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Review article

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Regulatory T cells in the face of the intestinal microbiota

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Abstract

Regulatory T cells (T_{reg} cells) are key players in ensuring a peaceful coexistence with microorganisms and food antigens at intestinal borders. Startling new information has appeared in recent years on their diversity, the importance of the transcription factor FOXP3, how T cell receptors influence their fate and the unexpected and varied cellular partners that influence T_{reg} cell homeostatic setpoints. We also revisit some tenets, maintained by the echo chambers of Reviews, that rest on uncertain foundations or are a subject of debate.

Sections

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Introduction

The immune system faces unique challenges in the digestive tract. It is exposed to a constant but ever-evolving array of food antigens, and a large, complex array of microorganisms, the gene products of which provide essential support for nutrient production, detoxification and necessary metabolites. Peaceful coexistence, while avoiding microbial breach and takeover as well as over-exuberant immune responses, is essential. Indeed, the gut is where the boundaries of self and non-self are the fuzziest. Physical barriers, such as mucus layers or epithelial tight junctions, ensure part of the separation, but the state of 'active tolerance' also involves immunoregulatory circuits, among which are FOXP3$^+$ regulatory T cells (T_{reg} cells). T_{reg} cells regulate mucosal immunity to both commensal and pathogenic microorganisms, via anti-inflammatory cytokines and small molecules that affect many types of immunocytes, and by modulating IgA responses. They also preserve intestinal physiology by promoting epithelial barrier functions and tissue repair. Intestinal T_{reg} cells have been reviewed in past years[1–7]. Here, we revisit intestinal T_{reg} cells and their diversity, functions, differentiation, microbial influences, T cell receptor (TCR) repertoires and genomic control, organized around recent results from mouse studies (mentioning corresponding results in human studies when possible). Although several T cell types with immunoregulatory properties exist in the gut, we focus on canonical CD4$^+$FOXP3$^+$ T_{reg} cells (not discounting the importance of type 1 regulatory T cells (T_R1 cells)[8] or CD8$^+$FOXP3$^+$ T_{reg} cells[9]).

Overview of intestinal T_{reg} cell subsets

Intestinal T_{reg} cells generally belong to the broad family of tissue-resident T_{reg} cells — FOXP3$^+$ cells that reside outside secondary lymphoid organs, mostly have activated and/or memory-like phenotypes, and whose properties and functions are moulded by the tissues in which they reside[5,10]. Intestinal T_{reg} cell pools have the key property, partially shared with skin T_{reg} cells, of being moulded by the microbiota[11–14] and diet, including food antigens[15–17]. Intestinal T_{reg} cells are an unusual mix of T_{reg} cells that differentiated from a conventional CD4$^+$ T cell lineage, acquiring the expression of FOXP3 and the stereotypic T_{reg} cell signature, either in the thymus ('tT_{reg} cells') or in peripheral tissues ('pT_{reg} cells') in the intestinal lamina propria or in draining lymph nodes (see Box 1). Several subsets of intestinal T_{reg} cells have been reported, usually identified by expression of a particular transcription factor, such as Helios (encoded by *Ikzf2*), GATA3, RORγ, c-MAF, T-bet or ZBTB20 (refs. [18–28]). Schematically, based on their properties and their sensitivity to microbial influence, we discuss two major subsets of intestinal T_{reg} cells, the Helios$^+$NRP1$^+$ subset and the RORγ$^+$ subset. With a few subtleties, GATA3$^+$ T_{reg} cells are a smaller subset within Helios$^+$ T_{reg} cells, whereas MAF$^+$ T_{reg} cells largely (but not entirely) overlap with RORγ$^+$ T_{reg} cells[19–21,25,28]. Two key elements support this dichotomy: RORγ$^+$MAF$^+$ T_{reg} cells are strongly tuned by gut bacteria[21,23–25,29], and were recently found to be only partially dependent on FOXP3 (refs. [30,31]). By contrast, Helios$^+$GATA3$^+$ T_{reg} cells strictly require FOXP3 but are not tuned by microorganisms. This partition is not unique, however: a population of RORγ$^-$NRP1$^-$ T_{reg} cells dominates in the small intestine and responds to dietary antigens[15]. Some Helios$^+$NRP1$^+$ cells do express MAF; and flow cytometry profiles show sizeable proportions of Helios$^-$RORγ$^-$ cells (although it is unclear whether they are transitional states or stable entities).

The balance in intestinal T_{reg} cell populations varies with anatomical location, driven by microbial or food cues. Helios$^+$ T_{reg} cells are more abundant in the small intestine, whereas RORγ$^+$ T_{reg} cells dominate in the colon (40–60% of colon T_{reg} cells versus 10–15% in the ileum and ~10% in gut-draining lymph nodes)[15,19,24], which is plausibly tied to differences in microbial load in these locations. Intestinal T_{reg} cells generally reside in the lamina propria, but they can enter the intraepithelial niche, subsequently losing FOXP3 expression[32]. RORγ$^+$ T_{reg} cells are also found in extraintestinal locations (spleen and subcutaneous lymph nodes) at lower frequencies (1–5%) and in non-lymphoid tissues (muscles, lungs and mammary glands)[22–24,33]. The genomic characteristics of these extraintestinal RORγ$^+$ T_{reg} cells are strongly related to their colonic counterparts[31], and their numbers tend to correlate with those of intestinal RORγ$^+$ T_{reg} cells during microbial or other perturbations[34,35]. Beyond these correlations, a recent study demonstrates directly that microbiota-dependent intestinal T_{reg} cells migrate to and help to promote tissue regeneration in injured skeletal muscle and liver[35]. Thus, fluctuations of intestinal RORγ$^+$ T_{reg} cells have corollaries elsewhere.

Intestinal T_{reg} cell subsets were originally defined by flow cytometry and genetic reporters; recent studies have revisited their definition using single-cell transcriptomics[30,31,36–41]. Beyond the regulatory networks detailed below, these data have confirmed the Helios versus RORγ dichotomy among intestinal T_{reg} cells, establishing more precise transcriptional signatures but also showing that the boundary is somewhat blurred.

In the current paradigm, Helios$^+$NRP1$^+$ T_{reg} cells are tT_{reg} cells that differentiated in the thymus, whereas RORγ$^+$ T_{reg} cells and related subsets are pT_{reg} cells that derived from conventional T cells, under particular conditions of activation[22–25,30,36,42] (whether this dichotomy actually holds is discussed in detail below). RORγ$^+$ T_{reg} cells seem to require continuous microbial stimuli: their proportions are very low in germ-free mice relative to specific pathogen-free mice, and after broad-spectrum antibiotic treatment[15,22–24,34,43]. Accordingly, RORγ$^+$ T_{reg} cells appear around weaning age in mice, at the time of explosive diversification of intestinal microorganisms, whereas Helios$^+$ T_{reg} cells are present from birth[24,44–46]. However, it is not the microbial diversification per se that drives RORγ$^+$ T_{reg} cell levels, as monocolonization of germ-free mice by any one of many single microorganisms can induce levels of RORγ$^+$ T_{reg} cells similar to those of specific pathogen-free mice[13,34], as detailed below.

