent. In addition, IL-33 induced expression of several chemokines in WAT and an increase in the proportion of infiltrating macrophages into WAT of obese mice. Whereas MCP-1 is reported to recruit M1 macrophages and have detrimental effects on obesity and metabolism, the IL-33–induced macrophages did not have a classic proinflammatory M1 phenotype. It is possible that M1 macrophages were recruited, which then switched to an M2 phenotype on encountering an IL-33–driven local Th2 environment in WAT. Consistent with this notion, IL-33 increased expression of M2 markers (CD206, Arginase 1, Ym1/ chitinase 3-like 3 and FIZZ/RELM, IL-10, and PPARδ) in adipose tissue and liver. Two recent studies document that PPARδ plays a key role in alternative activation of resident macrophages in WAT and liver. Furthermore, deletion of PPARδ in macrophages led to impaired glucose tolerance and exacerbation of insulin resistance. In liver, Odegaard et al demonstrated that PPARδ is critical for maintaining an M2 phenotype.
phenotype of Kupffer cells and this leads to hepatic dysfunction and insulin resistance. Alternatively activated M2 macrophages can regulate insulin resistance through the production of IL-10. This IL-10 can reverse the proinflammatory cytokine-induced activation of serine kinases, which phosphorylate IRS-1, thus inhibiting the action of insulin.

It is thus likely that one of the mechanisms by which IL-33 treatment induces protective effects is by promoting Th2 cytokine synthesis leading to the preferential differentiation of M2 macrophages in both adipose and liver.

Investigation of the metabolic phenotype of ST2−/− mice demonstrated that these mice have enhanced weight gain (11.5%) at 24 weeks of age on HFD and had more adipose tissue compared to WT controls, indicating a mature onset of obesity. However, the obese phenotype of the ST2−/− mice may not be as severe as that seen with other cytokine/cytokine receptor knock out mice, especially as the ST2 phenotype was only seen with high fat feeding. On normal diet, IL-18−/− mice were 18.5% heavier at 6 months, and both IL-1R1−/− and IL-6−/− mice displayed weight deviation from WT at 5 to 6 months of age with 20% weight difference by 9 months. This comparatively smaller effect in the ST2−/− mice may be

Figure 5. IL-33 induces accumulation of alternatively activated (M2) macrophages in WAT. Epididymal fat pads from ob/ob mice treated with PBS (open bars) or IL-33 (filled bars) were either formalin fixed and embedded in paraffin wax for immunohistochemistry or collagenase digested for SVF cell isolation and analyzed by FACS. A, Representative photomicrographs of F4/80+ macrophages (brown) in eWAT (original magnification, ×40). B, Representative FACS plots of CD45+ F4/80+ cells in eWAT with subsequent gating showing TLR2 marker expression of classically activated (M1) macrophages and the CD206 marker of alternatively activated (M2) macrophages. C, Quantification of the percentage of CD45+ F4/80+ macrophage cells and the percentage of those cells expressing either TLR2 or CD206 in eWAT. Data are means±SEM pooled from 2 independent experiments (n=8 to 10 mice/group). **P<0.01, ***P<0.001 (Student’s unpaired t test versus respective control).

Figure 6. IL-33 induces expression of M2 macrophage genes in liver. Ob/ob mice were treated with PBS (open bars) or IL-33 (filled bars) 3 times per week for 3 weeks and then euthanized, and livers were removed for subsequent quantitative RT-PCR analysis of total RNA extracted for expression of inducible nitric oxide synthase (NOS2) and arginase 1 (Arg1) (A), Chi313 and retinla (B), and PPARγ (C). Data are mean gene expression±SEM compared to TATA-binding protein (TBP) endogenous control pooled from 2 independent experiments (n=10 mice/group). **P<0.01, ***P<0.001 (Student’s unpaired t test).
a result of their BALB/c background, known to be more obesity resistant than the C57BL/6 background.

In summary, we report a novel function for IL-33 in obesity which is likely to be through the induction of the TH2 cytokines IL-5 and IL-13, which, in turn, can skew macrophages in adipose and liver from an M1 to M2 phenotype, and hence reduce the chronic inflammatory response associated with obesity. Many questions now arise from this work. IL-33 appears to have catabolic effects and experiments should be carried out to assess its effects on activity levels and energy expenditure, substrate utilization and metabolic rate. In addition, although we have clearly focused on the role of secreted IL-33, the nuclear expression of IL-33 in adipose may also indicate a separate function that is not dependent on its secretion. Further studies to address these points will allow a better understanding of the complex role of the role of IL-1 family cytokines in insulin resistance and obesity.

