The human small intestine comprises two subsets of regulatory Foxp3+ CD4 T cells with very different life span and functional properties

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ABSTRACT

Gut resident regulatory CD4+ T (Tregs) cells in mice are mainly specific for intestinal antigens and play an important role in the suppression of immune responses against harmless dietary antigens and the gut microbiota. In contrast, information about the phenotype and function of Tregs in the human gut is limited. Here, we performed a detailed characterization of Foxp3+ CD4 Tregs in human small intestine (SI). SI Foxp3+ CD4 T cells were CD45RA-CTLA4+CD127- and suppressed proliferation of autologous T cells. Approximately 60% of SI Tregs expressed the transcription factor Helios. When stimulated, Helios- Tregs produced IL-17, IFNγ and IL-10, whereas Helios+ Tregs produced very low levels of these cytokines. Sampling mucosal tissue from transplanted human duodenum we demonstrated that donor SI Helios+ Tregs have a rapid turnover rate whereas Helios- Tregs persisted for at least 1 yr post transplantation. In the normal SI, Foxp3+ Tregs constituted only 2% of all CD4 T cells, while in active celiac disease both subsets expanded 5-10-fold. Taken together, these findings suggest that human SI contains two phenotypically and functionally distinct Treg subsets (Helios+ and Helios- Tregs), which are reminiscent of rapidly renewed dietary antigen-specific Tregs and microbiota-specific Tregs resident in the mouse gut, respectively.

Keywords: regulatory T cells (Tregs); CD4 T cells; human small intestine; Foxp3+ Tregs, transplantation; Helios+ Tregs, Celiac disease

Introduction

The intestine is a challenging environment for the local immune system, which has to respond effectively to eliminate infectious pathogens while at the same time avoiding detrimental inflammatory responses to ubiquitous food antigens and the normal gut microbiome - a function termed oral tolerance (1).The mechanisms underlying oral tolerance are not completely clear, but it is thought that Foxp3+ CD4 Tregs residing in the intestinal mucosa play a central role in this process (1, 2).

Our current knowledge about gut Tregs is mainly derived from mouse models. Tregs are found in most organs of mice, but those residing in the intestinal mucosa appear to have gut-specific phenotypes and functions (2). While Tregs in other tissues are directed towards self-antigens (3), intestinal Tregs express a T cell receptor (TCR) repertoire specific for food and microbiota antigens (2). Intestinal Tregs therefore appear well suited to avoid unwanted immune reactions to such ubiquitous antigens (1, 2, 4). As can be expected, the majority of Tregs in the mouse colon are microbial-specific (5-7), whereas most Tregs in the mouse SI respond to food antigens (4).

Tregs specific for exogenous antigens originate from naïve CD4 T cells that are induced in the periphery (pTregs). Retinoic acid produced by intestinal dendritic cells (DC) induces both a regulatory T cell phenotype and upregulates gut homing receptors (1, 8). In contrast, Tregs specific for self-antigens are thymus-derived (tTregs) (3). It has been suggested that the expression of the transcription factor Helios can distinguish tTregs from pTregs, the former being Helios+ (9). However, in a recent elegant study analyzing the occurrence of Tregs specific for mucosal antigens in humans it was found that expanded memory Foxp3+ Tregs specific for grass, birch, mite and A. fumigatus all expressed high levels of Helios(8). Therefore, at least in humans, the expression of Helios does not appear to distinguish tTreg from pTregs (10).

The importance of Foxp3+ Tregs in humans is documented by the fact that mutations of the Foxp3 gene are causative of the IPEX syndrome (11). This syndrome is characterized by a detrimental inflammatory state in many organs, including the intestine. The receptor CTLA4 plays an important role for the suppressive effect of Foxp3+ Tregs, and interestingly colitis is the most prevalent adverse effect when anti-CTLA4 monotherapy is given to treat cancer

patients (12, 13). Together, these findings strongly suggest that Tregs are important for maintaining homeostasis in the gut mucosa. However, studies directly examining Tregs in the human gut are scarce; in particular in the SI.

