ARICLE

ICOS signaling limits regulatory T cell accumulation and function in visceral adipose tissue

Kristen L. Mittelsteadt1,2, Erika T. Hayes1,2, and Daniel J. Campbell1,2,3

A unique population of Foxp3+ regulatory T cells (Tₘ) resides in visceral adipose tissue (VAT) that regulates adipose inflammation and helps preserve insulin sensitivity. Inducible T cell co-stimulator (ICOS) is highly expressed on effector (e)Tₘ that migrate to nonlymphoid tissues, and contributes to their maintenance and function in models of autoimmunity. In this study, we report an unexpected cell-intrinsic role for ICOS expression and downstream phosphoinositide 3-kinase (PI3K) signaling in limiting the abundance, VAT-associated phenotype, and function of Tₘ specifically in VAT. Icos−/− mice and mice expressing a knock-in form of ICOS that cannot activate PI3K had increased VAT-TR abundance and elevated expression of canonical VAT-TR markers. Loss of ICOS signaling facilitated enhanced accumulation of Tₘ to VAT associated with elevated CCR3 expression, and resulted in reduced adipose inflammation and heightened insulin sensitivity in the context of a high-fat diet. Thus, we have uncovered a new and surprising molecular pathway that regulates VAT-TR accumulation and function.

Introduction

CD4+Foxp3+ regulatory T cells (Tₘ) are critical for maintaining immune tolerance and for the resolution of ongoing inflammation after infection (Dominguez-Villar and Hafler, 2018; Noval Rivas and Chatila, 2016; Smigiel et al., 2014a). Specialized subsets of tissue-specific Tₘ also function in tissue repair and homeostasis. For example, Areg+ Tₘ expand in skeletal muscle and the lungs in response to injury and are required for proper tissue repair (Arpaia et al., 2015; Burzyn et al., 2013), whereas Tₘ within hair follicles and skin are essential for hair generation and inhibiting fibrosis (Ali et al., 2017; Kalekar et al., 2019). Tₘ are also found in the visceral adipose tissue (VAT) of both mice and humans, where they regulate adipose inflammation and preserve metabolic homeostasis (Deiulius et al., 2011; Eller et al., 2011; Feuerer et al., 2009; Ilan et al., 2010). As such, Tₘ ablation in diphtheria toxin–treated Foxp3DTR mice results in increased inflammatory mediators in VAT and reduced insulin sensitivity, whereas augmentation of Tₘ by administration of IL-2 immune complexes or IL-33 results in improved metabolic readouts in mice on a high-fat diet (HFD; Feuerer et al., 2009; Han et al., 2015; Vasanthakumar et al., 2015).

Given that Tₘ modulate diverse responses in different anatomical and inflammatory settings, it is not surprising that they exhibit considerable phenotypic and functional heterogeneity. Expression of CD62L and CD44 broadly divides Tₘ into distinct subsets that are enriched in lymphoid and nonlymphoid tissues (Campbell, 2015; Smigiel et al., 2014b). We term these populations central Tₘ (cTₘ) and effector Tₘ (eTₘ), respectively. Differential activation of phosphoinositide 3-kinase (PI3K), unique epigenetic landscapes, and distinct transcriptional programs underlie the diversification of Tₘ, and selective expression of chemokine receptors and cell adhesion molecules give subsets of Tₘ access to specific tissues (Delacher et al., 2017; DiSpirito et al., 2018; Luo et al., 2016). The proper distribution of Tₘ across tissues is crucial for maintaining immune tolerance and tissue homeostasis (Sather et al., 2007; Yamaguchi et al., 2011). Hence, understanding the signals that control the development, maintenance, and function of tissue-specific Tₘ is vital to fully harnessing their therapeutic potential.

Tₘ occupancy in different tissues is met with unique homeostatic maintenance requirements. Generally, IL-2 signaling maintains cTₘ within T cell zones of secondary lymphoid tissues by driving pro-survival signals, whereas maintenance of eTₘ in nonlymphoid tissues can be IL-2 independent, and instead relies on TCR and costimulatory molecule engagement (Levine et al., 2014; Smigiel et al., 2014b; Tang et al., 2003). Inducible T cell co-stimulator (ICOS) is expressed on highly suppressive Tₘ and can control Tₘ abundance by inhibiting apoptosis and stimulating proliferation (Burmeister et al., 2008; Redpath et al., 2013). IL-10 is essential for control of local immune responses and is primarily expressed by Blimp-1+ICOS+ central Tₘ.
et al., 2013). ICOS costimulation in vitro superinduces IL-10 expression (Arimura et al., 2002; Hutloff et al., 1999), and this depends on its ability to activate PI3K (Feito et al., 2003; Okamoto et al., 2003). Although mice deficient in ICOS or ICOS ligand (ICOSL) do not develop spontaneous inflammatory or autoimmune disease, genetic or antibody (Ab)-mediated blockade of ICOS signaling promotes inflammation in mouse models of autoimmune infection as a result of reduced TR abundance and/or IL-10 production (Busse et al., 2012; Dong et al., 2001; Herman et al., 2004; Kohyama et al., 2004; Kornete et al., 2012; Landuyt et al., 2019; Miyamoto et al., 2005; Redpath et al., 2013).

ICOS potently activates PI3K and downstream AKT (also known as protein kinase B) via a unique YMFM motif in its cytoplasmic tail (Arimura et al., 2002; Fos et al., 2008). AKT can act on several different substrates, including the transcription factor Foxo1, to modulate transcription of genes involved in proliferation, survival, metabolic reprogramming, and migration/tissue tropism (So and Fruman, 2012). Indeed, inactivation of Foxo1 by AKT is essential for the differentiation of eTRs and their migration to nonlymphoid tissues (Luo et al., 2016). The mammalian target of rapamycin (mTOR) pathway is also activated by PI3K signaling and regulates TR metabolism and differentiation. Thus, engagement of ICOS may facilitate the development and/or maintenance of eTRs via multiple PI3K-dependent signaling pathways.

Accumulation of TRs in VAT is dependent on several signals. VAT-TRs consist of clonally expanded populations, suggesting that recognition of one or more adipose tissue antigens promotes their tissue residence (Feuerer et al., 2009; Kolodin et al., 2015). Additionally, mice lacking expression of IL-33 or its receptor ST2 are deficient in VAT-TRs, whereas injection of exogenous IL-33 results in increased VAT-TR abundance with little impact on lymphoid TRs (Han et al., 2015; Vasanthakumar et al., 2015). Recent work also identified a cell-intrinsic role for CCR2 in the ability of donor TRs to repopulate VAT (Vasanthakumar et al., 2020). Finally, TR expression of peroxisome proliferator–activated receptor γ, the master regulator of adipocyte differentiation, supports VAT-TR phenotype and accumulation and drives expression of factors important for lipid metabolism (Cipolletta et al., 2012; Cipolletta et al., 2015). However, despite having an eTR phenotype, the role of costimulatory molecule signaling in VAT-TRs is poorly understood.

In this study, we demonstrate an unexpected role for cell-intrinsic ICOS-dependent PI3K signaling in restricting VAT-TR accumulation and function. Moreover, we implicate the CCL11/24–CCR3 axis as an additional factor capable of driving recruitment of TRs to VAT, which is enhanced in the absence of ICOS signaling. These surprising findings challenge the current model regarding signals that support diverse TR subsets and highlight the cell- and tissue-specific effects of ICOS expression and signaling on TR development, accumulation, and function.

### Results

#### Mice lacking ICOS signaling have reduced TRs in lymphoid tissues and altered expression of PI3K targets

To determine how ICOS signaling impacts TR abundance in different tissues, we crossed Foxp3mRFP mice (Wan and Flavell, 2005) to Icos−/− (KO) mice and to IcosY181F (YF) mice, which carry a tyrosine-to-phenylalanine knock-in mutation in the YMFM motif in the cytoplasmic tail of ICOS, thereby specifically abolishing ICOS-mediated PI3K activation (Gigoux et al., 2009).

