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IL-33 Reverses an Obesity-Induced Deficit in Visceral Adipose Tissue ST2+ T Regulatory Cells and Ameliorates Adipose Tissue Inflammation and Insulin Resistance

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Obesity is associated with insulin resistance and inflammation thought to be caused by a visceral adipose tissue (VAT)–localized reduction in immunoregulatory cells and increase in proinflammatory immune cells. We previously found that VAT regulatory T cells (Tregs) normally express high levels of IL-10 and that expression of this cytokine in VAT Tregs is specifically reduced in mice fed a high-fat diet. In this study, we further investigated the phenotype of VAT Tregs and found that the majority of IL-10–expressing Tregs in the VAT of lean mice also expressed the ST2 chain of the IL-33R. In addition to high expression of IL-10, ST2+ Tregs in lean VAT expressed higher proportions of Th2-associated proteins, including GATA3 and CCR4, and Neuropillin-1 compared with ST2− Tregs. The proportion of ST2+ Tregs in VAT was severely diminished in obese mice that had been fed a high-fat/sucrose diet, and this effect could be completely reversed by treatment with IL-33. IL-33 treatment also reversed VAT inflammation in obese mice and resulted in a reduction of hyperinsulinemia and insulin resistance. These data suggest that IL-33 contributes to the maintenance of the normal pool of ST2+ Tregs in the VAT, and that therapeutic administration of IL-33 results in multiple anti-obesity effects, including the reversal of VAT inflammation and alleviation of insulin resistance. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: DIO, diet-induced obesity; HFD, high-fat/sucrose diet; HOMA-IR, homeostatic model assessment of insulin resistance; NCD, normal chow diet; Nrp1, neuropillin-1; SVF, stromal-vascular fraction; Tconv, conventional T cell; Treg, regulatory T cell; VAT, visceral adipose tissue.

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Materials and Methods

Mice and IL-33 injections

Four-week-old male C57BL/6 FOXP3-eGFP or C57BL/6 FOXP3-RFP × IL10-GFP mice (bred in house) were fed a normal chow diet (NCD; Lab Diets catalog 5053) or 58 kcal% fat with sucrose diet (Research Diets catalog D12331) for additional 14–16 wk. Diets were not matched for micronutrients. Where indicated, mice were then injected i.p. for consecutive 10 d with 0.5 μg/day rIL-33 or PBS (eBioscience), while maintained
on their respective diets, and were sacrificed 10 d after the start of treatment. To determine metabolic effects of IL-33, consecutive 10 d of injections were followed by one injection every 2–3 d of 0.5 μg IL-33 or PBS. Fasting blood glucose and plasma insulin levels were measured at 14 and 17 d after the start of treatment. All animal studies were approved by the Canadian Council on Animal Care.

**Cell isolation and in vitro generation of ST2+ Tregs**

Spleens and epididymal fat pads (VAT) were processed, as described (7). The stromal-vascular fraction (SVF) was obtained after centrifugation and passed through a cell strainer. Intestines were minced and digested with collagenase, and live cells were isolated using Percoll centrifugation. Bone marrow–derived dendritic cells were differentiated with GM-CSF and 10 ng/ml IL-4 in RPMI 1640 and 10% FCS for 7 d, and then purified as CD11c+ cells (STEMCELL Technologies) and rested overnight in complete media, with or without 10 ng/ml IL-33. CD4+ T cells were magnetically sorted (STEMCELL Technologies) from spleens. A ratio of 10 T cells to 1 DC was cultured with 0.01–0.1 μg/ml anti-CD3 (2C11; BD Biosciences) in the presence or absence of 10 ng/ml IL-33 for 5 d.

**Flow cytometry, RT-PCR, and cytokine analysis**

All flow cytometry and ELISA Abs were commercially obtained from BD Biosciences or ebioscience. A portion of VAT was immediately immersed in lysis buffer and dissociated using gentleMACS (Miltenyi Biotec) for analysis of gene expression. Data were normalized to 18 s. The SVF from digested VAT was cultured overnight at 2 × 10^6 cells/ml, and amounts of secreted IL-6 were measured in the culture media by cytometric bead array (BD Biosciences).