Although their anatomic preferences and ontogenic timing are known, we have only a limited grasp of the population dynamics of intestinal T_{reg} cells. Do the different T_{reg} cell subsets regulate each other? Perturbation of one T_{reg} cell subset (genetic or microbiological) generally results in a reciprocal increase in the other subsets (for example, increased Helios$^+$ T_{reg} cells in *Rorc*-deficient mice or germ-free), suggesting that they compete in the same niche[47], foremost for the availability of IL-2. There are subset-specific homeostatic controls as well: for example, IL-33 is a specific trophic factor for Helios$^+$GATA3$^+$ T_{reg} cells[20], although dispensable[48]. It is also unclear how much the population maintains a memory of past encounters, to be mobilized anew upon re-exposure, or whether it continuously adapts to current microbiota, as IgA-secreting B cells do[49]. Cell tracing with photoconvertible markers suggests that intestinal T_{reg} cells include both a pool with fast (24 h) turnover (owing to cell death or emigration) and a longer-lasting component[50–52]. Lineage tracing experiments showed that RORγ$^+$ T_{reg} cells labelled in the pre-weaning and/or peri-weaning periods were long-lived, whereas those labelled later in life were more transient[53]. After T_{reg} cell ablation in *Foxp3*–DTR mice (which express human diphtheria toxin receptor from the *Foxp3* locus), Helios$^+$GATA3$^+$ and RORγ$^+$MAF$^+$ populations recover at roughly similar rates (~1 week to full recovery), suggesting comparable homeostatic drivers. Upon

Box 1

Regulatory T cell nomenclature

After some confusing semantics early on — with use of the terms 'adaptive regulatory T cell', which is illogical as every regulatory T cell (T_{reg} cell) expresses a variably rearranged T cell receptor (TCR), and 'natural T_{reg} cell', which begs the question of what is an unnatural T_{reg} cell — a breakout session at the 2013 T_{reg} cell meeting in Shanghai, China, recommended a simplification of the nomenclature, particularly relating to their mode of differentiation[186].

T_{reg} cell
- With no additional qualifiers, T_{reg} cell is used to denote $FOXP3^+$ cells, the ontogeny of which is undetermined or not relevant to the topic at hand.

Thymus-differentiated T_{reg} cell (tT_{reg} cell)
- T_{reg} cells that differentiate in the thymus, then migrate to the periphery. Most T_{reg} cells in lymphoid organs are thought to belong to this category, but in most cases this origin is only assumed.

Peripherally differentiated T_{reg} cell (pT_{reg} cell)
- T_{reg} cells that differentiate in peripheral lymphoid or parenchymal organs from a mature, previously $FOXP3^-$, precursor. Caution: the term is occasionally and misleadingly misused for T_{reg} cells that simply reside in peripheral organs, but are likely tT_{reg} cells.

In vitro-induced T_{reg} cell (iT_{reg} cell)
- $FOXP3^+$ cells generated in culture, typically by TCR activation in the presence of IL-2 and transforming growth factor-β (TGFβ)[187]. These iT_{reg} cells are usually unstable with regards to FOXP3 expression, and of conjectural in vivo relevance. iT_{reg} cell is occasionally misused to mean 'induced T_{reg} cell', referring to pT_{reg} cells in vivo.

Two additional designations have been more recently introduced to indicate T_{reg} cell activation states:

Resting T_{reg} cell (rT_{reg} cell)
- A T_{reg} cell with markers typical of non-activated cells ($CD62L^+CD44^-CD45RA^+$).

Activated or effector T_{reg} cell (aT_{reg} or eT_{reg} cell)
- T_{reg} cells with a higher degree of activation and suppressive activity. Most intestinal T_{reg} cells are aT_{reg} cells.

elimination of bacteria by antibiotics or recovery after cessation of treatment, the proportion of $ROR\gamma^+$ T_{reg} cells adjusts gradually to the new levels (~2–4 weeks), again indicating some population inertia[54]. What controls the balance between the drivers of the different T_{reg} cell subsets, how they work in concert with each other and their mechanism of action need to be further explored.

This brings us to 'homeostatic setpoints', the notion that the proportion of T_{reg} cells and their $ROR\gamma^+$ or $Helios^+$ subsets are stably maintained, with cell populations returning to their starting points after perturbations (antibiotic treatment, T_{reg} cell depletion or infections). Setpoints for T_{reg} cells and T_{reg} cell subsets are under strong genetic control, with a fivefold range in proportions among inbred mouse strains[54–56], and they are also affected by many of the other factors discussed in this Review (maternal, microbial and metabolic). As for any cell population, steady-state levels of T_{reg} cells are set by the combination of input (de novo differentiation, homing from other locations and proliferation) and output (cell egress, further differentiation and death) (Fig. 1). At the conceptual level, it is fascinating that such stability can be achieved when so many control mechanisms and influences are involved. At the practical level, we should be very cautious in interpreting changes in T_{reg} cell frequencies in response to various regulators (cellular or molecular) as implying altered pT_{reg} cell differentiation. Effects on niche size, homing rate and proliferation, for example, may equally well be responsible.

Functions of intestinal T_{reg} cells

Intestinal T_{reg} cells have pleiotropic functions: they control responses to pathogenic and commensal microorganisms and food antigens by regulating both T cells and B cells[21,23–25,57,58] and regulate tissue repair by promoting barrier function and epithelial stem cell renewal[20,59]. Schematically, the microorganism-dependent $ROR\gamma^+$ T_{reg} cells are thought to mediate tolerance to commensal and pathogenic bacteria. Although the exact mechanisms of suppression are unclear, $ROR\gamma^+MAF^+$ T_{reg} cells have been reported to dampen production of interferon-γ, IL-4 or IL-17 by effector T cells[23–25,54]; different studies report different outcomes. These divergences may be a result of variable environments, but they also denote the breadth of $ROR\gamma^+$ T_{reg} cell action. Accordingly, $ROR\gamma^+$ T_{reg} cell deficiency leads to more severe disease in several models of colitis that depend on different flavours of effector T cells[21–26,30,47]. This control also involves the regulation of intestinal mast cells, as pT_{reg} cell deficiency provokes intestinal mastocytosis and subclinical type 2 immune responses[30,60]. Mice deficient in $ROR\gamma^+$ T_{reg} cells are more susceptible to food allergy, and microbial therapy that induces $ROR\gamma^+$ T_{reg} cells improves oral tolerance to food antigens[45]. $ROR\gamma^+$ T_{reg} cells repress IgA production, and deficiency in $ROR\gamma^+MAF^+$ T_{reg} cells yields elevated IgA^+ plasma cells in the intestine and a higher IgA coating of intestinal microorganisms[21,54]. Indeed, the proportion of $ROR\gamma^+$ T_{reg} cells and IgA coating of microorganisms are negatively correlated in several contexts, suggesting a double negative-feedback loop[21,40,54]. By contrast, earlier studies (primarily using transfer of T_{reg} cells or conventional T cells into T cell-deficient hosts) suggested a positive relationship between IgA production and T_{reg} cells[57,58], and particularly BCL-6-dependent follicular T_{reg} cells[58]. These discrepancies might stem from different experimental contexts or reflect balancing roles of T_{reg} cells in specifically limiting germinal centre responses and IgA^+ plasma cell maturation, whereas their generic anti-inflammatory properties might provide an environment that is more favourable to B cell differentiation.