In line with published data, we found a ~30% reduction in the frequency and number of TRs in the spleens of KO compared with WT Foxp3mRFP mice (Fig. 1 A; Burmeister et al., 2008; Landuyt et al., 2019). This was even more pronounced in older animals, as TRs accumulate with age (Fig. 1 B; Nishioka et al., 2006). Despite normal ICOS expression on the cell surface (Fig. S1 A) and intact MAPK signaling (Gigoux et al., 2009), we saw a similar reduction in splenic TR abundance in YF mice, indicating that activation of PI3K signaling is the critical pathway by which ICOS regulates TR abundance (Fig. 1, A and B). ICOS is expressed on eTRs, but its expression is further up-regulated on eTRs (Fig. S1 B). The loss of TRs we observed in YF and KO spleens was associated with a disproportionate reduction in the frequency and number of eTRs, whereas cTR abundance was unchanged, consistent with results using Ab blockade of ICOS signaling (Fig. 1 C; Smigiel et al., 2014b). Loss of TRs was also observed in the peripheral LNs (pLNs) of YF and KO mice, indicating that ICOS signaling is important for eTR maintenance in multiple secondary lymphoid organs (Fig. S1 C). Both YF and KO TRs in the spleen had elevated expression of the lymphoid homing molecules CD62L and CCR7, which are down-regulated upon TR accumulation with age (Fig. 1 B; Nishioka et al., 2006). Despite normal ICOS expression on the cell surface (Fig. S1 A) and intact MAPK signaling (Gigoux et al., 2009), we saw a similar reduction in splenic TRs as well as increased CD62L and CCR7 expression by gated CD44hi TRs in YF and KO mice. When examined directly ex vivo, YF and KO splenic TRs also exhibited reduced phosphorylation of the mTORC1 target, ribosomal protein S6, consistent with diminished activation of PI3K (Fig. 1 E). Thus, the absence of ICOS-mediated PI3K activation drives the altered lymphoid TR frequency and phenotype in YF and KO mice.

#### eTRs are enriched in VAT in the absence of ICOS signaling

As eTRs are highly enriched in nonlymphoid sites (Lee et al., 2007; Smigiel et al., 2014b), we next assessed whether maintenance of TRs in peripheral tissues was diminished in YF and KO mice. Using intravascular labeling to identify tissue-localized TRs (Anderson et al., 2014), we observed a reduction in the frequency and number of TRs in the skin and a decrease in the absolute number of TRs in the lungs of YF and KO mice (Fig. 2, A and B). However, TR frequency and total number were actually elevated in the VAT of both ICOS mutant strains (Fig. 2 C). This was specific to VAT, as frequency and total number of TRs in subcutaneous adipose tissue (SQAT) remained similar between genotypes (Fig. 2 D). Male and female YF and KO mice demonstrated increased VAT-TR abundance compared with WT mice of the same sex; however, the frequency and number of VAT-TRs in females was significantly less than that observed in males (Fig. 2, C and D).
Consistent with previous studies, we observed age-dependent accumulation of VAT-TRs across all three genotypes in male mice (Feuerer et al., 2009), but despite considerable mouse-to-mouse variability, VAT-TRs remained elevated in YF and KO mice across all age groups analyzed (Fig. 2 G).

We used male mice for the remainder of our studies as we found female mice harbor approximately fourfold to fivefold fewer TRs/gram VAT than age-matched males, and female mice are protected from the development of diet-induced metabolic syndrome (Ahnstedt et al., 2018; Pettersson et al., 2012; Vasanthakumar et al., 2020). The frequency of VAT-TRs expressing KLRG1 and CD69 was significantly elevated in YF and KO compared with WT controls (Fig. 3 A). By contrast, in the spleen there was a reduced frequency of YF and KO TR-expressed CD69, and very few TRs were KLRG1+ (Fig. S2 A). We assessed VAT-TR Foxp3 and CD44 expression and found no difference in the absence of ICOS (Fig. S2 B). We did, however, observe a modest increase in the expression of CTLA-4 and a slight reduction in the frequency of CD25+ TRs in YF and KO VAT (Fig. 3 A). Therefore, TRs in the VAT of YF and KO mice are enriched in expression of markers indicative of an eTR and VAT-TR phenotype (Feuerer et al., 2009; Smigiel et al., 2014b).

In addition to prototypical eTR markers, a fraction of VAT-TRs express the IL-33 receptor ST2 (Kolodin et al., 2015; Vasanthakumar et al., 2015). Signaling downstream of ST2 is required for the establishment and maintenance of VAT-TRs, and expansion of VAT-TRs through administration of IL-33 can reduce VAT inflammation and improve metabolic indices in models of diet-induced obesity (Han et al., 2015; Kolodin et al., 2015; Vasanthakumar et al., 2015). In addition to ST2-expressing TRs, we found a substantial population of CXCR3+ TRs in the VAT of lean mice, confirming previously reported findings (Fig. 3 B; Li et al., 2018). Although there were no significant differences in the frequency of WT, YF, or KO VAT-TRs singly expressing ST2 or CXCR3, we found a unique population of double-expressing ST2+CXCR3+ TRs in YF and KO VAT, which resembled canonical VAT-TRs in their expression of KLRG1 and CD69 (Fig. 3, B and C). Furthermore, whereas the abundance of splenic TRs in WT mice was dramatically reduced following treatment with the mTORC1 inhibitor rapamycin, there was no significant change in the number of TRs in the VAT (Fig. S2 C), indicating that ICOS-dependent PI3K activation regulates VAT TRs independently of mTORC1 activity.

ICOS signaling can potentiate IL-10 production (Busse et al., 2012; Dong et al., 2001; Herman et al., 2004; Hutloff et al., 1999; E and F). Consistent with previous studies, we observed age-dependent accumulation of VAT-TRs across all three genotypes in male mice (Feuerer et al., 2009), but despite considerable mouse-to-mouse variability, VAT-TRs remained elevated in YF and KO mice across all age groups analyzed (Fig. 2 G).

We used male mice for the remainder of our studies as we found female mice harbor approximately fourfold to fivefold fewer TRs/gram VAT than age-matched males, and female mice are protected from the development of diet-induced metabolic syndrome (Ahnstedt et al., 2018; Pettersson et al., 2012; Vasanthakumar et al., 2020). The frequency of VAT-TRs expressing KLRG1 and CD69 was significantly elevated in YF and KO mice compared with WT controls (Fig. 3 A). By contrast, in the spleen there was a reduced frequency of YF and KO TR-expressed CD69, and very few TRs were KLRG1+ (Fig. S2 A). We assessed VAT-TR Foxp3 and CD44 expression and found no difference in the absence of ICOS (Fig. S2 B). We did, however, observe a modest increase in the expression of CTLA-4 and a slight reduction in the frequency of CD25+ TRs in YF and KO VAT (Fig. 3 A). Therefore, TRs in the VAT of YF and KO mice are enriched in expression of markers indicative of an eTR and VAT-TR phenotype (Feuerer et al., 2009; Smigiel et al., 2014b).
Kohyama et al., 2004; Kornete et al., 2012; Landuyt et al., 2019; Miyamoto et al., 2005; Redpath et al., 2013). Surprisingly, compared with VAT-TRs from WT mice, a substantially higher frequency of YF and KO VAT-TRs were poised to produce IL-10 upon ex vivo stimulation with phorbo-myristate-acetate and ionomycin (PMA/I), with no appreciable differences in IL-10 expression by splenic TRs (Fig. 3 D). Thus, absence of ICOS-dependent PI3K signaling supports both the accumulation and suppressive phenotype of TRs in VAT.

