


REVIEW



Mucosal tissue regulatory T cells are integral in balancing immunity and tolerance at portals of antigen entry

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Foxp3⁺ regulatory T cells (Tregs) are a subset of CD4⁺ T cells that exert suppressive control over other immune cells. Tregs are critical for preventing systemic autoimmunity and maintaining peripheral tolerance, and yet they also assist in orchestration of immunity to pathogenic insult, wherein they limit collateral immunopathology and assist in facilitating a fine balance between immune tolerance and effector activity. Tregs have been extensively studied in lymphoid tissues, and a growing body of work has characterized phenotypically distinct Tregs localized in various nonlymphoid tissue compartments. These tissue Tregs can perform location-specific, alternative functions, highlighting their dynamic, context-dependent roles. Tregs have also been identified in mucosal tissues where specialized physiological functions are paramount, including helping the host to respond appropriately to pathogenic versus innocuous antigens that are abundant at mucosal portals of antigen entry. As in other tissue Treg compartments, mucosal Tregs in the respiratory, gastrointestinal, and genitourinary tracts are distinct from circulating counterparts and can carry out mucosa-specific functions as well as classic suppressive functions that are the hallmark of Tregs. In this review, we summarize current knowledge regarding mucosal Tregs in both health and disease.

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INTRODUCTION

Regulatory T cells (Tregs) are a subset of CD4⁺ T cells defined by expression of the transcription factor forkhead box P3 (Foxp3) and their ability to suppress conventional T cells and other immune cells^{1–3}. Early on, Tregs were demonstrated to be an essential cell type, as patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome and scurfy mice lacking a functional *Foxp3* gene suffered from various autoimmune-like conditions involving multiple tissues and organs, plus dysregulated effector T cell activity^{4–6}. Additional studies demonstrated that *Foxp3* is the master regulator of Treg development and is indeed required for both their differentiation as well as function as suppressive cells^{7–12}. Subsequently, a mouse model wherein *Foxp3* is ablated in mature Tregs demonstrated that continuous *Foxp3* expression is required to maintain the Treg developmental program and thus for Tregs to sustain suppressive function¹³.

Several decades of active research has since confirmed these findings and demonstrated Tregs to be critical mediators of the immune system. Immunosuppressive mechanisms utilized by Tregs are diverse and varied. Tregs express several immunosuppressive cytokines, including TGFβ and IL-10^{14,15}, and they have also been shown to express granzyme B, leading to death of immune cells including B cells, conventional T cells, and antigen-presenting cells (APC)^{16–18}. More recently, this has been shown to occur under particular inflammatory conditions such as infection¹⁹. In addition, Tregs express high levels of CTLA-4 at steady state, which allows them to limit availability of CD80 and CD86 on dendritic cells (DC) to conventional T cells²⁰. Tregs also express the

ectoenzymes CD39 and CD73, which facilitate the conversion of extracellular ATP to adenosine, which thus directly limits proliferation of effector T cells, as well as suppressing myeloid cells including DCs^{21–24}. Finally, through their high expression of CD25, a subunit of the IL-2R, Tregs can serve as an IL-2 sink, effectively depriving effector T cells of IL-2 and thus limiting their proliferative capacity²⁵. Not only do Tregs utilize these immunosuppressive mechanisms, and many others, to limit immune responses to self and other innocuous antigens to prevent autoimmunity at homeostasis^{2,26,27}, but they additionally mediate inflammatory responses to infection^{28,29}.

Since their discovery, Tregs have been primarily studied in the thymus, where they develop, and in peripheral lymphoid organs. However, a growing body of research has shown that Tregs also reside in nonlymphoid organs, where they can have unique phenotypes and execute highly specialized roles outside of the canonical regulatory functions, suggesting that Treg phenotype and activity is highly context dependent. In particular, tissue Tregs have been described in visceral adipose tissue (VAT), skin, and muscle^{30–32}. These nonlymphoid tissue Tregs are transcriptionally and phenotypically distinct from Tregs in spleen and lymph node (LN) and can perform highly specialized, location-dependent roles. Tregs in human skin, concentrated near hair follicles, regulate epithelial stem cell differentiation and display an activated memory phenotype, limiting inflammation and pro-fibrotic responses at steady state^{32–34}. In the mouse VAT, Tregs express the IL-33 receptor (IL-33R), where IL-33 expressed by mesenchymal stromal cells promotes Treg accumulation and proliferation^{35,36}. These

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Tregs acquire robust expression of the master regulator of adipose differentiation—peroxisome proliferator-activated receptor (PPAR)- γ —which maintains VAT insulin sensitivity^{31,35,37}. Muscle Tregs respond to IL-33 produced by fibroblast-like fibro/adipogenic progenitor (FAP) cells during muscle damage in mice and in turn produce amphiregulin (Areg), a growth factor that directly acts on satellite cells to facilitate muscle repair^{30,38}. Thus, phenotypically distinct and highly specialized Treg populations reside in various nonlymphoid tissue sites, underscoring phenotypic and functional heterogeneity within Tregs based on tissue location. Furthermore, these studies suggest that dynamic populations likely exist throughout the body in other discrete tissue types.

In addition to nonlymphoid organs such as the muscle, VAT, and skin, Tregs have also been characterized in several mucosal tissue sites. The mucosa has critical location-dependent physiological functions, including air exchange in the respiratory tract, nutrient absorption in the gastrointestinal tract, and reproduction in the genitourinary tract. The mucosal tissues also share a common barrier function in protecting the host from invading pathogenic stimuli, as the mucosa serves as a portal of entry for many pathogens of global health importance. The unique physiological and barrier roles of mucosal sites require a tightly coordinated immune response to allow for necessary tolerance to harmless antigens such as dietary antigens and sperm during reproduction, while also patrolling for and eliminating viral, bacterial, and fungal pathogens at infection entry sites. Given the canonical roles of Tregs as immune response regulators, as well as their more recently discovered location-specific functions, an increasing body of research demonstrates that Treg populations in mucosal tissues are crucial in both homeostasis and disease (Fig. 1).

