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Regulatory T Cells Occupy an Isolated Niche in the Intestine that Is Antigen Independent

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SUMMARY

Regulatory T cells (Tregs) are $CD4^+$ T cells that maintain immune homeostasis and prevent autoimmunity. Like all $CD4^+$ T cells, Tregs require antigen-specific signals via T cell receptor-major histocompatibility complex class II (TCR-MHCII) interactions for their development. However, the requirement for MHCII in Treg homeostasis in tissues such as intestinal lamina propria (LP) is unknown. We examined LP Treg homeostasis in a transgenic mouse model that lacks peripheral TCR-MHCII interactions and generation of extrathymic Tregs (iTregs). Thymically generated Tregs entered the LP of weanlings and proliferated independently of MHCII to fill the compartment. The adult LP was a closed niche; new thymic Tregs were excluded, and Tregs in parabiotic pairs were LP resident. The isolated LP niche was interleukin-2 (IL-2) independent but dependent on commensal bacteria. Thus, an LP Treg niche can be filled, isolated, and maintained independently of antigen signals and iTregs. This niche may represent a tissue-specific mechanism for maintaining immune tolerance.

In Brief

Regulatory T cells (Tregs) maintain immune homeostasis and prevent autoimmunity. Korn et al. describe a unique Treg niche in the intestinal lamina propria that does not require T cell receptor signals for development or maintenance and is physiologically isolated from the Tregs that circulate through lymphoid organs. Maintenance of this niche is dependent upon local commensal

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AUTHOR CONTRIBUTIONS

L.L.K. designed and performed all experiments. H.G.H. and L.G.B. assisted in performing experiments. Q.Y. and P.M.P. assisted with parabioses. T.M.L. and L.L.K. designed the study and wrote the manuscript.

SUPPLEMENTAL INFORMATION

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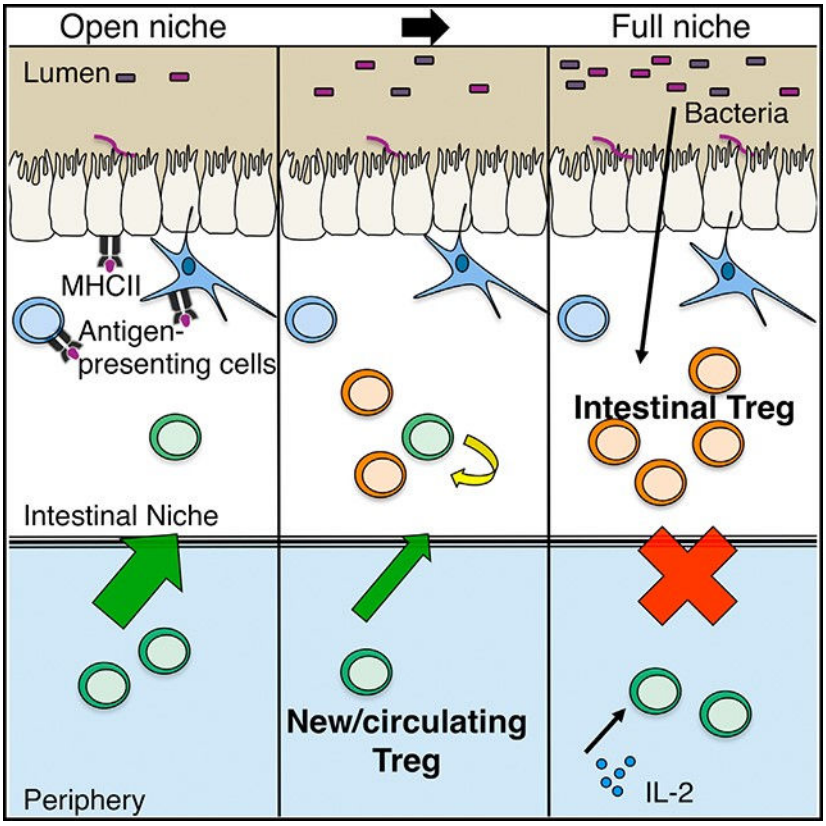
bacteria. The authors propose that this isolated niche may represent a tissue-specific mechanism for maintaining immune tolerance.

Graphical Abstract



INTRODUCTION

Proximity to the immunologic challenges of the outside world place mucosal tissue sites such as lung, skin, and intestine on the front lines of the mammalian immune response. The intestine is a unique immunologic site that exists in symbiosis with trillions of commensal microbes within the lumen and in concert with the metabolism and uptake of nutrients. Within the intestine, commensal, metabolite, and cytokine signals drive a balance between effector and regulatory elements to maintain homeostasis (Spencer and Belkaid, 2012).

