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Visceral adipose tissue Tregs and the cells that nurture them

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Abstract

Visceral adipose tissue (VAT) is a primary site for storage of excess energy, but it also serves as an important endocrine organ that impacts organismal metabolism. Chronic, low-grade inflammation of VAT, and eventually systemically, is one of the major drivers of obesity-associated insulin resistance and metabolic abnormalities. A unique population of regulatory T cells (Tregs), with a distinct transcriptional profile and antigen receptor repertoire resides in VAT, keeps inflammation in check and regulates organismal metabolism. Accumulation of these cells depends on interactions with other local immunocytes and, importantly, subtypes of VAT mesenchymal stromal cells (VmSCs) that are either immunomodulators or adipogenic. We summarize our current understanding of the phenotype, function, dependencies, derivation, and modulations of VAT Tregs, and review the heterogeneity and regulation of VmSCs as well as their cross talk with VAT Tregs. Lastly, we discuss imperative questions remaining to be answered.

KEYWORDS
adipose tissue obesity, IL-33, immunometabolism, mesenchymal stromal cells, Treg cells

1 | INTRODUCTION

It is becoming increasingly clear that the immune system's purview extends beyond the neutralization of microbial challenges to the regulation of tissue homeostasis. This concept first arose in the world of macrophages, highlighting their roles in a diversity of non-immunological processes, such as synaptic pruning in the central nervous system,[1] iron recycling in the liver,[2] and electrical conduction in the heart.[3] More recently, homeostatic roles have been attributed to distinct populations of Foxp3$^+$CD4$^+$ regulatory T cells (Tregs) operating in a variety of tissues, including visceral adipose tissue (VAT),[4] skeletal muscle,[5] the colon,[6] and skin.[7]

VAT Tregs are a paradigmatic "tissue-Treg" population. They were the first such population to be characterized in detail[4] and are the one we currently know the most about.[8] The decades-old discoveries that, first, obesity is associated with chronic, low-grade inflammation of VAT and eventually throughout the body,[9] and, second, that VAT is laden with macrophages[10,11] begged the question of what elements of the immune system's regulatory armamentarium are responsible for keeping these processes and cells in check to maintain metabolic homeostasis. The unique population of Tregs residing in VAT addresses this challenge.

Here, we will review the current knowledge on VAT Tregs and the cells that support them, primarily VAT mesenchymal stromal cells (VmSCs). As concerns the former, we will address their phenotype, functions, dependencies, derivation, and modulations. Concerning the latter, we will focus on their heterogeneity and regulation. Lastly, we will highlight open questions imperative to answer.

2 | VAT TREGS, IMPORTANT REGULATORS OF ORGANISMAL METABOLISM

2.1 | Phenotype and dependencies

Adipose tissue is a lipid-rich, loose connective tissue composed of adipocytes and a stromal vascular fraction that includes

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pre-adipocytes, fibroblasts, vascular endothelial cells, and many types of immunocytes.[12] Generally, adipose tissue is classified on the basis of histological and physiological characteristics into white and brown: The former plays a key role in lipid storage, while the latter is crucial for thermogenesis upon cold exposure. Such specialization permits a better performance of their particular activities. White adipose depots can be further divided according to their location into subcutaneous adipose tissue (SAT) that extends beneath the skin and VAT that is in close proximity to internal organs. To sustain and function in the unique microenvironment of adipose tissue, murine VAT Tregs or, more specifically, those that reside in the epididymal adipose depot, exhibit several features that are distinct from their lymphoid tissue counterparts.

First, VAT Tregs become highly enriched within the $CD4^+$ T cell compartment in lean mice. While Tregs from spleen and lymph nodes are typically maintained at 5%-15% of $CD4^+$ T cells throughout, VAT Tregs typically start to accumulate at 10-15 weeks of age in C57BL/6 (B6) mice and can reach as high as 40%-80% of $CD4^+$ T cells by 20-30 weeks.[4] This observation raises the question of what drives such a remarkable enrichment of VAT Tregs over time. Experiments utilizing short-term 5-ethynyl-2′-deoxyuridine (EdU) pulsing and long-term bromodeoxyuridine/5-bromo-2'-deoxyuridine (BrdU) pulse-chase approaches showed that, compared with splenic Tregs and $CD4^+$ $Foxp3^-$ T conventional cells (Tconvs) from VAT, the VAT-Treg population expands faster and decays more slowly.[13] Further analysis of Kaede/B6 transgenic mice that express a photoconvertible reporter, in combination with transfer and parabiosis experiments, showed that there is not much contribution of circulating Tregs to the VAT-Treg pool in adult mice, nor is there any evidence for conversion from the Tconv population.[13] Therefore, the accumulation of VAT Tregs over time seems to reflect a combination of enhanced proliferation and survival, rather than recruitment from the circulation or conversion from Tconvs.

Second, unlike lymphoid-organ Tregs that display very diverse T cell receptors (TCRs), VAT Tregs have a much more restricted and clonally expanded TCR repertoire. In mice engineered to have a restricted TCR diversity (a single β chain and limited α chain diversity), the complementary determining region (CDR3)α amino acid sequences from VAT Tregs are repeatedly generated by different nucleotide sequences, indicative of antigenic selection.[4] In B6 mice that are more than 25 weeks old, the vast majority of VAT Tregs form multiple micro clones. In addition, VAT Tregs are significantly reduced in major histocompatibility complex class II (MHCII)-deficient, but not CD1d-deficient mice, arguing against lipid antigens, and are found in close proximity to MHCII-expressing antigen-presenting cells (APCs).[13] These results suggest that VAT Tregs clonally expand in response to one or more local peptides displayed in the context of MHCII molecules.

However, direct proof of whether their TCR specificity is an essential determinant of VAT-Treg accumulation has been lacking. This question was recently addressed through the generation and analysis of vTreg53 TCR-transgenic (tg) mice engineered to express a TCR isolated from an expanded VAT-Treg clone.[14] In these mice, clonotype$^+$ Tregs are specifically enriched in VAT, but not in any other lymphoid or non-lymphoid tissue examined. Upon transfer into congenically marked B6 mice, only clonotype$^+$ Tregs from tg^+ mice were able to specifically accumulate in the VAT of recipients. Using a yeast display-based peptide-screening system, we and colleagues have recently identified several "surrogate peptides" that can stimulate vTreg53 TCR-tg Tregs in the context of the A^b MHCII allele (Fernandes, Li, et al, unpublished). Overall, these results strongly argue that VAT Tregs recognize a currently unknown peptide antigen (or antigens) bound to MHCII on the surface of VAT APCs and that this interaction is critical for their accumulation in VAT.

