Depletion of fat-resident T<sub>reg</sub> cells prevents age-associated insulin resistance

Sagar P. Bapat<sup>1,2</sup>, Jae Myoung Suh<sup>2,3</sup>, Sungsoon Fang<sup>2,4</sup>, Silhao Liu<sup>2</sup>, Yang Zhang<sup>1</sup>, Albert Cheng<sup>1</sup>, Carmen Zhou<sup>1</sup>, Yuqiong Liang<sup>1</sup>, Mathias LeBlanc<sup>2</sup>, Christopher Liddle<sup>5</sup>, Annette R. Atkins<sup>2</sup>, Ruth T. Yu<sup>2</sup>, Michael Downes<sup>2</sup>, Ronald M. Evans<sup>2,6</sup> & Ye Zheng<sup>1</sup>

Age-associated insulin resistance (IR) and obesity-associated IR are two physiologically distinct forms of adult-onset diabetes. While macrophage-driven inflammation is a core driver of obesity-associated IR<sup>1–6</sup>, the underlying mechanisms of the obesity-independent yet highly prevalent age-associated IR<sup>2</sup> are largely unexplored. Here we show, using comparative adipose-immune profiling in mice, that fat-resident regulatory T cells, termed fTreg cells, accumulate in adipose tissue as a function of age, but not obesity. Supporting the existence of two distinct mechanisms underlying IR, mice deficient in fTreg cells are protected against age-associated IR, yet remain susceptible to obesity-associated IR and metabolic disease. By contrast, selective depletion of fTreg cells via anti-ST2 antibody treatment increases adipose tissue insulin sensitivity. These findings establish that distinct immune cell populations within adipose tissue underlie ageing- and obesity-associated IR, and implicate fTreg cells as adipose-immune drivers and potential therapeutic targets in the treatment of age-associated IR.

The young, lean state is associated with insulin sensitivity, while both ageing and obesity can lead to the development of IR (Extended Data Fig. 1a). To explore key immune cell types that drive age- versus obesity-associated IR, we quantitatively profiled the immune cell components of adipose depots using a flow cytometry approach termed adipose-immune profiling (AIP) (Extended Data Fig. 1b–d and Extended Data Table 1). In contrast to the decrease in anti-inflammatory M2 adipose tissue macrophages and eosinophils observed in obesity-driven IR, AIP revealed that these cell populations are largely unperturbed in visceral adipose tissue (VAT) from aged mice<sup>8–12</sup> (M2 adipose tissue macrophages, aged: 33.6 ± 3.8% (mean ± s.d.), young: 29.8 ± 4.1%, obese: 22.9 ± 6.3%; eosinophils, aged: 4.4% ± 1.6%, young: 4.7% ± 0.7%, obese: 0.8% ± 1.0%; Fig. 1a). Instead, the relative portion of the non-macrophage compartment is significantly increased in aged compared to young or obese mice (aged: 24.3 ± 4.6%, young: 17.9 ± 2.8%, obese: 15.7 ± 3.8%; Fig. 1a), which is largely attributable to an ~12-fold expansion in the fTreg cell population<sup>13,14</sup> (aged: 5.0 ± 1.2%, young: 0.4 ± 0.1%, obese: 0.1 ± 0.1%; Fig. 1a, b). These condition-dependent AIP signatures of adipose tissue suggest that distinct pathophysiological processes drive age- and obesity-associated IR and specifically implicate fTreg cells in age-associated IR.

T<sub>reg</sub> cells in fat express Pparg at a high level, which allows them to expand their relative numbers approximately 6–7-fold<sup>15</sup>. Knockout of Pparg in T<sub>reg</sub> cells blocks this accumulation. Accordingly, we exploited this observation by creating Foxp3<sup>Cre<sub>–/–</sub></sup> (Foxp3–IREs-YFP-Cre) Pparg<sup>fl/fl</sup> mice in which T<sub>reg</sub> cells are selectively depleted (from 6.1% to 0.9%) from VAT<sup>15</sup> (fTreg knockout mice; Fig. 2a and Extended Data Fig. 2a, b), although the depletion of PPARγ-positive T<sub>reg</sub> cells in tissues such as muscle and liver cannot be ruled out. This depletion is achieved without significantly altering the immune profiles of subcutaneous adipose tissue (SAT) or spleen (Extended Data Fig. 2c, d). Importantly, the 6.8-fold VAT-specific loss of fTreg cells does not elicit any overt signs of systemic inflammation generally associated with T<sub>reg</sub> cell dysfunction. Aged fTreg knockout mice have normal-sized spleens and increased CD62L<sup>hi</sup>CD44<sup>hi</sup> naive CD4<sup>+</sup> T-cell populations compared to wild-type controls (Fig. 2c and Extended Data Fig. 3a). The normal intestinal histology provides additional evidence that the T<sub>reg</sub> cell population is not perturbed (Extended Data Fig. 3b, c). Furthermore, no differences are observed in the levels of inflammatory cytokines, including TNFα, IL-1β, IL-6, IFNγ and IL-17, in the serum of aged fTreg knockout compared to control mice (Extended Data Fig. 3d).