Regulatory T cells (Tregs), a subset of $CD4^+$ T cells defined by expression of the transcription factor Foxp3 (Hori et al., 2003), are central to the maintenance of intestinal tolerance. Their presence prevents the development of inflammatory bowel disease and suppresses immune-mediated inflammation during infection. In murine models and humans, absence of Tregs or Foxp3 causes fatal autoimmunity (Bennett and Ochs 2001; Kim et al., 2007). Like all $CD4^+$ T cells, Tregs recognize their cognate antigen by T cell receptor (TCR) interactions with peptide presented in the context of major histocompatibility complex class II (MHCII) on antigen-presenting cells. These signals are necessary for their development

in the thymus (natural or nTregs) and for their differentiation from naive $CD4^+$ T cells in the periphery (inducible or iTregs). In secondary lymphoid organs, maintenance of the Treg population and regulation of the size of the Treg niche rely on a combination of TCR-MHCII interactions and common gamma chain cytokine signals, particularly interleukin-2 (IL-2) (Setoguchi et al., 2005; Amado et al., 2013).

In contrast, the role of TCR-MHCII interactions in intestinal lamina propria (LP) Treg maintenance is unknown. Studies have indicated that MHCII (antigen)-dependent, commensal, or food-specific iTregs comprise a majority of the LP Treg pool (Atarashi et al., 2011; Lathrop et al., 2011; Josefowicz et al., 2012), although at least one recent study proposed otherwise (Cebula et al., 2013). The intestine is rich in antigen-presenting cells; dendritic cells, B cells, macrophages, small intestine epithelium, and type three innate lymphoid cells all express MHCII (Hepworth et al., 2013). However, LP Tregs are exposed to additional microbial and dietary metabolite signals that influence their development and expansion (Atarashi et al., 2011; Round and Mazmanian, 2010; Smith et al., 2013; Arpaia et al., 2013; Furusawa et al., 2013). Thus, we probed the contribution of MHCII-dependent signals to the homeostasis of intestinal Tregs and found that the intestine contains an isolated, antigen-independent niche for regulatory T cells.

RESULTS

MHCII-Independent Filling of an Intestinal Treg Niche

We previously showed that Tregs were present in the thymus, lymph nodes (LNs), and spleen of K14-Aβb (K14) mice, in which MHCII, $I\text{-}A^b$ expression is restricted to cortical thymic epithelium. $CD4^+$ T cells, including Tregs, develop in the K14 thymus but are not exposed to peripheral MHCII signals (Bensinger et al., 2001). In both small intestine LP (SI-LP) and mesenteric LNs (MLNs), across all ages examined (2–35 weeks), the frequency of total $CD4^+$ T cells ($Foxp3^+$ and $Foxp3^-$) as a percentage of lymphocytes was decreased in K14 mice compared to their $MHCII^{+/-}$ littermates (Figure 1A). If most LP Tregs were induced locally, the LP of K14 mice would contain few or no Tregs; however, $Foxp3^+$ $CD4^+$ T cells were present in the SI-LP of K14 mice. In 2- and 3-week-old mice, both SI-LP Treg frequencies and numbers in K14 mice were lower than in their $MHCII^{+/-}$ littermate controls (Figures 1A and 1B). However, by 5 weeks of age, a 2-fold increase in SI-LP Treg frequency in K14 mice compared to $MHCII^{+/-}$ littermates was established and then maintained (Figure 1A). In contrast, in LP-draining MLNs, K14 Treg frequencies were consistently reduced compared to $MHCII^{+/-}$ in mice less than 12 weeks old and approximately equivalent in older (>15-week-old) mice (Figure 1A). The numbers of Tregs in the SI-LP of 9- to 11-week-old $MHCII^{+/-}$ and K14 mice were equivalent (Figure 1B). A similar pattern of Treg accumulation was observed in the LI-LP (Figures S1A and S1B). Given the concurrent decreased $CD4^+$ T cell frequency in K14 SI-LP, the increase in Treg frequency that occurred with time in the K14 SI-LP led to the accumulation of equivalent numbers of Tregs in the LP of $MHCII^{+/-}$ and K14 mice. Treg numbers in the same mice in secondary lymphoid organs were reduced in K14 mice, with the exception of mesenteric LNs, where numbers were similar to $MHCII^{+/-}$ controls (Figure S1C). These data suggested that a Treg niche in the SI-LP could fill independently of MHCII signals.

Would SI-LP Tregs express activation markers in the absence of antigen-specific signals? SI-LP Tregs expressed CD103 (αe integrin) independent of MHCII (50%–70% in MHCII$^{+/-}$ and 80%–100% in K14 adult mice) (Figure 1C and not shown). Expression of CD44 on SI-LP Tregs and even on conventional T cells was also MHCII independent and increased with age. An activated phenotype, therefore, was MHCII independent.