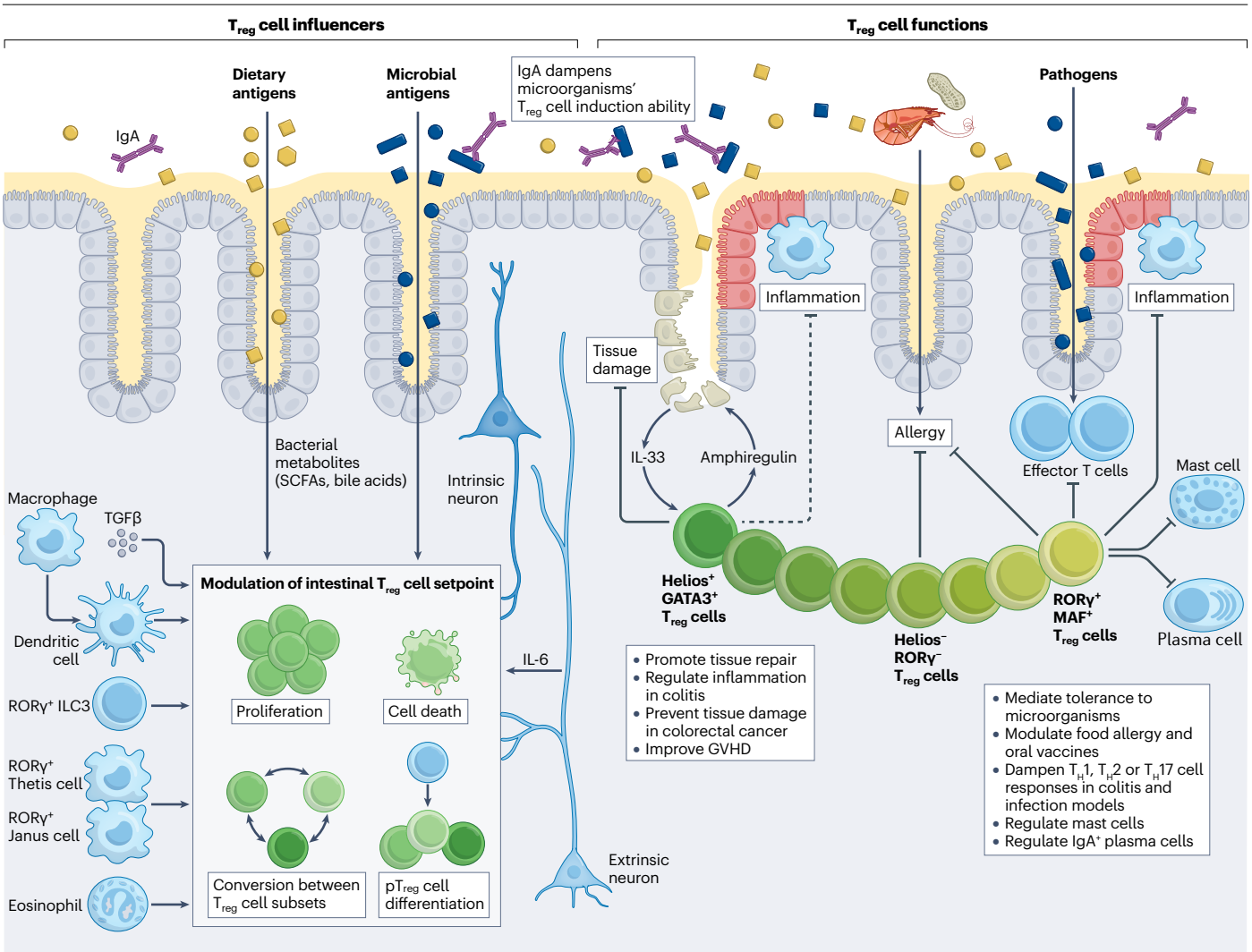

Fig. 1 | The many influencers and functions of intestinal regulatory T cell subsets. Intestinal regulatory T cells (T_{reg} cells) can be broadly categorized into three main subsets: Helios$^+$GATA3$^+$ T_{reg} cells that differentiate in the thymus, RORγ$^+$MAF$^+$ T_{reg} cells that differentiate in response to microorganisms or microbial antigens and Helios$^-$RORγ$^-$ T_{reg} cells that differentiate in response to dietary antigens. T_{reg} cell subsets are maintained at a homeostatic setpoint, which is influenced by many different cell types (macrophages, dendritic cells, RORγ$^+$ innate lymphoid cells (ILCs), RORγ$^+$ Thetis cells, RORγ$^+$ Janus cells, eosinophils and neurons) and microbial and non-microbial factors (short chain fatty acids (SCFAs), bile acids, IgA, transforming growth factor-β (TGFβ), IL-6 and IL-33). This setpoint maintenance is most likely achieved through a combination of regulating T_{reg} cell differentiation from conventional T cells, interconversion between T_{reg} cell subsets, T_{reg} cell proliferation and cell death. T_{reg} cell subsets have specific functions: Helios$^+$GATA3$^+$ T_{reg} cells promote tissue repair in colitis and colorectal cancer in an IL-33-dependent and amphiregulin-dependent manner, and ameliorate inflammation in colitis and graft-versus-host disease (GVHD); Helios$^-$RORγ$^-$ T_{reg} cells are required in the small intestine to prevent food allergy; and RORγ$^+$MAF$^+$ T_{reg} cells are required to maintain tolerance to microorganisms and prevent inflammation, particularly by inhibiting effector T cell responses to commensals and pathogens. RORγ$^+$MAF$^+$ T_{reg} cells also inhibit mastocytosis, regulate IgA$^+$ plasma cells and modulate responses to food allergy and oral vaccines. pT$_{reg}$ cell, peripherally differentiated regulatory T cell; T_H17 cell, T helper 17 cell.

Increased numbers of RORγ$^+$ T_{reg} cells were observed in a mouse model of environmental enteric dysfunction, with stunted growth and reduced efficacy of oral vaccination. Reduction of RORγ$^+$ T_{reg} cells by antibiotics restored the vaccine response of CD4$^+$ T cells, but worsened stunting associated with environmental enteric dysfunction[61]. Noxious effects of RORγ$^+$ T_{reg} cells are also encountered in oncology: RORγ$^+$ T_{reg} cells are increased in human colorectal cancer and in murine polyposis and are thought to contribute to tumour pathogenesis by paradoxically promoting T helper 17 cell (T_H17 cell)-mediated intestinal inflammation[62,63]. Thus, RORγ$^+$ T_{reg} cells have multifaceted roles in controlling inflammation.

The Helios$^+$GATA3$^+$ T_{reg} cell subset has been less well studied. In line with the initial descriptions of these cells[19,20,64], Helios$^+$GATA3$^+$ T_{reg} cells are often mentioned as promoting tissue repair, in good part because of their preferential expression of tissue repair transcripts (in particular *Areg*), under the control of IL-33 via the IL-33 receptor ST2 that they

express selectively. But this functional label may be too restrictive, as they also partake in the control of inflammation: mice deficient in either ST2 or GATA3$^+$ T_{reg} cells are more susceptible to the T cell transfer model of colitis[20], and in old age spontaneously develop mild intestinal inflammation[19,20,64]. Increased expression of ST2 in T_{reg} cells can also ameliorate enteric graft-versus-host disease (GVHD)[65,66]. Helios$^+$GATA3$^+$ T_{reg} cells may also improve the outcome of colorectal cancer by suppressing T_H17 cell responses and preventing excessive intestinal tissue damage[63,67–69], acting here in apparent opposition to RORγ$^+$ T_{reg} cells. It is unclear whether these anti-inflammatory properties of Helios$^+$ T_{reg} cells relate to generic T_{reg} cell functions or to secondary effects of epithelial cell protection via amphiregulin and similar mediators.

Human intestinal T_{reg} cells share many genomic features with corresponding mouse populations. Both mice and humans share reparative-like Helios$^+$ and RORγ$^+$ T_{reg} cell populations, with shared core gene expression signatures[38,70,71]. Although genomic and flow cytometry profiling have identified human RORγ$^+$ intestinal T_{reg} cells in pathological settings[24,38,71,72], more detailed investigations of this subpopulation, its repertoire and its phenotypes are called for.

Relationship of intestinal T_{reg} cells with microorganisms

Intestinal T_{reg} cells are deeply influenced by the intestinal microbiota, whether commensals or pathogens. Studies in germ-free and antibiotic-treated mice first revealed that intestinal T cell homeostasis is controlled by the local microbiota[73–75], followed by detailed studies aiming to identify the bacterial species, or combinations thereof, that modulate T_{reg} cells[11–13,23–25,34,45,76–78]. One of the first symbionts shown to modulate T_{reg} cells was *Bacteroides fragilis*, which induces IL-10-producing T_{reg} cells[11]. Some studies focused on combinations of microorganisms (altered Schaedler flora, Clostridia strain cocktails and probiotic formulations)[12,76,77,79], others demonstrated equivalent impact of individual microorganisms to influence intestinal T_{reg} cells, and particularly RORγ$^+$ T_{reg} cells[13,24,34,45,78,80]. Together, these studies show that the ability to influence intestinal T_{reg} cell accumulation or maintenance is variable but possessed by many different phyla and genera. Furthermore, strains that belong to the same species can have divergent effects[34]. Although not enough to power association studies, it is suggestive of an evolutionary balance, where the ability to influence T_{reg} cells may be favourable to bacteria in some circumstances but not others.