Baseline adipose inflammation is reduced in YF and KO mice
VAT inflammation is modulated through a balance of different immune cells. TRs, eosinophils, anti-inflammatory adipose tissue macrophages (ATMs), and type 2 innate lymphoid cells (ILC2s) maintain a type 2 immune environment that supports metabolic homeostasis in lean animals. Inflammation drives recruitment of neutrophils and cytotoxic T cells, differentiation of inflammatory ATM, and production of pro-inflammatory cytokines like TNFα (Burhans et al., 2019; Molofsky et al., 2013). Although there was no difference in mouse weights between WT, YF, and KO mice across different ages, we did observe a trend toward increased VAT weight in YF mice and a significant increase in KO mice (Fig. 4 A). By histology, we did not observe any overt morphological differences in VAT from WT, YF, and KO mice, including no observable differences in adipocyte size (Fig. S3 A). The increase in VAT weight, however, did correlate with a modest increase in the total number of CD4+ effector T cells (Teffs) per gram of tissue (Fig. 4 B). Despite this increase in VAT-Teff number, there was a substantially lower frequency of YF and KO VAT-Teffs expressing features of

![Figure 2. TR frequency and number are increased in VAT in the absence of ICOS signaling.](https://doi.org/10.1084/jem.20201142)
inflammatory T helper type 1 cells (Th1 cells), including CXCR3, IFN-γ, and TNFα (Fig. 4 C). Rather, ST2-expressing Teffs were enriched in YF and KO VAT (Fig. 4 C). Similar to VAT-TRs, YF and KO VAT-Teffs were superior at producing IL-10 upon stimulation, suggesting that absence of ICOS signaling in T effs supports a self-regulatory function (Fig. 4 C). IL-33 signaling induces production of Th2 cytokines (Schmitz et al., 2005), which maintain ATMs in an anti-inflammatory state. While we didn’t find any significant changes in ATM or ILC2 populations (Fig. S3, B and C), we did note an increased frequency of eosinophils in YF and KO VAT (Fig. 4 D), which produce IL-4 to maintain anti-inflammatory ATMs (Molofsky et al., 2013; Wu et al., 2011). Overall, global loss of ICOS expression and signaling impacts T effs along with other adipose-resident immune cells to maintain an anti-inflammatory state in VAT.

YF and KO mice are protected from HFD-induced insulin resistance

Given the abundance and suppressive phenotype of T effs and other anti-inflammatory immune cells in VAT in YF and KO mice, we asked if they were more resistant to developing HFD-induced adipose inflammation and metabolic changes. Mice placed on an 18-wk HFD steadily gained body mass, with a modest reduction in weight gained by KO mice compared with WT (Fig. 5 A). After 18 wk on HFD, YF and KO mice sustained elevated T eff frequencies in VAT compared with WT mice, with an increased fraction of cells expressing the canonical VAT-T eff markers ST2 and KLRG1 and a smaller proportion of T effs expressing CXCR3 (Fig. 5, B and C). Additionally, YF and KO mice placed on an HFD retained a greater frequency of eosinophils, and T effs skewed toward a type 2, ST2-expressing phenotype (Fig. 5, B and C). Fasting blood glucose levels (BGLs) were similar among genotypes after 18 wk, and WT, YF, and KO mice were able to clear glucose to the same extent, as measured by glucose tolerance test (GTT; Fig. 5 F). However, in an insulin tolerance test (ITT), YF and KO mice demonstrated improved insulin sensitivity compared with WT mice (Fig. 5 G). Thus, loss of ICOS supports T effs and other anti-inflammatory cells in VAT during an HFD, and this has functional consequences in maintaining metabolic homeostasis.
Absence of ICOS supports the accumulation and phenotype of VAT-T Rs in a cell-intrinsic manner

ICOS signaling supports a wide array of cellular processes in the context of adaptive immunity (Panneton et al., 2019). To determine which of the T cell phenotypes we observed in intact YF and KO mice were the result of cell-intrinsic ICOS expression and signaling, we generated mixed bone marrow chimeras using congenically marked CD45.1+ WT and CD45.2+ WT, YF, or KO donors (Fig. 6 A). Body and adipose depot weights were similar between groups of chimeric mice (Fig. S4 A). Consistent with the increased abundance of VAT-T Rs that we observed at baseline in intact YF and KO mice, both CD45.2+ YF and KO TRs display a competitive advantage compared with CD45.1+ WT TRs in repopulating VAT in our chimeric mice. Whereas WT:WT chimeras reconstituted VAT-T Rs at a ∼1:1 ratio, YF and KO TRs exhibited a significantly higher reconstitution ratio of ∼5.5:1 (Fig. 6 B). In addition to supporting VAT-TR abundance, absence of cell-intrinsic ICOS signaling also promoted a canonical VAT-TR phenotype. Within the same chimeras, a higher frequency of YF and KO VAT-Trs expressed CXCR3 and ST2 (Fig. 6 C). Functionally, a larger proportion of YF and KO VAT-Trs were poised to produce IL-10 after ex vivo stimulation (Fig. 6 C). Mixed bone marrow chimeras retain a population of radioresistant host TRs, which proliferate to replenish a portion of the available niche after irradiation (Komatsu and Hori, 2007), and this is particularly evident in many nonlymphoid tissues. Remarkably, in addition to preferentially reconstituting VAT compared with WT donor TRs, YF and KO VAT-Trs were able to outcompete endogenous WT VAT-T Rs as well, nearly replacing the entire TR compartment in some chimeras (Fig. 6 D).

Contrary to our findings in VAT, YF and KO TRs were at a selective disadvantage in the skin, spleen, and large intestine, with no difference in reconstitution in the lungs (Fig. 6 E and Fig. S4 B). TRs found in SQAT are phenotypically distinct from VAT-T Rs and do not appear to accumulate with age (Feuerer et al., 2009; Li et al., 2018). However, unlike in intact mice, we did note that YF and KO donor TRs displayed a competitive advantage in repopulating SQAT in chimeric mice at a ratio of ∼3:1 (Fig. 6 E). This could be due to low-level inflammation following irradiation of the recipient mice that drives reconstitution of the SQAT niche, with Trs exhibiting a VAT-Tr phenotype.

Although CD8+ T cells and ILC2s of either genotype were similarly represented (Fig. S4 C), we did find that YF and KO
Teffs were modestly, but significantly, enriched in VAT at a ∼2:1 ratio compared with WT Teffs, with no difference in reconstitution of other tissues (Fig. 6 F). A higher frequency of YF and KO VAT-Teffs were ST2+ within the same chimeras, with a reduction in CXCR3-expressing Teffs when stimulated ex vivo (Fig. 6 G), indicating that in addition to driving increased Tnf abundance, the
Figure 6. Cell-intrinsic ICOS signaling limits T_R and T_eff abundance and phenotype in VAT. (A) Schematic of mixed bone marrow chimera setup (left) and analysis (right). The dashed line in all subsequent graphs indicates a CD45.2/CD45.1 ratio of 1. (B) Representative gating of CD45.1 versus CD45.2 expression in Mittelsteadt et al. Journal of Experimental Medicine 80 of 17 ICOS signaling in VAT – TR https://doi.org/10.1084/jem.20201142
chimeric mice on tissue-localized T$_{8S}$ in VAT and quantification of donor T$_{8S}$ reconstitution in VAT. (C) Representative gating on CXCR3- and ST2-expressing CD45.1$^+$ and CD45.2$^+$ donor VAT-T$_{8S}$ in chimeric mice (top). Lines connect points indicating CD45.1$^+$ and CD45.2$^+$ cells within the same chimeric mouse. Histogram and quantification of CD69 expression in donor VAT-T$_{8S}$. Histogram and graphical summary of donor VAT-T$_{8S}$ expression of IL-10 after 4-h PMA/I + monensin stimulation ex vivo (bottom). (D) Frequency of endogenous CD45.1$^+$ VAT-T$_{8S}$ in chimeric mice. (E) Reconstitution of donor T$_{8S}$ in the indicated tissues. (F) Summary of donor T$_{eff}$ reconstitution in the indicated tissues. (G) CXCR3 and ST2 expression by gated VAT-T$_{eff}$ (top). Histograms and summary of donor VAT-T$_{eff}$ expression of IFN-γ, TNFα, and IL-10 after 4-h PMA/I + monensin stimulation ex vivo (bottom). Data are pooled from three independent experiments with $n = 3–5$ per group. Statistical significance for cell population reconstitution was determined using one-way ANOVA with Tukey’s post-test for each tissue analyzed. Data are presented as mean values ± SD. A two-tailed, paired Student’s t test was used to assess statistical significance when comparing expression in donor cells within the same chimeric mouse.

absence of ICOS signaling also promotes a regulatory phenotype in VAT-T$_{8S}$. Taken together, loss of ICOS signaling supports VAT-T$_{8S}$ and, to a lesser extent, T$_{eff}$ accumulation and phenotype through cell-intrinsic mechanisms.