We presumed that the Tregs present in an MHCII-independent periphery had developed in the thymus. Tregs in the SI-LP of K14 mice expressed the transcription factor Helios, which has been associated, albeit incompletely, with thymically derived nTregs (Thornton et al., 2010; Verhagen and Wraith, 2010) (Figure S2A). Nevertheless, we examined the potential for iTreg induction using an established model (Sun et al., 2007). Ovalbumin-specific OTII TCR transgenic CD4$^+$ T cells were transferred to MHCII$^{+/-}$, K14, or MHCII$^{-/-}$ mice that received soluble ovalbumin in their drinking water. Consistent with past work (Sun et al., 2007), antigen-dependent, induced Foxp3$^+$ OTII Tregs were present in the SI-LP and MLNs of MHCII$^{+/-}$ mice; however, no Tregs were induced in either MHCII$^{-/-}$ or K14 mice (Figure S2B). These data suggested that LP Tregs in K14 mice were thymically derived nTregs. The lack of iTreg induction implied that there was no functional MHCII present in the K14 LP, and flow cytometry of K14 intestinal epithelium did not reveal any MHCII expression (Figure S3A). To verify that the LP Treg accumulation in K14 mice did not result from ectopic transgenic MHCII expression, MHCII$^{-/-}$ mice were reconstituted with wild-type, MHCII-expressing B6 thymic grafts to rescue development of CD4+ T cells (Wu et al., 2011). SI-LP Treg frequency, Treg number, and CD4+ T cell frequency in MHCII$^{-/-}$ mice with WT thymic grafts phenocopied the K14 SI-LP (Figure S3B). We additionally asked if Tregs in the LP of K14 mice were MHCI-restricted. We previously showed that Tregs were still present in K14 mice lacking expression of MHCI molecules (b2 m KO) (Bensinger et al., 2001). The loss of b2 m in these mice did not lead to the specific loss of SI-LP Tregs (Figure S3C), suggesting that atypical MHC restriction was not responsible for the Treg accumulation there. Thus, an MHCII-negative niche in the K14 SI-LP niche can fill with nTregs.

Do nTregs fill an MHCII-independent Treg compartment via increased local proliferation? The proliferation of SI-LP Tregs, as assessed by Ki-67 positivity, was similar between MHCII$^{+/-}$ and K14 littermates and declined from approximately 50% in 2-week-olds to 20% in 10-week-olds (Figure 2A). Consistent with these data, equivalent proportions of SI-LP Tregs were bromodeoxyuridine (BrdU) positive 1 day after injection of BrdU in 7- to 10.5-week-old MHCII$^{+/-}$ and K14 mice (Figure 2B). There was the expected increased requirement for MHCII in the proliferation of Foxp3-negative CD4$^+$ cells (conventional CD4$^+$ T cells or Tconv) in 2- to 5-week-old mice (Figure 2A). Preserved local Treg proliferation in young mice thus allowed the LP niche to fill. In parallel with reduced Tconv proliferation, this proliferation led to the increased Treg frequency in K14 mice.

The Intestinal Treg Niche Is Isolated Independent of MHCII

Treg proliferation in an MHCII-deficient SI-LP could be augmented by continued influx of newly generated thymic nTregs. To examine this possibility, we asked how Tregs that were recent thymic emigrants (RTE) contributed to the intestinal Treg compartment. In

RAG2-GFP BAC reporter (RAG2-GFP) mice, RTEs retain GFP expression for 1–2 weeks after exit from the thymus and can be tracked into the periphery (Boursalian et al., 2004; Yu et al., 1999). Consistent with these data, GFP^+ Tregs could be identified in the thymus and periphery of wild-type (WT) and K14 RAG2-GFP mice (Figure 3A). The percentages of Tregs that were RTEs were equivalent in both the lymphoid organs and intestinal LP of 3-week-old K14 and $MHCII^{+/-}$ RAG2-GFP mice (Figure 3A). Thus, MHCII-independent filling of the intestinal Treg niche occurred without increased reliance on RTEs.

Interestingly, the SI-LP became inaccessible to RTE Tregs with age despite continued thymic Treg generation; the contribution of RTEs to intestinal Tregs declined to less than 1% of the compartment in 8-to 18-week-old $MHCII^{+/-}$ mice (Figure 3A). This precipitous fall in RTE contribution to the SI-LP Treg niche also occurred in K14 mice and thus was MHCII independent (Figure 3A). The percentage of MLN Tregs that were RTEs also fell in older $MHCII^{+/-}$ and K14 mice; however, RTE Tregs were still present in the MLNs of 8- to 18-week-old mice, and the relative RTE decrease was much greater in the SI-LP (10-fold versus 4-fold in MLNs) (Figure 3A). These findings stand in contrast to the preferential intestinal settling of $CD8^+$ and unconventional T cells (Staton et al., 2006; Guy-Grand et al., 2013).

