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Tissue-specific emergence of regulatory and intraepithelial T cells from a clonal T cell precursor

Angelina M. Bilate^{1,*}, Djenet Bousbaine^{1,2}, Luka Mesin¹, Marianna Agudelo¹, Justin Leube¹, Andreas Kratzert¹, Stephanie K. Dougan^{1,†}, Gabriel D. Victora¹, and Hidde L. Ploegh¹

¹Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Cambridge, MA 02142, USA.

²Department of Biology, Microbiology Graduate Program, Massachusetts Institute of Technology, Cambridge, MA 02142, USA.

Abstract

Peripheral Foxp3⁺ regulatory T cells (pT_{reg}s) maintain immune homeostasis by controlling potentially harmful effector T cell responses toward dietary and microbial antigens. Although the identity of the T cell receptor (TCR) can impose commitment and functional specialization of T cells, less is known about how TCR identity governs pT_{reg} development from conventional CD4⁺ T cells. To investigate the extent to which TCR identity dictates pT_{reg} fate, we used somatic cell nuclear transfer to generate a transnuclear (TN) mouse carrying a monoclonal TCR from a pT_{reg} (pT_{reg} TN mice). We found that the pT_{reg} TCR did not inevitably predispose T cells to become pT_{reg} but instead allowed for differentiation of noninflammatory CD4⁺CD8αα⁺ intraepithelial lymphocytes (CD4_{IEL}s) in the small intestine. Only when we limited the number of T cell precursors that carried the TN pT_{reg} TCR did we observe substantial pT_{reg} development in the mesenteric lymph nodes and small intestine lamina propria of mixed bone marrow chimeras. Small clonal sizes and therefore decreased intraclonal competition were required for pT_{reg} development. Despite bearing the same TCR, small intestine CD4_{IEL} developed independently of precursor frequency. Both pT_{reg} and CD4_{IEL} development strictly depended on the resident microbiota. A single clonal CD4⁺ T cell precursor can thus give rise to two functionally distinct and anatomically segregated T cell subsets in a microbiota-dependent manner. Therefore, plasticity of the CD4 T cell compartment depends not only on the microbiota but also on specialized environmental cues provided by different tissues.

Correspondence to: Angelina M. Bilate; Hidde L. Ploegh.

*Corresponding author. Email: ambilate@wi.mit.edu (A.M.B.); ploegh@wi.mit.edu (H.L.P.).

†Present address: Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215, USA.

Author contributions:

A.M.B. conceived the study and designed, performed, and analyzed the experiments. S.K.D. performed the SCNT. L.M. performed and analyzed the RNA-seq experiments. D.B., M.A., J.L., and A.K. helped with the experiments. D.B. helped with manuscript preparation. G.D.V. supervised RNA-seq analysis and helped with manuscript preparation. A.M.B. prepared the figures. H.L.P. supervised the study. A.M.B. and H.L.P. wrote the paper.

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INTRODUCTION

Foreign antigens derived from the diet and the microbiota pose a daily challenge to the gastrointestinal tract. Regulatory T cells (T_{regs}), abundant in the gut mucosa, prevent inflammatory bowel disease and food allergies through inhibition of harmful responses by effector T cells (1, 2). $CD4^+$ T cells exhibit plasticity and can differentiate into distinct subsets with diverse functional properties (3, 4). The intestinal lamina propria (LP), a location constantly exposed to commensal antigens, harbors many T cell subsets [e.g., T helper cell 1 (T_H1), T_H17 , and peripheral Foxp3⁺ regulatory T cell (pT_{reg})] living in relative harmony. How these T cell fates are determined in vivo is the subject of intense study, as is the role of T cell receptor (TCR) specificity in this process. The TCR repertoire of colonic T_{regs} shows little, if any, overlap with the repertoire of naïve or effector $CD4^+$ T cells present at the same location or with T_{regs} isolated from organs other than the intestine (5). Likewise, the repertoire of intestinal T_H17 differs substantially from that of other intestinal T cells (6). Whereas colonic commensals such as *Clostridium* spp. favor pT_{reg} development (5, 7), segmented filamentous bacteria (SFB) induce differentiation of conventional $CD4^+$ T cells (T_{convs}) into quasi-clonal T_H17 in the small intestine (6, 8). Thus, the repertoire and fate of $CD4^+$ T cells in the intestinal LP are determined by the microbiota. Fate decisions appear to be made at the clonal level so that different TCR specificities determine different developmental outcomes. The mechanism whereby TCR specificity drives T cell fate and function is unclear and may depend on the relative abundance of the antigens recognized, environmental cues, or a combination of both.

Development and expansion of thymus-derived or natural T_{regs} (nT_{regs}) are limited by intraclonal competition (9, 10). This process is driven by affinity for self-ligands: the higher the affinity for the antigen, the larger the nT_{reg} “niche” size (11). When antigen presentation occurs under subimmunogenic or noninflammatory conditions, T_{convs} may differentiate into pT_{regs} (1). If and how the identity of the TCR dictates cell fate among T_{convs} remains unsettled, as does the role of TCR specificity in determining the pT_{reg} phenotype.

To address these questions, we generated a monoclonal mouse line cloned from a pT_{reg} nucleus and thus bearing the prerrearranged TCR of a pT_{reg} . We found that this TCR facilitates conversion of $CD4^+$ T cells into $CD4^+CD8\alpha\alpha^+$ intraepithelial lymphocytes ($CD4_{\text{IELs}}$) while, at the same time, allowing development of pT_{reg} in the mesenteric lymph nodes (mLNs) and small intestine LP (siLP), all in a microbiota-dependent manner. Thus, a single TCR specificity can give origin to two distinct T cell phenotypes in two distinct anatomical locations.

RESULTS

We used somatic cell nuclear transfer (SCNT) to generate a transnuclear (TN) mouse line that carries a TCR cloned from the nucleus of a pT_{reg} lymphocyte, isolated from the intestine-draining mLNs of a healthy, unmanipulated Foxp3-GFP (green fluorescent protein) reporter mouse (fig. S1A). To simplify the identification of the TCR α in the SCNT-derived mouse, we sorted $CD4^+CD8\alpha^-$ Foxp3-GFP⁺ pT_{regs} that expressed Va2, Va8.3, or Va11 (fig. S1B) by fluorescence-activated cell sorting (FACS) and used them as donors of nuclei

for SCNT. Donor mLN pT_{regs} were further identified as neuropilin-1^{-low}, as previously reported (12). The resulting pT_{reg} TN mouse line carries pre-rearranged endogenous TCR α and TCR β loci that assemble into a functional receptor, as shown for other TN mouse lines (13, 14).

Resulting chimeras from SCNT were bred to C57BL/6 wild-type (WT) mice to obtain germline transmission (fig. S1C). TN mice were crossed to Rag1-deficient mice to preclude secondary TCR rearrangements (fig. S1A). We used these TN mice (fig. S1A) to investigate the role of the TCR in determining the fate of conventional CD4⁺ T cells.

Nearly all T cells in the peripheral lymphoid organs of these TN mice were CD4⁺CD8 α ⁻ and carried a monoclonal V α 2 V β 6 TCR pair with unique CDR3 sequences (referred to hereafter as pT_{reg} TN/RKO mice; fig. S1, A and D, and Fig. 1A). pT_{reg} TN/RKO mice are viable and fertile and show no signs of overt autoimmune or wasting disease (follow-up >8 months after birth). Despite having been cloned from a Foxp3⁺ pT_{reg}, no Foxp3⁺ cells could be detected in these mice in any of the organs analyzed (Fig. 1A and fig. S1E). To rule out an intrinsic inability of the TN cells to become Foxp3⁺ T_{reg}, we purified naïve CD4⁺ T cells from spleens of pT_{reg} TN/RKO mice and induced T_{reg} differentiation in vitro. Activation with anti-CD3 and anti-CD28 in the presence or absence of transforming growth factor- β (TGF- β) and interleukin-2 (IL-2) elicited proliferation of naïve TN CD4⁺ T cells to the same extent as WT polyclonal cells. Conversion into Foxp3⁺ T_{reg} was readily observed in the presence of TGF- β and IL-2 (fig. S1F), establishing the potential of these TN cells to become T_{reg}.