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Disclosures
None.

References
Chronic low-grade inflammation involving cytokines in adipose tissue

What Is Known?

- Chronic low-grade inflammation involving cytokines in adipose tissue may contribute to the metabolic consequences of obesity.

What New Information Does This Article Contribute?

- This is the first report that the new cytokine IL-33 has protective effects to adipose tissue inflammation and obesity.

The novel IL-1 family cytokine IL-33 and its receptor ST2 are expressed in adipose tissue but their role in adipose tissue inflammation during obesity is unclear. Here, we show that treatment of genetically obese diabetic (ob/ob) mice with recombinant IL-33 led to reduced adiposity and reduced fasting glucose, improved glucose and insulin tolerance, and induction of protective Th2 cytokines and M2 macrophages in adipose tissue. Furthermore, mice lacking endogenous ST2 have increased body weight and fat mass, impaired insulin secretion, and glucose regulation. This is the first report that the cytokine IL-33 has key protective metabolic effects and manipulation of IL-33 expression in adipose tissue may be a useful therapeutic strategy for treating or preventing type 2 diabetes in obese patients.
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METHODS

Glucose (GTT) and insulin tolerance tests (ITT)
Both GTT and ITT were carried out 1 day prior to cull of mice. Briefly, mice were fasted for 16 hrs (GTT) or 4hrs (ITT) injected intraperitoneally with filter-sterilized 2 g/kg glucose in 0.9% NaCl (GTT) or with insulin (0.75 U/kg, Sigma) in 25mM Hepes (ITT). A tail vein blood sample was taken before the injection and 30, 60, and 90 minutes after the injection for determination of blood glucose using the Accu-chek Compact test strips (Roche Diagnostics). GTT and ITT were performed on different sets of mice to prevent unnecessary repetitive stress that could interfere with measurements.

Stromal vascular fraction (SVF) cell isolation
Epididymal white adipose tissue (eWAT) from mice was excised and collagenase (C6885, Sigma) digested for 30 minutes at 37°C with shaking. Cells were filtered through a 100 μm filter and then spun at 300 g for 5 minutes to separate floating mature adipocytes from the SVF pellet. Cells were then cultured in 6-well plates (Corning) in high-glucose DMEM supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, 5 μg/ml insulin, 25 μg/ml sodium ascorbate, 10 mM Hepes, 100 units/ml penicillin and 100 μg/ml streptomycin for up to 8 days. After 24 hours any non-adherent cells (e.g. T cells) were removed. Analysis of the cellular composition of these cultures by FACS and immunocytochemistry at day 7 demonstrated no T cells (CD45+CD3+), no endothelial cells (CD31+), 3% macrophages (CD45+F4/80+), and 97% adipocyte-like cells (CD45-, vacuolated, ORO* lipid-droplet containing).

Analysis of cytokines and adipokines in cell supernatants
Supernatants were collected at various time points after addition of IL-33 (10-100ng/ml) and analyzed on a 20-plex mouse cytokine assay (Biosource) according to the manufacturer’s instructions, or by a Mouse Obesity Proteome Array (R&D Systems). For protein array supernatants were added to a membrane spotted with antibodies against 38 obesity-related proteins and following the addition of Streptavidin-HRP and chemiluminescence detections reagents the membrane was developed on X-ray film and quantitated by scanning on a transmission-mode scanner and analyzing the array image file using Quantity One® analysis software (Bio-Rad).

Flow cytometry and cell sorting
For flow cytometry and cell sorting by FACS Aria (BD Biosciences), SVF cells were resuspended in FACS buffer (PBS, 2% FCS, 2mm EDTA). Cells were incubated with Fc Block (BD Biosciences) for 15 minutes prior to staining with various conjugated antibodies or isotype controls: F4/80-APC (eBioscience), CD45-PE, CD3-FITC (both BD Biosciences), CD206-PE (Serotec), TLR2-PE (eBioscience), or ST2-FITC (MD Biosciences). For intracellular cytokine staining, cells were stimulated for 4 hours with PMA (50 ng/ml, Sigma) and Ionomycin (500 ng/ml, Sigma) in the presence of Golgi-Plug (1 mg/ml, BD Biosciences). The cells were fixed, permeabilized and stained with anti-F4/80 and anti-IL-5 or anti-IL-6 (BD Biosciences). For macrophage depletion experiments CD45"F4/80" macrophages were positively selected on the FACS Aria. Macrophage depleted populations were confirmed to contain < 1% CD45"F4/80" cells prior to further experiments.
Oil Red O Staining
Cells were grown in culture slides and then fixed with 10% neutral buffered formalin followed by incubation with newly filtered Oil Red O (ORO) staining solution (60 mls of saturated ORO/isopropanol solution was mixed with 40 mls of a 1% dextrin solution). The mean number of ORO positive cells in 5 randomly chosen microscope fields per slide was calculated using Scion Image software (Scion Corporation).