Third, compared with lymphoid-organ Tregs, VAT Tregs have a distinct transcriptome that is important for their homeostasis and function.[4,15] On the one hand, VAT Tregs do preserve the expression of classic Treg signature genes, including those encoding CD25, GITR, CTLA-4, and FOXP3. However, they also upregulate the expression of a unique set of genes encoding chemokine receptors (eg, CCR2, CCR3), cytokines and their receptors (eg, IL-10, IL-33R or ST2, IL-9R),[4,13,16,17] transcription factors (eg, PPARγ BATF, IRF4, BLIMP1, ID2),[13,16,18,19] and most interestingly, molecules involved in lipid metabolism (eg, LDLR, DGAT, CD36) and the circadian rhythm (eg, NR1D1, RORα.[18,20] On the other hand, the expression of genes encoding molecules involved in lymphoid-organ trafficking (eg, CCR7, S1PR1, CD62L) and several other transcription factors (eg, TCF1, LEF1, ID3)[21,22] is downregulated in VAT Tregs. Of note, although there is a certain degree of overlap in gene expression between VAT Tregs and activated Tregs (eg, *Cd44*, *Nr4a1*), a large fraction of VAT-Treg signature genes, particularly those involved in lipid metabolism, are not simply activation-related and are not shared with other "tissue-Treg" populations.[14]

PPARγ the "master regulator" of adipocyte differentiation, is the major driver of the unique VAT-Treg phenotype.[18] Recent analysis of double-reporter mice for PPARγ and FOXP3 showed that over 80% of VAT Tregs from 20-week-old mice highly express PPARγ while less than 10% of Tregs from lymphoid tissues do, and the latter express it at a much lower level.[14] Treg-specific PPARγ-deficient mice show a substantial reduction in the VAT-Treg compartment, and the remaining cells are devoid of typical VAT-Treg characteristics and gene expression. On the other hand, transduction of *Pparg* and *Foxp3* cDNAs into Tconvs induces the expression of most VAT-Treg signature genes, especially those involved in lipid metabolism, which can be further amplified by the addition of the PPARγ agonist, pioglitazone (Pio). Indeed, PPARγ and FOXP3 can be co-immunoprecipitated, indicating that they might control VAT-Treg gene expression within a common transcriptional complex.[18] In agreement with this notion, ectopic expression of PPARγ in the absence of FOXP3 in Tconvs from vTreg53 TCR-tg mice is insufficient to drive their accumulation in VAT.[14] Therefore, both FOXP3 and PPARγ are required to drive the unique phenotype and accumulation of VAT Tregs. To what extent these two transcription factors directly regulate the expression of VAT-Treg signature genes and which other transcriptional mediators are involved are issues that require further investigation.

What are the signals that drive PPARγ expression in Tregs? Recent data indicated that TCR activation is both necessary and sufficient for the induction of PPARγ expression in PPARγ$^-$ Tregs.[14] IL-33 can further enhance such induction and/or VAT-Treg expansion, but without TCR stimulation, it is insufficient to drive PPARγ induction (Li, et al, unpublished). IRF4 and BATF, both induced by TCR stimulation, directly bind to the *Pparg* locus, promote its expression, and are required for VAT-Treg accumulation.[13] However, since TCR and IL-33 signals are also abundant in several other tissues that do not contain high percentages of PPARγ$^+$ Tregs (eg, muscle, colon),[6,23] additional factors must be involved. One possibility is that adipose tissue is rich in natural PPARγ ligands, such as unsaturated fatty acids and prostaglandins. Activation of the lipid metabolism pathway by PPARγ might be particularly important for immunocytes [eg, Tregs, macrophages, innate lymphoid type 2 cells (ILC2s)] to survive in this lipid-rich microenvironment, which imposes a unique selective advantage to cells expressing PPARγ at high levels.

Fourth and lastly, VAT Tregs have growth factor dependencies that differ from those of lymphoid-organ Tregs, notably a strong dependency on the cytokine IL-33 and its receptor ST2.[13,16,17] IL-33 is best known for its function in inducing type 2 immunity in allergic diseases by promoting activation and expansion of Th2 cells and ILC2s. However, recent studies showed that it is also broadly involved in wound healing, tumor immune suppression, and metabolic processes such as beiging of white adipose tissue and thermogenesis in brown adipose tissue.[24] VAT-Treg homeostasis highly depends on the IL-33-ST2 axis. In 20- to 25-week-old mice, over 80% of VAT Tregs express ST2. Global ablation of IL-33 or ST2 results in a significant reduction in the VAT-Treg compartment, while lymphoid-organ Tregs are largely unaffected.[13,16,25] Injection of exogenous IL-33 substantially expands VAT Tregs, with only a moderate effect on Tregs in lymphoid organs. As a result, obese mice injected with IL-33 exhibit reduced VAT inflammation and improved metabolic indices.[16,17,25,26] Both direct and indirect mechanisms have been proposed. In one study, IL-33 promoted expansion and activation of ILC2 cells, which in turn drove accumulation of Tregs through Inducible T-cell costimulator (ICOS)–ICOS ligand (ICOSL) interactions.[25] However, several pieces of evidence indicate that Treg-intrinsic mechanisms also play a big role in IL-33's action. First, purified VAT Tregs expand significantly when IL-33 is added to an in vitro culture system,[16] (Li et al, unpublished). Secondly, mixed-bone marrow chimeras generated from wildtype and ST2-deficient cells show a significant reduction in VAT Tregs derived from ST2-deficient donors.[16] Lastly, mice with a Treg-specific deletion of *Il1rl1*, the gene encoding ST2, also have a significantly reduced KLRG1$^+$ *bona fide* VAT-Treg population.[14] Therefore, both cell-intrinsic and cell-extrinsic mechanisms may account for IL-33-mediated VAT-Treg accumulation. However, it is important to keep in mind that the current lack of a specific ILC2 marker may compromise previous conclusions concerning these cells' role. The major cell type producing IL-33 in VAT has also been under some debate. Several recent studies clearly demonstrate that subsets of mesenchymal stromal cells (mSCs) are the major IL-33 producers in VAT.[27-29] The characterization of these cells and their interaction with VAT Tregs will be discussed in more detail in later sections.

3 | FUNCTION

VAT Tregs play an important role in regulating adipose tissue homeostasis and organismal metabolism. Since their initial discovery over a decade ago, several groups have independently shown a clear insulin-sensitizing function for VAT Tregs in mice younger than 30-40 weeks of age. Initial correlative studies showed that both the frequencies and the total numbers of VAT Tregs are significantly reduced and inversely correlated with insulin resistance in genetic or diet-induced mouse models of obesity.[4,30] In addition, global manipulation of the total Treg population in mice supports an insulin-sensitizing role for VAT Tregs. Injection of diphtheria toxin (DT) into Foxp3^{DTR} mice or administration of anti-CD25 depleting antibodies to wildtype B6 mice increases VAT inflammation and worsens metabolic parameters [eg, fasting glucose and insulin levels, phosphorylation of the insulin receptor, the values for homeostatic model assessment of insulin resistance (HOMA-IR)].[4,31] On the other hand, global expansion of Tregs by injecting an IL-2/anti-IL-2 complex in mice fed a high-fat diet (HFD), repetitive transfer of Tregs into obese *db/db* mice that underwent uninephrectomy, or oral administration of anti-CD3 antibody and β-glucosylceramide in leptin-deficient *ob/ob* mice all improved insulin sensitivity.[4,31,32]

