Inflammation and Regeneration

REVIEW

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Localization and movement of Tregs in gastrointestinal tract: a systematic review

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Abstract

Background: The intestine is rich in food-derived and microbe-derived antigens. Regulatory T cells (Tregs) are an essential T-cell population that prevents systemic autoimmune diseases and inhibits inflammation by encountering antigens. Previously, it was reported that the functional loss of Tregs induces systemic inflammation, including inflammatory bowel disease and graft-versus-host disease in human and murine models. However, there is a dearth of information about how Tregs localize in different tissues and suppress effector cells.

Main body: The development of Tregs and their molecular mechanism in the digestive tract have been elucidated earlier using murine genetic models, infectious models, and human samples. Tregs suppress immune and other non-immune cells through direct effect and cytokine production. The recent development of *in vivo* imaging technology allows us to visualize how Tregs localize and move in the settings of inflammation and homeostasis. This is important because, according to a recent report, Treg characterization and function are regulated by their location. Tregs located in the proximal intestine and its draining lymph nodes induce tolerance against food antigens, and those located in the distal intestine suppress the inflammation induced by microbial antigens. Taken together, various Tregs are induced in a location-specific manner in the gastrointestinal tract and influence the homeostasis of the gut.

Conclusion: In this review, we summarize how Tregs are induced in the digestive tract and the application of *in vivo* Treg imaging to elucidate immune homeostasis in the digestive tract.

Keywords: Regulatory T cells (Tregs), Live imaging, Digestive tract, Multiphoton microscopy, Review

Background

Regulatory T cells (Tregs) are important for maintaining immune tolerance for self-antigens and suppressing inflammation. Loss of Tregs function induces autoimmune diseases and disrupts the overall homeostasis [1–4]. Tregs in inflammatory bowel disease (IBD) patients were less able to suppress the effector cells in the lamina propria [5, 6]. Moreover, the risk of graft-versus-host disease (GVHD) following bone marrow transplantation was associated with the depletion of Tregs in peripheral blood, while the introduction of Tregs into GVHD mice improved their survival [7, 8].

The importance of Tregs was elucidated using mice and human forkhead box p3 (*Foxp3*) gene mutation studies; human IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, caused by *Foxp3* mutation, induces early onset of T-cell-dependent lymphoproliferation with cytokine storm [9–12].

The mechanism of Treg suppression of other immune cells was studied extensively [13, 14]. Tregs express a substantial number of genes, including those of secreted proteins and molecules, on the cell surface. They suppress other immune cells via cell contact independent

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and dependent mechanisms. The suppression via a cell contact independent mechanism is through the secretion of the major cytokines, such as IL-10, IL-35, granzyme B, and TGFβ [15–17]. Lack of IL-10 and IL-35 production by Tregs induces inflammation in the colon and lungs [18, 19]. TGFβ, which is essential for the induction of the Tregs, suppresses helper T-cell 1 (Th1) response [20, 21].

Tregs-induced suppression via cell contact-dependent mechanism is performed through the major molecules expressed on Tregs surface, such as IL-2 receptor (IL-2R), CTLA-4, PD-1, LAG-3, GITR, and TIGIT. CD25, known as the original Treg cell marker, is highly expressed by Tregs (Fig. 1A). High levels of IL-2R expression deprive effector T cells of IL-2, resulting in inhibition of proliferation [22]. CTLA-4 downregulates the CD80/CD86 expression on the antigen-presenting cells (APCs) [23, 24]. Accordingly, patients with CTLA-4 haploinsufficiency have impaired Treg functions, leading to the development of Crohn's-like intestinal inflammation [25–27]. Moreover, patients treated with anti-CTLA-4 antibodies developed colitis as a side effect.

The PD-1 co-inhibitory receptor is highly expressed on Tregs. PD-1-mediated signaling inhibits CD28 costimulation by binding PD ligands 1 and 2, resulting in the inhibition of T-cell costimulation in the early phase after antigen stimulation [28].