Notably, the selective loss of fTreg cells attenuates many of the hallmarks of age-associated metabolic dysregulation<sup>16</sup>. They weigh less...
fTreg knockout mice (~13 months, fTreg knockout (~15 months) mice (of non-macrophage AIPs of young (12 weeks), aged (~15 months) and aged glucose tolerance tests and increased sensitivity during insulin tolerance are significantly reduced (Fig. 3b, c and Extended Data Fig. 4a). Furthermore, aged fTreg knockout mice display smaller glucose excursions during metabolic ageing. a

Although fTreg cells were previously implicated in the insulin-sensitizing function of the PPARγ agonists thiazolidinediones (TZDs)15, in our studies fTreg knockout mice display similar metabolic improvements to the TZD rosiglitazone as control mice. These beneficial effects of TZDs are evident with either direct treatment of obese mice (therapeutic intervention; Extended Data Fig. 6a–g) or prophylactic treatment (drug intervention coincident with high-fat diet (HFD) feeding; Extended Data Fig. 6h–l). Additionally, we find that the insulin-sensitizing effects of rosiglitazone precede the TZD-induced expansion of fTreg cells in HFD-fed mice (Extended Data Fig. 6m–q). While intercolony variation cannot be ruled out, our findings do not support a significant role for fTreg cells in the therapeutic mechanism of action of TZDs.

Histologically, aged fTreg knockout VAT depots appear similar to control mice, and inflammatory processes such as macrophage crowning are observed at comparable frequencies (Fig. 3g and data not shown). However, aged fTreg knockout VAT has increased levels of TNFα (Extended Data Fig. 7a), increased expression of Vegfa (implicated in adipose remodelling and insulin sensitivity17; Extended Data Fig. 7b) and decreased expression of extracellular matrix genes (including collagen VI implicated in adipose tissue rigidity18, and the wound response gene Sparc; Extended Data Fig. 7b, c) compared to control tissue. Accompanying these changes, several proteases involved in extracellular matrix remodelling and angiogenesis (members of the ADAM, ADAMTS, MMP and CELA families) are differentially expressed (Extended Data Fig. 7b, d). Of note, adipocytes from aged fTreg knockout mice are smaller than those in control mice (fTreg knockout: ~70% < 5,000 μm²; control: ~41% < 5,000 μm²; Fig. 3h and Extended Data Fig. 4b), and serum non-esterified free fatty acid levels are reduced to almost half those of control mice; both indicators of improved insulin sensitivity (Fig. 3i). In addition, circulating levels of the adipokine resistin, which positively correlates with mouse IR, are reduced in the aged fTreg knockout mice19,20 (Fig. 3j). Furthermore, aged fTreg knockout mouse present with decreased hepatic steatosis, as determined histologically and by decreased fasting hepatic and serum triglyceride content (Fig. 3l–n). In combination, these findings suggest that the loss of fTreg cells in adipose tissue alleviates many of the indications of age-associated IR in mice, a primary clinical manifestation of metabolic ageing.

To associate fTreg cells more directly with age-associated IR, we measured basal glucose uptake in adipose tissue ex vivo. Notably, VAT from fTreg knockout mice took up almost twice the amount of glucose as control tissue (Fig. 3k). Conversely, expansion of fTreg cells in wild-type mice via treatment with IL-2–IL-2–monoclonal antibody complex21 abrogates basal glucose uptake in VAT by ~50% (Fig. 3o, p). This inverse correlation between fTreg cell numbers and glucose uptake in adipose tissue supports a causal association between fTreg cells and IR during ageing.

Our findings of an association between fTreg cells and age-associated IR and metabolic ageing suggest that these cells are functionally distinct from splenic Treg cells. To investigate this notion, we compared the transcriptomes of Treg cells, as well as conventional CD4⁺ T (Tconv) cells, isolated from VAT and spleen. Comparative analyses revealed that while certain canonical genes are similarly expressed (for example, Foxp3, Ctla4 and Tigit), VAT and splenic Treg cells have discrete expression signatures, consistent with the suggested functional distinction. In particular, Pparγ, Gata3 and Irf4 are selectively enriched in VAT but not splenic Treg cells22 (Extended Data Fig. 8a). Furthermore, unbiased comparative gene expression analyses combined with hierarchical clustering defined extensive fat- and splenic-residence clusters (1,142 and 1,431 genes, respectively) relative to

Figure 2 | fTreg knockout mice are protected from general hallmarks of metabolic ageing. a, Representative FACS plots of fTreg knockout (KO) (Foxp3Cre Pparγfl/fl) and control (Foxp3Cre Pparγ+/+) mice depicting Treg cell enrichment in VAT and spleen (~15 months, CD45.2⁺ CD4⁺ gating). b, Total body weight (n = 15 per group), and lean and fat mass of control and fTreg knockout mice (~12 months, n = 8 per group). c, Mass of VAT, SAT and spleen in aged control and fTreg knockout mice (~15 months, n = 9 per group). d, Cumulative food consumption of control and fTreg knockout mice (~6–9 months, n = 8 per group). e, f. Average 24 h respiratory exchange ratio (RER) (e) and average oxygen consumption (VO₂) (f) of aged control and fTreg knockout mice (~11 months, n = 6 per group). g, Core body temperature of control and fTreg mice (~13 months, n = 9 per group). h, Principal component analysis of non-macrophage AIPs of young (12 weeks), aged (~15 months) and aged fTreg knockout (~15 months) mice (n = 9 per group). Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student’s t-test.

than control mice and are leaner (decreased VAT and SAT adiposity) despite increased food consumption (Fig. 2b–d). In addition, the respiratory exchange ratio (Fig. 2e), oxygen consumption (Fig. 2f) and core body temperature (Fig. 2g) are increased in aged fTreg knockout mice compared to control mice. These marked improvements suggest that the age-associated metabolic phenotype is closely linked with VAT immune responses, and that in the aged setting, a reduction in fTreg cell numbers may be protective. Indeed, the AIPs of aged fTreg knockout mice are shifted towards those of young mice, as visualized by principal component analysis (Fig. 2h).

