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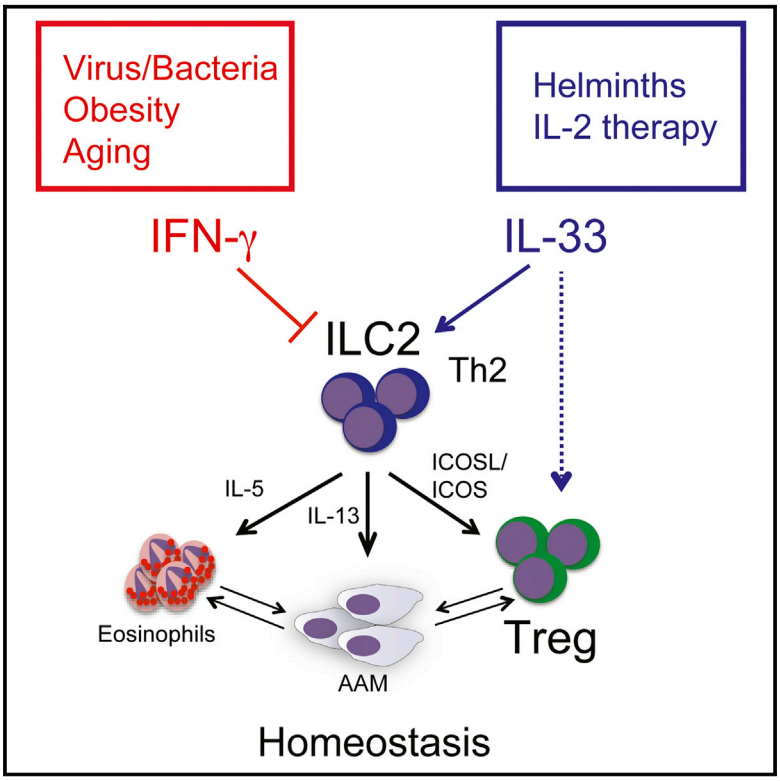
Graphical Abstract



Highlights

- IL-33-activated ILC2s are required for Treg accumulation in type 2 immune responses
- IL-33 induction of Tregs depends on interactions between Treg ICOS and ILC2 ICOSL
- IFN-γ represses IL-33-mediated ILC2 activation and limits Treg cell accumulation

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In Brief

Group 2 innate lymphoid cells (ILC2s) and regulatory T (Treg) cells are systemically induced by helminth infection but also sustain metabolic homeostasis and contribute to tissue repair. Locksley and colleagues describe how the cytokines IL-33 and IFN-γ counter-regulate ILC2 activation to control Treg cell numbers and type 2 immune responses.

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Interleukin-33 and Interferon-γ Counter-Regulate Group 2 Innate Lymphoid Cell Activation during Immune Perturbation

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SUMMARY

Group 2 innate lymphoid cells (ILC2s) and regulatory T (Treg) cells are systemically induced by helminth infection but also sustain metabolic homeostasis in adipose tissue and contribute to tissue repair during injury. Here we show that interleukin-33 (IL-33) mediates activation of ILC2s and Treg cells in resting adipose tissue, but also after helminth infection or treatment with IL-2. Unexpectedly, ILC2-intrinsic IL-33 activation was required for Treg cell accumulation in vivo and was independent of ILC2 type 2 cytokines but partially dependent on direct co-stimulatory interactions via ICOSL-ICOS. IFN-γ inhibited ILC2 activation and Treg cell accumulation by IL-33 in infected tissue, as well as adipose tissue, where repression increased with aging and high-fat diet-induced obesity. IL-33 and ILC2s are central mediators of type 2 immune responses that promote tissue and metabolic homeostasis, and IFN-γ suppresses this pathway, likely to promote inflammatory responses and divert metabolic resources necessary to protect the host.

INTRODUCTION

Allergic, or type 2, immunity occurs in response to parasitic helminths, restricting sites permissive for parasite reproduction and limiting tissue damage. Such responses involve activation of group 2 innate lymphoid cells (ILC2s) and adaptive $CD4^+$ Th2 cells, which together secrete the cytokines interleukin-4 (IL-4), IL-5, and IL-13 necessary for the accumulation of eosinophils and alternatively activated macrophages (AAMs) in involved tissues (Walker and McKenzie, 2013). In turn, chronic parasitism induces regulatory T (Treg) cells, which dampen immune pathology not only in helminth infection, but also in a variety of mouse and human conditions characterized by excess immune activation, such as autoimmunity, graft-versus-host disease, and metabolic syndrome (Johnston et al., 2014; McSorley and Maizels, 2012; Wiria et al., 2014). Indeed, one of the key issues during allergic pathology provoked by common environmental allergens might be the failure to induce or maintain Treg cells (Allen and Maizels, 2011). Although transcriptional networks are establishing connections between Treg cells and the subsets of effector T cells they restrain (Chaudhry and Rudensky, 2013), cellular networks that link these effector and regulatory modules are incompletely understood. Further insights will be important in considering therapeutic strategies to control chronic pathologic states characterized by loss (allergy, atopy) or gain (cancer, chronic infectious diseases) in this regulatory-to-effector cell balance.

Recent reports have called attention to the confluence of innate cells associated with type 2 immunity, including ILC2s, eosinophils, and AAMs, with Treg cells in visceral adipose tissue (VAT) of resting mice (Cipolletta et al., 2012; Feuerer et al., 2009; Molofsky et al., 2013; Odegaard et al., 2007; Qiu et al., 2014; Vasanthakumar et al., 2015; Wu et al., 2011). Maintaining this cellular architecture is necessary for metabolic homeostasis, and its loss during obesity is associated with increased inflammatory T cells, macrophages, and the development of insulin resistance and type 2 diabetes. Adipose ILC2s and Treg cells constitutively express interleukin-1 receptor-like 1, (IL1RL1, ST2), the regulated subunit of the receptor for the IL-1 family member IL-33, a cytokine maintained in the nucleus of some epithelial, endothelial, and mesenchymal cells (Pichery et al., 2012) (Cayrol and Girard, 2014). IL-33 is released during necrosis or possibly via other regulated mechanisms, and stimulates ILC2s to produce cytokines like IL-13 and IL-5, important for the response to helminths and allergens (Neill et al., 2010). Exogenous IL-33 induces expansion of adipose tissue ILC2 and Treg cells (Molofsky et al., 2013; Vasanthakumar et al., 2015) and beneficial metabolic effects (Miller et al., 2010), acting in part via ILC2-mediated induction of "beige" adipose tissue associated with increased heat production (Brestoff et al., 2014; Lee et al., 2015). IL-33 administration also expands systemic Treg cells that suppress the rejection of allogeneic cardiac transplants (Brunner et al., 2011; Turnquist et al., 2011), and IL-33 promotes

resolution of tissue damage in models of colitis (Duan et al., 2012; Schiering et al., 2014), hepatitis (Liang et al., 2013), cutaneous wounding (Yin et al., 2013), central nervous system injury (Gadani et al., 2015), and atherosclerosis (Miller et al., 2008). On the basis of these prior findings, here we determine the role of ILC2s in regulating Treg cells under conditions of elevated IL-33, including resting adipose tissue and during helminth infection. We found that ILC2-intrinsic IL-33 signaling and ICOSL expression promoted Treg cell accumulation, whereas the inflammatory cytokine IFN-γ counter-regulated the effects of IL-33, in part through direct effects on ILC2s.

RESULTS

Visceral Adipose Tissue IL-33 Promotes ILC2 Function and Treg Cell Maintenance

We documented high concentrations of IL-33 in adipose tissue as compared to lung or spleen (Figure 1A), corroborating prior studies (Miller et al., 2010; Vasanthakumar et al., 2015; Zeyda et al., 2012). IL-33 was expressed selectively in the nuclei of many adipose tissue endothelial cells (Figure 1B, Figure S1A and S1B, data not shown). Although we confirmed IL-33-positive cells in mouse lung and spleen, where expression has been reported for alveolar type 2 pneumocytes and fibroblastic reticular cells (FRC), respectively, endothelium from these tissues did not express IL-33 (Figure S1A, and Pichery et al., 2012). In contrast, human endothelial expression of IL-33 is more widespread (Pichery et al., 2012). VAT ILC2s express IL1RL1 (ST2) and localize near the adipose tissue vasculature (Figure S1B and Molofsky et al., 2013) suggesting they might be sensitized by endothelial IL-33. We used previously described "Red5" IL-5$^{tdtomato\text{-}cre}$ reporter mice (Nussbaum et al., 2013) to assess the expression of IL-5 in ILC2 without the need for ex vivo stimulation. ILC2 IL-5 production was diminished in adipose ILC2s from IL1RL1-deficient mice (Figure 1C, Figure S1C). Although less numerous than ILC2s, IL-5^{+} CD4^{+} Th2 cells that accumulate in adipose tissue also showed diminished IL-5 expression in the absence of IL-33 signals (Figures S1D and S1E). Despite their lower IL-5 production, the numbers of ILC2s and primed, IL-4-competent CD4^{+} Th2 cells in VAT were not decreased by the loss of IL-33 signaling (Figure S1C, data not shown). The attenuated IL-5 expression in VAT of IL1RL1-deficient mice resulted in a diminution in numbers of VAT eosinophils, consistent with a biologically relevant effect that was not evident in blood or lung (Figure 1D).