ICOS signaling impacts expression of homing receptors in T$_{8S}$ that allow access to VAT
The increased abundance of VAT-T$_{8S}$ from YF and KO mice could result from the combination of increased proliferation, increased survival, or increased migration to the VAT. The proliferation marker Ki67$^+$ was expressed by $10–20\%$ of VAT-T$_{8S}$, and we found no significant difference in the frequency of Ki67$^+$ VAT-T$_{8S}$ between WT, YF, and KO mice (Fig. 7 A). Similarly, WT, YF, and KO VAT-T$_{8S}$ expressed similar levels of the prosurvival protein Bcl2 (Fig. 7 B). Despite the lack of differences in Ki67 or Bcl-2 expression, we cannot rule out that changes in cellular turnover contribute to the differences we see, as T$_{8S}$ express other prosurvival factors. However, recent data demonstrating continuous recruitment of T$_{8S}$ to VAT via the circulation led us to assess potential differences in the migration of cells (Vasanthakumar et al., 2020).

In both intact and mixed bone marrow chimeric mice, YF and KO splenic T$_{8S}$ contained a small but significantly increased population of ST2$^+$ T$_{8S}$ (Fig. 8, A and B), a subset of T$_{8S}$ that is both transcriptionally and epigenetically poised to take up residence in peripheral tissues (Delacher et al., 2017; Delacher et al., 2020). Given this, we hypothesized that superior migration and accumulation could account for the increased frequency of VAT-T$_{8S}$ in YF and KO mice. Although CCR2 facilitates recruitment of T$_{8S}$ to VAT (Vasanthakumar et al., 2020), we detected no difference in CCR2 expression among genotypes and a modest decrease in CCR2$^+$ T$_{8S}$ in KO spleens (Fig. S5 A), arguing against enhanced CCR2-mediated recruitment of YF and KO T$_{8S}$ to VAT.

Previous studies identified an open chromatin landscape around the Ccr3 locus as well as high Ccr3 transcript levels in VAT-T$_{8S}$ (Cipolletta et al., 2012; Li et al., 2018). The frequency of CCR3-expressing splenic T$_{8S}$ was slightly elevated in both young (<8 wk) and older (>8 wk) YF and KO mice (Fig. S5 B), indicating the presence of a T$_{8S}$ population primed for both potential early seeding and continuous repopulation of peripheral tissues. In line with this, YF and KO VAT contained more CCR3$^+$ T$_{8S}$ compared with WT mice, and this population increased with age (Fig. 8 C). In mixed bone marrow chimeras, a significantly greater frequency of YF and KO donor T$_{8S}$ were CCR3$^+$ in spleen and VAT (Fig. S5 C). Additionally, YF and KO VAT contained a unique population of T$_{8S}$ that expressed CCR3 alone, while WT VAT-T$_{8S}$ expressing CCR3 were also positive for CCR2 (Fig. 8 D). CCR3 expression was unique to VAT-T$_{8S}$, unlike CCR2, which we found to be also highly expressed on VAT-T$_{eff}$ (Fig. 8 C). Additionally, the accumulation of CCR3$^+$ T$_{8S}$ was unique to VAT, as we detected very little CCR3 expression in skin, lungs, and large intestine T$_{8S}$, and no differences across genotypes (Fig. S5, D and E). We detected substantial expression of the CCR3 ligands Ccl7 and Ccl24 in the VAT, with no differences detected either by age or genotype and little expression of Ccl5, Ccl7, and Ccl26 (Fig. 8 E).

To determine if ICOS signaling could directly impact expression of CCR3 on T$_{8S}$, WT, YF, and KO VAT-Cd4$^+$ cells were cultured with αCD3/αCD28 in the presence or absence of an agonistic αICOS Ab (Fig. 8 F). We additionally added 500 U/ml IL-2 to all conditions to account for any deficiencies in IL-2 production by YF and KO VAT-T$_{eff}$. After 48 h in culture, T$_{8S}$ frequency remained unchanged in both groups. However, the frequency of CCR3$^+$ T$_{8S}$ in WT cultures significantly decreased after 2 d with addition of an ICOS agonist, whereas no changes

Figure 7. Increased abundance of YF and KO VAT-T$_{8S}$ is not due to enhanced proliferation or Bcl2 expression. (A and B) Ki67 (% A) and Bcl2 (gMFI) expression (B) in tissue-localized VAT-T$_{8S}$ as measured by flow cytometry directly ex vivo. Bcl2 gMFI was calculated as fold change compared with average expression in WT T$_{8S}$ for each individual experiment. Dashed line in B marks average value of WT control samples (Ki67: $n = 3–5$ per group from three independent experiments; Bcl-2: $n = 3–5$ per group from two independent experiments). Mice were age matched within independent experiments, and pooled data are from experiments using male mice aged 8–16 wk. Statistical significance was determined using one-way ANOVA with Tukey’s post-test. All data are presented as mean values ± SD.
Figure 8. Increased VAT-T<sub>R</sub> accumulation in the absence of ICOS signaling is associated with elevated CCR3 expression. (A) ST2 expression in splenic T<sub>R</sub>s (n = 3–5 per group from eight independent experiments). (B) ST2 expression in CD45.1<sup>+</sup> and CD45.2<sup>+</sup> donor splenic T<sub>R</sub>s in WT:YF and WT:KO chimeric mice.
were observed in YF or KO cultures, indicating that ICOS-dependent PI3K signaling can directly antagonize CCR3 expression in T$_R$s (Fig. 8 F).

To address the impact of CCR3 signaling on continuous T$_R$ seeding of VAT in vivo, we administered blocking antibodies to the CCR3 ligands CCL11 and CCL24 to 8–10-wk-old WT and YF mice. Blockade of CCL11/24 for ~2 wk resulted in a modest, although not statistically significant, decrease in the frequency of VAT-T$_R$s specifically in YF mice, with no differences in T$_R$ abundance in spleen or lungs between treated and untreated mice (Fig. 8 G). We additionally noted a slight reduction in VAT eosinophils in YF mice treated with αCCL11/24 (Fig. S5 F). Together, these results suggest a potential mechanism by which loss of ICOS-dependent PI3K signaling can drive increased recruitment of T$_R$s to VAT by CCR3-CCL11/24 interactions.

**Discussion**

We have identified surprising tissue-specific effects of ICOS signaling on T$_R$ abundance and phenotype. Although there was a significant loss of CD44$^+$ eTRs in the spleen, pLN, skin, and lungs of YF and KO mice compared with WT, there was a substantial increase in T$_R$ abundance in VAT. T$_R$ frequency and phenotype were similar in YF and KO mice, including comparably altered readouts of PI3K signaling, suggesting that ICOS primarily impacts T$_R$ abundance and homeostasis via PI3K-dependent mechanisms.