In this review, we summarize a selection of current knowledge of lung, small intestine, colonic, uterine, and vaginal Treg populations during health as well as infection, autoimmunity, and cancer.

RESPIRATORY TRACT TISSUE TREGS

The respiratory tract experiences constant exposure to inhaled antigens and pathogens, yet this uniquely delicate mucosal tissue must maintain a state of relative quiescence for the preservation of tissue architecture and oxygen exchange. In this regard, overt immune responses to perturbations such as infection must be tightly regulated, striking a balance between rapid pathogen clearance and tissue preservation (Fig. 1). Tregs employ multiple mechanisms to dictate respiratory tract tissue homeostasis, including promoting effector immune responses mitigating inflammation, and facilitating tissue preservation and healing^{15,19,29,39–47}. Other reviews have more comprehensively detailed the multifaceted roles for Tregs during infection^{28,29,42,48}. Here, we mention only a handful of key examples of various functions of Tregs in the respiratory tract with the intention of setting the stage to consider the effects of respiratory tract tissue-specific cues on Treg phenotype and function.

In the context of influenza virus infection in mice, Tregs guide the formation of antigen-specific T follicular helper (Tfh) cell and germinal center (GC) B cell responses in the lymph node through their consumption of excess IL-2⁴¹. Thus, by controlling IL-2 availability, Tregs promote appropriate effector immune responses for viral clearance^{41,49}. Additionally, respiratory tract Tregs have been demonstrated to mount rapid and robust antigen-specific primary and memory recall responses to influenza virus infection, acting to appropriately limit antiviral T cell responses and ameliorate excess inflammation in mice^{39,45}. Importantly, after secondary influenza challenge, naïve Tregs are insufficient in recapitulating CD8+ T cell regulation³⁹. Similarly, upon *Mycobacterium tuberculosis* (Mtb) challenge in mice, thymically derived Tregs specific to the immunodominant Mtb epitope expand robustly, exhibit an activated phenotype, and delay the priming

and accumulation of effector T cells in the lung. These Mtb-specific Tregs are then specifically culled though Th1 inflammation-derived IL-12, which promotes high Tbet expression in Mtb-specific Tregs^{50,51}. In other examples of respiratory tract infections such as respiratory syncytial virus (RSV) in mice and SARS-CoV-2 in humans, disease severity and pulmonary pathology have been linked to functional differences among Tregs, where a loss in Tregs leads to accelerated viral clearance at the expense of immune-mediated tissue damage and worsened disease outcomes^{19,52–54}. Notably, one study identified a decrease in airway Tregs in patients with COVID-19 compared to healthy controls⁵⁴, thereby raising the possibility that a Treg deficit in the lung could be contributing to disease and the dysregulated immune response in COVID-19, though this requires further study of the mucosal immune responses to natural SARS-CoV-2 infection.

Recent studies employing a combination of epigenetic, single-cell chromatin accessibility, gene expression, and T cell receptor (TCR) fate mapping methods have identified a generalizable tissue-specific signature for Tregs that is conserved among mice and humans^{55–61}. At homeostasis, roughly 10–20% of the total Tregs found in the lungs of specific pathogen-free (SPF) mice exhibit a tissue-specific profile⁵⁶. Phenotypically, mouse tissue Tregs, including those found in the lungs, can be identified by their expression of KLRG1 and IL-33R and are regulated by BATF^{56,57}. Interestingly, lung tissue Tregs expressing IL-33R appear to be particularly sensitive to IL-33 signaling, undergoing a 60-fold expansion in response to treatment with rIL-33 in vivo, as compared to other nonlymphoid tissue IL-33R+ Tregs isolated from peripheral tissues such as fat (10-fold) and skin (5-fold)⁵⁶. Other transcription factors contributing to the core tissue Treg signature in the lung include *Nfil3*, *GATA-3*, and *Maf*, as well as the Delta-Notch signaling pathway, among others^{55–57}. Notably, although tissue Tregs isolated from various nonlymphoid tissues share an overlapping “core” signature with lung tissue Tregs, tissue-specific cues also appear to influence their overall phenotype, revealing a possible relationship for site-specific functions.

Studies elucidating a comprehensive ascription of the functional capacities for respiratory tract tissue Tregs are incomplete. However, it is clear that lung tissue Tregs play a major role in respiratory immunity and resolution. One prominent example is the ability of lung tissue Tregs (defined as CD44^{hi}CD62L^{lo} with increased expression of CD103, PD-1, GITR, CTLA-4 and KLRG1) to promote tissue healing through their production of Areg after influenza infection in mice⁴⁴. Importantly, the selective deficiency of Areg production by Tregs results in profound tissue damage and decreased oxygen exchange but does not impact other Treg suppressive functions, demonstrating a non-redundant requirement for tissue Tregs to produce Areg. Perhaps not surprisingly, IL-18 and IL-33 signaling are pivotal in initiating the tissue repair program, including among non-infectious scenarios^{44,62}. Conversely, when IL-18 signaling is antagonized by Notch4, Tregs lose their ability to produce Areg resulting in severe morbidity, as has been recently demonstrated for COVID-19 disease⁶³. Themes of lung tissue-specific roles for Tregs are further recapitulated by a model of chronic inflammation and fibrosis established by repetitive *Aspergillus fumigatus* challenge wherein lung tissue Tregs (CD69^{hi}CD103^{hi}) ameliorate Th2-mediated pathology in mice, possibly through immunosuppressive functions and/or the production of amphiregulin⁴⁷ (Fig. 2). Notably, other mechanisms of tissue healing may also be mediated by Treg expression of the soluble growth factor, keratinocyte growth factor (KGF), which can be induced in Tregs derived from lung tissue in mice and humans⁶⁴. Characterization of additional functions for respiratory tract tissue Tregs are still forthcoming, but will likely include quintessential Treg functions, possibly with extra guidance from tissue-specific cues during homeostasis and insult.