The exclusion of RTEs from the LP in adult mice raised the question of whether the LP was inaccessible to all circulating Tregs in adult mice. We utilized parabiosis of congenic mice to assess circulation of Tregs over 8–11 days. As a control, B cell chimerism in the SI-LP of $MHCII^{+/-}$ and K14 pairs was comparable to that in the spleen, showing that the intestinal circulation was accessible to donor cells in the parabiosed mice (Figure S4). In $MHCII^{+/-}$ parabionts, chimerism of $Foxp3^+$ Tregs in the LP of the parabionts was limited, with 1.8%–2.5% chimerism, or approximately 14% relative to the chimerism in the spleen (Figure 3B). Importantly, this was not typical of all nonlymphoid tissues, as the chimerism of lung Tregs was greater than that of lymphoid organs (Figure 3B). In K14 parabionts, chimerism of Treg in the LP was again quite restricted, to 0.5%–1.5%, or 7% of chimerism in the spleen (Figure 3B). Chimerism of conventional $CD4^+$ T cells was also reduced in the LP, though not nearly as drastically as Treg chimerism (Figure S4). Therefore, circulation of Tregs to intestine is quite restricted regardless of the presence of MHCII.

MHCII-Independent Maintenance of the Intestinal Treg Niche

Which MHCII-independent signals might help maintain intestinal Tregs? IL-2 is required for Treg maintenance and proliferation, and its presence can facilitate Treg proliferation even in the absence of direct TCR-MHCII interactions (Zou et al., 2012). Recently, Smigiel et al. described IL-2-dependent “central Tregs,” preferentially found in secondary lymphoid tissues, and IL-2-independent “effector Tregs,” which expressed lower levels of the IL-2 receptor alpha chain, CD25, and were enriched in the LP (Smigiel et al., 2014). We examined CD25 expression in $MHCII^{+/-}$ and K14 SI-LP Tregs. CD25 expression on $MHCII^{+/-}$ and K14 splenic Tregs was comparable (Figure 4A). Consistent with Smigiel et al., we found that $MHCII^{+/-}$ SI-LP Tregs expressed lower levels of CD25 than splenic Tregs. However, K14 SI-LP Tregs expressed approximately 2-fold higher levels of CD25 than $MHCII^{+/-}$ SI-LP Tregs (Figure 4A). We therefore considered the possibility that, in

the absence of TCR signals, IL-2 would have an increased role in maintenance of MHCII-independent SI-LP Tregs. IL-2 availability was disrupted for 10 days with a blocking antibody as previously described (Setoguchi et al., 2005), and Treg frequencies were assessed. Treg frequencies in MHCII$^{+/-}$ mice fell in peripheral LNs, MLNs, and spleen, in agreement with previously published results (Setoguchi et al., 2005). Treg frequencies also fell in lymphoid organs of treated K14 mice (Figure 4B). Thus, Tregs in secondary lymphoid organs of K14 mice are IL-2 responsive, despite the lack of MHCII-CD4^{+} T cell interactions. In contrast to the peripheral lymphoid organs, Treg frequencies remained constant in the SI-LP of both MHCII$^{+/-}$ and K14 mice and similar results were obtained in the LI-LP (Figure 4B). Thus, relative IL-2 independence further defines the unique LP Treg niche, and this is also independent of CD25 expression.

Signals derived from the commensal microbiota also regulate the differentiation of LP Tregs (Atarashi et al., 2011; Round and Mazmanian, 2010). However, it is not clear if the maintenance of LP Tregs requires persistent commensal-derived TCR-MHCII signals. We recently reported that the composition of the intestinal microbiota is quantitatively and qualitatively indistinguishable in MHCII$^{-/-}$ and MHCII$^{+/-}$ mice (Korn et al., 2014), implying that MHCII$^{+/-}$ and K14 littermates have similar microbiomes. We treated MHCII$^{+/-}$ and K14 mice with a regimen of broad-spectrum antibiotics for 2 weeks, which led to 10^2- to 10^3-fold decreases in the total number of bacteria in the colon and cecum (not shown), and assessed the effect on local Treg frequencies. Consistent with previous work (Atarashi et al., 2011), antibiotic treatment reduced Treg frequencies in the LI-LP of MHCII$^{+/-}$ mice by one-third. This reduction also occurred in K14 mice (Figure 4C). Treg frequencies in the SI-LP of treated MHCII$^{+/-}$ mice were unchanged, consistent with data from germ-free mice (Atarashi et al., 2011). In contrast, antibiotic treatment was associated with decreased Treg frequency in the SI-LP of K14 mice; the mean Treg frequency fell on average by one-quarter (Figure 4C). There was no change in the Treg frequency in the spleen and MLNs of either MHCII$^{+/-}$ or K14 mice, suggesting that the MHCII-independent, commensal-dependent regulation of Treg homeostasis in K14 mice was restricted to the LP (Figure 4C). Therefore, MHCII-independent, microbial-derived signals can contribute to the maintenance of the intestinal Treg niche in both the SI-LP and the LI-LP, with a larger effect in the LI-LP.