Treg cells accumulate in VAT of 4- to 6-month-old mice and express high amounts of GATA3, IL1RL1, KLRG1, and CD25 (Figure 1E, Figure S1F, data not shown), consistent with an activated or "effector" tissue-resident phenotype (Burzyn et al., 2013b; Cipolletta et al., 2012; Feuerer et al., 2009; Vasanthakumar et al., 2015). Accumulation of Treg cells was attenuated by loss of IL-33 signals in VAT, but not in lung or spleen (Figures 1F and 1G, Figure S1G). Even on normal chow diet, IL1RL1-deficient animals develop significant increases in VAT CD8^{+} T cells after 12–16 weeks (wild-type 6,801+/−1221 cells, IL1RL1-deficient 10,670+/−1176 cells, $p = 0.03$, $n = 16–18$). Thus, IL-33 promotes VAT ILC2 cytokine expression associated with the accumulation of VAT eosinophils and activated Treg cells, and suppresses the accumulation of CD8^{+} T cells, thus potentially limiting adipose inflammation and obesity (Miller et al., 2010).

Next we tested the ability of isolated Treg cells and ILC2s to respond directly to IL-33. Although splenic Treg cells, which are largely IL1RL1^{-} at the time of isolation, were not affected by addition of IL-33 to short-term in vitro suppression assays, VAT IL1RL1^{+} Treg cells demonstrated enhanced suppression in the presence of IL-33, particularly at low Treg-to-Teffector ratios (Figure 2A). Given once in vivo, IL-33 rapidly enhances CD25 expression on VAT IL1RL1^{+} Treg cells and drives their entry into the cell cycle (Figures S2A and S2B). VAT IL1RL1^{+} ILC2 and Th2 also respond to IL-33 by increasing CD25, proliferating, and increasing IL-5 production, as assessed by MFI of the IL-5 reporter (Figures S2C–S2E). Non-IL-5^{+} CD4^{+} T cells and NK cells in VAT do not respond to IL-33 or IL-2 over this time period (data not shown). Thus, IL-33 can directly promote the proliferation, activation and function of IL1RL1^{+} lymphocytes, including VAT Treg cells, ILC2s, and rare IL-5^{+} Th2 cells. IL-33 also increases CD25 expression on Treg cells and ILC2s, potentially increasing their sensitivity to IL-2.

IL-2-Mediated Expansion of Treg Cells and ILC2 Is Augmented by Endogenous IL-33

Low-dose IL-2 has been used to expand Treg cells and treat patients with autoimmune disease and graft-versus-host disease (Liao et al., 2011). ILC2 and Treg cells constitutively express the high-affinity IL-2 receptor, including CD25 (IL-2Rα), and both expand in vivo to IL-2 (Van Gool et al., 2014). Because IL-33 maintains VAT Treg cells and promotes their expression of CD25 (Figure S2B), we assessed whether systemic responses to IL-2 are reinforced by endogenous IL-33 in vivo. IL-2 promotes systemic expansion of Treg cells that express IL1RL1 and high levels of CD25 (Figure 2B). IL-2 modestly increased Treg cell cycling (Figure S2F), and the greatest proliferation occurred in IL1RL1^{+} Treg cells (Figure 2C). The IL-2-mediated expansion of VAT and lung Treg cells, and their upregulation of CD25, was attenuated in IL1RL1-deficient mice (Figure 2D-E, Figure S2G). Co-administration of IL-33 with IL-2 further enhanced Treg cell accumulation (Figure S2H). ILC2 also expanded to IL-2 in VAT and lungs, and expansion was blunted in both tissues in the absence of IL1RL1 (Figure 2F). These data show that endogenous tissue IL-33 cooperates with IL-2 to promote the expansion of both Treg cells and ILC2.

ILC2s Mediate IL-33-Dependent Treg Cell Homeostasis In Vivo

Despite direct effects of IL-33 in promoting IL1RL1^{+} Treg cells (Figure 2, Figure S2) (Schiering et al., 2014; Vasanthakumar et al., 2015), our data did not exclude indirect effects of IL-33 in vivo through its ability to activate ILC2s. Unexpectedly, loss of ILC2s via IL-5^{cre}-mediated cell deletion (Molofsky et al., 2013) significantly impaired the age-related Treg cell accumulation in VAT (Figure 3A, Figure S3A); this was particularly apparent in the IL1RL1^{+} Treg cell population (Figure 3B). ILC2-deficient mice displayed no overt signs of autoimmunity and young mice had normal numbers of VAT, lung, and spleen Treg cells (data not shown). To assess whether ILC2 were required for IL-33-mediated induction of Treg cells, we administered IL-33 to mice rendered ILC2-deficient using IL-5^{cre} or IL-13^{cre} strains crossed to deleter alleles (Molofsky et al., 2013; Nussbaum et al., 2013).

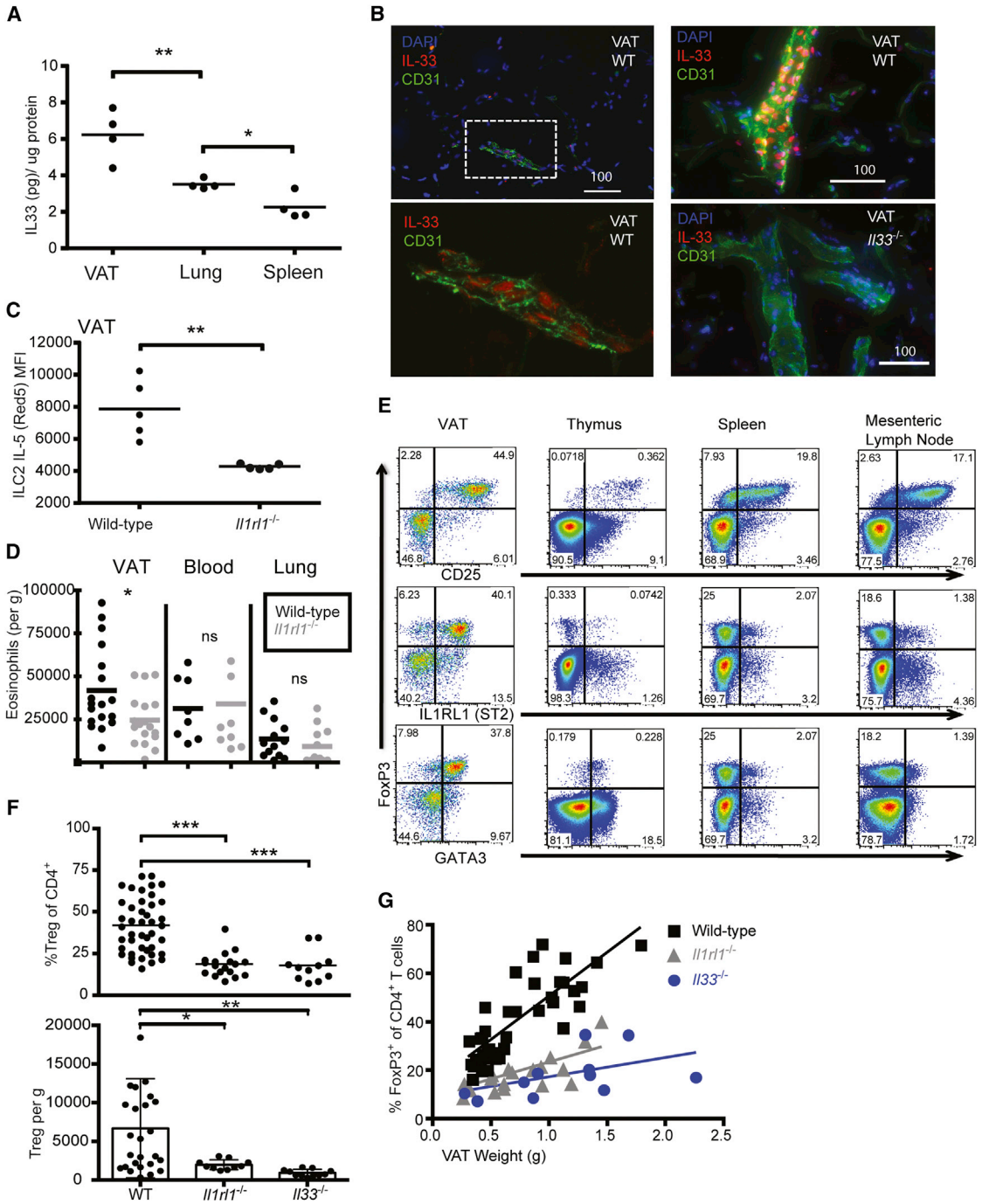

Figure 1. IL-33 Is an Endothelial Cytokine that Promotes ILC2 IL-5 Production, Eosinophilia, and Treg Cells in Visceral Adipose Tissue

(A) Total tissue IL-33 concentrations measured by ELISA.

(B) VAT immunofluorescence microscopy demonstrating IL-33 and CD31 endothelial cell co-localization in wild-type (WT) but not IL-33-deficient mice.

(C) Quantification of IL-5 reporter (Red5 tdtomato mean fluorescence intensity, MFI) from wild-type or IL1RL1-deficient (*Il1rl1*$^{-/-}$) *Il5*$^{tdtomato\text{-}cre/+}$ animals.

(D) Enumeration of total eosinophils in the indicated tissues and strains.

(E) Representative flow cytometric plots pre-gated on CD4$^+$ T cells of 4- to 6-month-old male animals with (F) quantification of percent (top) and total per gram (bottom) FoxP3$^+$ CD4$^+$ Treg cells from the tissues and strains indicated or (G) percent Treg cell expressed as a function of VAT weight. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant. Data are representative of three or more experiments (A–C, E) or pooled from three or more experiments (D, F, and G). Error bars represent SEM.

