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 multilayered array of common and tissue-distinct transcriptional programmes. Our deepening knowledge of tissue T_{reg} cell biology will inform ongoing attempts to harness T_{reg} cells for precision immunotherapeutics.

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

White adipocytes
Fat cells that store lipids as triglycerides. They are 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 antipathogen responses.

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Since their molecular definition about 20 years ago[1–3], the impressive power of FOXP3$^+$CD4$^+$ regulatory T (T_{reg}) cells has been increasingly appreciated. These cells initially attracted attention as regulators of the activities of other T cells but eventually were recognized to control the responses of most innate and adaptive immunocyte types. For a decade, almost all studies of 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 variety 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} cell 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 the roles of T_{reg} cells in tissue 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} cell populations functioning in non-lymphoid tissues emerged during the early years of T_{reg} cell research[4]. However, the notions that non-lymphoid tissues harbour 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 ensuring 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 flavour of the phenotypic heterogeneity exhibited by tissue T_{reg} cells, we begin with vignettes of three particular tissue T_{reg} cell 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 preadipocytes, diverse stromal cell types, vascular endothelial cells, sympathetic nerves and various 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 adipocytes 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} cell population to be identified and are the one we presently know most about.

The epididymal VAT depot of lean, 'middle-aged' mice harbours 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

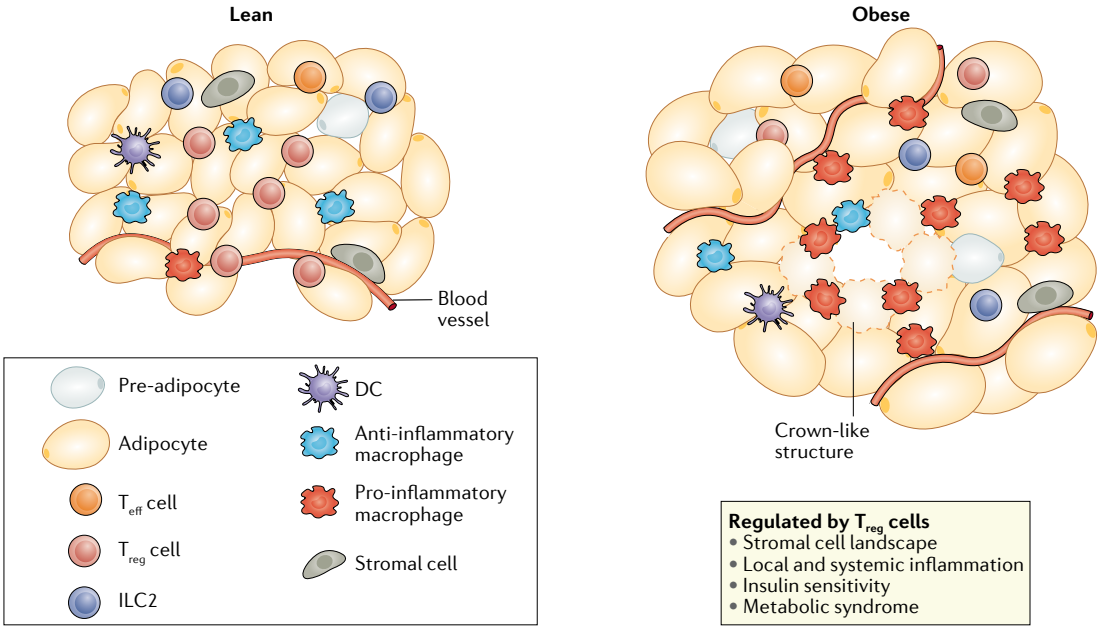

Fig. 1 | **Visceral adipose tissue biology and T_{reg} cells.** Schematic of the epididymal fat pad of 'middle-aged' lean versus obese mice. This visceral adipose tissue depot, composed mostly of white adipose cells, hosts a panoply of resident and recruited innate and adaptive immunocytes. Genetic or diet-induced obesity is associated with chronic, low-grade inflammation that entails secretion of pro-inflammatory cytokines (notably, tumour necrosis factor (TNF), IL-1, IL-6 and interferons) as well as accumulation of pro-inflammatory leukocytes, especially pro-inflammatory macrophages. Inflammation of visceral adipose tissue depots promotes type 2 diabetes and other features of metabolic syndrome, including insulin resistance, fatty liver disease and heart disease. DC, dendritic cell; ILC2, group 2 innate lymphoid cell; T_{eff} cell, effector T cell; T_{reg} cell, regulatory T cell.