DISCUSSION

Collectively, these studies demonstrate that the Treg niche in the intestine fills with RTEs and subsequent local proliferation, and, once full, excludes RTEs and circulating Tregs. The WT Treg intestinal niche is reliant on factors distinct from the lymphoid niche for maintenance. Maintenance of the niche is relatively IL-2 independent, but partially dependent on microbial signals. Signals derived from microbes or their metabolites regulate Treg homeostasis in WT mice, for example *Bacteroides fragilis*–driven TLR2 signals (Round and Mazmanian, 2010), LPS-driven TLR4 signals (Caramalho et al., 2003), and short chain fatty acid driven signals (Furusawa et al., 2013; Smith et al., 2013). Perhaps these signals are sufficient to maintain intestinal Tregs even in the absence of TCR signals.

Past studies have attributed SI-LP Treg expansion to antigen-dependent iTreg induction (Josefowicz et al., 2012). Studies presented here suggest that thymically derived nTregs are

capable of filling the LP niche, and that MHCII-independent proliferation and maintenance may also contribute to this homeostasis. Similar findings were present in the large intestine. As the small and large intestines are subject to unique regulation, these data may represent either similar underlying mechanism or, alternatively, a common result of differing processes.

Data in by Smigiel et al. suggested that TCR specificity, along with the presence of inflammatory signals, mediates the differentiation of effector Tregs with an IL-2-independent phenotype (Smigiel et al., 2014). Here, we show that Tregs that settle the LP acquire an activated phenotype and are IL-2 independent even in the absence of MHCII-TCR interactions. Further, our data establish that the LP Treg niche can fill, exclude new entrants, and be maintained independent of antigen. Thus, our data suggest a primacy of tissue-specific factors in defining local Treg homeostasis that overrides both IL-2 and MHCII signaling requirements.

Despite data consistent with a closed, inaccessible niche at steady state, past studies have shown that the LP Treg niche changes in infection and inflammatory bowel disease (Oldenhove et al., 2009; Holmén et al., 2006). In humans, studies have indicated that there are limited T cell RTEs in the healthy adult intestine but increased RTEs in patients with ulcerative colitis (Elgbratt et al., 2010). Further, newly induced iTregs accumulate in LP (Hadis et al., 2011), suggesting flexibility in the permissiveness of the niche. Combined with our data, these studies support the existence of a Treg niche with context-dependent accessibility. Perhaps the steady state isolation of the LP Treg niche protects the local environment by promoting the presence of a specialized Treg population best suited to the LP milieu. It is remarkable that TCR-MHCII interactions are not required to regulate the opening and closing of this LP Treg niche.

EXPERIMENTAL PROCEDURES

Mice

MHCII$^{+/-}$, MHCII$^{-/-}$, K14-Aβ^{b}, b2 $\beta^{-/-}$ (Bensinger et al., 2001), Foxp3-GFP mice (Bettelli et al., 2006), and RAG2-GFP reporter mice (Yu et al., 1999) were bred in-house. All mice were housed under specific-pathogen-free conditions, and procedures, postoperative monitoring, and pain control were in accordance with the University of Pennsylvania animal care and use guidelines. Experimental mice were littermates or age-matched and extensively cohoused.

Antibodies and Flow Cytometry

Antibodies were purchased from eBioscience, BioLegend, BD Biosciences, or Invitrogen. Live/Dead Fixable Aqua (Invitrogen) or DAPI was used for live/dead discrimination. The Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used for transcription factor stains, and Tregs were defined as CD19^{-}TCRβ^{+}CD4^{+}CD8α^{-}Foxp3^{+} (or GFP+ in Foxp3-GFP mice). To stain of the RAG-GFP reporters, samples were fixed with 1.6% paraformaldehyde and stained in 0.5% saponin with anti-GFP (Invitrogen) and Foxp3. For

BrdU staining, the BD BrdU Kit was used with the −80° freezing step. Samples were collected on a BD LSRII or FACs Canto and analyzed using FlowJo software (Tree Star).

Preparation of Organ Single-Cell Suspensions

Lymphoid organs were prepared by mechanical disruption. For LP, Peyer's patches were removed from intestines and LP and epithelial cells were obtained as described previously (Korn et al., 2014). Lungs were prepared as described elsewhere (Yang et al., 2013).

In Vivo IL-2 Blocking

Mice were injected IP with 1mg of S4B6–1 blocking antibody (Bio X Cell) or rat immunoglobulin G isotype control (Sigma) and sacrificed 10 days later as previously published (Setoguchi et al., 2005).

Parabiosis

Pairs were composed of age-matched females. Mice were anesthetized using continuous isoflurane, and longitudinal skin flaps from knee to olecranon of each member of the pair were created. Pairs were first joined behind the knee and olecranon using absorbable suture for stabilization. The skin flap edges were then everted together and sewn using absorbable suture or stapled using MikRon 9 mm autoclips (BD).

Antibiotic Treatment

Drinking water was supplemented with ampicillin (0.5 mg/ml), gentamicin (0.5 mg/ml), metronidazole (0.5 mg/ml), neomycin (0.5 mg/ml), and vancomycin (0.25 mg/ml) plus sucralose sweetener for 2 weeks.