Here, we performed a detailed characterization of Treg phenotypes, functions, lifespan, and response to inflammation using clinical material from normal and transplanted SI as well as tissue from patients with active celiac disease

Results

Foxp3+ CD4 Tregs are scarce but functional in the human SI

In order to investigate the Treg population in human SI, we obtained single cells from enzyme-digested mucosal tissue from histologically normal SI as well as from colon and peripheral blood for comparison. As reported before (14), we found that about 10% of memory CD4 T cells expressed Foxp3 in peripheral blood and in the lamina propria (LP) of colonic mucosa (Fig. 1a). However, only a median of 2% of LP CD4 T cells in the SI expressed Foxp3 (Fig. 1a). We detected very few Foxp3+CD4 T cells in the epithelium (Supplementary Fig. S1) and subsequent analysis therefore focused on Tregs in LP only. Most SI Foxp3+ CD4 T cells expressed a memory Treg phenotype being $CD45RA^{-}CD127^{-}CTLA4^{+}$ (Fig. 1b). The transcription factor Helios was expressed by approximately 60% of Tregs in the SI, which were similar to colon and somewhat lower that in peripheral blood mononuclear cells (PBMCs) (Fig. 1c). Finding that Tregs in SI were so scarce in comparison to blood and colon we analyzed whether Treg numbers depended on age. However, when examining a cohort of adults from 20-80 yrs old (n=33) we found that the percentage of both Helios+ and Helios- Foxp3+ Tregs were independent of age in adult individuals (Supplementary Fig. S2 b and c).

To test whether SI Foxp3+ Tregs had suppressive activity, we measured their ability to inhibit T-cell proliferation when SI-derived Tregs were co-cultured with autologous naïve CD4 T cells isolated from peripheral blood. High expression of CD25 is a hallmark of Tregs, however, surface CD25 was cleaved after enzymatic treatment during the sample preparation. In order to sort Tregs based on CD25 expression, we therefore cultured tissue-derived single cells for Tregs to regain CD25 expression. After overnight culture we found that 2-3% of CD4 T cells expressed CD25 and all of these were $CD127^{neg}$. Further phenotyping showed that the vast majority of CD25 +$CD127^{neg}$ cells expressed Foxp3 and more than half expressed Helios;

phenotype reminiscent of Tregs (Fig. 2a). Sorted $CD25^{+}CD127^{neg}$ CD4 Tregs were then stimulated with anti-CD3/CD28-conjugated beads for 48 h. Naïve CD4 T cells proliferated extensively in response to CD3/CD28 TCR stimulation, but when co-cultured with activated SI-derived Tregs the proliferative response was strongly suppressed (Fig. 2b).

Together, these findings show that although less abundant than in colon and blood, Foxp3+ CD4 Tregs in human SI have strong suppressive capacity.

Helios+ and Helios- Tregs display different replacement kinetics in the transplanted SI

Studies in mice have shown that a large population of SI CD4 Tregs are dynamic cells with tissue half-life of 4-6 weeks (4). To study the turnover of Tregs in human SI we examined their replacement kinetics in transplanted duodenum. We obtained duodenal biopsies from type I diabetes patients undergoing pancreas transplantation with a duodenal segment (Fig. 3a and (15)). Protocol biopsies were obtained from the duodenal graft and recipient (native) duodenum at 3, 6 and 52 weeks after transplantation. Only patients without clinical and histological signs of rejection were included. This approach provided a unique possibility to study the lifespan of tissue-resident cells directly because there is no recruitment of donor cells from the circulation (16-18). Because most donors and recipients express different HLA class I molecules, donor and recipient can be readily distinguished by flow cytometry (Fig. 3b) (16-18). We recently reported that the majority of CD8 and CD4 T cells were still of donor origin 52 weeks after transplantation (16)((19)). Here we showed that the persistency of Helios- CD4 Tregs was similar to total CD4 T cells (Fig 3c, (19)), whereas the lifespan of Helios+ CD4 Tregs was dramatically shorter with virtually no remaining cells 1 yr post-transplantation (Fig. 3c). Importantly, in the native (non-transplanted) duodenum both Treg subsets were present at all time-points (Fig. 3c).

