Review

Developmental Heterogeneity of Foxp3^{+} Regulatory T Cells in the Orchestration of Metabolic Homeostasis

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Posted Date: 9 May 2025

doi: 10.20944/preprints202505.0281.v2

Keywords: Foxp3; pTreg/tTreg; β cells; insulin; autoimmunity; adipose tissue; obesity; diabetes

 doi:10.20944/preprints202505.0281.v2

Review

Developmental Heterogeneity of Foxp3⁺ Regulatory T Cells in the Orchestration of Metabolic Homeostasis

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Abstract: Regulatory T (Treg) cells were first identified through the observation that *Foxp3* gene mutations in mice and humans can result in their dysfunction, leading to a catastrophic multi-organ autoimmune syndrome. Since then, it has become increasingly evident that Foxp3⁺ Treg cells serve functions extending well beyond dominant tolerance and the mere prevention of autoimmune pathology. Highlighting their pivotal role in metabolic regulation, dysfunction of Treg cells has been implicated in the pathogenesis of both type 1 and type 2 diabetes. Emerging evidence further suggests that Treg cells contribute to tissue homeostasis and regeneration by facilitating repair processes, modulating immune responses to curb excessive inflammation, and supporting stem cell function in key metabolic organs such as muscle, adipose tissue, and the liver. This review aims to highlight recent progress in elucidating the functional specialization of Treg cells in the regulation of metabolic homeostasis. It explores the distinct roles of thymic and peripheral Treg cells in constraining pancreatic β-cell autoimmunity and the inflammation of metabolic organs, while also underscoring the pathogenic potential of Treg cell instability and their dedifferentiation into pathogenic effector cells. Investigating the roles of thymic and peripheral Treg cells in both forms of diabetes is a valuable endeavor, offering insight into their distinct and shared contributions to disease progression, while shedding light on immune dysregulation, metabolic inflammation, and immune-metabolic crosstalk. These insights may provide a foundation for the development of targeted therapeutic approaches directed at specific Treg cell subsets, offering the potential to attenuate disease progression or even entirely prevent its onset.

Keywords: Foxp3; pTreg/tTreg; b cells; insulin; autoimmunity; adipose tissue; obesity; diabetes

Introduction

Type 1 diabetes (T1D) is characterized by insulin deficiency caused by the immune-mediated destruction of insulin-producing β-cells of the pancreas, primarily driven by autoreactive T cells and a breakdown in immune tolerance. The recognition of T1D as an autoimmune disease dates back to the mid-1970s, marking a significant paradigm shift in the understanding of its etiology at that time [1]: Pioneering studies identified the presence of autoantibodies targeting insulin-producing β-cells [2,3] and highlighted lymphocytic infiltration of the pancreatic islets of Langerhans (insulitis) as a hallmark of the disease. This autoimmune nature was further corroborated by research showing the role of cellular autoimmunity [4]. This groundwork paved the way for the broad acceptance of T1D as an autoimmune disease, distinguishing it from type 2 diabetes (T2D) due to its autoimmune pathogenesis and its primary onset in younger individuals, in contrast to T2D, which is characterized by insulin resistance and is more commonly associated with older age, obesity, and metabolic dysfunction. It wasn't until the late 1990s that T2D was first linked to the immune system, in particular to elevated cytokine responses and innate immune activation [5,6]. These findings paved the way for recognizing chronic low-grade inflammation, driven by innate and adaptive immune

 doi:10.20944/preprints202505.0281.v2

cells particularly in adipose tissue, as a key driver of obesity-related metabolic complications, including insulin resistance, T2D, cardiovascular diseases, and fatty liver disease. Thus, both T1D and T2D share fundamental pathophysiological characteristics, such as chronic hyperglycemia and β-cell dysfunction, albeit driven by distinct underlying mechanisms. Inflammation is a pivotal factor in both conditions: In T1D, autoimmune-mediated β-cell destruction is central, while in T2D, chronic low-grade inflammation exacerbates insulin resistance and contributes to β-cell failure. Another unifying link is represented by $CD4^+$ regulatory T (Treg) cells expressing the lineage specification factor Foxp3, which have emerged as central players in metabolic homeostasis and the pathogenesis of diabetes. In fact, the non-redundant role of $Foxp3^+$ Treg cells not only in maintaining immune tolerance to β-cells but also in regulating adipose tissue inflammation is now well established. However, the physiological $Foxp3^+$ Treg cell pool comprises both intrathymically (tTreg) and peripherally (pTreg) induced subsets [7,8]. Together, tTreg and pTreg cells are thought to act synergistically to maintain immune homeostasis and prevent severe autoimmunity [9]. However, their respective contributions to autoimmune β-cell protection and adipose tissue homeostasis have only begun to be elucidated. In this review, we highlight recent advances in our understanding of Treg cell function in these contexts, with particular emphasis on the distinct roles of tTreg and pTreg cells.

Thymic and Peripheral Pathways of Treg Cell Development

Before the identification of suppressive T cells through the Foxp3 transcription factor, early studies demonstrated that T cell receptor (TCR) transgenic $CD4^+$ T cells could acquire an immunoregulatory phenotype upon recognizing cognate antigen in peripheral tissues - whether the antigen was expressed as a transgene [10], administered exogenously as a free antigen peptide [11], or selectively delivered to dendritic cells (DCs) [12,13]. The subsequent demonstration that steady-state DCs can drive the extrathymic conversion of conventional $CD4^+$ T cells with a truly $Foxp3^-$ phenotype into $Foxp3^+$ Treg cells with potent suppressive function [14,15] raised the possibility that, even under physiological conditions (i.e., T cells with naturally occurring TCRs, in the absence of deliberate antigen stimulation), the generation of $Foxp3^+$ Treg cells may not be confined to the thymus [16,17]. Since then, it has become widely accepted that the physiological Treg cell pool is developmentally heterogeneous, consisting of tTreg and pTreg cells, which originate from distinct $CD4^+CD25^{high}Foxp3^-$ precursor cells residing in thymus [7] and peripheral lymphoid tissues [8], respectively. According to the prevailing view, tTreg cells are primarily positively selected by self-antigens during intrathymic development and are functionally specialized to control immune homeostasis and autoimmune responses [18,19]. In the thymus, distinct $CD4^+CD8^-$ single-positive precursor cells, which express low levels of Foxp3 protein prior to up-regulation of CD25 expression, further expand the mature tTreg cell repertoire toward TCRs with attenuated self-reactivity [20]. In contrast, pTreg cells are thought to be primarily induced by environmental antigens and the gut microbiota, and have been implicated in the regulation of immune responses at mucosal surfaces [21,22] as well as in feto-maternal tolerance [23]. Recently, pTreg cells dependent on the gut microbiota have also been proposed to mediate functions beyond dominant suppression by facilitating muscle regeneration [24]. The developmental heterogeneity of the mature Treg cell pool can be visualized in dual $Foxp3^{RFP/GFP}$ reporter mice, in which RFP is expressed in all cells of the mature Treg cell pool, while GFP expression is restricted to intrathymic Treg cell development and is stably maintained in their progeny within peripheral tissues [25,26]. This differential expression of Foxp3-linked fluorochrome reporters enables the tracking and discrimination of RFP^+GFP^- pTreg cells from RFP^+GFP^+ tTreg cells (Figure 1A), further corroborating that, under physiological steady-state conditions, both thymic and peripheral pathways contribute to the overall pool of mature Treg cells [25]. The concept of a division of labor between pTreg and tTreg cells, which continues to hold merit, was decisively shaped by elegant studies in 2011 by Haribhai et al., who investigated the functional specialization of Treg cell developmental subsets through adoptive transfer immunotherapy in newborn *scurfy* mice, which are completely deficient in functional Foxp3 protein

 doi:10.20944/preprints202505.0281.v2

and Treg cells [9]. In these experiments, transfer of total Foxp3$^+$ Treg cell populations, composed primarily of tTreg cells (~80%), prevented disease lethality but failed to suppress chronic inflammation and autoimmunity. This required the provision of Foxp3-sufficient CD4$^+$ T cells to enable the extrathymic conversion of initially CD4$^+$Foxp3$^-$ T cells into functional Foxp3$^+$ Treg cells [9]. More recently, studies in Foxp3$^{RFP/GFP}$ mice, which are selectively deficient in tTreg cell development but spare the pTreg population (ΔtTreg mice [26]), further underscored the critical role of pTreg cells in mediating bystander effects to maintain self-tolerance [27]. In adult ΔtTreg mice, pTreg cells replenished the Treg cell pool and adopted a highly activated suppressor phenotype, thereby largely preventing early mortality and the fatal autoimmunity typically observed in Foxp3-deficient models of complete Treg cell deficiency [27]. Overall, these studies illustrate the synergistic roles of pTreg and tTreg cells in maintaining systemic immune homeostasis in the adult organism, although their respective contributions during the neonatal period remain incompletely understood. The following sections focus on Treg cells and their developmental subsets in the context of pancreatic β-cell autoimmunity and adipose tissue inflammation.

Figure 1. Developmental Treg cell subsets in the spontaneous NOD mouse model of T1D. (A, B) Representative flow cytometry of dual Foxp3 fluorochrome reporter expression in pancreatic CD4$^+$ T cells from Foxp3$^{RFP/GFP}$ reporter mice on the (A) C57BL/6 and (B) NOD background following >14 backcross generations. **(C)** Histological assessment of insulitis in Treg cell-proficient (wild-type) and pTreg-deficient (ΔpTreg) mice prior to diabetes onset. Representative H&E-stained pancreatic sections from female (top) and male (bottom) mice illustrates increased leukocytic infiltration in the islets of Langerhans of ΔpTreg mice. **(D)** Cumulative diabetes incidence in Treg cell-proficient (wild-type9, pTreg-deficient (ΔpTreg), and tTreg-deficient (ΔtTreg) mice (n = 12-20 per group). All mice in panels (C) and (D) were backcrossed onto the NOD background for a minimum of four generations.

Treg Cells in b Cell Autoimmunity and T1D

The discovery of Foxp3 gene mutations as the cause of fatal autoimmune disease in mice [28,29] and humans [30,31] not only established the molecular basis of dominant Treg cell-mediated tolerance but also provided a first hint for a critical role of Foxp3$^+$ Treg cells in controlling β cell autoimmunity. Notably, T1D is a hallmark [32] of the fatal multiorgan autoimmune syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), which affects approximately 80% of individuals with disrupted Treg cell function due to *FOXP3* gene mutations [30,31]. This underscores that a profound Treg cell defect is sufficient to drive destructive β cell autoimmunity, independent of other genetic or environmental factors. Beyond *FOXP3*, additional monogenic autoimmune lymphoproliferative syndromes that involve reduced peripheral tolerance and manifestations such as autoimmune diabetes can result from mutations in genes that are constitutively expressed in Treg cells [33]. These include *IL2RA* (encoding CD25, the high-affinity subunit of the IL-2 receptor) and *CTLA4*, which limits immune activation by blocking CD28 binding to CD80/CD86 on antigen-presenting cells (APCs), and may also directly inhibit TCR signaling. In T1D, disease manifestation is under complex genetic control, with over 50 risk-associated genes, many of which influence the development and function of Treg cells [34]. Notable examples in both humans and the non-obese diabetic (NOD) mouse model of T1D include *IL2RA*, *PTPN2*, and *CTLA4*, which collectively contribute to the dysregulation of immune tolerance. Foxp3$^+$ Treg cells were first implicated in T1D in 2005, when Lindley and colleagues reported that Treg cells from individuals with T1D were less effective at controlling autologous effector T cell proliferation, which was accompanied by shifted cytokine expression from anti-inflammatory cytokines, such as IL-10, to a pro-inflammatory profile, such as IFN-g [35]. While some subsequent studies have confirmed impaired Treg cell function in T1D patients [36–40], others have reported no significant differences in Treg frequency or suppressive

 doi:10.20944/preprints202505.0281.v2

capacity compared to healthy individuals [41–43]. In prediabetic NOD mice, however, even transient Treg cell depletion is sufficient to induce severe insulitis and persistent hyperglycemia, despite the rapid reconstitution of the Treg compartment following diphtheria toxin-mediated ablation [44]. These findings suggest that brief abrogation in Treg-mediated tolerance may be sufficient to initiate irreversible autoimmune processes. Therefore, the manifestation of T1D may not require sustained Treg cell dysfunction, but rather a temporary lapse in their regulatory control during a critical window of disease initiation. Overall, it is widely accepted that the breakdown of peripheral immune tolerance, particularly the failure of Treg cells to control autoreactive responses, is central to the onset and progression of T1D, ultimately leading to β-cell destruction and insulin deficiency.