The development of thymus-derived nT_{reg} can occur only when the number of precursors bearing the same TCR rearrangement is small (9, 10). To determine whether this is the case for pT_{reg} development from our TN TCR, we reconstituted sublethally irradiated TCR $\alpha\beta$ KO recipients with bone marrow preparations containing a mixture of cells from polyclonal (WT) and monoclonal pT_{reg} TN/RAGKO donors [or ovalbumin (OVA)-specific OTII RAGKO donors as a control] in different proportions and analyzed T cell development at 12 weeks after reconstitution (see Materials and Methods and Fig. 1, B to D). No T_{reg} development was observed in any organ when the monoclonal precursors were OTII RAGKO, regardless of the degree of chimerism (Fig. 1, B and C), consistent with the idea that generation of pT_{reg} requires exposure to antigen (12,15). In contrast, T_{reg} development from TN precursors was readily observed but only when the TN/WT ratio (2.5/97.5) was low. Polyclonal precursors gave rise to T_{reg} regardless of the degree of chimerism (Fig. 1, B to E). Although low precursor frequency facilitated some T_{reg} development in inguinal LNs (iLNs) and spleen, T_{reg} frequencies at these sites were much lower than those in mLN (fig. S2, A to C). No TN T_{regs} were found in the thymus, regardless of the extent of chimerism (Fig. 1E and fig. S2, A and B), indicating that this particular TCR does not support nT_{reg} development, a further argument in favor of the notion that the quality of the TCR contributes to setting T cell fate.

We next analyzed whether TN pT_{reg} could also accumulate at the intestinal mucosa, given that their TCR was originally derived from a pT_{reg} in the intestine-draining mLN. Again, the development of pT_{reg} was dependent on low precursor frequencies and was seen only in the

siLP, whereas very few pT_{regs} were found in the large intestine LP (liLP) (Fig. 1, B and C). We conclude that, for this particular TCR, low clonal frequencies are a prerequisite for generation of pT_{reg} in peripheral lymphoid organs and in the siLP. Given a previous report of niche-dependent pT_{reg} generation in the colon (5), it is likely that precursor frequency effects are a common property of pT_{reg} development in the gut.

The absence of pT_{reg} in the monoclonal pT_{reg} TN/RKO mice (Fig. 1 and fig. S1) prompted us to investigate whether CD4⁺ TN cells could adopt a phenotype other than that of a T_{reg} in vivo. In lymphoid organs (mLN, iLN, and spleen), the vast majority of the TN cells retained a naïve CD44^{low} phenotype (Fig. 2A). Because the mLN drains the intestine, we analyzed LP and intraepithelial (IE) compartments for the presence and phenotype of the TN cells. The few TN cells present in the liLP had a naïve phenotype, whereas CD4⁺ TN T cells were abundant in the siLP and IE and expressed high levels of CD44 (Fig. 2A). Although no Foxp3⁺ T_{regs} were present in the siLP and IE compartments (Fig. 2, B and C), unexpectedly, >50% of TN CD4⁺ T cells at these sites co-expressed CD8 α (but not CD8 β), and almost all of these produced interferon- γ (IFN- γ) (Fig. 2, B to D). Only mice older than 10 weeks of age displayed these traits (Fig. 2, C and D, and fig. S3, A and B). In younger mice, CD4⁺ T cells remained CD4 single-positive (SP) with a naïve phenotype and did not produce IFN- γ (Fig. 2, A and D). The trigger for differentiation and IFN- γ production is therefore likely to be a foreign antigen that accumulates over time, rather than a self-antigen present since birth. We did not detect production of IL-17A by TN cells isolated from siLP or IE (Fig. 2D), yet these cells had no intrinsic defect in making IL-17 when stimulated in vitro under T_H17-polarizing conditions (fig. S3C). Therefore, in the small intestine, the TN TCR naturally favors the efficient conversion of CD4⁺ T cells into IFN- γ -producing CD4_{IELs} rather than into Foxp3⁺ pT_{regs}. Production of IFN- γ is consistent with the cytotoxic phenotype reported for IEL (16). Despite massive expansion of these IFN- γ producers in the small intestine, pT_{reg} TN/RKO mice showed no signs of overt inflammatory or wasting disease. Tissue architecture was preserved with no signs of epithelial disruption (Fig. 2E). Levels of fecal lipocalin-2 (LCN2), a sensitive marker of intestinal inflammation (17), were low and similar to those in WT mice (Fig. 2F), confirming the absence of intestinal inflammation in our pT_{reg} TN/RKO mice.

Using the mixed bone marrow chimera approach (Fig. 1), we analyzed the niche dependency of the conversion of TN precursors into CD4_{IELs}. In sharp contrast to pT_{reg} development (Fig. 1), large numbers of CD4⁺CD8 β ⁻CD8 α ⁺ T cells emerged not only in the IE but also in the siLP (and less so in the liLP), irrespective of the extent of chimerism (Fig. 2, G to I). As expected, no CD4⁺CD8 β ⁻CD8 α ⁺ T cells emerged from the OTII bone marrow (Fig. 2G). In the majority of mice analyzed, TN CD4_{IEL} outcompeted their polyclonal counterparts (CD45.1⁺), as evidenced by the increased proportion of TN CD45.2⁺ cells among the CD4⁺CD8 β ⁻CD8 α ⁺ population (Fig. 2, G and H). Thus, in the microenvironment of the small intestine, CD4⁺ TN T cells engage a T cell program that preferentially leads to the expression of CD8 α . A single clonal CD4⁺ T cell precursor originating from a naïve unmanipulated mouse can therefore spontaneously develop along two distinct cell fates (pT_{reg} versus CD4_{IEL}), with different niche requirements.

TN cells cloned from an mLN pT_{reg} yield not only pT_{reg} but also CD4_{IEL} in the small intestine, in the absence of any exogenous stimulation or immunization (Figs. 1, B to E, and 2, A to D, H, and I). The TN TCR might therefore recognize an antigen constitutively present at these sites. We tested the ability of TN cells to undergo homeostatic expansion in lymphopenic hosts (18), which requires TCR engagement. We adoptively transferred naïve CD4⁺ T cells isolated from spleens and mLNs of pT_{reg} TN/RKO or WT mice into TCRαβKO recipients and analyzed the recovery of TN cells from different anatomical sites 6 to 8 weeks after transfer. We found TN cells to be abundant in the siLP and IE but less so in the liLP (fig. S4, A and B). Moreover, transferred cells converted into pT_{reg} in mLN and siLP, but pT_{regs} were almost entirely absent from the IE, where CD4_{IEL} was the dominant population (fig. S4, A and B). Because of the prerrearranged TCR in TN mice, TCRα is expressed prematurely during thymic selection, resulting in an increased frequency of SP cells at the expense of double-positive (DP) precursors, as seen also in TCR transgenic mice. Conversion into CD4_{IEL} from TN precursors might have been a result of aberrant thymic selection. To exclude the unlikely possibility that “spontaneous” conversion of mature CD4⁺CD8α⁻ T cells (isolated from spleen and mLN) into CD4_{IEL} is an inevitable outcome in TN mice that carry a monoclonal CD4 compartment, we transferred CD4⁺ T cells from an unrelated TN line, cloned from pancreatic tumor-infiltrating CD4⁺ T cells, into TCRαβKO hosts and analyzed their fate 6 weeks after transfer. As expected, these control TN T cells (also purified as CD4⁺CD8α⁻) migrated poorly to the intestine compared with pT_{reg} TN cells and did not convert into CD4_{IELs} (fig. S4C).