LAG-3, which binds MHC class 2, is required for the suppressive activity of Tregs [29]. LAG-3 suppresses IL-23 on Cx3cr1$^+$ macrophage, enhancing IL-22 production from group 3 innate lymphoid cells in an anti-CD40 colitis model [30]. TIGIT promotes IL-10 production on APCs [31]. GITR, one of the TNF receptor family members, is highly expressed on Tregs [32, 33].

More details about the functioning of Tregs have been elucidated recently, yet their action *in vivo* is still unknown. We review how the Tregs are generated and summarize Treg localization and movement in the gut using an *in vivo* imaging system.

Importance of Tregs in the gut

Tregs develop in either the thymus or peripheral tissue. Tregs that develop in the thymus are called "tTregs," and transcription factors such as Helios or Neuropilin are used to identify tTregs [34–40]. The other Tregs originating extrathymically in peripheral tissues are called "pTregs." pTregs develop from T conventional cells (Foxp3$^-$ cells) [41, 42]. Based on their location and function, pTregs can be classified into three groups: central, effector, and tissue-resident Tregs. Central Tregs that express CD62L^{high} and CCR7$^+$ are the major population of naïve Tregs, and they localize in secondary lymph nodes. Effector Tregs are CD62L^{low} CCR7^{low} Tregs. Tissue-resident Tregs reside in nonlymphoid organs, especially the colon. Most of the Tregs in the gut in a steady state are tissue-resident pTregs [13, 43]. Like conventional T cells, Tregs are governed by specific transcriptional factors. T-bet$^+$ Tregs, IRF4$^+$ Tregs, and STAT3$^+$ Tregs suppress Tbet$^+$ T cells (Th1), IRF4$^+$ T cells (Th2), and STAT3$^+$ T cells (Th17), respectively [44–46]. Other Tregs express unique transcription factors to adapt to their microenvironment. For instance, some GATA3$^+$Helios$^+$Tregs expanded during tissue damage response to IL-33 [47, 48].

In the gut, some of the pTregs express the transcriptional factor retinoic acid-related orphan receptor-γt (Rorγt), which was initially described as the essential transcriptional factor for Th17 cell development [49] (Fig. 1B).

Rorγt$^+$ pTregs develop under the existence of microbial antigens; they inhibit Th17 response during gut inflammation [50]. Rorγt$^-$ Helios$^-$ pTregs are induced by dietary antigens [51]. According to a recent report, Treg characterization and function are regulated by location. Tregs located in the proximal intestine and its draining lymph node induce tolerance against the food antigens, while those in the distal intestine suppress inflammation induced by the microbial antigens [52] (mentioned in the "Treg localization and movement in the gut," see Fig. 1C). Taken together, various Tregs are induced in a location-specific manner in the gastrointestinal tract.

Treg *in vivo* imaging

CD4 imaging of lymphoid tissue is used to be challenging; now, two-photon intravital microscopy makes *in vivo* imaging possible (Fig. 2). Two-photon imaging enables us to visualize how the antigen is transferred from DCs to T cells in lymph nodes [53]. To visualize Tregs *in vivo*, two-photon microscopy was used to visualize CFSE-labeled CD4$^+$CD25$^+$T cells in lymphoid tissues [54, 55]. An antigen-specific model was developed to demonstrate that the contact of Tregs with dendritic cells (DCs) is required to inhibit Th cell activation. Imaging analysis revealed that Tregs in lymph nodes stopped for longer durations when they encountered antigen-bearing DCs and did not interact with CD4 non-Tregs. These data suggest that Tregs directly suppress the function of APCs, resulting in the inhibition of Th cell activation. CFSE-labeled CD4$^+$CD25$^+$T cells are injected during *in vivo* imaging to visualize the connection between other immune cells and cell proliferation. This system enables us to visualize Treg movement in an antigen-specific model. However, it is difficult to envisage where transferred cells do not migrate to, as the florescent activity fades in a time-dependent manner. Moreover, ex vivo cultured CFSE-labeled CD4$^+$CD25$^+$T cells may not have the same movement as internal Tregs.