reside in the spaces between adipocytes, often where several of them intersect, a site where macrophages, dendritic cells and other leukocytes also tend to accumulate[5,15]. In genetically or diet-induced models of obesity, VAT T_{reg} cells are strikingly reduced in number 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 the transcriptomes of its lymphoid organ counterparts[5], with thousands of transcripts being upregulated or downregulated according to recent RNA sequencing (RNA-seq) data[11]. Enriched transcripts include those encoding transcription factors (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*), co-stimulatory molecules (such as *Pdcd1*, *Ctla4* and *Cd80*) and, most interestingly, a set of molecules associated with lipid metabolism (such as *Dgat1, Dgat2* and *Cd36*). The VAT T_{reg} cell signature is driven largely 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 obtained with a transgenic mouse model wherein the TCR repertoire is highly skewed for a particular VAT T_{reg} cell specificity: these mice have an overabundance 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 increase insulin sensitivity in an adoptive-transfer system[17].

Growth and survival factors. Unexpectedly, VAT enrichment of the aforementioned TCR-transgenic T_{reg} cells also requires FOXP3 expression[14], perhaps

Beige adipocytes
Thermogenic adipocytes 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 programme dependent on expression of UCP1. They are activated by release of β-adrenoreceptor agonists from sympathetic neurons and the adrenal gland.

reflecting a need for chemokine or adhesion receptors, the expression of which is regulated by this transcription factor. 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 IL-2–anti-IL-2 complexes into obese mice[5,19]. However, in contrast to those in lymphoid organs, T_{reg} cells in VAT respond much more strongly to IL-33 (REFS[15,19,20]). Not surprisingly then, most VAT T_{reg} cells express high levels of the IL-33 receptor, ST2, gradually increasing to more than 80% of the population at 30 weeks of age, in comparison with less than 10% of splenic T_{reg} cells. The importance of IL-33 signalling 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 the number and proportion of VAT T_{reg} cells but not lymphoid organ 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 programmes[19,23]. The current consensus is that T_{reg} cells and ILC2s in VAT collaborate to perform 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 (REF.[22]) interactions and to be inhibited by interferon-γ (IFNγ)[21].

The importance of IL-33 for VAT T_{reg} cell homeostasis has inspired several investigators to identify its major cellular source or sources in the mouse epididymal fat depot[15,23–28]. Imaging and single-cell RNA-seq (scRNA-seq) revealed that cells of phenotype $CD45^-CD31^-PDGFR\alpha/PDPN^+SCA1^+$ are the major IL-33 producers in lean mice. Consistent with this designation, mice lacking IL-33 expression specifically in $PDGFR\alpha^+$ cells have a significantly reduced VAT T_{reg} cell population[25]. This stromal cell population is heterogeneous in lean mice, composed of three or four $IL\text{-}33^+$ subtypes that may be either mesothelial or mesenchymal in nature[25,27]. Many of the $IL\text{-}33^+$ 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. $IL\text{-}33^+$ stromal cells, in particular certain subtypes, increase in number with age[25], in males versus females[23,25] and in response to a long-term challenge with a high-fat diet[25].

In humans. Essentially all of the findings mentioned so far pertain to mice. In considering data on the human VAT T_{reg} cell compartment — as well as on human T_{reg} cell 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 few 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 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 T_{reg} cells — such as *Pparg*, *Ccr4*, *Prdm1* and *Cxcl2* — are also upregulated 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 programme[34] (FIG. 2). The T_{reg} cell 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 (REF.[6]). Uninjured hindlimb muscles of healthy young mice harbour a small T_{reg} cell population[6,35,36], but it has proven difficult to study because of its small size. Subsequent to acute injury, the muscle T_{reg} cell population rapidly expands, peaking at around day 3 or 4, rapidly declining until about day 14, and tapering off thereafter, but remaining detectably