When transferred into immunocompetent WT hosts, pT_{reg} TN CD4⁺ cells proliferated in the mLN but not in the iLN, as measured by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution 5 days after adoptive transfer (Fig. 3, A and B). We also observed expansion and differentiation into CD4_{IEL} in the intestinal mucosa as well as into pT_{reg} in the mLN (Fig. 3, C and D) 6 to 8 weeks after cell transfer. Antigen presentation in the mLN and small intestine, but not in the iLN, supports proliferation and differentiation of TN cells into pT_{reg} and CD4_{IEL}, and this is not a result of aberrant expansion driven by the lymphopenic environment in T cell-deficient hosts. TN CD4⁺ T cells failed to expand when adoptively transferred into major histocompatibility complex class II (MHCII)-deficient (MHCIKO) recipients (fig. S5, A and B) and are thus MHCII-restricted. Likewise, pT_{reg} TN/RKO mice crossed to MHCIKO mice develop few, if any, CD4⁺ or CD8⁺ T cells (fig. S5C).

The late development of CD4_{IEL} observed in the pT_{reg}/RKO mice, together with the absence of any overt disease, suggested that the antigen recognized by the TN TCR was neither a dietary nor a self-antigen. We therefore investigated whether expansion and conversion of TN cells in TCRαβKO hosts depended on the resident microbiota. We treated TCRαβKO recipients of TN cells with a cocktail of antibiotics to deplete the microbiota (19). Efficiency of treatment was confirmed by the absence of bacterial colony growth under aerobic and anaerobic conditions and by a reduction of at least one log in 16S ribosomal DNA amplification from fecal samples when compared with untreated mice (see Materials and Methods) (fig. S6, A and B). We adoptively transferred naïve CD4⁺ T cells isolated from pT_{reg} TN/RKO mice into antibiotic-treated or untreated recipients (Fig. 4A). TN cells expanded and converted into pT_{reg} and CD4_{iel} in untreated mice, but TN T cells failed to do so in antibiotic-treated recipients. The few cells that did migrate to the small intestine

remained Foxp3⁺ and CD8α⁺ (Fig. 4B). Conversion into pT_{reg} and CD4_{IEL} thus depends on the microbiota, suggesting that this TN TCR recognizes a bacterial antigen. To confirm this, we adoptively transferred CFSE-labeled CD4⁺ TN cells into TCRαβKO recipients previously treated or not with antibiotics for 7 to 14 days (Fig. 4C). As expected, transferred TN cells proliferated extensively in the mLN of untreated mice but not in antibiotic-treated mice. Proliferation of TN cells in antibiotic-treated mice was rescued by immunization with sonicated bacterial extracts obtained from fecal pellets or cecum lavage of Taconic mice with excluded flora (EF) (see Materials and Methods) (Fig. 4D). Extensive TN T cell proliferation was also triggered in iLN by subcutaneous immunization of both TCRαβKO and WT recipients, whereas no proliferation was observed in iLN of nonimmunized mice (Fig. 4, E to H). Last, TN T cell proliferation was also inducible in vitro by coculture with dendritic cells (DCs) in the presence of sonicated fecal extracts from Taconic EF but not from germfree (GF) mice (Fig. 4, I and J). Bacterial antigen extracts derived from feces of mice housed in different animal facilities induced different degrees of proliferation in TN cells, but never to the same extent as the Taconic EF (fig. S7, A to C), possibly reflecting differential abundance of the targeted commensal in mice with different microbiota. No proliferation was observed in the absence of DCs or when DCs were deficient in MHCII (Fig. 4, I and J, and fig. S7A). Proliferation induced by Taconic EF bacterial extracts was abolished by treatment with trypsin and by heat inactivation (fig. S7D). The relevant microbial antigen is therefore likely to be a protein that requires internalization, processing into peptides, and presentation at the cell surface via MHCII products. When RAGKO mice purchased from Taconic Biosciences or Jackson Laboratory (and kept in microisolators) were adoptively transferred with naïve CD4⁺ T cells isolated from pT_{reg}/RKO mice, TN T cells expanded and converted into CD4_{IEL} in Taconic recipients but not in Jackson Laboratory recipients. Expansion and conversion were readily observed in Jackson Laboratory mice cohoused with Taconic mice (fig. S8A). We conclude that the antigen recognized by the pT_{reg} TN TCR is a protein derived from the intestinal microbiota prevalent in Taconic mice.

We used RNA sequencing (RNA-seq) to characterize the pT_{reg} and CD4_{IEL} that had differentiated from the same clonal TN precursor and recovered from the intestinal mucosa. We adoptively transferred CD4⁺CD8α⁺ Foxp3-GFP⁺ isolated from pT_{reg} TN/ RKO/Foxp3-GFP mice into TCRαβKO hosts. After 6 weeks, we sorted CD4⁺CD8α⁺ Foxp3⁺ pT_{regs} and “unconverted” CD4⁺CD8α⁺ Foxp3-GFP⁺ cells from the siLP and IE compartments for whole-transcriptome analysis by RNA-seq (Fig. 5A). Comparison of gene expression profiles from our sorted populations with signatures of pT_{reg} and CD4⁺CD8α⁺ cells in the published record (Fig. 5, B to F) (12, 16, 20, 21) confirmed the similarity between differentiated TN cells and polyclonal pT_{reg} and CD4_{IEL}. As expected, CD4⁺CD8α⁺ cells down-modulated expression of the transcription factor ThPOK in comparison to CD4⁺CD8α⁺ Foxp3-GFP⁺ cells and up-regulated the surface marker CD103 (Fig. 6, A to E), corroborating results obtained for WT IELs (16, 22). In our system, converted pT_{regs} did not up-regulate retinoid-related orphan receptor γ t (RORγt) expression (Fig. 6F), the transcription factor of T_H17 and expressed by a fraction of intestinal pT_{regs} (21,23,24). We conclude that adoptive transfer of TN T cells leads to differentiation and expansion of bona fide pT_{reg} and CD4_{IEL} T cells from the same clonal precursors.

The preferential conversion into pT_{reg} or IFN- γ -producing CD4_{IEL} in vivo and the absence of T_H17 conversion were not due to a lack of SFB colonization of our recipient mice, as a quantitative polymerase chain reaction (qPCR) assay specific for SFB readily detected them in the TCR $\alpha\beta$ KO mice housed in our facility, but less so or not at all in EF mice purchased from Taconic (fig. S9A).

The ability of TN T cells to differentiate into both the pT_{reg} and the CD4_{IEL} phenotypes raised the possibility that CD4_{IEL} development may require transient expression of Foxp3⁺. We therefore crossed the pT_{reg} TN/RKO mice to RAGKO/scurfy (Foxp3-deficient) mice and used the resulting offspring as donors of CD4⁺ T cells that were adoptively transferred into TCR $\alpha\beta$ KO hosts. Although TN cell expansion was unaffected in these hosts (Fig. 7, A to C), CD4⁺ T cells no longer converted into pT_{reg}. However, differentiation of CD4_{IEL} occurred to the same extent as in Foxp3-sufficient mice (Fig. 7D). Development of CD4_{IEL} is thus Foxp3-independent.

The massive expansion of IFN- γ -producing TN T cells in the siLP and IE compartment of pT_{reg} TN/RKO mice or in TCR $\alpha\beta$ KO mice transferred with TN T cells causes neither wasting disease nor disruption of epithelial integrity (Figs. 2, D and E, and 7, E and G). This is in contrast to the colitis model induced by T_{reg}-depleted polyclonal T cells transferred into immunodeficient hosts (25). Thus, it was possible that the pT_{reg} generated in TCR $\alpha\beta$ KO mice transferred with TN T cells might have prevented excessive inflammation or disease caused by the CD4_{IEL}. Despite increased production of IFN- γ in the IE compartment, mice that received Foxp3-deficient TN cells showed no signs of disease 8 to 12 weeks after transfer (Fig. 7, E to H). Histopathological analysis of the intestine showed extensive lymphocyte infiltration of the LP in both groups to the same extent but no signs of epithelial disruption (Fig. 7G). Mice that received either Foxp3-deficient or Foxp3-sufficient TN CD4⁺ T cells also had overall similar levels of LCN2 in the feces (Fig. 7H). Therefore, despite their activated status and robust production of IFN- γ , CD4_{IELs} derived from the pT_{reg} TN/RKO mice are not overtly pathogenic, even in the absence of Foxp3⁺ T_{reg}, presumably related to the absence of recognition of a self-antigen in the gut and to their potential regulatory properties.