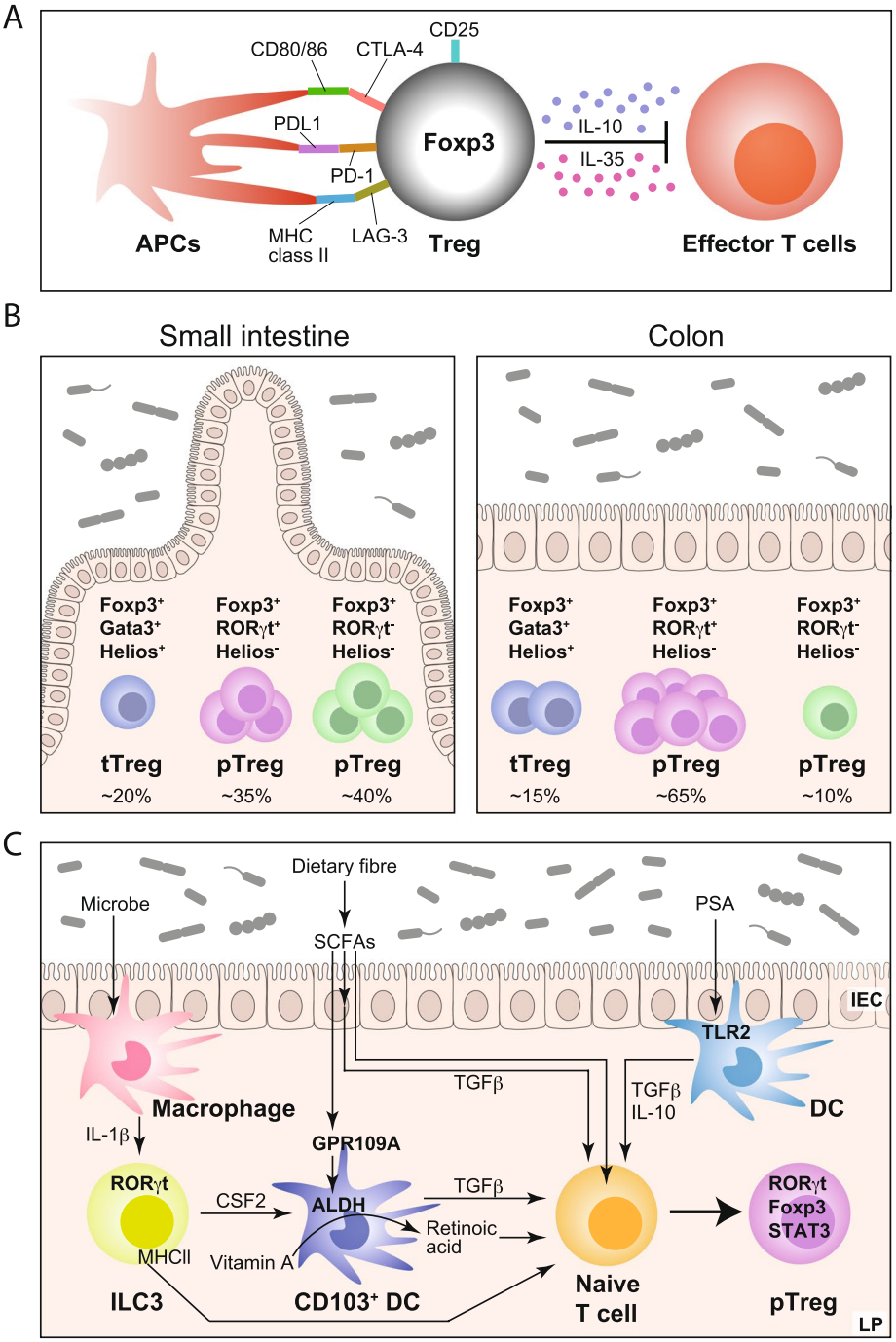

Fig. 1 Characteristics of Treg in gut. **A** Tregs suppress effector cells and antigen-presenting cells via surface protein and cytokine. **B** Helios^{+}Gata3^{+}Foxp3^{+}Tregs (thymic Tregs: tTregs), Helios^{-}Roryt^{+}Foxp3^{+}Tregs (peripheral Tregs: pTregs), Helios^{-}Roryt^{-}Foxp3^{+}Tregs (peripheral Tregs: pTregs) in small intestine (left) and colon (right). The percentage of each population in total Tregs was listed below. **C** Schema of generation of peripheral Tregs in gut