**Determination of blood glucose, insulin, and homeostatic model assessment of insulin resistance**

Mice were fasted for 4–6 h at days 14 and 17 after the start of treatment, respectively. Blood glucose was measured using a OneTouch Ultra 2 glucometer, and plasma insulin was measured by ELISA (ALPCO). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as fasting blood glucose (mmol/l) × fasting insulin (mU/l)/22.5, as described (10).

**Statistical analysis**

Unpaired Student t tests were used to analyze significance. The p values are indicated as follows: *p < 0.05 and **p < 0.01.

**Results**

A majority of Tregs in lean VAT express ST2 and Th2 cell–associated markers.

We previously found that Tregs resident in VAT express high levels of IL-10, which is most likely involved in restraining the production of proinflammatory cytokines from macrophages (7). To further characterize the phenotype and function of VAT Tregs, we asked whether these cells also express ST2, one of the chains of the IL-33R, because expression of this protein has recently been reported on Tregs resident in other tissues (11, 12). Single-cell suspensions from the VAT of 20-wk-old mice maintained on NCD were analyzed by flow cytometry. We found that 54.5 ± 14.3% (n = 14) of VAT Tregs expressed ST2, compared with just 2.9 ± 0.9% (n = 14) of splenic Tregs (Fig. 1A). In addition, only 6.8 ± 4.2% (n = 14) of CD4+FOXP3+ Tcrons in the VAT expressed ST2. CD4+FOXP3+ Tregs in the VAT also had high expression of CD25, CTLA-4, and CD39 regardless of whether they express ST2 (Supplemental Fig. 1A). VAT Tcrons expressed significantly higher levels of CD25, CTLA-4, and CD39 compared with splenic Tcrons, albeit at lower levels than on Tregs, and interestingly, ST2+ Tcrons in the VAT expressed increased levels of CD25 compared with ST2− Tcrons (Supplemental Fig. 1A). The proportion of Tregs expressing ST2 in lean VAT was significantly higher than in the intestinal lamina propria, in which an average of 17.7 ± 0.4% (n = 3) of Tregs expressed ST2 (Supplemental Fig. 1B). ST2 expression has also been previously linked to Th2 cells (13, 14); thus, we measured expression of Th2-associated proteins CCR4 and GATA3 in VAT Tregs. We found that VAT Tregs expressed significantly higher levels of CCR4 and GATA3 than splenic Tregs (Fig. 1B), but did not express Th2-related cytokines such as IL-13 and IL-5 (data not shown). VAT Tcrons expressed slightly higher levels of GATA3, but not of CCR4, compared with splenic Tcrons (Fig. 1B). By comparison, VAT Tregs did not express increased levels of Th1 (TBET, CXCR3)- or Th17 (retinoic acid-related orphan receptor γt)-associated proteins (Supplemental Fig. 1C). However, VAT Tregs expressed significantly more Th17-associated CCR6 than splenic Tcrons (Supplemental Fig. 1C), but so did VAT Tcrons, indicating that this change is not Treg specific.

To ask whether expression of Th2 markers is associated with expression of ST2, we examined expression of CCR4 and GATA3 in ST2+ versus ST2− VAT Tregs. As shown in Fig. 1C, VAT-specific expression of GATA3 and CCR4 was restricted to ST2+ Tregs, indicating that ST2 appears to specifically mark Tregs with a Th2-like phenotype. Of note, ST2+ Tcrons also expressed significantly more CCR4 than ST2− Tcrons (Fig. 1C). Because GATA3 enhances the expression of IL-10 in CD4+ T cells (15), and VAT Tregs express high levels of IL-10 (7), we used FOXP3-RFP × IL10-GFP mice to determine the correlation between ST2+ and IL-10. Indeed, the vast majority (90.8 ± 6.7%, n = 6) of IL-10–expressing Tcrons in VAT also expressed ST2, and by corollary, ST2+ Tcrons in the VAT expressed significantly more IL-10 than did ST2− Tcrons (Fig. 1D); 68.1 ± 13.1% of ST2+ Tcrons expressed IL-10, compared with only 17.9 ± 9.1% of ST2− Tcrons.