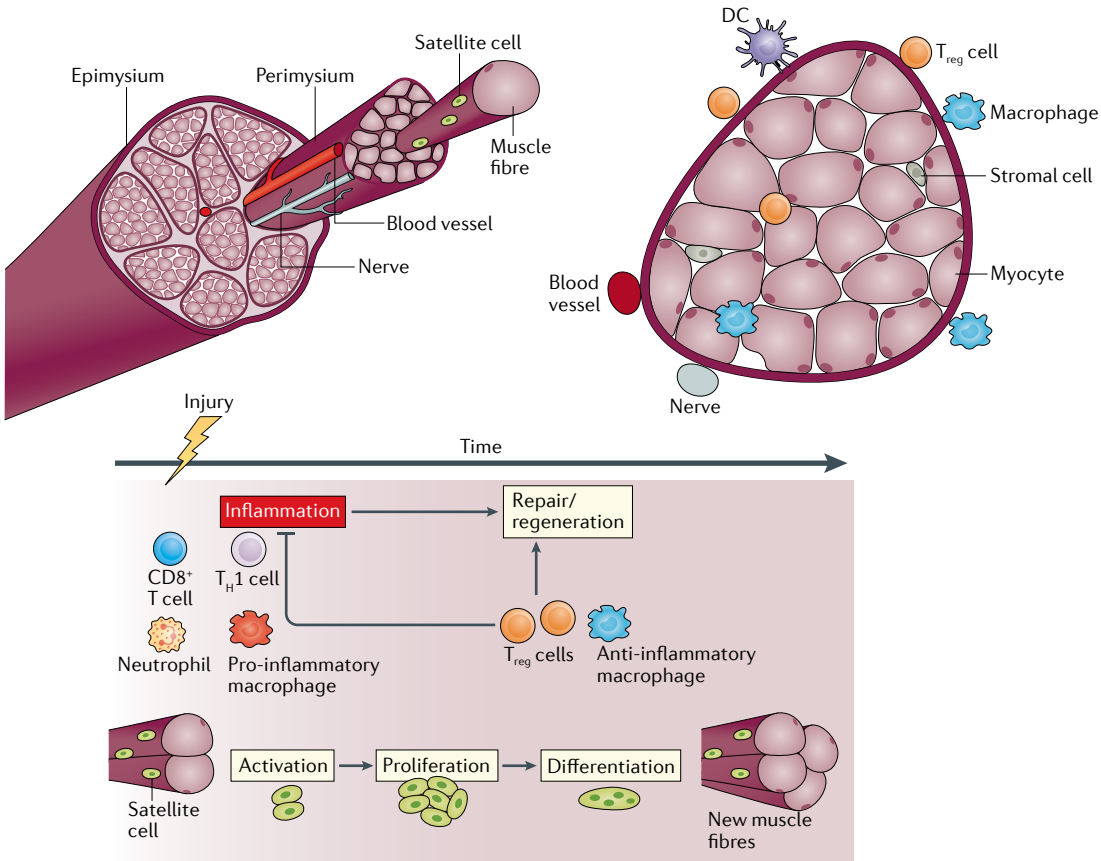

Fig. 2 | **Skeletal muscle biology and T_{reg} cells.** Schematic of hindlimb muscle from a young mouse (top) and its response to acute injury (bottom). Upon injury, mostly quiescent muscle progenitor cells (termed 'satellite cells') are activated, undergo asymmetric division and differentiate into postmitotic precursors, which then fuse to form multinucleated myotubes. Myotubes engender new myofibres or fuse to existing ones, followed by a stage of terminal differentiation and growth. This regenerative programme 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 macrophages, $CD8^+$ 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.

elevated even 1 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 fibres. Increased T_{reg} cell levels are also a feature of the diaphragm and hindlimb muscles, but not lymphoid organs, of mouse models of Duchenne 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 injury 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 the expression of 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 and 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 population but not the latter population communicates extensively with the circulating T_{reg} cell pool[39].

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} cell 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 in adoptive-transfer derivatives of it[38]. These findings argue that muscle T_{reg} cell recognition of a local antigen or antigens drives the specific accumulation of T_{reg} cells 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, more than 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} cell 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-fibre nerve bundles and small-fibre 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 biopsy samples of patients with Duchenne muscular dystrophy compared with healthy controls[35]. We also know that $IL\text{-}33^+$ stromal and vascular cells in close apposition to nerve fibres are readily detectable in uninjured muscle biopsy samples from healthy individuals[39].