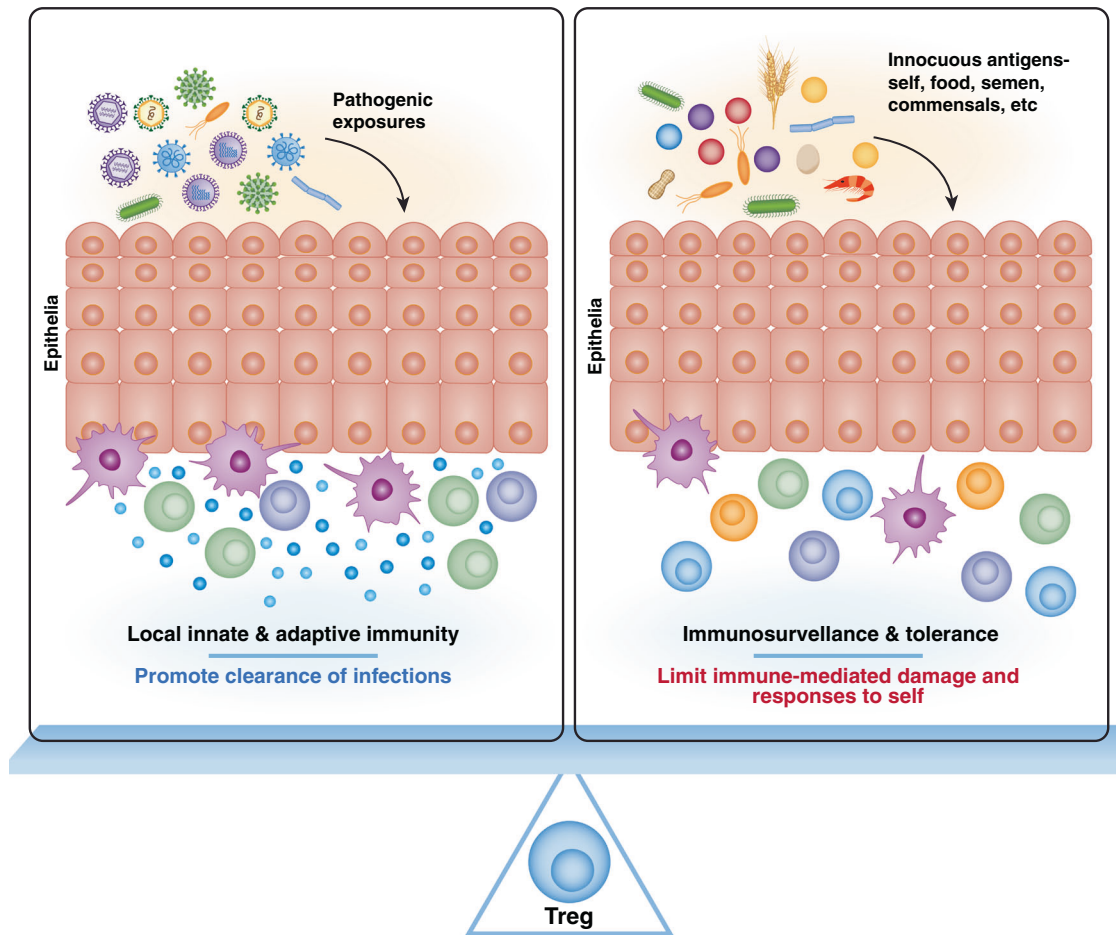


Fig. 1 Tregs in mucosal tissue sites help to balance active immunity versus immunological tolerance in the face of diverse antigenic exposures. Mucosal barrier tissues serve as portals of entry to a wide variety of antigens, both harmful and harmless to the host. Thus, the mucosal immune system has the complex job of balancing tolerance to innocuous antigens such as food, allergens, semen, and commensals while at the same time patrolling for pathogens so that an appropriate immune response can be generated to benefit but not harm the host. Tregs, depicted here at the fulcrum of this balance, participate in this process of maintaining a homeostatic state of active immunosurveillance and tolerance rather than persistent mucosal inflammation.

In addition to immune responses to infection, respiratory tract Tregs have been implicated in restraining immune reactions to innocuous antigens to restrict allergic responses including asthma^{65,66}. In humans with loss-of-function mutations in *Foxp3* resulting in IPEX, clinical presentation includes not only autoimmune manifestations, but also severe allergic inflammatory conditions^{67,68}, and mice with mutant *Foxp3* also spontaneously develop allergic airway inflammation⁶⁹. Indeed, mouse model studies have demonstrated that Tregs can suppress Th2 responses to allergens through an IL-10-dependent mechanism and that Treg-mediated suppression of DC activation is also involved in restriction of the response^{70,71}. Furthermore, peripherally induced Tregs play a crucial role in restricting Th2-pathologies such as allergic inflammation and asthma in the lungs⁷². Consistent with this finding, patients with atopic asthma were documented to have decreased abundance of Tregs in bronchoalveolar lavage fluid⁷³.