The different turnover rate between Helios- and Helios+ Tregs may have several explanations, including different egress rate into the draining lymphatics and/or different proliferative capacity *in situ*. SI CD4 resident memory T (Trm) cells are CD69+ CD103+/- CCR7- (19); receptors involved in retention and tissue egress (20) (21, 22)). In order to indirectly compare the potential of the two Treg subsets to egress from the tissue we analyzed the expression of these markers on CD4 T cells isolated from histologically normal SI mucosa. Interestingly, Helios+ Tregs expressed significantly lower levels of CD69 and

CD103 than Foxp3- CD4 T cells and Helios- Tregs, while both Tregs subsets expressed significantly more CCR7 than Foxp3- CD4 T cells (Fig. 4a). To determine their proliferative capacity we analyzed the expression of the proliferation marker Ki-67. Whereas less than 2% of Foxp3-Helios- CD4 T cells expressed Ki67, approximately 10% of both of Treg subset expressed this marker (Fig. 4b).

Together this shows that Helios+ and Helios- Tregs have different survival rates in the transplanted SI. Helios- Tregs persist for more than one year whereas Helios+ Tregs are more rapidly depleted from the tissue. This may to some extent depend on their expression of retention and migration elements such as CD69 and CD103 and CCR7.

Both SI Treg subsets increase dramatically in active celiac disease

Next, we wanted to examine how SI Tregs responded under inflammatory conditions. To this end, we obtained SI biopsies from patients with newly diagnosed active celiac disease (n=8); a common chronic inflammatory condition in human SI caused by intake of dietary gluten in genetically predisposed individuals. Analysis of tissue-derived single cell preparations from active celiac lesions showed that nearly 20% of all CD4 T cells expressed Foxp3, of which 60-70% co-expressed Helios (Fig. 5a).

We were not able to sort sufficient numbers of Tregs from celiac tissue to test their suppressive capacity. However, we and others have shown that Helios+ and Helios- Tregs can be functionally separated by their ability to produce cytokines (23-25). To test their cytokine producing capacity we stimulated tissue-derived single cells from normal SI and active celiac lesions with PMA/ionomycin. Helios+ Tregs from both normal and celiac SI produced very low levels of the cytokines measured, including IFNγ, IL-17 and IL-10 (Fig 5b). In contrast, Helios- Tregs produced significant levels of all cytokines tested compared to Helios+ Tregs. Interestingly, Helios- Tregs from normal SI produced significantly more IL-17 than Foxp3- CD4 T cells and their counterparts in the celiac lesion. Moreover, a significantly higher fraction of Helios- Tregs produced IL-10 compared to Foxp3- CD4 T cells; the highest percentage found in Tregs derived from celiac lesions. Helios- Tregs produced similar amounts of IFNγ in normal and celiac tissue, but less than Foxp3- CD4 T cells (Fig. 5b).

Taken together, these findings show that there was a large increase in both Helios+ and Helios- Tregs in celiac lesions. Their ability to produce cytokines closely mirrored their

counterparts in the normal SI, strongly suggesting that Foxp3+ CD4 T cells in the celiac lesion are functionally similar to Foxp3+ Tregs derived from in the normal SI.

Discussion

Here we show that the human SI contains suppressive Foxp3+ CD4 Tregs that comprise two functionally distinct subsets distinguished by expression of the transcription factor Helios. Helios- Tregs show long-term residency in SI and produce substantial levels of both pro- and anti-inflammatory cytokines in response to stimulation, whereas Helios+ Tregs are rapidly replaced in the mucosa and produce very low levels of cytokines.

Recent reports have demonstrated that a large proportion of effector T cells in non-lymphoid tissues are long-lived Trm cells without the capacity to recirculate (26). However, to what extent tissue Tregs are migratory or resident is less studied. Using a unique human transplantation setting, in which a segment of the duodenum is grafted, we find that approximately 60% of CD4 T cells were still of donor origin 1 yr after transplantation (19), suggesting that the majority of CD4 T cells are long-lived Trm cells. In this study, we find that the fraction of donor-derived Helios- CD4 Tregs constituted 1-2% of donor CD4 T cells at 3, 6, and 52 weeks post-transplantation, demonstrating that SI Helios- Tregs are very persistent. Helios+ Tregs, in contrast, turned over much faster and at 1 yr post-transplantation donor-derived Helios+ Tregs were virtually absent in the transplanted duodenum. CD69 has been shown to inhibit lymphocyte egress from tissues (20) and whereas virtually all CD4 Trm cells expressed CD69 (19), less than 40% of Helios+ Tregs expressed this protein. Moreover, Helios+ Tregs were virtually negative for CD103, but expressed significantly higher levels of CCR7 than CD4 Trm cells. Conversely, more than half of Helios- Tregs were CD69+, and a significant fraction expressed CD103. This phenotypic signature suggest that Helios+ Tregs may have enhanced capacity for tissue egress, which may partly explain why Helios+ Tregs were depleted much faster from the tissue than their Helios- counterparts.