In addition to supporting VAT-T$_R$ abundance, loss of ICOS signaling promoted a highly suppressive VAT-T$_R$ phenotype, including an increased proportion of T$_R$s expressing KLRG1, CD69, and IL-10. In vitro, addition of ICOS stimulation augments IL-10 production in CD4$^+$ T cells, while loss of ICOS signaling in vivo results in reduced T$_R$ abundance and IL-10 expression in models of autoimmunity, allergy, and infection (Basse et al., 2012; Hutloff et al., 1999; Kohyama et al., 2004; Kornete et al., 2012; Landuyt et al., 2019; Redpath et al., 2013). Although ICOS expression is associated with IL-10–producing T$_R$s, ICOS signaling does not appear to be intrinsically required for IL-10 production. CD4$^+$ T cells from KO mice are capable of producing WT levels of IL-10 in vitro and, interestingly, appear to produce more IL-10 than WT counterparts under Th2-polarizing conditions (Dong et al., 2001). Furthermore, recent studies assessing T$_R$s in the brain during chronic Toxoplasma gondii infection and lamina propria T$_R$s at baseline report no changes in IL-10 production in the presence or absence of ICOS signaling (Landuyt et al., 2019; O’Brien et al., 2019). Therefore, the reduction in IL-10$^+$ T$_R$s with ICOS blockade observed in specific in vivo models is likely due to insufficient activation or access to specific environmental cues within tissues rather than an inherent requirement for ICOS signaling to drive IL-10 production.

VAT is a complex tissue, consisting of triglyceride-containing adipocytes and a variety of immune cells that function to maintain metabolic homeostasis. In YF and KO mice, other immune cells in addition to T$_R$s showed evidence of an anti-inflammatory phenotype. Although ATM and VAT-ILC2 abundance was similar across genotypes at baseline, eosinophil frequency was significantly elevated in YF and KO VAT. In YF and KO mice, VAT-T$_R$s were skewed toward a Th2 phenotype in a cell-intrinsic manner, with an increased frequency of ST2-expressing and IL-10–producing cells. Therefore, in addition to promoting an anti-inflammatory state by sustaining VAT-T$_R$s, global loss of ICOS signaling supports the abundance and phenotype of other anti-inflammatory adipose-resident immune cells. These combined changes in cellular composition in VAT in the absence of ICOS signaling were maintained in the setting of a long-term HFD and rendered YF and KO mice more resistant to HFD-induced loss of insulin sensitivity. Changes in insulin responsiveness were subtle, despite a significant increase in T$_R$s in YF and KO mice. A recent study demonstrated that ablation of IL-10 improved insulin sensitivity in mice (Rajbhandari et al., 2018). Thus, it is possible that the increased IL-10 production we observed in VAT-T$_R$s and VAT-T$_R$s in the absence of ICOS signaling offsets the insulin-sensitizing effects of increased VAT-T$_R$ abundance in these mice.

YF and KO donor T$_R$s preferentially repopulated VAT in mixed bone marrow chimeras and were able to outcompete both donor and endogenous WT T$_R$s. ST2 is expressed on a large fraction of VAT-T$_R$s (Vasanthakumar et al., 2015), and although the frequency of ST2-expressing VAT-T$_R$s was not changed in intact mice, YF and KO VAT-T$_R$s in mixed bone marrow chimeras were composed of significantly more ST2$^+$ cells compared with WT donor T$_R$s, suggesting that the competitive advantage of YF and KO donor T$_R$s in chimeric VAT is in part due to an enhanced ability to access available IL-33 through increased ST2 expression. The transcriptional regulators BATF and IRF4 cooperate to induce expression of ST2 in T$_R$s, possibly downstream of TCR signals as well as through IL-33 signaling itself (Vasanthakumar et al., 2015). Additionally, IL-2 can work synergistically with IL-33 to up-regulate ST2 and specifically expand ST2$^+$ T$_R$s (Guo et al., 2009; Matta et al., 2014). T$_R$s are unable to produce IL-2 themselves due to Foxp3-mediated
transcriptional repression at the I2 locus (Wu et al., 2006; Ono et al., 2007) and therefore are dependent on paracrine sources of IL-2 produced by, for example, autoreactive T cells (Setoguchi et al., 2005; Stolley and Campbell, 2016). However, KO Tg mice are defective in production of IL-2 compared with WT mice (Dong et al., 2001). The absence of an enriched population of ST2-expressing YF and KO VAT-Tg mice at baseline may be explained by this lack of IL-2, which is rescued in the presence of WT IL-2-producing cells in mixed bone marrow chimeras.

Molofsky et al. (2015) have argued that IL-33 signaling on Tg mice in VAT is mediated through ICOS/ICOSL interactions with ILC2s and that absence of this interaction results in loss of VAT-Tg. In their system, blockade of ICOS signaling was assessed in Icos−/− mice. Consistent with their data in Icos−/− mice, we did not observe any differences in ILC2 frequency or number in VAT of KO or YF mice. However, given that ILC2s express both ICOS and ICOSL, loss of one versus the other could differentially impact ILC2 function, resulting in unique cell-extrinsic impacts on Tg mice. Additionally, recent work revealed ligand-independent, constitutive ICOS signaling through PI3K-dependent pathways, which relies on p85 association with the ICOS cytoplasmic YMFM motif and interactions between the ICOS transmembrane domain and the tyrosine kinase Lck (Feito et al., 2003; Wan et al., 2020). Thus, the reported differences observed in our study and Molofsky’s may be the result of low-level, cell-intrinsic PI3K activation in ICOS-expressing Tg mice from Icos−/− mice.

Although CCR2 plays an important role in recruitment of Tg mice to VAT, we did not detect any differences in CCR2 expression in the absence of ICOS signaling. However, the frequency of CCR3+ Tg mice was increased in the spleen and VAT of YF and KO mice, which was due to cell-intrinsic loss of ICOS signaling and was enhanced with age. CCR3 is primarily associated with type 2 responses (Danilova et al., 2015; Francis et al., 2007; Humbles et al., 2002; Ma et al., 2002; Nagakubo et al., 2016), and lean, metabolically healthy VAT is maintained in an anti-inflammatory state by the presence of Th2-associated cells. In vivo blockade of CCL11/24 resulted in a modest reduction of Tg mice in YF VAT with no changes in other tissues analyzed. Parabiosis experiments demonstrated recruitment of circulating parabiont-derived Tg mice to VAT after months of shared circulation (Kolodin et al., 2015; Vasanthakumar et al., 2020), indicating that recruitment of Tg mice to VAT occurs throughout adulthood. Thus, longer-term Ab-mediated blockade may be necessary to observe significant effects on Tg accumulation in our system. Additionally, addressing the role of CCR3 is further complicated by its promiscuity, and whether compensation through other ligands occurs during in vivo blockade is unknown. In vitro, we found that ICOS stimulation resulted in reduced CCR3 expression in WT VAT-Tg mice, with no changes in YF or KO cultures, suggesting that ICOS-dependent PI3K signaling inhibits expression of CCR3. Control of CCR3 expression in T cells has not been extensively characterized; however, there is evidence that the transcription factor GATA3 is able to bind to a regulatory region in the Ccr3 locus (Kong et al., 2013). Nonlymphoid Tg mice, including VAT-Tg mice, express high levels of GATA3 (Cipolletta et al., 2012; Delacher et al., 2020). However, whether ICOS signaling impacts CCR3 expression by modulating GATA3 expression or activity and whether GATA3 is involved in controlling CCR3 expression in Tg mice remains to be explored.

Most studies assessing VAT-Tg biology use older male mice due to an increased abundance of canonical VAT-Tg with age (Cipolletta et al., 2015). However, mediators of tissue inflammation, including IL-6, TNFα, and IL1β, are up-regulated in the VAT of aged male mice (older than 24 wk; Wu et al., 2007). We observed consistent accumulation of VAT-Tg from 5 wk on in all three genotypes, which was correlated with an increase in CCR3+ Tg up to ~20 wk of age. CCR2 regulates T cell access to sites of inflammation (Lee et al., 2007), and studies assessing the contribution of CCR2 on Tg migration to VAT use mice aged 25–30 wk, when measures of VAT inflammation are increasing. CCR2 and CCR3 likely both have important roles for promoting recruitment of Tg to VAT; however, it will be necessary to evaluate whether these receptors are used differentially based on age, sex, and inflammation. For example, CCR3 may be one important factor for early seedling and continuous recruitment of Tg to VAT in comparatively younger mice when VAT inflammation is low. With increasing age and inflammation (particularly in male mice), a switch to CCL2-CCR2-mediated migration may dominate.