Finally, whereas tissue-specific Tregs represent only a fraction of the total respiratory tract Treg population in SPF mice⁵⁶, it remains likely that this frequency would be impacted by antigen experience, such as in the context of “dirty/pet store” mice⁷⁴. Interestingly, the frequency and distribution of tissue Tregs in humans appears to be related to age, wherein pediatric tissues demonstrate the highest frequencies of Tregs in mucosal and nonlymphoid tissues followed by a shift toward accumulation in

lymphoid tissues during adulthood⁷⁵. In this regard, whereas the total frequency of Tregs among total CD4⁺ T cells in the lung tissue of pediatric donors has been estimated to be roughly 15%, in adult lung tissue that number is reduced to roughly 5%. Phenotypically, among both children and adults, human lung tissue Tregs are mostly CD45RA⁻ and CD69⁺, with a smaller fraction dually expressing CD103⁷⁵. Importantly, each individual’s past history of antigen experience is also likely to impact their current status^{76–78}. Studies examining the tissue-specific Treg TCR repertoire in the human respiratory tract will be influential⁷⁹. Notably, among unmanipulated SPF mice, tissue context significantly impacts the Treg TCR repertoire wherein clonal diversity tends to be more restricted in peripheral tissues as compared to the spleen⁵⁷. A final consideration, specific to the lung, is the elucidation of recent evidence demonstrating that respiratory tract tissue-resident memory T cells (Trm) may be more transient than has been reported for other peripheral tissues^{80–82}. For example, the local draining lymph node may serve as a more permanent reservoir for respiratory Trm in lieu of long-term maintenance within the delicate pulmonary tissue⁸⁰, thereby raising the notion that respiratory tract Tregs may not persist long-term within the tissue parenchyma. In conclusion, studies further elucidating the functional capabilities, antigen-specificities, and longevity of respiratory tract-specific tissue Tregs among both mice and humans will be informative.

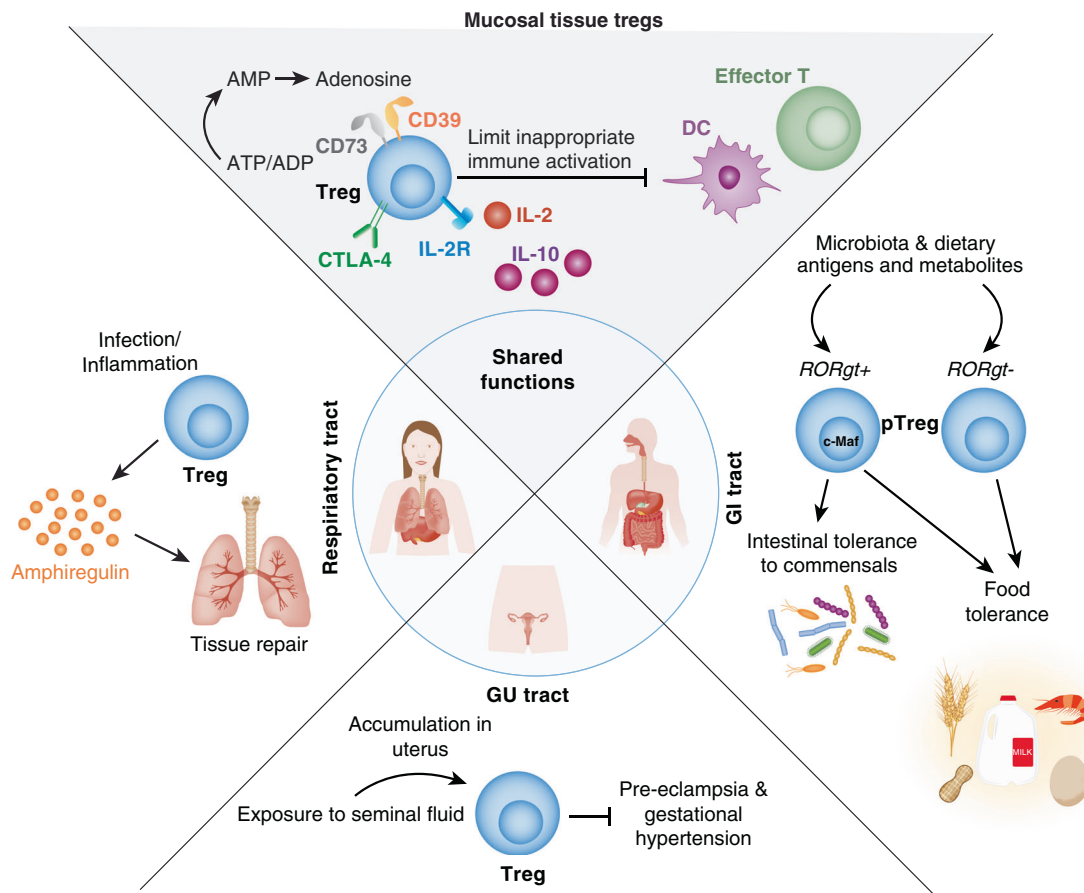


Fig. 2 Mucosal tissue Tregs have both shared and specialized phenotypes and functions dependent on the tissue location. *Foxp3*⁺ Tregs found throughout the body have immunosuppressive roles and can limit inappropriate inflammatory responses to self- and foreign antigens through a variety of mechanisms, including but not restricted to CTLA-4, ectoenzymes CD39 and CD73, IL-2 deprivation, and secretion of soluble regulatory mediators such as IL-10 and TGF β . In addition, as has been demonstrated in non-mucosal and nonlymphoid tissues such as the visceral adipose tissue, skin, and muscle, Tregs can execute additional specialized functions that are induced by and meet the needs of their unique tissue environment. Here we highlight one example from each of the major mucosal tissue sites. In the respiratory tract, infection or inflammation can induce Tregs to produce amphiregulin, which promotes lung tissue repair. In the gastrointestinal tract, ingestion of dietary antigens early in life induces peripherally generated Tregs that are neuropilin-1 (NRP1) and Helios double-negative, as well as negative for the transcription factor ROR γ t. These cells have been shown to be critical for tolerance to dietary antigens, and so are major players in the prevention of food allergy. In addition, microbiota induces ROR γ t⁺c-Maf⁺ pTregs that are crucial to maintain tolerance to both food and commensal bacteria. Finally, exposure to sperm and seminal fluid induces an increase in the number of Tregs present in uterine tissues, and Tregs have a demonstrated role in preventing pre-eclampsia and gestational hypertension. Future work to characterize the unique phenotypic and functional traits of mucosal tissue Tregs will surely reveal additional novel roles for these cells in these barrier tissue compartments.