A recent report studying Tregs in the SI of mice showed that SI Tregs could be separated based on their expression of the transcription factor RORγt (4). Moreover, whereas the RORγt + Tregs was induced from conventional T cells in the periphery (pTregs) by commensal microbiota, the RORγt- subset was induced by ingested protein antigens and repressed an underlying strong immunity to such proteins. Interestingly, these dietary antigen-specific

RORγt- pTregs were continuously generated and replaced with a half-life of 4 to 6 weeks (4), reminiscent of SI Helios+ Tregs in humans. Many studies have suggested that Helios is a marker for natural (thymus-derived) tTregs (9) . However, an elegant recent study showed that the vast majority of human Tregs specific for various aeroantigens expressed Helios (8). Thus, it is tempting to speculate that SI Helios+ Tregs are human equivalents of dietary antigen-specific pTregs in the SI of mice.

Human SI Helios- Tregs, on the other hand, displayed turnover dynamics similar to non-circulating CD4 and CD8 Trm cells (19) (16), and persisted for >1 year in transplanted SI. Moreover, whereas few Helios+ Tregs produced cytokines following activation, Helios- Tregs cells produced substantial levels of IL-17, IFNγ and IL-10. In fact, Helios- Tregs derived from normal SI showed higher capacity for cytokine production than their CD4 Trm counterparts. Several studies have shown that human Tregs with suppressive functions produce high levels of IL-17 (27-29), in particular in inflammatory diseases such as ulcerative colitis and Crohns disease (27, 28). Interestingly, it has been reported that treatment with anti-IL-17 exacerbate inflammatory bowel disease (30), suggesting that IL-17 may have a protective function in the gut. Moreover, a large fraction of IL-17-producing Helios- Tregs express the Th17 transcription factor RORγt, both in the human and mouse gut (29, 31). In contrast to dietary antigen-specific RORγt- pTregs (4), RORγt + Tregs in the intestine of mice are microbiota-specific (4, 32) and this subset has been shown produce IL-17 in the SI, but not in the colon (31).

IL-10 is thought to play an important role in Treg-mediated suppression. Surprisingly, very few SI Tregs produced IL-10 in response to activation, compared to Tregs in colon and airway mucosa (25, 33). Interestingly, when IL-10 was selectively deleted in Foxp3+ Tregs in mice, the animals developed severe colitis and inflammation in the lungs, but no overt inflammation in the SI (34). Moreover, inherited deficiencies of IL-10 orIL-10 receptor lead to life-threatening early onset colitis and airway inflammation, but no inflammation in the SI (35). In the latter situation allogeneic stem cell transplantation proved to be effective, indicating that the IL-10/IL-10 receptor deficiency affects hematopoietic cells (35). Also, IL-10 producing Foxp3-CD4 T cells, termed Tr1 cells, were found to be very infrequent in the SI in comparison to the colon and nasal mucosa (25, 33). Together, this suggests that IL-10, produced by Tregs and Tr1, play a less important role to maintain homeostasis in the SI

compared with other mucosal sites. It is well documented that the microbiota regulates the number and function of intestinal Tregs (32), and given the enormously different numbers of commensal bacteria between the SI and colon (36), this may explain why the density of Tregs and their capacity to produce IL-10 is lower in the SI.

Tregs in the SI of human adults (>20 yrs of age) constituted only 2% of all CD4 T cells, which is very low compared to approximately 5-10% in colon and other peripheral tissues (25, 37). However, in a recent study comparing tissue-resident Tregs in pediatric and adult organ donors it was found that the fractions of Tregs were age-dependent (38). In fact, the percentage of Tregs in the SI of infants under 2 yrs were 15-20% of all CD4 T cells, whereas adult SI contained approximately 2% of CD4 T cells as Tregs. Together, these results suggest that maintenance of homeostasis (oral tolerance) in the SI is less dependent on Tregs in adulthood compared with the first years of life.