DISCUSSION

We show that T cell precursors of TN mice cloned from and bearing the TCR rearrangements of a mLN peripheral T_{reg} can adopt two distinct fates (pT_{reg} and CD4_{IEL}) upon encounter with microbial antigen in the intestinal environment. We believe that our experiments have identified a compelling example of a naturally occurring pT_{reg} TCR that promotes spontaneous CD4_{IEL} conversion in response to a component of the microbiota.

The unimpaired differentiation of CD4_{IEL} from Foxp3-deficient TN precursors suggests that the pathways that lead to CD4_{IEL} differentiation in this case are independent of pT_{reg} conversion. However, pT_{reg} can acquire the CD4_{IEL} phenotype in a microbiota-dependent manner (26), indicating that there is more than one pathway for CD4_{IEL} development. Although the TN pT_{reg} and CD4_{IEL} presumably recognize the same bacterial species, these populations accumulate in distinct anatomical locations—mLN and intestinal epithelium,

respectively. Whether this dichotomy is due to the independent priming of pT_{reg} and CD4_{IEL} in situ at each of these locations or is due to migration to distinct sites after priming in the mLN is unclear. One possibility is that priming in the mLN may give rise simultaneously both to pT_{reg} and to undifferentiated but activated T cells that will further acquire the CD4_{IEL} phenotype upon a second encounter with the bacterial antigen in the intestine. Alternatively, antigen presentation by different antigen-presenting cell (APC) subsets in distinct microenvironments may promote independent differentiation of the two cell types. Previous work using a TCR transgenic model showed that the extracellular commensal SFB drive the intestinal T cells to adopt the T_H17 program, whereas infection with the intracellular pathogen *Listeria monocytogenes* expressing the SFB epitope directs the same SFB-specific cells toward T_H1 (6). Thus, in this model, monomorphic differentiation of T cells is directed by the changes in the environment in which T cell priming occurs. The constantly stimulated epithelial environment could be both inhospitable for pT_{reg} and favorable for CD4_{IEL} differentiation, whereas the mLN favors pT_{reg} differentiation and allows the accumulation of pT_{regs} at that site. Whether and how the relative abundance of ligands (5, 6), TCR-independent factors such as microbial metabolites (27,28), and cell-intrinsic mechanisms, as well as the different types of APCs (29–31), cooperate to define T cell fate in the gut environment remain to be determined.

Although pT_{reg} development is dependent on low precursor frequencies, CD4_{IELs} carrying the identical TCR are not limited in any way by clonal size. Intrinsic properties of the T cell subset concerned can thus directly affect their expansion. Development of nT_{reg} requires small niche sizes (9,10), possibly due to intraclonal competition for a limited amount of ligands. Development of *Clostridium*-specific pT_{reg} also appears to be dependent on small clonal sizes (5). In contrast, when clonal sizes are larger, CD4⁺ T cells no longer convert into T_{regs} and remain as effector or naïve T cells. Therefore, the small niche requirement may be a common property of T_{reg} populations. Although relative abundance for ligands is suspected to play a major role in Treg development, the precise mechanisms at play remain to be unveiled.

Dietary antigens may stimulate the bulk of pT_{regs} present in the small intestine (24), whereas induction of colon pT_{regs} depends almost exclusively on the microbiota (5,7). However, constant stimulation by the microbiota in the small intestine can also allow for the generation of microbiota-specific pT_{regs} at this site. On the basis of experiments using GF and antigen-free mice, it is estimated that a sizable proportion of pT_{reg} in the siLP is induced by the microbiota and expresses ROR γ t (21,23,24). We report a different lineage of pT_{regs} that does not up-regulate ROR γ t expression but does recognize bacterial antigens present in the intestinal microbiota. The T cell precursors that give rise to these pT_{regs} do not allow for differentiation of T_H17 in vivo, despite SFB colonization and the ability to produce IL-17 under polarizing conditions in vitro. Instead, these precursors showed a strong tendency to develop into CD4_{IELs}. It remains to be determined whether other CD4⁺ T cell precursors that can simultaneously differentiate into pT_{reg} and CD4_{IEL}, or pT_{reg} that can also differentiate into CD4_{IEL} (26), express ROR γ t and/or could interconvert into T_H17-like cells.

Premature expression of TCR α in transgenic mice may contribute to the development of unconventional IELs (32, 33). However, unlike the TN cells in our mice, these cells predominantly consist of unconventional IELs that expressed only CD8 $\alpha\alpha$. Moreover, we show microbiota-dependent CD4_{IEL} differentiation from mature CD4⁺ T cells isolated from secondary lymphoid organs (spleen and mLN), thus discounting a potential role for direct development of CD4_{IEL} from DN (double-negative) or DP thymic precursors in our model. Accordingly, CD4_{IELs} are absent in GF and antibiotic-treated WT mice (16). The microbiota must play a specific role in the generation/ expansion of this T cell subset. CD4⁺ T cells isolated from an unrelated TN line cloned from a pancreatic tumor-infiltrating lymphocyte fail to convert to CD4_{IELs}, demonstrating that CD4_{IEL} differentiation is directed by TCR specificity.

Mice harboring microbiota-specific TN cells remain healthy despite massive accumulation of IFN- γ -expressing CD4_{IEL} and irrespective of the presence of T_{regs}. This suggests that additional factors may suppress pathogenic inflammation in the intestine, for example, by inducing a regulatory response related to the CD4_{IEL} phenotype. IFN- γ -producing IELs can display regulatory functions rather than proinflammatory activity (34). Using a model antigen (OTII and OVA) in which antigen-specific T_{reg} development was impaired by the scurfy mutation, antibody-mediated depletion of CD4_{iel} led to intestinal inflammation and diarrhea (26), suggesting a regulatory role for CD4_{IEL}. Although the mechanisms by which CD4_{IEL} and other IELs regulate the immune response at intestinal surfaces remain to be fully elucidated, our studies showcase the importance of location in determining T cell fate and functions.

MATERIALS AND METHODS

Study design

The present study was designed to investigate the extent to which TCR identity dictates pT_{reg} fate in vivo. We conducted adoptive transfer experiments and in vitro proliferation assays and used FACS analyses and RNA-seq as described in the figure legends. As indicated in the figures and figure legends, the number of mice per experiments varied from 2 to 14 per experimental group. All experiments were repeated at least once. Mice were arbitrarily assigned to each experimental group, and both males and females were used for the experiments. All data obtained are presented, including outliers. For the in vitro proliferation assays, experiments were performed in duplicate and repeated two to three times.

Mice

TN mice for pT_{reg} TCR (pT_{reg} TN) were generated by SCNT in a mixed C57BL/6 and 129 background as described (fig. S1) (14). All mice were housed at the Whitehead Institute for Biomedical Research (WIBR) under specific pathogen-free conditions, and all procedures were approved and performed according to the Institutional Animal Care and Use Committee of the Massachusetts Institute of Technology (MIT). C57BL/6 CD45.1, *Rag1*^{-/-}, MHCII^{-/-}, *Tcra*^{-/-}, *Tcrb*^{-/-}, and scurfy mice were purchased from Taconic Biosciences and Jackson Laboratory and maintained at our facility. *Tcra*^{-/-} and *Tcrb*^{-/-} were intercrossed to

originate *Tcra*^{-/-}*b*^{-/-} (TCRabKO). Foxp3^{gfp} mice were generated as described (35) and provided by V. Kuchroo and M. Oukka. Scurfy mice and pT_{reg} TN mice were crossed to *Rag1*^{-/-}. pT_{reg} TN/RAG1KO and scurfy/RAG1KO were interbred to generate pT_{reg} TN/RAG1KO/scurfy. pT_{reg} TN/RAG1KO mice were crossed to Foxp3^{gfp} and MHCII^{-/-} to generate pT_{reg} TN/RAG1KO/Foxp3^{gfp} mice and pT_{reg} TN/RAG1KO/MHGI^{-/-}. pT_{reg} TN mice were backcrossed at least for six generations into C57BL/6 RAG1KO background. PancCD4 TN mouse was cloned from pancreatic tumor-infiltrating CD4⁺ T cells and used as a control in fig. S4. They were generated by SCNT in a mixed C57BL/6 and 129 background as described (14) and backcrossed at least for six generations into C57BL/6 RAG2KO background.