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 multilayered organ, consisting of the epidermis, then the 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} cell 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 T_{reg} cells are absent or impaired[7,46–49]. Their restraint of antipathogen 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, ranging from 20% to 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 such as dermatitis and 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. Furthermore, they upregulate expression of transcripts specifying known effector molecules, such as IL-10, granzyme B, amphiregulin (AREG), Jagged 1 and the neuropeptide PENK, whose expression is increased upon UVB irradiation. More generally, UVB exposure induces programmes of neuropeptide signalling and wound healing in skin T_{reg} cells[56].

Among the transcriptionally characterized tissue T_{reg} cell 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 transcription factors, such as BATF, IRF4, GATA3, MAF, BLIMP1 and RORα[11,51,55]. This and other

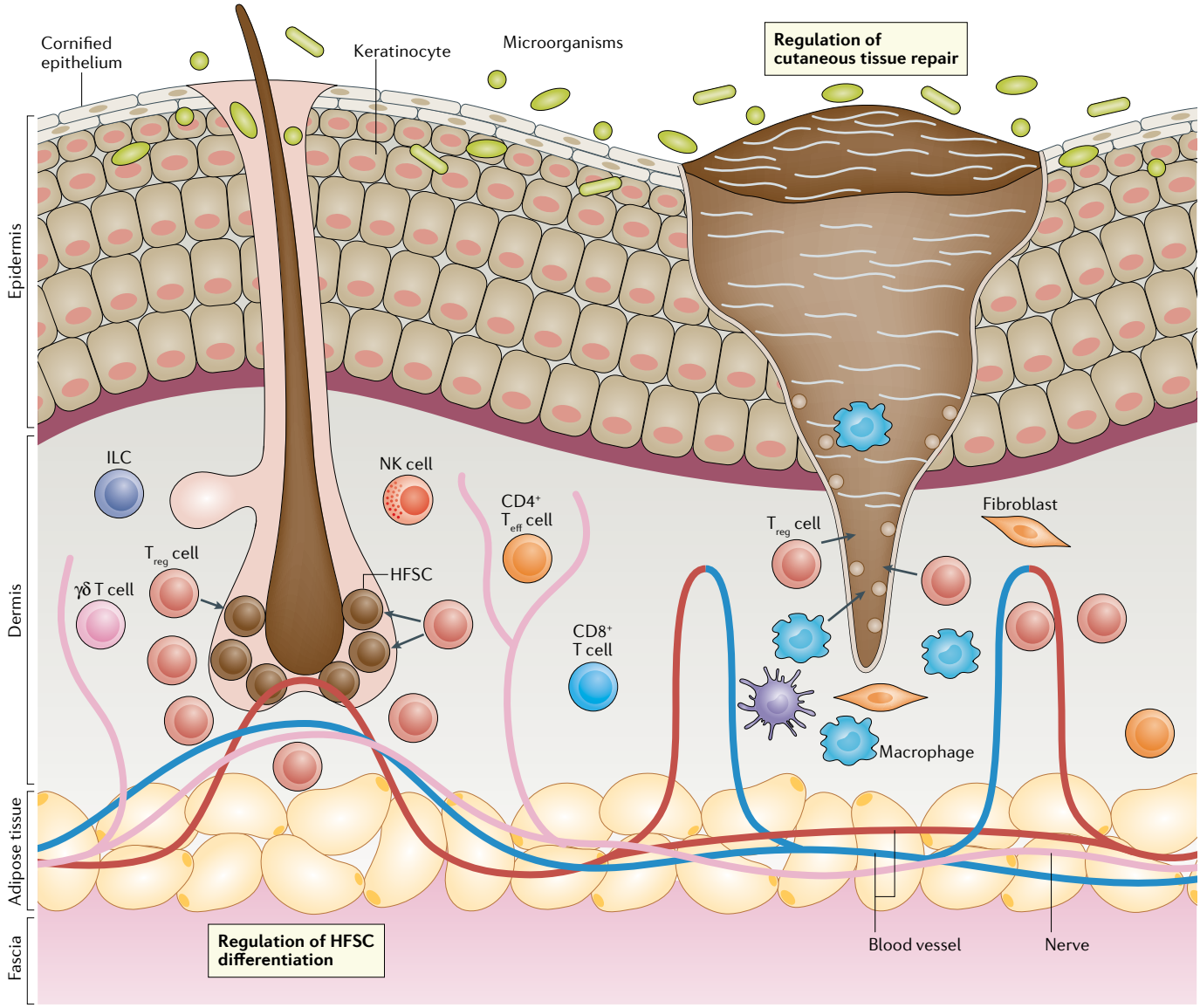

Fig. 3 | **Skin biology and T_{reg} cells.** Schematic of skin showing a hair follicle and a full-thickness wound. The skin epidermis is composed primarily of keratinocytes that differentiate from basal-layer stem cells while migrating upwards, culminating in a cornified layer that interfaces with the 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; and regulation of HFSC proliferation, differentiation and fate. They exert their influences by impacting the activities of local immune, stromal and stem cells. DC, dendritic cell; ILC, innate lymphoid cell; NK cell, natural killer cell; T_{eff} cell, effector T cell.

features of the transcriptomes are reminiscent of the T helper 2 (T_H2) cell transcriptional programme 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 VAT T_{reg} cell compartments are overwhelmingly dominated by an ST2-marked subtype that preferentially expresses these T_H2 cell-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 cell[53] and T_H17 cell[50,54] responses, so the story does not end there.

TCR repertoire. The skin T_{reg} cell 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 T_{reg} cells is largely distinct from the TCR repertoires of lymphoid organ T_{reg} cell and other tissue T_{reg} cell 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 Jagged 1 (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 (REFS[56,58]). By contrast, they do require signalling 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 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 near hair follicles, as in mice, and are most abundant in regions of high hair density[60]. The corresponding T_{reg} cell population in fetal skin is less frequent and less activated, suggesting that skin T_{reg} cells may accumulate over time in response to a local antigen or local antigens[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) syndrome[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 (REF.[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, psoriatic skin T_{reg} cells are highly proliferative and produce low levels of IL-17 (REFS[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 signalling[65], which is known to be detrimental to T_{reg} cell accumulation and activities in mice[68].

Tissue T_{reg} cell functional heterogeneity