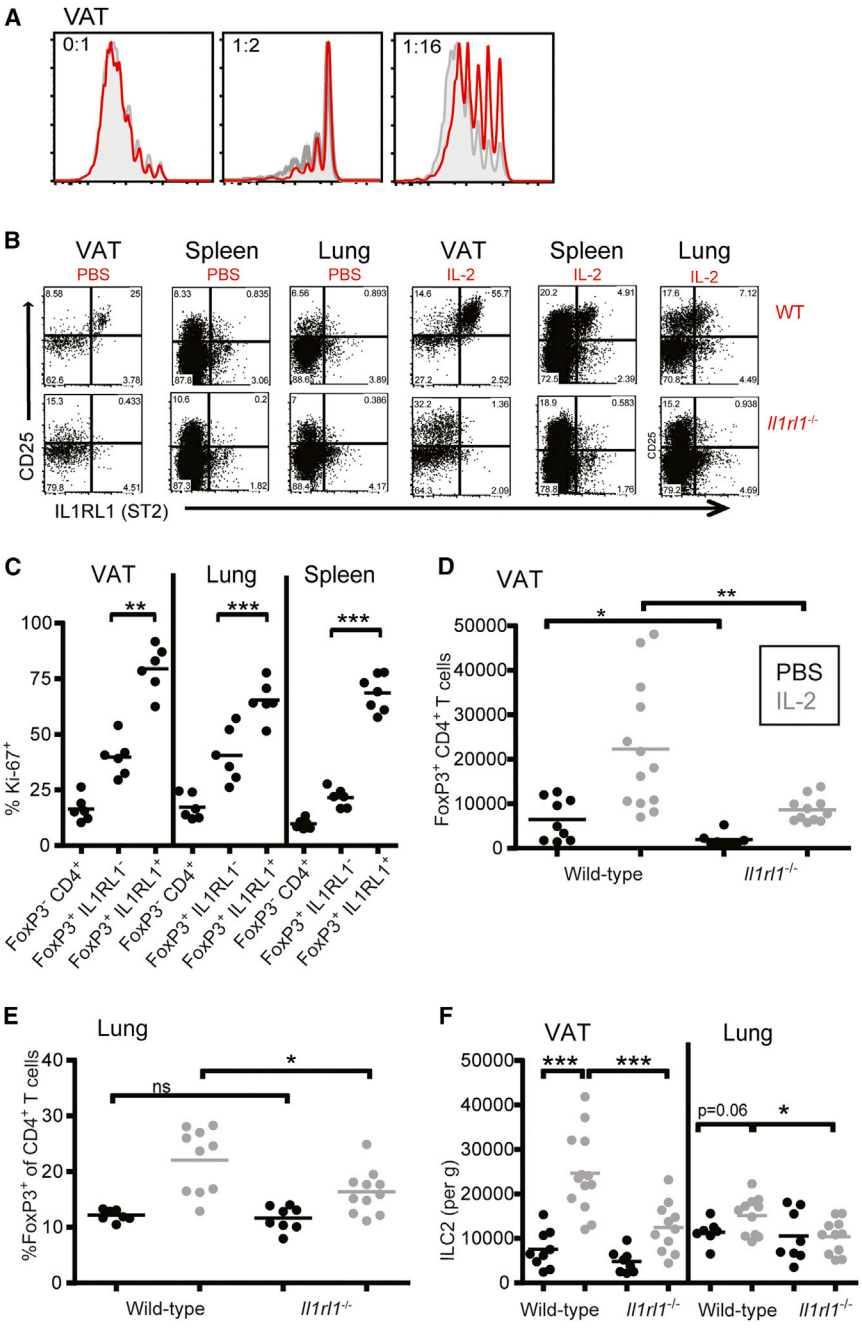

Figure 2. IL-2 Coordinates with IL-33 to Promote IL1RL1 (ST2)$^+$ Treg Cells and ILC2 Expansion and Function

(A) VAT FoxP3GFP$^+$ Treg cells from multiple mice were sort-purified and used in an in vitro suppression assay at the indicated Treg/Teff ratios by assessing CTV dilution in naive CD4$^+$ T cells in the presence (red lines) or absence (gray lines) of IL-33.

(B) Flow cytometric plots pre-gated on CD4$^+$ T cells from the indicated tissues of wild-type (top) or IL1RL1-deficient (*Il1rl1*$^{-/-}$) mice (bottom) treated with PBS (left) or IL-2 complexes (right), as indicated.

(C) Quantitation of Ki-67$^+$ proliferative cells from the indicated CD4$^+$ T cell subsets of IL-2-treated mice.

(D–F) FoxP3$^+$ CD4$^+$ Treg cells (D and E) or ILC2 (F) were quantitated from the indicated tissues after PBS or IL-2 complexes in wild-type or IL1RL1-deficient mice, as indicated. Black dots (PBS), gray dots (IL-2 complex). Data are representative of two to three experiments (A and B) or pooled from three experiments (C–F). Error bars represent SEM.

IL-33 robustly increased ILC2 in VAT, lung, and spleen of wild-type mice; IL-33 also promoted Treg cells comparably to IL-2 (Figures S3B and S3C, data not shown). In contrast, in ILC2-deficient mice, IL-33-induced Treg cell expansion was impaired (Figures 3C–3E, Figure S3D–S3F), and this was particularly marked in the subset of "activated" GATA3$^+$ IL1RL1$^+$ KLRG1$^+$ Treg cells (data not shown). MyD88 is a shared adaptor for TLR and IL-1 family signaling and is required for IL-33 signaling. To assess the cell-intrinsic role of IL-33 signaling in ILC2-directed Treg cell accumulation, we gave IL-33 to mice lacking the adaptor protein MyD88 in IL-5$^+$ ILC2s (IL-5$^{tdtomato\text{-}cre}$ × MyD88 flox). In multiple tissues, ILC2 expansion and proliferation were impaired and Treg cell accumulation was blunted (Figures 3F–3H, Figure S3G, data not shown). In contrast, mice lacking MyD88 in FoxP3$^+$ Treg cells (*Foxp3*$^{YFP\text{-}cre}$ × *Myd88* flox) showed normal proliferation and accumulation of ILC2 and Treg cells in response to IL-33, although a modest reduction in the KLRG1$^+$ IL1RL1$^+$ Treg cell subset was noted (Figures 3G–3I). These ILC2-mediated effects of IL-33 on Treg cell accumulation were not mediated by IL-5, IL-4, IL-13, or IL-9; Treg cell expansion to IL-33 was normal in mice lacking these cytokines (Figure 3C, Figures S3D and S3E, data not shown). FoxP3$^+$ Treg cells, in contrast to CD4$^+$ Th2 cells, did not express reporters for either IL-5 or IL-13 (data not shown). Thus, ILC2-intrinisic responses to IL-33, but not ILC2 canonical cytokines, are required for optimal IL-33-mediated expansion of Treg cells in vivo.

We next determined whether ILC2s mediate the normal expansion of Treg cells during helminth infection, a challenge associated with elevated IL-33. During primary infection with the nematode *Nippostrongylus brasiliensis*, which transiently passes through the lung before reaching the small intestine, lung KLRG1$^+$ Treg cell accumulation was significantly diminished in ILC2-deficient (IL-5 deleter) mice (Figure 3J); similar trends were observed in mice lacking the adaptor MyD88 in IL-5$^+$ cells (Figure S3H, data not shown). Although smaller numbers of IL-5-expressing Th2 cells were also depleted in these mice, and might contribute to these ILC2-dependent effects, we noted that after secondary helminth infection, accumulation of lung ILC2,

