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Tissue regulatory T cells: regulatory chameleons

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Abstract

The FOXP3$^+$CD4$^+$ regulatory T (T_{reg}) cells located in non-lymphoid tissues differ in phenotype and function from their lymphoid organ counterparts. Tissue-T_{reg} cells have distinct transcriptomes, T cell receptor repertoires and growth and survival factor dependencies that arm them to survive and operate in their home tissue. Their functions extend beyond immune surveillance to tissue homeostasis, including regulation of local and systemic metabolism, promotion of tissue repair and regeneration, and control of the proliferation, differentiation and fate of non-lymphoid-cell progenitors. T_{reg} cells in diverse tissues share a common FOXP3$^+$CD4$^+$ precursor located within lymphoid organs. This precursor undergoes definitive specialization once in the home tissue, following a multi-layered array of common and tissue-distinct transcriptional programs. Our deepening knowledge of tissue-T_{reg}-cell biology will inform ongoing attempts to harness them for precision immunotherapeutics.

INTRODUCTION

Since their molecular definition in the early 2000s [1–3], the impressive power of FOXP3$^+$CD4$^+$ regulatory T (T_{reg}) cells has been increasingly appreciated. These cells initially garnered attention as regulators of other T cells' activities but eventually were recognized to control the responses of most innate and adaptive immunocyte types. For a decade, almost all studies on FOXP3$^+$CD4$^+$ T cells focused on those circulating through the blood and peripheral lymphoid organs but, more recently, unique populations of T_{reg} cells have been discovered in a diversity of non-lymphoid tissues, opening our eyes to the true breadth of T_{reg}-cell phenotypic and functional diversity.

This Review focuses on the T_{reg} populations in non-lymphoid tissues, which we term tissue T_{reg} cells. We discuss tissue-T_{reg}-cell phenotypic heterogeneity, highlighting three examples of specific interest; tissue T_{reg}-cell functions, with an emphasis on their roles in tissue

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homeostasis rather than immunity; the cellular derivation and molecular diversification of tissue T_{reg} cells; and, finally, a set of issues imperative to address.

Tissue-T_{reg}-cell phenotypic heterogeneity

A few reports of T_{reg} populations functioning in non-lymphoid tissues did emerge during the beginning years of T_{reg}-cell research [4]. However, the notions that non-lymphoid tissues harbor unique populations of T_{reg} cells adapted to survive and operate in their home tissue and that the purview of these cells extends beyond dealing with infections to assuring tissue homeostasis were not advanced until 2009, with the discovery of visceral adipose tissue (VAT) T_{reg} cells [5]. Subsequently, tissue T_{reg} cells with unique phenotypes have been identified at a multiplicity of sites, including the skeletal muscle [6], skin [7], colonic lamina propria [8], cardiac muscle [9], lungs [10], liver [11] and central nervous system (CNS)[12]. To provide a flavor of the phenotypic heterogeneity exhibited by tissue T_{reg} cells, we begin with vignettes of three particular tissue T_{reg} populations, each chosen to illustrate a specific point of interest.

VAT T_{reg} cells

Adipose tissue is a loose connective tissue made up of adipocytes and a complex stromal vascular fraction that includes pre-adipocytes, diverse stromal cell types, vascular endothelial cells, sympathetic nerves and a multiplicity of immunocyte types [13] (Fig. 1). Fat depots occur throughout the body, for example, surrounding internal organs (VAT); around the neck, especially in neonates (brown adipose tissue (BAT)); beneath the skin (subcutaneous adipose tissue (SAT)); and embedded within certain tissues, such as the bone marrow, liver and skeletal muscle. The three major adipocyte classes — white adipocytes , beige and brown adipocytes — have different developmental origins, distributions within the body and functions. The T_{reg} cells operating in VAT merit special attention because they were the first tissue-distinct T_{reg} population to be identified and are the one we presently know most about.

The epididymal VAT depot of lean, "middle-aged" mice harbors a unique population of $FOXP3^{+}CD4^{+}$ T cells[5]. These cells begin to accrue at 3-4 months of age and continue to do so until they constitute the large majority of $CD4^{+}$ T cells by 5-7 months. They do not accumulate in other VAT depots, nor in SAT [5,14]. T_{reg} cells reside in the spaces between adipocytes, often where several of them intersect, a site where macrophages, dendritic cells (DCs) and other leukocytes also tend to aggregate [5,15]. In genetically or diet-induced models of obesity, VAT-T_{reg} cells are strikingly reduced and are more concentrated in "crown-like" structures at multi-adipocyte junctures [5]. Such changes were the first clue that VAT-T_{reg} cells are important regulators of local and systemic metabolism. Their distinct transcriptome, T cell receptor (TCR) repertoire and set of growth and survival factor dependencies arm VAT-T_{reg} cells for this specialized function.

Transcriptome.

The VAT.-.T_{reg}-cell transcriptome is strikingly different from those of its lymphoid organ counterparts [5], with thousands of transcripts being upregulated or downregulated according

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to recent RNA sequencing (RNA-seq) data [11]. Enriched transcripts include those encoding transcription factors (TFs) (such as *Pparg*, *Rora* and *Gata3*), chemokines and their receptors (such as *Cxcl2*, *Cxcr6*, *Ccr1* and *Ccr2*), cytokines and their receptors (such as *Il10*, *Il5*, *Il1rl1* and *Il9r*), costimulatory molecules (such as *Pdcd1*, *Ctla4* and *Cd80*) and, most interestingly, a set of molecules associated with lipid metabolism (such as *Dgat* and *Cd36*). The VAT T_{reg} cell signature is largely driven by peroxisome proliferator-activated receptor-γ (PPARγ), the "master" transcriptional regulator of adipocyte differentiation [16]. Mice lacking expression of PPARγ specifically in FOXP3^{+} cells have few T_{reg} cells in VAT and the residual cells do not show the characteristic VAT-T_{reg}-cell signature. By contrast, animals treated with a PPARγ agonist have an expanded VAT, but not splenic, T_{reg}-cell compartment.

TCR repertoire.

Like most CD4^{+} αβ T cells, T_{reg} cells from mouse lymphoid organs are highly polyclonal, repeated TCR usage being very rare except in old mice or in individuals undergoing an immune response. By contrast, the VAT-T_{reg}-cell compartment exhibits a much more constrained TCR repertoire, with each mouse showing multiple private clonal expansions [5,15]. Such clones argue that VAT T_{reg} cells are responding to one or more local antigens.

Accumulation of VAT T_{reg} cells within the epididymal fat depot depends on TCR recognition of peptide–MHC class II complexes displayed by local antigen-presenting cells [15,17]. The critical importance of TCR specificity is best illustrated by recent findings using a transgenic mouse model wherein the TCR repertoire is highly skewed for a particular VAT-T_{reg}-cell specificity: these mice have an over-abundance of FOXP3^{+}CD4^{+} T cells in epididymal VAT but not elsewhere; and transfer of splenic FOXP3^{+}CD4^{+} T cells from these mice into non-transgenic recipients results in accumulation of the transferred cells specifically in epididymal VAT [14]. Moreover, potent mimotopes capable of stimulating these TCR-transgenic T_{reg} cells were recently identified and used to improve insulin sensitivity in an adoptive-transfer system [17].

Growth and survival factors.

Unexpectedly, VAT enrichment of the above-mentioned TCR-transgenic T_{reg} cells also requires FOXP3 expression [14], perhaps reflecting a need for chemokine or adhesion receptors, the expression of which is regulated by this TF. Indeed, transcripts encoding many chemokine receptors are differentially transcribed in VAT.T_{reg} cells compared with lymphoid-organ T_{reg} cells [5], a profile that changes with obesity [18].

As is typical of FOXP3^{+}CD4^{+} T cells, VAT.T_{reg} cells are IL-2 dependent. They express high levels of CD25 and proliferate upon *in vitro* incubation with IL-2 or *in vivo* injection of obese mice with IL-2–anti-IL-2 complexes [5,19]. However, in contrast to those in lymphoid organs, T_{reg} cells in VAT respond much more strongly to IL-33 [15,19,20]. Not surprisingly, then, most VAT.T_{reg} cells express high levels of the IL-33 receptor, ST2, gradually increasing to >80% of the population at 30 weeks of age, in comparison with <10% of splenic T_{reg} cells. The importance of IL-33 signaling for VAT-T_{reg}-cell accumulation was solidified by results from lean mice with a constitutive knockout of either IL-33 or ST2 expression, both of which show a strong reduction in VAT T_{reg} cells but not lymphoid-organ

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T_{reg} cells [15,19]. Initially there was disagreement over whether the IL-33 effect on VAT-T_{reg} cells is a direct one because mice engineered to lack ST2 on group 2 innate lymphoid cells (ILC2s) showed a reduction in both FOXP3$^+$CD4$^+$ T cells and ILC2s in VAT[21,22]. However, studies of both mixed bone marrow chimeras [19] and T_{reg}-cell-specific ST2-deficient mice [14] have demonstrated a T_{reg}-cell-intrinsic effect. IL-33 directly impacts VAT T_{reg} cells by inducing their proliferation and modifying their transcriptional programs [19,23]. The current consensus is that T_{reg} cells and ILC2s in VAT collaborate to carry out the functions of IL-33, which is consistent with the substantial transcriptional overlap these two cell types exhibit [22]. Collaboration between ILC2s and T_{reg} cells has been reported to operate via ICOSL–ICOS [21] and/or OX40L–OX40 [22] interactions and to be inhibited by IFNγ [21].