In striking contrast, we found that the number of Tregs increased dramatically in active celiac disease lesions and constituted almost 20% of total CD4 T cells. A role for Tregs in celiac disease has been disputed, but a recent study reported that the vast majority of gluten-specific CD4 T cells in blood of gluten-challenged celiac patients were Foxp3+ Tregs, albeit with impaired suppressive function (39). Other studies have shown a similar increase of Foxp3+ Tregs numbers in the celiac lesion, and found that the suppressive action of Tregs is reduced by local production of IL-15 (40). Our study clearly shows that SI Tregs are highly expanded in response to inflammation, and in active celiac disease both Tregs and Foxp3- CD4 T cells (putative Tr1) contained significantly higher fractions of IL-10 producing cells than in normal tissue.

Together, in line with the current knowledge about the function of Treg populations in the mouse gut, we show that the human SI contains two distinct Treg subsets: a dynamic population of Helios+ Tregs that may play an important role in tolerance to food antigens, and a population of tissue-resident Helios- Tregs that contribute to immune tolerance to the gut microbiota. Differences in Treg abundance and functions between different mucosal sites should be taken into consideration when Tregs are targeted to treat inflammatory disorders.

Materials and methods

Subjects and biological material

Proximal jejunum tissue was resected from non-pathological SI during surgery for cancer in the pancreas or distal bile duct with a Whipple procedure (pancreatoduodenectomy) (n = 18) or from donor and recipient duodenum during pancreas transplantation of type I diabetes mellitus patients (donors: n = 14; recipients, n=12; Horneland et al., 2015). Endoscopic biopsies were obtained from donor and patient duodenum at 3, 6, and 52 wk after transplantation. Colonic biopsies were obtained by colonoscopy of individuals with unexplained stomach pain with normal histology (n=3). All samples were evaluated by an experienced pathologist and only material with normal histology was included. Duodenal biopsies were also obtained from newly diagnosed untreated celiac patients (n=8). The study was approved by the Regional Committee for Medical Research Ethics in Southeast Norway (# 2012/341, 2010/2720, and 2012/2278) and the Privacy Ombudsman for Research at Oslo University Hospital–Rikshospitalet and complies with the Declaration of Helsinki. All participants gave their written informed consent. Resected SI was opened longitudinally and rinsed thoroughly in PBS, and mucosal folds were dissected off the submucosa. To obtain single-cell suspensions, epithelial cells were removed by washing in PBS containing 2 mM EDTA three times for 20 min at 37°C, and the lamina propria was minced and digested in RPMI medium containing 2.5 mg/ml Liberase and 20 U/ml DNase I (both from Roche) at 37°C for 1 h. Digested tissue was passed through 100-µm cell strainers (Falcon) and washed three times in PBS. PBMC were isolated by lymphoprep gradient centrifugation of blood from patients or buffycoats from healthy donors from the Oslo University Hospital Blood Center. Identically treated PBMCs served as controls for the effect of collagenase on epitopes recognized by antibodies used in flow cytometry.

Flow cytometric analysis

Cells were stained with Fixable Viability Dye eFluor 780 (1 µl/10^6 cells, eBioscience, San Diego, CA) for 30 min at 4°C, followed by surface staining with antibodies to CD3 (clone SK7), CD4 (clone OKT4), CD8 (clone SK1) and CD127 (clone M21). To detect intracellular cytokines, cells were treated with FOXP3/transcription factor staining buffer set according to the manufacturers protocol (eBioscience) and stained with antibodies to FOXP3 (clone 236A/E7),

Helios (clone 22F6), and IL-17 (clone 64DEC) from eBioscience, or CTLA-4 (clone L3DTO), IFN-g (clone 4S.B3), and IL-10 (clone JES3-9D7) from Biolegend. All antibodies were incubated for 30 min at 4°C. Flow cytometry was performed on a BD LSRFortessa (BD Biosciences), and analyzed using FlowJo 10.3 software (Tree Star, Eugene, OR). Gates for surface markers were set based on FMO controls, and cytokines gates was based on untreated cells.

Cytokine analysis

To assess the cytokine production by mucosal T cells, dispersed cells were cultured for 4 h in RPMI/10% fetal calf serum (FCS) with 1.5 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin (all from Sigma-Aldrich, St Louis, Mo) with Golgi-Stop (BD Bioscience, San Jose, CA) added after 1 h of stimulation to allow intracellular accumulation of cytokines.