To determine whether the Th2-like phenotype and IL-10 production by ST2+ Tcrons were related to IL-33 signaling, we used bone marrow–derived dendritic cells and IL-33 to differentiate ST2+ Tcrons in vitro from splenic CD4+ T crons, which had very low levels of ST2 expression prior to stimulation (16). This culture system with IL-33 resulted in the induction of ST2+ Tcrons, which had increased expression of both CCR4 and IL-10 (Supplemental Fig. 2A, 2B).

Interestingly, ST2+ Tcrons also had increased expression of neutropilin-1 (Nrp1) (Fig. 1E), which is known to promote Treg survival and function (17). In contrast, analysis of Helios revealed no significant differences in expression between ST2+ and ST2− Tcrons (Fig. 1F), suggesting that these cells are comprised of similar proportions of thymically and peripherally derived cells.

**Obesity diminishes VAT-resident ST2+ Tregs**

DIO results in a reduced proportion of VAT Tregs due to an expansion of proinflammatory Tcrons (5, 6). In addition, Tregs from obese VAT have diminished expression of IL-10, whereas other suppressive mechanisms, including CD39, CTLA-4, and LAP, remain unchanged compared with cells in lean VAT (7). To investigate the effect of DIO on ST2+ Tcrons, littersmates to the lean NCD mice shown in Fig. 1 were fed with a 58 kcal% fat with sucrose diet (high-fat/sucrose diet [HFD]). We found that obese VAT had a substantially reduced proportion of ST2+ Tcrons (Fig. 2A); 54.5 ± 14.3% (n = 14) of VAT Tcrons expressed ST2 in lean VAT, compared with just 16.3 ± 7.1% (n = 14) in obese VAT from HFD mice. There was a similar HFD-induced reduction in the minor population of VAT Tcrons that also expressed ST2.

In parallel, VAT Tcrons from HFD mice also had reduced expression of multiple proteins that were associated with ST2 expression, including CCR4, GATA3, and Nrp1 (Fig. 2B–D). VAT Tcrons in HFD mice had a 56.9 ± 19.7% (n = 12) reduction in CCR4, and a 43.6 ± 12.9% (n = 6) reduction in the expression of GATA3. VAT Tcrons had a similar percentage reduction of GATA3, but no reduction in the expression of CCR4. Interestingly, HFD induced a large 84 ± 11.6% (n = 7) reduction of Nrp1 in VAT Tcrons, compared with 53.4 ± 34.8% (n = 7) in VAT Tcrons. The expression of Th1 (TBET, CXCR3)- or Th17 (retinoic acidi-
related orphan receptor γt, CCR6-associated proteins on VAT Tregs was not affected by HFD (Supplemental Fig. 1C).

Within the populations of ST2+ Treg and ST2+ Tconvs in the VAT, DIO significantly reduced the expression of CCR4 (Fig. 2E) and Nrp1 (Fig. 2G), but not GATA3 (Fig. 2F). The expression of Nrp1 in ST2+ Tregs and ST2+ Tconvs was also downregulated in HFD mice (Fig. 2G), indicating that both ST2+ and ST2+ cells were affected by DIO. In addition, although the overall expression of CCR4, GATA3, and Nrp1 in total VAT Tregs and/or Tconvs was decreased in HFD mice, ST2+ Tregs and Tconvs retained higher expression of these proteins compared with ST2+ cells (Supplemental Fig. 3).

**IL-33 reverses the reduction of ST2+ Tregs in obese VAT**

Injection of IL-33 is known to expand peripheral ST2+ Tregs in a model of cardiac transplantation (18), so we asked whether in vivo administration of IL-33 could reverse the diet-induced reduction of ST2+ Tregs in VAT. Mice were fed a NCD or HFD for 16 wk, then injected i.p. with IL-33, or PBS as a control, for consecutive 10 d. Notably, 10 d of IL-33 treatment did not change the body weights of NCD or HFD mice (Supplemental Fig. 4A, 4B). Mice fed an HFD had a significant reduction in the proportion of Tregs, and injection of IL-33 completely reversed this effect, resulting in restoration of the normal proportion of Tregs (Fig. 3A). IL-33 also significantly increased the absolute number of Tregs per gram of VAT. However, IL-33 did not increase the numbers of Tconvs, and, contrary to a previous report (18), IL-33 injections did not expand Tregs in the spleen.