The importance of IL-33 for VAT-T_{reg}-cell homeostasis has inspired several investigators to identify its major cellular source(s) in the mouse epididymal fat depot [15,23–28]. Imaging and single-cell RNA sequencing (scRNA-seq) revealed that cells of phenotype CD45$^-$CD31$^-$PDGFRα/PDPN$^+$SCA1$^+$ are the major IL-33 producers in lean mice. Consistent with this designation, mice lacking IL-33 expression specifically in PDGFRα$^+$ cells have a significantly reduced VAT.T_{reg} population [25]. This stromal cell population is heterogeneous in lean mice, composed of three or four IL-33$^+$ subtypes that may be either mesothelial or mesenchymal in nature [25,27]. Many of the IL-33$^+$ cells are found within a ring of connective tissue at the circumference of the depot, presumably the mesothelium [29]. Others are located in the interior of the depot – often, but not always, in close association with blood vessels and/or neurons. IL-33$^+$ stromal cells, in particular certain subtypes, increase with age [25], in males versus females [23,25] and in response to a long-term challenge with a high-fat diet (HFD) [25].

In humans.

Essentially all of the findings mentioned above pertain to mice. In considering data on the human VAT-T_{reg}-cell compartment — as well as on human T_{reg} populations in other non-lymphoid tissues — it is important to be aware of several differences in the two systems: greater genetic heterogeneity in humans than in the mice studied to date; more extensive and more heterogeneous environmental exposures in humans than in laboratory mice; molecular and cellular variations between the immune systems of the two species. Thus, one must ask to what extent human tissue-T_{reg} cells should mimic their mouse counterparts even if they were to perform the same functions. In the particular case of VAT T_{reg}-cells, differences in the sizes and locations of fat depots in the two species, and divergences in the relative contributions of the three adipocyte types to these depots, further confound the issue.

Nonetheless, T_{reg} cells have been found in the omental fat depot of lean individuals, in fractions of the CD4$^+$ T cell compartment higher than in human blood but lower than in lean mice [30,31]. However, so little data are currently available that it is not possible to determine whether this difference reflects true biology or whether the analysis of human VAT has not yet been optimized — as concerns age, depot (for example, no reports on human epididymal VAT) and extraction conditions. As in mice, a negative correlation between body-mass index (BMI) and either *FOXP3* transcript level [5,32] or T_{reg}-cell representation [30] in VAT has repeatedly been observed. Some of the transcripts upregulated in mouse VAT

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T_{reg} cells – such as *Pparg*, *Ccr4*, *Prdm1* and *Cxcl2* – are also augmented in their human counterparts [30]. Strikingly different from mice, the IL-33 receptor, ST2, encoded by *Il1rl1*, was found to be absent from human VAT T_{reg} cells by one group [30,33], although another has reported its expression [19]. Again, the difference may be rooted in experimental details such as patient selection, choice of fat depot and/or the precise T_{reg}-cell extraction procedure used. Alternatively, it may reflect true differences in cell surface markers of or functions exerted by VAT-T_{reg} cells in the two species. It may be worth noting that variations in the VAT-T_{reg}-cell compartments of different mouse strains have also recently been described [31].

Skeletal muscle T_{reg} cells

Skeletal muscle, one of the largest vertebrate organs, is frequently injured due to its size, superficial location and vulnerability to mechanical stress. It undergoes regeneration in healthy individuals, in muscular dystrophies and in response to acute injury, for example after administration of the neurotoxin cardiotoxin (CTX). Damage to skeletal muscle initiates a highly orchestrated and stereotyped repair and regeneration program [34] (Fig. 2). The T_{reg} population found in skeletal muscle merits a focused discussion because it highlights the important role local T_{reg} cells can play in tissue repair and regeneration.

A population of T_{reg} cells located in skeletal muscle was first reported in 2013 [6]. Uninjured hindlimb muscles of healthy young mice harbor a small T_{reg} population [6,35,36], but it has proven difficult to study because of its small size. Subsequent to acute injury, the muscle T_{reg} population rapidly expands, peaking around day 3 or 4, rapidly declining until about day 14, and tapering off thereafter, but remaining detectably elevated even a month after the injurious event [6,36]. No changes are seen in the FOXP3^{+}CD4^{+} populations of lymphoid organs or of control uninjured muscle in the same mice. In acutely injured muscle, T_{reg} cells are located within the inflammatory lesion as well as between proximal muscle fibers. Increased T_{reg}-cell levels are also a feature of the diaphragm and hindlimb muscles, but not lymphoid organs, of mouse models of Duchenne's muscular dystrophy (DMD), that is, *mdx* or *Dysf*$^{-/-}$ mice [6,35]. Muscle T_{reg} cells are important regulators of tissue repair and regeneration after both acute and chronic injury. Their tissue-adapted transcriptome, TCR repertoire and growth and survival factor dependencies render them competent to perform their specialized tasks.

Transcriptome.

Skeletal muscle T_{reg} cells have a distinct transcriptome, with thousands of transcripts increased or decreased in comparison with the transcriptomes of lymphoid-organ T_{reg} cells [6,37,38]. The muscle -T_{reg}-cell-upregulated and downregulated gene signatures share many transcripts with the corresponding VAT-T_{reg}-cell signatures, but each tissue-T_{reg}-cell signature also has a private component. The most striking element of the muscle-T_{reg}-cell upregulated gene signature is the preponderance of pathways related to cell cycle proliferation, which is consistent with the elevated proliferation rate of these cells [6,39]. Muscle T_{reg} cells are more similar to splenic T_{reg} cells than are their VAT counterparts , a finding that concurs with the observation that the former but not the latter population communicates extensively with the circulating T_{reg}-cell pool [39].

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TCR repertoire,

Skeletal muscle T_{reg} cells have a clonally expanded TCR repertoire in both acute and chronic injury settings [6]. Almost all of the muscle T_{reg} clones are private; however, one clone (or a conservative variant of it) was found in all 11 individuals examined 2 or 4 days after CTX-induced injury. In TCR-transgenic mice carrying this clone's rearranged *Tcra* and *Tcrb* genes, $FOXP3^{+}CD4^{+}$, but not $FOXP3^{-}CD4^{+}$, T cells displaying the transgene-encoded TCR preferentially accumulate in injured hindlimb muscle in a TCR-dependent manner, both in the transgenic model and adoptive-transfer derivatives of it [38]. These findings argue that muscle-T_{reg}-cell recognition of a local antigen or antigens drives their specific accumulation within muscle.

Growth and survival factors.

Skeletal muscle T_{reg} cells express a range of CD25 levels [6]. Indeed, the muscle-T_{reg}-cell compartment expands or contracts in response to injection of IL-2–anti-IL-2 complexes or anti-CD25, respectively, which improves or worsens, respectively, indices of muscle damage in the *mdx* model [6,35]. But, just like their VAT counterparts, skeletal muscle T_{reg} cells are much more responsive to IL-33 than to IL-2, a preference first discovered in the context of ageing [39]. Ageing of skeletal muscle, like that of most mammalian tissues, is accompanied by a steady decline in function and regenerative capacity due at least in part to an age-associated decrease in satellite cell frequency and function [40]. The T_{reg}-cell compartment is strikingly diminished in CTX-injured skeletal muscle of old mice, reflecting defects in recruitment, proliferation and retention [39]. In injured mice, both young and old, over half of the accumulating muscle, but not splenic, T_{reg} cells express ST2. T_{reg}-cell-specific loss of IL-33 signalling in young mice dampens T_{reg} cell accumulation specifically in muscle but not spleen, likely at the level of local proliferation, and compromises muscle repair. The major IL-33-producers in skeletal muscle are mesenchymal stromal cells [25,39]; there are fewer of these cells in aged mice and, consequently, less *Il33* expression. Remarkably, co-injection of IL-33 and CTX into old mice augments the expanding muscle-T_{reg} population and reverses the age-associated defect in muscle repair [39].

IL-33-producing muscle stromal cells are often found in close proximity to both large-fiber nerve bundles and small-fiber sensory neurons [39,41]. Moreover, these stromal cells transcribe an array of genes encoding neuropeptides, their receptors and other nerve-related factors [41]. Of particular interest, one stromal cell subtype expresses both IL-33 and the two subunits of the receptor for the neuropeptide CGRP (calcitonin-gene-related-peptide). Upregulation or downregulation of CGRP signals increases or decreases, respectively, IL-33 production by muscle stromal cells and, eventually, muscle (but not spleen) T_{reg}-cell accumulation.

In humans.

Little information about skeletal muscle T_{reg} cells in humans is currently available. We do know that $FOXP3^{+}CD4^{+}$ T cells are present at elevated levels in muscle biopsies of patients with DMD compared with healthy controls [35]. We also know that IL-33^{+} stromal and vascular cells in close apposition to nerve fibers are readily detectable in uninjured muscle biopsies from healthy individuals [39].

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Skin-T_{reg} cells

Skin forms a physical barrier between the organism and its environment, protecting against chemical, mechanical and microbial insults and guarding against excessive water loss. It is a multi-layered organ, consisting of the epidermis, then dermis above a bed of adipose tissue and fascia (Fig. 3). The population of T_{reg} cells operating in skin is a fascinating example of tissue-T_{reg} cells because of the diversity of functions these cells perform [42]. Given their accessible location, skin-T_{reg} cells are also the tissue-T_{reg} population that is best characterized in humans.

The existence and functional importance of skin-T_{reg} cells have been recognized for at least two decades [43]; however, their distinct nature and impressive range of activities became evident only relatively recently. There is an abrupt accrual of activated FOXP3^{+}CD4^{+} T cells in skin of perinatal mice, constituting as much as 90% of local CD4^{+} cells, an accumulation that both depends on and promotes tolerance to the skin microbiota [44,45]. Skin-T_{reg} cells also ensure life-long tolerance to local self-antigens, which is a classical T_{reg}-cell function' as evidenced by the severe autoinflammatory disease that develops when they are absent or impaired [7,46–49]. Their restraint of anti-pathogen responses to minimize collateral tissue damage [43,50] and their control of innate and adaptive allergic reactions [51] are also classical T_{reg}-cell functions. T_{reg} cells remain at elevated levels in skin of healthy adult mice, varying from 20–60% of CD4^{+} T cells according to the stage of the hair cycle; they concentrate around hair follicles and control the activities of hair follicle stem cells (HFSCs) [52].