Future studies are required to understand how ICOS exerts tissue-specific effects. The skin and lungs are under chronic stimulation by environmental antigens and commensal microbes, whereas the sterile VAT is maintained in a Th2-skewed environment in metabolically healthy mice. The milieu of chemokines and cytokines expressed at baseline in these tissues is likely very different. Indeed, Tg express distinct patterns of homing receptors and adhesion molecules to appropriately access specific nonlymphoid tissues (reviewed in Campbell and Koch, 2011). Absence of ICOS signaling may support expression of chemokine receptors that preferentially drive Tg to VAT due to constitutive expression of unique chemokines within this tissue. Additionally, VAT-Tg may rely less on PI3K signals than Tg found from other tissues, due to the presence of other supporting factors in the VAT of naive mice, such as high levels of IL-33. Indeed, a recent study demonstrated that insulin receptor deletion in Tg, which also results in reduced PI3K signaling, led to an increased suppressive phenotype in VAT-Tg mice and enhanced insulin sensitivity (Wu et al., 2020).

We describe a surprising, antagonistic role for ICOS-dependent PI3K signaling in the accumulation, prototypical phenotype, and function of Tg specifically in VAT. We suggest that absence of ICOS signaling supports enhanced recruitment of Tg to VAT associated with increased expression of CCR3. Further studies will be needed to identify the molecular mechanisms by which ICOS inhibits the VAT-Tg phenotype and CCR3 expression. This highlights the complexity of tissue-specific Tg development and accumulation and the importance of considering how signals from the immune environment elicit both cell- and tissue-specific effects.

Materials and methods

Mice
CD45.1+ B6.SJL (B6.SJL-PtprcaPepcb/Boyj), B6.Icos−/− (B6.129P2-Icostm1mak/j), and B6.Foxp3mRFP (C57BL/6-Foxp3tmFlw/f) mice
were purchased from The Jackson Laboratory. B6.1cosY18IF mice were provided by M. Pepper (University of Washington, Seattle, WA). All mice were backcrossed to a C57BL/6J background for at least 10 generations. Foxp3mRFP mice were used as WT controls, and mice from different genotypes used were cohoused at weaning. CD45.1+ B6.SJL mice were crossed to B6.Foxp3mRFP mice to generate Foxp3mRFP mice expressing CD45.1, CD45.2, and CD45.1/2 allelic variants. B6.1cos−/− and B6.1cosY18IF mice were crossed to B6.1cos−/− and B6.1cosY18IF mice expressing Foxp3mRFP. All animals were bred and housed in specific pathogen–free conditions under the approval of the Institutional Animal Care and Use Committee of the Benaroya Research Institute. Males were used unless specified otherwise. Age of mice is indicated per experiment.

**Mixed bone marrow chimeras**

Bone marrow cells were prepared from donor mice by flushing femurs and tibias with PBS. RBCs from filtered cells were lysed in ammonium-chloride-potassium lysis buffer (Life Technologies) for 5 min at room temperature (RT). Recipient mice were lethally irradiated (2 × 600 rad separated by ≥ 4 h). Mixed bone marrow chimeras were generated by retro-orbitally injecting a 1:1 ratio (≥ 2 × 10^6 total cells) of bone marrow cells of the appropriate genotypes into anesthetized recipient mice. Chimeric mice were rested for ≥10 wk.

**Intravascular labeling**

Mice were anesthetized with 4% isoflurane, and 3 μg BV510- or BV650-conjugated CD45 (30-F11) was injected into mice retro-orbitally in 200 μl PBS 5 min before sacrifice. Single-cell suspensions were prepared for flow cytometry as described below, and localization of cells was determined by positive (blood-exposed) and negative (tissue-restricted) staining.

**Cell isolation**

Single-cell suspensions were prepared from spleen and pLNs (pooled inguinal, axillary, brachial, and superficial cervical nodes) by tissue disruption with frosted glass slides into RPMI with 10% bovine calf serum (BCS; R-10) and filtered through nitex mesh. Blood was collected via cardiac puncture into PBS containing 2 mM EDTA. Epididymal VAT and inguinal SQAT were excised, finely minced with scissors, and digested in RPMI basal medium with 0.14 U/ml Liberase (Roche) and 10 U/ml DNase I (Roche) for 30 min at 37°C with shaking (200 rpm). Supernatants were filtered through a 100-μM cell strainer and washed several times to remove mature adipocytes from the stromal vascular fraction. Ears were harvested for skin-infiltrating cells. Dorsal and ventral sides were separated using forceps and digested as described for VAT/SQAT for 45 min. Lungs were digested as described for skin. Large intestines were cleaned, stripped of fatty tissue, and inverted. Tissue was placed into extraction media (RPMI basal medium, 2 mM dithiothreitol, 1 mM EDTA, and 2% BCS) and shaken at 37°C for 20 min to release intraepithelial lymphocytes (IELs). IEL-containing supernatant was removed and filtered into 50-ml conical tubes. For lamina propria lymphocyte isolation immediately following incubation with extraction media, tissue was finely minced and placed into digestion media (RPMI basal media, 300 U/ml collagenase I [Worthington Biochemical Corporation], and 1% BCS), shaken for 30 min at 37°C, and filtered into 50-ml conical tubes. Isolated cells from all tissues were incubated in ammonium-chloride-potassium lysis buffer for 5 min, washed with R-10, and stained for flow cytometry or cultured for intracellular cytokine or chemokine receptor staining.

**Flow cytometry and intracellular cytokine staining**

Single-cell suspensions were stained with Fixable Viability Dye eFluor 780 (eBioscience) in PBS for 10 min at RT. For surface staining, cells were incubated at 4°C for 30 min in FACS buffer (PBS, 2% BCS) with directly conjugated Abs for murine proteins. Abs were purchased from BioLegend unless noted: CD4 (RM4-5), CD8 (53–6.7), CD11b (M1/70), CD11c (N418), CD25 (PC6), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD69 (H1.2F3), CD206 (C068C2), CCR3 (CXR3-173), F4/80 (BM8), Gr1 (RB6-8C5), ICOS (15F9), KLRG1 (2F1/KLRG1), Siglec-F (E50-2440; BD Biosciences), ST2 (U29-93; BD Biosciences), and TCRβ (H57-597). For intracellular staining, cells were stained for surface antigens as described above, washed, and permeabilized for 20 min with eBioscience Fix/Perm buffer at 4°C. Cells were washed and stained in PermWash buffer (eBioscience) for 30 min at 4°C with Abs (purchased from BioLegend unless noted) against Bcl2 (BCL-10C4), CTLA-4 (UCI0-49B), Foxp3 (JFK-16s; Invitrogen), IFN-γ (XMG1.2; BD Biosciences), IL-10 (JES5-16E3), Ki67 (11F6), and TNPα (MP6-XT22). For intracellular cytokine staining following restimulation, cells were incubated in FACS tubes with PMA (50 ng/ml) and ionomycin (1 μg/ml) plus monensin (2 μM) in 0.3 ml complete RPMI medium (RPMI with 10% [vol/vol] fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml gentamycin, 1 mM sodium pyruvate, 10 mM Hepes, 2 mM L-glutamine, and 55 μM β-ME) for 4 h at 37°C, 5% CO2 before staining as described above. Loss of monomeric RFP expression occurred with our fixation/permeabilization protocols, requiring intracellular Foxp3 staining. Data were acquired on LSR II or FACS Canto flow cytometers (BD Biosciences) and analyzed using FlowJo software (TreeStar). Due to intraexperimental variability, geometric mean fluorescence intensity (gMFI) was normalized as fold change compared with average expression in WT samples per experiment. Polybead polystyrene nonfluorescent microspheres (15 mm; Polysciences) were used to determine absolute cell numbers in flow cytometry samples. 100 μl of a fixed concentration (C_<SUB>0</SUB>) of beads was mixed with 100 μl cells to be counted. Samples were acquired on a flow cytometer, with gates drawn on lymphocyte and bead populations based on their forward- and side-scatter properties. The ratio of lymphocyte gate events (N_<SUB>i</SUB>) to bead gate events (N_<SUB>b</SUB>) was determined and used to calculate the concentration (C_i) of the original cell suspension as follows: C_i = (N_i/N_b) × C_0.