To visualize live internal Tregs in tissue, we developed the tamoxifen-inducible *Foxp3*$^{\text{eGFPcreERT2}}$: *Rosa26*$^{\text{t-dTomato}}$ mice [56, 57]. Tamoxifen can visualize the bona fide Tregs. Some unstable Tregs differentiate to other cells such as Th17, Th1, and CD8a expressing cells in the peripheral tissue, called exTregs [2, 57–61]. We visualized Tregs after 24 h of the tamoxifen induction, as more than 99% of tomato-positive cells still expressed Foxp3 protein

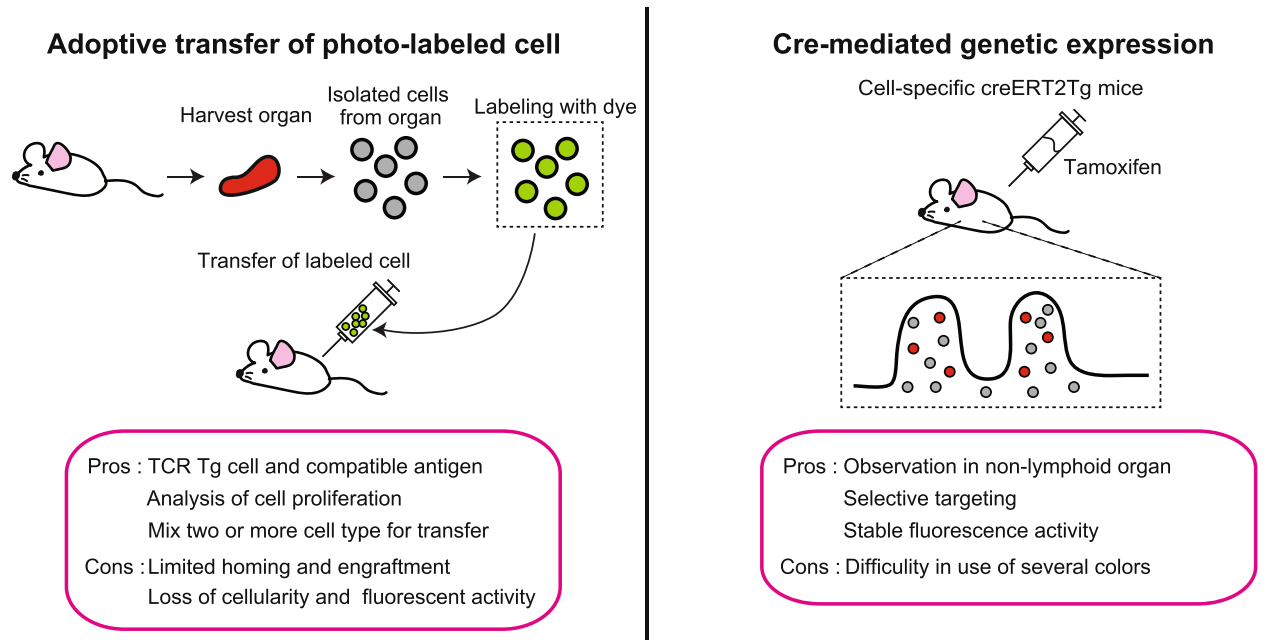

Fig. 2 Two different techniques for *in vivo* imaging. In the procedure shown to the left, cells are isolated from organs harvested from mice, labeled with a specific dye, and transferred into mice. On the other hand, the procedure shown on the right establishes mice transgenic with the creERT2 gene and fluorescent protein targeting specific cells. The mice are then injected with the hormonal substance tamoxifen, which induces the activity-dependent expression of the fluorescent protein by Cre recombinase, allowing *in vivo* observation. Pros and cons for each method are listed at the lower part of the figure

[56, 57]. The benefit of this model is to visualize the internal bona fide Tregs, thus enabling us to image nonlymphoid organs. However, it is not quite feasible to image Tregs and immune/nonimmune cells simultaneously.

Treg localization and movement in the gut

The distribution of Tregs in the gastrointestinal tract was different among different organs [62–65].