Beyond their protective and homeostatic roles in healthy tissue, skin-T_{reg} cells promote tissue repair after a variety of cutaneous insults: such as full-thickness wounding [53], epidermal abrasion by tape stripping [54], bleomycin-induced sclerosis [55] and ultraviolet-B (UVB) light exposure [56]. Their activation and accumulation in association with conditions like dermatitis or psoriasis may also reflect, at least in part, their reparative activities [42]. After wounding, highly activated T_{reg} cells accumulate in the skin, where they hasten re-epithelialization and wound closure [53,54,56], rein in inflammation [53,56] and dampen fibrosis [55]. The skin-T_{reg}-cell transcriptome, TCR repertoire and growth and survival factor dependencies are adapted for effective performance of these diverse functions.

Transcriptome.

The skin-T_{reg}-cell transcriptome has been studied in diverse contexts: in comparison with conventional CD4^{+} T cells in skin and/or with T_{reg} cells in lymph nodes from healthy or UVB-exposed mice[11,50–52,55,56]. As anticipated, transcripts encoding activation or memory markers are upregulated in skin-T_{reg} cells. They preferentially express transcripts encoding a skin-specific set of chemokine receptors, including CCR2, CCR6, CCR8, CCR10, CXCR4 and CXCR6. And they upregulate expression of transcripts specifying known effector molecules, such as IL-10, granzyme B, amphiregulin (AREG), Jagged1 and the neuropeptide Penk, which is increased upon UVB irradiation. More generally, UVB exposure induces programs of neuropeptide signaling and wound healing in skin-T_{reg} cells [56].

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Amongst the transcriptionally characterized tissue-T_{reg} populations, skin and VAT-T_{reg} cells are the most alike, at least at the population level [11,56]. This similarity likely reflects shared dependence on mutually upregulated TFs such as BATF, IRF4, GATA3, MAF, BLIMP1 and RORα [11,51,55]. This and other features of the transcriptomes are reminiscent of the T helper 2 (T_H2) transcriptional program of effector T cells, and it has been argued that skin-T_{reg} cells are primed to regulate type 2 immune responses such as those that drive fibrosis [55]. The skin and VATT_{reg}-cell compartments are overwhelmingly dominated by an ST2-marked subtype that preferentially expresses these T_H2-associated molecules [11,15,19], driving their transcriptional concordance. Yet, scRNA-seq has revealed seven skin-T_{reg}-cell subtypes [55], and skin-T_{reg} cells can regulate both T_H1 [53] and T_H17 cell [50,54] responses, so the story does not end there.

TCR repertoire.

The skinT_{reg} population exhibits clonal expansion, a feature that has been noted for the $ST2^+FOXP3^+CD4^+$ population of healthy skin [57] and the $CD25^{hi}CD4^+$ cell population of UVB-exposed skin [56]. The degree of clonal expansion is greater than that of corresponding $CD25^-CD4^+$ T cell populations in skin. But it is not as extensive as that observed for $ST2^+FOXP3^+CD4^+$ T cells in VAT of lean mice, perhaps reflecting the advanced age at which VAT is routinely prepared. While the TCR repertoire of skin Treg cells is largely distinct from those of lymphoid organs and other tissue-T_{reg} populations, sharing of rare clones with VAT and/or colonic lamina propria T_{reg} cells has been documented [57].

Growth and survival factors.

T_{reg} cells seed the skin perinatally and localize near HFSCs in hair follicles [52]. Their accumulation at that site depends on development of follicles, more specifically their production of the chemokine CCL20 (a CCR6 ligand), which is further induced by the skin microbiota [45]. Once installed, skin-T_{reg} cells interact directly with HFSCs to drive their proliferation and differentiation, dependent on expression of Jagged1 (by T_{reg} cells) and Notch (by HFSCs) [52]. It is not yet known whether HFSCs talk back to T_{reg} cells.

Skin-T_{reg} cells show a somewhat perplexing cytokine dependency. Most of them express CD25 and ST2 but their steady-state maintenance does not seem to depend critically on either IL-2 or IL-33 [56,58]. By contrast, they do require signaling through the IL-7 receptor, which most of them display quite prominently [58]. This dependency likely reflects the effector/memory phenotype of adult skin-T_{reg} cells [7], and this is fitting given the relatively high level of IL-7 production by local keratinocytes and relatively low level constitutive IL-2 secretion in skin. However, IL-2 or IL-33 could still play a role in skin-T_{reg} cell phenotypic adaptation to particular stimuli, especially since both of these cytokines are well able to expand the skin-T_{reg}-cell compartment [11].

In humans.

Skin-T_{reg} cells are one of the few human tissue-T_{reg}-cell compartments about which we have more than just a rudimentary knowledge. Healthy adult human skin hosts a substantial population of $FOXP3^+CD4^+$ T cells, on average about five times their frequency in the blood [59,60]. These cells, highly activated and non-migratory, are preferentially localized

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near hair follicles, as in mice, and are most abundant in regions of high hair density [60]. The corresponding T_{reg} population in fetal skin is less frequent and less activated, suggesting that skin-T_{reg} cells may accumulate over time in response to local antigen(s) [60,61]. The functional importance of human skin-T_{reg} cells is suggested by the severe autoinflammatory skin disease manifested in patients with IPEX (immune-dysregulation, polyendocrinopathy, enteropathy, X-linked) [62]. In addition, development of alopecia areata, a common autoimmune disease resulting from T cell attack on hair follicles, is positively associated with a *FOXP3* promoter polymorphism [63], and augmentation of T_{reg} cells via low-dose IL-2 treatment is an effective alopecia therapy [64].

As in mice, healthy human skin-T_{reg} cells show elevated expression of cell surface, activation and memory markers compared with blood T_{reg} cells [51,60]. But differences between human and mouse T_{reg} cells do exist — for example, human but not mouse T_{reg} cells express the mitochondrial enzyme arginase 2 [65]; and mouse but not human T_{reg} cells display high levels of and depend on CD127, the IL-7 receptor [60].

The skin T_{reg}-cell compartment expands notably in the inflamed lesions of patients with psoriasis and, in contrast to healthy skin T_{reg} cells, they are highly proliferative and produce low levels of IL-17 [50,60,66,67]. There is a negative correlation between IL-17 and CD27 expression in patients' skin-T_{reg} cells, perhaps reflecting a role for CD27 in dampening effector T cell cytokine expression, as has been documented in mice [50]. Psoriatic skin T_{reg} cells show low-level expression of transcripts encoding arginase 2, an enzyme that has been linked to the high accrual, distinct identity and heightened suppressor activity of healthy skin T_{reg} cells through downregulation of mTOR signaling [65], which is known to be detrimental to T_{reg}-cell accumulation and activities in mice [68].

Tissue-T_{reg}cell functional heterogeneity

Analogous to studies on tissue-resident macrophages [69], explorations of tissue-T_{reg}-cell function have extended their purview beyond the regulation of local immune responses. For example, they have been reported to exert control over tissue and systemic metabolism, repair and regeneration of multiple tissues, and the proliferation, differentiation or fate of non-lymphoid cell progenitors. In part, these activities reflect indirect effects via neighboring immunocytes, often macrophages or ILCs, but they also issue from direct impacts of T_{reg} cells on stromal, parenchymal or progenitor cells. As exemplars, we detail tissue-T_{reg}-cell influences on organismal metabolism and on tissue repair and regeneration.

Control of organismal metabolism

VAT-T_{re-} cell control of metabolic tenor.

Epididymal VAT-T_{reg} cells regulate local and systemic metabolic indices, including phosphorylation of proteins downstream of insulin signaling, glucose tolerance, insulin tolerance and the HOMA-IR (homeostatic model assessment for insulin resistance). Most studies have demonstrated a positive effect of VAT-T_{reg} cells on the insulin sensitivity (and correlated parameters) of lean male mice [5,14,19,23,70,71]; however, one report argued that they have a negative influence [72]. The root of this divergence remains unknown but could

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lie in experimental divergences, such as different age of mice, different housing conditions, different microbiota and dissimilar approaches to manipulate T_{reg} cells. The key VAT-T_{reg} cell-deficient comparator strain in the latter case exhibited substantial reductions in body weight and fat mass, which does not typically occur in the absence of VAT T_{reg} cells and could, in and of itself, account for improved metabolic indices.

By contrast, there is universal consensus that VAT-T_{reg} cells promote metabolic health under obesogenic conditions. They are strikingly reduced in genetically or diet-induced models of obesity, which provokes local and systemic inflammation and insulin resistance[5,19,72]. This loss does not reflect a dearth of IL-33 — in fact, there is more of this cytokine in the epididymal fat pad of obese than of lean mice [19,25]. Rather some inhibitory or toxic factor(s) must be coming into play. Candidates include IFNγ[21,73], TNF[74] and a soluble ST2 decoy molecule [74], all of which are increased in obese mice, but additional factors are almost certainly involved. Somewhat surprisingly, then, injection of IL-33 into obese mice augments their VAT-T_{reg}-cell compartment and improves organismal metabolism [19–21,23].