Genetic Ablation of pTreg and tTreg Subsets in the NOD Model

Before genetically engineered mice with selective Treg subset deficiencies were available, studies using NOD.Foxp3$^{RFP/GFP}$ mice supported a model in which pTreg and tTreg cells act synergistically to prevent autoimmune β-cell destruction [25]: Co-transfer of either RFP^{+}GFP^{-} pTreg or RFP^{+}GFP^{+} tTreg cells into lymphopenic recipient mice - alongside diabetogenic CD4^{+} T cells to induce diabetes - initially protected the mice from developing hyperglycemia. However, pTreg cells provided long-term protection against β-cell autoimmunity, whereas all mice that received tTreg cells eventually developed disease within two weeks [25]. These findings illustrate that pTreg and tTreg cells possess distinct functional capacities consistent with their differential Treg signature gene expression [25], although the experimental setup may not fully reflect their behavior under physiological conditions. In fact, in otherwise unmanipulated NOD mice, genetic tTreg cell ablation precipitated an especially severe form of autoimmune diabetes, characterized by early onset, high incidence, and a loss of the female bias typically observed in spontaneous disease manifestation within the NOD model [27]. Destructive β-cell autoimmunity and overt diabetes in such DtTreg mice were critically dependent on *Idd1* (I-Ag7 MHC class II) and a small set of additional *Idd* genes, some of which unfold their diabetogenic activity directly in pTreg cells, such as PD-1 [27]. Interestingly, Treg cell-specific genetic deletion of the gene encoding PD-1 has been shown to protect NOD mice from developing autoimmune diabetes [45]. These findings establish tTreg cells as key regulators of β-cell autoimmunity, in line with their central role in maintaining immune tolerance. In contrast to the catastrophic β-cell autoimmunity observed in DtTreg mice, several independent loss-of-function studies consistently suggest that pTreg cells play a comparatively dispensable role in the control of β-cell autoimmunity. Nevertheless, given the therapeutic potential of pTreg cells in T1D, it is worth examining the similarities and sometimes contradictory results regarding their relative contribution in more detail. To investigate the role of pTreg cells in restraining genetically preprogrammed β-cell autoimmunity, selective pTreg deficiency has been modeled in NOD mice using different genetic strategies. Two independent studies [46,47] employed CRISPR-Cas-9 for targeted deletion of *conserved non-coding sequence 1* (*CNS1*) within the *Foxp3* gene locus in the NOD background, an approach previously used in the C57BL/6 background to selectively abrogate pTreg cell development while preserving tTreg cell differentiation [48]. To generate pTreg cell-deficient mice, we employed Foxp3$^{RFP/GFP}$ mice expressing a thymus-specific GFP–Cre recombinase fusion protein, in conjunction with a Cre-activatable Foxp3.STOP cassette [27]. This strategy enabled selective activation of Foxp3 expression in tTreg lineage-committed thymocytes, while precluding the extrathymic induction of pTreg cells [27]. On a non-autoimmune-prone background, both Foxp3.CNS1$^{-/-}$ mice [23,48] and Foxp3$^{RFP/GFP}$ mice with selective pTreg cell deficiency (ΔpTreg) [27] failed to reveal severe autoimmune symptoms. While selective pTreg cell deficiency was consistently found to exacerbate insulitis in Foxp3.CNS1$^{-/-}$ NOD females [46,47], the impact on the progression to overt diabetes varied considerably, ranging from an unchanged or even decreased incidence in male and female mice, respectively [46], to a significantly increased incidence in both sexes [47]. In the pancreas of Treg cell-proficient Foxp3$^{RFP/GFP}$ mice, both the size of the Foxp3^{+} Treg cell compartment and the pTreg-to-tTreg

 doi:10.20944/preprints202505.0281.v2

ratio were comparable between the non-autoimmune-prone C57BL/6 (Figure 1A) and pure NOD (Figure 1B) backgrounds, and were also not affected by glycemic status (see below). In Foxp3$^{RFP/GFP}$ mice with selective pTreg cell deficiency, six (C57BL/6>NOD) backcross generations were sufficient to promote enhanced insulitis in both female and male mice (Figure 1C), but resulted in early onset of overt diabetes only in rare cases, and exclusively in females (Figure 1D) [27]. These differences may, in part, be attributed to variations in the degree of pTreg cell depletion and differences in microbiota composition across mouse models. Notably, CNS1 deletion impairs, but does not completely abrogate pTreg generation [46,47], and even residual pTreg cells may be sufficient to sustain immune tolerance under certain conditions. More compelling, however, is the hypothesis that the strong dependence of pTreg cell development on microbial cues [49–51] renders autoimmune outcomes highly sensitive to even subtle shifts in microbiota composition. In conclusion, elucidating the mechanistic basis underlying the apparent discrepancies in the impact of pTreg cell deficiency on the manifestation of autoimmune diabetes remains an important objective for future research.

Treg Cells in Adipose Tissue Inflammation, Obesity, and T2D

While earlier studies suggested associations between Treg cells and T2D without specifically focusing on Foxp3$^+$ Treg cells, several key publications have since played a pivotal role in shaping our current understanding of the critical functions of Treg cells in adipose tissue and metabolic homeostasis, as well as obesity. Seminal studies by Feuerer et al. (2009) established that lean adipose tissue in mice and men is enriched with a unique population of Foxp3$^+$ Treg cells, suggesting a role in maintaining insulin sensitivity [52]. These findings paved the way for the concept of harnessing the anti-inflammatory properties of Treg cells to inhibit elements of the metabolic syndrome, as exemplified by Eller et al. (2011) demonstrating that enhancing Treg cell function could reverse obesity-linked insulin resistance and diabetic nephropathy in mice [53], further suggesting a potential therapeutic value of Treg cells to improve insulin resistance and end organ damage in T2D by limiting the proinflammatory milieu. While these studies have been pivotal, since then numerous studies have convincingly demonstrated a central role of Treg cells in adipose tissue homeostasis and metabolic control. The critical role of the metabolic-immune interplay for systemic metabolic homeostasis has been recently underscored by studies showing that surgical removal of murine visceral adipose tissue (VAT) prevents obesity-induced multiorgan insulin resistance [54]. Beyond VAT, tissue-resident Treg cells have emerged as central mediators of immuno-metabolic crosstalk across various metabolic organs, including other adipose depots [55], the liver [56], and skeletal muscle [57], where they support local functional adaptation to metabolic demands. This integration is essential for maintaining systemic metabolic homeostasis, as highlighted by a recent study demonstrating that Treg cells in the brain modulate hypothalamic immune responses and thereby promote systemic metabolism [58]. However, these studies have not addressed the relative contributions of pTreg and tTreg cells to these tissue-specific functions.

Phenotypic Adaptation of VAT Treg Cells

To persist and function within their distinct microenvironment, VAT Treg cells exhibit several features that set them apart from their lymphoid tissue counterparts, including a unique transcriptomic profile, antigen receptor repertoire, and specific growth factor dependencies [59,60]. Along with Foxp3, VAT Treg cells constitutively co-express the nuclear transcription factor PPAR-γ [52,61]. Foxp3$^+$PPAR-γ^+ Treg cells in VAT exhibit upregulated expression of unique sets of genes encoding transcription factors (e.g., RORα, GATA-3, BATF, and IRF4), chemokine/cytokine receptors (e.g., CXCR6, CCR1, CCR2, ST-2, and IL-9R), chemokines/cytokines (e.g., CXCL2, IL-10, and IL-5), and co-stimulatory molecules (e.g., PD-1 and CTLA4) [52,61–69]. To facilitate their survival in the otherwise toxic lipid-rich environment, VAT Treg cells are further characterized by a set of highly expressed molecules associated with lipid metabolism, including DGAT1, LDLR, and CD36 [61,63,67]. Conversely, VAT Treg cells show down-regulated expression of lymphoid tissue-associated genes encoding molecules involved in lymphoid tissue trafficking, such as CD62L, CCR7,

 doi:10.20944/preprints202505.0281.v2

and S1PR1 and lymphoid tissue transcription factors, such as TCF1, LEF1, and ID3 [63,67,70]. The recruitment and maintenance of VAT Treg cells is largely driven by PPAR-γ [61,71]: Mice lacking PPAR-γ expression selectively in Foxp3$^+$ cells exhibit a substantial reduction in the VAT Treg cell compartment size, and the residual cells lack the expression of typical VAT Treg cell signature genes [61]. Consistently, mice treated with a PPAR-γ agonist (pioglitazone) exhibit an increased Treg cell population size specifically in the VAT but not lymphoid tissues [61]. Within VAT, Treg cells are positioned between adipocytes in close proximity to MHC class II$^+$ APCs [66] and display a distinct TCR repertoire characterized by restricted diversity from the accumulation of micro-clones, in contrast to their lymphoid tissue counterparts [52,66], suggesting the capacity of VAT Treg cells to recognize and respond to locally presented antigenic peptides. Consistently, analysis of TCR transgenic mice, which express a TCR derived from a Treg cell clone that expanded within the VAT, but whose exact agonist ligand remains unidentified, has revealed that the transgenic TCR-expressing Treg cells specifically accumulate in the VAT, without similar accumulation at other anatomical sites [71]. VAT Treg cells are believed to undergo stepwise, multi-site differentiation: Splenic Foxp3$^+$ tissue-Treg precursors bearing an ST-2$^+$KLRG1$^+$ phenotype and initially lacking PPAR-γ expression migrate to VAT, where local antigen recognition induces PPAR-γ upregulation [66,71], with full acquisition of the VAT-Treg cell transcriptional signature being driven by TCR engagement and IL-33 signaling. Although IL-2-dependent survival and proliferation are preserved in VAT Treg cells, they exhibit additional growth factor dependencies not shared by their lymphoid tissue counterparts. Notably, VAT Treg cell homeostasis is highly dependent on IL-33 and its receptor ST-2, as evidenced by the constitutive ablation of either IL-33 or ST-2, which results in a marked reduction of the VAT Treg cell compartment without impacting Treg cells in lymphoid tissues [62,66,69].