More recent work using particular genetic models has allowed the manipulation of VAT Tregs in a more specific manner. As mentioned above, mice with Treg-specific ablation of PPARγ show a significant reduction in the VAT-Treg population but no effect on Tregs in lymphoid tissues. As a result, VAT inflammation and metabolic parameters in these mice worsen.[18] In addition, the PPARγ agonist, Pio, a known insulin-sensitizing agent, is able to prevent VAT-Treg loss during obesity. The insulin-sensitizing effect of Pio is at least in part attributable to its effect on VAT Tregs as this drug is much less effective in HFD-fed mice that lack PPARγ specifically in Tregs.[18] The modulation of VAT Tregs by IL-33 has also supported an insulin-sensitizing role for these cells. Mice deficient in IL-33 or ST2 have a substantially reduced VAT-Treg population and show greater insulin resistance and glucose intolerance even on a normal-chow diet (NCD).[16] Conversely, injection of IL-33 into HFD-fed mice provokes significant expansion of VAT Tregs and an improvement in insulin sensitivity.[16,17,25,26] The relative contributions of VAT Tregs versus ILC2s in this model require further investigation. Lastly, vTreg53 TCR-tg mice showing preferential accumulation of VAT Tregs, but not other tissue Tregs, are also more insulin-sensitive and glucose-tolerant.[14] These data have also been supported by results from studies showing that several other VAT immunocytes exert their effect on metabolic parameters through modulation of VAT Tregs; for example, iNKT cells sustain VAT Tregs to promote insulin sensitivity,[33] and IFN-γ-producing Th1 cells inhibit VAT Tregs to drive insulin resistance.[34]

Mechanistically, both immunocytes and adipocytes are modulated by VAT Tregs. As concerns immunocytes, VAT Tregs play a critical role in keeping a balance between anti-inflammatory and pro-inflammatory macrophages, promoting differentiation of the former while suppressing the latter.[14,33] VAT Tregs also highly express the enzyme hydroxy-prostaglandin dehydrogenase (HPGD), which generates 15-keto prostaglandin E_2 (PGE_2) from PGE_2 to suppress activation and proliferation of VAT Tconvs.[35] Concerning adipocytes, VAT Tregs produce large amounts of IL-10, which can inhibit the expression of inflammatory genes, block down-modulation of insulin-dependent tyrosine phosphorylation of insulin receptor substrate 1, and reverse downregulation of the glucose transporter Glut4 by TNF-α.[4]

Although most loss-of-function approaches have demonstrated an insulin-sensitizing role for VAT Tregs in lean mice below 30-40 weeks of age, also supported by gain-of-function studies in genetically and diet-induced obese mice, one study has reported that VAT Tregs may also promote insulin resistance in lean mice over 50 weeks of age.[36] In this age-induced insulin resistance model, expansion of VAT Tregs using IL-33 or IL-2/anti-IL-2 complexes in mice over 50 weeks of age leads to increased insulin resistance, while depletion of VAT Tregs via Treg-specific PPARγ ablation or anti-ST2 antibody improved metabolic indices. The mechanisms by which VAT Tregs promote insulin resistance in this aging model are currently unclear, although the TGFβ pathway has been implicated.[36] We have repeatedly observed that mice from different vendors and maintained at different facilities have distinct VAT-Treg population dynamics over time; for example, they begin to expand at different ages. In certain studies, VAT Tregs keep expanding beyond 50 weeks of age, while in many other cases, they start to curtail and lose their unique characteristics after 40 weeks of age. These differences might reflect variable compositions of the chow diet, distinct microbiota, or even how "clean" the animal facility is, which all critically influence metabolic indices.[8]

4 | DERIVATION

An important, but difficult to answer, set of questions relates to the precise life history of VAT Tregs: "Where do they come from?", "when do they seed VAT?", and "when, where, and how do they acquire their unique phenotype?". Using a collection of immunologic, genetic, and "-omic" approaches, two recent studies have gone far to fill in this knowledge gap.[13,14]

As concerns their origin, several lines of evidence suggest that VAT Tregs are primarily thymus-derived cells (tTregs), rather than being peripherally converted cells (pTregs). First, TCR sequencing analysis showed no overlap of the TCR repertoires between VAT Tregs and lymphoid-organ or VAT Tconvs.[4] Second, almost all VAT Tregs are positive for Helios and Neuropilin (Nrp)-1, both thought to be markers that can distinguish tTregs from pTregs, though imperfectly.[13] Third, compared with lymphoid tissue Tregs that are mostly tTregs, VAT Tregs do not show preferential expression of gene signatures derived from either pTregs induced in vivo or generated in vitro.[13] Lastly, adoptive transfer of either polyclonal Tconvs or vTreg53 TCR-tg Tconvs leads to no enrichment of these cells in VAT, nor do they convert to VAT Tregs.[13,14] Collectively, these data point clearly to a thymus origin for VAT Tregs.

Several lines of evidence indicate that seeding of VAT by Tregs occurs during the first weeks of life. When B6 mice are thymectomized at 3 to 4 or 13.5 weeks of age and then aged until 25 weeks, there is no significant reduction in the quantity of VAT Tregs, indicating that thymic output beyond 3-4 weeks is not required for establishment of the VAT-Treg compartment.[13] In addition, when Tregs are punctually ablated by injecting DT into $Foxp3^{DTR}$ mice at 16/17 days or 3 weeks of age and then left until 25 weeks, the VAT-Treg compartment is not affected. In contrast, when Tregs are ablated beyond 8 weeks of age, the VAT-Treg population is significantly compromised, indicating that the pool of Tregs generated in the adult thymus is impoverished in VAT-Treg precursors.[13] Lastly, in vTreg53 TCR-tg mice, when all T cells are forced to express a VAT-Treg TCR, the clonotype$^+$ Treg cells are most efficiently selected during the first weeks of life, after which Treg cells with secondary TCR rearrangements start to emerge at elevated levels.[14] Overall, these data suggest that VAT Tregs are generated in the thymus and seed the VAT early in life. Whether this skewing occurs because the perinatal thymus preferentially expresses particular antigens promoting the selection of VAT Tregs or due to distinct biological factors associated with this developmental stage (eg, a lymphopenic environment, availability of a certain cytokine) requires further investigation.