Males vs females

Male and female mice have very different gonadal VAT-T_{reg}-cell compartments, leading to differences in their metabolic tenors in response to obesogenic challenges and perhaps under steady-state conditions as well. Mouse ovarian VAT lacks the expanded T_{reg} population characteristic of males of the same age [14,23,25,75]. In addition, the gonadal T_{reg}-cell transcriptomes differ by thousands of transcripts in the two sexes [23]. These differences in accumulation and transcription are specific to gonadal VAT as T_{reg} cells from other adipose tissues or from lymphoid organs do not diverge in these respects [14,23]. Unexpectedly, lean female mice were reported to be more insulin-sensitive and glucose-tolerant and to have less VAT inflammation than their male counterparts [23]. However, such metabolic improvements in female mice were not evident in another study [75]. It may be relevant that in the former, but not the latter, case lean females also weighed substantially less than males did, confounding interpretation of the metabolic divergences: is the T_{reg}-cell gain or weight loss causal? And, even in this study, experimental reduction of T_{reg} cells in lean females impaired organismal metabolism, as it does in males [23].

Again in contrast with the behavior of their epididymal counterparts, ovarian VAT-T_{reg} cells increase under obesogenic conditions [75,76]. Moreover, obese females exhibit reduced VAT inflammation and improved metabolic indices compared with lean females, in opposition to the changes observed in males. This sexual dichotomy reflects the actions of sex hormones, as the increase in ovarian VAT-T_{reg} cells is abolished in ovariectomized mice but is subsequently restored by oestrogen supplementation [76].

IL-33 levels are key to the sex differences observed under steady-state conditions [23,25]. Indeed, injection of IL-33 into young females induces levels of T_{reg} cells in ovarian VAT that are similar to those in epididymal VAT of unmanipulated, age-matched males. Lean male mice have more IL-33^{+} stromal cells in their gonadal VAT depot, and the landscapes of stromal cell subtypes are quite dissimilar in the two sexes, as are the transcriptomes of their gonadal VAT (but not other) T_{reg}-cell compartments. The transcriptional differences include programs responding to the sex hormones oestrogen and androgen. Female mice

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with a genetically or pharmacologically induced deficit in oestrogen receptor signaling have more gonadal T_{reg} cells and improved metabolic indices, whereas females lacking androgen receptor signaling show the opposite effects. Moreover, oestrogen-treated male mice have reduced gonadal VAT T_{reg} cell levels whereas testosterone-treated females show an increase. The sexual dichotomy in T_{reg} cell compartments is not T_{reg} cell intrinsic, which is consistent with a central role for differential IL-33 production by stromal cells.

SAT-T_{reg}-cell control of thermogenesis.

VAT is composed primarily of white adipocytes, the major function of which is to store excess energy. The inflammation and metabolic dysregulation provoked by nutrient overload are initiated in VAT depots, and we have explored how VAT T_{reg} cells regulate these aberrant processes. However, mammals also host two other major adipocyte types, brown and beige, which do not store energy but rather dissipate it as heat, a process termed adaptive thermogenesis.

In lean, young mice, both BAT and SAT host a readily detectable population of T_{reg} cells [5,25,77–79]. Before 3 months of age, SAT-T_{reg} cells constitute a fraction of $CD4^+$ T cells similar to or even greater than that of VAT -T_{reg} cells (i.e. ~10–20%) [5,25,77]. However, SAT-T_{reg} cells neither increase with age nor substantially decrease with obesity [5]. Their phenotype appears to be different from that of their VAT counterparts, more like that of splenic T_{reg} cells — for example, SAT-T_{reg} cells express low levels of ST2 and KLRG1 and seem not to depend much on IL-33 at steady state [25]. Their transcriptome is yet to be characterized. On the other hand, the transcriptome of BAT T_{reg} cells, which makes up a fraction of $CD4^+$ T cells similar to that of their SAT counterparts, has been reported [77,79]. Most transcripts upregulated in SAT-T_{reg} cells compared with lymphoid organ T_{reg} cells are elements of the VAT T_{reg}-cell signature, although a small fraction is not, raising the possibility of depot-adapted T_{reg}-cell compartments.

Data from three studies argue that the T_{reg} populations in SAT and/or BAT can respond to environmental cues — such as cold exposure, short-term high-calorie diet or β-3 adrenergic receptor agonism – to promote thermogenesis, whereas their counterpart in VAT cannot or can only minimally do so [77–79]. Application of such stimuli expands the local T_{reg} population; induces expression of the mitochondrial protein UCP1 and additional elements of the thermogenic program; promotes beiging of SAT, increases lipolysis, and stifles heat production. The centrality of T_{reg} cells to this process has been demonstrated through *in vivo* loss- and gain-of-function experiments.

Tissue repair and regeneration

Studies on skeletal -muscle T_{reg} cells advanced the concept that a dedicated population of T_{reg} cells can promote the repair and regeneration of injured tissue through combined effects on immunological and non-immunological processes [6]. Such influences are both widespread and evolutionarily conserved. Local T_{reg}-cell impacts on tissue regeneration have been documented subsequent to multiple types of acute or chronic injury of many other mouse tissues, for example the skin [53,54], lungs [10,80,81], heart [9,82,83], CNS [12,84], intestines [85] and peripheral vascular system [86]. Zebrafish also have $FOXP3^+$ T cells that infiltrate

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damaged tissues such as the spinal cord, heart and retina; these cells promote regeneration by secreting tissue-specific regulators of progenitor cell proliferation [87]. In essentially all of these contexts, tissue -T_{reg}-cells play the dual roles of dampening inflammation and promoting tissue recovery.

An early inflammatory response is a necessary element of the tissue repair andregeneration process as it serves to sterilize the wound, to remove dead cells and debris, and to enhance proliferation of parenchymal cell precursors. Major contributors from the innate immune system are neutrophils and inflammatory macrophages, which are soon joined by a variety of adaptive immune-system cells – T_H1 cells, T_H2 cells, T_H17 cells, $CD8^+$ T cells, γδ T cells and NK cells, depending on the particular context. On the other hand, an overexuberant or over-long inflammatory response is detrimental to tissue recovery as it can interfere with the eventual change in immunocyte tenor required for effective repair, notably the emergence of pro-reparative macrophages [88]. Tissue -T_{reg} cells rein in the early inflammatory response and promote the transition to a tissue milieu that favors regeneration [9,53,54,89]. T_{reg}-cell production of IL-10 seems to be a major effector mechanism [90,91], although the importance of this cytokine has too often just been assumed from its augmented expression and too little effort has been devoted to identifying other mechanisms involved, for which transcriptomic analyses have now provided many candidates (such as the proteins GZMB, METRNL, FGL2, Serpin B1a and LTB4R1).

Tissue T_{reg} cells impact non-immunological processes at several points along the regeneration pathway. First, they can promote repair of tissue barriers, as skin T_{reg} cells do by inhibiting a local CXCL5–IL-17 axis of inflammation and thereby diverting HFSC differentiation towards the epidermal cell lineage [54]. Second, they foster the proliferation and/or differentiation of non-lymphoid cell precursors in several injured tissues: such as muscle [6], heart [83], skin [54] and the CNS [84] in mice; and heart, CNS and retina in zebrafish [87]. Third, they promote healthy tissue remodeling, dampening pathological processes such as fibrosis and astrogliosis [6,9,12,55,83].

The pro-regenerative effects of tissue-T_{reg} cells are multivariate. They can operate directly or indirectly on non-lymphoid cells or their precursors — even within the same tissue. On the one hand, a major influence of local T_{reg} cells early after muscle injury is to restrain IFNγ production by T_H1 and $CD8^+$ T cells [89], which could protect quiescent satellite cells from collateral attack by limiting their display of MHC class I molecules, as has been documented in other contexts [92,93]. On the other hand, AREG, produced by muscle T_{reg} cells later during the healing process, binds to epidermal growth factor receptor (EGFR) on muscle cell progenitors and enhances their differentiation [6]. The pro-regenerative effects of tissue T_{reg} cells can reflect cell–cell contacts — for example, elevated HFSC proliferation and differentiation induced by Jagged1–Notch interactions in skin [52] — or can be mediated by soluble factors. These factors can be general or tissue-specific. AREG exemplifies a general factor with documented regenerative activity in the skeletal muscle [6], lungs [10], skin [53], CNS [12] and neonatal heart in mice [83]. There are also many examples of tissue -T_{reg}-cell-produced soluble factors implicated in the regeneration of a particular tissue: the matricellular protein CCN3 in the CNS [84], the pro-angiogenic peptide apelin in the peripheral vascular system

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[86] and keratinocyte growth factor in the lungs [81] of mice; neurotrophin 3 in the spinal cord, neuregulin 1 in the heart and insulin-like growth factor 1 in the retina of zebrafish.

On a distinct but related note, several examples of tissue -T_{reg} cells promoting the homeostatic proliferation and/or differentiation of non-lymphoid-cell precursors have been reported. Through Jagged1–Notch interactions, skin-T_{reg} cells regulate the hair follicle cycle by driving HFSC division and differentiation [52]. Intestinal T_{reg} cells use IL-10 to support intestinal stem cell renewal, restraining their proliferation and aberrant differentiation [91]. Bone -marrow-T_{reg} cells are frequently located next to haematopoietic stem cells, assuring their numbers and quiescence by regulating local adenosine production [94,95]. And VAT-T_{reg} cells exert control over the numbers and relative representation of adipocyte-generating stromal-cell subtypes in their microenvironment [25].

Tissue T_{reg} cell origin and diversification

Cellular derivation

The distinctiveness of the various tissue T_{reg} populations raises the issue of their origin. When are they made? Do they migrate from the thymus as T_{reg} cells or are they generated through peripheral conversion of conventional $CD4^+$ T cells? Where do their specialized phenotypes emerge?