**Phospho-flow cytometry staining**

Approximately one fourth of each spleen was immediately disrupted between frosted glass slides into BD Fix/Perm buffer (BD Biosciences). Cells were incubated for 20 min at RT, washed in FACS buffer, and resuspended in 90% ice-cold methanol for ≥30 min. Cells were washed with BD Perm/Wash buffer (BD Biosciences) and stained for surface and intracellular antigens,
Chemokine receptor staining
Freshly isolated cells were incubated for 2.5 h at 37°C, 5% CO₂ in RPMI-C. For CCR7 expression, cells were incubated with CCL19-human IgGFc fusion protein for 20 min at 4°C, washed, and then incubated with PE-conjugated goat anti-human IgGFc (Jackson ImmunoResearch Laboratories) for 20 min at 4°C. To detect CCR2 (475301; R&D Systems) and CCR3 (J073E5; BioLegend), cells were incubated for 30 min at 37°C with directly conjugated antibodies diluted in FACS buffer. Cells were washed twice with FACS buffer before being acquired on the flow cytometer.

Histology
VAT was excised, immediately fixed in 10% formalin for 24 h, and then paraffin embedded. H&E staining was performed on 6-μm tissue sections by the Benaroya Research Institute Histology Core. Slides were imaged at RT with a Leica DMM brightfield microscope with a 10× eyepiece and 20× objective lens (together, 200×). Images were captured with a Leica EC3 3.1 megapixel digital color camera and imported into Leica Application Suite EZ imaging software.

RNA extraction and quantitative PCR (qPCR)
VAT was dissected, immediately stabilized in RNAlater (Thermo Fisher), and frozen at −80°C until ready for processing. ~100 mg tissue was homogenized in 1 ml Qiazol, and RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA quantity and quality were determined using an ND-1000 spectrophotometer (NanoDrop; Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan Gene Expression Master Mix (Applied Biosystems) for amplification and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan Gene Expression Master Mix (Applied Biosystems) for amplification and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan Gene Expression Master Mix (Applied Biosystems) for amplification and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan Gene Expression Master Mix (Applied Biosystems) for amplification and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan Gene Expression Master Mix (Applied Biosystems) for amplification and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan Gene Expression Master Mix (Applied Biosystems) for amplification and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 500 ng template RNA was reverse transcribed with random hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3 with nuclease-free water. qPCR was performed using 2 μl diluted cDNA and presynthesized TaqMan Gene Expression assays in TaqMan Gene Expression Master Mix (Applied Biosystems) for amplification and presynthesized TaqMan Gene Expression assays in TaqMan nuclease-free water. qPCR was performed using 2 μl diluted cDNA Synthesis Kit (Thermo Fisher) and subsequently diluted 1:3.3 with hexamer primers in 20 μl using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher).
Core; P. Johnson in the Benaroya Research Institute Histology/ Imaging Core; and D. Hackney and S. Zraika from the University of Washington Diabetes Research Center Cell Function Analysis Core for help with metabolic profiling tests. M. Pepper generously provided IcoS /81F mice with kind permission from W.-K. Suh. A. Burich, C. Toledano, and the Benaroya Research Institute vivarium staff helped maintain mouse colonies. We thank members of the Campbell laboratory for helpful discussion and laboratory support.

This work was supported by the National Institutes of Health (grants R01AI24693 and R01AI36475 to D.J. Campbell) and National Institutes of Health National Institute of Allergy and Infectious Diseases T32 grant AI06677 and National Institute of General Medical Sciences T32 grant GM007270 (to K.L. Mittelsteadt), and the University of Washington Diabetes Research Center Samuel and Althea Strom Endowed Graduate Fellowship (2P30 DK17047) to K.L. Mittelsteadt.

Author contributions: K.L. Mittelsteadt and D.J. Campbell conceptualized the study and designed experiments. K.L. Mittelsteadt, E.T. Hayes, and D.J. Campbell performed experiments. K.L. Mittelsteadt and D.J. Campbell analyzed data and wrote the manuscript.

Disclosures: The authors declare no competing interests exist.

Submitted: 2 June 2020
Revised: 24 August 2020
Accepted: 15 March 2021

References

Ahnstedt, H., M. Roy-O’Reilly, M.S. Szychala, A.S. Mobley, J. Bravo-Alegria, A. Chauhan, J. Aronowski, S.P. Marrelli, and L.D. McCulloch. 2018. Sex differences in adipose tissue CD8+ T cells and regulatory T cells in middle-aged mice. Front. Immunol. 9:659. https://doi.org/10.3389/fimmu.2018.00659

Ali, N., B. Zirak, R.S. Rodriguez, M.L. Pauli, H.-A. Truong, K. Lai, R. Ahn, K. Corbin, M.M. Lowe, T.C. Scharschmidt, et al. 2017. Regulatory T cells in skin facilitate epithelial stem cell differentiation. Cell. 169:1119–1129.e11. https://doi.org/10.1016/j.cell.2017.05.002

Anderson, K.G., K. Mayer-Barber, H. Sung, L. Beura, B.R. James, J.J. Taylor, L. Ahnstedt, H., M. Roy-O. 2014. Contribution of adipose tissue inflammation to the development of type 2 diabetes mellitus. Compr. Physiol. 5.1-58. https://doi.org/10.1002/cphy .c170040

Burmeister, Y., T. Lischke, A.C. Dahler, H.W. Mages, K.-P.P. Lam, A.J. Coyle, R.A. Kroczek, and A. Hutloff. 2008. ICO S controls the pool size of effector-memory and regulatory T cells. J. Immunol. 180:774–782. https://doi.org/10.4049/jimmunol.180.2.2774

Buryn, D., W. Kuswanto, D. Kolodin, J.L. Shadrach, M. Cerletti, Y. Jang, E. Seifkin, T.G. Tan, A.J. Wagers, C. Benoist, and D. Mathis. 2013. A special population of regulatory T cells potentiates muscle repair. Cell. 155:1282–1295. https://doi.org/10.1016/j.cell.2013.10.054
Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. Mcclanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, et al. 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 23:479–490. https://doi.org/10.1016/j.immuni.2005.09.015

Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J. Exp. Med. 201:723–735. https://doi.org/10.1084/jem.20041982

Smigiel, K.S., S. Srivastava, J.M. Stolley, and D.J. Campbell. 2014a. Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation. ImmunoL Rev. 259:40–59. https://doi.org/10.1111/imr.12170

Smigiel, K.S., E. Richards, S. Srivastava, K.R. Thomas, J.C. Dudda, K.D. Klokowski, and D.J. Campbell. 2014b. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. J. Exp. Med. 211:121–136. https://doi.org/10.1084/jem.20131142

So, L., and D.A. Fruman. 2012. PI3K signalling in B- and T-lymphocytes: new developments and therapeutic advances. Biochem. J. 442:465–481. https://doi.org/10.1042/Bj2012092

Stolley, J.M., and D.J. Campbell. 2016. A 33D1+ dendritic cell/autoreactive CD4+ T cell circuit maintains IL-2-dependent regulatory T cells in the spleen. J. Immunol. 197:2635–2645. https://doi.org/10.4049/jimmunol.1600974

Tang, Q., K.J. Henriksen, E.K. Boden, A.J. Tooley, J. Ye, S.K. Subudhi, X.X. Zheng, T.B. Strom, and J.A. Bluestone. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. J. Immunol. 171:3348–3352. https://doi.org/10.4049/jimmunol.171.7.3348