Function of VAT Treg Cells

Treg cells have been implicated in both immunoregulatory and non-immune functions within VAT, with multiple independent lines of evidence supporting their insulin-sensitizing role. In obese mice, various approaches that promote the proliferative expansion of Foxp3$^+$ Treg cells - such as the administration of IL-2/anti-IL-2 mAb immune complexes [52], anti-CD3 mAbs [72], exogenous IL-33 [62], or a PPAR-γ agonist [61] - have consistently been shown to ameliorate VAT inflammation and improve metabolic indices [52,61,62,72]. Conversely, Foxp3-dependent genetic ablation of PPAR-γ, resulting in the selective loss of VAT Treg cells, exacerbated insulin resistance and glucose intolerance [61,73], while also abolishing the metabolic improvements typically induced by PPAR-γ agonist treatment in obese mice [61]. Mechanistically, VAT Treg cells can modulate both immune cells and adipocytes to maintain metabolic homeostasis [52]. VAT Treg cells have been shown to use a unique mechanism of suppression via the enzyme hydroxyprostaglandin dehydrogenase (HPGD), which converts prostaglandin E_2 (PGE_2) into 15-keto PGE_2 suppressing activation and proliferation of conventional T cells [74]. VAT Treg cells are also potent producers of IL-10, which fosters anti-inflammatory immune responses while suppressing pro-inflammatory ones. In adipocytes, IL-10 inhibits the expression of inflammatory genes, preserves insulin signaling by preventing the downregulation of tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), and counteracts the reduction of the glucose transporter GLUT4 [52]. More recently, VAT Treg cells have been implicated in non-immune functions by restraining the differentiation of stromal adipocyte precursors into adipocytes through the oncostatin-M (OSM) signaling pathway, thereby directly influencing adipose tissue remodeling to support insulin sensitivity and systemic metabolic homeostasis [75].

pTreg Cells - Central Regulators of Adipose Tissue Function and Systemic Metabolism

The VAT Treg cell compartment is seeded from the thymus during the early postnatal period, followed by local proliferative expansion of specific tTreg cell clonotypes [66,71]. While tTreg cell-deficient mouse models have only recently become available [26,27], the use of Foxp3.CNS1$^{-/-}$ mice -

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characterized by selective pTreg cell deficiency - for loss-of-function studies in metabolic homeostasis is confounded by the presence of an N-terminal Foxp3-GFP fusion protein, which functions as a hypomorph [76] and impairs Treg cell accumulation in VAT [77]. Consequently, earlier studies have relied on rather indirect evidence, suggesting that pTreg cell development may contribute only marginally to the VAT Treg cell pool, thereby indirectly supporting the notion of a predominantly thymic origin of these cells. Evidence that pTreg cells may play only a minor role in the generation of the VAT Treg cell pool included the observation of distinct TCR repertoires of VAT-residing $CD4^+Foxp3^+$ Treg cells and conventional $CD4^+Foxp3^-$ Teff cells [52], high expression levels of the putative tTreg cell markers Helios and Nrp-1 by VAT Treg cells [66], and a transcriptional signature of VAT Treg cells distinct from that of $CD4^+$ T cells with an artificially induced $Foxp3^+$ phenotype in vitro [66]. Additionally, $Foxp3^+$ cells originating from the conversion of adoptively transferred, initially naive $CD4^+Foxp3^-$ T cells failed to accumulate in the VAT of recipient mice [66,71]. However, under physiological conditions, studies employing the dual $Foxp3^{RFP/GFP}$ reporter model indicated that VAT of adult mice is seeded by both pTreg and tTreg cells [78]. Overall, the composition of the VAT Treg cell pool mirrors that observed in other anatomical locations, with pTreg cells comprising approximately 20-30% and tTreg cells accounting for 70-80% of the compartment in lean mice (Figure 2B). The first evidence linking pTreg cells to adipose tissue homeostasis came from previous transcriptomic analyses, which revealed striking similarities between the core transcriptional signature of splenic pTreg cells (but not tTreg cells) [25] and that of VAT Treg cells [63,67,79], including elevated expression of ST-2 and KLRG1 [25]. More recently, we found that tissue-type Treg precursors - characterized by co-expression of CD25, KLRG1, ST-2, OX40, and PD-1 [63] - are readily detectable within both the pTreg and tTreg cell subsets in the spleen, but are significantly enriched within the splenic pTreg cell compartment [78]. Observations in $Foxp3^{RFP/GFP}$ mice with selective pTreg cell deficiency further substantiated a critical functional role for pTreg cells in preserving adipose tissue immune equilibrium and systemic metabolic homeostasis: despite being maintained on a standard normocaloric vegetarian diet, the adipose tissues of both male and female ΔpTreg mice exhibited clear signs of immune dysregulation, including a macrophage profile skewed toward a pro-inflammatory M1 phenotype at the expense of anti-inflammatory M2 macrophages. Strikingly, normocaloric diet-fed ΔpTreg mice spontaneously developed pronounced obesity, characterized by increased overall body and adipose tissue weight, alongside elevated ERK phosphorylation, a molecular hallmark in VAT previously linked to insulin resistance and obesity [78].

Figure 2. Proposed model illustrating specialized functions of pTreg and tTreg cells in autoimmune diabetes and obesity, including the potential for pathogenic Treg dedifferentiation. Both the pancreas and lean adipose tissue are populated by $Foxp3^+$ Treg cells derived from peripheral (pTreg) and thymic (tTreg) origins, with a distribution of approximately 20-30% pTreg and 70-80% tTreg cells. (A) In NOD mice, which carry a genetic predisposition to autoimmune diabetes, tTreg cells are key regulators in controlling β cell autoimmunity by suppressing pathogenic $CD4^+$ and $CD8^+$ T cell responses. In contrast, the functional contribution of pTreg cells remains less well defined (see main text). Early in disease progression, M1-like macrophages within the islets of Langerhans produce low levels of pro-inflammatory cytokines, while a concurrent decline in M2-like macrophage activity contributes to the establishment and amplification of chronic inflammation (insulitis). This evolving pro-inflammatory milieu undermines immune homeostasis, enhancing Th1 and cytotoxic $CD8^+$ T cell responses that ultimately drive β cell dysfunction and destruction. Notably, the frequency of Treg cells among pancreatic $CD4^+$ T cells is comparable between C57BL/6 and NOD mice and remains stable throughout the transition from normoglycemia to insulitis and overt diabetes. Nevertheless, dedifferentiation of pTreg and/or tTreg cells may contribute to the expanding diabetogenic Th1 effector pool, thereby exacerbating insulitis. (B) In non-autoimmune-prone mice, the immune compartment in lean adipose tissue is predominantly composed of anti-inflammatory M2-like macrophages, Th2-polarized $CD4^+$ T cells, and $Foxp3^+$ Treg cells co-expressing PPAR-γ. Within this environment, the pTreg cell subset acquires a tissue-type Treg phenotype and plays a crucial role in maintaining adipose tissue homeostasis by suppressing pro-inflammatory immune responses and restraining the differentiation of stromal adipocyte precursors. Under conditions of metabolic stress, such as a hypercaloric diet, this regulatory balance is disrupted. Key features of this breakdown include a shift from anti-inflammatory

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M2- to pro-inflammatory M1-like macrophages, enhanced Th1 polarization, and the selective loss of tTreg cells. Elevated levels of inflammatory cytokines, particularly IL-6 and TNF, destabilize the tTreg cell compartment, driving their clonal expansion into pathogenic Foxp3^{-}IFN-γ^{+} T effector cells. This dedifferentiation further amplifies type 1 inflammation and contributes to the loss of immune regulation within obese adipose tissue.

Roles of VAT pTreg and tTreg Cells in Obesity

In contrast to genetic obesity models such as *ob*/*ob* mice [80,81], the C57BL/6 model of high-fat diet (HFD)-induced obesity offers the advantage of incorporating a combination of dietary, genetic, and environmental factors, which collectively influence the manifestation of obesity symptoms, thereby more closely recapitulating the complexities of human obesity [82,83]. Unsurprisingly, HFD feeding of ΔpTreg mice enhanced the pre-established pro-inflammatory type 1 immune response in VAT, exacerbating obesity symptoms and contributing to a severe form of hepatic steatosis [78]. Accordingly, selective pTreg cell deficiency induced distinct 'transcriptional holes' in the so-called 'VAT-Treg' signature of HFD-fed ΔpTreg mice highlighting the non-redundant contribution of pTreg cell development to the overall VAT pool of functional Treg cells [78]. Further underscoring the key role of pTreg cells in VAT homeostasis, HFD-fed ΔtTreg mice exhibited an increased compartment size of VAT pTreg cells and concomitantly increased expression of many genes of the 'VAT-Treg' signature, including key transcription factors (e.g., *Pparg*, *Gata3*) as well as mediators of Treg cell survival (e.g., *Il2ra*, *Il1rl1*), metabolic fitness (e.g., *Dgat1*) and suppressor function (e.g., *Areg*, *Lgals1*, *Tgfb1*). Consequently, selective tTreg cell deficiency not only amplified anti-inflammatory type 2 immune responses in the VAT of Foxp3$^{RFP/GFP}$ mice maintained on a normocaloric diet, but also, quite strikingly, significantly mitigated HFD-induced obesity symptoms. This attenuation was accompanied by improved metabolic parameters, including enhanced glucose tolerance and insulin sensitivity [78]. Consistently, genetic tTreg cell ablation in ΔtTreg mice reversed the typical HFD-driven immune shift from anti-inflammatory type 2 to pro-inflammatory type 1 responses usually observed in VAT of Treg cell-proficient mice (Figure 2B).

Non-Immune Functions of VAT pTreg Cells

VAT pTreg cells exert their key function in maintaining metabolic homeostasis not only via immune regulation, but also through direct modulation of adipogenesis. Our single-cell transcriptomic analysis pinpointed the previously reported expression of OSM within the total VAT Treg cell pool [75] specifically to the pTreg subset, with minimal or no expression observed in tTreg cells [78]. Notably, the VAT of tTreg-deficient mice, which retain and even expand a functional pTreg compartment with elevated expression levels of OSM, exhibits an accumulation of adipogenic mesenchymal stromal cells alongside a 'hypertrophic' adipose phenotype, characterized by fewer but enlarged adipocytes [78]. These findings support a model in which pTreg-derived OSM signals through the OSM receptor on mesenchymal stromal cells to constrain adipogenesis (Figure 2B). The hypertrophic shift in adipocyte morphology may represent a compensatory mechanism and aligns with the improved metabolic profiles observed in ΔtTreg mice [78], consistent with reports linking larger adipocyte size to enhanced insulin sensitivity and glucose tolerance [84–87]. Consistently, Foxp3.PPAR-$\gamma^{-/-}$ mice - which are lacking the entire VAT Treg cell compartment, including pTreg cells - display a 'hyperplastic' phenotype characterized by more numerous but smaller adipocytes, indicative of increased adipogenic differentiation [75]. Collectively, these data highlight the unique ability of VAT pTreg cells to finely regulate adipose tissue architecture, linking their immunoregulatory and adipogenic functions to systemic metabolic health. Given the emerging roles of Treg cells in mediating immuno-metabolic crosstalk in other metabolic organs such as pancreas, skeletal muscle and the brain, future investigations into the presence and function of pTreg cells in these tissues may yield novel insights into their systemic role in metabolic regulation.

 doi:10.20944/preprints202505.0281.v2

Treg Cell Plasticity: Pathogenic Threat in b Cell Autoimmunity and Obesity?