Contrasting with this depth of information on microbial identity, mechanistic information on the regulation of intestinal T_{reg} cells by the microbiota is much less extensive. As discussed below, it is often unclear what proportion of these effects correspond to de novo differentiation of pT_{reg} cells, as opposed to recruitment, amplification or changes in homeostatic setpoints. Microorganisms may exert their T_{reg} cell-modulatory activity directly, by producing molecules that influence T_{reg} cells themselves, or indirectly by controlling signals coming from epithelial cells, dendritic cells or other T_{reg} cell-regulating cells (see below). For example, colonization with Clostridia spp. generates an environment rich in transforming growth factor-β (TGFβ) and indoleamine-2,3 dioxygenase (IDO) that can positively regulate T_{reg} cells[12]. Microbial effects on T_{reg} cell-controlling neurons also belong to the indirect category.

At the molecular level, two main mechanisms have been highlighted. First, microorganisms can influence intestinal T_{reg} cells by exposing different molecular structures that activate innate immune sensors and/or adaptive immune receptors. As discussed below, specific recognition of microbial antigens by TCRs expressed by T_{reg} cells (or their conventional T cell precursors) is clearly required in some instances. Among possible innate immune system ligands, an early study pinpointed *B. fragilis* polysaccharide A as triggering Toll-like receptor 2 (TLR2) on T_{reg} cells through plasmacytoid dendritic cells[81,82]. Capsule polysaccharides were also reported as RORγ$^+$ T_{reg} cell inducers in two studies, albeit with very different mechanisms[40,83]. In one study, the *Bifidobacterium bifidum* capsule was proposed to act via TLR2 on dendritic cells[83]. In the other, capsule components encoded by the *Kfi* operon in *Escherichia coli* also induced of RORγ$^+$ T_{reg} cells[40], but through a very different mechanism: without shielding by the capsule, the bacteria appeared shunted into IgA$^+$ intraluminal casts, thus avoiding exposure to the immune system. More generally, variation between bacterial species in their ability to affect T_{reg} cells might result from their degree of exposure to the immune system (shielding by IgA or the mucus layer, for example), and not solely from variable production of bioactive molecules that we often consider first. Secondly, intestinal T_{reg} cells can be induced by microbial metabolites or by-products. The parasite *Heligmosomoides polygyrus* can expand T_{reg} cells by secreting a molecule that mimics TGFβ[84,85]. Short chain fatty acids (SCFAs), bacterial fermentation bioproducts, have been invoked as T_{reg} cell regulators[77,86–91] but are a subject of debate (Box 2). More recently, secondary bile acids produced by bacterial metabolism have been shown to potentiate T_{reg} cell differentiation by engaging transcriptional regulators of the nuclear hormone receptor family[92–94].

Overall, the multitudinous influences of the microbiota on intestinal T_{reg} cells are clear, and likely involve several molecules and pathways that are yet to be identified. However, it is still unclear how signals from the more than a thousand microbial species that coexist inside a single adult mammal are integrated by the T_{reg} cells and, conversely, what those microorganisms gain by inducing T_{reg} cells. Are we fooled into believing that mucosal T_{reg} cells are one of the host's mechanisms to enforce tolerance of commensals or are they really a mechanism for the microbiota to manipulate the host?

More generally, it will be essential to understand what is hidden under the cliché 'T_{reg} cells enforce tolerance to symbionts', stated in this Review and in countless introductions to articles. Is it a non-specific 'speed limit' on any overactive innate and adaptive immune response, regardless of specificity, symbionts and pathogens being distinguished by the injury they cause (T_{reg} cells as generic damage controllers)? Or does specific recognition of microbial antigens channel response and tolerance in a microorganism-specific manner?

T_{reg} cell antigen specificity and TCR repertoire

Directly related to the question of specific recognition of microbial or food antigens by T_{reg} cells is the TCR they express, and its central role in their differentiation and function. Several studies have tackled the TCR diversity of intestinal T_{reg} cells, and the interplay with antigenic peptides generated in the intestinal ecosystem. Overall, the intestinal T_{reg} cell repertoire is polyclonal and quite diverse, albeit not as broad as that found in T_{reg} cells of secondary lymphoid organs. Studies using transgenic mouse models with constrained TCR diversity (such as single-chain TCRβ transgenic mice and TCR^{mini} mice, which express a constricted TCR repertoire) indicated that the T_{reg} cell TCR repertoires in the colon lamina propria or draining lymph nodes are comparable in diversity with those of conventional T cells in the same locations[14,95–97]. RORγ$^+$ and RORγ$^-$ T_{reg} cell subsets also showed comparable diversity in this context[97]. With the broader informativity afforded by single-cell transcriptomics, enabling the determination of αβTCR pairs in mice

Box 2

Are we sure?

Several tenets about factors that influence intestinal regulatory T cells (T_{reg} cells) circulate in the field, often restated from Review to Review, but not always resting on solid or uncontroversial evidence. Incomplete evidence does not necessarily imply error but some of these accepted truths may warrant circumspection or deeper experimental re-evaluation.

RORγ$^+$ T_{reg} cells are peripherally differentiated T_{reg} cells (pT_{reg} cells), and vice versa

- As discussed in this Review (see 'Origins of intestinal T_{reg} cells'), there are reasons to think that this tenet is not absolute.

Retinoic acid

- Several reports showed a strong impact of retinoic acid on in vitro-induced T_{reg} (iT_{reg}) cell generation[108,111,151], through a debated mechanism[188–190]. From there, it has been assumed that retinoic acid similarly bolsters peripherally derived T_{reg} cell (pT_{reg} cell) conversion in vivo, a plausible hypothesis as retinoic acid levels are high in the intestine, but supporting data are scarce and contradictory. Ohnmacht et al. reported low numbers of RORγ$^+$ T_{reg} cells in the context of vitamin A deficiency[23]. However, mice deficient in the main retinoic acid receptor (RARα) in T cells actually have higher T_{reg} cell proportions in the gut[191], in part because retinoic acid is required by conventional T cells. T_{reg} cells in which retinoic acid signalling is curtailed by a dominant-negative RAR or by overexpressing a retinoic acid-catabolic enzyme have normal T_{reg} cell proportions[192]. Retinoic acid may stabilize existing T_{reg} cells[193], further confounding interpretations of an effect on pT_{reg} cell conversion.

Short chain fatty acids (SCFAs)

- SCFAs such as butyrate, succinate and propionate have been proposed as small molecular mediators for microbial control of T_{reg} cells, acting via inhibition of histone deacetylases (modulating histone or FOXP3 acetylation[86]) or via metabolite-sensing G-protein-coupled receptors GPR43 or GPR109a (refs. [88–90,194]). Unfortunately, these results have not all been reproducible[17,24,93], or when positive there were contradictions as to whether SCFAs affected T_{reg} cell expansion[90] or pT_{reg} cell differentiation[88], or which SCFA was most effective. It is unclear what elements account for the divergent results.

Transforming growth factor-β (TGFβ)

- TGFβ is perhaps less controversial. Its spectacular effects at inducing iT_{reg} cells in culture[187], reproduced in hundreds of studies, argue strongly for a role in T_{reg} cell biology, with several lines of evidence for in vivo relevance. Early transfer studies showed some impact of TGFβ signalling on pT_{reg} cell generation in extraintestinal tissues[106], and mice with deficient TGFβ signalling show decreased T_{reg} cell or RORγ$^+$ T_{reg} cell levels[195,196]. But reductions in colonic T_{reg} cells are only partial in these studies (twofold to threefold), and stronger effects of TGFβ inhibition have paradoxically been demonstrated in conventional T cells[197]. It would be worth re-addressing the importance of TGFβ, particularly on actual pT_{reg} cell differentiation, not only on RORγ$^+$ T_{reg} cell frequencies.

Germ-free and gnotobiotic mice

- These are attractive models to assess the impact of the microbiota (or absence thereof) or the immunomodulatory potential of individual cell types. They are, however, highly reductive 'circus animals', with other perturbations only indirectly related to the microbiota (such as a fourfold-enlarged caecum). The irruption of microorganisms in a previously germ-free mouse is likely to have general consequences in itself, beyond the specific effects of the colonizing microorganisms.