Stomach

The role of Tregs in the stomach is not well understood. Infection with *Helicobacter* spp. is the trigger for chronic gastric inflammation, resulting in gastric cancer. However, in the mouse model, *Helicobacter* spp. induced inflammation in the small intestine and colon, especially in IL-10 knockout mice [66–68]. Tregs were observed to localize near the bottom of the glandular epithelium but not near the luminal side (Fig. 3). Further research is required to understand the function of Tregs in the stomach.

Small intestine

Oral antigen ovalbumin (OVA) is the commonly used model food antigen to understand Treg induction. Oral tolerance to OVA is dependent on the resident intestinal DCs, resulting in the induction of Tregs in the small intestine [69]. $CD103^+$ DCs have more potential to induce Tregs than $CD103^-$ DCs do, as $CD103^+$ DCs highly express retinal aldehyde dehydrogenase (RALDH) [70]. The enriched retinol, vitamin A, and its metabolite (retinoic acid (RA)) are highly concentrated in the small intestine. RA produced by the enzyme RALDH is essential for the induction of Tregs [21]. Moreover, TGFβ itself induces $CD11b^+CD103^+$ DCs.

The distribution of the DC subsets is different in the small intestine and colon. The proportion of $CD11b^+CD103^+$ DCs is higher in the small intestine than that in the colon, whereas the $CD11b^-CD103^+$ DCs are the major population in the colon [63]. Consistent with the abundance of $CD11b^+CD103^+$ DCs in the small intestine, RALDH activity is the highest in the small intestine. These findings highlight the fact that the induction of Tregs in the small intestine is established by the relationship between the $CD103^+$DCs and environmental factors, including RA and TGFβ.

TGFβ is produced by cells other than $CD103^+$DCs. For instance, $TLR2^+$ DCs produce TGFβ and IL-10 by polysaccharide A (PSA), and epithelial cells (ECs) also produce TGFβ by short-chain fatty acids (SCFAs) (i.e., metabolite of dietary fiber) [71, 72]. SCFAs also act