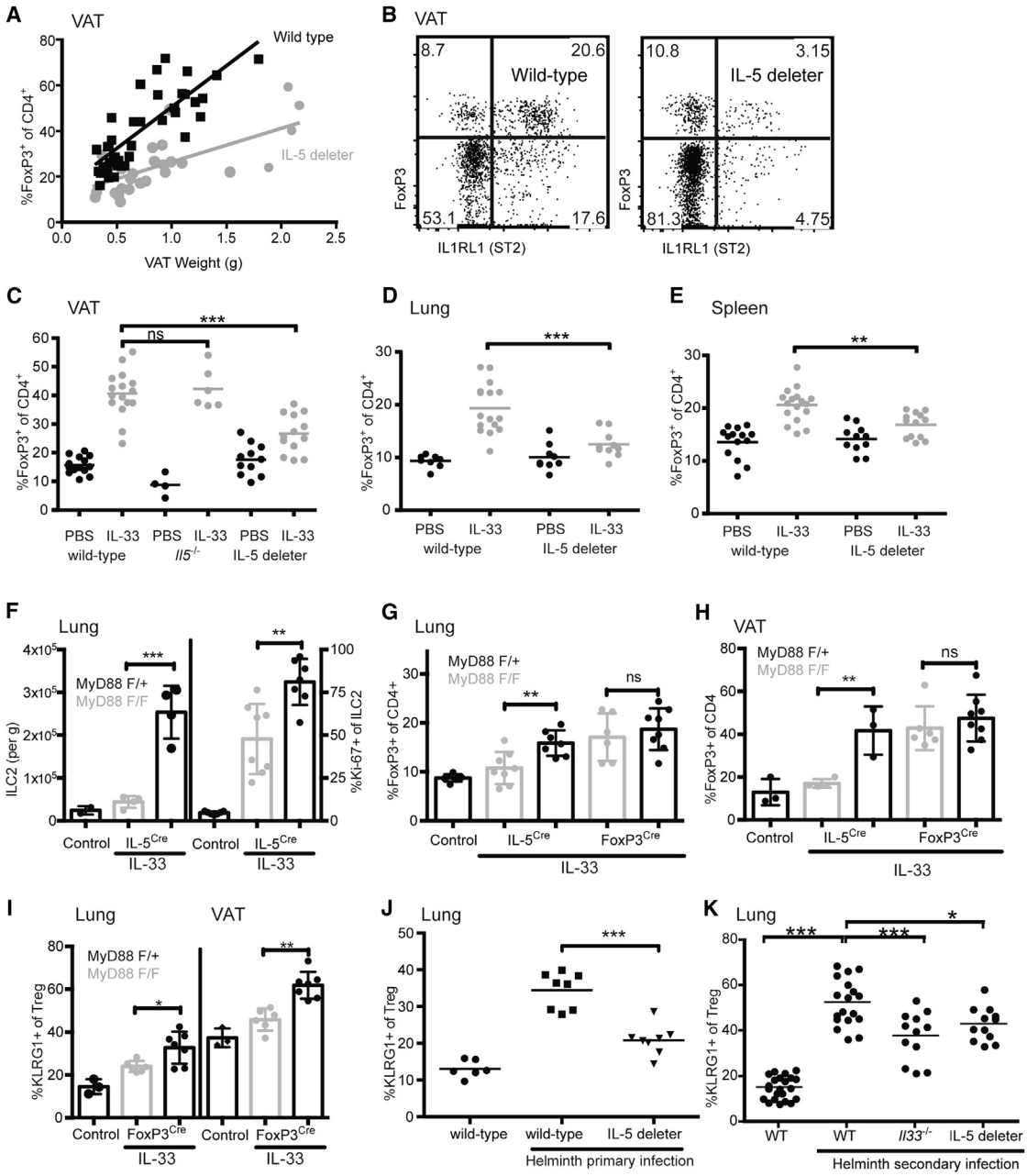

Figure 3. ILC2 Are Required for IL-33- and Helminth-Dependent Treg Cell Accumulation In Vivo

(A) FoxP3$^+$ CD4$^+$ Treg cells from VAT of 2- to 6-month-old male wild-type or IL-5 deleter (*Il5*$^{tdtomato\text{-}cre/tdtomato\text{-}cre}$ × *Rosa26*$^{-DTA/DTA}$) animals expressed as a correlation with VAT weight.

(B) Representative flow cytometric plots of resting VAT CD4$^+$ T cells from wild-type or IL-5 deleter mice.

(C–E) Percent FoxP3$^+$ Treg cells of CD4$^+$ T cells measured in wild-type, IL-5-deficient (*Il5*$^{tdtomato\text{-}cre/tdtomato\text{-}cre}$), or IL-5 deleter animals after control PBS (black dots) or IL-33 treatment (gray dots) from the indicated tissues.

(F–I) Cells were enumerated from the indicated tissues (VAT, Lung) and strains (*Il5*$^{tdtomato\text{-}cre/tdtomato\text{-}cre}$ or FoxP3$^{YFP\text{-}cre/y}$ × MyD88 flox/flox or flox/$^+$) after three doses of IL-33 or from untreated controls.

(J and K) Percent KLRG1$^+$ of FoxP3$^+$ Treg cells in lung was enumerated on (J) day 7–8 of primary infection or (K) 2 weeks after secondary infection with *N. brasiliensis* from the indicated strains. Data represent three or more experiments (B) or pooled from two (J and K) or three or more experiments (A, C–I). Note the double y axis in (F). Error bars represent SEM.

but not total CD4$^+$ Th2 cells, remained impaired in IL-33-deficient and IL-5 deleter mice (Figures S3I and S3J). KLRG1$^+$ Treg cells proportionately increased in lung, mesenteric lymph node (MLN) and VAT, and optimal accumulation depended on both IL-33 signaling and IL-5$^+$ ILC2s (Figure 3K, Figures S3K and S3L). Thus, IL-33, whether induced endogenously in

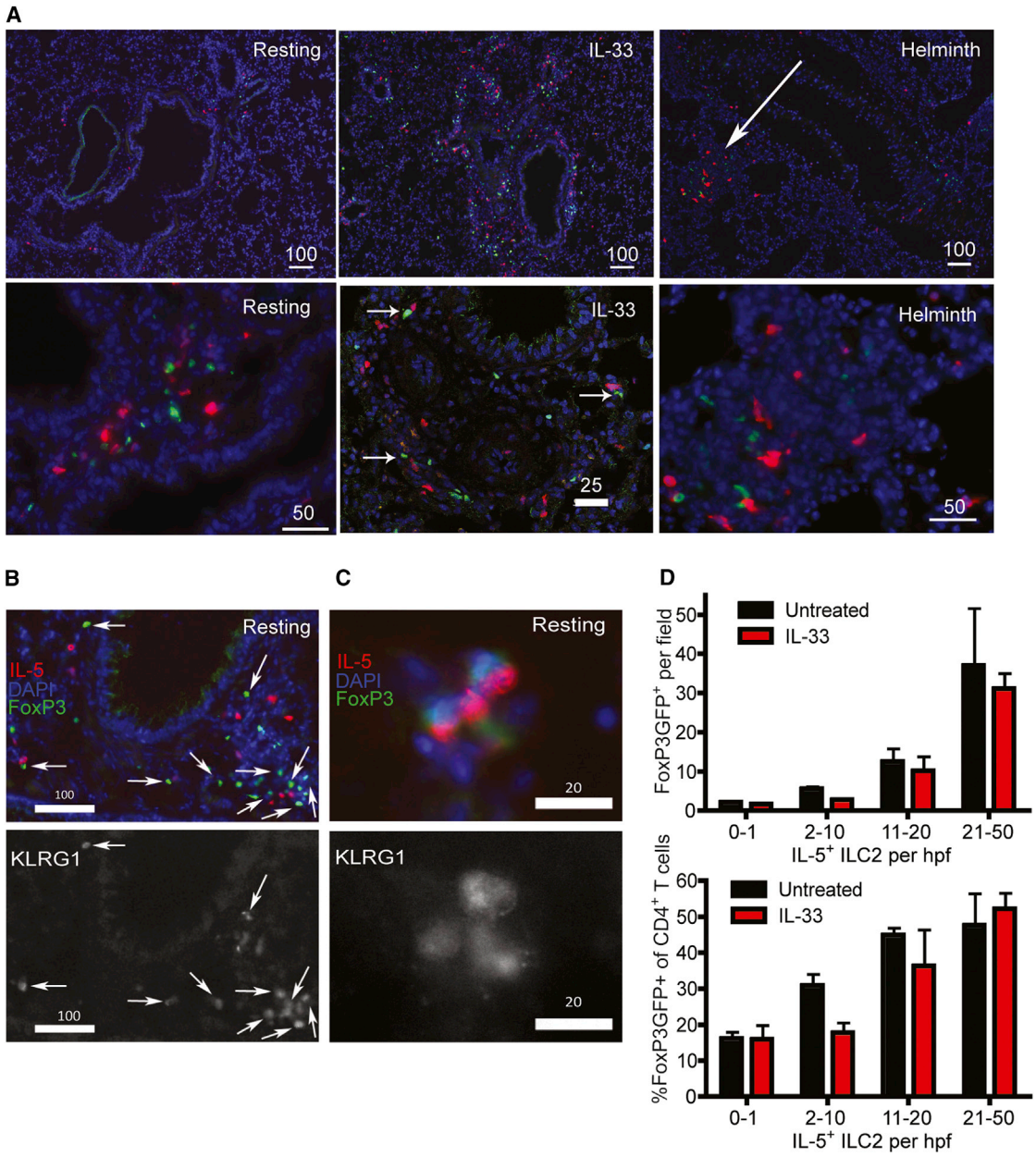

Figure 4. ILC2 and Treg Cells Co-Localize In Vivo

(A–C) Representative immunofluorescence microscopy images of lung under resting conditions (Resting), after IL-33 administration ×3 doses (IL-33), or 2 weeks after *N. brasiliensis* infection (Helminth) identifying IL-5$^+$ (tdtomato$^+$) cells, FoxP3$^+$ (GFP$^+$) cells, and (B and C) KLRG1$^+$ cells from Red5 (*Il5*$^{tdtomato\text{-}cre/+}$) Foxp3GFP double reporter animals.

(D) Quantification of total FoxP3GFP$^+$ cells and IL-5$^+$ ILC2 (top) or percent FoxP3GFP$^+$ of CD4$^+$ T cells and total IL-5$^+$ ILC2 (bottom) per high-powered field (400× total magnification). Data are expressed as total or percent FoxP3$^+$ Treg cells per IL-5$^+$ ILC2 and grouped as bins. (A–D) Representative data from two experiments with two or mice per group. Error bars represent SEM.

response to migratory helminths or provided exogenously, promoted ILC2-dependent increases in "activated" Treg cells in multiple tissues.