How VAT Tregs acquire their unique characteristics is a fundamental question. Do they emerge from the thymus already behaving like VAT Tregs, indicative of some type of thymic imprinting, or do they acquire their definitive phenotype only after installation within VAT, reflecting a response to tissue-specific cues? The lack of an appropriate cell-transfer system or lineage-tracing tool for VAT Tregs was a major roadblock to answer such questions. The generation and analysis of the vTreg53 TCR-tg mouse line and a tdTomato reporter line for the VAT-Treg diagnostic marker PPARγ (PPARγ-Tdt) finally solved these problems.[14] Neither of the two above-mentioned scenarios is entirely accurate. Rather, the differentiation of VAT Tregs undergoes an important intermediate stage in secondary lymphoid organs, particularly the spleen. In vTreg53 TCR-tg PPARγ-Tdt mice, thymic Tregs are mostly PPARγ$^-$, while VAT Tregs are mostly PPARγ$^+$. However, in the spleen (Figure 1), a small proportion (<10%) of Tregs have already turned on PPARγ expression, although at a much lower level than that in the VAT.[14]

Transcriptomic analysis of these splenic PPARγ^{lo} cells revealed them to have "turned on" part of the VAT-Treg signature (144/270 genes), including loci associated with Treg activation (eg, *Cd44*, *Klrg1*, *Prdm1*), response to cytokines (eg, *Il1rl1*, *Il9r*), and migration to non-lymphoid tissues (eg, *Ccr2*, *Ccr3*, *Ccr8*). In contrast, they downregulated genes associated with lymphoid tissue trafficking (eg, *Ccr7*, *Sell*) and the resting state (eg, *Tcf7*, *Lef1*). However, these splenic PPARγ^{lo} Tregs are clearly not yet true VAT Tregs, as they lack expression of many genes encoding enzymes involved in lipid metabolism

(eg, *Dgat1*, *Nsdhl*, *Coq10b*, *Dhcr24*).[14] A population resembling these PPARγ^{lo} cells can also be identified by single-cell RNA-seq (scRNA-seq) analysis. Transfer experiments further showed that this splenic PPARγ^{lo} population is derived from PPARγ^{-} cells and that they engender PPARγ^{hi} cells in VAT much more efficiently. Lastly, the open chromatin landscape of this splenic PPARγ^{lo} population is much more similar to that of splenic PPARγ^{-} cells than PPARγ^{hi} cells in VAT, arguing that they are not recirculating cells.[14]

Collectively, these results strongly argue that the splenic PPARγ^{lo} Treg population hosts VAT-Treg precursors. The precise trajectory for the differentiation of this precursor population in the spleen is currently unclear, but its generation can be regulated by TCR activation, Foxp3 levels, or IL-33 stimulation[14] (Figure 1). Interestingly, there seems to be a limiting niche for the generation and/or maintenance of the PPARγ^{lo} splenic population, as it is consistently maintained below 10% of the total splenic Treg compartment and does not further increase in vTreg53 TCR-tg mice.[14] Limited antigen exposure, IL-33 supply, or PPARγ ligand availability can potentially contribute to this limited niche. Using other markers (ST2, ID3, TCF1), results from several recent studies also support a stepwise model for the differentiation of tissue Tregs.[21,22,37] In the future, it is important to further investigate what factors control the differentiation of the PPARγ^{lo} splenic population, and how it might be related to other tissue-Treg populations.

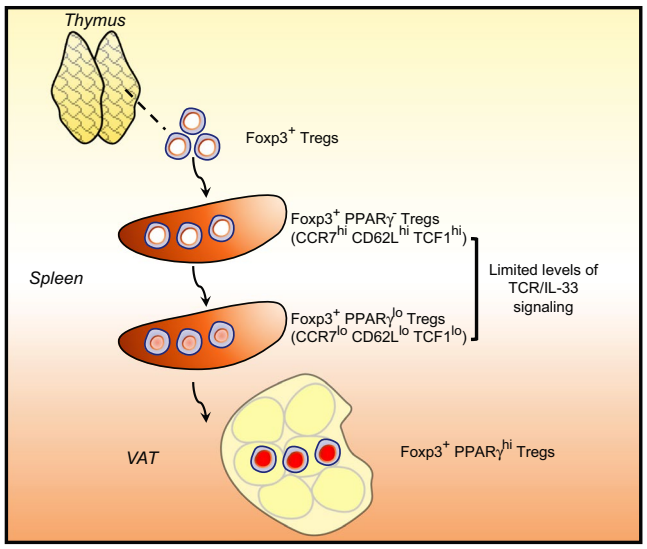

FIGURE 1 Derivation of VAT Tregs. After emerging from the thymus, a small population of Tregs in secondary lymphoid organs, particularly those in the spleen, "turn-on" *Pparg* expression at a low level, partially acquire the VAT-Treg phenotype, and downregulate the expression of molecules involved in lymphoid tissue trafficking (eg, CCR7, CD62L) and resting cell state (eg, TCF1), in response to limited amount of TCR and IL-33 stimulation. This allows the PPARγ^{lo} Tregs to exit lymphoid organs and surveil non-lymphoid tissues. Upon recognition of particular cognate antigen(s) in the VAT, these VAT-Treg precursor cells are retained in the tissue, upregulate *Pparg* at a high level, and fully establish the VAT-Treg transcriptome and chromatin landscape in response to microenvironmental cues

5 | MODULATIONS

Several physiological or pathological variables modulate the accumulation and/or phenotype of VAT Tregs. The best known of these are age, sex, depot, and obesity.

5.1 | Age

As discussed above, the VAT-Treg population typically begins to expand at around 10-15 weeks of age and can attain over 40%-80% of the CD4^{+} T cell compartment at 20-30 weeks of age. A key driver of this expansion is a parallel increase in the numbers of IL-33-expressing VAT stromal cells. The characteristics and regulation of these IL-33-expressing cells and the means by which they interact with VAT Tregs will be discussed in more detail in a later section.

5.2 | Sex and depot

Males and females have many differences in adipose tissue physiology and organismal metabolism.[38,39] For example, males accumulate more visceral fat, while females have more subcutaneous fat. Under HFD, males show increased weight gain and a greater propensity for developing metabolic diseases than females do.

Gonadal VAT depots (ie, epididymal and ovarian VAT) constitute one of the major and most extensively studied white adipose compartments in mice[40] and attract special interest because perturbations affecting these sites are associated with the development of metabolic disease in both rodents[41] and humans.[42] We recently reported that gonadal VAT hosts a much larger population of Tregs in males than in females.[14] In addition, when Tregs from female vTreg53 TCR-tg mice are transferred into female wildtype recipients, they accumulate less in gonadal VAT and express much lower levels of ST2 than is the case when Tregs from males are transferred into male recipients.[43] Within males, there is also a reduction in Tregs and their ST2 expression in inguinal SAT compared with gonadal VAT.[4,14]

The sex and depot dimorphisms of VAT Tregs can potentially be explained by different compositions of IL-33-expressing stromal cells, which were significantly reduced in female gonadal VAT or inguinal SAT from both genders compared with male gonadal VAT.[14,27] The mechanisms underlying the differential representation of IL-33-expressing stromal cells in males versus females are not completely known but could be caused by signaling through the sex hormone receptors.[27,43] In addition, a basal heightened inflammatory state in male gonadal VAT may also drive the preferential accumulation of VAT Tregs[43] (see below for more details). Consistent with this scenario, VAT Tregs in females, but not in males, are increased in response to weight gain by HFD treatment.[44] The heightened basal VAT-Treg accumulation in males may serve as an important anti-inflammatory brake as males are more prone to obesity-induced inflammation and insulin resistance.