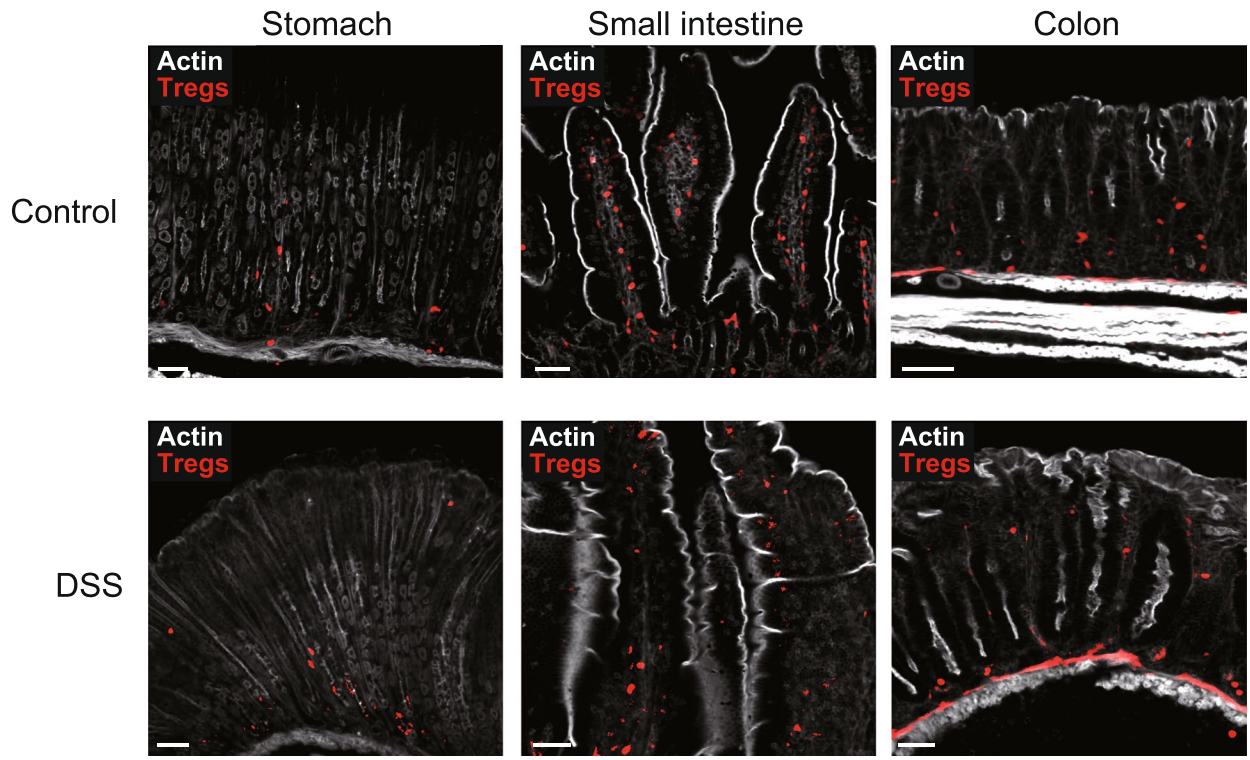

Fig. 3 The distribution of Tregs in the gastrointestinal tract. (upper panel) Localization of tdTomato^{+} Tregs (red) within the stomach (left), small intestine (middle), and colon (right). (lower panel) Localization of tdTomato^{+} Tregs (red) within stomach (left), small intestine (middle), and colon (right) after 7-day DSS administration. Vibratome sections of each tissues from *Foxp3*^{eGFPcreERT2}*Rosa26*^{tdTomato} mice were stained with phalloidin to show actin (white)

directly on CD103^{+}DCs via GPR109A [13, 73]. Recently, Rorγt^{+} innate lymphoid cells (ILC3) directly and indirectly produce Rorγt^{+} Tregs. ILC3 are activated by IL-1β^{+} macrophage [30]. MHC class 2-positive ILC3 directly induce Rorγt^{+} Tregs in colon, while CSF2 production from ILC3 indirectly induces Rorγt^{+} Tregs through the activation of CD103^{+}DCs [74–77].

A small number of Tregs in the small intestine are localized in the intraepithelial compartment, while most Tregs in the small intestine are localized in the lamina propria. Moreover, unlike stomach and colonic Tregs, Tregs in the small intestine are localize through the middle of the villi to the bottom (Fig. 3).

Large intestine

Colonic Tregs are mainly induced by microbes and their metabolites. Germ-free mice have fewer colonic Tregs compared with specific pathogen-free mice [41, 42]. *Clostridia* species induce colonic Tregs [78]. SFCAs, such as butyrate, are a fermentation by-product of fiber digestion by commensal bacteria [79].

In a recent study, T cells in the thymus recognize colonic microbe antigen, indicating that parts of the colonic Tregs are generated in the thymus [80, 81]. Colonic Treg TCRs cloned into hybridomas reactive against fecal extract were found in thymic Tregs; this supports the conjecture that some colonic Tregs are of thymic origin [82]. Colonic Tregs are located around the bottom of the crypts (Fig. 3C).

In vivo imaging enables us to track the cell movement in each tissue (movie 1). Tregs in the small intestine move in the lamina propria at a velocity of 40 μm/s, and Tregs in the colon move at a velocity of 20–30 μm/s. It is unclear why Tregs migrate at various speeds in different tissues, but one possibility may be the difference in the spaces through which they migrate among different organs; another possibility may be due to the space in which they contact with surrounding immune and non-immune cells.

TCRγδ T cells, abundant in the intraepithelial compartment, move at a speed of 30 μm/s. TCRγδ T cells interact with the intestinal epithelial cells (IECs) to detect microbe invasion and IEC damage, such as colon cancer [83, 84]. However, it remains unclear how Tregs interact with APCs and how they suppress other immune cells.

Treg localization and intestinal disease

The 3D construction of Tregs in the peripheral tissue reveals that Tregs suppress not only immune cells but also other types of cells as well, such as fibroblasts, ECs, and neurons [85, 86]. Some Tregs are located near the intestinal stem cells, which induce IL-10 to sustain the intestinal stem cells. Some Tregs located near the enteric nerve and neuronal IL-6 induce the Rorγt^{+} Tregs to sustain the intestinal homeostasis [87].