Antibodies, reagents, and FACS analysis

Fluorescent dye-conjugated antibodies were purchased from BD Biosciences (anti-Vβ6, RR4-7; anti-CD69, H1.2F3; anti-CD3, 145-2C11; anti-Vβ5, MR9-4; anti-IL-17, TC11-18H10; and anti-ThPOK, T43-94), eBioscience (anti-CD45.1, A20; anti-Foxp3, FJK-16s; anti-Ki-67, Sol A15; anti-CD4, RM4-5; Vα2, B20.1; anti-CD19, eBio1D3; anti-CD11c, N418; anti-IFN-γ, XMG1.2; and anti-Vα11, RR 8-1), or BioLegend (anti-CD45.2, 104; anti-CD8α, 53-6.7; anti-CD8β, YTS 156.7.7; anti-CD44, IM7; anti-I-A/I-E, M5/114.15.2; and anti-Vα8.3, KT50). Cell dyes CFSE and CellTrace Violet for proliferation assays were purchased from Sigma-Aldrich and Life Technologies, respectively. Cell viability dye 7-AAD (Via-Probe) was purchased from BD Biosciences. Flow cytometry data were acquired on an LSRFortessa (Becton Dickinson) instrument and analyzed with the FlowJo software package (Tri-Star). Staining with cell proliferation dyes was performed according to the manufacturer's instructions. Staining for surface markers was performed at 4°C for 25 to 30 min. Intracellular staining of Foxp3 and Ki-67 staining were performed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience), and intra-cellular cytokine staining was performed with Cytofix/Cytoperm Kit (BD Biosciences), according to the manufacturer's instructions. For the cytokine staining, cells were incubated with phorbol 12-myristate 13-acetate (100 ng/ml; Sigma), ionomycin (200 ng/ml; Sigma), and 2 μM monensin (Sigma) for 4 hours at 37°C. Monensin was added for the last 2 hours of incubation.

Antibiotic treatment

Vancomycin (0.5 g/liter), ampicillin (1.0 g/liter), neomycin (1.0 g/liter) (Amresco), and metronidazole (1.0 g/liter) (Sigma) cocktail was given ad libitum in the drinking water for 7 to 14 days or for 6 to 8 weeks as indicated in the figure legends.

Quantification of fecal LCN2

LCN2 was measured in fecal homogenates as described (17) using the Mouse Lipocalin-2/NGAL DuoSet ELISA (enzyme-linked immunosorbent assay) kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Isolation of mononuclear cells from intestine

Small and large intestines were harvested, and mononuclear cells were isolated from LP as previously described (36). IELs were recovered from the supernatants of 1 mM dithiothreitol and 30 mM EDTA washes.

16S and SFB qPCR of fecal DNA

DNA was extracted from fecal pellets using the Zymo Research Fecal DNA Isolation Kit, according to the manufacturers' protocol. qPCR was performed using SYBR Green Master Mix (Bio-Rad). Primer sequences used for 16S were 5'-gtgStgcaYggYgtcgtca-3' and 5'-acgtcRtccMcaccttctc-3' (37), and those used for SFB were SFB225F 5'-aggaggagtctgcggcacattagc-3' and SFB558R 5'-cgcatcctttacgccagc- tattc-3' (38).

Stool test for bacterial growth

Single fecal pellets were resuspended into 500 µl of phosphate-buffered saline (PBS) and spun briefly to remove fibers. Supernatant was diluted 1:10 and 1:100 and then streaked on brain-heart infusion plates and incubated under aerobic and anaerobic conditions. After 48 to 72 hours, plates were examined for colony growth.

Bacterial extracts for proliferation assays

Feces (2 to 10 pellets) from healthy WT or *Rag1*^{-/-} mice housed at different animal facilities (WIBR, Jackson Laboratory, and Taconic Biosciences) were homogenized in PBS and shaken at 2000 rpm for 20 min at 4°C. Large debris was removed by centrifugation (700g, 1 min). Supernatants were centrifuged at 13,500g for 10 min at 4°C, and bacterial pellet was resuspended in 1 ml of PBS containing protease inhibitors (cOmplete Mini, Roche; 1 tablet per 10 ml of PBS following the manufacturer's instructions) and deoxyribonuclease I (Roche). Bacteria were lysed by three rounds of sonication (Branson Sonifier 450) for 1 min, 60 pulses, followed by 5 min of cooling on ice between each round (output control, 4; duty cycle, 50%). Cellular debris was removed by centrifugation at 14,000g for 30 min at 4°C. Protein concentration was measured by bicinchoninic acid assay (BCA) and used for in vitro and in vivo proliferation assays as indicated. In some experiments (fig. S7), bacterial extracts were subjected to trypsin or heat inactivation treatment before being used in in vitro proliferation assays. For trypsin treatment, bacterial extract (50 µg/ml; prepared in the absence of protease inhibitor) was incubated with trypsin (2.5 µg/ml) for 4 hours at 37°C. Trypsin was inactivated by adding protease inhibitor as described above. For heat inactivation, bacterial extracts were boiled for 20 min and then centrifuged at 14,000g for 10 min at 4°C. Supernatant was collected, and pellet was resuspended in PBS containing protease inhibitors and sonicated in a water bath sonicator for 10 min. Supernatants and resolubilized pellets were used for the in vitro proliferation assays at a maximum of 20 µl per well.

In vitro proliferation assay

DCs were purified with the Pan Dendritic Cell Isolation Kit (Miltenyi Biotec) from spleens of WT mice injected with 1×10^6 B16-FLT3L cells. T cells were purified with the Naïve CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) from the spleens and mLN of pT_{reg} TN/RKO

mice and labeled with 2.5 μ M CFSE (Sigma). Purity was always greater than 98% for both DCs and T cells. DCs (1×10^5) were incubated with sonicated bacterial extracts (25, 50, or 100 μ g/ml) for 2 hours before the addition of 1×10^5 TN CD4⁺ T cells. Cell division was assessed by CFSE dilution and analyzed by flow cytometry after 3.5 days of coculture.

In vivo proliferation assay

TCR $\alpha\beta$ KO or WT mice were treated with a cocktail of antibiotics (ampicillin, 1 g/liter; metronidazole, 1 g/liter; neomycin, 1 g/liter; and vancomycin, 0.5 g/liter) for 1 to 2 weeks and adoptively transferred with 5×10^5 CFSE- or CellTrace Violet-labeled T cells. One day later, mice were immunized with 50 μ g of sonicated bacterial extract adsorbed in alum (Imject, Thermo Fisher Scientific). Bacterial extracts used for immunization were obtained from fecal pellets of WT mice (EF health status) purchased from Taconic. Cell division and activation markers were measured 5 days after adoptive transfer by flow cytometry.

Isolation of CD4⁺ T cells

Naïve CD4⁺ T cells were purified from the spleen and mLN using the Naïve CD4 Isolation Kit according to the manufacturer's instructions (Miltenyi, Germany). Purity was always greater than 98%. In some experiments as indicated in the figure legends, naïve CD4⁺ T cells were FACS-sorted on a FACS Aria instrument (BD Biosciences) by the Flow Cytometry Core Facility at the WIBR. Purity was always greater than 98%. Purified CD4⁺ T cells were used in adoptive transfer (by intravenous injection) experiments or in vitro proliferation assays as indicated in the figure legends.