IL-33 administration also increased the proportion of ST2+ Tregs in the VAT by 3-fold in HFD mice, resulting in restoration of the normal proportion of Tregs (Fig. 3A). IL-33 also significantly increased the absolute number of Tregs per gram of VAT. However, IL-33 did not increase the numbers of Tconvs, and, contrary to a previous report (18), IL-33 injections did not expand Tregs in the spleen.

**FIGURE 1.** Phenotypically distinct ST2+ Tregs populate lean VAT. (A) The percentages of ST2+ cells (n = 14) and (B) mean fluorescence intensities of CCR4 (n = 14) and GATA3 (n = 6) on gated CD4+FOXP3+ Tregs and CD4+FOXP3+ Tconvs from the spleen and VAT of 20-wk-old NCD mice were analyzed by flow cytometry. The mean fluorescence intensities of (C) CCR4 (n = 14) and GATA3 (n = 6), (D) percentages of IL-10hi (n = 6), (E) mean fluorescence intensities of Nrp1 (n = 7), and (F) percentages of Helios+ cells (n = 7) in ST2+ versus ST2- FOXP3+ Tregs in the VAT of 20-wk-old NCD mice are shown. Plots and histograms shown are representative data. Graphs show averages (A, D, and F) or paired biological replicates (B, C, and E) from the number of mice indicated analyzed in three to four (A–C) or two (D–F) independent experiments. Error bars represent SD. *p < 0.05, **p < 0.01.
ST2+ cells in these populations always remained significantly lower than that of VAT Tregs.

**IL-33 treatment reduces VAT inflammation**

We next asked whether the IL-33–induced restoration of ST2+ Tregs in the VAT of HFD mice was accompanied by a reduction in inflammation. As expected, compared with NCD mice, HFD mice had an increase in F4/80+ macrophages infiltrating the VAT, and this was almost completely reversed by IL-33 administration (Fig. 4A). By comparison, IL-33 did not significantly alter the proportion of CD4+, CD8+, or CD11c+ cells in the VAT (Supplemental Fig. 4C). RT-PCR analysis of the VAT revealed that IL-33 treatment of HFD mice also reduced the expression of Tnf mRNA in the VAT (Fig. 4B), but did not significantly affect levels of Ccl2 or Il10 mRNA (Supplemental Fig. 4D). We also isolated the SVF from the VAT, which contains all VAT-resident immune cells, and measured the amount of IL-6 released in the culture media. SVF cells isolated from the VAT of HFD mice that had been injected with IL-33 produced significantly less IL-6 than did SVF cells isolated from HFD mice receiving PBS (Fig. 4C). Indeed, IL-33 administration was able to reduce TNF-α and IL-6 in the VAT to the level of expression found in lean NCD control mice, as indicated by the dashed lines (Fig. 4B, 4C).

**IL-33 treatment ameliorates insulin resistance in diet-induced obese mice**

To determine whether IL-33–induced resolution of VAT inflammation could also lead to an improvement of metabolism, mice fed an HFD for 16 wk were injected with IL-33 for consecutive 10 d to expand VAT Tregs and reduce VAT inflammation, followed by one injection every 2–3 d. At days 14 and 17, mice were fasted for 4–6 h, and their fasting blood glucose and serum insulin were measured, and the HOMA-IR, a mathematical equation used to estimate insulin resistance using fasting blood glucose and insulin levels (19), was calculated. There was no significant difference in body weights between PBS- and IL-33–treated mice before and after treatment (Fig. 5A); however, paired analysis revealed IL-33 injections resulted in a reduction of body weight after 14 and 17 d of treatment, whereas PBS injections did not (Fig. 5B). IL-33 significantly reduced fasting glycemia and amounts of plasma insulin (Fig. 5C, 5D). Consequently, the HOMA-IR was reduced to normal levels in mice receiving IL-33 treatment (Fig. 5E), indicating a significant improvement in insulin resistance.

**Discussion**

A deficit in FOXP3+ Tregs in the VAT is thought to be a key factor in the development of adipose inflammation, resulting in the ac-
cumulation of proinflammatory macrophages and cytokines that promote insulin resistance (1, 3, 6, 20–23). In this work, we show that the VAT contains a large proportion of ST2+ Tregs that have a phenotype that is distinct from ST2− Tregs and consistent with cells likely to have superior stability and IL-10–dependent suppressive function. IL-33 can be used therapeutically to completely reverse the DIO-associated reduction in the proportion of ST2+ Tregs, resulting in reduced VAT inflammation and improved metabolic health of obese mice. Thus, manipulation of ST2+ Tregs may be a new approach to control obesity-associated inflammation and insulin resistance.