Although the fTreg knockout phenotype is most pronounced in aged mice, a reduction in fTreg cell levels can also be found in obese fTreg knockout mice (Fig. 3a). However, the beneficial metabolic effects of fTreg ablation are only significant in age-associated metabolic dysregulation, in which fasting serum glucose and insulin levels are significantly reduced (Fig. 3b, c and Extended Data Fig. 4a). Furthermore, aged fTreg knockout mice display smaller glucose excursions during glucose tolerance tests and increased sensitivity during insulin tolerance tests compared to weight-matched control mice (Fig. 3e). Again, these improvements in glucose homeostasis are observed only in aged mice; no significant differences are seen in young or obese fTreg knockout mice (Fig. 3d, f), which is consistent with the largely unchanged AIPs of obese fTreg knockout mice (Extended Data Fig. 5a, b).

Letter

LETTER

© 2015 Macmillan Publishers Limited. All rights reserved
much smaller pan-Treg clusters 1 and 2 (56 and 162 genes, respectively). Transcriptionally, Treg KO cells cluster more closely with fat Tconv cells than splenic Treg cells (Fig. 4a), suggesting that the functional specification of Treg cells is informed by their anatomical location within adipose tissue, as well as the expression of the Treg cell-lineagespecifying transcription factor Foxp3 (refs 23, 24 and 4b). Importantly, aged Treg cells maintain their suppressive functionality (~14 months). i, Ad-libitum–fed serum non-esterified fatty acid (NEFA) levels in 14-month-old control (n = 9) and Treg knockout mice (n = 10). j, Serum resistin levels in 14-month fasted control and Treg knockout mice (n = 4 pooled samples (2 mice per sample) per group). k, Postprandial glucose uptake in VAT of aged control (n = 5) and Treg knockout mice (n = 4). i, Representative H&E staining of VAT from control (n = 3) and Treg knockout mice (n = 5). Scale bars, 50 μm. m, Hepatic triglyceride levels in 14-month-old control (n = 5) and Treg knockout mice (n = 3). n, Fasting serum triglycerides in 14-month-old control (n = 9) and Treg knockout mice (n = 10). o, Treg cells, expressed as percentage of total CD45.2+ cells, in control (PBS) and IL-2–anti-IL-2–treated mice (n = 3 mice per group). p, Relative glucose uptake in VAT of 16-week-old control and IL-2–anti-IL-2–treated mice (n = 4 mice per group). Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student’s t-test.

to explore the therapeutic potential of the IL-33/ST2 signalling pathway, aged mice were initially injected with IL-33 (0.5 μg intraperitoneally (i.p.) on days 0, 2, and 4, analysis on day 6) to expand the Treg cell population (Fig. 4i–k). In agreement with Treg cell expansion driven by IL-2–anti-IL-2 treatment, mice injected with IL-33 display signs of IR (basal glucose uptake in VAT reduced to ~60% of control mice; Fig. 4i). In the converse approach, acute treatment with an anti-ST2 antibody (200 μg per mouse i.p. on days 0 and 2, analysis on day 3) is able to significantly deplete Treg cells (~50% reduction), with a smaller percentage reduction of splenic Treg cells (Fig. 4m).

Notably, the partial depletion of Treg cells achieved with acute anti-ST2 treatment coincides with an increase in insulin-stimulated glucose uptake in VAT (~25% increase in glucose uptake compared to control treated mice; Fig. 4n), suggesting a link between Treg cell depletion and increased adipose insulin sensitivity. Furthermore, this increase in insulin sensitivity is achieved without any signs of Tconv cell activation associated with systemic Treg cell dysfunction (Extended Data Fig. 8c–e).

As obesity and ageing often associate in humans, we challenged aged Treg knockout and control mice with HFD. While initially protected against HFD-induced weight gain and associated metabolic dysregulation, the metabolic benefits attributed to the loss of Treg cells were progressively lost over 8 weeks (Extended Data Fig. 9a–e), further
Hierarchical clustering of differentially expressed genes between fat Treg and Tconv and splenic Treg and Tconv cells from Foxp3<sup>CreERT2</sup> mice (47 weeks, cells pooled from 3–4 mice, same data set used in b, e). b, Fragments per kilobase of transcripts per million mapped reads (FPKM) values of selected genes important for Treg cell identity and canonical suppressive function. c, d, In vitro suppression assay of T<sub>reg</sub> cells (pooled from retired breeders, added at 1:1 ratio with splenic T<sub>conv</sub> cells, conducted in triplicate). e, Representative carboxyfluorescein succinimidyl ester (CFSE) tracings of T<sub>conv</sub> cells with or without fTreg cells. Gating indicates percentage of dividing cells. d, Expansion index of T<sub>reg</sub> cells. e, Fold change in expression levels of differentially expressed genes across fat T<sub>reg</sub> and T<sub>conv</sub> and splenic T<sub>reg</sub> and T<sub>conv</sub> cells. Fat Treg cell cluster genes are labelled in red. Position of St2 (also known as Il1r1) is marked.

Figure 4 | T<sub>reg</sub> cell depletion improves adipose glucose uptake.

a, Hierarchical clustering of differentially expressed genes between fat T<sub>reg</sub> and T<sub>conv</sub> and splenic T<sub>reg</sub> and T<sub>conv</sub> cells from Foxp3<sup>CreERT2</sup> mice (47 weeks, cells pooled from 3–4 mice, same data set used in b, e). b, Fragments per kilobase of transcripts per million mapped reads (FPKM) values of selected genes important for Treg cell identity and canonical suppressive function. c, d, In vitro suppression assay of T<sub>reg</sub> cells (pooled from retired breeders, added at 1:1 ratio with splenic T<sub>conv</sub> cells, conducted in triplicate). e, Representative carboxyfluorescein succinimidyl ester (CFSE) tracings of T<sub>conv</sub> cells with or without fTreg cells. Gating indicates percentage of dividing cells. d, Expansion index of T<sub>reg</sub> cells. e, Fold change in expression levels of differentially expressed genes across fat T<sub>reg</sub> and T<sub>conv</sub> and splenic T<sub>reg</sub> and T<sub>conv</sub> cells. Fat Treg cell cluster genes are labelled in red. Position of St2 (also known as Il1r1) is marked.

suggesting that distinct pathophysologies drive obesity- versus age-associated IR.