5.3 | Obesity

It was very clear from early observations that severe obesity leads to substantial reduction in the VAT-Treg population, in both diet-induced (over 8-20 weeks of HFD feeding) and genetically induced (*ob/ob*, A^y/a) mouse models.[4] In addition, the Tregs persisting in VAT of obese mice were no longer *bona fide* VAT Tregs. They express much lower levels of signature genes such as *Klrg1*, *Ccr2*, *Il1rl1*, and *Il10*, instead gaining expression of a cluster of genes defined as the obese mice (om)VAT-Treg signature.[15] The omVAT-Treg up-signature is enriched, while the down-signature is impoverished in PPARγ-deficient Tregs, indicating that obesity-associated transcriptional changes in VAT Tregs are at least partially attributable to loss of either PPARγ expression or activity. In an in vitro culture system, supplementation of TNF-α, which is highly induced in VAT during obesity, activated cyclin-dependent kinase (Cdk5) that phosphorylates the serine residue at position 273 (Ser273) of PPARγ and induces expression of the omVAT-Treg signature in T cells.[15]

IL-21, STAT3, and IFN-γ have also been proposed to drive the dysregulation of VAT Tregs during obesity.[34,45,46] Levels of IL-21 and IFN-γ and the activity of STAT3 are all elevated in VAT during obesity. Ablation of total IL-21 or specific deletion of STAT3 results in a significant increase in VAT Tregs, reduced VAT inflammation, and improved insulin sensitivity in mice fed a HFD.[45,47] However, given that these genetically modified mice are also resistant to diet-induced obesity, the increase in VAT Tregs could be secondary to the changes in body weight and/or the local VAT microenvironment, which themselves could strongly influence VAT-Treg homeostasis. In the case of IFN-γ, YETI mice that have a constitutive increase in IFN-γ levels due to aberrant bicistronic *Ifng-Ires-Yfp* mRNA regulation show a significant reduction in VAT Tregs on NCD.[25] On the contrary, adipocyte-specific MHCII-deficient mice exhibit significantly reduced IFN-γ production in VAT on HFD and correspondingly elevated numbers of VAT Tregs.[34] It is not entirely clear whether the effect of IFN-γ on VAT Tregs is due to Treg-intrinsic mechanisms or rather to modulation of the numbers of ILC2s, which may promote Treg accumulation through the ICOS–ICOSL axis.[25] In addition to these factors, Rab4-B, a small GTPase controlling endocytic trafficking, is reduced in VAT T cells from obese individuals and may also contribute to the reduction in VAT Tregs during obesity.[48]

One common caveat of most of these obesity studies has been that they all focus on either mice fed HFD long-term (>8-20 weeks) or genetically obese mice, so only a late snapshot is taken. We still lack a comprehensive view of the exact kinetics of VAT Tregs, their transcriptional changes, and modulations of local environmental factors during the onset, progression, and late phases of obesity, which can reflect distinct physiological or pathological processes (eg, adipocyte differentiation and expansion, hypoxia, endoplasmic reticulum and reactive oxygen species stress, and fibrosis). A better understanding of the kinetics and interplay between VAT Tregs and these obesity-associated processes is crucial for better designing Treg-based immunotherapies to improve insulin sensitivity in obesity-associated metabolic diseases.

6 | HUMANS

While most of the findings mentioned above are derived from mouse studies, several reports have also examined the VAT (in this case omental fat) Treg population of humans (reviewed in Ref. 49). In several studies, either the levels of *FOXP3* mRNA (normalized to *CD3e*) or numbers of Tregs in VAT were reduced in obese individuals and had an inverse correlation with body mass index and fasting glucose.[4,30,50] However, in a few other studies, an increase in *FOXP3* level and Tregs in VAT from obese individuals was observed.[51-53] It is worth noting that in many inflammatory settings, such as autoimmune diseases, Tregs often increase in numbers in response to heightened inflammation as a means to counteract and protect against immune pathology. In fact, we have observed that in mice, VAT Tregs do not reduce, but rather increase in response to short-term HFD, which is accompanied by largely healthy VAT expansion (Li, et al, unpublished). Therefore, without functional perturbation experiments, one should always be cautious when interpreting results from these correlative studies.

7 | VAT MSCS, BOTH IMMUNOCYTE PROMOTING AND ADIPOCYTE GENERATING

Locally produced IL-33 is a key determinant of VAT-Treg accumulation and function.[13,16,17] This cytokine, constitutively present in the nucleus, belongs to the IL-1 family and acts as an alarmin,[54] and its deficiency in mice is associated with metabolic alterations.[16]

However, characterization of IL-33's main cellular sources within VAT received less attention and remained elusive until recent years. Whereas some early reports initially claimed that endothelial cells are the main producers of this cytokine,[25] our laboratory and others reported that a cadherin-11$^+$ fibroblast-like population is a more relevant source.[13,55] These cells are encompassed within the mSC compartment. mSCs have often been referred to as mesenchymal "stem" cells, but this is a terminology that should be abandoned given the absence of in vivo proof that all of the cellular components encompassed within this designation display the fundamental features of stem cells (ie, self-renewal and differentiation into multiple cell lineages).[56]

scRNA-seq studies on isolated VAT stromal cells lacking hematopoietic and endothelial cell markers and expressing the mSC markers PDGFRα, Sca-1, and Podoplanin (PDPN) uncovered the existence of two VAT (V)mSC subtypes (VmSC4 and VmSC5) capable of engendering adipocytes and another three (VmSC1, VmSC2, and VmSC3) expressing *Il33*.[27] These findings paralleled earlier observations showing mSCs to be key IL-33 expressers in skeletal muscle, alternatively named fibroblast/adipogenic progenitor cells (FAPs), that promotes critical tissue-Treg expansion upon injury.[23] Similarly, IL-33-expressing mSCs were found to sustain ILC2 function in fat-associated lymphoid clusters,[57] liver,[58] and lung.[59] Taken together, mounting evidence supports the notion of mSCs as the major source of IL-33 conserved across a wide array of mouse tissues, and capable of modulating the phenotype of Tregs as well as other ST2-expressing immunocytes (eg, ILC2s).

8 | FUNCTIONAL HETEROGENEITY

Intra-VmSC heterogeneity seems to be based on the expression of cell-lineage markers, tissue location relative to other cellular components, and their functional contribution to adipose tissue homeostasis.[60,61] However, the advent of higher resolution methods, such as scRNA-seq, during recent years, has revealed cryptic mSC heterogeneity in unprecedented detail. In this section, we will mainly refer to the latest efforts in understanding the dissimilarities of mSCs and related stromal cells residing in various adipose depots that contribute to modulation of fat Tregs and other immunocytes, with a focus on VmSC heterogeneity.