BrdU Labeling

Mice were injected with 1 mg BrdU (Sigma) per mouse and analyzed 24 hr later.

Thymic Transplants

Thymic transplants were performed as previously described (Wu et al., 2011). Recipients were euthanized and examined 9 weeks after surgery.

Ova Feeding

iTreg conversion following ovalbumin feeding was assessed as previously described (Sun et al., 2007).

Statistics

Statistical significance was determined by two-tailed Student's t test. Welch's correction was applied if an F test to compare variances reached statistical significance. Error bars show SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- There is a unique niche for regulatory T cells in the intestinal lamina propria (LP)
- This niche can be filled and maintained independently of MHC class II and IL-2
- Parabiosis shows that the niche is closed as lamina propria Tregs do not circulate
- Maintenance of MHCII-independent LP Tregs does depend upon commensal bacteria

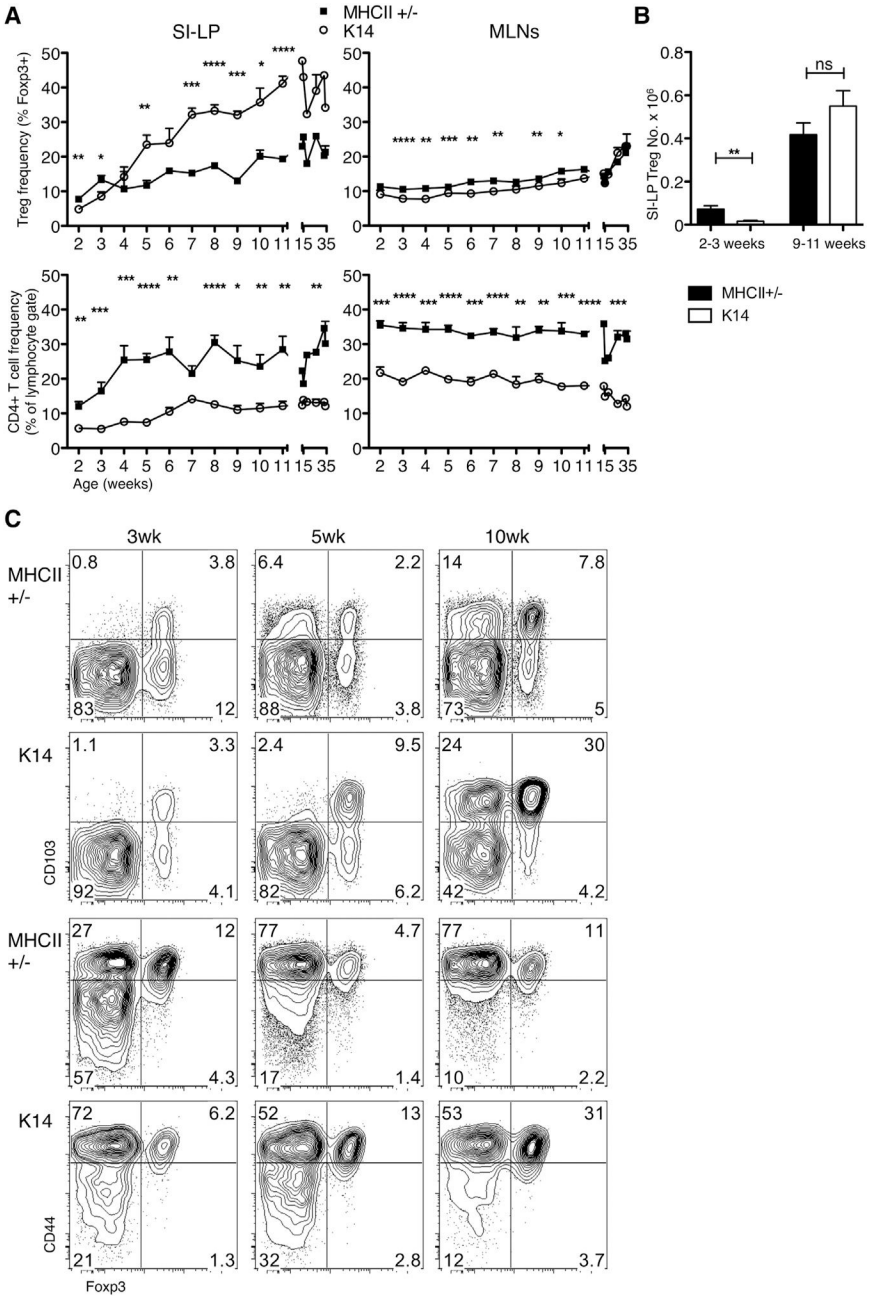

Figure 1. The Intestinal Compartment for Tregs Is MHCII Independent

(A) Treg frequency among $CD4^+$ T cells and $CD4^+$ T cell frequency among lymphocytes in SI-LP and MLNs of $MHCII^{+/-}$ and K14 mice at indicated ages.