Taken together, our data provide evidence that distinct adipose immune populations orchestrate unique features of age- and obesity-associated IR. We show in the non-obese setting that Pparγ-positive T<sub>reg</sub> cells accumulate to unusually high levels (6.7%) as a function of age, exacerbating both the decline of adipose metabolic function as well as the rise in IR (Fig. 4o). These results are in marked contrast to the increased role of M1 adipose tissue macrophages in metabolic dysfunction linked to obesity, coupled with a suppression of T<sub>reg</sub> levels to 0.9%. Thus, these studies highlight contrasting roles of the immune compartment in contributing to key aspects of adipose health and disease.

Given the classical immune suppressive and anti-inflammatory nature of T<sub>reg</sub> cells, we speculate that the chronic inflammatory processes that drive obesity-associated IR seem unlikely to be driving age-associated IR. Indeed, there is increasing appreciation that maintaining a certain degree of inflammation is beneficial for adipose tissue remodelling and its metabolic function.<sup>29</sup> Failure to preserve an optimal immune state in the aged adipose tissue may directly contribute to metabolic disorders such as IR and age-associated diabetes. We suggest type IV diabetes as a designation for non-obese-dependent T<sub>reg</sub>-driven metabolic disease of the elderly.

In this context, it is of particular significance that T<sub>reg</sub> cells in aged adipose tissue express the cytokine receptor ST2 at ~30–60-fold higher levels than in other sites such as spleen, making the T<sub>reg</sub> cell population sensitive to depletion via anti-ST2 treatment. While ST2 has been implicated in other physiological processes and immune cell types that may also affect glucose homeostasis, this simple T<sub>reg</sub> cell depletion approach increases adipose tissue insulin sensitivity suggesting the potential of selective T<sub>reg</sub> cell depletion therapy in the prevention of age-related IR.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 23 May; accepted 28 October 2015.
Published online 18 November 2015.
1. Ferrante, A. W. Jr. Macrophages, fat, and the emergence of immunometabolism. *J. Clin. Invest.* **123**, 4992–4993 (2013).
2. Lumeng, C. N. & Saltiel, A. R. Inflammatory links between obesity and metabolic disease. *J. Clin. Invest.* **121**, 2111–2117 (2011).
3. Mathis, D. Immunological goings-on in visceral adipose tissue. *Cell Metab.* **17**, 851–859 (2013).
4. Osborn, D. & Olefsky, J. M. The cellular and signaling networks linking the immune system and metabolism in obesity-related insulin resistance. *J. Clin. Invest.* **112**, 1821–1830 (2003).
5. CDC. 2014 National Diabetes Statistics Report; http://www.cdc.gov/diabetes/data/statistics/2014statisticsreport.html (2014).
6. Wu, D. & Olefsky, J. M. The cellular and signaling networks linking the immune system and metabolism in disease. *Annu. Rev. Immunol.* **32**, 307–312 (2014).
7. Weisberg, S. P. Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. *Cell Metab.* **15**, 1279–1291 (2012).
8. Lumeng, C. N. et al. Aging is associated with an increase in T cells and inflammatory macrophages in visceral adipose tissue. *J. Immunol.* **187**, 6208–6216 (2011).
9. Miano, M. J. & Olefsky, J. M. Eosinophils sustain adipose alternatively activated macrophages with glucocorticoid-dependent metabolic function. *Cell Metab.* **13**, 363–374 (2011).
10. Qiu, Y. et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* **157**, 1292–1308 (2014).
11. Odgaard, J. L. et al. Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. *Nature* **447**, 1116–1120 (2007).
12. Fujisaka, S. et al. Regulatory mechanisms for adipose tissue m1 and m2 macrophages in diet-induced obese mice. *Diabetes* **58**, 2574–2582 (2009).
13. Feurer, M. et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells. *Nature Med.* **18**, 1142–1151 (2012).
14. Weisberg, S. P. et al. Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. *Cell Metab.* **15**, 1279–1291 (2012).
15. Liu, Q. et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell Metab.* **15**, 1279–1291 (2012).
16. Liu, Q. et al. Eosinophils sustain beige fat metabolism and improves insulin resistance. *Proc. Natl Acad. Sci. USA* **109**, 1575–1591 (2009).
17. Khan, T. et al. Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol. Cell. Biol.* **30**, 271–275 (2014).
18. Khan, T. et al. Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol. Cell. Biol.* **30**, 271–275 (2014).
19. Houtkooper, R. H. et al. The metabolic footprint of aging in mice. *Sci. Rep.* **1**, 134 (2011).
20. Sun, K. et al. Diane 33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–568 (2014).
21. Asterholm, I. W. et al. Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. *Cell Metab.* **20**, 103–118 (2014).

**Author Contributions** S.P.B., J.M.S., M.D., R.M.E. and Y. Zheng designed the research. S.P.B., J.M.S., S.L., A.R.A., R.T.Y., M.D., R.M.E. and Y. Zheng performed research. S.P.B., J.M.S., M.L., C.L., A.R.A., R.T.Y., M.D., R.M.E. and Y. Zheng analysed data. Y. Zheng wrote the manuscript.