Epididymal VAT hosts five VmSC populations that exhibit distinct transcriptional profiles. Functional analyses support their partitioning into two principal groups: immunocyte-promoting mSCs characterized by the expression of *Il33* and having poor adipogenic potential (VmSC1, VmSC2, and VmSC3) and adipocyte-generating mSCs that express *Pparg* and other markers of pre-adipocytes (VmSC4 and VmSC5).[27] A similar dichotomy was reported in another study.[62] VmSCs exhibiting *Il33* expression are most obviously linked to Treg homeostasis as they are the major source of IL-33 production within VAT and regulate Treg expansion in vivo, as demonstrated by mSC-specific *Il33*-ablation experiments.[27] In addition, these cells are able to control ILC2 activity (notably IL-5 and IL-13 secretion) via IL-33 production.[29]

An additional, albeit minority, IL-33 source is a population of mesothelial cells—characterized as PDGFRα^{-}PDPN^{hi}Mesothelin^{+}—exclusively present in epididymal VAT but not in other adipose depots.[29] These cells appear to represent a functional specialization, given that their anatomical distribution along the edges of VAT allows them to better sense signals derived from the tissue surroundings and to upregulate IL-33 in response to infection, as demonstrated in a model of *Nippostrongylus brasiliensis*. Additionally, in the course of early stages of high-fat feeding, when the adipose tissue rapidly expands and acquires an acute pro-inflammatory tenor, IL-33 production by mesothelial cells remained unaltered, unlike the down-regulation of IL-33 expression exhibited by VmSCs.[27,29] The VmSC1 subset resembles this mesothelial stromal-cell subtype, according to transcriptomic and surface-marker analyses.[27] Interestingly, VAT mesothelial cells have been previously reported to give rise to adipocyte progenitors.[63] Altogether, these data indicate that heterogeneous stromal sources of IL-33 production are affected under diverse physiological and pathological situations and modulate VAT Tregs and other immunocytes.

9 | REGULATION

9.1 | Age

Aging is associated with a higher risk of metabolic abnormalities, in particular type-2 diabetes, characterized by the incapacity to appropriately lower glucose levels after insulin exposure (insulin resistance) or as a consequence of impaired insulin secretion.[64] In this regard, VAT stromal and immunocyte constituents are subjected to progressive changes that ultimately affect the inflammatory and metabolic status both locally and systemically. Gradual VAT-Treg accumulation in aging mice seems to primarily reflect increasing IL-33 levels in the local environment, as demonstrated at both the transcriptional[13] and protein[16] levels in whole tissue. Significant age-dependent accrual of IL-33-producing mSCs, in particular the VmSC1 and VmSC2 subtypes, has also been documented.[27] These cells appeared to be the biologically relevant supporters of the VAT Treg increase with age as specific *Il33* depletion using a *Pdgfra-Cre* driver to target the mSC compartment exhibited a significant reduction in VAT Tregs during the age interval in which these cells typically experience their expansion.[27] Mechanistically, TNF-α and IL-17A derived from γδ T cells act on VmSCs and PDGFRα^{-}PDPN^{hi} mesothelial cells to induce IL-33 expression.[28] Thus, both increased numbers of IL-33 producers and induction of its expression account for the overall rise of this cytokine in VAT during aging (Figure 3A).

However, the existence of additional pathways modulating VAT IL-33 during aging cannot be ruled out as certain cytokines such as IL-1β and IFN-γ have been reported to induce IL-33 in other locations.[65] Additionally, as VmSC subsets were identified using scRNA-seq technology in relatively young mice aged 8 weeks, it is possible that age-associated progression of the stromal compartment leads to the expansion of under-represented mSC populations not detected in young individuals. In addition, precisely how the transcriptional profiles of these cells vary with age, beyond IL-33 production, are still open questions.

9.2 | Sex

As described above, there are sex-specific dissimilarities in gonadal VAT-Treg abundance, with male mice harboring higher numbers of Tregs than females do.[14] This difference is detected already at 8-10 weeks of age and becomes even more apparent by 18-20 weeks.[27] Although the basis of this divergence is not known, recent observations indicate that environment-associated factors might be pivotal drivers, as female gonadal VAT Tregs have a similar capacity to proliferate and accumulate upon systemic injection of IL-33.[27] The VmSC subtypes expressing *Il33* are augmented in males, in particular VmSC1 and VmSC3, suggesting that they may contribute to preferential VAT-Treg expansion.[27]

An RNA-seq comparison of each of the VmSC subtypes in males vs females revealed that over-representation of the early/late estrogen response pathways is one of the hallmarks of all five of the female subtypes; conversely, over-representation of genes related to the androgen response pathway is characteristic of male VmSCs (Figure 2).[27] In line with these data, sex-specific differences in VmSC subtype distribution were also found based on CD90 and CD73 expression. Female mice with a null mutation of the estrogen receptor gene (*Esr1*) exhibited a male-like phenotype associated with increased VAT inflammation and higher numbers of CD73^{+} stromal

cells, which overlap with the VmSC1 subset as they both express *Il33* and lack CD90 expression. Correspondingly, estrogen administration was associated with reduced *Il6* and *Ccl2* transcript levels within VAT, resulting in less Treg accumulation.[43] Although the precise cellular target of estrogen is unknown, mSCs are likely to be involved, as previous evidence indicated that a subset of VAT stromal cells ($CD45^-CD31^-CD29^+CD34^+Sca\text{-}1^+CD24^-$) is a relevant source of CCL2 upon high-fat feeding.[66] In contrast, male mice lacking androgen receptor gene (*Ar*) expression, exhibited fewer IL-33-producing $CD73^+$ stromal cells, paired with lower numbers of VAT Tregs compared with their wildtype counterparts. Likewise, testosterone treatment in female mice expanded $CD73^+$ stromal cells, which was associated with expansion of the VAT-Treg population.[43] These observations support the notion that sex hormones influence the function of VmSC subtypes and this, in turn, might set niche-specific characteristics, most notably IL-33 availability (Figure 3B).

Nevertheless, the existence of additional factors defining sex-dependent VAT-Treg variations and whether sex hormones are exerting direct or indirect effects on IL-33 expression by VmSCs remain to be explored. The latter issue may represent a challenging task given that whole-body *Esr1*- or *Ar*-deficient mice show dramatic changes in adipose tissue development and expansion that could constitute confounding factors.[67,68] Thus, the use of mice with mSC-specific ablation of the androgen or estrogen pathway may prove necessary to unveil the underlying mechanisms involved in the sexual dimorphism of the regulation of Tregs by mSCs in VAT. Finally, studies addressing the existence of such sex-dependent differences in human visceral adipose depots will be of great interest.

9.3 | Depot

Stromal cells dwelling in diverse adipose depots show functional heterogeneity that relies on their distinct developmental roots[63] and, more importantly, depot-specific cell-autonomous mechanisms.[12] As noted above, whereas Tregs are highly enriched in epididymal VAT, where *Il33* transcripts are expressed at higher levels, SAT has comparatively fewer Tregs paired with much lower *Il33* expression, further supporting the notion of this cytokine as a pivotal driver of Treg enrichment.[14] Moreover, mSCs are the major producers of IL-33 in all adipose compartments analyzed as follows: gonadal VAT, SAT, omental, and brown adipose tissue, in both male and female mice,[27] and mesenteric adipose tissue in males.[29] Interestingly, VAT stromal cells exhibit additional immunomodulatory properties that may directly or indirectly impact Treg biology, including a heightened secretion of pro-inflammatory cytokines (IL-6 among others) under in vitro conditions, compared with stromal cells isolated from SAT in both humans[69] and mice.[70]

Despite recent characterization efforts of VAT and SAT stromal cells at the single-cell level,[27,62,71-73] a more comprehensive analysis using a standardized tissue preparation protocol that yields an accurate representation of all stromal components, as well as consensus markers to render results more comparable, would be very valuable. Interestingly, certain poorly adipogenic stromal-cell subtypes have the capacity to inhibit adipogenesis of precursors in SAT[72] and VAT.[62] Relatedly, new layers of complexity uncovered by high-resolution techniques such as scRNA-seq will be useful in future studies to explore biologically relevant interaction networks among the different mSC subtypes and Tregs residing in diverse adipose compartments.