GASTROINTESTINAL TRACT TISSUE TREGS

The gastrointestinal (GI) tract, including the small intestine and colon, is the site of food ingestion and metabolite transport in addition to hosting a complex microbiome. Therefore, the mucosal GI immune system has the extraordinary task of tolerating orally ingested dietary antigens (a phenomenon known as oral tolerance)^{83,84} as well as beneficial commensal bacterial species (spp.), while concurrently providing barrier immunity against ingested and enteric pathogens (Fig. 1). Tregs are crucial mediators of GI tract tolerance⁸⁴ and are maintained in high numbers in the GI tract, making up 20–30% of total CD4⁺ cells in the lamina propria of the small intestine and colon^{85,86}. Ablation of Tregs or mutations in *Foxp3* leads to systemic autoimmunity in mice and humans, including in the GI tract^{5,26,87,88}. Moreover, Tregs are necessary for prevention of GI tract inflammation and Inflammatory Bowel Diseases (IBD) such as colitis^{89,90}. Due to the increased need for tolerance in the GI tract, Tregs have been widely studied in the small intestine and colon, and more than any other mucosal site, a large quantity of research has been published⁹¹. Here we provide a broad rather than exhaustive overview of the vast and nuanced field of GI tract Treg biology in both health and disease.

As we have briefly introduced, Tregs canonically arise in the thymus, where *Foxp3* is expressed during thymic differentiation following relatively high avidity interactions of the TCR with self antigens^{92–94}. These thymically derived Tregs have thus been termed tTreg⁹⁵ and are generally defined by the expression of the transcription factor Helios and cell surface receptor neuropilin1 (NRP1)^{96,97}. However, Tregs also arise in the periphery (pTreg) in response to particular signals generated under tolerogenic conditions, including TGF β ^{98–101}, and can sometimes express Helios despite having extra-thymic origins¹⁰². Given the major tolerogenic requirements of the GI tract, the colon and small intestine are a major site of pTreg induction^{85,95}. In the mouse and human colon, ~30–35% of Tregs are NRP1⁺ and Helios⁺^{103,104}, suggesting that the majority of GI tract Tregs are induced locally^{96,97,105}. However, some colonic Tregs share TCR repertoires with Tregs in the thymus, suggesting that the colon hosts a combination of both pTregs and tTregs¹⁰⁶. Likewise, Helios⁺ Treg proportions are mouse strain-dependent and can vary greatly¹⁰⁷. Numerous studies have identified pTreg to be critical for tolerance to dietary antigens and the microbiota: mice lacking pTregs present with increased type 2 immunity and pathologies as well as

dysbiosis in the GI tract^{72,108}, and pTreg have been shown to be important for preventing inappropriate responses to food antigens in the GI tract¹⁰⁹.

Given the demonstrated critical roles for pTreg in the GI tract, these cells have been intensely studied in recent years. pTregs in the GI tract can be further classified by expression of transcription factors associated with CD4⁺ effector T cell lineages. We will first discuss ROR γ t, the transcription factor associated with T-helper 17 (Th17) lineage differentiation⁸⁵. In the mouse small intestine and colon, around 40% and 10%, respectively, of Tregs are negative for both ROR γ t and NRP1^{85,109}. In SPF mice weaned onto an antigen-free diet, small intestine NRP1⁺ Treg numbers are reduced, mostly due to a loss of ROR γ t⁺ Tregs¹⁰⁹. Interestingly, SPF mice treated with antibiotics are depleted of ROR γ t⁺, but not ROR γ t⁻ Tregs, demonstrating that dietary, but not microbial, antigens are crucial for induction of ROR γ t⁻ Tregs¹⁰⁹. Furthermore, SPF mice weaned onto an antigen-free diet followed by immunization and challenge with Ova develop intestinal allergy, implicating ROR γ t⁻ pTregs in the prevention of food allergy¹⁰⁹ (Fig. 2).

Thus, ROR γ t⁻ Tregs in the small intestine, and, in smaller numbers, in the colon, are induced by dietary antigens and are also necessary for continued tolerance to food-derived antigens. Although preservation of dietary tolerance is mainly attributed to ROR γ t⁻ Tregs, food allergy in human infants has been associated with a lack of microbiota-induced ROR γ t⁺ Tregs, and administration of oral antigen in mice increases ROR γ t⁺ Treg numbers, demonstrating a role for ROR γ t⁺ Tregs in maintaining tolerance to dietary antigens as well as to commensals^{110–112}. Additionally, microbiota-induced pTregs arising around the age of weaning are later required to prevent spontaneous Th2-mediated allergic responses to dietary antigens and protect from dextran sodium sulfate (DSS)-mediated severe colitis in mice^{113–115}.