Analogously to studies on tissue-resident macrophages[69], explorations of tissue T_{reg} cell functions 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 neighbouring 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 the influences of tissue T_{reg} cells on organismal metabolism and on tissue repair and regeneration.

Control of organismal metabolism

VAT T_{reg} cell control of metabolic tenor. Epididymal VAT T_{reg} cells regulate local and systemic metabolic indices, including phosphorylation of proteins downstream of insulin signalling, glucose and insulin tolerance, and insulin resistance as defined by homeostatic model assessment (HOMA-IR). 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 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 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 — there is actually more of this cytokine in the epididymal fat pad of obese mice than of lean mice[19,25]. Rather some inhibitory or toxic factor or factors must be coming into play. Candidates include IFNγ[21,73], TNF[74] and a soluble ST2 decoy molecule[74], the levels of which are all increased in obese mice, but additional factors are almost certainly involved. Somewhat surprisingly then, injection of IL-33 into obese mice increases their VAT T_{reg} cell compartment and improves organismal metabolism[19–21,23].

Males versus 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} cell 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 T_{reg} cells, as T_{reg} cells from other adipose tissues or from lymphoid organs do not diverge in these respects[14,23]. Unexpectedly, in the study by Vasanthakumar et al., 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 by Pettersson et al.[75]. It may be relevant that in Vasanthakumar et al., but not Pettersson et al., lean female mice also weighed substantially less than male mice, confounding interpretation of the metabolic divergences: is the T_{reg} cell gain or weight loss causal? Furthermore, even in Vasanthakumar et al., experimental reduction of T_{reg} cells in lean female mice impaired organismal metabolism, as it does in male mice[23].

Again in contrast with the behaviour of their epididymal counterparts, ovarian VAT T_{reg} cells increase under obesogenic conditions[75,76]. Moreover, obese female mice exhibit reduced VAT inflammation and improved metabolic indices compared with the changes observed in male mice. 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 female mice induces levels of T_{reg} cells in ovarian VAT that are similar to those in epididymal VAT of unmanipulated, age-matched male mice. 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 programmes responding to the sex hormones oestrogen and androgen. Female mice with a genetically or pharmacologically induced deficit in oestrogen receptor signalling have more gonadal T_{reg} cells and improved metabolic indices, whereas female mice lacking androgen receptor signalling show the opposite effects. Moreover, oestrogen-treated male mice have reduced gonadal VAT T_{reg} cell levels, whereas testosterone-treated female mice 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 have 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 (that is, ~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 in the 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[79]. Most transcripts upregulated in BAT T_{reg} cells compared with lymphoid organ T_{reg} cells are elements of the VAT T_{reg} cell signature, although a small fraction are not, raising the possibility of depot-adapted T_{reg} cell compartments.