IBD, including ulcerative colitis and Crohn's disease, is characterized by chronic inflammation of the gastrointestinal tract. Although its exact mechanism is still unknown, the colitis mouse model suggests the importance of Tregs in colitis. Tregs are essential for the inhibition of colitis in the T-cell-adoptive transfer model [2, 88, 89]. IL-10-deficient mice spontaneously developed colitis [90, 91]. Despite the surge in the number of Tregs in the inflamed tissues [92], the suppressive function of Tregs is defected in these tissues.

The dextran sulfate sodium (DSS) model induced the localization of Tregs at the site of the chemical epithelial cell damage (Fig. 3). The localization of Tregs in the stomach, small intestine, and large intestine was not so different under DSS administration. Furthermore, previous studies observed that there was no change in the number of Tregs after DSS administration [2, 89]. These findings suggest that the acute epithelial damage itself does not immediately alter the localization of Tregs. We analyzed Treg localization in the early stages of the epithelial damage but not in the late or recovery stages. Moreover, we did not induce other types of inflammation, such as *Citrobacter* infection or adoptive T-cell transfer models.

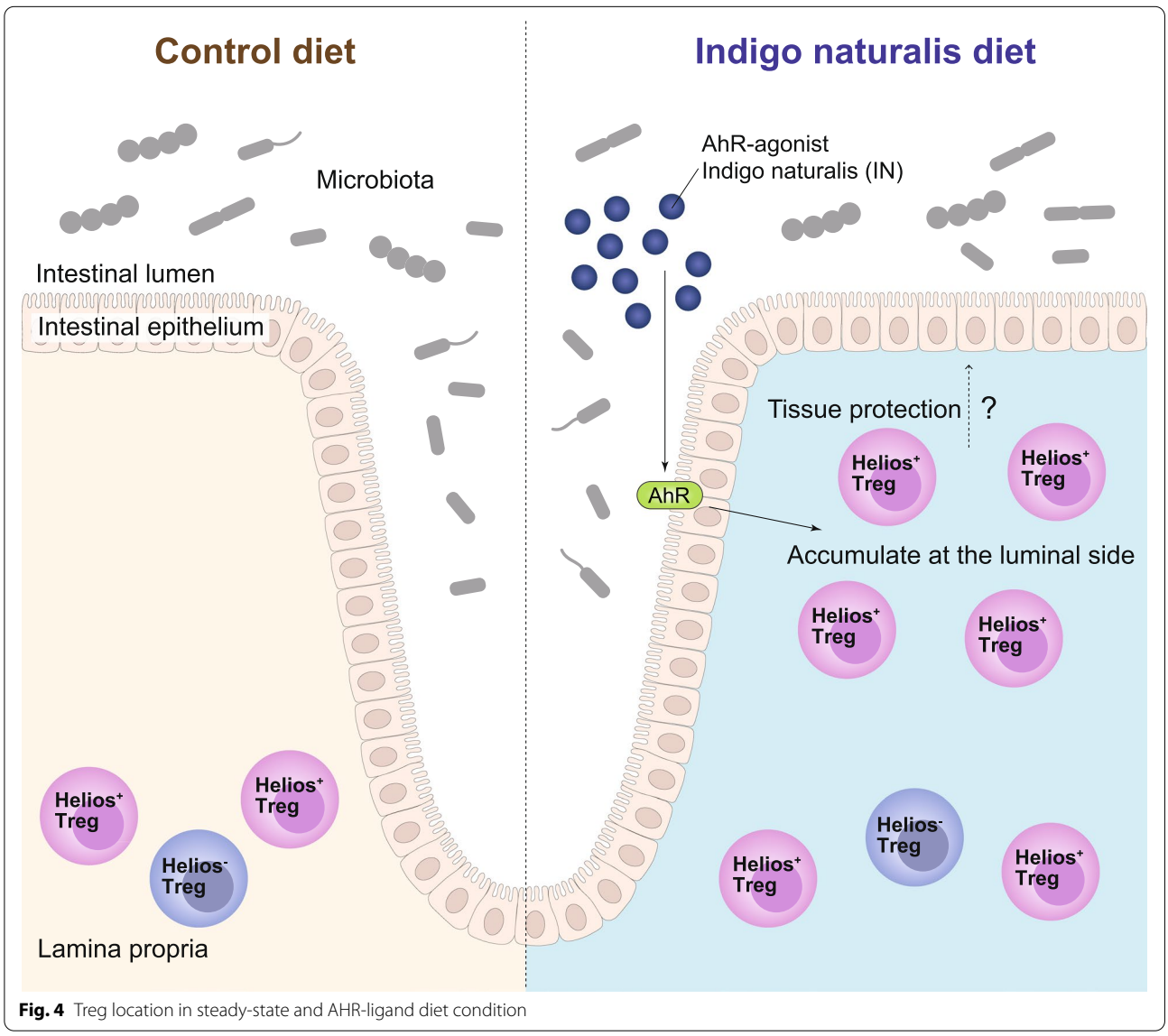

Fig. 4 Treg location in steady-state and AHR-ligand diet condition

Accordingly, further research is required to decipher how their localization is manipulated.

As previously mentioned, intestinal Tregs are generated by environmental cues (see "Treg localization and intestinal disease"); other nonimmune cells also contribute to the induction of Tregs. Stromal cells produce microbiome-dependent RA [93]. IECs educate the immune system, especially tolerogenic DCs by TGFβ and RA, to induce Tregs in humans [94].

Aryl-hydrocarbon receptor (AhR) agonist, which is fermented by green vegetables, is one of the pivotal factors in maintaining gut homeostasis. Lack of the AhR signal induces chronic inflammation in the colon [95]. Indigo naturalis (IN) is an AhR agonist containing indole derivatives, such as indigo, indirubin, and indole-3-aldehyde. IN diet increased GATA3^{lo} Helios^{+} Rorγt^{-} Tregs in the colon (IN-Tregs). Intriguingly, these IN-Tregs were located next to MHC class 2-positive ECs near the luminal side in IN-fed mice, and they moved faster than the colonic Tregs in normal diet-fed mice [56] (Fig. 4). Thus, *in vivo* imaging will help decipher how the cells migrate not only in the steady state but also in the treatment or disease state.