As briefly mentioned above, a large fraction of GI tract pTregs are ROR γ t positive. ROR γ t⁺ Tregs require expression of transcription factor c-Maf for development and function as highly suppressive Tregs that specifically regulate distinct T-helper subsets in different inflammatory contexts, such as limiting pathogenic Th17 inflammatory responses to *Helicobacter hepaticus*^{103,112,116–118}. Many ROR γ t⁺ Tregs display a highly activated effector phenotype defined by expression of CD44, ICOS, and CTLA-4^{111,112,116,119}. IL-10, a critical anti-inflammatory cytokine, is constitutively expressed on a large percentage of ROR γ t⁺ Tregs in both the small intestine and colon, and a loss of IL-10 results in spontaneous colitis in mice and humans, demonstrating the importance of Treg-derived IL-10 in preventing GI tract inflammation and immunopathology^{15,120–122}.

In the mouse colon, 40–60% of Tregs are ROR γ t⁺, while ROR γ t⁺ Tregs make up a smaller fraction of small intestine Tregs^{85,103,107,112,116}. In humans, IL-17-producing Tregs are found in the circulation and in the microenvironment of chronic ulcerative colitis¹²³, and circulating ROR γ t⁺ memory Tregs can be found in humans¹²⁴. ROR γ t⁺ Treg abundance in small intestine and colon is dramatically reduced in germ-free mice or mice treated with antibiotics^{103,109,112,125}, demonstrating that the commensal microbiota is required for the generation of ROR γ t⁺ Tregs. A loss of ROR γ t⁺ Tregs in mice results in increased Type-2 immune responses and more severe oxazolone-induced colitis, suggesting that commensals regulate intestinal immune responses through induction of ROR γ t⁺ Tregs¹¹². Furthermore, local bacterial antigens mediate colonic pTreg selection, especially for ROR γ t⁺ Tregs^{104,109,125,126}. In support of Treg induction by commensal antigens, mouse colonic Tregs express TCR specific for *Clostridium* and commensal spp. that normally colonize the GI mucosal layer and expand in the presence of commensal bacteria¹²⁶. Other commensals such as lactobacilli and bifidobacteria also increase induction and maturation of GI tract Tregs⁸⁵. In summary, the small intestine Treg compartment is predominantly ROR γ t⁻ and responsible for dietary tolerance, while colonic ROR γ t⁺ Tregs facilitate

tolerance to commensal microbiota, but this distinction is not finite and GI tract Treg phenotype and function overlap.

GI tract pTregs are also heavily influenced by dietary and commensal-derived metabolites. Compared to Tregs in other tissues or lymphoid organs, Tregs in the mouse small intestine and colon express more aryl hydrocarbon receptor (AhR)—a nuclear sensor of dietary, microbiota, and other derivatives¹²⁷—suggesting an elevated AhR-mediated metabolite dependence in GI tract Tregs^{91,103,128}. Tryptophan, which can be metabolized by commensal microbiota into various AhR ligands¹²⁷, promotes Treg development through various mechanisms^{91,129}. Retinoic acid (RA), the bioactive derivative of vitamin A, is abundantly found in the GI tract and induces pTregs in mice when in combination with TGF β ^{91,130–132}. Likewise, mice given Vitamin A-deficient diets or RA inhibitors display reduced ROR γ t⁺ but not Helios⁺ Treg numbers, suggesting a specific requirement for Vitamin A in the ROR γ t⁺ Treg population in the GI tract^{91,112}. Among others, vitamins D3 and B9 promote expression of Foxp3 and anti-apoptotic BCL-2 on colonic Tregs^{85,133,134}. Although some Treg:metabolite processes function independently of the microbiota, others rely heavily on bacteria. Commensals facilitate metabolite production by fermenting dietary fiber to produce short chain fatty acids (SCFA) and other products that promote pTreg induction, expansion, and function, especially in the mouse colon^{91,112,135–137}. Bacterial products can also function as Toll-like receptor (TLR) ligands, adjuvanting the induction and function of GI tract pTregs^{85,91,138–141}. Recently, gut bacteria have been shown transform bile acids into bioactive products that modulate local Treg function and promote pTreg induction in mouse and humans^{142–144}.

A third population of GI Tregs expressing GATA3, the master transcription factor for T-helper 2 (Th2) CD4⁺ T cell differentiation, make up around 20 and 15%, respectively, of mouse small intestine and colon Tregs^{85,112,145,146}. In humans, GATA3⁺ Tregs have thus far only been identified in blood⁹¹. GATA3 directly interacts with Foxp3 to regulate Foxp3 expression and transcriptional programs^{111,147,148}. GATA3⁺ GI tract Tregs express Helios and are stable under germ-free conditions, suggesting thymic origins^{103,112}. GATA3⁺ Tregs express IL-33R, which recognizes IL-33, an alarmin produced by intestinal epithelial cells during inflammation^{145,146}. IL-33R ligation, along with TCR activation and IL-2, activates GATA3¹⁴⁵, which drives Treg-mediated suppression. A loss of GATA3 expression abrogates accumulation of Tregs in the small intestine, particularly in the context of inflammation, where GATA3⁺ Tregs are critical to protect mouse GI tissues from collateral immunopathology during enteric infection with pathogenic microbes¹⁴⁵. Additionally, small intestinal Tregs maintain epithelial barrier integrity by preserving epithelial cells (IEC) through various IL-10-mediated mechanisms in mice¹⁴⁹, where the IEC physically separates lamina propria immune cells and the microbiota in the intestinal lumen¹¹¹. Thus, GATA3⁺ Tregs are important for regulation of type-2 immunity and respond to cognate antigen and alarmins during inflammatory events to preserve GI tract tissues¹¹¹. Further research is warranted to resolve whether GATA3⁺ Tregs also directly contribute to tissue repair, as is seen in other IL-33R⁺ Tregs^{30,38,44,111}.