The plasticity and potential instability of Foxp3⁺ Treg cells remain subjects of ongoing debate, with major implications for immune tolerance, disease progression, and Treg-based therapies. In fact, pTreg cells are particularly prone to losing Foxp3 expression, a marker of stable Treg identity, due to their dependence on post-thymic acquisition of a distinct epigenetic program - most notably, demethylation of the *Treg-specific demethylated region* (*TSDR*), or *conserved noncoding sequence 2* (*CNS2*), within the *Foxp3* gene locus. A prominent example is the early finding that in vitro TGF-β-induced iTreg cells exhibit inefficient *TSDR* demethylation and an inherently unstable Foxp3⁺ phenotype [88]. Insufficient epigenetic imprinting leaves pTreg cells vulnerable to reprogramming into effector-like cells, potentially contributing to β cell autoimmunity and obesity-related inflammation. The clinical success of strategies to induce a Foxp3⁺ phenotype in naïve CD4⁺Foxp3⁻ T cells will depend on the long-term stability and sustained suppressive function of the *de novo* induced Treg phenotype. Encouragingly, prior studies have also demonstrated that the *de novo* generation of *Foxp3*⁺ pTreg cells in vivo can achieve efficient *TSDR* demethylation, persist long-term even in the absence of the inducing antigen, and maintain a robustly stable *Foxp3*⁺ phenotype with preserved suppressive capacity, even under highly inflammatory conditions [89]. While Foxp3 loss can affect both pTreg and tTreg cells, instability in tTreg cells is particularly concerning given their selection for self-antigen recognition during thymic development, as their loss of Foxp3 may result in autoreactive CD4⁺ T effector cells. Emerging evidence suggests that tTreg plasticity is not merely a theoretical risk, but a contributing factor in the pathogenesis of both b cell autoimmunity and obesity-dependent adipose tissue inflammation.

Transdifferentiation of Treg Cells into Pathogenic T Effector Cells

The stability of Treg cells is shaped by a combination of extrinsic factors, including inflammatory cytokines [90–92], metabolic stress [93–96], and tissue-specific cues [56,96]. In the absence of sustained Foxp3 expression, robust epigenetic maintenance, and appropriate metabolic support, Treg cells may undergo functional reprogramming and adopt effector-like, pro-inflammatory phenotypes [90–92,97–100]. However, demonstrating the physiological significance of these processes has proven more challenging. The loss of Treg cell identity in vivo and their transdifferentiation into pathogenic CD4⁺ T effector cells has been proposed to occur in a highly inflammatory environment [92,97,98], potentially contributing to autoimmune pathology. This phenomenon has been implicated in various experimental autoimmune disease models, including arthritis [92], encephalitis [98], psoriasis [101], as well as in b cell autoimmunity and T1D [97,102] (Figure 2A). However, this idea remains controversial [103,104]. In fact, conclusions regarding Foxp3 loss in vivo were largely driven by genetic lineage tracing [92,97,98], where Foxp3-dependent Cre recombinase irreversibly activates the expression of a fluorochrome reporter, such as YFP, which remains stably expressed regardless of the Foxp3 expression status of the cell [26]. Some of these studies indicated that a substantial fraction (up to 15%) of cells within the Foxp3⁺ Treg population exhibit a Foxp3⁻ 'ex-Treg' phenotype. These findings were contested by the demonstration that Foxp3⁺ Treg cells are predominantly stable under physiological steady-state and a broad range of inflammatory conditions [104]. This included IL-2 deprivation, radiation-induced lymphopenia, and Th1 inflammation following infection with *Listeria monocytogenes* or Ab-mediated cross-linking of CD40 [104]. Lending further support to an overwhelmingly stable Foxp3⁺ phenotype among physiological Treg cell populations, Treg plasticity following adoptive transfer into a lymphopenic environment is confined to a small subset of the overall Treg population, primarily enriched for naïve, Nrp-1⁻ Treg cells, including pTreg cells and recent thymic emigrants [105]. Technical limitations inherent to fluorochrome-based lineage tracing - such as spectral overlap between reporters like GFP and YFP [26] - may, at least in part, account for the observed discrepancies in the reported extent of Foxp3 loss. Interpretation of these data is further complicated by the possibility that the accumulation of CD4⁺ T cells with an apparently Foxp3⁻ 'ex-Treg' phenotype in peripheral tissues may result from transient Foxp3 expression and Cre

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recombinase activity during thymic development, rather than from true Foxp3 downregulation in situ [103]. This scenario of intrathymic 'abortive' Treg cell development gives rise to Foxp3^{-} cells that are unable to reacquire Foxp3 expression upon in vitro restimulation, a distinguishing feature from *bona fide* 'ex-Treg' cells, which readily re-express Foxp3 following brief in vitro stimulation [103]. Clearly, fluorochrome-based genetic lineage tracing has been instrumental in elucidating T cell lineage fate decisions, though its interpretation warrants careful consideration.

Of direct relevance to β cell autoimmunity, early studies on pancreatic Treg cell dedifferentiation in the NOD model [97,102] have recently been corroborated by the observation that the costimulatory receptor CD226 contributes to Treg instability and b cell autoimmunity [106]. Whereas, studies in the NOD.Foxp3$^{RFP/GFP}$ model [27] failed to reveal significant differences in pancreatic Treg cell abundance between C57BL/6 (Figure 1A) and both non-diabetic and diabetic NOD mice (Figure 1B), suggesting that the progression to overt diabetes may not be associated with an acute loss of Foxp3^{+} Treg cells in the pancreas due to in situ dedifferentiation, whether in pTreg or tTreg subsets [27]. In contrast, the loss of Foxp3^{+} Treg cells represents a hallmark of VAT inflammation in the pathogenesis of obesity [52,65,107,108]. The VAT Treg cell loss can be attributed to the selective loss of tTreg cell identity and their dedifferentiation into 'ex-Treg' cells with an effector/memory T cell phenotype (Figure 2B), while preserving the pTreg cell compartment [78]. Mechanistically, dysregulated cytokine signaling pathways – in particular, the selective loss of expression of the high-affinity component of the IL-2 receptor (CD25) and the IL-33 receptor (ST-2), combined with enhanced TNF-α and IL-6 receptor signaling - drive the destabilization of VAT-resident tTreg cells (Figure 2B) [78]. In obese mice, VAT Treg cells have been shown to progressively lose their tissue-specific identity, with downregulation of key signature genes - including *Pparg*, *Klrg1*, *Il1rl1* (ST-2), and *Il10* - and gain expression of gene clusters enriched in PPARγ-deficient Treg cells [65]. This transcriptional reprogramming has been attributed, at least in part, to reduced PPAR-γ activity, which is inhibited by cyclin-dependent kinase-5–mediated phosphorylation at serine 273 of PPAR-γ [65,109]. These changes can be mimicked in adipocyte cultures treated with TNF-α [65], directly linking the pro-inflammatory milieu to impaired PPAR-γ function. Consistently, enhanced obesity symptoms in ΔpTreg mice are associated with dysregulated 'VAT-Treg' signature expression, including the downregulated expression of PPAR-γ in VAT tTreg cells [78]. Together, these findings highlight how obesity-induced inflammation undermines the transcriptional and functional stability of tTreg cells, contributing to their progressive loss within adipose tissue. Similarly, IFN-γ has been implicated in the reduction of Treg cells in the adipose tissue of obese human subjects [110].

In the pancreas of NOD mice, the majority of Treg cells may preserve Foxp3 expression while progressively losing their suppressive function, underscoring a dissociation between phenotypic and functional stability. In line with this, Treg cells residing within pancreatic islets are thought to undergo functional impairment primarily due to limited IL-2 availability [111–114], which compromises STAT5 signaling, destabilizes Foxp3 expression, and ultimately diminishes suppressive capacity [115–118]. These studies collectively indicate that defects in the IL-2/CD25 pathway impair Treg cell function in NOD mice, contributing to the development of autoimmune diabetes, positioning IL-2 as both a central mechanistic regulator and a promising therapeutic target [113,114,119].

However, the destabilization of even a small fraction of Treg cells may be sufficient to amplify autoimmune responses - particularly when transdifferentiating Treg cells bearing highly pathogenic TCR specificities undergo proliferative expansion within the effector T cell compartment. Indeed, single-cell mRNA and TCR transcriptomic analyses of CD4^{+} T cells have been instrumental in firmly demonstrating that immunological and metabolic stress can compromise tTreg cell stability in the VAT of HFD-fed mice. This destabilization initiates a cascade culminating in Foxp3 downregulation and transdifferentiation into pro-inflammatory, obesogenic CD4^{+} T effector cells (Figure 2B), thereby fueling adipose tissue inflammation, obesity, and systemic metabolic dysfunction [78]. In these studies, the integration of single-cell mRNA and TCR transcriptomics data has enabled the identification of intermediate transdifferentiation states - such as *Foxp3* mRNA^{low} Teff-like cells.

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Additionally, TCR clonotype trajectory analyses revealed that destabilized tTreg clonotypes selectively accumulate within the pro-inflammatory Th1 compartment, but not Th2, providing direct evidence for lineage conversion into pathogenic effector cells [78].

Concluding Remarks

The dual Foxp3$^{RFP/GFP}$ mouse model and its derivatives with selective pTreg- and tTreg-deficiency have proven valuable for dissecting the distinct contributions of Foxp3$^+$ Treg subsets to immune homeostasis, particularly in the context of pancreatic β-cell autoimmunity and obesity-associated inflammation in adipose tissue. While tTreg cells are key regulators of pancreatic β-cell autoimmunity, pTreg cells have emerged as crucial players in maintaining VAT homeostasis by regulating local immune responses and adipocyte differentiation. This functional specialization among Treg subsets may help explain why, despite the central role of Treg cells in both diseases and evidence of shared etiological features, the overlap in genetic risk between T1D and T2D remains limited, especially for genes whose pathogenic effects are mediated through Treg cell dysfunction [120,121]. Recent advances in single-cell transcriptomics have provided valuable insights into the cascade of events within the highly pro-inflammatory adipose tissue microenvironment that drive Treg cell destabilization and their transdifferentiation into pathogenic T effector cells. While the physiological relevance of Foxp3 loss in pancreatic Treg cells in T1D requires further scrutiny, the integration of single-cell transcriptomics with TCR sequencing and chromatin accessibility profiling (e.g., scATAC-seq) offers a powerful framework for exploring Treg cell plasticity within the pancreatic and adipose tissue microenvironments. These multi-omic trajectory analyses hold great promise for uncovering lineage relationships, as well as the transcriptional and epigenetic stability of Treg cells and the clonal architecture of their subsets. Such insights will help illuminate the mechanisms of pathogenic reprogramming in β-cell autoimmunity and adipose tissue inflammation. Furthermore, these studies may extend the concept of developmental and functional Treg cell heterogeneity to the human setting. Ultimately, a deeper understanding of the specialized functions and potential risks associated with Treg developmental subsets will facilitate the rational design of subset-specific Treg cell-based therapies. These therapies may also involve strategies to stabilize Treg cells - such as through low-dose IL-2, IL-2 muteins, or epigenetic modulators - or selectively ablate unstable or dedifferentiating Treg cells. Alternatively, CRISPR-based engineering or the use of engineered Treg cells expressing chimeric antigen receptors could bypass stability concerns entirely, providing promising new avenues for therapeutic Treg cell-based intervention.

Funding: This work was supported with funds from the Technische Universität Dresden (TUD), Center for Molecular and Cellular Bioengineering (CMCB), Center for Regenerative Therapies Dresden (CRTD); from the German Ministry of Education and Research to the German Center for Diabetes Research (DZD e.V.); from the European Commission and EUREKA Eurostars-3 joint program (siaDM, E!1856), and from the DFG (German Research Foundation) (FOR2599) to KK. Dimitra Maria Zevla was financially supported by the Deutsche Forschungsgemeinschaft (DFG) under project number 288034826 –IRTG2251: 'Immunological and Cellular Strategies in Metabolic Disease'. Additionally, AY received financial support from the Graduate Academy, supported by Federal and State Funds.