Previous studies have shown that the VAT of lean mice is populated with a high proportion of Tregs that are phenotypically unique compared with peripheral Tregs due to high expression of PPARγ (6, 9) and IL-10 (7). Our study adds to this knowledge by showing that the majority of Tregs in lean VAT express ST2, and that they can be therapeutically expanded with IL-33. Thus, VAT Tregs may be a new approach to control obesity-associated inflammation and insulin resistance.

 FIGURE 3. IL-33 treatment reverses the deficit of ST2− Tregs in obese VAT. (A–D) Mice were fed an HFD for 16 wk, then injected with PBS or IL-33 for 10 d. (A) The percentage of FOXP3+ Tregs of CD4+ cells in the spleen and VAT (n = 9) and the absolute number of cells per gram of VAT (n = 7) were determined by flow cytometry. (B) The percentage (n = 9) and absolute number (n = 7) of ST2− cells. (C) Mean fluorescence intensity of GATA3 (n = 4), and (D) the percentage of Ki67− cells (n = 5–6) in gated CD4+FOXP3+ Tregs and CD4+FOXP3− Tconv in the spleen and VAT were measured. Dot plots and histograms shown are representative data from VAT. Representative dot plots of NCD mice injected with PBS for 10 d are also shown for (A) and (B). Percentages and mean fluorescence intensity shown are averages from the number of mice indicated analyzed in three (A and B) or two (C and D) independent experiments. Absolute numbers in (A) and (B) are representative data from one of two independent experiments. Error bars represent SD. *p < 0.05, **p < 0.01.

ST2+ Tregs as well as ST2+ Tconv in lean VAT expressed increased levels of the Th2-related proteins GATA3 and CCR4 (24, 25). Similar to VAT Tregs, ST2 expression on colonic Tregs is also associated with GATA3 expression (11). As GATA3 promotes Treg stability and function (26, 27), VAT-resident ST2+ Tregs may be more stable and potent than their ST2− counterparts. In support of this hypothesis, ST2+ Tregs have enhanced expression of IL-10 compared with ST2− Tregs. Because IL-10 produced by Tregs inhibits TNF-α production by macrophages (7), and IL-10 is well known to limit obesity-associated inflammation (28, 29) and TNF-α–induced insulin resistance (4), ST2−IL-10+ Tregs are most likely key regulators of immune responses in the VAT. Evidence that ST2+ Tregs also express increased levels of Nrp1, which is

 FIGURE 4. IL-33 administration reduces VAT inflammation. (A–C) Mice were fed an HFD for 16 wk, then injected with PBS or IL-33 for 10 d. (A) The percentages of F4/80+ macrophages of total live cells in the spleen and VAT were analyzed by flow cytometry (n = 6–7). (B) The relative amounts of Tnfα mRNA in the VAT were detected by quantitative RT-PCR (n = 6–7). (C) The average amounts of IL-6 produced by SVF cells during overnight culture were measured by cytometric bead array (n = 4). The dashed lines indicate the average values from the VAT of NCD mice injected with PBS for 10 d. Graphs shows averages from the number of mice indicated analyzed in two independent experiments. Error bars represent SD. *p < 0.05, **p < 0.01.
critical for the maintenance and function of Tregs (17), further supports the hypothesis that ST2+ Tregs possess superior stability and function. Although ST2+ Tregs are not more suppressive than ST2- Tregs in vitro suppression assays (16, 18), evidence that ST2-deficient Tregs have attenuated suppressive function in vivo (11) supports the notion that this subset may have unique functional properties.