Morphometric and immunohistochemical analysis of WAT
For morphometric analysis 5 μm sections of formalin-fixed paraffin-embedded eWAT were stained with hematoxylin and eosin (H&E). Forty adipocytes were randomly chosen in each section and the mean adipocyte size was determined using Scion Image software at x20 magnification. Immunohistochemical staining was performed using molecule-specific and isotype-control antibodies as negative controls: anti-mouse F4/80 (Clone CI:A3-1, Serotec) for macrophages; anti-mouse ST2 (MD Biosciences); anti-mouse IL-33 (R&D Systems); anti-human IL-33 (Axxora, NESSY-1); anti-human ST2L (MAB10041, R&D Systems). Staining was visualized using biotinylated secondary antibodies and detection with the ABC/DAB system or Avidin FITC (Vector).

Quantitative PCR
Total RNA from eWAT and liver was prepared by tissue homogenization in 1 ml of Qiazol (Qiagen) or Trizol (Invitrogen) respectively. Gene expression was analyzed on the Applied Biosystems ABI7900HT machine with SDS 2.2 software. Gene expression was normalized to TATA binding protein (TBP) using Assays on Demand and QPCR master mix (Applied Biosystems). The following TaqMan® Gene Expression Assays (Applied Biosystems) were used: TBP (Mm00446973_m1), phosphoenolpyruvate carboxykinase 1 (PCK1, Mm00440636_m1), CAAT enhancer–binding protein-α (C/EBP-α, Mm01265914_s1), peroxisome proliferator–activated receptor γ and δ (PPAR-γ/δ, Mm01184322_m1 and Mm01305434_m1), peroxisome proliferator–activated receptor γ co-activator 1α (PGC-1α, Mm00447183_m1), liver X receptors α- and β (LXRα and β, Mm00443454_m1 and Mm00437262_m1), fatty acid synthase (FAS, Mm00662319_m1), sterol regulatory element binding protein 1c (SREBP-1c, Mm01138344_m1), ATP-binding cassette transporter-1 (ABCA-1, Mm00442646_m1), arginase 1 (ARG1, Mm00475988_m1), resistin-like molecule/found in inflammatory zone (retnla, RELM/FIZZ, Mm00445109_m1), inducible nitric oxide synthase (NOS2, Mm01309897_m1v) and Ym1/ chitinase 3-like 3 (Chi3l3, Mm00657889_mH).

Serum Analysis
Total serum cholesterol and triglyceride levels (mmol/L) were measured by enzymatic assay (Roche Diagnostics). Serum cytokines were analyzed in a 20-plex mouse cytokine assay (Biosource), and metabolic and hormonal patterns were analyzed using the mouse adipocyte kit (Insulin, Resistin, tPAI-1) (Millipore) on the Bio-Plex 100 system (Bio-Rad).

Magnetic Resonance Spectroscopy (MRS)
Mice underwent whole body MRS on a 7T Bruker Biospec system (Bruker Biospec, Karlsruhe, Germany), using a 15cm diameter proton birdcage radio frequency coil, for both transmitting and receiving at the proton resonance of 300MHz. The large RF coil ensured the B1 field was uniform over the whole mouse, thus giving uniform signal detection. The free induction decay was detected following a 75μs RF pulse, and 4 signal averages taken (Repetition time Tr= 15s, 50kHz sweep width, 8192 point
digitization). The resultant signal was then Fourier transformed and phased automatically to give the proton spectra (Paravision 4.0, Bruker, Germany). At 7 Tesla the water and lipid resonances of the whole body MRS spectra are well separated allowing the areas under the curve (AUC) to be obtained by simple integration. The AUC of the water peak (AUCH₂O) was determined over a 3000Hz range below the relative minimum cutoff, and the AUC of the lipid peak over a 3000Hz range above the relative minimum cutoff. These AUC’s were then used to determine percentages of body fat (%FAT) and fat free mass by MRS (%FFM).