(B) Number of Tregs per SI-LP in 2- to 3-week-old and 9- to 11-week-old $MHCII^{+/-}$ and K14 mice. Error bars show SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Each dot or bar represents 4–18 mice.

(C) Representative staining of CD103 and CD44 on SI-LP $CD4^+$ T cells ($CD4^+CD8^-TCR\beta^+CD19^-$) in $MHCII^{+/-}$ and K14 littermates at the indicated ages.

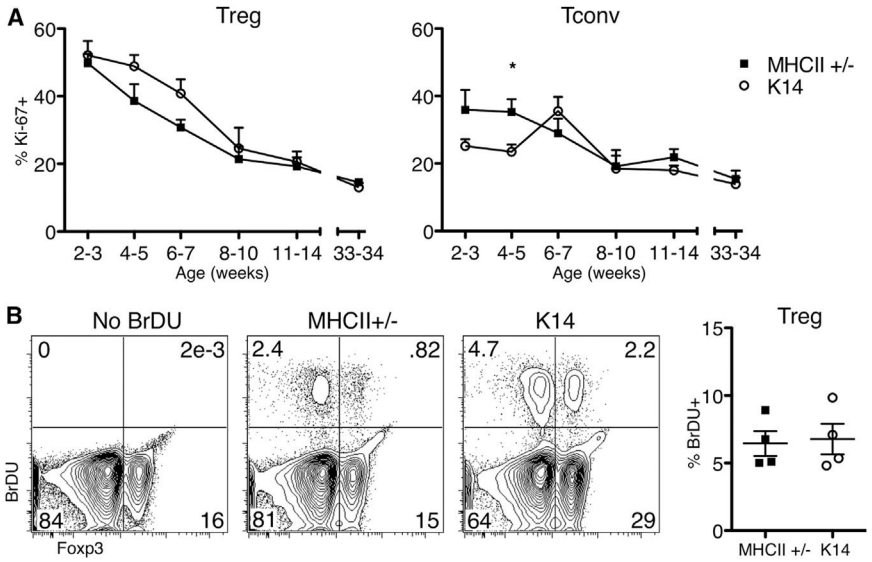

Figure 2. Treg Proliferation within the SI-LP Treg Niche Is MHCII Independent

(A) Ki-67 positivity among SI-LP Tregs and Tconv in MHCII$^{+/-}$ and K14 mice with age. Each dot represents six to nine mice.

(B) Representative staining of BrdU on SI-LP CD4^{+} T cells and BrdU+ percent among SI-LP Tregs in 8- to 10-week-old MHCII$^{+/-}$ and K14 mice 1 day post-BrdU injection. Each dot represents one mouse.

Error bars show SEM; *p < 0.05, **p < 0.01.