Treg suppression assay

Tregs were defined as $CD25^{+}CD127^{-}$ $CD4^{+}$ T cells. Autologous $CD25^{-}CD127^{+}CD45RA^{+}$ naïve CD4+ T cells were used as responder T cells (Tresp). Sorted Tregs were pre-activated for 48h using α-CD3/CD28 coated beads (Dynabeads Human T-Activator, Thermo Fisher scientific) in a 1:1 ratio (beads to cells). Tregs and CFSE- (1.5µM, Thermo Fisher scientific) labeled Tresp cells were mixed in a 1:2 (Treg:Tresp) ratio and stimulated with α-CD3/CD28 coated beads in a 1:2 ratio (beads:cells). CFSE dilution in Tresp was analyzed after 4 days of co-culture by flow cytometry.

Figure Legends

Fig. 1: CD4 Tregs are scarce in the SI. a) Representative contour plots showing the expression of CD4 and Foxp3 on CD3+ T cells in the lamina propria (LP) SI, colon, and PBMCs (gated on CD45RO). Compiled data are shown (right panel, gated on CD4). b) Representative contour plots showing the expression of Foxp3, CD45RA, CD127 and CTLA-4 in SI (LP) CD4+ T cells. **c)** Representative contour plots showing the expression of Helios and Foxp3 on CD4+ T cells from SI (LP), colon and PBMCs (gated on CD45RO). Compiled data are shown (right panel, gated on Foxp3+CD4 T cells). One-way ANOVA with Dunnett's multiple comparisons test, *** $p<0.001$; non significant (ns).

Fig. 2: SI Tregs suppress autologous naïve T cells. a) Contour plot showing the expression of CD127 and CD25 on SI CD4 T cells cultured overnight (left) and expression of Foxp3 and

Helios gated on CD25+ CD127- cells (right). b) Histograms showing the expression of CFSE on blood-derived CFSE-labelled conventional CD4 T cells ($CD25^{-}CD127^{+}CD45RA^{+}$) stimulated with CD3/CD28 beads for 4 days without (left) or with autologous SI -derived Tregs ($CD25^{+}CD127^{-}CD4^{+}$). Representative of three independent experiments.

Fig. 3: Helios+ Tregs are short-lived in transplanted SI. a) Drawing showing the pancreaticoduodenal transplantation procedure. b) Donor and recipient Foxp3+CD4 T cells in transplanted duodenum were distinguished based on disparate HLA class I. Representative contour plot showing the expression of Helios and HLA-A3 on SI-derived Foxp3+ CD4 T cells 52 weeks after transplantation (donor $HLA\text{-}A3^{+}$; recipient $HLA\text{-}A3^{-}$). c) Percentage of Helios+Foxp3+ and Helios- Foxp3+ CD4 T cells in transplanted (left, gated on donor CD4 T cells) and recipient duodenum (right), respectively, at 0, 3, 6, and 52 weeks after transplantation. One-way ANOVA with Dunnett's multiple comparisons test, *** $p<0.001$; *$p<0.05$; non significant (ns).

Fig. 4: Helios+ Tregs express less CD69 and CD103 and more CCR7 than Trm cells. a) Representative contour plots of SI-derived cells showing the expression of Foxp3, CD69, CD103, and CCR7 on CD4 T cells (upper) and expression of Helios, CD69, CD103, and CCR7 on Foxp3+ CD4 T cells (middle). Compiled data are shown (lower). b) Representative contour plots showing the expression of Ki67 on SI-derived CD4 and Foxp3+ CD4 T cells. Compiled data are shown (right). One-way ANOVA with Dunnett's multiple comparisons test, *** $p<0.001$; ** $p<0.01$; *$p<0.05$; non significant (ns).

Fig. 5: Helios+Foxp3+ and Helios-Foxp3+ CD4 T cells are dramatically increased in untreated celiac disease. a) Representative contour plots showing expression of Helios and Foxp3 on CD4 T cells derived from normal SI (left) and untreated celiac disease (right). Compiled data are shown (lower). b) Cytokine production in Foxp3-Helios-, Foxp3+Helios+, and Foxp3+Helios- CD4 T cells derived from normal and untreated celiac SI following 4h stimulation with PMA/ionomycin. Student's t-test, *** $p<0.001$; ** $p<0.01$; *$p<0.05$; non significant (ns).