References

1. Gale, E.A. The Discovery of Type 1 Diabetes. *Diabetes* **2001**, *50*, 217–226, doi:10.2337/diabetes.50.2.217.
2. Bottazzo, G.F.; Florin-Christensen, A.; Doniach, D. Islet-Cell Antibodies in Diabetes Mellitus with Autoimmune Polyendocrine Deficiencies. *Lancet (London, England)* **1974**, *2*, 1279–1283, doi:10.1016/s0140-6736(74)90140-8.
3. MacCuish, A.C.; Irvine, W.J.; Barnes, E.W.; Duncan, L.J. Antibodies to Pancreatic Islet Cells in Insulin-

 doi:10.20944/preprints202505.0281.v2

Dependent Diabetics with Coexistent Autoimmune Disease. *Lancet (London, England)* **1974**, *2*, 1529–1531, doi:10.1016/s0140-6736(74)90281-5.

4. Cudworth, A.G.; Woodrow, J.C. Genetic Susceptibility in Diabetes Mellitus: Analysis of the HLA Association. *Br. Med. J.* **1976**, *2*, 846–848, doi:10.1136/bmj.2.6040.846.
5. Pickup, J.C.; Crook, M.A. Is Type II Diabetes Mellitus a Disease of the Innate Immune System? *Diabetologia* **1998**, *41*, 1241–1248, doi:10.1007/s001250051058.
6. Pickup, J.C.; Mattock, M.B.; Chusney, G.D.; Burt, D. NIDDM as a Disease of the Innate Immune System: Association of Acute-Phase Reactants and Interleukin-6 with Metabolic Syndrome X. *Diabetologia* **1997**, *40*, 1286–1292, doi:10.1007/s001250050822.
7. Lio, C.-W.J.; Hsieh, C.-S. A Two-Step Process for Thymic Regulatory T Cell Development. *Immunity* **2008**, *28*, 100–111, doi:10.1016/j.immuni.2007.11.021.
8. Schallenberg, S.; Tsai, P.-Y.; Riewaldt, J.; Kretschmer, K. Identification of an Immediate Foxp3− Precursor to Foxp3+ Regulatory T Cells in Peripheral Lymphoid Organs of Nonmanipulated Mice. *J. Exp. Med.* **2010**, *207*, 1393–1407, doi:10.1084/jem.20100045.
9. Haribhai, D.; Williams, J.B.; Jia, S.; Nickerson, D.; Schmitt, E.G.; Edwards, B.; Ziegelbauer, J.; Yassai, M.; Li, S.-H.; Relland, L.M.; et al. A Requisite Role for Induced Regulatory T Cells in Tolerance Based on Expanding Antigen Receptor Diversity. *Immunity* **2011**, *35*, 109–122, doi:10.1016/j.immuni.2011.03.029.
10. Apostolou, I.; Sarukhan, A.; Klein, L.; von Boehmer, H. Origin of Regulatory T Cells with Known Specificity for Antigen. *Nat. Immunol.* **2002**, *3*, 756–763, doi:10.1038/ni816.
11. Apostolou, I.; von Boehmer, H. In Vivo Instruction of Suppressor Commitment in Naive T Cells. *J. Exp. Med.* **2004**, *199*, 1401–1408, doi:10.1084/jem.20040249.
12. Hawiger, D.; Inaba, K.; Dorsett, Y.; Guo, M.; Mahnke, K.; Rivera, M.; Ravetch, J. V; Steinman, R.M.; Nussenzweig, M.C. Dendritic Cells Induce Peripheral T Cell Unresponsiveness under Steady State Conditions in Vivo. *J. Exp. Med.* **2001**, *194*, 769–779, doi:10.1084/jem.194.6.769.
13. Hawiger, D.; Masilamani, R.F.; Bettelli, E.; Kuchroo, V.K.; Nussenzweig, M.C. Immunological Unresponsiveness Characterized by Increased Expression of CD5 on Peripheral T Cells Induced by Dendritic Cells in Vivo. *Immunity* **2004**, *20*, 695–705, doi:10.1016/j.immuni.2004.05.002.
14. Kretschmer, K.; Apostolou, I.; Hawiger, D.; Khazaie, K.; Nussenzweig, M.C.; von Boehmer, H. Inducing and Expanding Regulatory T Cell Populations by Foreign Antigen. *Nat. Immunol.* **2005**, *6*, 1219–1227, doi:10.1038/ni1265.
15. Kretschmer, K.; Heng, T.S.P.; von Boehmer, H. De Novo Production of Antigen-Specific Suppressor Cells in Vivo. *Nat. Protoc.* **2006**, *1*, 653–661, doi:10.1038/nprot.2006.105.
16. Jaeckel, E.; Kretschmer, K.; Apostolou, I.; von Boehmer, H. Instruction of Treg Commitment in Peripheral T Cells Is Suited to Reverse Autoimmunity. *Semin. Immunol.* **2006**, *18*, 89–92, doi:10.1016/j.smim.2006.01.011.
17. Kretschmer, K.; Apostolou, I.; Jaeckel, E.; Khazaie, K.; von Boehmer, H. Making Regulatory T Cells with Defined Antigen Specificity: Role in Autoimmunity and Cancer. *Immunol. Rev.* **2006**, *212*, 163–169, doi:10.1111/j.0105-2896.2006.00411.x.
18. Hsieh, C.-S.; Lee, H.-M.; Lio, C.-W.J. Selection of Regulatory T Cells in the Thymus. *Nat. Rev. Immunol.* **2012**, *12*, 157–167, doi:10.1038/nri3155.
19. Pohar, J.; Simon, Q.; Fillatreau, S. Antigen-Specificity in the Thymic Development and Peripheral Activity of CD4(+)FOXP3(+) T Regulatory Cells. *Front. Immunol.* **2018**, *9*, 1701, doi:10.3389/fimmu.2018.01701.
20. Owen, D.L.; Mahmud, S.A.; Sjaastad, L.E.; Williams, J.B.; Spanier, J.A.; Simeonov, D.R.; Ruscher, R.; Huang, W.; Proekt, I.; Miller, C.N.; et al. Thymic Regulatory T Cells Arise via Two Distinct Developmental Programs. *Nat. Immunol.* **2019**, *20*, 195–205, doi:10.1038/s41590-018-0289-6.

 doi:10.20944/preprints202505.0281.v2

21. Josefowicz, S.Z.; Niec, R.E.; Kim, H.Y.; Treuting, P.; Chinen, T.; Zheng, Y.; Umetsu, D.T.; Rudensky, A.Y. Extrathymically Generated Regulatory T Cells Control Mucosal TH2 Inflammation. *Nature* **2012**, *482*, 395–399, doi:10.1038/nature10772.
22. Campbell, C.; Dikiy, S.; Bhattarai, S.K.; Chinen, T.; Matheis, F.; Calafiore, M.; Hoyos, B.; Hanash, A.; Mucida, D.; Bucci, V.; et al. Extrathymically Generated Regulatory T Cells Establish a Niche for Intestinal Border-Dwelling Bacteria and Affect Physiologic Metabolite Balance. *Immunity* **2018**, *48*, 1245-1257.e9, doi:10.1016/j.immuni.2018.04.013.
23. Samstein, R.M.; Josefowicz, S.Z.; Arvey, A.; Treuting, P.M.; Rudensky, A.Y. Extrathymic Generation of Regulatory T Cells in Placental Mammals Mitigates Maternal-Fetal Conflict. *Cell* **2012**, *150*, 29–38, doi:10.1016/j.cell.2012.05.031.
24. Hanna, B.S.; Wang, G.; Galván-Peña, S.; Mann, A.O.; Ramirez, R.N.; Muñoz-Rojas, A.R.; Smith, K.; Wan, M.; Benoist, C.; Mathis, D. The Gut Microbiota Promotes Distal Tissue Regeneration via RORγ(+) Regulatory T Cell Emissaries. *Immunity* **2023**, *56*, 829-846.e8, doi:10.1016/j.immuni.2023.01.033.
25. Petzold, C.; Steinbronn, N.; Gereke, M.; Strasser, R.H.; Sparwasser, T.; Bruder, D.; Geffers, R.; Schallenberg, S.; Kretschmer, K. Fluorochrome-Based Definition of Naturally Occurring Foxp3+ Regulatory T Cells of Intra- and Extrathymic Origin. *Eur. J. Immunol.* **2014**, *44*, 3632–3645, doi:https://doi.org/10.1002/eji.201444750.
26. Simonetti, M.; Yilmazer, A.; Kretschmer, K. Genetic Tools for Analyzing Foxp3+ Treg Cells: Fluorochrome-Based Transcriptional Reporters and Genetic Fate-Mapping. In *Methods in Molecular Biology*; 2023; Vol. 2559, pp. 95–114 ISBN 978-1-0716-2647-4.
27. Yilmazer, A.; Zevla, D.M.; Malmkvist, R.; Rodríguez, C.A.B.; Undurraga, P.; Kirgin, E.; Boernert, M.; Voehringer, D.; Kershaw, O.; Schlenner, S.; et al. Selective Ablation of Thymic and Peripheral Foxp3+ Regulatory T Cell Development. *Front. Immunol.* **2023**, *14*, 1298938, doi:10.3389/fimmu.2023.1298938.
28. Chatila, T.A.; Blaeser, F.; Ho, N.; Lederman, H.M.; Voulgaropoulos, C.; Helms, C.; Bowcock, A.M. JM2, Encoding a Fork Head-Related Protein, Is Mutated in X-Linked Autoimmunity-Allergic Disregulation Syndrome. *J. Clin. Invest.* **2000**, *106*, R75-81, doi:10.1172/JCI11679.
29. Brunkow, M.E.; Jeffery, E.W.; Hjerrild, K.A.; Paeper, B.; Clark, L.B.; Yasayko, S.-A.; Wilkinson, J.E.; Galas, D.; Ziegler, S.F.; Ramsdell, F. Disruption of a New Forkhead/Winged-Helix Protein, Scurfin, Results in the Fatal Lymphoproliferative Disorder of the Scurfy Mouse. *Nat. Genet.* **2001**, *27*, 68–73, doi:10.1038/83784.
30. Bennett, C.L.; Christie, J.; Ramsdell, F.; Brunkow, M.E.; Ferguson, P.J.; Whitesell, L.; Kelly, T.E.; Saulsbury, F.T.; Chance, P.F.; Ochs, H.D. The Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome (IPEX) Is Caused by Mutations of FOXP3. *Nat. Genet.* **2001**, *27*, 20–21, doi:10.1038/83713.
31. Wildin, R.S.; Ramsdell, F.; Peake, J.; Faravelli, F.; Casanova, J.L.; Buist, N.; Levy-Lahad, E.; Mazzella, M.; Goulet, O.; Perroni, L.; et al. X-Linked Neonatal Diabetes Mellitus, Enteropathy and Endocrinopathy Syndrome Is the Human Equivalent of Mouse Scurfy. *Nat. Genet.* **2001**, *27*, 18–20, doi:10.1038/83707.
32. Bacchetta, R.; Barzaghi, F.; Roncarolo, M.-G. From IPEX Syndrome to FOXP3 Mutation: A Lesson on Immune Dysregulation. *Ann. N. Y. Acad. Sci.* **2018**, *1417*, 5–22, doi:10.1111/nyas.13011.
33. Johnson, M.B.; Cerosaletti, K.; Flanagan, S.E.; Buckner, J.H. Genetic Mechanisms Highlight Shared Pathways for the Pathogenesis of Polygenic Type 1 Diabetes and Monogenic Autoimmune Diabetes. *Curr. Diab. Rep.* **2019**, *19*, 20, doi:10.1007/s11892-019-1141-6.
34. Hull, C.M.; Peakman, M.; Tree, T.I.M. Regulatory T Cell Dysfunction in Type 1 Diabetes: What's Broken and How Can We Fix It? *Diabetologia* **2017**, *60*, 1839–1850, doi:10.1007/s00125-017-4377-1.
35. Lindley, S.; Dayan, C.M.; Bishop, A.; Roep, B.O.; Peakman, M.; Tree, T.I.M. Defective Suppressor Function in CD4(+)CD25(+) T-Cells from Patients with Type 1 Diabetes. *Diabetes* **2005**, *54*, 92–99,

 doi:10.20944/preprints202505.0281.v2

doi:10.2337/diabetes.54.1.92.