Generation of mixed bone marrow chimeras

TCR $\alpha\beta$ KO host mice were sublethally irradiated (6 Gy) 4 hours before bone marrow reconstitution. Bone marrow cells were harvested from CD45.2 pT_{reg} TN/RKO or CD45.2 OTII Tg/RKO and CD45.1 WT mice and depleted of T cells using CD90.2 MACS beads (Miltenyi) according to the manufacturer's instructions. A total of 5×10^6 cells (composed of 2.5 to 10% of CD45.2 cells and 90 to 97.5% of CD45.1 cells) were intravenously injected into host mice. After 12 weeks of reconstitution, T cell development was analyzed in different organs of recipient mice by flow cytometry.

Histopathological analysis

Small and large intestines were fixed in 10% buffered formalin and subjected to hematoxylin and eosin (H&E) staining. Slides were scored blindly by a pathologist from the Division of Comparative Medicine of MIT.

RNA-seq library preparation and sequencing

Sorted cells were lysed in a guanidine thiocyanate buffer (Qiagen) supplemented with 1% β -mercaptoethanol. RNA isolated by solid-phase reversible immobilization bead cleanup was reversely transcribed into complementary DNA and preamplified as described (39). Nextera XT was used to prepare a pooled library of fragmented and uniquely indexed samples. Sequencing was performed on an Illumina HiSeq 2000. For RNA-seq analysis, 40-base pair

single-end reads were aligned to the mouse reference genome (mm10) using TopHat. Gene read count was performed using HTseq-count with RefSeq mm10 annotation.

Statistical analysis

Mean and SD values were calculated with GraphPad Prism (GraphPad Software). Unpaired Student's *t* test was used to compare two variables, as indicated in each figure legend. *P* values of <0.05 were considered significant and are indicated in the figure or figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES AND NOTES

1. Bilate AM, Lafaille JJ, Induced CD4⁺Foxp3⁺ regulatory T cells in immune tolerance. *Annu. Rev. Immunol.* 30, 733–758 (2012). [PubMed: 22224762]
2. Shale M, Schiering C, Powrie F, CD4⁺ T-cell subsets in intestinal inflammation. *Immunol. Rev.* 252, 164–182 (2013). [PubMed: 23405904]
3. Zhou L, Chong MMW, Littman DR, Plasticity of CD4⁺ T cell lineage differentiation. *Immunity* 30, 646–655 (2009). [PubMed: 19464987]
4. Brucklacher-Waldert V, Carr EJ, Linterman MA, Veldhoen M, Cellular plasticity of CD4⁺ T cells in the intestine. *Front. Immunol.* 5, 488 (2014). [PubMed: 25339956]
5. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio C-W, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh C-S, Peripheral education of the immune system by colonic commensal microbiota. *Nature* 478, 250–254 (2011). [PubMed: 21937990]
6. Yang Y, Torchinsky MB, Gobert M, Xiong H, Xu M, Linehan JL, Alonzo F, Ng C, Chen A, Lin X, Szczesnak A, Liao J-J, Torres VJ, Jenkins MK, Lafaille JJ, Littman DR, Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. *Nature* 510, 152–156 (2014). [PubMed: 24739972]
7. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K, Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500, 232–236 (2013). [PubMed: 23842501]
8. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR, Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139, 485–498 (2009). [PubMed: 19836068]

9. Bautista JL, Lio C-W, Lathrop SK, Forbush K, Liang Y, Luo J, Rudensky AY, Hsieh C-S, Intracloal competition limits the fate determination of regulatory T cells in the thymus. *Nat. Immunol.* 10, 610–617 (2009). [PubMed: 19430476]
10. Leung MWL, Shen S, Lafaille JJ, TCR-dependent differentiation of thymic Foxp3+ cells is limited to small clonal sizes. *J. Exp. Med.* 206, 2121–2130 (2009). [PubMed: 19737865]
11. Lee H-M, Bautista JL, Scott-Browne J, Mohan JF, Hsieh C-S, A broad range of self-reactivity drives thymic regulatory T cell selection to limit responses to self. *Immunity* 37, 475–486 (2012). [PubMed: 22921379]
12. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, Xiong H, Dolpady J, Frey AB, Ruocco MG, Yang Y, Floess S, Huehn J, Oh S, Li MO, Nier RE, Rudensky AY, Dustin ML, Littman DR, Lafaille JJ, Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J. Exp. Med.* 209, 1723–1742 (2012). [PubMed: 22966001]
13. Hochedlinger K, Jaenisch R, Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* 415, 1035–1038 (2002). [PubMed: 11875572]
14. Kirak O, Frickel E-M, Grotenbreg GM, Suh H, Jaenisch R, Ploegh HL, Transnuclear mice with predefined T cell receptor specificities against *Toxoplasma gondii* obtained via SCNT. *Science* 328, 243–248 (2010). [PubMed: 20378817]
15. Curotto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ, Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 29, 114–126 (2008). [PubMed: 18617425]
16. Mucida D, Husain MM, Muroi S, van Wijk F, Shinnakasu R, Naoe Y, Reis BS, Huang Y, Lambolez F, Docherty M, Attinger A, Shui J-W, Kim G, Lena CJ, Sakaguchi S, Miyamoto C, Wang P, Atarashi K, Park Y, Nakayama T, Honda K, Ellmeier W, Kronenberg M, Taniuchi I, Cheroutre H, Transcriptional reprogramming of mature CD4+ helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. *Nat. Immunol.* 14, 281–289 (2013). [PubMed: 23334788]
17. Chassaing B, Srinivasan G, Delgado MA, Young AN, Gewirtz AT, Vijay-Kumar M, Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. *PLOS One* 7, e44328 (2012). [PubMed: 22957064]
18. Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ, CD25– T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion. *J. Immunol.* 173, 7259–7268 (2004). [PubMed: 15585848]
19. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R, Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229–241 (2004). [PubMed: 15260992]
20. Haribhai D, Williams JB, Jia S, Nickerson D, Schmitt EG, Edwards B, Ziegelbauer J, Yassai M, Li S-H, Relland LM, Wise PM, Chen A, Zheng Y-Q, Simpson PM, Gorski J, Salzman NH, Hessner MJ, Chatila TA, Williams CB, A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity* 35, 109–122 (2011). [PubMed: 21723159]
21. Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, Burzyn D, Ortiz-Lopez A, Lobera M, Yang J, Ghosh S, Earl A, Snapper SB, Jupp R, Kasper D, Mathis D, Benoist C, Individual intestinal symbionts induce a distinct population of ROR γ + regulatory T cells. *Science* 349, 993–997 (2015). [PubMed: 26272906]
22. Reis BS, Rogoz A, Costa-Pinto FA, Taniuchi I, Mucida D, Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4+ T cell immunity. *Nat. Immunol.* 14, 271–280 (2013). [PubMed: 23334789]
23. Ohnmacht C, Park J-H, Cording S, Wing JB, Atarashi K, Obata Y, Gaboriau-Routhiau V, Marques R, Dulauroy S, Fedoseeva M, Busslinger M, Cerf-Bensussan N, Boneca IG, Voehringer D, Hase K, Honda K, Sakaguchi S, Eberl G, The microbiota regulates type 2 immunity through ROR γ T cells. *Science* 349, 989–993 (2015). [PubMed: 26160380]
24. Kim KS, Hong S-W, Han D, Yi J, Jung J, Yang B-G, Lee JY, Lee M, Surh CD, Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science* 351, 858–863 (2016). [PubMed: 26822607]