Conclusion

We summarized the localization and movement of Tregs in the gut, especially in the small intestine and colon. The novel technique of live imaging and the genetic development of animals have enabled us to visualize cell movement and localization. Integrating analysis of the localization/movement of immune cells with their function is required in the future. The next step in visualizing the condition of the cells will help us to understand how the immune homeostasis is regulated *in vivo*.

Abbreviations

Foxp3: Forkhead box p3; IBD: Inflammatory bowel disease; IL: Interleukin; IPEX: Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked; TGFβ: Transforming growth factor β; Tregs: Regulatory T cells; Th: T-helper cells; IL-2R: IL-2 receptor; APCs: Antigen-presenting cells; MHC: Major histocompatibility complex; TNF: Tumor necrosis factor; Roryt: Retinoid-related orphan receptor gamma t; DCs: Dendritic cells; OVA: Oral antigen ovalbumin; CTLA-4: Cytotoxic lymphocyte antigen 4; PD-1: Programmed death receptor 1; LAG3: Latent activation gene 3; RALDH: Retinal aldehyde dehydrogenase; RA: Retinoic acid; GF: Germ-free; SPF: Specific pathogen-free; SFCAs: Short-chain fatty acids; TCRs: T-cell receptor; AhR: Aryl-hydrocarbon receptor; IN: Indigo naturalis; ECS: Epithelial cells.

Supplementary Information

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Additional file 1. Movie 1. Intra-vital microscopy imaging of Tregs in small intestine and colon.

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Authors' contributions

TS wrote the manuscript. YH and KM made the figures. AC, YK, ATO, and YY reviewed the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All the data and materials are stocked in the Keio University.

Declarations

Ethics approval and consent to participate

All the experiments in this manuscript have been approved by the Keio ethical committee.

Consent for publication

Not applicable.

Competing interests

KM is an employee of the Miyarisan Pham. Co. Ltd. The other authors declare that they have no competing interests.

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