36. Long, S.A.; Cerosaletti, K.; Wan, J.Y.; Ho, J.-C.; Tatum, M.; Wei, S.; Shilling, H.G.; Buckner, J.H. An Autoimmune-Associated Variant in PTPN2 Reveals an Impairment of IL-2R Signaling in CD4(+) T Cells. *Genes Immun.* **2011**, *12*, 116–125, doi:10.1038/gene.2010.54.
37. Ferraro, A.; D'Alise, A.M.; Raj, T.; Asinovski, N.; Phillips, R.; Ergun, A.; Replogle, J.M.; Bernier, A.; Laffel, L.; Stranger, B.E.; et al. Interindividual Variation in Human T Regulatory Cells. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, E1111-20, doi:10.1073/pnas.1401343111.
38. Brusko, T.M.; Wasserfall, C.H.; Clare-Salzler, M.J.; Schatz, D.A.; Atkinson, M.A. Functional Defects and the Influence of Age on the Frequency of CD4+ CD25+ T-Cells in Type 1 Diabetes. *Diabetes* **2005**, *54*, 1407–1414, doi:10.2337/diabetes.54.5.1407.
39. Long, S.A.; Cerosaletti, K.; Bollyky, P.L.; Tatum, M.; Shilling, H.; Zhang, S.; Zhang, Z.-Y.; Pihoker, C.; Sanda, S.; Greenbaum, C.; et al. Defects in IL-2R Signaling Contribute to Diminished Maintenance of FOXP3 Expression in CD4(+)CD25(+) Regulatory T-Cells of Type 1 Diabetic Subjects. *Diabetes* **2010**, *59*, 407–415, doi:10.2337/db09-0694.
40. Aghili, B.; Amirzargar, A.A.; Rajab, A.; Rabbani, A.; Sotoudeh, A.; Assadiasl, S.; Larijani, B.; Massoud, A. Altered Suppressor Function of Regulatory T Cells in Type 1 Diabetes. *Iran. J. Immunol.* **2015**, *12*, 240–251.
41. Brusko, T.; Wasserfall, C.; McGrail, K.; Schatz, R.; Viener, H.L.; Schatz, D.; Haller, M.; Rockell, J.; Gottlieb, P.; Clare-Salzler, M.; et al. No Alterations in the Frequency of FOXP3+ Regulatory T-Cells in Type 1 Diabetes. *Diabetes* **2007**, *56*, 604–612, doi:10.2337/db06-1248.
42. McClymont, S.A.; Putnam, A.L.; Lee, M.R.; Esensten, J.H.; Liu, W.; Hulme, M.A.; Hoffmüller, U.; Baron, U.; Olek, S.; Bluestone, J.A.; et al. Plasticity of Human Regulatory T Cells in Healthy Subjects and Patients with Type 1 Diabetes. *J. Immunol.* **2011**, *186*, 3918–3926, doi:10.4049/jimmunol.1003099.
43. Putnam, A.L.; Vendrame, F.; Dotta, F.; Gottlieb, P.A. CD4+CD25high Regulatory T Cells in Human Autoimmune Diabetes. *J. Autoimmun.* **2005**, *24*, 55–62, doi:10.1016/j.jaut.2004.11.004.
44. Watts, D.; Janßen, M.; Jaykar, M.; Palmucci, F.; Weigelt, M.; Petzold, C.; Hommel, A.; Sparwasser, T.; Bonifacio, E.; Kretschmer, K. Transient Depletion of Foxp3(+) Regulatory T Cells Selectively Promotes Aggressive β Cell Autoimmunity in Genetically Susceptible DEREG Mice. *Front. Immunol.* **2021**, *12*, 720133, doi:10.3389/fimmu.2021.720133.
45. Tan, C.L.; Kuchroo, J.R.; Sage, P.T.; Liang, D.; Francisco, L.M.; Buck, J.; Thaker, Y.R.; Zhang, Q.; McArdel, S.L.; Juneja, V.R.; et al. PD-1 Restraint of Regulatory T Cell Suppressive Activity Is Critical for Immune Tolerance. *J. Exp. Med.* **2021**, *218*, doi:10.1084/jem.20182232.
46. Holohan, D.R.; Van Gool, F.; Bluestone, J.A. Thymically-Derived Foxp3+ Regulatory T Cells Are the Primary Regulators of Type 1 Diabetes in the Non-Obese Diabetic Mouse Model. *PLoS One* **2019**, *14*, e0217728, doi:10.1371/journal.pone.0217728.
47. Schuster, C.; Jonas, F.; Zhao, F.; Kissler, S. Peripherally Induced Regulatory T Cells Contribute to the Control of Autoimmune Diabetes in the NOD Mouse Model. *Eur. J. Immunol.* **2018**, *48*, 1211–1216, doi:10.1002/eji.201847498.
48. Zheng, Y.; Josefowicz, S.; Chaudhry, A.; Peng, X.P.; Forbush, K.; Rudensky, A.Y. Role of Conserved Non-Coding DNA Elements in the Foxp3 Gene in Regulatory T-Cell Fate. *Nature* **2010**, *463*, 808–812, doi:10.1038/nature08750.
49. Lathrop, S.K.; Bloom, S.M.; Rao, S.M.; Nutsch, K.; Lio, C.-W.; Santacruz, N.; Peterson, D.A.; Stappenbeck, T.S.; Hsieh, C.-S. Peripheral Education of the Immune System by Colonic Commensal Microbiota. *Nature* **2011**, *478*, 250–254, doi:10.1038/nature10434.
50. Arpaia, N.; Campbell, C.; Fan, X.; Dikiy, S.; van der Veeken, J.; deRoos, P.; Liu, H.; Cross, J.R.; Pfeffer, K.;

 doi:10.20944/preprints202505.0281.v2

Coffer, P.J.; et al. Metabolites Produced by Commensal Bacteria Promote Peripheral Regulatory T-Cell Generation. *Nature* **2013**, *504*, 451–455, doi:10.1038/nature12726.

51. Sefik, E.; Geva-Zatorsky, N.; Oh, S.; Konnikova, L.; Zemmour, D.; McGuire, A.M.; Burzyn, D.; Ortiz-Lopez, A.; Lobera, M.; Yang, J.; et al. MUCOSAL IMMUNOLOGY. Individual Intestinal Symbionts Induce a Distinct Population of RORγ^{+} Regulatory T Cells. *Science* **2015**, *349*, 993–997, doi:10.1126/science.aaa9420.
52. Feuerer, M.; Herrero, L.; Cipolletta, D.; Naaz, A.; Wong, J.; Nayer, A.; Lee, J.; Goldfine, A.B.; Benoist, C.; Shoelson, S.; et al. Lean, but Not Obese, Fat Is Enriched for a Unique Population of Regulatory T Cells That Affect Metabolic Parameters. *Nat. Med.* **2009**, *15*, 930–939, doi:10.1038/nm.2002.
53. Eller, K.; Kirsch, A.; Wolf, A.M.; Sopper, S.; Tagwerker, A.; Stanzl, U.; Wolf, D.; Patsch, W.; Rosenkranz, A.R.; Eller, P. Potential Role of Regulatory T Cells in Reversing Obesity-Linked Insulin Resistance and Diabetic Nephropathy. *Diabetes* **2011**, *60*, 2954–2962, doi:10.2337/db11-0358.
54. Franczyk, M.P.; He, M.; Yoshino, J. Removal of Epididymal Visceral Adipose Tissue Prevents Obesity-Induced Multi-Organ Insulin Resistance in Male Mice. *J. Endocr. Soc.* **2021**, *5*, bvab024, doi:10.1210/jendso/bvab024.
55. Kälin, S.; Becker, M.; Ott, V.B.; Serr, I.; Hosp, F.; Mollah, M.M.H.; Keipert, S.; Lamp, D.; Rohner-Jeanrenaud, F.; Flynn, V.K.; et al. A Stat6/Pten Axis Links Regulatory T Cells with Adipose Tissue Function. *Cell Metab.* **2017**, *26*, 475-492.e7, doi:10.1016/j.cmet.2017.08.008.
56. Muñoz-Rojas, A.R.; Mathis, D. Tissue Regulatory T Cells: Regulatory Chameleons. *Nat. Rev. Immunol.* **2021**, *21*, 597–611, doi:10.1038/s41577-021-00519-w.
57. Becker, M.; Joseph, S.S.; Garcia-Carrizo, F.; Tom, R.Z.; Opaleva, D.; Serr, I.; Tschöp, M.H.; Schulz, T.J.; Hofmann, S.M.; Daniel, C. Regulatory T Cells Require IL6 Receptor Alpha Signaling to Control Skeletal Muscle Function and Regeneration. *Cell Metab.* **2023**, *35*, 1736-1751.e7, doi:10.1016/j.cmet.2023.08.010.
58. Becker, M.; Kälin, S.; Neubig, A.H.; Lauber, M.; Opaleva, D.; Hipp, H.; Salb, V.K.; Ott, V.B.; Legutko, B.; Kälin, R.E.; et al. Regulatory T Cells in the Mouse Hypothalamus Control Immune Activation and Ameliorate Metabolic Impairments in High-Calorie Environments. *Nat. Commun.* **2025**, *16*, 2744, doi:10.1038/s41467-025-57918-z.
59. Li, C.; Spallanzani, R.G.; Mathis, D. Visceral Adipose Tissue Tregs and the Cells That Nurture Them. *Immunol. Rev.* **2020**, *295*, 114–125, doi:10.1111/imr.12850.
60. Elkins, C.; Li, C. Deciphering Visceral Adipose Tissue Regulatory T Cells: Key Contributors to Metabolic Health. *Immunol. Rev.* **2024**, *324*, 52–67, doi:10.1111/imr.13336.
61. Cipolletta, D.; Feuerer, M.; Li, A.; Kamei, N.; Lee, J.; Shoelson, S.E.; Benoist, C.; Mathis, D. PPAR-γ Is a Major Driver of the Accumulation and Phenotype of Adipose Tissue Treg Cells. *Nature* **2012**, *486*, 549–553, doi:10.1038/nature11132.
62. Vasanthakumar, A.; Moro, K.; Xin, A.; Liao, Y.; Gloury, R.; Kawamoto, S.; Fagarasan, S.; Mielke, L.A.; Afshar-Sterle, S.; Masters, S.L.; et al. The Transcriptional Regulators IRF4, BATF and IL-33 Orchestrate Development and Maintenance of Adipose Tissue-Resident Regulatory T Cells. *Nat. Immunol.* **2015**, *16*, 276–285, doi:10.1038/ni.3085.
63. Delacher, M.; Imbusch, C.D.; Hotz-Wagenblatt, A.; Mallm, J.-P.; Bauer, K.; Simon, M.; Riegel, D.; Rendeiro, A.F.; Bittner, S.; Sanderink, L.; et al. Precursors for Nonlymphoid-Tissue Treg Cells Reside in Secondary Lymphoid Organs and Are Programmed by the Transcription Factor BATF. *Immunity* **2020**, *52*, 295-312.e11, doi:10.1016/j.immuni.2019.12.002.
64. Delacher, M.; Imbusch, C.D.; Weichenhan, D.; Breiling, A.; Hotz-Wagenblatt, A.; Träger, U.; Hofer, A.-C.; Kägebein, D.; Wang, Q.; Frauhammer, F.; et al. Genome-Wide DNA-Methylation Landscape Defines Specialization of Regulatory T Cells in Tissues. *Nat. Immunol.* **2017**, *18*, 1160–1172, doi:10.1038/ni.3799.