25. Mottet C, Uhlig HH, Powrie F, Cutting edge: Cure of colitis by CD4+CD25+ regulatory T cells. *J. Immunol.* 170, 3939–3943 (2003). [PubMed: 12682220]
26. Sujino T, London M, Hoytema van Konijnenburg DP, Rendon T, Buch T, Silva HM, Lafaille JJ, Reis BS, Mucida D, Tissue adaptation of regulatory and intraepithelial CD4+ T cells controls gut inflammation. *Science* 352, 1581–1586 (2016). [PubMed: 27256884]
27. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS, The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* 341, 569–573 (2013). [PubMed: 23828891]
28. Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, Suda W, Imaoka A, Setoyama H, Nagamori T, Ishikawa E, Shima T, Hara T, Kado S, Jinnohara T, Ohno H, Kondo T, Toyooka K, Watanabe E, Yokoyama S.-i., Tokoro S, Mori H, Noguchi Y, Morita H, Ivanov II, Sugiyama T, Nuñez G, Camp JG, Hattori M, Umesaki Y, Honda K, Th17 cell induction by adhesion of microbes to intestinal epithelial cells. *Cell* 163, 367–380 (2015). [PubMed: 26411289]
29. Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Förster R, Agace WW, Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J. Exp. Med.* 202, 1063–1073 (2005). [PubMed: 16216890]
30. Diehl GE, Longman RS, Zhang J-X, Breart B, Galan C, Cuesta A, Schwab SR, Littman DR, Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX3CR1hi cells. *Nature* 494, 116–120 (2013). [PubMed: 23334413]
31. Esterhazy D, Loschko J, London M, Jove V, Oliveira TY, Mucida D, Classical dendritic cells are required for dietary antigen-mediated induction of peripheral Treg cells and tolerance. *Nat. Immunol.* 17, 545–555 (2016). [PubMed: 27019226]
32. Baldwin TA, Sandau MM, Jameson SC, Hogquist KA, The timing of TCR α expression critically influences T cell development and selection. *J. Exp. Med.* 202, 111–121 (2005). [PubMed: 15998791]
33. McDonald BD, Bunker JJ, Ishizuka IE, Jabri B, Bendelac A, Elevated T cell receptor signaling identifies a thymic precursor to the TCR $\alpha\beta$ +CD4–CD8 β – intraepithelial lymphocyte lineage. *Immunity* 41, 219–229 (2014). [PubMed: 25131532]
34. Cheroutre H, Lambolez F, Mucida D, The light and dark sides of intestinal intraepithelial lymphocytes. *Nat. Rev. Immunol.* 11, 445–456 (2011). [PubMed: 21681197]
35. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK, Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235–238 (2006). [PubMed: 16648838]
36. Valdez Y, Grassl GA, Guttman JA, Coburn B, Gros P, Vallance BA, Finlay BB, Nramp1 drives an accelerated inflammatory response during Salmonella-induced colitis in mice. *Cell. Microbiol.* 11, 351–362 (2009). [PubMed: 19016783]
37. Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, Takashiba S, Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunol. Med. Microbiol.* 39, 81–86 (2003). [PubMed: 14557000]
38. Ge Z, Feng Y, Woods SE, Fox JG, Spatial and temporal colonization dynamics of segmented filamentous bacteria is influenced by gender, age and experimental infection with *Helicobacter hepaticus* in Swiss Webster mice. *Microbes Infect.* 17, 16–22 (2015). [PubMed: 25448636]
39. Trombetta JJ, Gennert D, Lu D, Satija R, Shalek AK, Regev A, Preparation of single-cell RNA-Seq libraries for next generation sequencing. *Curr. Protoc. Mol. Biol.* 107, 4.22.1–4.22.17 (2014). [PubMed: 24984854]

Fig. 1. Low clonal frequency of pT_{reg} precursors allows development of T_{regs}.

(A) Flow cytometry analysis of the indicated organs from WT and pT_{reg} TN/RKO. Dot plots on the left show CD3⁺ cells. Middle and right dot plots show CD3⁺CD4⁺CD8 α ⁻ cells. (B) Mixed bone marrow chimeras. Sublethally irradiated TCR α β KO mice were reconstituted with a total of 5×10^6 T cell-depleted bone marrow cells from congenically marked WT (CD45.1) along with pT_{reg} TN/RKO or OTII Tg/RKO (CD45.2) mice at different TN/WT ratios (2.5:97.5 or 10:90). Cells from the indicated locations were harvested 12 weeks after reconstitution and analyzed by flow cytometry. For each indicated location, dot plots on the left show the proportion of CD4⁺CD8 α ⁻ T cells among the monoclonal (CD45.2) and polyclonal (CD45.1) compartments of two chimeric mice, and right plots show the

frequency of T_{reg} in the monoclonal compartment (top plots, reconstituted with p T_{reg} TN/RKO bone marrow; bottom plots, with OTII Tg/RKO). Dot plots are representative of four to six mice per group and show mice reconstituted with 2.5:97.5 ratio. **(C)** Quantification of the frequency of $Foxp3^+ T_{regs}$ among $CD4^+CD8\alpha^-V\alpha 2^+V\beta 6^+$ (TN) or $V\beta 5^+$ (OTII) cells in the indicated locations of the mixed bone marrow chimeric mice. Graph shows the mean and SD of all mice analyzed in one experiment. **(D)** Mixed bone marrow chimeras as in (B). Dot plots show two chimeric mice with different degrees of chimerism of TN/WT in the mLN. **(E)** Graph shows the frequency of $Foxp3^+ T_{regs}$ among TN lymphocytes recovered from the indicated locations from all mice analyzed in three independent experiments (different TN/WT ratios). Each symbol represents a mouse, and horizontal bars represent the means.

Fig. 2. pTreg TCR allows for the development of CD4iel.

(A) Flow cytometry analysis of CD44 and CD8α expression on CD45⁺CD4⁺CD8β⁻Vα2⁺Vβ6⁺ TN cells in the indicated lymphoid organs and intestinal compartments of 8-week-old (8 wko) and 24-week-old (24 wko) pTreg TN/RKO mice. (B) Dot plots show the frequencies of CD8α⁺ and Foxp3⁺ T cells among CD45⁺CD4⁺CD8β⁻Vα2⁺Vβ6⁺ cells in the siLP and siLE compartment in C57BL/6 WT and pTreg TN/RKO mice. (C and D) Dot plots gated as in (B) showing the comparison between young (8-week-old) and old (24-week-old) pTreg TN/RKO mice. (E) Photomicrographs of the small intestine and colon H&E staining

of WT C57BL/6 (24-week-old) and pT_{reg} TN/RKO (8- and 24-week-old) mice. Original magnification, $\times 200$; scale bar, 200 μm . (A to E) Representative of at least five mice per age group. (F) Fecal LCN2 levels measured by ELISA. Feces were collected from pT_{reg} TN/RKO mice 4 to 6 months old and age-matched C57BL/6 WT mice. Colitis denotes positive controls for ELISA (feces from colitic mice). (G) Mixed bone marrow chimeras. Sublethally irradiated TCR $\alpha\beta$ KO mice were reconstituted with a total of 5×10^6 T cell-depleted bone marrow cells from congenically marked WT mice (CD45.1) along with pT_{reg} TN/RKO or OTII Tg/RKO (CD45.2) mice at the proportions indicated on the graph. Dot plots show the frequency of CD4⁺CD8 α ⁺ TN cells in the LP of two chimeric mice reconstituted with the indicated bone marrow cells. Graph shows the mean and SD of all mice analyzed in one experiment. (H) Mixed bone marrow chimeras as in (G). Dot plots show two chimeric mice with different degrees of chimerism of TN/WT among the siIEL. (I) Graph shows the frequency of CD4⁺CD8 α ⁺ in the IE and LP in all mice analyzed in three independent experiments (different TN/WT ratios). Each symbol represents a mouse, and the horizontal bars represent the means.

Fig. 3. Expansion and differentiation of pT_{reg} TN T cells in WT polyclonal hosts.