Ontogeny.—T_{reg} cells generated early and later in a mouse's life are different [96]. $FOXP3^+CD4^+$ T cells can be detected in the thymus from as early as two days after birth and in the peripheral lymphoid organs shortly thereafter. Perinatally generated T_{reg} cells are quite stable, persisting for months. Selection of about one third to one half of the perinatal T_{reg}-cell repertoire depends on thymic expression of the transcriptional regulator AIRE and these cells are crucial for avoidance of autoimmune attack of a number of organs. Peptides recognized by several perinatal T cell clones were recently identified, and certain of them are tissue-preferentially expressed [97]. Moreover, the transcriptome of perinatally generated T_{reg} cells is more similar to those of various tissue -T_{reg} populations than is the transcriptome of T_{reg} cells generated in adult mice, including upregulation of typical tissue-T_{reg}-cell transcripts such as *Il1rl1, Pdcd1, Ccr2, Icos, Fgl2* and *Il9r* [96]. These observations suggest that tissue-T_{reg} cells might be generated perinatally.

Indeed, T_{reg}-cell seeding of several tissues (VAT, skin, lungs, colon and liver) is active during the first two weeks of life [15,44,57,98]. The installed skin- and VAT-T_{reg} populations are minimally migratory, and the population in VAT is inadequately replenished if depleted in an adult [5,15]. Thus, at least some tissue-T_{reg}-cell compartments are seeded perinatally.

Derivation.—T_{reg} cells can emerge from the thymus as $FOXP3^+CD4^+$ cells (thymus-derived T_{reg} cells) or can come from $FOXP3^-CD4^+$ cells by peripheral conversion (peripherally derived T_{reg} cells). While not perfect markers, $FOXP3^+CD4^+$ cells expressing little or no helios or neuropilin 1 are generally considered to be peripherally derived. Placental T_{reg} cells and a major subtype of T_{reg} cells in the colonic lamina propria are notable examples of peripherally derived T_{reg} cells, and the primary phenotype of mice with a genetically engineered deficiency in peripherally derived T_{reg} cells is intestinal

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inflammation, at least at steady state [99,100]. Given their generally high level expression of helios and/or neuropilin 1, most tissue-T_{reg} cells are probably thymus-derived. And where the issue has been addressed more rigorously via transcriptomics, TCR sequencing and/or adoptive-transfer experiments (for example for VAT and muscle T_{reg} cells [6,15]), the same conclusion was reached. It remains possible, however, that peripherally derived T_{reg} cells contribute to the Treg compartments of these tissues as a minor component, for example in response to inflammatory challenges.

Specification.—The emergence of distinct tissue-T_{reg} populations was anticipated to follow one of two scenarios: they exit ready-made from the thymus or they take on their specialized phenotype once installed within their home tissue. However, the actual scenario turns out to be more complex (Fig. 4): future tissue-T_{reg} cells emigrate from the thymus to lymphoid organs without (so far) detectible distinction; there they undergo an activation event of unknown origin, which permits them to leave the circulation and access the tissues; those of them whose TCRs recognize a local antigen are retained and undergo definitive phenotypic adaptation. This scenario was originally established using a TCR-transgenic mouse model harboring the rearranged *Tcra* and *Tcrb* genes of a VAT-T_{reg}-cell clone coupled with a *Pparg* reporter line [14]. It has subsequently been generalized to the skin [57,101–102], colon [57,101], liver [57] and lungs [57,102].

Various markers can be used to distinguish the tissue- T_{reg}-cell precursor residing in lymphoid organs: PPARγ^{low} [14], ID3^{low} [102], NFIL3^{+} [57] and TCF1^{low} [103]. Although the activation event that incites their maturation in the spleen is not yet known, precursor accumulation *in vivo* depends on TCR–MHC class II interactions, FOXP3 and IL-33 [14]; and they can be induced *in vitro* from naive splenic T_{reg} cells by addition of IL-4, IL-6 or IL-33. The transcriptome and BATF dependence of splenic tissue-T_{reg}-cell-precursors are reminiscent of the ST2^{+} subtype of tissue-T_{reg} cells that is dominant in VAT and skin but present to at least some degree in all tissues so far examined [57]. It remains to be determined whether other tissue-T_{reg}-cell subtypes arise from the same precursor population.

Molecular diversification

The transcriptomes of T_{reg} cells located in various non-lymphoid tissues diverge substantially from those of lymphoid-organ T_{reg} cells and are also quite distinct from each other. To illustrate this point, we have compiled a set of robust published RNA-seq datasets from diverse tissue-T_{reg} populations (Fig. 5) and have distilled lists of genes expressed differentially compared with control lymphoid-organ-T_{reg} populations from the same mice. (Note that this approach avoids issues of dissimilar mice, T_{reg}-cell isolation procedures and/or RNA analysis platforms). Principal components analysis (PCA) on the differentially expressed gene sets shows that VAT- and skin Treg cells have the most distinct transcriptomes, whereas brain- and liver-T_{reg} cells are more similar to, yet still distinct from, lymphoid organ T_{reg} cells (Fig. 5a).

The establishment and regulation of these distinct tissue-T_{reg}-cell transcriptomes is complex and multi-layered. Differential-transcript analysis reveals gene sets that are upregulated or downregulated in all tissue-T_{reg}-cell compartments (pan-tissue), shared by more than

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one tissue's T_{reg} cells (tissue-preferential) and unique to a particular tissue- T_{reg}-cell compartment (tissue-specific) (Fig. 5b and c). The pan-tissue upregulated signature includes transcripts encoding genes that are likely to be involved in the accumulation and functions of tissue-T_{reg}-cells in general: TFs (for example, *Irf4*, *Nfil3*, *Id2*, *Rora* and *Hif1a*), ST2 (*Il1rl1),* several chemokine receptors (*Ccr1*, *Ccr2*, *Ccr8*, *Ccrl2* and *Cxcr6*), effector molecules (*Il10*, *Ebi3*, *Areg*, *Gzmb*, *Fgl2, Metrnl* and *Ltb4r1*) and co-inhibitory molecules (such as *Ctla4* and *Cd274*). Pathway analysis of this signature reveals enrichment of cellular responses to hypoxia, a possible adaptation to the slightly more hypoxic environments inside tissues; cytokine-mediated signalling, consistent with the importance of soluble factors in tissue-T_{reg}-cell recruitment and survival; and the circadian clock. The much smaller pan-tissue downregulated signature includes transcripts encoding TFs (such as *Id3* and *Tcf7*) and molecules implicated in lymphocyte trafficking to lymph nodes (*Icam2* and *Cxcr5*). Key members of these pan-tissue upregulated and downregulated transcript sets are already modulated during the transition of naive T_{reg} cells to tissue-T_{reg}-cell precursors in the lymphoid organs [14,57,101,102,103]. On the other hand, tissue-specific signatures are enriched for pathways that are important for T_{reg}-cell accumulation or function in unique tissue environments: for example, regulation of cell matrix adhesion pathways in the skin-specific upregulated signature, which is useful for T_{reg} cells residing in the collagen-rich dermis; or lipid metabolism pathways in the VAT-specific upregulated signature, which is important for survival in the otherwise toxic lipid-rich environment of fat. This multi-layered transcriptional landscape reflects influences both by epigenetic factors and by an interconnected network of positively and negatively acting TFs. However, we are just beginning to identify and integrate the activities of the key factors involved.

Epigenetic factors.—Methylation of CpG residues on DNA is an epigenetic mark that has been correlated with gene expression or silencing. A genome-wide analysis revealed that the DNA methylomes of VAT- and skin Treg cells differ substantially from that of lymph node T_{reg} cells, whereas the liver and lymph-node T_{reg}-cell methylation patterns are much more similar [11]. Analogous to their transcription profiles, the DNA methylation landscapes of VAT- and skin Treg cells have both shared and distinct components. Indeed, there is the expected negative correlation between most genes' methylation and transcription statuses. Whereas tissue-T_{reg}-cell signature genes (both pan-tissue and tissue-specific) are often hypermethylated in the bulk population of lymphoid organ T_{reg} cells, this modification declines in the small population of tissue T_{reg} cell precursors found within lymphoid organs.

Another class of epigenetic marks that shows correlations with the level of gene expression is post-translational histone modification, especially methylation and acetylation. Open-chromatin regions (OCRs), often measured by the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq), represent an integration of positively and negatively associated histone marks. Focusing on the differentially expressed sets of genes, tissue T_{reg}-cell OCRs again fall into three classes [37,57]: pan-tissue, tissue-preferential and tissue-specific. Interestingly, more than half of these OCRs — whether pan-tissue or tissue-specific — are already accessible in the bulk T_{reg} population of lymphoid organs, though not in other T cells. Such priming might serve to facilitate rapid changes

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in gene expression once the T_{reg} cells are installed in their home tissue and/or to create a restrained framework on which tissular cues can act.

Transcription factors.—Along with more classical approaches, ATAC-seq analyses have provided important information on TFs that regulate the tissue-T_{reg} cell differentiation pathway. BACH2, IRF4 and BATF are all key TFs for entry into the tissue-T_{reg}-cell precursor pool in lymphoid organs. BACH2 promotes the expression of transcripts that are downregulated in tissue-T_{reg} cells and their immediate precursors, and restrains their expression of upregulated transcripts [37,104]; whereas IRF4 and BATF show the opposite activities [11,57,105]. Consequently, populations of tissueT_{reg} cells and their precursors are expanded in BACH2-deficient mice and are absent or contracted in mice lacking IRF4 or BATF. A model has emerged whereby BACH2, induced by TCR engagement, directly enhances the expression of certain tissue-T_{reg}-cell downregulated signature loci, but also competes with BATF/JUN complexes for AP-1 binding sites at upregulated signature loci, thereby blocking recruitment of IRF4 [104].