ILC2 and Treg Cells Interact In Vitro and In Vivo

IL-5$^+$ ILC2 and KLRG1$^+$ FoxP3$^+$ Treg cells localize to similar areas of the lung and VAT under resting conditions and in multiple tissues after induction by IL-33 or helminth infection (Figures 4A–4D, Figure S4A–S4E), suggesting these cells may interact in vivo. The co-stimulatory protein ICOS was reported to function in sustaining tissue Treg cells, as opposed to the role for IL-2 in promoting Treg cell survival in lymphoid organs (Smigiel et al., 2014). When assessed directly ex vivo, ILC2s express ICOS ligand (ICOSL) at amounts comparable to B cells

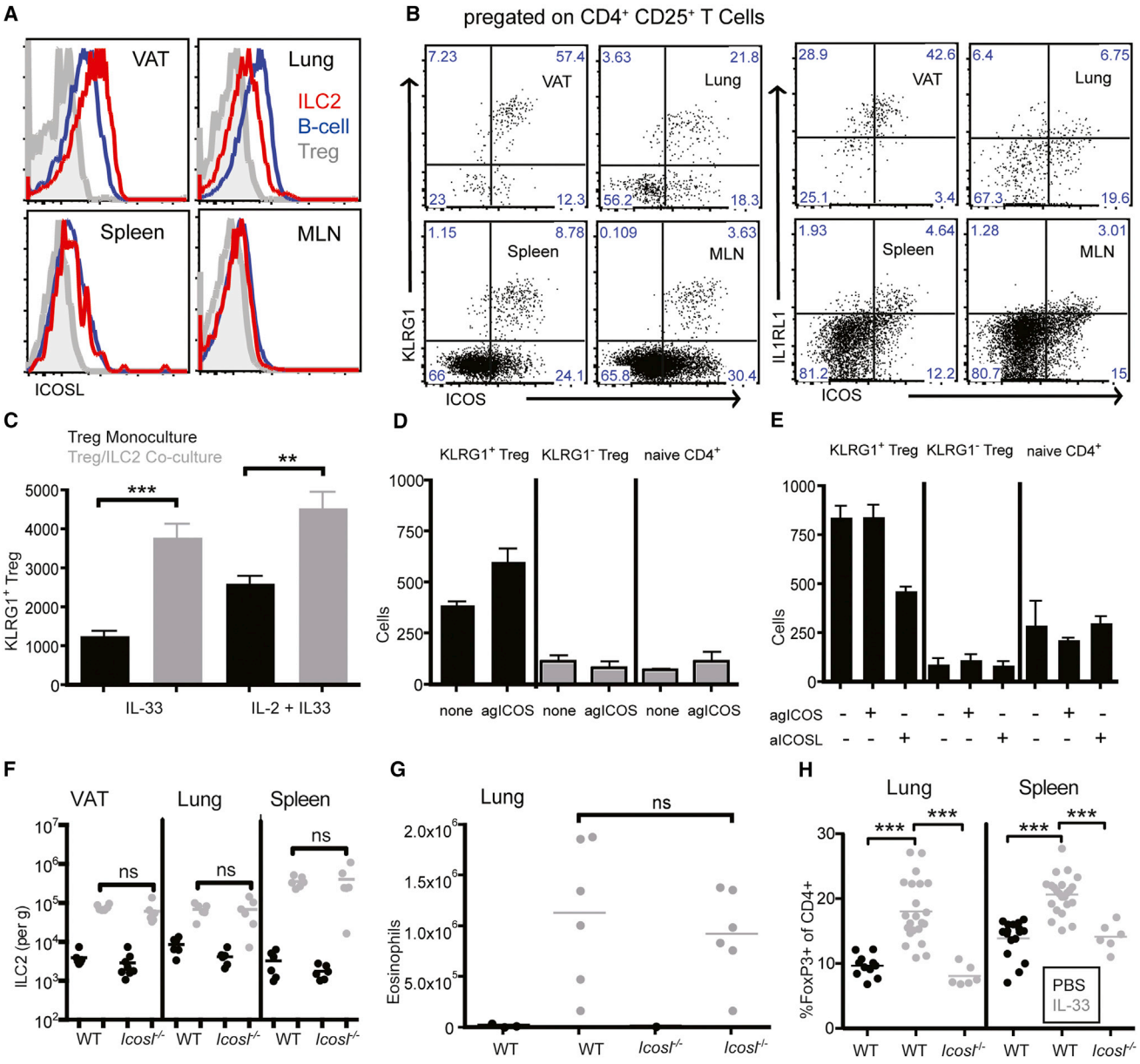

Figure 5. ILC2 ICOSL Engages Treg Cell ICOS to Promote Treg Cell Tissue Accumulation

(A and B) Flow cytometric analysis of (A) ICOSL or (B) ICOS, KLRG1, and IL1RL1 expression from the indicated tissues and populations.

(C–E) In vitro 3 day culture of (C) KLRG1$^+$ Treg cells ± ILC2, (D) KLRG1$^+$ Treg, KLRG1$^-$ Treg, or naive CD4$^+$ T cells alone ± agonist ICOS antibody, or (E) CD4$^+$ populations indicated co-cultured with ILC2 ± blocking ICOSL antibody, enumerating Treg cell survival.

(F and G) Treatment with three doses of IL-33 (gray dots) or control PBS (black dots) and (F) total ILC2 or ILC2 per g (VAT), (G) eosinophils, or (H) percent FoxP3$^+$ Treg cells of CD4$^+$ T cells were enumerated from wild-type and ICOSL deficient (*Icosl*$^{-/-}$) animals. Data are representative of two to three experiments (A–E) or pooled from two or more experiments (F–H). Error bars represent SEM.

(Figure 5A); expression is particularly high in VAT (Figure S5A). ICOSL was also expressed on the small numbers of IL-5$^+$ KLRG1$^+$ GATA3-hi Th2 cells in VAT, but not on CD8$^+$ T cells, FoxP3$^+$ CD4$^+$ Treg, other CD4$^+$ T cells, or NK cells (Figures S5B and S5C; and data not shown). In peripheral tissues, Treg cells comprised the major ICOS^{hi} lymphocytes, and, among these, KLRG1$^+$ IL1RL1$^+$ Treg cells were uniformly ICOS^{hi} (Figure 5B). ICOS^{hi} Treg cells have been associated with IL-2 therapy, helminth infection, and neoplasms, and are activated and highly suppressive Treg cells (Busse et al., 2012; Redpath et al., 2013; Sim et al., 2014).

After administration of IL-33 or infection with *N. brasiliensis*, ICOSL$^+$ ILC2 and ICOS^{hi} Treg cells accumulate in tissues and persist for prolonged periods (Figures S5D–S5F, data not shown). Although ILC2 and Th2 both express ICOS, the expression was much lower than on Treg cells, was inversely correlated with ICOSL expression, and was induced in response to IL-2 but not IL-33 (data not shown). Genetic deficiency or

antibody blockade of ICOSL led to increased lymphocyte expression of ICOS (data not shown), suggesting the possibility of ICOSL/ICOS shedding after binding. Whereas ICOS activation (agICOS) supported KLRG1$^+$ Treg cell survival in vitro, co-culture of ILC2 and KLRG1$^+$ IL1RL1$^+$ Treg cells, but not other CD4$^+$ lymphocytes, promoted Treg cell survival that was blocked by ICOSL antibody (aICOSL) (Figures 5C–5E). Following administration of systemic IL-33, ILC2 and eosinophil expansion was normal in ICOSL-deficient mice, but Treg cell expansion was impaired (Figures 5F–5H). In VAT there was a trend toward impaired total Treg cell accumulation ($p = 0.1$, data not shown). We conclude that ILC2s can interact with ICOS^{hi}, KLRG1$^+$ Treg cells and promote Treg cell accumulation via ICOSL-ICOS interactions, although additional mechanism(s) also contribute, particularly in VAT. After secondary helminth infection, a subset of IL-5$^+$ Th2 cells that express ICOSL also increase (Figures S5D–S5F) and may contribute additionally to enhance Treg cell accumulation.

Interferon-γ Inhibits ILC2 Activation by IL-33

Although VAT IL-33 is increased in obesity (data not shown and Zeyda et al., 2012), ILC2s, Treg cells, eosinophils, and AAMs decline (Feuerer et al., 2009; Molofsky et al., 2013), suggesting that loss of IL-33 does not account for this effect. However, interferon-γ-producing T cells and NK cells increase in obese VAT and IFN-γ can promote inflammation and systemic insulin resistance (Nishimura et al., 2009; Stolarczyk et al., 2013; Wensveen et al., 2015; Winer et al., 2009). ILC2s express both components of the IFN-γ receptor (Robinette et al., 2015; data not shown), and we found IFN-γ directly represses ILC2 activation, cytokine production, and proliferation in vitro (Figure 6A, Figures S6A–S6C). Although ILC2s express receptors for IL-10, IL-18, and IL-27 (data not shown), these cytokines did not impair ILC2 activation by IL-33 (Figure S6D). In vivo, lung tissue ILC2 proliferation and accumulation in response to IL-33 were blocked by co-administration of IFN-γ (Figure 6B), and this was accompanied by decreases in ILC2 and Th2 IL-5 production (Figure 6C) and in Treg cell accumulation (Figure 6D). Similar effects were noted in VAT (Figures S6E–S6G), although the ability of a short course of IFN-γ to repress IL-33-driven ILC2 and Treg cell activation was less marked, possibly due to the constitutively high expression of IL1RL1 by VAT ILC2s and Treg cells. We could not detect IL1RL1 on NK cells or CD8$^+$ T cells either at rest or following challenge with IL-33 or IL-33 and IFN-γ; although IFN-γ promoted CD8$^+$ T cell and NK cell proliferation, IL-33 alone had minimal effects (data not shown).

To assess effects of endogenous IFN-γ on ILC2 function, we used Yeti mice (Stetson et al., 2003), in which the 3′ untranslated region (UTR) of IFN-γ is stabilized by a YFP-bovine growth hormone poly-A construct, leading to constitutive increases in IFN-γ; heterozygous mice were used here, thus avoiding overt IFN-γ-induced auto-inflammation (Reinhardt et al., 2015). Similar to wild-type mice on high-fat diet (Molofsky et al., 2013), young heterozygous Yeti mice on normal diet have fewer total adipose tissue ILC2s, eosinophils, and Treg cells, show attenuated IL-5 and ICOSL expression by ILC2s and rare Th2 cells, and accumulate NK cells, Th1 CD4$^+$ T cells, and CD8$^+$ T cells (Figures 6E–6I, Figure S6H, and data not shown). VAT Treg cells in Yeti mice express diminished levels of GATA3, IL1RL1, KLRG1, and CD25 (Figure 6H). In the lung, where endogenous IL-33-driven type 2 immunity is constitutively less active as compared to VAT, ILC2 numbers, and IL-5 production, eosinophils and Treg cell were minimally affected in Yeti mice (Figures S6I and S6J).