 doi:10.20944/preprints202505.0281.v2

65. Cipolletta, D.; Cohen, P.; Spiegelman, B.M.; Benoist, C.; Mathis, D. Appearance and Disappearance of the MRNA Signature Characteristic of Treg Cells in Visceral Adipose Tissue: Age, Diet, and PPARγ Effects. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 482–487, doi:10.1073/pnas.1423486112.
66. Kolodin, D.; van Panhuys, N.; Li, C.; Magnuson, A.M.; Cipolletta, D.; Miller, C.M.; Wagers, A.; Germain, R.N.; Benoist, C.; Mathis, D. Antigen- and Cytokine-Driven Accumulation of Regulatory T Cells in Visceral Adipose Tissue of Lean Mice. *Cell Metab.* **2015**, *21*, 543–557, doi:10.1016/j.cmet.2015.03.005.
67. DiSpirito, J.R.; Zemmour, D.; Ramanan, D.; Cho, J.; Zilionis, R.; Klein, A.M.; Benoist, C.; Mathis, D. Molecular Diversification of Regulatory T Cells in Nonlymphoid Tissues. *Sci. Immunol.* **2018**, *3*, doi:10.1126/sciimmunol.aat5861.
68. Frias, A.B.J.; Hyzny, E.J.; Buechel, H.M.; Beppu, L.Y.; Xie, B.; Jurczak, M.J.; D'Cruz, L.M. The Transcriptional Regulator Id2 Is Critical for Adipose-Resident Regulatory T Cell Differentiation, Survival, and Function. *J. Immunol.* **2019**, *203*, 658–664, doi:10.4049/jimmunol.1900358.
69. Han, J.M.; Wu, D.; Denroche, H.C.; Yao, Y.; Verchere, C.B.; Levings, M.K. IL-33 Reverses an Obesity-Induced Deficit in Visceral Adipose Tissue ST2+ T Regulatory Cells and Ameliorates Adipose Tissue Inflammation and Insulin Resistance. *J. Immunol.* **2015**, *194*, 4777–4783, doi:10.4049/jimmunol.1500020.
70. Sullivan, J.M.; Höllbacher, B.; Campbell, D.J. Cutting Edge: Dynamic Expression of Id3 Defines the Stepwise Differentiation of Tissue-Resident Regulatory T Cells. *J. Immunol.* **2019**, *202*, 31–36, doi:10.4049/jimmunol.1800917.
71. Li, C.; DiSpirito, J.R.; Zemmour, D.; Spallanzani, R.G.; Kuswanto, W.; Benoist, C.; Mathis, D. TCR Transgenic Mice Reveal Stepwise, Multi-Site Acquisition of the Distinctive Fat-Treg Phenotype. *Cell* **2018**, *174*, 285-299.e12, doi:10.1016/j.cell.2018.05.004.
72. Ilan, Y.; Maron, R.; Tukpah, A.-M.; Maioli, T.U.; Murugaiyan, G.; Yang, K.; Wu, H.Y.; Weiner, H.L. Induction of Regulatory T Cells Decreases Adipose Inflammation and Alleviates Insulin Resistance in Ob/Ob Mice. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 9765–9770, doi:10.1073/pnas.0908771107.
73. Torres, S.V.; Man, K.; Elmzzahi, T.; Malko, D.; Chisanga, D.; Liao, Y.; Prout, M.; Abbott, C.A.; Tang, A.; Wu, J.; et al. Two Regulatory T Cell Populations in the Visceral Adipose Tissue Shape Systemic Metabolism. *Nat. Immunol.* **2024**, *25*, 496–511, doi:10.1038/s41590-024-01753-9.
74. Schmidleithner, L.; Thabet, Y.; Schönfeld, E.; Köhne, M.; Sommer, D.; Abdullah, Z.; Sadlon, T.; Osei-Sarpong, C.; Subbaramaiah, K.; Copperi, F.; et al. Enzymatic Activity of HPGD in Treg Cells Suppresses Tconv Cells to Maintain Adipose Tissue Homeostasis and Prevent Metabolic Dysfunction. *Immunity* **2019**, *50*, 1232-1248.e14, doi:10.1016/j.immuni.2019.03.014.
75. Wang, G.; Muñoz-Rojas, A.R.; Spallanzani, R.G.; Franklin, R.A.; Benoist, C.; Mathis, D. Adipose-Tissue Treg Cells Restrain Differentiation of Stromal Adipocyte Precursors to Promote Insulin Sensitivity and Metabolic Homeostasis. *Immunity* **2024**, *57*, 1345-1359.e5, doi:https://doi.org/10.1016/j.immuni.2024.04.002.
76. Bettini, M.L.; Pan, F.; Bettini, M.; Finkelstein, D.; Rehg, J.E.; Floess, S.; Bell, B.D.; Ziegler, S.F.; Huehn, J.; Pardoll, D.M.; et al. Loss of Epigenetic Modification Driven by the Foxp3 Transcription Factor Leads to Regulatory T Cell Insufficiency. *Immunity* **2012**, *36*, 717–730, doi:10.1016/j.immuni.2012.03.020.
77. Darce, J.; Rudra, D.; Li, L.; Nishio, J.; Cipolletta, D.; Rudensky, A.Y.; Mathis, D.; Benoist, C. An N-Terminal Mutation of the Foxp3 Transcription Factor Alleviates Arthritis but Exacerbates Diabetes. *Immunity* **2012**, *36*, 731–741, doi:10.1016/j.immuni.2012.04.007.
78. Yilmazer, A.; Eugster, A.; Zevla, D.M.; Helbich, S.S.; Boernert, M.; Torun, B.; Marsela, E.; Kirgin, E.; Dahl, A.; Petzold, A.; et al. Functional Dichotomy of Developmental Foxp3+ Treg Cell Subsets in the Visceral Adipose Tissue of Lean and Obese Mice. *bioRxiv* **2025**, 2025.03.12.642664, doi:10.1101/2025.03.12.642664.
79. Miragaia, R.J.; Gomes, T.; Chomka, A.; Jardine, L.; Riedel, A.; Hegazy, A.N.; Whibley, N.; Tucci, A.; Chen,

 doi:10.20944/preprints202505.0281.v2

X.; Lindeman, I.; et al. Single-Cell Transcriptomics of Regulatory T Cells Reveals Trajectories of Tissue Adaptation. *Immunity* **2019**, *50*, 493-504.e7, doi:10.1016/j.immuni.2019.01.001.

80. Martins, T.; Castro-Ribeiro, C.; Lemos, S.; Ferreira, T.; Nascimento-Gonçalves, E.; Rosa, E.; Oliveira, P.A.; Antunes, L.M. Murine Models of Obesity. *Obesities* 2022, *2*, 127–147.
81. Zhang, Y.; Proenca, R.; Maffei, M.; Barone, M.; Leopold, L.; Friedman, J.M. Positional Cloning of the Mouse Obese Gene and Its Human Homologue. *Nature* **1994**, *372*, 425–432, doi:10.1038/372425a0.
82. Suleiman, J.B.; Mohamed, M.; Bakar, A.B.A. A Systematic Review on Different Models of Inducing Obesity in Animals: Advantages and Limitations. *J. Adv. Vet. Anim. Res.* **2019**, *7*, 103–114, doi:10.5455/javar.2020.g399.
83. Collins, S.; Martin, T.L.; Surwit, R.S.; Robidoux, J. Genetic Vulnerability to Diet-Induced Obesity in the C57BL/6J Mouse: Physiological and Molecular Characteristics. *Physiol. Behav.* **2004**, *81*, 243–248, doi:https://doi.org/10.1016/j.physbeh.2004.02.006.
84. McLaughlin, T.; Sherman, A.; Tsao, P.; Gonzalez, O.; Yee, G.; Lamendola, C.; Reaven, G.M.; Cushman, S.W. Enhanced Proportion of Small Adipose Cells in Insulin-Resistant vs Insulin-Sensitive Obese Individuals Implicates Impaired Adipogenesis. *Diabetologia* **2007**, *50*, 1707–1715, doi:10.1007/s00125-007-0708-y.
85. Khan, T.; Muise, E.S.; Iyengar, P.; Wang, Z. V; Chandalia, M.; Abate, N.; Zhang, B.B.; Bonaldo, P.; Chua, S.; Scherer, P.E. Metabolic Dysregulation and Adipose Tissue Fibrosis: Role of Collagen VI. *Mol. Cell. Biol.* **2009**, *29*, 1575–1591, doi:10.1128/MCB.01300-08.
86. Fang, L.; Guo, F.; Zhou, L.; Stahl, R.; Grams, J. The Cell Size and Distribution of Adipocytes from Subcutaneous and Visceral Fat Is Associated with Type 2 Diabetes Mellitus in Humans. *Adipocyte* **2015**, *4*, 273–279, doi:10.1080/21623945.2015.1034920.
87. Krautbauer, S.; Neumeier, M.; Rein-Fischboeck, L.; Haberl, E.M.; Tilg, H.; Eisinger, K.; Buechler, C. Adipocyte Hypertrophy and Improved Postprandial Lipid Response in Beta 2 Syntrophin Deficient Mice. *Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol.* **2019**, *52*, 1151–1165, doi:10.33594/000000078.
88. Floess, S.; Freyer, J.; Siewert, C.; Baron, U.; Olek, S.; Polansky, J.; Schlawe, K.; Chang, H.-D.; Bopp, T.; Schmitt, E.; et al. Epigenetic Control of the Foxp3 Locus in Regulatory T Cells. *PLoS Biol.* **2007**, *5*, e38, doi:10.1371/journal.pbio.0050038.
89. Polansky, J.K.; Kretschmer, K.; Freyer, J.; Floess, S.; Garbe, A.; Baron, U.; Olek, S.; Hamann, A.; von Boehmer, H.; Huehn, J. DNA Methylation Controls Foxp3 Gene Expression. *Eur. J. Immunol.* **2008**, *38*, 1654–1663, doi:10.1002/eji.200838105.
90. Alvarez, F.; Istomine, R.; Shourian, M.; Pavey, N.; Al-Aubodah, T.A.-F.; Qureshi, S.; Fritz, J.H.; Piccirillo, C.A. The Alarmins IL-1 and IL-33 Differentially Regulate the Functional Specialisation of Foxp3(+) Regulatory T Cells during Mucosal Inflammation. *Mucosal Immunol.* **2019**, *12*, 746–760, doi:10.1038/s41385-019-0153-5.
91. Hua, J.; Inomata, T.; Chen, Y.; Foulsham, W.; Stevenson, W.; Shiang, T.; Bluestone, J.A.; Dana, R. Pathological Conversion of Regulatory T Cells Is Associated with Loss of Allotolerance. *Sci. Rep.* **2018**, *8*, 7059, doi:10.1038/s41598-018-25384-x.
92. Komatsu, N.; Okamoto, K.; Sawa, S.; Nakashima, T.; Oh-hora, M.; Kodama, T.; Tanaka, S.; Bluestone, J.A.; Takayanagi, H. Pathogenic Conversion of Foxp3+ T Cells into TH17 Cells in Autoimmune Arthritis. *Nat. Med.* **2014**, *20*, 62–68, doi:10.1038/nm.3432.
93. Fu, Z.; Ye, J.; Dean, J.W.; Bostick, J.W.; Weinberg, S.E.; Xiong, L.; Oliff, K.N.; Chen, Z.E.; Avram, D.; Chandel, N.S.; et al. Requirement of Mitochondrial Transcription Factor A in Tissue-Resident Regulatory T Cell Maintenance and Function. *Cell Rep.* **2019**, *28*, 159-171.e4, doi:10.1016/j.celrep.2019.06.024.