**Acknowledgements** We would like to thank L. Chong, J. Alvarez, Y. Dai, S. Kaufman and B. Collins for technical assistance, L. Ong and C. Brondos for administrative assistance, and J. Simon for assistance with graphics. S.P.B. is supported by National Institutes of Health (NIH) grants F30 DK096828 and T32 GM007198. C.L. and M.D. are funded by grants from the National Health and Medical Research Council of Australia Project grants 121535 and 1043199. R.M.E. is an Investigator of the Howard Hughes Medical Institute (HHMI) at the Salk Institute and March of Dimes Chair in Molecular and Developmental Biology, and is supported by NIH grants DK057978, DK090962, HL088093, HL105278 and ES010337, the Glenn Foundation for Medical Research, the Leona M. and Harry B. Helmsley Trust, the California Institute for Regenerative Medicine and The Ellison Medical Foundation. Y. Zheng is supported by the Nomis Foundation, the Rita Allen Foundation, the Emerald Foundation, the Hearst Foundation, the National Multiple Sclerosis Society, and National Institutes of Health (AI099295 and AI107027). This work was also supported by National Cancer Institute funded Salk Institute Cancer Center core facilities (CA014195) and the James B. Pendleton Charitable Trust.

**Author Information** RNA-Seq data can be accessed in the NCBI Sequence Read Archive under the accession SRP053799. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.M.E. (evans@salk.edu) or Y.Z. (yzheng@salk.edu).
Methods

Mice. All mice were housed in the specific pathogen-free facilities at The Salk Institute for Biological Studies or purchased from Taconic Biosciences. C57BL/6NTac mice were purchased from Taconic Biosciences for comparative AIPs or studies that required wild-type aged mice. Age-matched retired breeders were purchased for AIPs of aged adipose tissue, and diet-induced obese C57BL/6NTac mice were purchased for profiling of obese adipose tissue. Foxp3−/− knockouts were generated by crossing Foxp3−/− and Ppap2Δβ (ref. 31) mice. We used the Foxp3CreER(T2) (ref. 32) reporter mice when isolating Treg and Tcon cells from spleen and fat for subsequent RNA-seq analysis. Mice within the Salk Institute for Biological Studies received autoclaved normal chow (M laboratory rodent diet 5011, Harlan Teklad), irradiated HFD (60 kcal% fat, Research Diets), or irradiated HFD with rosiglitazone (30 mg kg−1 of food, Research Diets). All mice used for studies were male. All procedures involving animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) and Animal Resources Department (ARD) of the Salk Institute for Biological Studies.

AIPs. Visceral (epidymal) and subcutaneous (inguinal) adipose depots were dissected from mice after 10 ml PBS perfusion through the left ventricle. Inguinal lymph nodes resident in inguinal adipose tissue were removed. Adipose tissue was minced into fine pieces (2–5 mm3) and digested in stromal vascular isolation buffer (100 mM HEPES, pH 7.4, 120 mM NaCl, 50 mM KCl, 5 mM glucose, 1 mM CaCl2, and 1.5% BSA) containing 1 mg ml−1 collagenase at 37 °C with intermittent shaking for 1.5 h. The suspension was then passed through a 100-μm mesh to remove undigested clumps and debris. The flow-through was allowed to stand for 10 min to separate the floating adipocyte fraction and infranatant containing the stromal vascular fraction. The infranatant was removed while minimally disturbing the floating adipocyte fraction and centrifuged at 400g for 10 min. The pellet containing the stromal vascular fraction was washed once in 10 ml RPMI. The resultant isolated cells were subjected to FACs analysis. The following antibodies were used to examine the adipose-immune profile. BioLegend: CD45.2 (104), CD44 (IM7), C626L (MEL-14), TCGR/d (GL3), CD19 (6D5), CD25 (PC61), CD206 (C066/2C), CD301 (LOM-14); eBioscience: CD3 (145-2C11), CD25 (PC61), CD4 (RM4–5), CD86 (RB6-8C5); BD Pharmingen: Siglec-F (E50–2440); BD Biosciences: CD8a (53-6.7), Foxp3 (FJK-16s), F4/80 (BM8), CD11c (N418), CD11b (M1/70); Tonbo: CD4 (RM4–5), TCRb (H57–597), B220 (RA3–6B2), NK1.1 (PK136), CD49b (C068C2), CD301 (LOM-14); eBioscience: CD3 (145-2C11), CD25 (PC61), CD4 (RM4–5), CD86 (RB6-8C5); BD Pharmingen: Siglec-F (E50–2440); BD Biosciences: CD8a (53-6.7). Cells were analysed using BD FACSaria instrument (Becton Dickenson) and FlowJo software (Tree Star).

Body composition and adipocyte size analyses. Body composition was measured with an Echo MRI-100 body composition analyser (Echo Medical Systems). VAT (epidymal) was dissected, and the wet weight was determined. Adipose tissues were fixed in 10% formalin, sectioned, and stained in haematoxylin and eosin. An adipocyte cross-sectional area was determined from photomicrographs of VAT using ImageJ.

In vivo metabolic phenotype analysis. Real-time metabolic analyses were conducted in a Comprehensive Lab Animal Monitoring System (Columbus Instruments). Oxygen consumption and ambulatory counts were determined for at least three consecutive days and nights after at least 24 h for adaptation before data recording.

Principal component analysis of AIPs. Non-macrophage immune cell populations, described as percentage of the total CD45.2+ immune compartment, were inputted into MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/ MetaboAnalyst/) for PCA. No normalizations, transformations, or scalings were implemented.

Glucose homeostasis studies. Fasting was induced for 6 h, except for glucose tolerance tests, which were conducted after overnight fasting. Glucose (1–2 g kg−1, i.p.) and insulin (0.5–1.0 U kg−1, i.p.) was injected for glucose tolerance tests and insulin tolerance tests, respectively. Blood glucose was monitored using a Nova Max Plus glucometer.

Histological analyses. Sections (4 μm) of fixed tissues were stained with haematoxylin and eosin according to standard procedures. Histopathological scores were graded on blinded samples for severity and extent of inflammation and morphological changes by a pathologist.