References:

1. Mowat AM. 2018. To respond or not to respond - a personal perspective of intestinal tolerance. *Nat Rev Immunol* 18: 405-15
2. Tanoue T, Atarashi K, Honda K. 2016. Development and maintenance of intestinal regulatory T cells. *Nat Rev Immunol* 16: 295-309
3. Sakaguchi S, Powrie F, Ransohoff RM. 2012. Re-establishing immunological self-tolerance in autoimmune disease. *Nat Med* 18: 54-8
4. Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, Lee JY, Lee M, Surh CD. 2016. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science* 351: 858-63
5. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov, II, Umesaki Y, Itoh K, Honda K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 331: 337-41
6. Campbell C, Dikiy S, Bhattarai SK, Chinen T, Matheis F, Calafiore M, Hoyos B, Hanash A, Mucida D, Bucci V, Rudensky AY. 2018. Extrathymically Generated Regulatory T Cells Establish a Niche for Intestinal Border-Dwelling Bacteria and Affect Physiologic Metabolite Balance. *Immunity* 48: 1245-57 e9
7. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS. 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature* 478: 250-4
8. Bacher P, Heinrich F, Stervbo U, Nienen M, Vahldieck M, Iwert C, Vogt K, Kollet J, Babel N, Sawitzki B, Schwarz C, Bereswill S, Heimesaat MM, Heine G, Gadermaier G, Asam C, Assenmacher M, Kniemeyer O, Brakhage AA, Ferreira F, Wallner M, Worm M, Scheffold A. 2016. Regulatory T Cell Specificity Directs Tolerance versus Allergy against Aeroantigens in Humans. *Cell* 167: 1067-78.e16
9. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *Journal of immunology* 184: 3433-41
10. Thornton AM, Shevach EM. 2019. Helios: still behind the clouds. *Immunology* 158: 161-70
11. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27: 20-1
12. Alissafi T, Hatzioannou A, Legaki AI, Varveri A, Verginis P. 2019. Balancing cancer immunotherapy and immune-related adverse events: The emerging role of regulatory T cells. *J Autoimmun* 104: 102310
13. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363: 711-23
14. Saito T, Nishikawa H, Wada H, Nagano Y, Sugiyama D, Atarashi K, Maeda Y, Hamaguchi M, Ohkura N, Sato E, Nagase H, Nishimura J, Yamamoto H, Takiguchi S, Tanoue T, Suda W, Morita H, Hattori M, Honda K, Mori M, Doki Y, Sakaguchi S. 2016. Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med* 22: 679-84
15. Horneland R, Paulsen V, Lindahl JP, Grzyb K, Eide TJ, Lundin K, Aabakken L, Jenssen T, Aandahl EM, Foss A, Oyen O. 2015. Pancreas transplantation with enteroanastomosis to native duodenum poses technical challenges--but offers improved endoscopic access for scheduled biopsies and therapeutic interventions. *Am J Transplant* 15: 242-50