 doi:10.20944/preprints202505.0281.v2

94. Huynh, A.; DuPage, M.; Priyadharshini, B.; Sage, P.T.; Quiros, J.; Borges, C.M.; Townamchai, N.; Gerriets, V.A.; Rathmell, J.C.; Sharpe, A.H.; et al. Control of PI(3) Kinase in Treg Cells Maintains Homeostasis and Lineage Stability. *Nat. Immunol.* **2015**, *16*, 188–196, doi:10.1038/ni.3077.
95. Shrestha, S.; Yang, K.; Guy, C.; Vogel, P.; Neale, G.; Chi, H. Treg Cells Require the Phosphatase PTEN to Restrain TH1 and TFH Cell Responses. *Nat. Immunol.* **2015**, *16*, 178–187, doi:10.1038/ni.3076.
96. Shi, H.; Chi, H. Metabolic Control of Treg Cell Stability, Plasticity, and Tissue-Specific Heterogeneity. *Front. Immunol.* **2019**, *10*, 2716, doi:10.3389/fimmu.2019.02716.
97. Zhou, X.; Bailey-Bucktrout, S.L.; Jeker, L.T.; Penaranda, C.; Martínez-Llordella, M.; Ashby, M.; Nakayama, M.; Rosenthal, W.; Bluestone, J.A. Instability of the Transcription Factor Foxp3 Leads to the Generation of Pathogenic Memory T Cells in Vivo. *Nat. Immunol.* **2009**, *10*, 1000–1007, doi:10.1038/ni.1774.
98. Bailey-Bucktrout, S.L.; Martinez-Llordella, M.; Zhou, X.; Anthony, B.; Rosenthal, W.; Luche, H.; Fehling, H.J.; Bluestone, J.A. Self-Antigen-Driven Activation Induces Instability of Regulatory T Cells during an Inflammatory Autoimmune Response. *Immunity* **2013**, *39*, 949–962, doi:10.1016/j.immuni.2013.10.016.
99. Lal, G.; Zhang, N.; van der Touw, W.; Ding, Y.; Ju, W.; Bottinger, E.P.; Reid, S.P.; Levy, D.E.; Bromberg, J.S. Epigenetic Regulation of Foxp3 Expression in Regulatory T Cells by DNA Methylation. *J. Immunol.* **2009**, *182*, 259–273, doi:10.4049/jimmunol.182.1.259.
100. Ohkura, N.; Hamaguchi, M.; Morikawa, H.; Sugimura, K.; Tanaka, A.; Ito, Y.; Osaki, M.; Tanaka, Y.; Yamashita, R.; Nakano, N.; et al. T Cell Receptor Stimulation-Induced Epigenetic Changes and Foxp3 Expression Are Independent and Complementary Events Required for Treg Cell Development. *Immunity* **2012**, *37*, 785–799, doi:10.1016/j.immuni.2012.09.010.
101. Bovenschen, H.J.; van de Kerkhof, P.C.; van Erp, P.E.; Woestenenk, R.; Joosten, I.; Koenen, H.J.P.M. Foxp3+ Regulatory T Cells of Psoriasis Patients Easily Differentiate into IL-17A-Producing Cells and Are Found in Lesional Skin. *J. Invest. Dermatol.* **2011**, *131*, 1853–1860, doi:10.1038/jid.2011.139.
102. Setoguchi, R.; Hori, S.; Takahashi, T.; Sakaguchi, S. Homeostatic Maintenance of Natural Foxp3(+) CD25(+) CD4(+) Regulatory T Cells by Interleukin (IL)-2 and Induction of Autoimmune Disease by IL-2 Neutralization. *J. Exp. Med.* **2005**, *201*, 723–735, doi:10.1084/jem.20041982.
103. Miyao, T.; Floess, S.; Setoguchi, R.; Luche, H.; Fehling, H.J.; Waldmann, H.; Huehn, J.; Hori, S. Plasticity of Foxp3(+) T Cells Reflects Promiscuous Foxp3 Expression in Conventional T Cells but Not Reprogramming of Regulatory T Cells. *Immunity* **2012**, *36*, 262–275, doi:10.1016/j.immuni.2011.12.012.
104. Rubtsov, Y.P.; Niec, R.E.; Josefowicz, S.; Li, L.; Darce, J.; Mathis, D.; Benoist, C.; Rudensky, A.Y. Stability of the Regulatory T Cell Lineage in Vivo. *Science* **2010**, *329*, 1667–1671, doi:10.1126/science.1191996.
105. Junius, S.; Mavrogiannis, A. V; Lemaitre, P.; Gerbaux, M.; Staels, F.; Malviya, V.; Burton, O.; Gergelits, V.; Singh, K.; Tito Tadeo, R.Y.; et al. Unstable Regulatory T Cells, Enriched for Naïve and Nrp1(Neg) Cells, Are Purged after Fate Challenge. *Sci. Immunol.* **2021**, *6*, doi:10.1126/sciimmunol.abe4723.
106. Thirawatananond, P.; Brown, M.E.; Sachs, L.K.; Arnoletti, J.M.; Yeh, W.-I.; Posgai, A.L.; Shapiro, M.R.; Chen, Y.-G.; Brusko, T.M. Treg-Specific CD226 Deletion Reduces Diabetes Incidence in NOD Mice by Improving Regulatory T-Cell Stability. *Diabetes* **2023**, *72*, 1629–1640, doi:10.2337/db23-0307.
107. Li, C.; Wang, G.; Sivasami, P.; Ramirez, R.N.; Zhang, Y.; Benoist, C.; Mathis, D. Interferon-α-Producing Plasmacytoid Dendritic Cells Drive the Loss of Adipose Tissue Regulatory T Cells during Obesity. *Cell Metab.* **2021**, *33*, 1610-1623.e5, doi:10.1016/j.cmet.2021.06.007.
108. Muñoz-Rojas, A.R.; Wang, G.; Benoist, C.; Mathis, D. Adipose-Tissue Regulatory T Cells Are a Consortium of Subtypes That Evolves with Age and Diet. *Proc. Natl. Acad. Sci. U. S. A.* **2024**, *121*, e2320602121, doi:10.1073/pnas.2320602121.
109. Choi, J.H.; Banks, A.S.; Estall, J.L.; Kajimura, S.; Boström, P.; Laznik, D.; Ruas, J.L.; Chalmers, M.J.;

 doi:10.20944/preprints202505.0281.v2

Kamenecka, T.M.; Blüher, M.; et al. Anti-Diabetic Drugs Inhibit Obesity-Linked Phosphorylation of PPARgamma by Cdk5. *Nature* **2010**, *466*, 451–456, doi:10.1038/nature09291.

110. Bradley, D.; Smith, A.J.; Blaszczak, A.; Shantaram, D.; Bergin, S.M.; Jalilvand, A.; Wright, V.; Wyne, K.L.; Dewal, R.S.; Baer, L.A.; et al. Interferon Gamma Mediates the Reduction of Adipose Tissue Regulatory T Cells in Human Obesity. *Nat. Commun.* **2022**, *13*, 5606, doi:10.1038/s41467-022-33067-5.
111. Goudy, K.S.; Johnson, M.C.; Garland, A.; Li, C.; Samulski, R.J.; Wang, B.; Tisch, R. Reduced IL-2 Expression in NOD Mice Leads to a Temporal Increase in CD62Llo FoxP3+ CD4+ T Cells with Limited Suppressor Activity. *Eur. J. Immunol.* **2011**, *41*, 1480–1490, doi:10.1002/eji.201040890.
112. Yamanouchi, J.; Rainbow, D.; Serra, P.; Howlett, S.; Hunter, K.; Garner, V.E.S.; Gonzalez-Munoz, A.; Clark, J.; Veijola, R.; Cubbon, R.; et al. Interleukin-2 Gene Variation Impairs Regulatory T Cell Function and Causes Autoimmunity. *Nat. Genet.* **2007**, *39*, 329–337, doi:10.1038/ng1958.
113. Grinberg-Bleyer, Y.; Baeyens, A.; You, S.; Elhage, R.; Fourcade, G.; Gregoire, S.; Cagnard, N.; Carpentier, W.; Tang, Q.; Bluestone, J.; et al. IL-2 Reverses Established Type 1 Diabetes in NOD Mice by a Local Effect on Pancreatic Regulatory T Cells. *J. Exp. Med.* **2010**, *207*, 1871–1878, doi:10.1084/jem.20100209.
114. Tang, Q.; Adams, J.Y.; Penaranda, C.; Melli, K.; Piaggio, E.; Sgouroudis, E.; Piccirillo, C.A.; Salomon, B.L.; Bluestone, J.A. Central Role of Defective Interleukin-2 Production in the Triggering of Islet Autoimmune Destruction. *Immunity* **2008**, *28*, 687–697, doi:10.1016/j.immuni.2008.03.016.
115. Burchill, M.A.; Yang, J.; Vogtenhuber, C.; Blazar, B.R.; Farrar, M.A. IL-2 Receptor Beta-Dependent STAT5 Activation Is Required for the Development of Foxp3+ Regulatory T Cells. *J. Immunol.* **2007**, *178*, 280–290, doi:10.4049/jimmunol.178.1.280.
116. Li, X.; Liang, Y.; LeBlanc, M.; Benner, C.; Zheng, Y. Function of a Foxp3 Cis-Element in Protecting Regulatory T Cell Identity. *Cell* **2014**, *158*, 734–748, doi:10.1016/j.cell.2014.07.030.
117. Feng, Y.; Arvey, A.; Chinen, T.; van der Veeken, J.; Gasteiger, G.; Rudensky, A.Y. Control of the Inheritance of Regulatory T Cell Identity by a Cis Element in the Foxp3 Locus. *Cell* **2014**, *158*, 749–763, doi:10.1016/j.cell.2014.07.031.
118. Passerini, L.; Allan, S.E.; Battaglia, M.; Di Nunzio, S.; Alstad, A.N.; Levings, M.K.; Roncarolo, M.G.; Bacchetta, R. STAT5-Signaling Cytokines Regulate the Expression of FOXP3 in CD4+CD25+ Regulatory T Cells and CD4+CD25- Effector T Cells. *Int. Immunol.* **2008**, *20*, 421–431, doi:10.1093/intimm/dxn002.
119. Dwyer, C.J.; Ward, N.C.; Pugliese, A.; Malek, T.R. Promoting Immune Regulation in Type 1 Diabetes Using Low-Dose Interleukin-2. *Curr. Diab. Rep.* **2016**, *16*, 46, doi:10.1007/s11892-016-0739-1.
120. Aylward, A.; Chiou, J.; Okino, M.-L.; Kadakia, N.; Gaulton, K.J. Shared Genetic Risk Contributes to Type 1 and Type 2 Diabetes Etiology. *Hum. Mol. Genet.* **2018**, ddy314, doi:10.1093/hmg/ddy314.
121. Nyaga, D.M.; Vickers, M.H.; Jefferies, C.; Fadason, T.; O'Sullivan, J.M. Untangling the Genetic Link between Type 1 and Type 2 Diabetes Using Functional Genomics. *Sci. Rep.* **2021**, *11*, 13871, doi:10.1038/s41598-021-93346-x.