(A) Naïve CD4⁺ T cells from pT_{reg} TN/RKO were labeled with CFSE, and 5×10^6 cells were transferred into WT CD45.1 mice. Five days later, the indicated LNs were harvested, and CFSE dilution was measured by flow cytometry. Dot plots are representative of three mice and show CD45.1⁻CD45.2⁺V α 2⁺V β 6⁺ cells. (B) Graph shows all mice analyzed. (C) Naïve CD4⁺ T cells from pT_{reg} TN/RKO/Foxp3^{GFP} were bead-purified, and 5×10^6 cells were transferred into congenically marked WT hosts (CD45.1). Conversion of transferred cells was analyzed 8 weeks after cell transfer at the indicated locations. Dot plots are representative of four to five mice. (D) Graph shows the mean and SD of all the mice analyzed. *P* values were calculated with Student's *t* test. *P* values of <0.05 were considered statistically significant.

Fig. 4. pT_{reg} and CD4_{IEL} development and expansion are dependent on the microbiota.

(A) Experimental design. TCR $\alpha\beta$ KO mice were treated or not with antibiotics (Abx) for 6 to 8 weeks and adoptively transferred with 5×10^5 naïve CD4 $^+$ T cells isolated from pT_{reg} TN/RKO mice. (B) Dot plots show the frequency of pT_{reg} in mLN and CD4_{IEL} TN cells (gated on CD45 $^+$ CD8 β $^-$ CD4 $^+$ V α 2 $^+$ V β 6 $^+$) 6 to 8 weeks after adoptive transfer. Graphs show all mice analyzed in four independent experiments. *P* values were determined with unpaired Student's *t* test. (C) Experimental design. TCR $\alpha\beta$ KO mice were treated or not with antibiotics for 7 to 14 days and adoptively transferred with 5×10^5 CFSE-labeled naïve

CD4⁺ T cells. One day later, mice were immunized intraperitoneally (imm i.p.) with 50 µg of bacterial antigen extract adsorbed in alum. Bacterial extract was obtained from feces and cecum of Taconic mice (EF health status). **(D)** Dot plots show CFSE dilution in CD4⁺Vα2⁺Vβ6⁺ T cells in the LNs of the indicated mice. Graph shows all mice that were analyzed in two independent experiments. **P* = 0.0015, iLN, treated with antibiotics versus untreated; **P* = 0.0008, mLN, treated with antibiotics versus untreated. **(B and D)** Each symbol represents a mouse, and the horizontal bars represent the means. **(E)** Experimental design. **(F)** Frequency of activated (CD44^{high}) and divided (CFSE^{low}) TN cells in the iLN of TCRαβKO mice transferred with 5×10^5 CFSE-labeled naïve CD4⁺ T cells and immunized subcutaneously (imm s.c.) or not with 50 µg of bacterial antigen extract adsorbed in alum as in **(C)**. Graph shows the frequency of divided cells in all mice analyzed in two experiments. **(G)** Experimental design. WT CD45.1 mice were adoptively transferred with CellTrace Violet-labeled naïve CD4⁺ T cells isolated from pT_{reg} TN/RKO mice (CD45.2) and subcutaneously immunized or not with 50 µg of bacterial antigen extract adsorbed in alum as in **(C)** 1 day later. Four days after the immunization, iLNs were harvested and analyzed. **(H)** Dot plots show CellTrace Violet dilution in CD45.1⁻CD45.2⁺CD4⁺Vα2⁺Vβ6⁺ T cells in the iLN of the indicated mice. Graph shows all mice analyzed in one experiment. **(I)** In vitro culture of CFSE-labeled CD4⁺ T cells isolated from pT_{reg} TN/RKO mice in the presence or absence of DCs purified from B16-Flt3L-injected mice and in the presence of fecal bacterial antigen extract (50 µg/ml) from Taconic EF or GF mice. Proliferation was measured 3.5 days after coculture by CFSE dilution. **(J)** Graph shows the mean and SD of one experiment (representative of two independent experiments). *P* values were calculated with Student's *t* test. *P* values of <0.05 were considered statistically significant.

Fig. 5. Gene expression analysis of pTreg and CD4_{IEL} TN cells.

TCRαβKO mice were transferred with 5×10^5 naïve CD4⁺CD8α⁻ Foxp3-GFP⁺ T cells purified from the spleen of pTreg TN/RKO/Foxp3^{gfp} mice. Small intestine lymphocytes were harvested 6 weeks after transfer, and the indicated donor T cell subsets were sorted for RNA-seq library preparation and sequencing. (A) Gating strategy for FACS sorting of the indicated populations. (B) Principal components analysis of the top 300 differentially expressed genes by plotting contribution of each T cell population to the first two principal components (PC1 and PC2) (left). Gene set enrichment analysis (GSEA) of Foxp3⁺ pTregs

versus “rest” within each intestinal compartment defined by the PC2 shows significant enrichment ($P < 0.001$) of transcriptional signatures associated with Foxp3⁺ T_{regs} (right). (C) Heat map of fold change expression in log scale of major genes contributing to the PC2 shown in triplicate for each T cell population from the LP versus IE compartment. (D) Diversity of gene expression plotted as correlation of transcript expression levels (log-scale, normalized counts) between each sorted T cell population. Differentially expressed genes described to be characteristic of different T cell subsets are shown in red. (E) GSEA showing a highly significant enrichment ($P < 0.001$) of genes previously found to be up-regulated in CD4⁺CD8αα⁺ IEL for both IE (left; enrichment score, 0.71) and LP (CD4⁺CD8α⁺ versus CD4⁺ lymphocytes; right; enrichment score, 0.69). (F) Heat map of normalized gene expression across three biological replicates of LP CD4⁺CD8α⁺ and CD4⁺ lymphocytes. Differentially expressed transcripts (log₂ fold change) of selected genes known to be up-regulated in the indicated T cell subsets are shown.

Fig. 6. Low levels of ThPOK and high levels of CD103 in TN CD4_{IEL}.

ThPOK, CD103, and ROR γ t expression in the indicated populations were measured by flow cytometry. TCR α β KO mice were adoptively transferred as described in Fig. 4, and small intestine or mLN lymphocytes were analyzed by flow cytometry 6 to 8 weeks after transfer. Unless otherwise stated, cells were gated on CD45⁺CD8 β ⁻ singlets. Histograms in (A) to (E) are representative of five mice. Numbers indicate the geometric median fluorescence intensity (gMFI). Histograms in (C) and (D) also show levels of CD103 in polyclonal T cells from WT mice for comparison. Dot plots in (F) are representative of three mice.

Fig. 7. Foxp3 deficiency does not confer pathogenicity to CD4_{IEL}.

TCRαβKO mice were adoptively transferred with 5×10^5 naive CD4⁺ T cells isolated from the spleen and mLN of pT_{reg} TN/RKO (TN^{wt}) or pT_{reg} TN/RKO/scurfy (TN^{sly}) mice. Recovery of transferred cells was analyzed after 8 to 12 weeks at the indicated locations. (A) Dot plots show the frequency of TN cells recovered in the intestine and in the mLN among CD45⁺CD8β⁺CD4⁺ cells. Graphs show the quantification of three independent experiments. Each symbol represents a mouse, and the horizontal bars represent the means. (B) Cell counts from mice shown in (A). (C) Frequency of Ki-67 cells among transferred TN cells

identified as CD45⁺CD8β⁻CD4⁺Vα2⁺Vβ6⁺. **(D)** Frequency of CD8α⁺ cells among transferred TN cells. **(E)** Frequency of IFN-γ- and IL-17A-producing cells among transferred TN cells identified as CD45⁺CD8β⁻CD4⁺Vα2⁺Vβ6⁺. Graphs show data from two independent experiments. **(F)** Animals transferred with TN^{wt} or TN^{sfy} cells were cohoused (four to five mice per cage), and weight was recorded periodically up to 12 weeks after transfer. **(G)** Photomicrographs of H&E staining of the indicated organs of TCRαβKO mice transferred with TN^{wt} or TN^{sfy}. Original magnification, ×200; scale bars, 200 μm. **(H)** Fecal levels of LCN2 were measured by ELISA. Colitis denotes positive controls for ELISA (feces from colitic mice). *P* values were calculated with Student's *t* test. NS, nonsignificant. *P* values of <0.05 were considered statistical significant.