Data from three studies argue that the T_{reg} cell populations in SAT and/or BAT can respond to environmental cues — such as cold exposure, a 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} cell population, induces expression of the mitochondrial protein UCP1 and additional elements of the thermogenic programme, promotes beiging of SAT, increases lipolysis, and stifles heat production. The centrality of T_{reg} cells in this process has been demonstrated through in vivo loss-of-function 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 impacts of T_{reg} cells 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 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 and regeneration 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 include neutrophils and inflammatory

macrophages, which are soon joined by a variety of adaptive immune cells — T_H1 cells, T_H2 cells, T_H17 cells, $CD8^+$ T cells, γδ T cells and natural killer cells, depending on the particular context. On the other hand, an overexuberant or overlong 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 proreparative macrophages[88]. Tissue T_{reg} cells rein in the early inflammatory response and promote the transition to a tissue milieu that favours 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], the heart[83], skin[54] and the CNS[84] in mice, and the heart, CNS and retina in zebrafish[87]. Third, they promote healthy tissue remodelling, by dampening pathological processes such as fibrosis and astrogliosis[6,9,12,55,83].

The proregenerative 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 cells 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 proregenerative effects of tissue T_{reg} cells can reflect cell–cell contacts — for example, elevated HFSC proliferation and differentiation induced by Jagged 1–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 of 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 proangiogenic peptide apelin in the peripheral vascular system[86] and keratinocyte growth factor in the lungs[81] of mice; and 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 Jagged 1–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, ensuring their numbers and quiescence by regulating local adenosine production[94,95]. Furthermore, 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} cell 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 2 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 the transcriptomes of various tissue T_{reg} cell 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} cell 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 inflammation, at least in the steady state[99,100]. Given their generally high-level expression of Helios

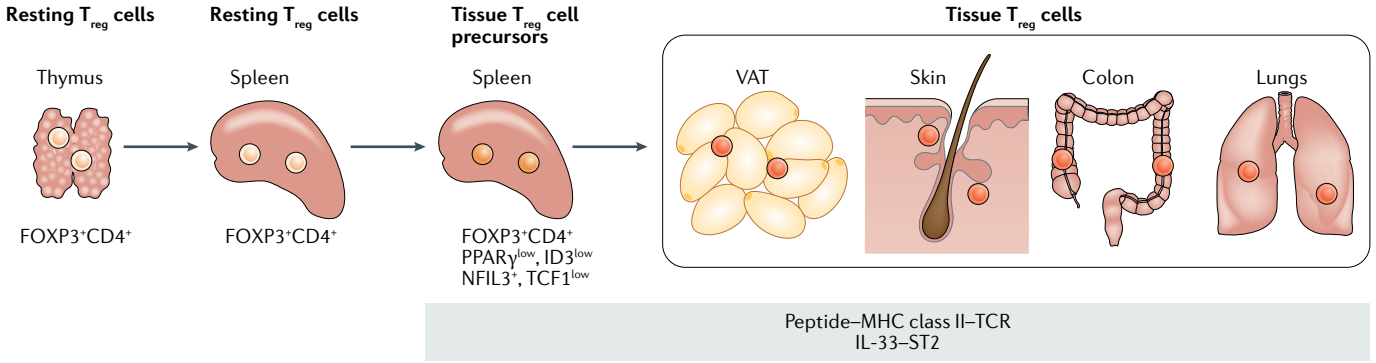

Fig. 4 | **The cellular derivation of tissue T_{reg} cells.** FOXP3⁺CD4⁺ T cells exit the thymus and enter the circulation, including lymphoid organs such as the spleen. Less than 10% of resting lymphoid organ regulatory T (T_{reg}) cells undergo an unknown activation event that allows them to escape 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. NFIL3, nuclear factor IL-3-regulated protein 3; PPARγ, peroxisome proliferator-activated receptor-γ; ST2, IL-33 receptor; TCF1, T cell factor 1; VAT, visceral adipose tissue.

and/or neuropilin 1, most tissue T_{reg} cells are probably thymus derived. Furthermore, 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 T_{reg} cell compartments of these tissues as a minor component, for example in response to inflammatory challenges.