Clostridia communities

- From early studies[12,76,77] it is often stated that bacterial effects on intestinal T_{reg} cells reflect the complementary activity of species within bacterial communities. Many subsequent studies showed that single microorganisms are as effective[11,13,34]. Cooperativity between microorganisms remains an attractive notion, but requires demonstration.

Bile acids

- Bile acids are steroidal lipids involved in essential nutrient absorption, synthesized in the liver as primary bile acids and released into the duodenum, where they help to emulsify dietary lipids and mostly shuttle back from the ileum to the liver. A fraction escape reabsorption and are transformed by bacteria in the colon into various 'secondary bile acids'. Recent results from several laboratories have shown that bile acids influence intestinal T cells[92–94,198]. The unresolved dilemma is that different secondary bile acids appear active, and different receptors (VDR, FKR and NR4A1) are involved, in the different reports, begging reconciliation.

with full TCR diversity, these conclusions have largely held: intestinal T_{reg} cells have polyclonal repertoires, with some degree of clonal expansion (typically, sequencing a hundred different T_{reg} cells will show 5–10% of cells sharing expanded clonotypes)[36–38,40]. Perhaps surprisingly, distributions of TCR clonotypes are comparable in specific pathogen-free mice with a complex microbiota and in germ-free mice colonized by a single microorganism[40,47].

In terms of clonotype sharing, studies in limited-repertoire mice showed T_{reg} cells and conventional T cells to have mostly distinct repertoires, albeit with significant overlap between intestinal T_{reg} cells

and conventional T cells[14,96–98]. For instance, of the 25 most frequent clonotypes in conventional T cells, 5–10 were also observed in T_{reg} cells. This is perhaps not unexpected if many intestinal T_{reg} cells are pT_{reg} cells derived from conventional T cells. But marked clonal sharing was also observed between RORγ$^+$ and RORγ$^-$ T_{reg} cells[97], a finding reproduced in polyclonal mice[40,47] – a puzzling observation if these represent pT_{reg} cells and tT_{reg} cells, respectively. Instead, this clonal overlap implies that RORγ$^+$ and Helios$^+$ T_{reg} cells may interconvert or have a common precursor.

Pathogens elicit vigorous immune responses, and a central question is whether symbionts also elicit T cells whose TCRs are specific for their antigens, and whether these specific T cells partake in commensalism. Indeed, commensal bacteria prompt specific antigenic recognition by T cells (including Clostridia, *Bacteroides*[99,100], *Lactobacillus*[14,96], *Akkermansia muciniphila*[101,102] and *Helicobacter*[25,98]). Peptide–MHC complexes derived from processing of microbial antigens have been identified that activate T cell hybridomas or transgenic T cells (in vitro or by transfer into microorganism-colonized mice). In several cases, corresponding TCRs can drive the conversion of conventional T cells into pT_{reg} cells[14,25,98,102,103]. This potential does not apply to all commensals; for instance, in one study *Helicobacter* spp. could activate cognate T cells whereas *Bacteroides ovatus* could not, plausibly because mucosa-associated *Helicobacter* is more visible to T cells than the luminal *B. ovatus*[98]. More generally, commensals also show a bewildering diversity in the type of T cell they trigger in an antigen-specific manner: *Helicobacter hepaticus* preferentially activates RORγ$^+$ T_{reg} cells (at least in a non-inflammatory context)[25], *A. muciniphila* drives T follicular helper cell-like conventional T cells more than T_{reg} cells[101], commensal Cryptosporidia induce either T_H1 cells or T_{reg} cells depending on the presence of dendritic cells[104], whereas an epitope from *Bacteroidetes* promotes differentiation of tolerogenic CD4$^+$CD8αα$^+$ intraepithelial lymphocytes[100]. It will be important to better understand the root of these specific relationships – that is, why some commensals but not others drive the clonal expansion of antigen-specific T_{reg} cells. The route and manner through which proteins of a given microorganism are processed and presented by activating or tolerogenic antigen-presenting cells (APCs) likely interplays with the non-specific modes through which microorganisms affect the T_{reg} cell pool. These questions are tied to the more general conundrum faced by T_{reg} cells in the intestine: given the thousands of microbial species in the gut, how can one specific signal not be drowned out by the noise. For focused immune responses, T_{reg} cells are thought to suppress specifically by inactivating APCs or effector T cells in a local micro-domain[105]. This scenario is difficult to entertain in a sea of microbial peptides. Ultimately, the question is why do T_{reg} cells bother with an antigen-specific TCR in this context. It may be that they use their TCR for retention in a relevant location, rather than to elicit the T cell activation and differentiation cascade that we associate with acute immune responses. In addition, immunoregulation may only care about high-abundance signals from the most dominant microbial or food antigens, and by controlling these responses T_{reg} cells maintain order in the intestine and the peptidic chatter does not matter.

Origins of intestinal T_{reg} cells

The question of T_{reg} cell origin is of central importance to intestinal T_{reg} cell physiology and key to understanding the relation to commensal microorganisms and food antigens. Overall, most T_{reg} cells differentiate in the thymus (tT_{reg} cells), but an alternative pathway results from 'conversion' of conventional T cells into FOXP3$^+$ T_{reg} cells in peripheral organs. This conversion is typically induced by high-affinity ligands under conditions of ineffective TCR activation (low dose of antigen, low levels of co-stimulatory signals or tolerogenic APCs)[106,107] or by homeostatic drive in conditions of T_{reg} cell deficiency[108,109]. Helios$^+$GATA3$^+$ T_{reg} cells are considered to be tT_{reg} cells, whereas microorganism-responsive RORγ$^+$MAF$^+$ T_{reg} cells are pT_{reg} cells that differentiate from conventional T cells in the gut. The actual data are more nuanced, however, and the true quantitative contribution of thymic versus peripheral differentiation paths to intestinal T_{reg} cells is still unresolved. Further, from experiments showing that conventional T cell to pT_{reg} cell conversion in response to antigens administered via the gut mainly occurs in gut-associated lymphoid tissue[108,110,111], our field has extrapolated with some degree of circular reasoning that the gut-associated lymphoid tissue is inherently a preferential site of pT_{reg} cell conversion – and, from there, that microorganism-induced changes in intestinal T_{reg} cells result from this conversion pathway. However, clonal amplification, selective homing or alterations in homeostatic controls (cell survival, turnover rate and proliferation) are equally plausible mechanisms through which microorganisms may quantitatively influence intestinal T_{reg} cell populations.

Definitive experimental evidence related to the intestinal tT_{reg} cell–pT_{reg} cell balance has been elusive. First, the markers initially used to define tT_{reg} cells, Helios[42] and NRP1 (refs. [43,112]), proved to be incompletely reliable[113], as both can by upregulated by TCR-driven activation[114–116]. Conversely, RORγ can be upregulated in tT_{reg} cells under inflammatory conditions (such as by IL-6)[117,118]. It is probably valuable to consider these markers as suggestive of T_{reg} cell origin, but not as formally establishing the origin of a given cell. Second, the comparison of TCR sequences used by intestinal and thymic T_{reg} cells from transgenic mice with restricted TCR diversity yielded conflicting results: one study found a high degree of similarity between TCRs in colonic and tT_{reg} cells[96]; another found little similarity between them, and showed that TCRs from intestinal T_{reg} cells failed to facilitate T_{reg} cell differentiation[14] (not necessarily a definitive argument, as many T_{reg} cell-derived TCRs lead to little or no thymic selection in transgenic mice). Third, mice with a mutation in the CNS1 enhancer element of the *Foxp3* locus show a strong delay in pT_{reg} cell conversion in response to oral antigen[103,119]. But this block is not complete, and CNS1-deficient mice have a quasi-normal contingent of colonic RORγ$^+$ T_{reg} cells[113,120]. Thus, the presence or absence of T_{reg} cells in the face of CNS1 deficiency also cannot be considered a definitive criterion for thymic or peripheral origin.