In obese VAT, not only is the frequency of Tregs reduced, but also the proportion of the remaining Tregs expressing ST2 is severely diminished. As a result, there was an overall reduction in the expression of CCR4, GATA3, and Nrp1 in VAT Tregs. It remains unclear whether this reduction is due to the loss of ST2+ Tregs, selective expansion of ST2- Tregs, and/or an intrinsic change in the phenotype of VAT-resident ST2+ Tregs. Because, as discussed above, the expression of ST2, GATA3, and Nrp1 have all been associated with Treg expansion, stability, and function, the phenotype of Tregs in obese VAT suggests these cells may be less potent. This possibility is supported by our previous finding that Tregs from obese VAT have diminished expression of IL-10 (7). Because the HFD and NCD were not micronutrient matched, we cannot exclude the possibility that there may also be obesity-independent, but diet-related effects.

Therapeutic IL-33 administration corrected the obesity-associated deficit in VAT ST2+ Tregs, suggesting that IL-33 is sufficient to maintain a pool of functionally specialized VAT-resident ST2+ Tregs. IL-33 increased both the proportion and absolute numbers of ST2+ Tregs, indicating that IL-33 induced the active expansion of ST2+ Tregs. In contrast, although IL-33 treatment increased the proportion of ST2+ Tconv, their absolute number did not significantly change. Evidence that IL-33 reprograms DCs to selectively expand ST2+ Tregs (16), and the paucity of ST2+ Tregs that could respond to IL-33 in HFD mice raises the possibility that, at least initially, the effect of IL-33 on the expansion of ST2+ Tregs may be indirect. Contrary to a previous report (18), we found that IL-33 injections did not expand Tregs in the spleen, possibly because the 20-wk-old mice used in these experiments had a higher age-related (30) proportion of Tregs in the spleen than the younger mice reported by Turnquist et al. (18).

IL-33 therapy also reversed VAT inflammation in obese mice, reducing expression of TNF-α and IL-6 to the levels found in the VAT of healthy lean mice. Because macrophages express almost all of the TNF-α, and a significant amount of IL-6, in the VAT (29), changes in these cytokines most likely originate from these cells. Although the reversal of VAT inflammation was correlated with the expansion of ST2+ Tregs in the VAT, IL-33 could also have many other effects in the adipose tissue, including the activation of eosinophils (31) and type 2 innate lymphoid cells (ILC2)-mediated beiging of VAT (32). IL-33 also affects macrophages; however, it is unclear whether IL-33 promotes the polarization of alternatively activated macrophages (33), or enhances inflammatory cytokine production (34), and studies of the effects of IL-33 specifically on VAT-resident macrophages remain to be done. Thus, the IL-33-mediated reduction of inflammation is most likely due to a combination of anti-inflammatory effects of this cytokine on multiple cell types.

Both TNF-α and IL-6 have previously been shown to induce insulin resistance in adipocytes (35, 36), leading us to the hypothesis that IL-33 may also improve the metabolic parameters of obese mice. One hallmark of obesity is an overproduction of insulin, which at high levels can inhibit IL-10–dependent Treg function (7). IL-33-induced reduction of hyperinsulinemia may thus dampen the inhibitory effect that insulin exerts on Tregs in obesity. Using HOMA-IR to estimate the severity of insulin resistance (19), we found that IL-33 decreased the severity of insulin resistance in obese mice. Notably, we observed the metabolic effects of IL-33 at day 14, but not at day 10 (data not shown). Because IL-33 had already reversed VAT inflammation at day 10, the delayed improvement in insulin sensitivity may thus be subsequent to the effect of reduced inflammation. Longitudinal analysis of individual mice revealed that IL-33 treatment reduced body weight at day 14, coincident with improved metabolic parameters. Although the difference between the body weights of PBS versus IL-33–treated mice was not significant, alleviation of insulin resistance may be partly related to the reduction of body weight.

Our data are consistent with a recent report that the majority of VAT-resident Tregs express ST2, and IL-33 treatment promoted the increase of VAT Tregs, reduced VAT inflammation, and improved glucose tolerance of HFD mice (37). In addition, we show that DIO diminishes ST2+ Tregs and the expression of ST2-associated proteins such as CCR4, GATA3, and Nrp1. IL-33–mediated cor-