IRF4 and/or BATF induce the expression of additional important TFs — such as BLIMP1, MAF, GATA3 and ID2 — in tissueT_{reg}-cell precursors [57,105,106]. BLIMP1, encoded by *Prdm1*, is crucial for the induction of tissue-T_{reg}-cell effector molecules, notably IL-10 [106]. It also inhibits the activity of the DNA methyltransferase DNMT3a, resulting in hypermethylation of the *Foxp3* locus, amongst others. This effect ensures high level FOXP3 expression and, consequently, maintenance of T_{reg}-cell stability under inflammatory conditions [107]. ID3 and ID2 are TFs of the basic helix-loop-helix class that regulate E-protein function. ID3 is highly expressed in resting T_{reg} cells but not tissue-T_{reg} cells or their immediate precursors, and its loss favors tissue-T_{reg}-cell differentiation; whereas ID2 has the opposite distribution and impact [37,57,102,104,108].

TFs with tissue-preferential or tissue-specific effects on T_{reg}-cell pools have also been identified [37]. Interestingly, integrated ATAC-seq and RNA-seq analysis has revealed that most of the consequential tissue-specific TFs are members of just a few major families — such as bZIP, ETS, nuclear receptor and RHD — but that different family members are dominant in different tissues. For example, within the nuclear receptor family, PPARγ, RORα and RARα are associated with VAT-T_{reg}-cell transcriptional regulation, whereas RORγ, VDR and others are associated with colon T_{reg}-cell transcriptional regulation. A paradigmatic example of tissue-specific control of T_{reg}-cell expression is PPARγ. This nuclear receptor family member is expressed at low levels in splenic tissue-T_{reg}-cell progenitors but at high levels only after their installation in VAT [14]. VAT-T_{reg} cells require PPARγ for normal accumulation and function [16]. Given that expression of *Pparg* is regulated by BATF and IRF4 in VAT [19], its transcription in tissue-T_{reg}-cell progenitors and tissue-T_{reg} populations other than that in VAT [11,37] is not surprising. However, a direct comparison between VAT and skin T_{reg} cells revealed substantially lower DNA methylation and higher *Pparg* transcription levels in VAT cells [11]. Moreover, PPARγ was not found to influence the accumulation of T_{reg} cells in skeletal muscle, the only non-VAT tissue-T_{reg} population functionally examined to date [37].

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Lastly, major features of the tissue-T_{reg}-cell diversification pathway are likely conserved between mice and humans. Many of the core skin and colon-T_{reg}-cell identity genes, or their paralogues, are shared between the two species [101]. Moreover, an IPEX-associated FOXP3 mutation expands the DNA-recognition profile of FOXP3 when introduced into mice, thereby repressing *Batf* transcription and impairing tissue-T_{reg}-cell fitness [105].

Summary and perspectives

The tissue-T_{reg}-cell concept has seen tremendous advances over the past decade. We have learned that many non-lymphoid tissues host phenotypically and functionally distinct T_{reg}-cell compartments, and that they have done so through vertebrate evolution. Exploring the distinct T_{reg} populations of diverse tissues has uncovered extensive heterogeneity in their provenance, transcriptomes, TCR repertoires, growth and survival factor dependencies and effector mechanisms. Their impacts on more and more non-immunological processes are becoming evident. Yet, we still have a lot to learn. Some areas that seem particularly ripe for exploration are listed below. Certain of these endeavors will benefit from, or may even require, technological advances (such as those discussed in Box 1).

We cited many examples of T_{reg} cells driving division and/or differentiation of non-lymphoid cell precursors during tissue regeneration. More surprising (and intriguing) are the observations of T_{re-} cell homeostatic impacts on progenitor cells of diverse organs in healthy animals. To what extent are these direct effects? What soluble mediators and cell-surface ligands are involved? Are these molecular pathways the same as those mobilized subsequent to acute or chronic injury? Do T_{reg}-cell–progenitor-cell interactions play any roles during postnatal organogenesis?

The identification of antigens recognized by tissue-T_{reg} cells would render several aspects of experimental work easier or more precise and could open new avenues of preclinical exploration. Currently, the best-characterized examples are two prostate T_{reg}-cell clones that recognize distinct peptides from the prostatic protein TCAF3 [109]. The identification of other tissue-T_{reg}-cell targets has proven challenging so far, as is true for most $CD4^+$ T cell self-antigens.

Understanding the true capabilities of tissue-T_{reg} cells requires “capturing” them within their microenvironment: their definitive phenotype is set therein, they respond to signals from neighboring cells, and they influence the behavior of cells in their vicinity. Thus, we must no longer confine explorations of molecular and cellular interaction partners to the realm of immunocytes. We have already seen that tissue-T_{reg} cells receive key signals from nerve and stromal cells [12,15,39,41,56,110] and provide signals to stem [6,52,91] and vascular [86] cells.

Information on human tissue-T_{reg}-cell compartments is woefully scant, with the relative exceptions of the skin and colon T_{reg} populations. This dearth of information reflects the poor accessibility of most human non-lymphoid tissues and, more recently, the artefactually poor representation of tissue-T_{reg} cells (and other T cells) achieved with whole-tissue scRNA-seq platforms or single-nuclear variants thereof. Precision targeting of designated tissue-T_{reg}-cell compartments stands to be both more effective and less risky.

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GLOSSARY

Immunocyte
A cell, such as a lymphocyte, that has an immunologic function

White adipocytes
Type of fat cell that stores lipids as triglycerides. They are mostly found in visceral adipose tissue, which has functions beyond lipid storage, including cushioning and insulating the body; serving as an endocrine organ through secretion of adipokines, cytokines and other mediators; and in anti-pathogen responses.

Beige adipocytes
A type of thermogenic adipocyte that can be induced in white adipose tissue – in particular, subcutaneous depots – in response to environmental cues such as cold or short-term nutrient excess.

Brown adipocytes
Lipid storage cells that play a crucial role in non-shivering thermogenesis. They are confined to brown adipose tissue. Like beige adipocytes, they have an elevated mitochondrial content and transcribe a thermogenic program dependent on expression of UCP1. They are activated by release of β-adrenoreceptor agonists from sympathetic neurons and the adrenal gland.

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Box 1:

A wish-list of technological advances

A tissue T_{reg} single-cell atlas

Single-cell RNA sequencing (scRNA-seq) data on tissue T_{reg} populations have begun to emerge [37,55,57,83,101,112]. They have reinforced the global distinctiveness of the T_{reg}-cell compartments in different tissues but have also revealed the existence of analogous, though still distinct, subtypes within the T_{reg}-cell compartments of the various tissues. Since the data remain relatively limited and have been generated using different platforms, filtered using different quality-control measures and analyzed via different algorithms, a more comprehensive, stereotypically generated tissueT_{reg} cell atlas stands to provide immense stimulus to the field. Such a compendium should not only encompass the T_{reg}-cell compartment of a diversity of tissues at homeostasis but also include dynamic data on T_{reg} populations subsequent to classical non-immunological challenges. (Note that existing dynamic data on tumor-T_{reg} cells argue the value of such an endeavor [112]). Spatial transcriptomics approaches could also be valuable in localizing diverse tissue-T_{reg}-cell subtypes within their host organ. Achieving this ambitious goal would not only demand will and wealth, but also improvements in existing technologies. Lastly, the power of zebrafish as a vertebrate model argues for an ancillary, analogous tissue-T_{reg} cell atlas under steady-state and challenged conditions.

Tissue-specific targeting of T_{reg} cells.

An issue that has often arisen in studying tissue -T_{reg} cells is the inability, with a few exceptions, to either expand or diminish the T_{reg} population specifically in a designated tissue rather than organism-wide. Experimental explorations would greatly benefit from such a targeted approach, while clinical studies may well depend on it. It is imperative, then, to invest efforts in approaches such as: combinatorial conditional knockouts [113,114], bispecific (T_{reg} cells plus tissue) delivery modalities, data mining for targets on designated tissue-T_{reg} populations, and arming of transferred cells or nanoparticles with tissue-relevant T cell receptors.

More sophisticated *in vitro* models

Classical functional tests like the *in vitro* suppression assay are largely irrelevant to tissue-T_{reg}-cell biology, so we must rely on *in vivo* or more complex *in vitro* systems (reviewed in [115]) for meaningful insights. One approach is to use *ex vivo* tissue slices, which maintain integrity over several days and permit convenient additions and subtractions of cells and molecules. Organoids, whether derived from stem cells or constructed from existing tissue components, are an increasingly used alternative. While complex and not perfect reproductions of the tissue being modeled, such systems permit easier and more punctual manipulation than is possible with living organisms. And they can often be more readily translated to the analogous human tissue.