Cell transfer studies offer the most direct evidence for pT_{reg} cell conversion, as the starting cell type is defined, and donor-derived pT_{reg} cell progeny are formally identified with a congenic marker[25,37,97,103]. With conventional T cells from CT2 transgenic mice that express a microorganism-reactive TCR, Hsieh and colleagues[97,103] observed the generation of pT_{reg} cells with a predominant (but not exclusive) RORγ$^+$Helios$^-$ yet, paradoxically, NRP1$^+$ phenotype. Xu et al. showed conversion of *Helicobacter*-specific conventional T cells from HH7 transgenic mice into RORγ$^+$ T_{reg} cells[25]. In the setting of T_{reg} cell complementation in T_{reg} cell-ablated hosts, where conversion is driven by a strong homeostatic drive to restore T_{reg} cell pools, naive conventional CD4$^+$ T cells from normal adult donors also differentiated into RORγ$^+$ pT_{reg} cells in the colon, but into Helios$^+$ pT_{reg} cells when donor T cells stemmed from perinatal mice or were recent thymic emigrants[37]. Although the homeostatic drive inherent to the latter system might influence the outcome, here again the notion that pT_{reg} cells are all Helios$^-$NRP1$^-$RORγ$^+$ is not absolute.

Most recently, van der Veeken et al. engineered an ingenious reporter model — combining a fluorescent genetic marker to pulse-label conventional T cells, a *Foxp3*–DTR construct for T_{reg} cell ablation and control of inflammation by third-party T_{reg} cells — and observed strong pT_{reg} cell generation driven by acute recovery from deprivation of microbial and food antigens[30]. pT_{reg} cell generation could be observed over a period of a few weeks. The pT_{reg} cells generated in this context were unequivocally RORγ$^+$. Interestingly, conventional T cell to pT_{reg} cell conversion was visualized almost exclusively in the gut with this system, and not in other locations of tolerance induction where pT_{reg} cell generation had been suspected (such as allogeneic pregnancy, parasitic infection and grafted tumours), perhaps because the temporal drivers of conversion in these experiments (sudden exposure to food and microbial antigens) mostly concerned the gut. This system is unfortunately limited over time (as tT_{reg} cells re-emerge) and cannot establish the contribution of pT_{reg} cells and tT_{reg} cells in unchallenged mice.

Finally, TCR repertoire analysis can provide clues to the relationship between conventional T cell pools and putative pT_{reg} cells in the colon. In limited-diversity mice, TCR clonotypes were abundantly shared between Helios$^+$ and Helios$^-$RORγ$^+$ T_{reg} cells[97,113]. In more recent studies of monocolonized germ-free mice, whose normal TCR loci allow unambiguous identification of clonal lineages (full identity of both TCRα and TCRβ at the nucleotide level, beyond matching by chance), TCR clonotypes were shared by colonic conventional T cells and RORγ$^+$ T_{reg} cells, but also with Helios$^+$ T_{reg} cells[40,47]. Although no directionality can be inferred from TCR sharing (just that cells somehow have some common origin, via ancestry or interconversion), these results negate the strict Helios$^+$ = tT_{reg} cell, RORγ$^+$ = pT_{reg} cell formula.

Another central question concerns the mechanisms that drive pT_{reg} cell conversion in the intestine. Many mediators specific to the intestinal milieu have been invoked (such as TGFβ, bile acids, retinoic acid, SCFAs, TLR2 ligands, tryptophan metabolites and prostaglandins). Beyond the debates that surround some of these mediators (Box 2), it is fair to state that most of these drivers have been implicated either by extrapolation from in vitro generated T_{reg} cells (iT_{reg} cells), the relevance of which to true pT_{reg} cells remains conjectural, or by changes observed in vivo but that were not explicitly connected to pT_{reg} cell conversion, versus changes in T_{reg} cell homeostatic setpoints. How the effects of these candidate mediators would be integrated is also unclear. Thus, the molecular mechanisms underlying conventional T cell to pT_{reg} cell conversion remain largely to be determined.

Genomic characteristics and control of intestinal T_{reg} cells

Intestinal T_{reg} cells have layered genomic programmes that distinguish them from T_{reg} cells in other lymphoid and non-lymphoid organs. Most genomic studies of intestinal T_{reg} cells have focused on colonic T_{reg} cells, which share an activation and homing gene programme with other tissue T_{reg} cells, including upregulation of T_{reg} cell effector molecules, activation-associated transcription factors (such as *Irf4*, *Fos* and *Nr4a1*) and chemokine receptors and downregulation of typical resting T_{reg} cell (rT_{reg} cell) transcripts that promote retention in lymphoid organs (such as *Sell* and *Tcf7*)[10,38,39,121]. This common tissue T_{reg} cell programme is overlaid by colonic T_{reg} cell-specific features, presumably in response to environmental cues specific to the intestine, such as constitutively high expression of *Il10* (refs. [24,27,38,122]).

RORγ$^+$ and Helios$^+$ T_{reg} cells differ in their genomic programmes. Helios$^+$ T_{reg} cells share gene expression and chromatin accessibility signatures with ST2$^+$ tissue T_{reg} cells from other organs, co-expressing other transcription factor transcripts such as *Gata3* and *Rora*, and inducing many elements of the TNFRSF–NF-κB pathway[10,20,38,39]. These molecular signatures arise in a stepwise manner, sharing trajectories with ST2$^+$ T_{reg} cells in other organs. As initially characterized and validated by tracking T_{reg} cells expressing transgenic TCRs and by transfer experiments, tissue T_{reg} cells establish molecular programmes in two steps: initial priming in lymphoid organs followed by programme completion upon arrival in the tissues[123–125] (computational trajectory analysis, although not yet experimentally validated, reached the same conclusion[38]).

By contrast, RORγ$^+$ T_{reg} cells show increased expression of T_H17 cell-like transcripts (*Il23r*), chemokine and cytokine receptors (*Ccr2, Ccr4* and *Ccr9*), co-inhibitory receptors (*Havcr2*, *Lag3* and *Ctla4*) and *Il10* (refs. [10,22,24,38,40]). The steps leading to RORγ$^+$ T_{reg} cells are less clear. As discussed above, RORγ expression may be acquired at the time of conventional T cell to pT_{reg} cell conversion[30,37,97,103], but RORγ expression can also be induced in pre-formed T_{reg} cells[117,118]. Mechanistic links remain uncertain, but extracellular inputs may directly induce RORγ expression: STAT3-dependent signals (involving the IL-6 or IL-23 receptor p19 subunit) modulate RORγ$^+$ T_{reg} cell generation in vitro and in vivo[23,24,126,127], and WNT signalling may also promote RORγ expression in T_{reg} cells[63,72,128,129], as might the secondary bile acids[92–94].

Multiple inputs impact the ability of RORγ to regulate its target genes. RORγ is required for accessibility at its binding sites across diverse immunological lineages, suggesting function as a pioneer factor[130]. However, RORγ function varies by context, with different signature genes induced across different RORγ$^+$ cells (such as T_H17 cells, RORγ$^+$ T_{reg} cells, group 3 innate lymphoid cells (ILC3s), γδ T cells and lymphoid tissue inducer cells)[24,130]. In T_{reg} cells, FOXP3–RORγ interactions are required for specific gene expression modules, and ectopic overexpression of FOXP3 in conventional T cells induces *Rorc* expression, suggesting that FOXP3 and RORγ may form a regulatory complex[131–133]. Which transcriptional and extracellular factors in T_{reg} cells versus other cell types condition RORγ targets remains an open question.

Beyond RORγ, numerous transcription factors contribute specific facets to the intestinal T_{reg} cell programme. Aryl hydrocarbon receptor interacts with MAF to promote IL-10 production in both T_R1 cells and T_{reg} cells, and ZBTB20 expression also marks an IL-10^{hi} intestinal T_{reg} cell subset[27,134,135]. Other transcription factors, such as IRF4 and JUNB, are associated with generic activation programmes[136,137]. Similarly, BATF and BACH2 are important for pan-tissue T_{reg} cell programmes[10,124,138]. BLIMP1 (encoded by *Prdm1*) favours CNS2 demethylation and binds *Il17a* and *Il17f* loci to decrease their accessibility and expression in RORγ$^+$ T_{reg} cells[139–141]. Some transcription factors prevent alternative cell fates: a THPOK (encoded by *Zbtb7b*) regulatory loop controls the balance between T_{reg} cells in the lamina propria and intraepithelial lymphocytes[41,142]. Many such studies have focused on single transcription factors in intestinal T_{reg} cells, proposed as drivers of a phenotype or specific function, but a systematic perspective on how these transcription factors are integrated has been missing. In contrast to classic discrete, 'master transcription factor'-mediated differentiation cascades, recent single-cell genomics have revealed imbricated transcription factor configurations, in which overlapping combinations of multiple transcription factors operate across the intestinal T_{reg} cell phenotypic space[31] (Fig. 2). Speculatively, these interlaced transcription factor activities may reflect differential exposure to extracellular signals or distinct spatial positioning within the tissue, and it is unclear whether these states are stable or whether cells fluctuate between them.