Specification. The emergence of distinct tissue T_{reg} cell 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 is 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 whose TCRs recognize a local antigen are retained and undergo definitive phenotypic adaptation. This scenario was originally established using a TCR-transgenic mouse model harbouring 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} (REF.[14]), ID3^{low} (REF.[102]), NFIL3⁺ (REF.[57]) and TCF1^{low} (REF.[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 (REF.[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 examined so far[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 data sets from diverse tissue T_{reg} cell populations (FIG. 5) and have distilled lists of genes expressed differentially compared with control lymphoid organ T_{reg} cell 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 performed on the differentially expressed gene sets shows that VAT and skin T_{reg} 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 multilayered. Differential transcript analysis reveals gene sets that are upregulated or downregulated in all tissue T_{reg} cell compartments (pan-tissue), shared by more than one tissue's T_{reg} cells (tissue preferential) and unique to a particular tissue T_{reg} cell compartment (tissue specific) (FIG. 5b,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: transcription factors (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 transcription factors (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[14,57,101–103]. 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 multilayered transcriptional landscape reflects influences both by epigenetic factors and by an interconnected network of positively and negatively acting transcription factors. However, we are just beginning to identify and integrate the activities of the key factors involved.

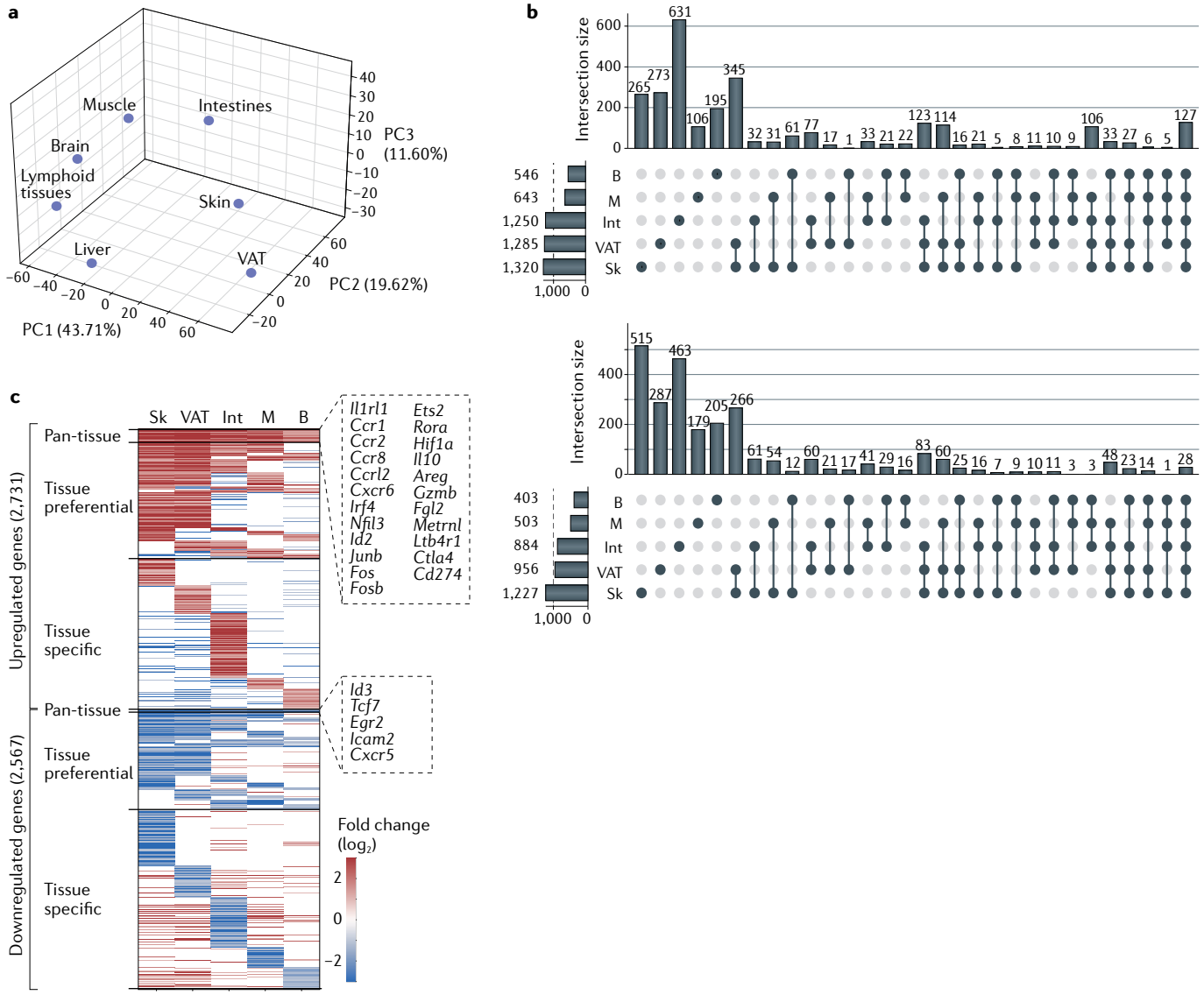

Fig. 5 | **Analysis of tissue T_{reg} cell transcriptomes.** Transcripts differentially expressed between each tissue regulatory T (T_{reg}) cell population and its corresponding lymphoid tissue control (fold change greater than 2 and false discovery rate less than 0.10) were calculated with use of the edgeR package. RNA sequencing data sets for visceral adipose tissue (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 change) for each of the tissue T_{reg} cell populations was scaled and used to perform principal component analysis. Principal components (PCs) 1, 2 and 3 with their proportions of explained variance are plotted. **b** | UpSet plot depicting intertissue 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} cell 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 change) for each tissue T_{reg} cell population, separated into pan-tissue, tissue-preferential and tissue-specific gene sets. For each tissue T_{reg} cell population, the log_2(fold change) of transcripts not in the signature (for example, fold change less than 2 or false discovery rate greater than 0.10) is 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 from https://cbdm.hms.harvard.edu/img/resources/SignaturesAndDatasets/Tissue-Treg%20signatures.xlsx. B, brain; Int, intestines; M, muscle; Sk, skin.