FIGURE 5. IL-33 treatment reverses insulin resistance in diet-induced obese mice. (A–E) Mice were fed an HFD for 14–15 wk, then injected with PBS or IL-33 for 10 consecutive days, followed by one IL-33 injection every 2–3 d. (A) Averaged body weights were measured before treatment (d0) and after 14 and 17 d (n = 6). (B) Paired analysis of body weights before and after PBS or IL-33 treatment with each line representing one mouse (n = 6). (C) Fasting plasma insulin (n = 4) and (D) fasting blood glucose (n = 4) were measured at days 14 and 17 after initial treatment. (E) HOMA-IR (n = 4) was determined as an indicator of insulin resistance. Dashed lines indicate normal levels found in control NCD mice. Graphs show averaged data from the indicated number of mice per group analyzed in two independent experiments. Error bars represent SD. *p < 0.05, **p < 0.01.
rection of the Treg deficit and VAT inflammation lasted as long as 25 d after initial treatment (data not shown), suggesting a prolonged therapeutic effect. IL-33 has also been reported to reduce genetically driven insulin resistance in leptin-deficient (ob/ob) mice (38). However, because ob/ob mice have an abnormal T cell compartment (39) and leptin has direct effects on T cells, including Treg proliferation (40), it is difficult to interpret these findings as they relate to T cells.

In summary, we show that more than half of the Tregs in normal VAT have a unique Th2-like and ST2+ phenotype and express markers associated with potent and activated Tregs (i.e., IL-10, Foxp3, and T-bet) (39). Thus, IL-33–induced therapeutic expansion of ST2+ Tregs may be a new approach to control obesity-associated inflammation and insulin resistance.

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Disclosures
The authors have no financial conflicts of interest.

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Supplemental Figure S1. Characterization of VAT CD4⁺FOXP3⁺ Tregs. (A) The expression of CD25, CTLA-4 and CD39 amongst CD4⁺ FOXP3⁺ and CD4⁺ FOXP3⁻, and their ST2⁺ and ST2⁻ subpopulations in the spleen and VAT of 20 weeks old NCD mice were analyzed by flow cytometry (n = 3-5). (B) The percentage of ST2⁺ cells on gated CD4⁺FOXP3⁺ Tregs and CD4⁺FOXP3⁻ Tconvs from the spleen, VAT and intestinal lamina propria of 20 weeks old NCD mice were analyzed by flow cytometry (n = 3-12). (C) Mice were fed a NCD or HFD for 16 weeks and the expression of CXCR3, TBET, CCR6 and RORγt on Tregs and Tconvs in spleen and VAT was measured by flow cytometry (n = 4). Graphs show averaged data. Plots and histograms show representative data. Error bars represent SD.
Supplemental Figure S2. *In vitro differentiated ST2*+ Tregs have increased expression of CCR4 and IL-10. CD4+ T cells were co-cultured with bone marrow-derived DCs in the presence of 10ng/ml IL-33 and 0.01-0.1µg/ml anti-CD3 at a ratio of 10 T cells to 1 DC. (A) Representative plot depicting ST2 expression in Tregs is shown, and (B) the expression of CCR4 and IL-10 were measured in ST2+ and ST2- Tregs by flow cytometry. Averaged data from n = 6 are shown. Error bars represent SD.
Supplemental Figure S3. VAT ST2+ Tregs have increased expression of CCR4, GATA3 and Nrp1 in HFD mice. Mean fluorescence intensities of CCR4 (n = 14), GATA3 (n = 6) and Nrp1 (n = 7) in ST2+ versus ST2- Tregs in the VAT of 20 weeks old HFD mice were analyzed by flow cytometry. Graphs show paired biological replicates.
Supplemental Figure S4. 10 days of IL-33 administration does not affect the body weights, the proportions of CD4+, CD8+ and CD11c+ cells, or the expression of CCL2 and IL10 mRNAs in the VAT. Mice were fed a NCD (n = 2-3) or HFD (n = 8) for 16 weeks, and then injected with PBS or IL-33. Weights were measured before (d0) and after treatment (d10), and shown as (A) averaged data. Error bars represent SD. (B) Paired analysis comparing the weights of mice before and after treatment of each replicate. Each line represents one mouse. (C) The percentages of CD4+, CD8+ and CD11c+ cells of total live cells in the spleen and VAT were determined by flow cytometry (n = 4-8). (D) The relative amounts of CCL2 and IL10 mRNA in the VAT were detected by quantitative RT-PCR (n = 4-6). The dashed lines indicate the average values from the VAT of NCD mice injected with PBS for 10 days. All graphs show averaged data. Error bars represent SD.