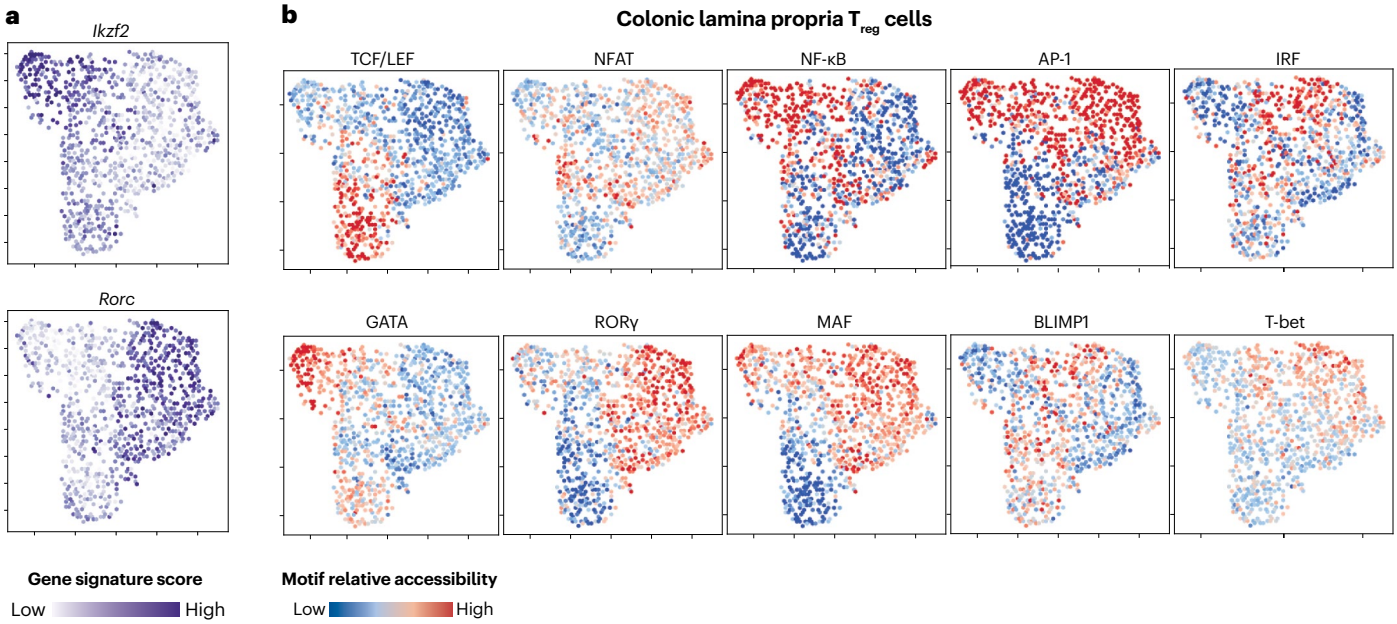

Fig. 2 | Transcription factor activities across colonic regulatory T cell populations. Uniform manifold approximation and projection (UMAP) visualization of single-cell chromatin accessibility of colonic lamina propria regulatory T cells (T_{reg} cells). **a**, Expression of *Ikzf2* and *Rorc*, which encode Helios and RORγ, respectively, delineating Helios$^+$ and RORγ$^+$ T_{reg} cells. **b**, Relative accessibility per cell (chromVAR scores) of open chromatin regions containing the indicated transcription factor motifs. Some transcription factors have opposing activity patterns across T_{reg} cell subpopulations (for example, GATA accessibility does not overlap with that of RORγ). However, in contrast to previous models, which generally posit that each T_{reg} cell subpopulation is controlled by the specific activity of defining 'master transcription factors', these higher-resolution data indicate that the spectrum of T_{reg} cell variability is instead marked by interwoven gradients of multiple transcription factors. For example, BLIMP1, T-bet and RORγ have overlapping accessibility patterns, and activation-related factors such as NF-κB, NFAT or AP-1 incompletely overlap.

Quite surprisingly, RORγ$^+$ T_{reg} cells in the colon behave as if they are FOXP3-independent[30,31]. Cells with T_{reg} cell-like characteristics are found in the absence of FOXP3 expression in mice and humans[143–148], which are usually outcompeted by normal T_{reg} cells in heterozygous females. In the colon, however, microorganism-dependent RORγ$^+$ T_{reg} cells devoid of FOXP3 expression persist in normal numbers and are not outcompeted by FOXP3-proficient RORγ$^+$ T_{reg} cells[30,31]. This observation is particularly puzzling as inflammatory bowel disease is one of the prototypic manifestations of FOXP3 deficiency disease (known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). Importantly, these FOXP3-deficient RORγ$^+$ T_{reg} cells acquired some effector T cell characteristics (production of IL-17 or IL-4 and/or IL-5), in line with the known role of FOXP3 in suppressing cytokine expression[143,144,149,150]. Very speculatively, this observation might suggest that RORγ can not only collaborate with FOXP3 but also sustain some aspects of the T_{reg} cell programme in its absence.

Control of T_{reg} cells by other cells

Several immunological and non-immunological cell types influence T_{reg} cell differentiation and homeostasis. Foremost, perhaps, are MHC class II-positive myeloid cells. Classic CD103$^+$ dendritic cells can promote TGFβ-dependent iT_{reg} cell differentiation in vitro via their high metabolism of retinoic acid[108,111,151,152] and by their expression of ITGB8, which activates latent TGFβ[153]. However, the exact role of CD103$^+$ dendritic cells in pT_{reg} cell differentiation in vivo is less clear: induction of oral tolerance using transfer of ovalbumin-specific CD4$^+$ T cells in different models of dendritic cell depletion yielded conflicting conclusions[154,155]. In a transfer model of *Helicobacter*-specific T cells, reducing CD103$^+$ classical dendritic cells in favour of CD103$^-$ dendritic cells did not affect total colonic T_{reg} cells or the RORγ$^+$ fraction[156]. It may be that newly identified RORγ$^+$ APCs (see below) complicate the interpretation of these experiments, as the *Zbtb46*-Cre/DTR and *Cd11c*-Cre drivers used in some of this work are also active in Thetis and Janus cells[157,158]. 'Dendritic cell-specific' Cre drivers (*Clec9a*) showed that MHC class II-mediated antigen presentation by classic dendritic cells did not affect RORγ$^+$ T_{reg} cell levels at steady state[158,159]. CX_3CR1^+ macrophages can transfer luminal antigens to CD103$^+$ dendritic cells and indirectly promote the expansion of intestinal T_{reg} cells in response to ovalbumin[160,161]. CX_3CR1^+ macrophages also regulate food antigen-specific colonic T_{reg} cells[97,162], whereas T_{reg} cells feed back by inhibiting IL-1 and IL-23 production by CX_3CR1^+ macrophages[163]. During bacterial infections or antigenic challenges, eosinophils reside close to intestinal T_{reg} cells and their production of TGFβ may partake in RORγ$^+$ T_{reg} cell expansion[164]. Whether the influence of these myeloid cell populations on intestinal T_{reg} cells is through direct or indirect mechanisms involving other cell types and/or microbial populations needs to be further explored. In addition, different types of antigen (food or microbial) are likely to be ferried to different locations (mesenteric lymph node versus lamina propria) and presented by different types of APC; thus, the nature of the APC that determines T_{reg} cell responses in different experiments may result from the type of antigen as much as from any intrinsic property of the APC itself.