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 T_{reg} cells differ substantially from the DNA methylome 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 T_{reg} 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, 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, we find tissue T_{reg} cell open-chromatin regions again fall into three classes[37,57]: pan-tissue, tissue preferential and tissue specific. Interestingly, more than half of these open-chromatin regions — whether pan-tissue or tissue specific — are already accessible in the bulk T_{reg} cell population of lymphoid organs but not in other T cells. Such priming might serve to facilitate rapid changes 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 transcription factors that regulate the tissue T_{reg} cell differentiation pathway. BACH2, IRF4 and BATF are all key transcription factors 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 tissue T_{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 (REF.[104]).

IRF4 and/or BATF induces the expression of additional important transcription factors — such as BLIMP1, MAF, GATA3 and ID2 — in tissue T_{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 (REF.[106]). It also inhibits the activity of the DNA methyltransferase DNMT3A, resulting in hypermethylation of the *Foxp3* locus, among other loci. This effect ensures high-level FOXP3 expression and, consequently, maintenance of T_{reg} cell stability under inflammatory conditions[107]. ID3 and ID2 are transcription factors 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 favours tissue T_{reg} cell differentiation, whereas ID2 has the opposite distribution and impact[37,57,102,104,108].

Transcription factors 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 transcription factors 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 gene expression is PPARγ. This nuclear receptor

Box 1 | **A wish list of technological advances**

A tissue-T_{reg} single-cell atlas

Single-cell RNA sequencing data on tissue regulatory T (T_{reg}) cell 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 analysed via different algorithms, a more comprehensive, stereotypically generated tissue T_{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 should also include dynamic data on T_{reg} cell populations subsequent to classical non-immunological challenges (note that existing dynamic data on tumour T_{reg} cells argue for the value of such an endeavour)[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 demand not only 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} cell 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} cell 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 REF.[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 modelled, such systems permit easier and more punctual manipulation than is possible with living organisms. Furthermore, they can often be more readily translated to the analogous human tissue.

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} cell 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} cell population functionally examined to date[37].

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} cell 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 endeavours 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_{reg} 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 (REF.[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 neighbouring cells and they influence the behaviour 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 cells[6,52,91] and vascular cells[86].

Information on human tissue T_{reg} cell compartments is woefully scant, with the relative exceptions of the skin and colon T_{reg} cell 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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