Next, we used knockin IFN-γ reporter mice to identify NK cells, CD4$^+$ T cells, and CD8$^+$ T cells as IFN-γ-producing adipose tissue cells under conditions of excess (Yeti mice; Figure S6K) and normal IFN-γ (Great mice; Figure S6L) (Price et al., 2012); accumulation of IFN-γ-expressing CD4$^+$ Th1 and CD8$^+$ T cells was particularly dynamic in resting VAT as compared to lung. Indeed, wild-type IL-5 reporter mice older than 15 months showed decreases in VAT, but not lung, ILC2 numbers, IL-5 production, and eosinophil accumulation, which correlated with increased inflammatory CD4$^+$ and CD8$^+$ T cells (Figure 7A, Figures S7A–S7C). VAT Tregs, which accumulate in animals 4–9 months of age, decline with further aging (Figure S7D). Similarly, young animals fed high-fat diet (HFD) develop increased VAT IFN-γ-producing T cells (Nishimura et al., 2009; Stolarczyk et al., 2013; Winer et al., 2009) and VAT-specific loss of IL-5$^+$ ILC2 and Th2, eosinophils, and Treg cells (Figure 7B, Figures S7E and S7F, data not shown). Together, we conclude that VAT IL-33 promotes ILC2 activation previously associated with metabolic homeostasis, including the accumulation of Treg cells, and that IFN-γ-producing lymphocytes infiltrate VAT in response to aging and high-fat diet and repress ILC2-mediated function.

To assess the role of IFN-γ in repressing ILC2 activation during infection, we challenged heterozygous Yeti mice with *N. brasiliensis*. Although helminth infection induces IL-33 (Moro et al., 2010), ILC2 proliferation, ILC2 and Th2 ICOSL expression and IL-5 production, and accumulation of KLRG1$^+$ Treg cells, each of these responses was attenuated in the lungs of Yeti mice (Figures 7C–7E, Figures S7G–S7I). Helminth clearance from the gastrointestinal tract was also delayed (Figure 7F). To assess the effects of biologically induced IFN-γ, we infected mice with *Listeria monocytogenes*, intracellular gram-positive bacteria that elicit potent IFN-γ-mediated responses necessary to clear infection. *Listeria* co-infection with *N. brasiliensis* repressed lung ILC2s and KLRG1$^+$ Treg cell expansion, ILC2 and rare Th2 IL-5 production, and eosinophilia (Figures 7G–7K), and promoted the accumulation of CD8$^+$ T cells (Figure 7G). These effects were dependent on IFN-γ, as IFNγR1 deficient co-infected mice did not display impaired ILC2 activation or Treg cell expansion (data not shown). Helminth infection is protective in mouse models of type 2 diabetes (Hussaarts et al., 2015; Wu et al., 2011; Yang et al., 2013), promoting short-term VAT ILC2 activation and eosinophilia (Molofsky et al., 2013). We found that VAT ILC2s, Th2, and eosinophils remain elevated 1 month after helminth infection, and VAT eosinophils are elevated up to 10 months post-infection (Figures S7J and S7K). In contrast, self-limited *Listeria* infection promotes persistent elevations in VAT IFN-γ-producing CD8$^+$ T cells and decreased VAT ILC2 IL-5 production (Figure S7L). Together, we conclude that IFN-γ inhibits ILC2 activation and limits both the constitutive IL-33-dependent maintenance of activated ILC2 and Treg cells in VAT and the helminth- and IL-33-induced activation of ILC2s

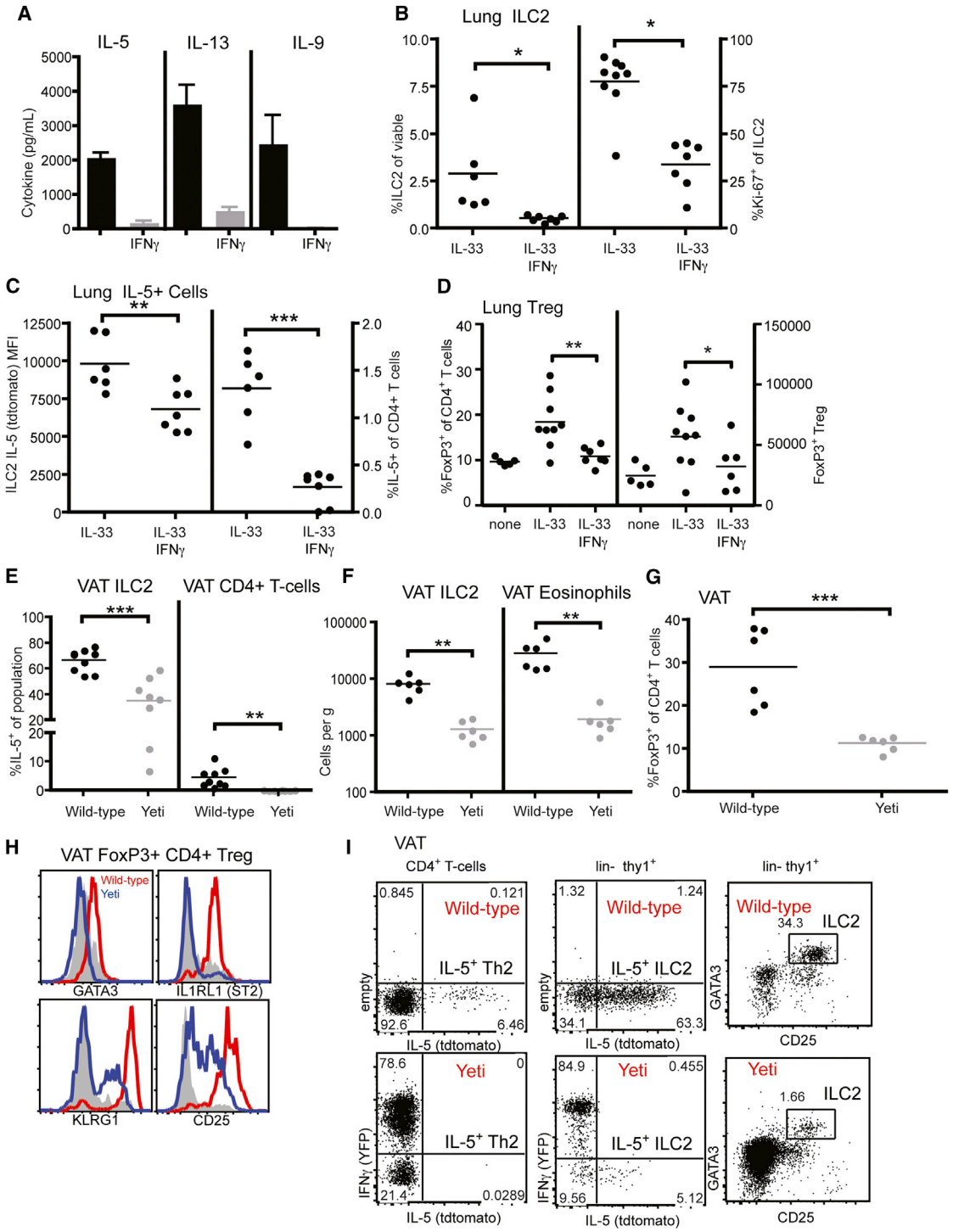

Figure 6. IFN-γ Represses ILC2 Activation and Limits IL-33-Driven ILC2s and Treg Cells

(A) Naive lung ILC2s (lin- thy1$^+$ KLRG1$^+$ CD25$^+$) were sort purified and cultured in vitro with IL-2 and IL-33 with or without IFN-γ for 3 days, and supernatants were assayed for the indicated cytokines.

(B–D) Mice were treated with IL-33 ± IFN-γ for three doses and lungs were assayed using flow cytometry for (B) ILC2 and Ki-67$^+$ proliferating ILC2, (C) IL-5 production from ILC2 and CD4$^+$ Th2, and (D) percent FoxP3$^+$ Treg cells and total Treg cells (note secondary y axis on each).

(E–G) VAT from animals with constitutive excess IFN-γ (Yeti × Red5, gray dots) or wild-type (Red5) controls (black dots) were assayed by flow cytometry for (E) IL-5$^+$ ILC2 and IL-5$^+$ Th2, (F) total ILC2 and eosinophils, or (G) percent FoxP3$^+$ Treg cells of CD4$^+$ T cells.

(H) VAT Treg cell expression of markers indicated from Yeti and wild-type animals.

(I) Representative FACS plots of VAT from wild-type IL-5 reporter and Yeti × IL-5 reporter mice. Data are representative of two to three experiments (H and I) or pooled from two to three experiments (A–G). Error bars represent SEM.