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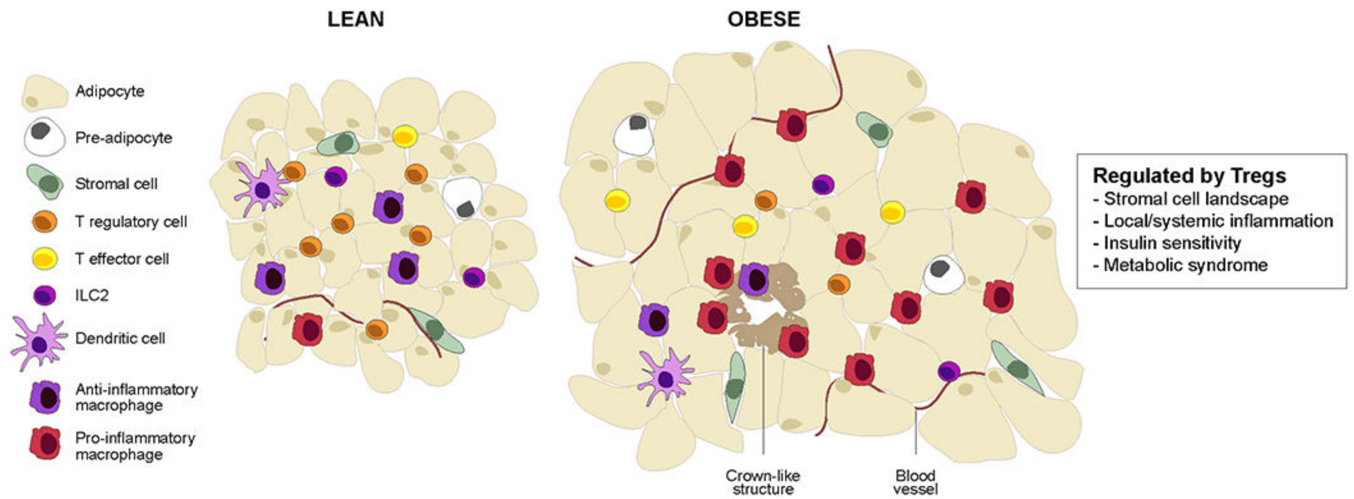

Figure 1: Visceral adipose tissue biology and regulatory T cells.
Schematic of the epidydimal fat pad of "middle-aged" lean versus obese mice. This visceral adipose tissue (VAT) depot, mostly composed of white-adipose cells, hosts a panoply of resident and recruited innate and adaptive immunocytes. Genetic or diet-induced obesity is associated with chronic, low-grade inflammation that entails secretion of inflammatory cytokines (notably, tumor necrosis factor (TNF), IL-1, IL-6 and interferons) as well as accumulation of inflammatory leukocytes, especially pro-inflammatory macrophages. Inflammation of VAT depots promotes type-2 diabetes and other features of the metabolic syndrome, including insulin resistance, fatty liver disease and heart disease. ILC, innate lymphocyte cell.

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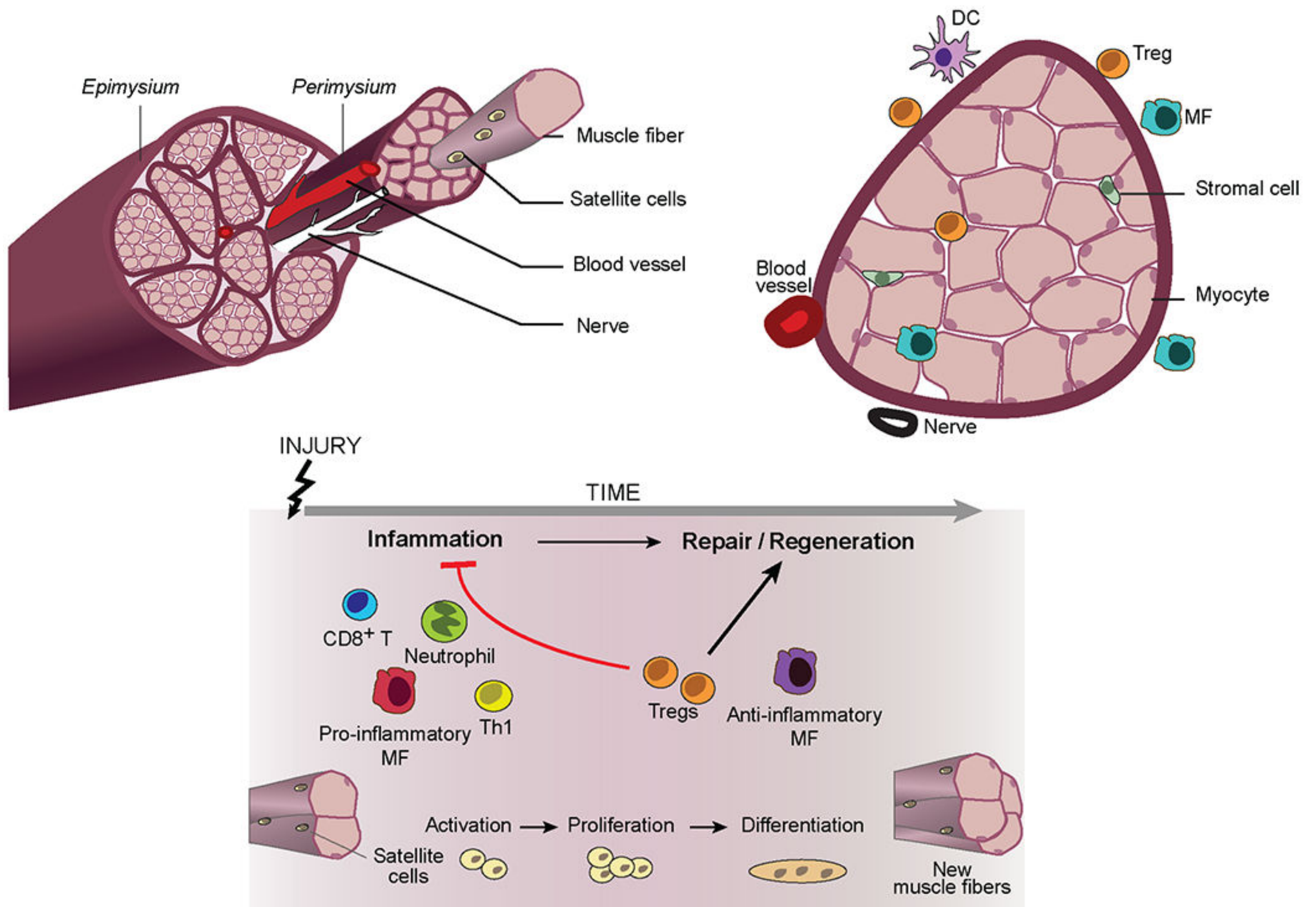

Figure 2: Skeletal muscle biology and regulatory T cells.

Schematic of hindlimb muscle from a young mouse (top) and its response to acute injury (bottom). Upon injury, mostly quiescent muscle progenitor cells (termed satellite cells) are activated, undergo asymmetric division, and differentiate into post-mitotic precursors, which then fuse to form multi-nucleated myotubes. Myotubes engender new myofibres or fuse to existing ones, followed by a stage of terminal differentiation and growth. This regenerative program is landmarked by a well-defined series of myogenic transcription factor changes. Both innate and adaptive immune-system cells positively or negatively regulate skeletal muscle regeneration. Neutrophils are the earliest of responders, followed by pro-inflammatory macrophagess, $CD8^+$ T cells and T helper 1 (T_H1) cells. This initial inflammatory response, which is a requirement for effective regeneration, is followed by a reparative stage dominated by anti-inflammatory macrophages and regulatory T (T_{reg}) cells, both cell types essential for effective regeneration. DC, dendritic cell.

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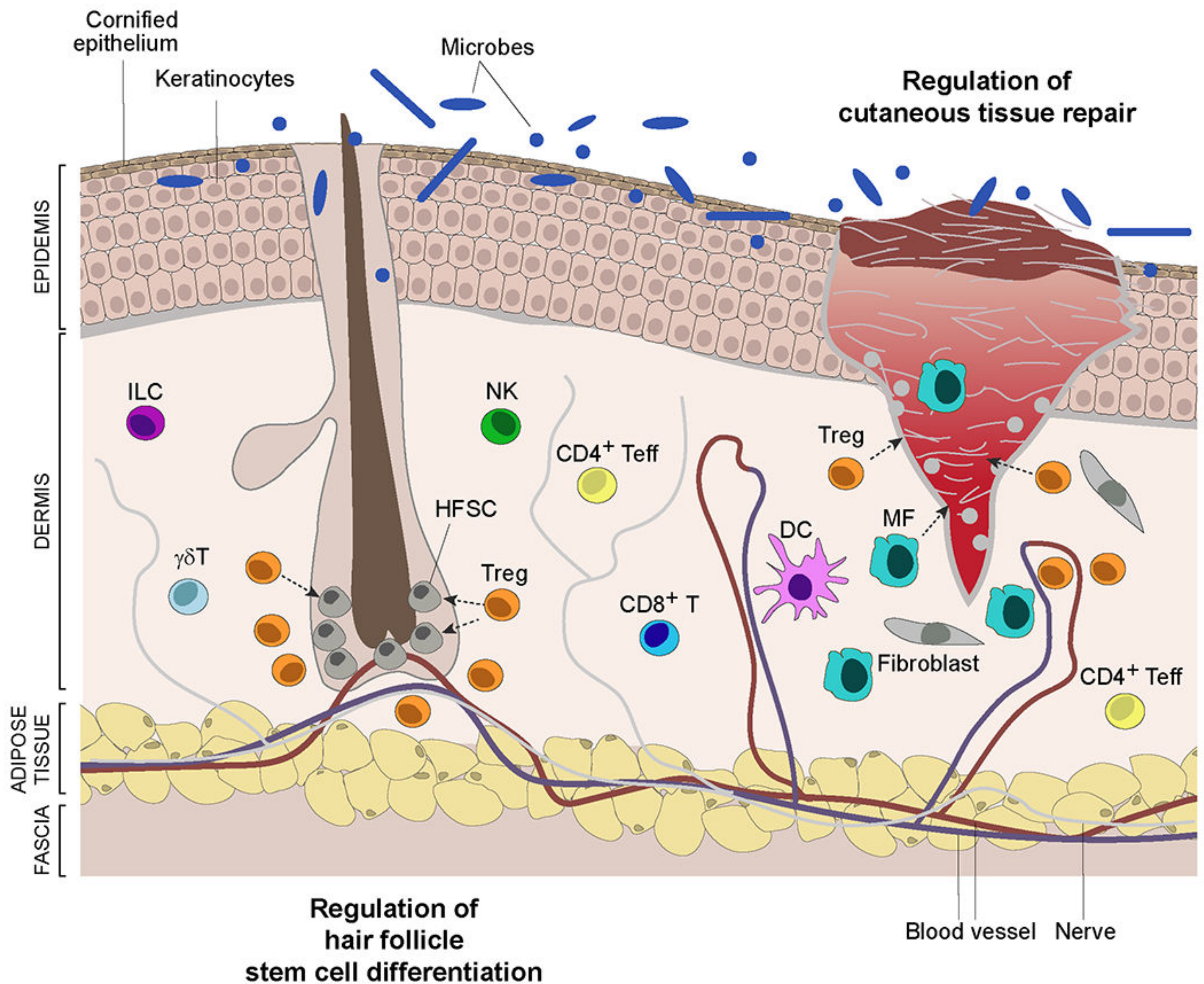

Figure 3: Skin biology and regulatory T cells.