Serum analyses. Blood was collected by tail bleeding or right atrial puncture. Non-esterified fatty acids (Wako) and triglycerides (Thermo) were measured using colorimetric methods. Serum insulin levels (Ultra Sensitive Insulin, Crystal Chem) were measured by ELISA. Serum cytokine and metabolic hormone levels were analysed by the Luminex Bio-Plex system using the Mouse Cytokine 23-Plex Panel and Diabetes Panel, respectively, according to the manufacturer's instructions (Bio-Rad).

Core body temperature. Mice were single housed, and core body temperature was measured with a clinical rectal thermometer (Thermalert model TH-5; Physitemp) during the middle of the light cycle. The probe was dipped in a room temperature lubricating glycerol before insertion.

Ex vivo 2-DG uptake assays. Adipose tissue was dissected from mouse, cut into small pieces with scissors, washed and incubated for 30 min with Krebs-Ringer Bicarbonate HEPES buffer (KRBH, 120 mM NaCl, 4 mM KH2PO4, 1 mM MgSO4, 0.75 mM CaCl2, 30 mM HEPES and 10 mM NaHCO3, pH 7.4, supplemented with 1% fatty-acid-free BSA). For determination of exogenous insulin-stimulated 2-deoxy-d-glucose (2-DG) uptake, adipose tissue was incubated in KRBH with 100–200 μg ml−1 insulin for 30 min at 37 °C. Cold 2-DG and hot 2-DG-1,2-3H(N) was added to incubated adipose tissue such that the final concentration of cold 2-DG was 0.1 mM and final quantity of hot 2-DG-1,2-3H(N) was 0.1 μCi (assuming total reaction volume ~400 μl). Adipose tissue was added for 20 min at 37 °C, then washed three times with PBS before being lysed by scintillation fluid. 2-DG uptake was determined by measuring scintillation counts normalized to adipose tissue mass used for assay. Non-specific 2-DG uptake levels were determined by co-treating adipose tissue with troglitazin B (0.1 μM final concentration) with the addition of cold and hot 2-DG.

IL-2–anti-IL-2 complex and IL-33 injections. IL-2–anti-IL-2 complexes were prepared by incubating 2 μg of mouse IL-2 (Biolegend) with 1 μg of anti-IL-2 antibody (JES6.1, Bexcell) in a total volume of 200 μl PBS for 30 min at 37 °C (amounts given per injection). Mice were injected i.p. three times (days 0, 1 and 2) and analysed on day 8. For IL-33 expansion assays, mice were injected i.p. with 0.5 μg of recombinant mouse IL-33 in PBS (R&D systems) three times (days 0, 2 and 4) and analysed on day 6. PBS was used for control injections.

RNA-Seq library generation. Total RNA was isolated from sorted cells or whole tissues using TRIzol reagent (Invitrogen) as per the manufacturer’s instructions and treated with DNasel (Qiagen) for 30 min at 22 °C. Sequencing libraries were prepared from 10–100 ng of total RNA using the TruSeq RNA sample preparation kit v2 (Illumina) according to the manufacturer’s protocol. In brief, mRNA was purified, fragmented and used for first- and second-strand cDNA synthesis followed by adenylation of 3′ ends. Samples were ligated to unique adaptors and subjected to PCR amplification. Libraries were then validated using the 2100 BioAnalyzer (Agilent), normalized and pooled for sequencing. RNA-Seq libraries prepared from two biological replicates for each experimental condition were sequenced on the Illumina HiSeq 2500 using barcoded multiplexing and a 100-bp read length.

High-throughput sequencing and analysis. Image analysis and base calling were done with Illumina CASAVA 1.8.2. This yielded a median of 29.9 M usable reads per sample. Short read sequences were mapped to a UCSC mm9 reference sequence using the RNA-Seq aligner STAR35. Known splice junctions from mm9 were supplied to the aligner and de novo junction discovery was also permitted. Differential gene expression analysis, statistical testing and annotation were performed using Cuffdiff 2 (ref. 34). Transcript expression was calculated as gene-level relative abundance in fragments per kilobase of exon model per million mapped fragments and employed correction for transcript abundance bias36. RNA-Seq results for genes of interest were also explored visually using the UCSC Genome Browser.

Hierarchical clustering. Differentially expressed gene names and corresponding FPKM values across samples were inputted into GENE-E (Broad Institute) for hierarchical clustering analysis (implemented one minus pearson correlation for sample and gene distance metrics and the average linkage method) and visualization. Gene cluster names were created to describe the gene expression characteristics within each cluster (that is, fat-residence cluster refers to the gene cluster in which genes are expressed at greater levels in T cells residing in fat). Fat-Treg Cluster refers to the gene cluster in which genes are expressed highest in only the Freg cells.

In vitro suppression assay. Freg cells were isolated from aged wild-type mice (CD45.2+) treated with IL-2–anti-IL-2 complexes to expand Freg cell numbers, as described above with isolation conducted on day 6. Stromal vascular fractions were isolated from VAT as described above, and Freg cells were sorted from stromal vascular fractions using the BD FACSaria instrument (Becton Dickenson), gating on CD45.2+ CD4+ CD25+ cells. CD45.1+ mice were used to isolate splenic responder T cells, which were purified by positive selection using CD4-specific Dynabeads (Invitrogen), followed by sorting on a BD FACSaria cell sorter, gating on CD45.1+ CD4+ CD25+ C626L+ CD44hi cells. Antigen-presenting cells were prepared from wild-type B6 splenocytes by T cell depletion using Thy1-specific MACS beads. CFSE labelled effector T cells (5 × 104 cells well−1) were added to Freg cells at the indicated ratio in the presence of irradiated (30 Gy) antigen-presenting cells (1 × 105 cells well−1) in 96-well plates in complete RPMI1640 medium supplemented with 10% FBS and CD3 antibody (1 μg ml−1). Cell proliferation and expansion index were determined 96 h later using the BD FACSaria instrument and analysed with the FlowJo software package (Tree Star).
ST2 studies and anti-ST2-depleting antibody treatment. Antibody for ST2 FACS analysis was purchased from MD Bioproducts, clone DJ8. For Treg cell depletion, mice were injected i.p. with 200 μg anti-ST2 antibodies (R&D systems, clone 245707) or isotype control (Bioxcell) twice (days 0 and 2) and euthanized for analysis on day 3.