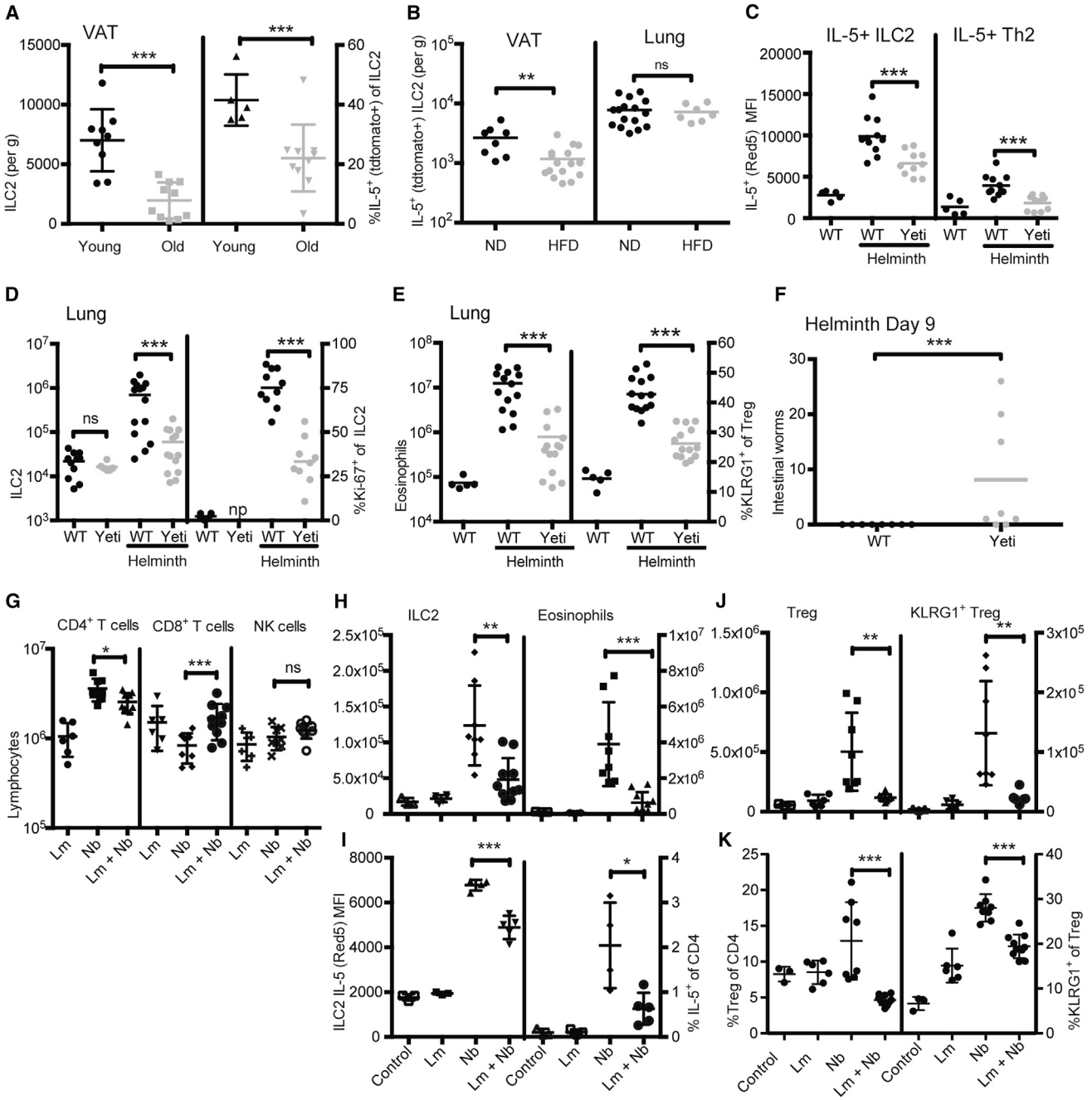

Figure 7. IFN-γ Associated with Age, Obesity, and *Listeria* Infection Limit ILC2-Mediated Immunity in Adipose Tissue and Helminth-Infected Lung

(A) VAT ILC2 and IL-5 expression (tdtomato MFI) were assessed in 15-month-"old" and 8- to 12-week-"young" IL-5 reporter (*Il5*$^{tdtomato\text{-}cre/+}$) male mice.

(B) Mice were placed on normal diet or high fat diet (60% kCal fat) for 16 weeks and IL-5$^+$ ILC2 were enumerated in VAT versus lung.

(C–E) Wild-type or Yeti IL-5 reporter mice were infected with *N. brasiliensis* and lung tissue harvested on days 7–9 post-infection, with the populations quantitated by flow cytometry as indicated (note secondary y axis on each).

(F) *N. brasiliensis* were enumerated on day 9 post infection from small intestine of wild-type and Yeti mice.

(G–K) Wild-type IL-5 reporter mice were infected with *N. brasiliensis*, *L. monocytogenes*, or co-infected, and lung tissue harvested on day 7 post-infection, with the populations quantitated by flow cytometry as indicated (note secondary y axis on each). Data are pooled from two to three experiments (A–J). Error bars represent SEM.

and Treg cells in lung. Further, our findings suggest adipose tissue is a dynamic immunologic organ that retains a prolonged "metabolic memory" of diverse infections and may lead to lasting metabolic alterations for the organism.

DISCUSSION

ILC2 cytokines, particularly IL-5 and IL-13, are critical in sustaining the accumulation of eosinophils and AAMs necessary to

maintain adipose tissue homeostasis and local tissue responses to perturbations mediated by allergens and helminths (Molofsky et al., 2013; Odegaard et al., 2007; Walker and McKenzie, 2013; Wu et al., 2011). Adipose tissue homeostasis and immunity to helminths also involve tissue accumulation of Treg cells (Cipolletta et al., 2012; Feuerer et al., 2009; McSorley and Maizels, 2012), but mechanisms that link ILC2 activation and Treg cells remain incompletely understood. Here, we identify IL-33 in coordinating ILC2s and Treg cell residence in resting VAT, but also during tissue perturbations induced by systemic IL-2 treatment or during migratory helminth infection. Although Treg cell activation by IL-33 was in part direct, as suggested by recent studies (Schiering et al., 2014; Vasanthakumar et al., 2015), optimal activation in vivo was ILC2-dependent by a process requiring intrinsic ILC2 MyD88 and interactions between ICOSL on ILC2s and ICOS on Treg. Although this IL-33-dependent pathway may serve to protect adipose tissue or injured tissues during allergen- or helminth-mediated injury, IFN-γ produced during acute inflammatory responses associated with infection inhibited these processes, potentially providing an overriding safeguard following invasion by rapidly replicating organisms, such as bacteria or viruses. Chronic inflammation linked to IFN-γ in the setting of obesity and aging also attenuated the normal organization of ILC2s and Treg cells in adipose tissue. These data position IL-33, IFN-γ, and ILC2s as critical regulators in the balance between tissue injury, metabolic homeostasis, and host defense.

Normal VAT contains high levels of IL-33 (Miller et al., 2010; Zeyda et al., 2012), which we demonstrate in endothelial cell nuclei in close proximity to resident ILC2s. Although others have reported IL-33 in both endothelium (Zeyda et al., 2012) and human adipocytes (Wood et al., 2009), our studies identify endothelium as a predominant source in resting mice. IL-33 was necessary to sustain normal ILC2 function and Treg cell numbers and function in VAT, as assessed in both IL1RL1- and IL-33-deficient mice. Although necrotic cell death can release IL-33, where it can be further activated by inflammatory proteases (Cayrol and Girard, 2014), the mechanisms by which VAT IL-33 titrates ILC2 and Treg cell function remain unknown. It is intriguing that VAT, a dynamic storage for high-energy fuels, would be a site constitutively impacted by IL-33, but perhaps such localization serves to protect the host from the detrimental metabolic effects of VAT inflammation. Recent reports have called attention to the role of IL-33 in promoting adipose tissue "beiging" via activation of ILC2s, leading to increased heat production and a loss of adipose tissue mass (Brestoff et al., 2014; Lee et al., 2015). The relative metabolic contribution of IL-33-induced beiging during normal physiology or cold-exposure is unknown.

We previously reported that IL-2, which is used therapeutically to expand Treg cells, activates ILC2 to proliferate, increase IL-5 production and promote eosinophilia (Van Gool et al., 2014). Here, we also demonstrate that IL-2 synergizes with endogenous IL-33 to promote expansion of IL1RL1$^+$ KLRG1$^+$ Treg cells, a subpopulation with high suppressive capacity (Burzyn et al., 2013b; Cheng et al., 2012; Feuerer et al., 2010; Wohlfert et al., 2011). Such a mechanism may work to expand the local Treg cell population and limit damage at immunologically active sites where IL-2 and IL-33 co-localize following tissue injury and recruitment or activation of IL-2 producing cells. IL-7, TSLP, and IL-9 are cytokines that also signal through STAT5 and, similar to IL-2, could cooperate with local IL-33 to enhance Treg cell and ILC2 function.

Unexpectedly, expansion of Treg cells by IL-33 was optimally dependent on ILC2s, as revealed using both IL-5- and IL-13-driven cell deletion. Although we cannot rule out contributions by small numbers of Th2 cells, which are partially deleted in these mice, ILC2s comprise the vast majority of cells secreting high levels of these cytokines under the conditions examined. ILC2 cell-intrinsic IL-33 signaling was required for this effect, as assessed using IL-5-mediated deletion of the MyD88 adaptor protein. Treg-intrinsic IL-33 signaling was not necessary for Treg cell expansion but was important in optimally sustaining KRLG1$^+$ "effector" Treg cells. Intriguingly, IL-4, IL-5, IL-9, and IL-13 were not required to mediate effects of ILC2s on Treg cells, suggesting that the downstream cellular targets of these cytokines, eosinophils and AAMs, are also not necessary. Nonetheless, it is possible that these cellular targets of ILC2s indirectly contribute to Treg cell accumulation in certain models. We were unable to implicate other ILC2-produced soluble mediators affecting Treg cell activation, including IL-2, IL-10, and amphiregulin (data not shown). Instead, we demonstrate that ILC2s and Treg cells co-localize in tissues, raising the possibility that direct interactions occur between these cells. Prior studies have called attention to a role for ICOSL:ICOS in maintaining the survival of tissue Treg cells (Redpath et al., 2013; Smigiel et al., 2014), and we demonstrate that activated ILC2s express high levels of ICOSL and are maintained in tissues for prolonged periods after administration of IL-33 or migratory helminth infection. A recent study also observed high levels of ICOSL on ILC2s, finding ICOSL/ICOS autocrine signals can promote ILC2 STAT5 signaling to promote ILC2 numbers and function in an asthma model (Maazi et al., 2015). We do not observe impaired ILC2 or eosinophil numbers at rest of after systemic IL-33 administration in ICOSL-deficient C57BL/6 mice, although autocrine signaling in ILC2s may be important in other contexts or strains. We found that treatment with ICOSL-Fc and IL-33 failed to promote Treg cell accumulation in ILC2-deficient mice (data not shown), and Treg cell expansion was compromised, but not abolished, in the absence of ICOSL. Together, these findings suggest ILC2s contribute to Treg cell expansion or maintenance through additional pathways. Recent studies have reported that innate lymphoid cells can express MHCII and perhaps present antigen to CD4$^+$ T cells (Hepworth et al., 2013; Oliphant et al., 2014); such interactions, in combination with ICOSL co-stimulation, could also contribute to Treg cell maintenance. Other VAT immune cells, such as NKT cells, can produce IL-2 and may also cooperate with ILC2 to maintain Treg cells (Lynch et al., 2015). Of note, IL-33 alone can induce significant Treg cell proliferation independently of ILC2s, suggesting that ILC2s act primarily to promote the survival of Treg cells.