In addition to maintaining tolerance to commensal and dietary antigens, GI Tregs also contribute to humoral mucosal functions such as control of germinal center reactions to promote secretion of immunoglobulin A (IgA), which blocks invading pathogens from attaching to the mucosal epithelium¹⁵⁰. Conversely, a loss of c-Maf⁺ ROR γ t⁺ Tregs leads to excessive, Th17 and IgA responses¹¹⁸, suggesting that GI tract pTregs balance protective vs pathogenic mucosal humoral responses. Mouse Tregs expressing B cell lymphoma 6 (BCL-6) migrate via CXCR5 to Peyer's patches germinal centers where these Follicular Tregs (Tfr) control Tfh responses, thereby encouraging IgA production¹⁵¹. Small intestine Tregs specific for commensal flagellin antigens dampen mucosal uptake of commensal antigens through supporting production of

IgA, in turn limiting local T cell activation and further maintaining tolerance to the microbiota in mice¹⁵². Finally, some GI Foxp3+ Tregs that receive environmental cues within Peyer's patches may convert into Tfh cells, in the process losing Foxp3 expression, to then interact with B cells in mice¹⁵³.

While Tregs in the small intestine and colon are indispensable for maintaining appropriate immune tolerance during homeostasis, colonic Tregs within the aberrant context of a cancer environment can be counterproductive. Tregs, including those expressing cytotoxic molecule granzyme B, are enriched in colorectal cancer (CRC), colon-draining lymph nodes, and other tumor sites and are associated with poorer disease prognosis in mouse and humans, likely related to the suppression of anti-tumor effector T cells^{154–159}. Tumor environment-mediated immune dysbiosis may abnormally increase Treg recruitment to the tumor through chemotactic receptors or by expanding Tregs through TGF β or IL-10^{160,161}, where they dampen anti-tumor immune responses. To combat tumor-driven accumulation of Tregs in CRC tumors, therapeutic strategies to eliminate Tregs or block their suppressive functions may help to promote anti-tumor effector T cell immunity¹⁵⁵. However, the role of Tregs in CRC is nuanced and context-dependent, as Tregs can also protect the mouse and human host from cancer-associated inflammation^{162,163} and BLIMP-1+ Tregs and Treg-derived IL-10 have been associated with decreased polyps and increased CRC survival in both mice and humans^{164–166}. Together, these conflicting results suggest that distinct Treg subsets may perform different functions in intestinal cancers^{167,168}. For example, in mice, Treg-specific loss of transcription factor TCF-1 expression, a suppressor of genes co-bound by FoxP3, increases Treg-mediated T cell suppression and promotes tumor growth in polyposis, demonstrating a role for Treg TCF-1 expression in tumor clearance¹⁶⁹. Additionally, tumor-infiltrating Tregs in human CRC express low TCF-1 expression, suggesting that TCF-1 expression levels may predict CRC outcomes¹⁶⁹. Therefore, Treg involvement in mucosal tumor immunity warrants further study to understand which Treg subsets may predict improved CRC outcomes and which subsets could be targeted therapeutically to increase survival.

Treg dysfunction has major implications for chronic and debilitating GI tract disease; therefore, additional research is necessary to elucidate potential Treg-targeting therapies to ameliorate IBD and allergy. Furthermore, oral vaccine delivery is an attractive route as a non-invasive vaccine strategy that could induce protective immune responses at the mucosal barrier, as tissue-resident memory T cells (Trm) are known to be indispensable for protection from mucosal infections such as herpes simplex virus 2 (HSV-2)¹⁷⁰. However, further studies are needed to understand how to best leverage mucosal immune responses, including Tregs, at the site of infection while overcoming the tolerogenic tendencies of the oral mucosa and GI tract^{84,171–174}.

GENITOURINARY TRACT TISSUE TREGS

The genitourinary (GU) tract, comprised of the urinary and reproductive organs, requires paradoxical immune responses. The mucosal GU tissues such as the vagina, cervix, and uterus serve as the entry point for both reproductive sperm and sexually transmitted infections (STIs). Therefore, the GU tract must be at once tolerant to select foreign antigens and commensal bacteria, while hostile to invading STI, poisoning the GU tract as a unique and complex immunological site. Mucosal Tregs are likely to be critical for facilitating the balance between tolerance and immunity.

Uterine Tregs

A healthy mammalian pregnancy requires careful immune tolerance at every stage, from the introduction of male sperm to the end of successful gestation. During sexual reproduction, the GU tract immune system must allow sperm, a foreign antigen, and

a fetus—essentially a semi-allogeneic graft—to be tolerated for an extended period. Tregs in the blood and at the maternal-fetal interface in the placenta increase during pregnancy and are known mediators of fetal tolerance, preventing spontaneous abortion, fetal resorption, and preeclampsia in mice and humans^{175–178}. Given the critical need for Treg-mediated tolerance during pregnancy, it is unsurprising that Tregs resident in the mucosal GU tract also play a role in pregnancy. As early as first exposure to male seminal antigens and before embryonic implantation, Tregs accumulate in the uterus and uterine-draining lymph nodes in mice^{179–181}. During pregnancy, CD25+ cells increase two-fold in the mouse iliac and inguinal lymph nodes and make up 30% of uterine CD4+ T cells, and in both allogeneically and syngeneically mated mice, uterine *Foxp3* mRNA concentration is 1000-times higher than in age-matched non-pregnant mouse uterine tissue, suggesting a robust increase in uterine Tregs during pregnancy¹⁷⁵. A reduction in Tregs in early pregnancy causes uterine artery dysfunction in mice, demonstrating a tissue-specific role for Treg-mediated prevention of gestational hypertension and preeclampsia¹⁸² (Fig. 2). Given that immune responses in both mouse and human have been shown to be dampened during the luteal phase of the estrus cycle and in response to seminal extracellular vesicles, it is possible that GU mucosal Tregs in the uterus and vagina have additional immunosuppressive roles to promote pregnancy at the early stages of conception^{183–187}. Treg-focused cellular therapies could be useful in preventing and treating preeclampsia and other pregnancy complications, but further research in humans is needed to design diagnostic tests for Treg function in early pregnancy and to identify the temporal window in which intervention is safe and effective¹⁸⁸.