Statistical analyses. Statistical analyses were performed with Prism 6.0 (GraphPad). P values were calculated using two-tailed unpaired or paired Student’s t-test. When analysing AIPs, we used a false discovery rate approach to avoid the problem of an inflated false positive rate due to the substantial number of hypothesis tests. Mice cohort size was designed to be sufficient to enable statistical significance to be accurately determined. When applicable, mice were randomly assigned to treatment or control groups. No animals were excluded from the statistical analysis, with the exception of exclusions due to technical errors, and the investigators were not blinded in the studies. Appropriate statistical analyses were applied, assuming a normal sample distribution, as specified in the figure legends. No estimate of variance was made between each group. All in vivo metabolic and glucose homeostasis experiments, ex vivo glucose uptake experiments, and AIP experiments were conducted with at least two independent cohorts. RNA-Seq experiments, Luminex profiling and histological analyses were conducted using multiple biological samples (as indicated in figure legends) from indicated cohorts.

Rubtsov, Y. P. et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546–558 (2008).

He, W. et al. Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc. Natl Acad. Sci. USA* **100**, 15712–15717 (2003).

Liston, A. et al. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. *Proc. Natl Acad. Sci. USA* **105**, 11903–11908 (2008).

Dobin, A. et al. STAR: ultrastable universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

Trapnell, C. et al. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature Biotechnol.* **31**, 46–53 (2013).

Roberts, A., Pimentel, H., Trapnell, C. & Pachter, L. Identification of novel transcripts in annotated genomes using RNA-Seq. *Bioinformatics* **27**, 2325–2329 (2011).

Monticelli, L. A. et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature Immunol.* **12**, 1045–1054 (2011).
Extended Data Figure 1 | Schematic outlining study premise and selected gating strategies used to generate AIPs. a, Schematic outlining study premise. b–d, AIPs were generated through the use of several distinct antibody cocktails. Here, using Foxp3<sup>Cre</sup> (Foxp3-IRES-YFP-Cre) reporter mice, we show how the stromal vascular fraction of VAT was analysed by flow cytometry to identify several T cell subtypes (b), macrophage subsets (c) and eosinophils and neutrophils (d).
Extended Data Figure 2 | AIPs of aged fTreg knockout and control mice in VAT, SAT and spleen. a, AIPs of aged (~14 months) fTreg knockout and control male mice depicting immune cell abundance, expressed as percentage of CD45.2⁺ cells. Entirety of immune compartment (top) is further divided into pan-macrophage (middle) and non-macrophage (bottom) pie charts (n = 9 mice per group). b–d, Immune cell abundance between fTreg knockout and control mice, expressed as cells per gram of VAT (b), SAT (c) and spleen (d) (n = 9 mice per group). Data are mean ± s.e.m. #, false discovery rate < 2%.
Extended Data Figure 3 | Aged fTreg knockout mice do not show signs of systemic autoimmunity or breakdown in peripheral tolerance.

a, Percentage of splenic naive CD4+ T cells as defined by CD62hi CD44lo relative to total CD4+ CD25− Foxp3Cre population (n = 9 mice per group).
b, Representative histology of gastrointestinal tract—duodenum, jejunum, ileum and colon (left to right) (n = 3 mice per group). There were no significant lesions observed or differences in inflammation, epithelial changes, or mucosal architecture between the two groups (H&E, original magnification, ×100). Scale bar, 50 μm.
c, Histopathology score in the small intestine and colon of fTreg knockout and control mice. The severity and extent of inflammation and epithelial changes as well as mucosal architecture were each graded on a score of 1 (minimal) to 5 (severe) and added to obtain an overall score over 20. There were minimal inflammatory changes with no significant differences between groups.
d, Multiplex inflammation panel of serum from fTreg knockout and control mice (n = 4 pooled samples (3 mice per sample) per group). Data are mean ± s.e.m. *P < 0.05, ***P < 0.001, Student’s t-test.
Extended Data Figure 4 | Weight-matched cohorts’ body weights and adipocyte size frequency in VAT of aged control and fTreg knockout mice. 

**a**, Body weights of fTreg knockout and control male mice used in weight-matched metabolic studies in young (12 week; control n = 9; fTreg KO n = 9), aged (36 week; control, n = 9 mice; fTreg KO, n = 11 mice) and obese (diet-induced obese, 12 weeks of HFD starting at 12 weeks; control n = 10; fTreg KO n = 10) settings. 