The interactions between myeloid cells and T_{reg} cells could be regulated by other intestinal immune cells such as RORγ$^+$ ILC3s. For

example, CD103$^+$ dendritic cells can be conditioned by ILC3s to produce more TGFβ and IL-10, which in turn may induce colonic T_{reg} cells[165]. In addition, ILC3s may directly influence intestinal T_{reg} cells: ILC3-derived IL-2 and OX40L modulate intestinal T_{reg} cell homeostasis[166,167].

There has been much interest recently in a population of MHC class II$^+$RORγ$^+$ cells in mesenteric lymph nodes that co-localize with and control intestinal T_{reg} cells. In three studies, RORγ$^+$ APCs were shown to control the balance between pathogenic T_H17 cells and tolerogenic T_{reg} cells by promoting microbiota-specific RORγ$^+$ T_{reg} cells (*Helicobacter*-specific[157,159] and general gut microbiota[158]). The spectrum of candidate pT_{reg} cell-inducing RORγ$^+$ APCs revealed by single-cell genomics included classical ILC3s, previously identified RORγ$^+$ cells expressing the thymic tolerance-inducing factor AIRE (referred to as RORγ$^+$ extrathymic AIRE-expressing cells or Janus cells[157,159,168]) and a class of APCs known as Thetis cells that included both AIRE$^+$ (presumably Janus cells or extrathymic AIRE-expressing cells) and AIRE$^-$ subsets[158]. Although these cells shared markers with both dendritic cells and ILCs, *Rora*-Cre and *Clec9a*-Cre driven lineage tracers demonstrated that both RORγ$^+$AIRE$^+$ populations and AIRE$^-$ Thetis cells were distinct from ILCs and dendritic cells[158,159]. *Rorc*-Cre mediated ablation of *H2-Ab*, *Itgav*, *Itgb8* or *Ccr7* diminished the numbers of RORγ$^+$ T_{reg} cells[157–159].

Box 3

Maternal influences on regulatory T cells

Maternal environments (microbiota, infections and genetics) can have long-term imprints on microbial, immunological, metabolic and neurological characteristics in the offspring. In the intestine, maternal microbiota and/or infection-driven IL-6 expression in utero and maternal antibodies in early life can influence effector T cell responses[199–201]. In a similar manner, intestinal regulatory T cells (T_{reg} cells) also reflect maternal influences.

In early life, dietary and microbial antigens are encountered by the intestinal immune system through goblet cell-associated antigen passages[44]. Antigen delivery via these passages is maternally and temporally controlled by changes in epidermal growth factor in breast milk, which leads to long-lived antigen-specific peripherally differentiated T_{reg} cells (pT_{reg} cells) in the offspring intestine[53].

Genetics of the mother (or foster mother) seem to influence the long-term setpoint of colonic T_{reg} cells (RORγ$^+$ T_{reg} cells) in a manner dependent on early-life IgA coating of intestinal microorganisms. Reciprocal setpoints of RORγ$^+$ T_{reg} cells and IgA coating of luminal microorganisms are both maternally transmitted, an influence that tracks for multiple generations via the entero-mammary axis[54].

These studies validate the notion that maternal influences in early life can promote long-term imprinting of T cell populations, with important implications on long-term offspring health, and on the understanding of heredity in autoimmune diseases. The exact mechanisms of maternal control are unresolved, and whether humans exert similar control of effector T cells and/or T_{reg} cells in utero or via breast milk needs to be established.

However, conditional ablation of MHC class II with *Aire*-Cre drivers[159] or diphtheria toxin-mediated ablation of peripheral AIRE$^+$ cells via an *Aire*-DTR construct[157] did not impact RORγ$^+$ T_{reg} cell numbers, suggesting that AIRE expression may be a red herring. Thetis cells in the mesenteric lymph nodes appear in a wave that roughly coincides with the appearance of RORγ$^+$ T_{reg} cells and diversification of the microbiota, suggesting a temporally controlled function[158], especially as the ablation of MHC class II in RORγ$^+$ APCs has much less impact on RORγ$^+$ T_{reg} cells in adults. The mechanisms of T_{reg} cell control by this population (or populations) requires antigen presentation by RORγ$^+$ APCs, but not by classic dendritic cells, and may involve integrin processing of TGFβ and competition for IL-2. More generally, the co-occurrence of RORγ in both T_{reg} cells and the APCs that influence them is presumably not a coincidence. One might speculate that *Rorc* expression is induced in both cell types by the same triggers or that the convergence is evolutionarily selected such that cells destined to interact respond to some of the same cues (for example, chemokine receptors).

Parenchymal cells in the intestine can also influence T_{reg} cell proportions and populations. As mentioned above, IL-33 secreted by intestinal epithelial cells and enteroendocrine cells drives the expansion of GATA3$^+$ T_{reg} cells and promotes tissue repair[20,169–171]. Intestinal epithelial cells also secrete cytokines such as IL-18, which promote intestinal T_{reg} cell function[172]. In addition, apoptotic intestinal epithelial cells and crosstalk between intestinal epithelial cells and dendritic cells influence intestinal T_{reg} cell abundance[173]. In conclusion, the differentiation of intestinal T_{reg} cells involves a concerted effort between many different cell types, the involvement of which is further modulated by the microbiome. It will be interesting to decipher how these various actors conspire to establish the puzzling maternal transmission of intestinal T_{reg} cell setpoints (Box 3).

Neuroimmune interactions affecting intestinal T_{reg} cells

The gastrointestinal tract is overseen by the nervous system, with an autonomous enteric nervous system and extrinsic projections from ganglia, including sympathetic and parasympathetic neurons[174,175]. Neuroimmune interactions have been described for several gut immunocytes. Intestinal ILC2s are regulated by neuropeptides (such as neuromedin U[176], adrenaline[177] and calcitonin gene-related peptide[178]) and the choline acetyltransferase–acetylcholine pathway[179], ILC3s are influenced by neurons expressing vasoactive intestinal peptide[180,181], and muscularis macrophages and enteric neurons have reciprocal interactions through colony-stimulating factor 1 and bone morphogenetic protein 2 (refs. [182,183]). Recent developments have pointed to an influence of the nervous system on intestinal T_{reg} cells. In an intestinal organ culture system, the early responses to several commensals included a shutdown of transcripts associated with neurons (*Tac1* and other nociceptive neuron-specific genes), which correlated with the bacteria's ability to induce RORγ$^+$ T_{reg} cells. Accordingly, *Tac1*-deficient mice showed increased proportions of colonic RORγ$^+$ pT_{reg} cells, whereas capsaicin, which activates sensory neurons, reduced colonic T_{reg} cells[126]. Teratani et al. reported that vagal gut–brain communication controls intestinal T_{reg} cells, with surgical section of the vagal nerve or its hepatic branch reducing colonic T_{reg} cells and RORγ$^+$ T_{reg} cell proportions[184]. Direct regulation of intestinal T_{reg} cells by intestinal neurons was demonstrated in iT_{reg} cell co-cultures with enteric neurons; neuron-produced IL-6 lowered the total iT_{reg} cell output but increased the RORγ$^+$ fraction[127]. Monocolonization of germ-free mice with the symbiont *Clostridium ramosum* diminishes colonic neuron density,

as do enteric pathogens[185], suggesting triangular crosstalk between gut microbiota, enteric neurons and T_{reg} cells[127]. Beyond these early leads, it will be fascinating to see how the sensory and discriminatory capabilities of the nervous and immune systems integrate around T_{reg} cells to manage coexistence with the microbiota.

Concluding remarks

This Review has provided a comprehensive overview of several aspects of intestinal T_{reg} cell biology, with some degree of Cartesian scepticism over some of the accepted tenets. Influencers and functions of intestinal T_{reg} cell subsets are clearly multifaceted. Given their importance in regulating intestinal tissue homeostasis, it may not be surprising that it takes concerted effort between many different players to fine-tune intestinal T_{reg} cells.

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