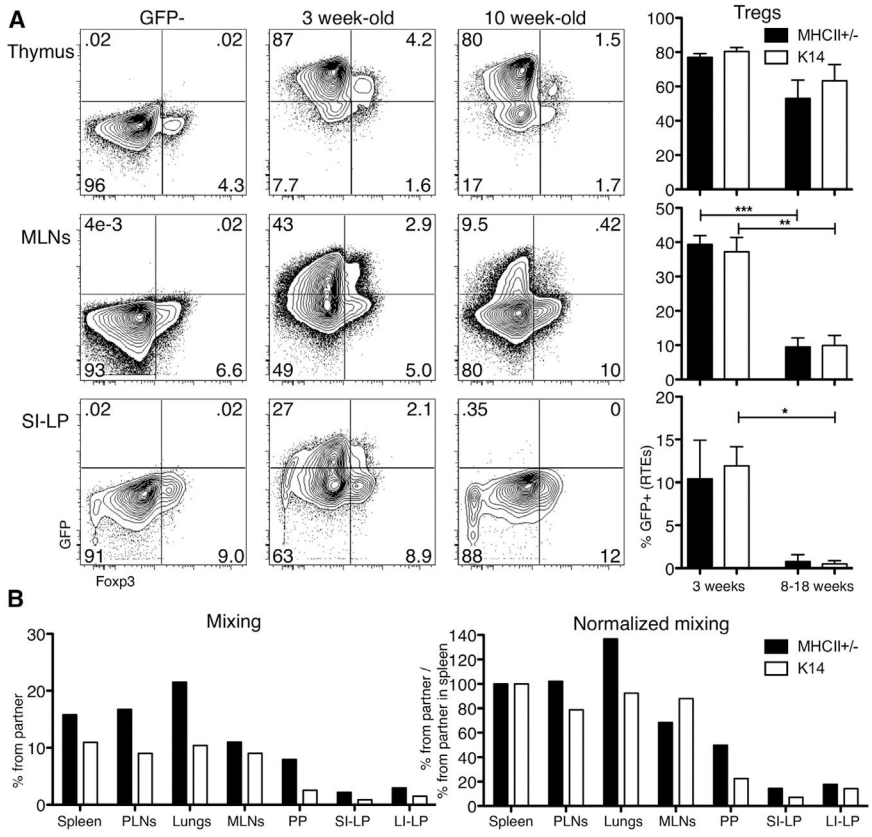

Figure 3. RTEs and Circulating Tregs Are Excluded from the Adult LP Treg Niche

(A) Analysis of recent thymic emigrants (RTEs). Left: representative staining of GFP+ RTEs by GFP and Foxp3 on $CD4^+$ T cells in either control (GFP−) or GFP+ $MHCII^{+/-}$ mice at indicated organs and ages. Right: Graphs show the average percentage of Tregs that are RTEs in GFP+ $MHCII^{+/-}$ and K14 mice. Error bars show SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Congenically marked $MHCII^{+/-}$ or K14 mice were parabiosed to form $MHCII^{+/-}$.CD45.2-$MHCII^{+/-}$.CD45.1 and K14.CD45.2-K14.CD45.1 pairs. Pairs were analyzed 8–11 days later. Left bar graph shows the average percentage of Tregs that were of congenic (partner) origin in the indicated organs. Right bar shows the same percentage, normalized to the percentage in the spleen. Data are averaged from two $MHCII^{+/-}$ pairs and two K14 pairs.

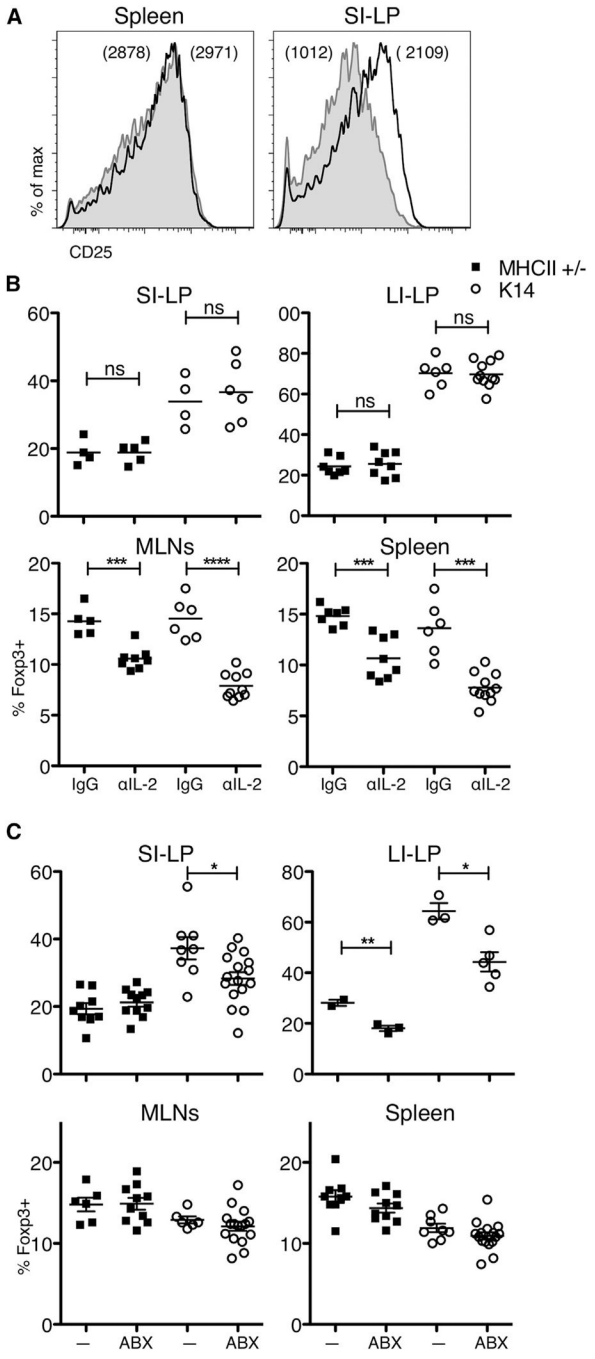

Figure 4. MHCII-Independent Intestinal Tregs Are Relatively IL-2 Independent and Microbe Dependent

(A) Representative flow cytometry of CD25 expression on splenic and SI-LP Tregs in MHCII$^{+/-}$ (gray shaded) and K14 (black line) mice. Numbers are representative MFIs. Plots are representative of >15 mice of each genotype.

(B) MHCII$^{+/-}$ and K14 mice were treated with IL-2 blocking antibody (S4B6) or rat immunoglobulin G control and sacrificed 10 days later. Graphs show Treg frequencies among CD4^{+} T cells in indicated organs.

(C) MHCII$^{+/-}$ and K14 mice were treated with broad-spectrum antibiotics in their drinking water for 2 weeks or left untreated. Graphs show Treg frequencies among CD4^{+} T cells in indicated organs.

Error bars show SEM; *p < 0.05; ***p < 0.001, ****p < 0.0001. Each dot represents one mouse. Data in (B) are pooled from six experiments, and data in (C) are pooled from eight experiments.