Vasanthakumar, A., K. Moro, A. Xin, Y. Liao, R. Gloury, S. Kawamoto, S. Fagarasan, L.A. Mielke, S. Afshar-Sterle, S.L. Masters, et al. 2015. The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. Nat. Immunol. 16:276–285. https://doi.org/10.1038/ni.3085

Vasanthakumar, A., D. Chisanga, J. Blume, R. Gloury, K. Britt, D.C. Hensridge, Y. Zhan, S.V. Torres, S. Liene, N. Collins, et al. 2020. Sex-specific adipose tissue imprinting of regulatory T cells. Nature. 579:581–585. https://doi.org/10.1038/s41586-020-2040-3

Wan, Y.Y., and R.A. Flavell. 2005. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc. Natl. Acad. Sci. USA. 102:5126–5131. https://doi.org/10.1073/pnas.0501701102

Wan, Z., X. Shao, X. Ji, L. Dong, J. Wei, Z. Xiong, W. Liu, and H. Qi. 2020. Transmembrane domain-mediated Lck association underlies bystander and costimulatory ICOS signaling. Cell. Mol. Immunol. 17:143–152. https://doi.org/10.1038/s41423-018-0183-z

Wu, Y., M. Borde, V. Heissmeyer, M. Feuerer, A.D. Lapan, J.C. Stroud, D.L. Bates, L. Guo, A. Han, S.F. Ziegler, et al. 2006. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell. 126:375–387. https://doi.org/10.1016/j.cell.2006.05.042

Wu, D., Z. Ren, M. Pao, W. Guo, X. Cui, A.H. Merrill, and S.N. Meydani. 2007. Aging up-regulates expression of inflammatory mediators in mouse adipose tissue. J. Immunol. 179:4829–4839. https://doi.org/10.4049/jimmunol.179.7.4829

Wu, D., A.B. Molofsky, H.-E.E. Liang, R.R. Ricardo-Gonzalez, H.A. Jouihan, J.K. Bando, A. Chawla, and R.M. Locksley. 2011. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science. 332:243–247. https://doi.org/10.1126/science.1201475

Wu, D., C.K. Wong, J.M. Han, P.C. Orban, Q. Huang, J. Gillies, M. Mojibian, W.T. Gibson, and M.K. Levings. 2020. T reg-specific insulin receptor deletion prevents diet-induced and age-associated metabolic syndrome. J. Exp. Med. 217:e20191542. https://doi.org/10.1084/jem.20191542

Yamaguchi, T., J.B. Wing, and S. Sakaguchi. 2011. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. Semin. Immunol. 23:424–430. https://doi.org/10.1016/j.smim.2011.10.002
Supplemental material

Figure S1. Despite normal surface expression of ICOS, YF mice phenocopy KO with equivalent loss of lymphoid T<sub>T</sub> (goes with Fig. 1). (A) ICOS expression on gated WT, YF, and KO splenic T<sub>T</sub>. Fold change of ICOS gMFI is compared with average expression in WT T<sub>T</sub> for each individual experiment. Dashed line marks average value of WT control samples (n = 3 per group from five independent experiments). (B) Representative ICOS expression on gated splenic KO and WT CD4<sub>lo</sub>CD62L<sub>hi</sub> cT<sub>T</sub> and CD4<sub>hi</sub>CD62L<sub>lo</sub> eT<sub>T</sub> as measured by flow cytometry (n = 3 per group from five independent experiments). (C) T<sub>T</sub> frequencies of total CD4<sup>+</sup> T cells in pLN s (n = 2–5 per group from five independent experiments). Mice were age matched within individual experiments, and quantified data are pooled from experiments using male mice 8–16 wk of age. Statistical significance was determined using one-way ANOVA with Tukey’s post-test. All data are presented as mean values ± SD.
Figure S2. **Expression of activation markers on splenic and VAT-TRs (goes with Fig. 3).** (A) KLRG1 and CD69 expression in gated WT, YF, and KO splenic TRs (n = 3–6 per group from three independent experiments). (B) CD44 and Foxp3 expression in tissue-localized VAT-TRs. gMFI was calculated as fold change compared with average expression in WT TRs for each individual experiment (n = 3–6 per group from six independent experiments). (C) Mice were age matched within individual experiments, and pooled data are from experiments using 8–16-wk-old male mice. Statistical significance was determined using one-way ANOVA with Tukey’s post-test. (C) Number of CD4+Foxp3+ TRs in spleen (left) and VAT (right) in mice treated with rapamycin (1 mg/kg) or vehicle as indicated (n = 5 mice per group from one experiment). Statistical significance was determined using two-tailed unpaired Student’s t-test. All data are presented as mean values ± SD.
Figure S3. Adipocyte, ATM, and ILC2 populations are unchanged in YF and KO VAT (goes with Fig. 4). (A) H&E staining of VAT from 8-wk-old male mice. (B) Representative flow cytometry plots and quantification of tissue-localized ATM in WT, YF, and KO mice (top). Representative gating of ATM on CD11c and CD206 to identify M1-like and M2-like macrophages, respectively (bottom; \( n = 2–6 \) per group from six independent experiments). (C) Frequency of tissue-localized ILC2s in VAT as measured by flow cytometry (\( n = 2–6 \) per group from six independent experiments). Mice were age matched within individual experiments, and pooled data are from experiments using 8–16-wk-old male mice. Statistical significance was determined using one-way ANOVA with Tukey’s post-test. All data are presented as mean values ± SD.
Figure S4.  **Cell- and tissue-specific changes in reconstitution in ICOS mutant mixed bone marrow chimeras (goes with Fig. 6).** (A) Summary of total mouse (left), VAT (middle), and SQAT (right) weights in mixed bone marrow chimeras. (B) Representative gating of CD45.1 versus CD45.2 expression on tissue-restricted T_{T_{h}}s in IELs from the large intestine. Graphs summarize reconstitution of donor T_{T_{h}}s of IELs and lamina propria lymphocytes (LPLs) in the large intestine. (C) Representative gating of CD45.1 versus CD45.2 expression in chimeric mice on total CD8^{+} T cells (top) and ILC2s (bottom) in VAT. Graphs summarize reconstitution of donor CD8^{+} T cells (top) and ILC2s (bottom) in VAT. Dashed lines in all graphs indicate a CD45.2/CD45.1 ratio of 1. Data are pooled from three independent mixed bone marrow chimera experiments with n = 3–5 male chimeric mice per group. Statistical significance was determined using one-way ANOVA with Tukey’s post-test for each individual tissue. All data are presented as mean values ± SD.
Figure S5. Increased accumulation of CCR3+ T$\text{Rs}$ in the absence of ICOS signaling is specific to VAT (goes with Fig. 8). (A) CCR2 expression by splenic (top) and VAT-T$\text{Rs}$ (bottom) as measured by flow cytometry ($n$ = 3–5 per group from two independent experiments). (B) Expression of CCR3 in splenic T$\text{Rs}$ in mice ≤8 wk and >8 wk of age ($n$ = 3–5 per group from seven independent experiments). (C) Expression of CCR3 by gated CD45.1+ and CD45.2+ donor VAT-T$\text{Rs}$ in chimeric mice. Graphs summarize CCR3 expression by donor T$\text{Rs}$ in VAT and spleen. Line connects point representing CD45.1+ and CD45.2+ cells within the same chimeric mouse ($n$ = 2–4 per group from two independent experiments). (D) CCR3 expression by tissue-localized skin T$\text{Rs}$ as measured by flow cytometry ($n$ = 2–4 per group from two independent experiments). (E) Frequency of tissue-restricted VAT eosinophils with and without in vivo CCL11/24 blockade as measured by flow cytometry ($n$ = 3 or 4 mice per group). Mice were age matched within individual experiments, and pooled data are from experiments using 8–16-wk-old male mice. Statistical significance was determined using one-way ANOVA with Tukey’s post-test (A, B, and D); two-tailed, paired Student’s t test for expression in donor cells within the same chimeric mouse (C and E); and two-tailed Student’s t test (F). All data are presented as mean values ± SD. LPL, lamina propria lymphocyte.