9.4 | Obesity

Obesity is associated with chronic, systemic, low-grade inflammation, initiated in the VAT, that leads to a number of metabolic alterations (eg, type 2 diabetes, insulin resistance, etc) linked to cardiovascular disease and cancer, among other detrimental consequences. This condition is thus an important topic for investigation and many advances focused on both mSCs, and immunocytes that populate VAT have been performed over the last two decades. As previously mentioned, VAT Tregs are critical regulators of tissue inflammation, and they are mostly lost in terms of numbers and transcriptional identity after prolonged HFD feeding under chronic inflammatory conditions.[4] In this section, we will concentrate on the effects of pathological perturbations that impact VmSCs in obese individuals.

As for VAT Tregs, the progression of obesity in a diet-induced model is accompanied by gradual changes in VmSC subsets. The IL-33-producing fraction of the VmSC compartment contracts during the initial 4 weeks after commencement of HFD, even though VAT-Treg proportions are not yet reduced at this point. This finding raises the question of whether the reduction in $IL\text{-}33^+$ cells represents a numerical contraction or a loss of intracellular cytokine due to its release into the extracellular space, where it can sustain VAT-Treg function. Later, at 8 weeks of HFD, IL-33-producing VmSCs bounce back to levels comparable with those of lean mice fed a low-fat diet, and VAT Tregs are fractionally and numerically reduced (Spallanzani et al, unpublished). Finally, in chronic obesity (16 weeks of HFD), when VAT exhibits fibrosis and an impaired capacity to expand, the VAT-Treg population is profoundly diminished. Interestingly, $IL\text{-}33^+$ VmSCs are significantly enriched compared with age-matched lean individuals at this late stage of HFD. These observations indicate that the positive association between levels of $IL\text{-}33^+$ mSCs and VAT Treg characteristic of physiological conditions (eg, age and sex) is abolished with long-term obesity.

Nonetheless, many questions regarding the complex intertwining of VmSCs, VAT Tregs, and other immunocytes in the context of obesity remain open: What is the availability of IL-33 and other factors regulating Tregs over the course of HFD-induced obesity? What phenotypic changes do VmSCs display in obese subjects and how do they influence VAT Tregs? Are other immunocytes and stromal cells implicated in the coordination of adaptive changes during obesity development? Do VmSC populations absent or under-represented under physiologic conditions take over VAT in the context of obesity-associated inflammation? Or, alternatively, how drastically

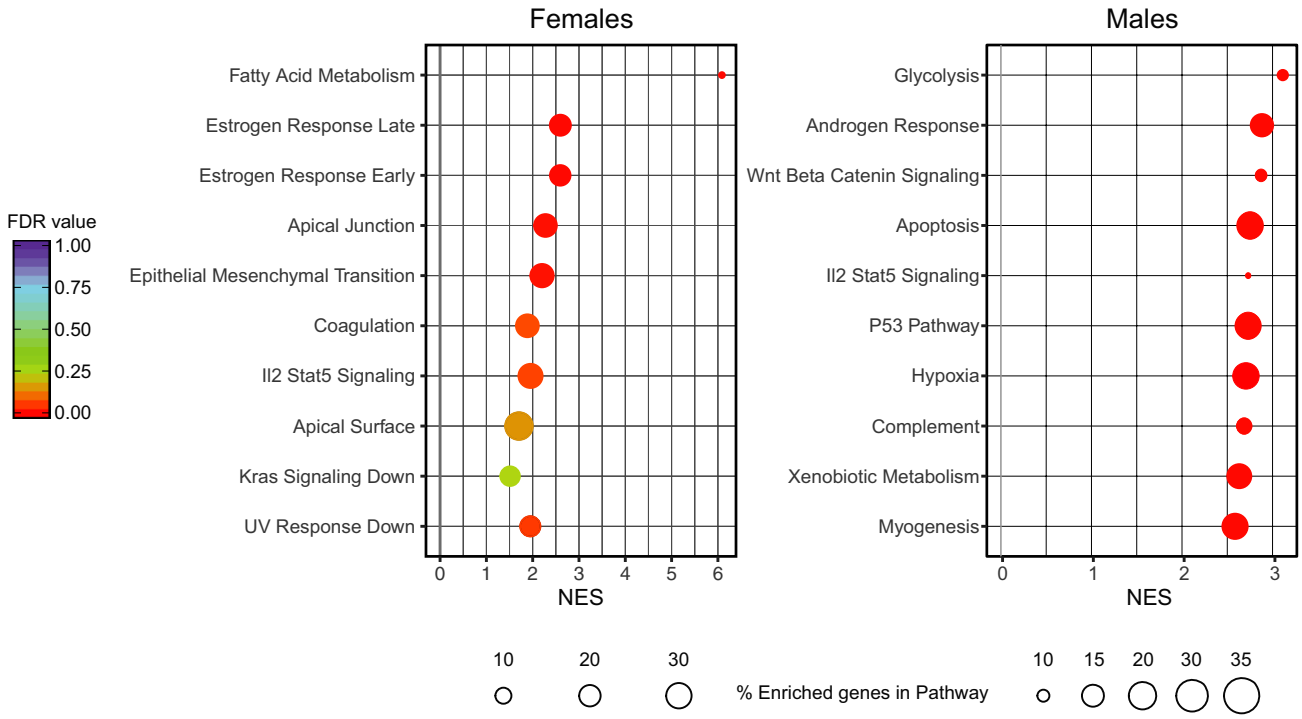

FIGURE 2 Over-represented pathways in female and male VmSCs. Top 10 significantly ($P < .05$) enriched pathways in all female VmSC subtypes compared with those of males (left) and in all male VmSC subtypes compared with those of females (right). NES, normalized enriched score; FDR, false discovery rate