**b**, Frequency of small (0–5,000 μm²), medium (5,000–10,000 μm²) and large (>10,000 μm²) adipocytes in VAT of aged control and fTreg knockout mice (n = 3 mice per group, 850 adipocytes counted from control mice, 269 adipocytes counted from fTreg knockout adipose). Data are mean ± s.e.m.
Extended Data Figure 5 | VAT AIPs of obese ftreg knockout and control mice. a, AIPs of diet-induced obese (16 weeks high fat diet started at 12 weeks) control (n = 6 mice) and ftreg knockout (n = 8 mice) male mice depicting immune cell abundance, expressed as percentage of CD45.2+ cells. Entirety of immune compartment (top) is further divided into pan-macrophage (middle) and non-macrophage (bottom) pie charts. b, Immune cell abundance between ftreg knockout and control mice, expressed as cells per gram of VAT (n = 9 mice per group). Data are mean ± s.e.m.
Extended Data Figure 6 | fTreg cells are dispensable for TZDs to exert their therapeutic insulin-sensitizing effect. a, Scheme used for longitudinal interventional study of control and fTreg knockout mice which indicates when particular assays were conducted and whose results are described in b–g, in which rosiglitazone (Rosi) was introduced in diet after firmly establishing obesity with a HFD alone for 12 weeks (n = 8 mice per group). b, Cohort weights during course of study. Black arrow indicates introduction of rosiglitazone to the diet. c, Homeostatic model assessment of IR (HOMA-IR). d, e, Glucose tolerance test (d) and glucose excursions of glucose tolerance test (e) described as area under curve (AUC). f, g, Insulin tolerance test (f) and bar-graph quantitation of relative serum glucose decrease during insulin tolerance test (g) described as area above curve (AAC). h, Scheme used for parallel prophylactic study of control and fTreg knockout mice, the results of which are described in i–l, in which mice were placed on a HFD or HFD with rosiglitazone for 12 weeks (n = 8 mice per group). i, Cohort weights at end of study. j, HOMA-IR. k, l, Glucose and insulin tolerance tests of control (k) or fTreg knockout (l) mice fed HFD or HFD with rosiglitazone. m, Scheme used to determine temporal relationship of TZD-induced fTreg expansion and TZD-induced insulin-sensitization in wild-type mice, the results of which are described in n–q, where mice were fed HFD or HFD with rosiglitazone for up to 11 weeks (n = 10 mice per group, 5 mice of each group were euthanized at 5 weeks after diet introduction and remaining 5 mice were euthanized at 11 weeks). n, HOMA-IR at 4 weeks. o, p, Glucose (o) and insulin (p) tolerance tests at 5 weeks. q, Relative fTreg cell enrichment of mice fed HFD with rosiglitazone versus mice fed HFD alone at 5 and at 11 weeks. Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.
Extended Data Figure 7 | Increased TNFα levels and gene expression pattern of aged fTreg knockout adipose tissue is consistent with an improved adipose remodelling capacity. a, TNFα levels quantified by ELISA of whole adipose lysate (~40 weeks, \( n = 6 \) per group). b–d, FPKM values of all differentially expressed genes (b), differentially expressed collagens (c) and differentially expressed extracellular matrix proteases (d) in VAT from aged fTreg knockout and control mice (~40 weeks, \( n = 3 \) mice per group). Data are mean ± s.e.m. ***\( P < 0.001 \), Student's t-test.
Extended Data Figure 8 | \( \text{T}_{\text{reg}} \) cell gene expression and depletion with anti-ST2 antibody treatment. a, Expression of several canonical \( \text{T}_{\text{reg}} \) cell genes across fat and splenic \( \text{T}_{\text{reg}} \) and fat and splenic \( \text{T}_{\text{conv}} \) cells. Cells were pooled from 3 and 4 mice before isolating RNA for subsequent RNA-Seq analysis. b, Expression of \( \text{St2} \) across all haematopoietic cells catalogued in the ImmGen database. Position of adipose CD4\(^+\) CD25\(^+\) T cells is marked. c, Total weight before beginning course of anti-ST2 or isotype control antibodies (day 0) and after terminal analysis (day 3) (\( n = 4 \) mice per group). d, Spleen weight (d) and percentage of splenic naive CD4\(^+\) T cells as defined by CD62L\(^{hi}\) CD44\(^{lo}\) relative to total splenic CD45\(^{+}\) T cell population (e) of mice after terminal analysis (day 3, \( n = 4 \) mice per group). Data are mean ± s.e.m.
Extended Data Figure 9 | Aged ftreg knockout mice are resistant to short-term, but not persistent, HFD-induced weight gain and IR.
a–e, Aged control and ftreg knockout mice were placed on HFD and monitored throughout course of diet for weight (a), fasting glucose levels (b), fasting serum insulin levels (c), performance on glucose tolerance test (d), and on insulin tolerance test (e). (Control, n = 10; ftreg KO, n = 11; mice were aged 27–29 weeks and weight-matched before HFD was introduced.) Data are mean ± s.e.m. ns, non-significant, *P < 0.05, **P < 0.01, Student’s t-test.
Extended Data Table 1 | Antibodies used to identify the given immune cell type molecularly

| Immune Cell Type          | Molecular Identification Scheme                  |
|---------------------------|--------------------------------------------------|
| TCRyδ                      | CD45.2^+ F4/80^− CD3^+ TCRyδ^− TCRyδ^+            |
| CD8^+                     | CD45.2^+ F4/80^− CD3^+ TCRyδ^+ CD4^− CD8^+        |
| Treg CD4^+                | CD45.2^+ CD4^+ CD25^− Foxp3^+                   |
| Naive CD4^+               | CD45.2^+ CD4^+ CD25^− Foxp3^− CD62L^H CD44^lo  |
| Activated CD4^+           | CD45.2^+ CD4^+ CD25^− Foxp3^− CD62L^lo CD44^hi  |
| NKT                       | CD45.2^+ NK1.1^+ TCRyδ^+                        |
| NK                        | CD45.2^+ NK1.1^+ TCRyδ^−                        |
| B                         | CD45.2^+ NK1.1^− CD19^+                         |
| Eosinophil                | CD45.2^+ F4/80^− Siglec-F^+                     |
| Neutrophil                | CD45.2^+ F4/80^− CD11c^+ CD11b^− Ly6G^+          |
| M2 ATM                     | CD45.2^+ F4/80^− CD11c med CD206^+              |
| M1 ATM                     | CD45.2^+ F4/80^− CD11c H CD206^−                |
| DN (Double-negative) ATM   | CD45.2^+ F4/80^− CD11c^− CD206^−                |