Schematic of skin showing a hair follicle and a full-thickness wound. The skin epidermis is composed primarily of keratinocytes that differentiate from basal-layer stem cells while migrating upwards, culminating in a cornified layer that interfaces with the environment. This layer is permeated by hair follicles that cycle through resting (telogen) and growth (anagen) phases, reflecting the activity of hair follicle stem cells (HFSCs). The collagen-rich dermis hosts a variety of stromal-cell types and innate and adaptive immunocyte populations. The deeper adipocyte layer functions as mechanical support and in thermoregulation. Regulatory T (T_{reg}) cells integrate with skin cells to perform a diversity of functions: maintenance of tolerance to local self-antigens; promotion of tolerance to the skin microbiota; prevention of collateral damage during pathogen infections; optimization of cutaneous tissue repair; control of fibrosis; regulation of hair follicle stem cell proliferation, differentiation and fate. They exert their influences by impacting the activities of local immune, stromal and stem cells. DC, dendritic cell.

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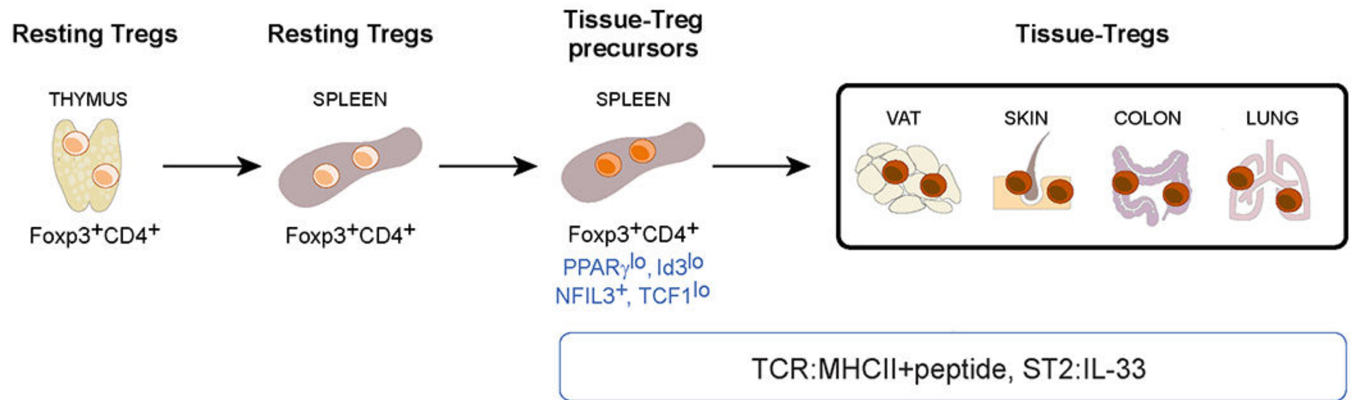

Figure 4: The cellular derivation of tissue regulatory T cells.

FOXP3^{+}CD4^{+} T cells exit the thymus and enter the circulation, including lymphoid organs such as the spleen. Less than 10% of resting lymphoid-organ regulatory T (T_{reg}) cells undergo an unknown activation event that allows them to escape the circulation and filter through tissues. T_{reg} cells are retained in a tissue that expresses peptide–MHC class II complexes recognized by their T cell receptors (TCRs) and therein undergo definitive specialization. VAT, visceral adipose tissue. NFIL3, nuclear factor interleukin-3-regulated protein 3; TCF1, T cell factor 1; ST2, IL-33 receptor.

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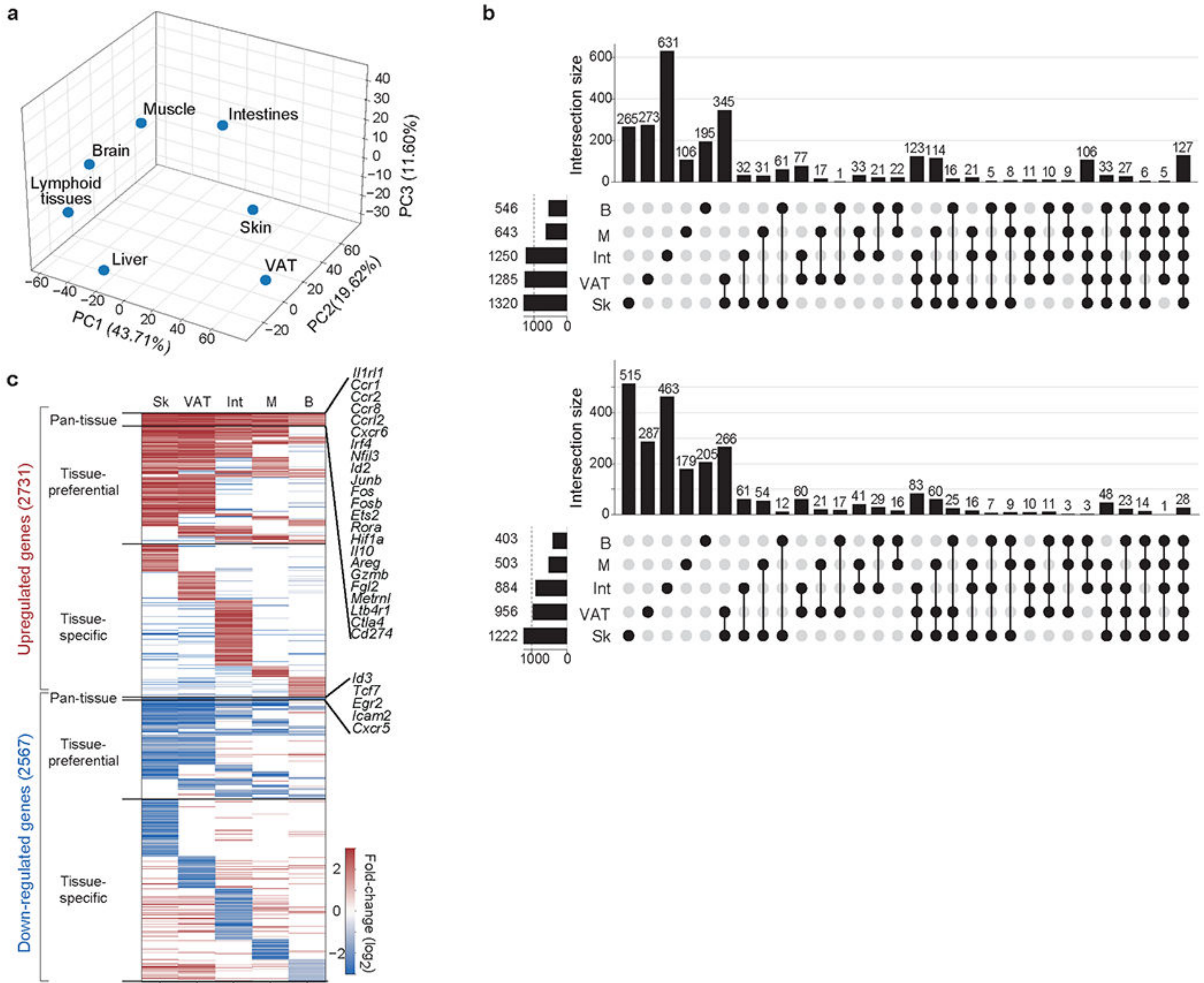

Figure 5: Analysis of tissue regulatory T cell transcriptomes.

Transcripts differentially expressed between each tissue regulatory T (T_{reg}) cell population and its corresponding lymphoid-tissue control (fold change >2 and FDR <0.10) were calculated using the edgeR package. RNA sequencing datasets for VAT, skin and liver T_{reg} cells are from REF. 11; skeletal-muscle T_{reg}-cell data are from REF. 38; brain T_{reg} cell-data are from REF. 107; and intestinal T_{reg} cell-data are from REF. 111. **a.** The compiled matrix of $\log_2$ (fold-changes) for each of the tissue-T_{reg}-cell populations was scaled and used to perform principal components analysis (PCA). Principal components 1, 2 and 3 with their proportions of explained variance are plotted. **b.** UpSet plot depicting inter-tissue intersections for upregulated (top) and downregulated (bottom) transcripts from tissue-T_{reg} cells that had more than 500 differentially expressed genes. The horizontal bar graph indicates the total number of upregulated or downregulated transcripts for each tissue-T_{reg} population. The vertical bar graph shows the number of transcripts corresponding to the particular intersection or set of intersections delineated on the dot matrix. **c.** Heatmap of $\log_2$ (fold-changes) for each tissue-T_{reg} population, separated into

pan-tissue, tissue-preferential or tissue-specific gene sets. For each tissue T_{reg} population, the log_2 (fold-changes) of transcripts not in the signature (for example, fold-change <2 or FDR >0.10) are set to 0 for that tissue. Example transcripts from the upregulated and downregulated pan-tissue signatures are highlighted on the right. The matrix containing all of the tissue-T_{reg}-cell signatures, whether pan-tissue, tissue-preferential or tissue-specific, can be downloaded at: [https://cbdm.hms.harvard.edu/img/resources/SignaturesAndDatasets/Tissue-Treg%20signatures.xlsx].

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