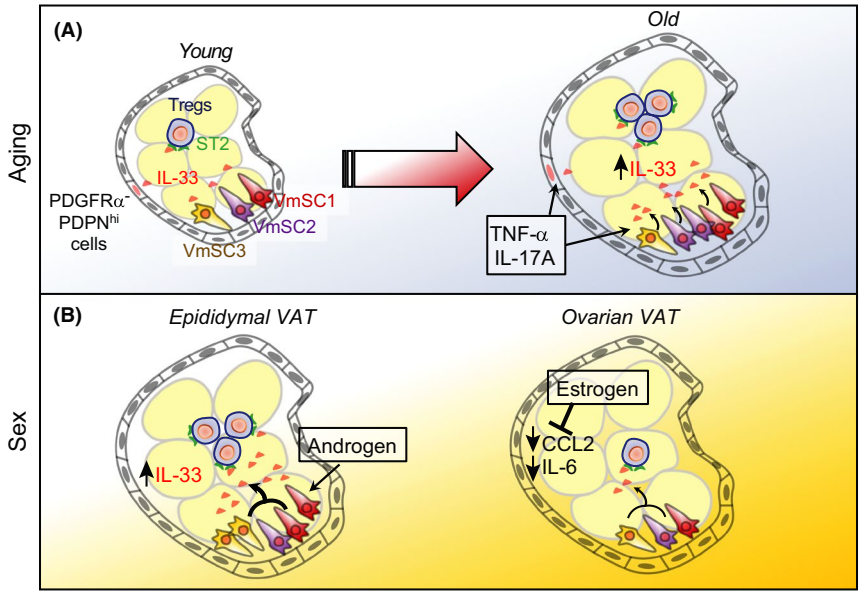

FIGURE 3 VmSC:VAT-Treg dynamics under physiological conditions. A, Aging is associated with increased IL-33 levels as well as VmSC1 and VmSC2 accumulation, which promotes VAT-Treg expansion. TNF-α and IL-17A produced by γδ T cells act on VmSCs and PDGFRα$^-$ PDPN^{hi} stromal cells to drive increased IL-33 production. B, Mice from different sexes differ in their proportion of VmSC subtypes, IL-33 levels, and VAT-Treg numbers. Epididymal VAT is characterized by higher IL-33 concentrations, associated with comparatively higher VmSC1 and VmSC3 fractions, leading to heightened VAT-Treg accumulation compared with females. Androgen may act, directly or indirectly, on IL-33$^+$ VmSCs to promote their accumulation (in particular, VmSC1). Estrogen signaling reduces CCL2 and IL-6 expression by ovarian VAT that exhibits an attenuated inflammatory environment, coupled with fewer IL-33-producing VmSCs and VAT Tregs

are VmSC subsets' transcriptomic programs affected under these circumstances? To address these questions, a comprehensive single-cell analysis at various points during the progression of HFD would be very useful to detect such changes and to trace interaction networks that might highlight ill-defined pathways involved in the pathogenesis of obesity-associated abnormalities.

10 | VAT TREG:MSC INTERACTIONS, A BIDIRECTIONAL DIALOGUE

As discussed above, currently available evidence indicates that, under physiological conditions (ie, aging and sex), there is a positive association between IL-33-producing VmSC subtypes and VAT Tregs. In addition, mSC-targeted ablation of *Il33* reduces VAT-Treg accumulation. These observations reflect unidirectional communication, wherein VmSCs promote VAT-Treg accrual accomplished through IL-33 production. However, disruption of this balance in pathological situations associated with chronic obesity causes dysregulation of this relationship, resulting in a strong enrichment of IL-33$^+$ VmSCs coupled with a quantitative and qualitative loss of *bona fide* VAT Tregs.[15,27] Mice lacking the IL-33 receptor, ST2, specifically on Tregs did show a robust accumulation of IL-33$^+$ VmSCs upon IL-33 injection, compared with wildtype littermates, suggesting a VAT-Treg/VmSC regulatory loop wherein stimulation of the ST2/IL-33 axis triggers Treg particular mechanisms that keep IL-33-expressing VmSCs under check by preventing their accumulation.[27] Thus, VAT Tregs would be able to "talk back" to stromal components and, by so doing, regulate adipose tissue homeostasis. It is presently unknown what molecules and pathways participate in the regulation of VmSCs by Tregs, and whether an indirect or direct mechanism is involved. The fact that VmSCs appear not to express ST2, at least under physiological circumstances, begs the question of what additional cells are promoting their accrual when animals are treated with IL-33 in the absence of an ST2-mediated signal on VAT Tregs. Macrophages or ILC2s are potential candidates as they express ST2.

10.1 | Humans

Human VmSC subtypes—encompassed within the CD45$^-$CD31$^-$CD34$^+$CD44$^+$PDGFRα$^+$ cell compartment—have been identified on the basis of CD9 expression in omental fat from patients with morbid obesity. Whereas CD9^{hi} VmSCs exhibit a fibro-inflammatory transcriptomic profile, are associated with type 2 diabetes, and correlate with fibrosis, CD9^{lo} VmSCs express higher *PPARG* transcript levels and are reduced in diabetic individuals. Such a classification resembles the murine immunocyte-promoting and adipocyte-generating VmSC subtypes and holds true in an obesity mouse model.[74] How human VmSC subtypes interact with VAT Tregs under physiologic and pathologic conditions, and the involvement of IL-33 in homeostasis and VAT-associated alterations during chronic obesity are unsolved questions currently. Evidence of an association between inflammation-driven increases in levels of IL-33 and fibrosis in other tissues such as liver, lung, kidney, and skin[75] suggest mSC-derived IL-33 could play a pathogenic role in chronic obesity.

11 | MORE TO COME…

Since the initial report of their discovery in 2009, we have learned a lot about the phenotype, functions, dependencies, derivation, and modulations of murine VAT Tregs. They have served as a paradigm for tissue Tregs as opposed to their lymphoid-organ counterparts, which had been the focus of almost all prior studies on Tregs. We have also started to learn about their major supporting cells within VAT, mSCs. This stromal compartment is highly heterogeneous, including both immunocyte-modulating and adipocyte-generating compartments. We have only the beginnings of an understanding of the bidirectional cross talk between VAT Tregs and the two sets of VmSCs. To date, most studies in this area have focused on gonadal adipose tissue in male mice. It is imperative to obtain a comparable knowledge-base concerning other adipose tissue depots, particularly in humans.

As concerns VAT Tregs, the most important questions to address in the coming years are as follows:

1. What is the inventory of transcriptional programs specifying the VAT-Treg phenotype?
2. What is the cast of effector molecules driving VAT-Treg regulation of neighboring inflammatory, parenchymal, stromal, and progenitor cells?
3. What antigen(s) do VAT Tregs see?
4. What factors underlie the loss of VAT Tregs in obese individuals?
5. How does the VAT-Treg compartment change in response to relevant infections?

Concerning VmSCs, questions imperative to address include the following:

1. What are the spatial relationships between the various VmSC subtypes?
2. Where and when do VmSCs interact with resident immunocyte populations?
3. What factors control IL-33 synthesis and how is this cytokine released?
4. What is the origin of the five VmSC subtypes and to what extent can they interconvert?
5. Can we learn to promote or repress the accumulation or function of particular VmSC subtypes?

Answers to these questions should go far in enriching our view of VAT-Treg and VmSC biology. They should also arm us with the knowledge to permit therapeutic manipulation of these critical elements of the VAT microenvironment and systemic correlates.

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CONFLICT OF INTEREST

None.

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