Although few inhibitory signals for ILC2s have been described, IFN-γ proved to be a potent inhibitor of ILC2 activation. We demonstrate that resting ILC2s respond directly to IFN-γ in vitro, restricting both cytokine production and cellular proliferation. Although the mechanisms by which IFN-γ restricts ILC2s activation are unknown, IFN-γ signals through STAT1 and STAT2 and could repress the expression and/or phosphorylation

of GATA3 (Schiering et al., 2014). SOCS1 is highly elevated in cells treated with IFN-γ, and could also repress IL-33 signaling. Although IL1RL1$^+$ Treg cells in the colon were repressed directly by IL-23 (Schiering et al., 2014), naive ILC2s are not inhibited by IL-23 or IL-12 in vitro (data not shown). Lack of IFN-γ signaling has been associated with enhanced IL-5 and eosinophilia in allergic models (Coyle et al., 1996) and patients with defects in the IFN-γ receptor have elevated immunoglobulin E and increased atopic disease (Wood et al., 2005). Conversely, IFN-γ is effective in the treatment of atopic dermatitis (Reinhold et al., 1993). Together, these results suggest that IFN-γ is potent at limiting both resting and pathologic type 2 immune responses, although further work will be required to determine the specific role of ILC2s and other leukocytes in these processes. IFN-γ-mediated immunity is beneficial in the context of life-threatening infection, when pathogens must be contained and metabolic energy diverted to glycolytic support of host defense. Indeed, our data with bacterial/helminth co-infections suggest bacterial infections limit helminth-induced ILC2 expansion and function. In chronic obesity and aging, however, invasion of adipose tissues by IFN-γ-expressing lymphocytes, such as CD8$^+$ T cells and Th1 cells, correlates with loss of adipose tissue ILC2s, Treg cells, eosinophils, and AAMs (Molofsky et al., 2013) and the development of systemic insulin resistance. Our data suggest these IFN-γ-expressing lymphocytes can directly repress ILC2s, leading to disruption of VAT metabolic homeostasis. As such, therapeutic interruption of IFN-γ signaling, possibly coupled with activation of ILC2s, may provide a strategy for re-establishing adipose immune homeostasis after obesity-driven dysregulation.

Our findings reveal novel interactions between ILC2s and Treg cells, and raise questions regarding the underlying role and function of "allergic immunity." Increasingly, studies of these pathways have uncovered interactions with basal metabolism, mucosal homeostasis, and tissue repair (Burzyn et al., 2013b; 2013a; Cipolletta et al., 2012; Heredia et al., 2013; Molofsky et al., 2013; Qiu et al., 2014; Schiering et al., 2014; Wu et al., 2011). Not surprisingly, these pathways proceed independently from activation of adaptive effector cells, which focus on pathogen-derived peptides presented during microbial invasion. Prior studies have called attention to the destructive forms of type 2 immunity that occur in the absence of Treg cells (Brunkow et al., 2001; Fontenot et al., 2003) or following deletion of GATA3 or IRF4 in FoxP3$^+$ lineage cells (Wang et al., 2011; Wohlfert et al., 2011; Zheng et al., 2009). Although we have been unable to document overt autoimmunity in IL-33- or IL1RL1-deficient mice (data not shown), we speculate that the IL-33-ILC2-Treg cell interactions we define may exist in part to protect local tissues during injury or microbial invasion and may be particularly important at sites where excess or chronic inflammation is detrimental. In the right setting, however, IFN-γ can suppress this pathway to enable development of host defense against rapidly proliferating microbes and perhaps contributes to the metabolic shifts in fuel utilization that accompany inflammation. The thresholds that define activation of these fundamental pathways in distinct tissues may be underpinned by the endogenous levels and regulation of IL-33, thus positioning this cytokine as a key regulator linking ILC2 and Treg cells in protecting the host during pathogen invasion or other challenges that disrupt tissue integrity.

EXPERIMENTAL PROCEDURES

Flow Cytometry

ILC2 are identified as lineage negative (CD11b$^-$, F4/80$^-$, CD3ε$^-$, CD4$^-$, CD8α$^-$, CD19$^-$, Siglec F$^-$, FcεR1$^-$, NK1.1$^-$), FSC/SSC-low-to-moderate, CD45$^+$, CD127 (IL7Rα)$^+$ or Thy1.2 (CD90.2)$^+$, and IL1RL1 (ST2)$^+$, CD25 (IL-2Rα)$^+$, or KLRG1$^+$, as indicated. CD4$^+$ T cells are identified as FSC/SSC-lo, CD45$^+$, CD3ε$^+$, CD4$^+$. CD8$^+$ T cells are identified as FSC/SSC-lo, CD45$^+$, CD3ε$^+$, CD8$^+$. Eosinophils are identified as CD45$^+$, side-scatter high, DAPI-lo, CD11b$^+$, and Siglec F$^+$. NK cells are identified as CD45$^+$ CD3ε$^-$ CD4$^-$ CD8$^-$ NK1.1$^+$ CD11b variable. Treg cell are identified as CD45$^+$ CD3ε$^+$ CD4$^+$ FoxP3$^+$. In some cases, FoxP3GFP mice were used to identify Treg cell as GFP$^+$ CD4 cells. Populations were back-gated to verify purity and gating. Samples were analyzed on an LSR II or, for cell sorting, a FACSAria II (both BD Biosciences). Live lymphocytes were gated by DAPI exclusion, size, and granularity based on forward- and side-scatter. Data were analyzed using FlowJo software (TreeStar) and compiled using Prism (Graphpad Software). Visceral adipose tissue (VAT) was normalized per gram adipose or as a percent of total viable cells or percent of CD45$^+$ hematopoietic cells, as indicated.

Immunofluorescence Microscopy

Animals were anesthetized and injected in vivo with 4% paraformaldehyde (PFA). Tissues were harvested (VAT, lung, spleen, small intestine), fixed for 3 hr in 2% PFA, washed overnight with PBS, cryoprotected with 30% sucrose for 12–36 hr, and embedded in OCT (Sakura Finetek) prior to freezing in blocks. For whole mounts, tissues were fixed as above and imaged after permeabilization with 0.4% triton X and DAPI nuclear counterstaining. Frozen sections were processed on a Leica CM 3050S cryomicrotome (45 μm in VAT, 8 μm all others), dried on slides for 30 min, and kept at −80° until staining. Tissues were blocked with 5% goat or horse serum, and maintained in PBS + 5% serum + 0.4% triton X throughout antibody treatments. Primary and secondary antibodies were incubated for 1 hr at room temperature. Primary antibodies used include goat-anti IL-33 (R&D Systems, 1:100), hamster-anti-KLRG1 (eBioscience, 1:50), anti-CD4-APC (BD Biosciences, 1:50), rat-anti-Siglec F (BD Biosciences, 1:100), rat anti-CD31 (BD Biosciences, 1:100), chicken anti-GFP (Aves labs, 1:500), or rabbit anti-dsRed (Clontech, 1:500). When necessary, secondary antibodies were used at 1:1000 dilution. Slides were mounted with Vectashield hardset mounting media. Where indicated, 5 min before sacrifice, animals were injected with 20 μg anti-CD31-APC (Clone 390, eBioScience) to label the vasculature. Whole-mount tissue or slides were examined with a Zeiss AxioVision M2 fluorescent microscope or a laser-scanning confocal microscope (Nikon C1si), as indicated. Confocal images were resolved to 1.2 μm per pixel in the xy plane and 1.0 μm in the z plane.

Infections

500 third-stage larvae of *N. brasiliensis* were injected subcutaneously as described (Voehringer et al., 2006). Mice were killed at the indicated time points and tissues were harvested and analyzed. Wild-type *Listeria monocytogenes* strain 10403s was infected intravenously (i.v.) at 3,000–4,000 CFU per mouse.

Experimental Design and Statistical Analysis

All data were analyzed by comparison of means using unpaired two-tailed Student's t tests using Prism (GraphPad Software), with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Figures display means ± SE of the mean unless otherwise noted. When possible, results from independent experiments were pooled. All data points reflect individual biological replicates.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.05.019.

AUTHOR CONTRIBUTIONS

A.B.M. designed experiments, performed research, analyzed data, and wrote the manuscript. F.V.G. designed experiments, performed research, and

analyzed data. J.C.N., H.-E.L., S.J.V.D., and J.L. provided reagents, performed research, and analyzed data. J.A.B. and R.M.L. designed experiments, analyzed data, and wrote the manuscript.

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