Cervical and vaginal Tregs

Historically, Tregs are understudied in the cervical and vaginal mucosa. However, as a barrier tissue site with constitutive exposure to commensal microbiota, plus the potential for exposures to male seminal antigens and microbial pathogens, the dynamics of the vaginal immune system must be carefully orchestrated, likely in part by Tregs.

In humans, the healthy vaginal mucosa hosts a microbiome dominated by *Lactobacillus*, and dysbiosis of the vaginal microbiome causes harmful overgrowth of fungal and bacterial pathogens, leading to bacterial vaginosis (BV), vaginal candidiasis, urinary tract infections, and dysregulated vaginal pH, all of which increase susceptibility to STIs and infertility^{189,190}. As in the GI tract, the vaginal immune system must remain tolerogenic to commensal bacteria while allowing for appropriate immune responses to deleterious microbes; therefore, we hypothesize that Tregs in the vagina may also facilitate tolerance to commensal microbiota. In support of this, vaginal isolates of *Lactobacillus crispatus*, a predominant species of healthy vaginal bacteria, induces Tregs from conventional CD4+ T cells in a human mixed leukocyte reaction¹⁹¹. Vaginal dysbiosis such as BV or abnormal vaginal dominance by anaerobic bacterial communities is associated with increased pro-inflammatory cytokines in the vagina and decreased peripheral Treg numbers, suggesting that a breach in normal vaginal commensals triggers a switch from tolerogenic to anti-microbial immune responses^{190,192,193}. Thus, further studies are necessary to determine how Tregs may facilitate the tolerance: inflammation axis based on the presence of either commensals or harmful bacterial spp.

Vaginal and uterine Tregs help maintain a healthy mucosal environment during homeostasis and pregnancy, but they also facilitate appropriate immune responses to harmful pathogens. In vaginal herpes simplex virus 2 (HSV-2) infection in mice, Tregs in the vaginal-draining lymph nodes (dLN) are necessary to promote proper antigen-bearing dendritic cell (DC) migration from the vagina to the dLN, and a loss of these Tregs delays HSV-2-specific

CD4⁺ T cell priming and results in worsened disease^{194,195}. This demonstrates that Tregs in the dLN tune the antiviral CD4 T cell responses in the nearby vaginal tissue. Moreover, Tregs accumulate in the mouse vaginal mucosa early after HSV-2 infection, suggesting an additional need for Treg-mediated immune regulation at the site of infection^{194,195}. Given the multitude of pathogens that may contact the vaginal mucosa, vaginal tissue Tregs likely have important roles in immune response coordination, such as limiting excessive inflammation during infection to prevent collateral immune-mediated tissue damage. In support of this, Treg-related cytokines exert anti-inflammatory effects during infection with *Trichomonas vaginalis*¹⁹⁶. In the human endocervix, Tregs are inversely correlated with inflammatory cytokine concentrations and abundance of CD4⁺ T cells, suggesting that Tregs in the GU tract prevent genital inflammation and could potentially lower HIV acquisition risk through limiting HIV target cell availability¹⁹⁷. Importantly, the roles of vaginal and uterine Tregs must be considered when designing mucosal vaccines, as Tregs promote anti-pathogenic immunity in some contexts, but dampen mucosal Trm responses in others. For example, intra-uterine immunization with non-adjuvanted UV-killed *Chlamydia trachomatis* in mice induces uterine Tregs that abrogate the effects of local effector T cells elicited by the vaccine¹⁹⁸. Additionally, vaginal Tregs may directly contribute to tissue repair through production of Areg after infection or injury, as has been shown in lung and muscle-resident Tregs in mice^{30,38,44}. Further vaginal Treg studies in both mouse and especially in human are necessary to determine how vaginal Tregs modulate local anti-pathogen immune responses, prevent mucosal tissue damage, and potentially execute unique, location-specific functions.

CONCLUSIONS

Peripheral Tregs are key mediators of systemic immune tolerance and immune orchestration, and mucosal tissue Tregs are no exception. In fact, Tregs at mucosal sites are charged with the paradoxical and highly nuanced role of facilitating protective immune responses to invading pathogens, while also allowing for immune quiescence in the context of sexual reproduction, inhaled or ingested harmless antigens, and commensal microbiota (Fig. 1). This immune balance is especially critical within the very delicate and highly specialized mucosal tissues. Although Tregs found in various mucosal tissues share the responsibility of balancing inflammatory immune responses, residency in distinct tissues is associated with previously unappreciated location-specific functions that lie beyond canonical Treg roles (Fig. 2). These specialized mucosal Tregs have implications for conditions such as allergy, autoimmunity, and cancer, where aberrant mucosal immune responses drive disease progression. Furthermore, the role of mucosal Tregs in anti-microbial immunity—especially respiratory infections and STIs—should not be underestimated. Importantly, immunity elicited by mucosal vaccination, depending on the context, may be either hindered or helped by Tregs. Therefore, further studies are necessary to elucidate how to best leverage Tregs to overcome oral tolerance and elicit protective resident T cell responses at the sites of bacterial and viral infection. Based on this growing body of mucosal Treg data, there is precedent to continue to characterize Tregs in other mucosal and nonlymphoid tissue sites, where they may perform distinct, yet-unknown tissue-specific functions.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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