16. Bartolome-Casado R, Landsverk OJB, Chauhan SK, Richter L, Phung D, Greiff V, Risnes LF, Yao Y, Neumann RS, Yaqub S, Oyen O, Horneland R, Aandahl EM, Paulsen V, Sollid LM, Qiao SW, Baekkevold ES, Jahnsen FL. 2019. Resident memory CD8 T cells persist for years in human small intestine. *J Exp Med* 216: 2412-26
17. Bujko A, Atlasy N, Landsverk OJB, Richter L, Yaqub S, Horneland R, Oyen O, Aandahl EM, Aabakken L, Stunnenberg HG, Baekkevold ES, Jahnsen FL. 2018. Transcriptional and functional profiling defines human small intestinal macrophage subsets. *J Exp Med* 215: 441-58
18. Landsverk OJ, Snir O, Casado RB, Richter L, Mold JE, Reu P, Horneland R, Paulsen V, Yaqub S, Aandahl EM, Oyen OM, Thorarensen HS, Salehpour M, Possnert G, Frisen J, Sollid LM, Baekkevold ES, Jahnsen FL. 2017. Antibody-secreting plasma cells persist for decades in human intestine. *J Exp Med* 214: 309-17
19. Bartolome-Casado R, Landsverk OJB, Chauhan SK, Yaqub S, Oyen O, Horneland R, Aandahl EM, Paulsen V, Baekkevold ES, Jahnsen FL. 2019. CD4+ T cells persist for years in the human small intestine and mediate robust TH1 immunity. *BioRxiv*
20. Shiow LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, Cyster JG, Matloubian M. 2006. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 440: 540-4
21. Baekkevold ES, Yamanaka T, Palframan RT, Carlsen HS, Reinholt FP, von Andrian UH, Brandtzaeg P, Haraldsen G. 2001. The ccr7 ligand elc (ccl19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J Exp Med* 193: 1105-12.
22. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. 1994. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* 372: 190-3
23. Raffin C, Pignon P, Celse C, Debien E, Valmori D, Ayyoub M. 2013. Human Memory Helios-FOXP3+ Regulatory T Cells (Tregs) Encompass Induced Tregs That Express Aiolos and Respond to IL-1beta by Downregulating Their Suppressor Functions. *Journal of immunology* 191: 4619-27
24. Mercer F, Khaitan A, Kozhaya L, Aberg JA, Unutmaz D. 2014. Differentiation of IL-17-producing effector and regulatory human T cells from lineage-committed naive precursors. *J Immunol* 193: 1047-54
25. Ballke C, Gran E, Baekkevold ES, Jahnsen FL. 2016. Characterization of Regulatory T-Cell Markers in CD4+ T Cells of the Upper Airway Mucosa. *PLoS One* 11: e0148826
26. Masopust D, Soerens AG. 2019. Tissue-Resident T Cells and Other Resident Leukocytes. *Annu Rev Immunol* 37: 521-46
27. Hovhannisyan Z, Treatman J, Littman DR, Mayer L. 2011. Characterization of interleukin-17-producing regulatory T cells in inflamed intestinal mucosa from patients with inflammatory bowel diseases. *Gastroenterology* 140: 957-65
28. Jung MK, Kwak JE, Shin EC. 2017. IL-17A-Producing Foxp3(+) Regulatory T Cells and Human Diseases. *Immune Netw* 17: 276-86
29. Pesenacker AM, Broady R, Levings MK. 2015. Control of tissue-localized immune responses by human regulatory T cells. *Eur J Immunol* 45: 333-43
30. Pappu R, Rutz S, Ouyang W. 2012. Regulation of epithelial immunity by IL-17 family cytokines. *Trends Immunol* 33: 343-9
31. 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. 2015. MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. *Science* 349: 993-7
32. Hegazy AN, Powrie F. 2015. MICROBIOME. Microbiota RORgulates intestinal suppressor T cells. *Science* 349: 929-30
33. Alfen JS, Larghi P, Facciotti F, Gagliani N, Bosotti R, Paroni M, Maglie S, Gruarin P, Vasco CM, Ranzani V, Frusteri C, Iseppon A, Moro M, Crosti MC, Gatti S, Pagani M, Caprioli F, Abrignani

S, Flavell RA, Geginat J. 2018. Intestinal IFN-gamma-producing type 1 regulatory T cells coexpress CCR5 and programmed cell death protein 1 and downregulate IL-10 in the inflamed guts of patients with inflammatory bowel disease. *J Allergy Clin Immunol* 142: 1537-47 e8

34. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR, Jr., Muller W, Rudensky AY. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28: 546-58
35. Engelhardt KR, Shah N, Faizura-Yeop I, Kocacik Uygun DF, Frede N, Muise AM, Shteyer E, Filiz S, Chee R, Elawad M, Hartmann B, Arkwright PD, Dvorak C, Klein C, Puck JM, Grimbacher B, Glocker EO. 2013. Clinical outcome in IL-10- and IL-10 receptor-deficient patients with or without hematopoietic stem cell transplantation. *J Allergy Clin Immunol* 131: 825-30
36. Mowat AM, Agace WW. 2014. Regional specialization within the intestinal immune system. *Nat Rev Immunol* 14: 667-85
37. Sharabi A, Tsokos MG, Ding Y, Malek TR, Klatzmann D, Tsokos GC. 2018. Regulatory T cells in the treatment of disease. *Nat Rev Drug Discov* 17: 823-44
38. Thome JJ, Bickham KL, Ohmura Y, Kubota M, Matsuoka N, Gordon C, Granot T, Griesemer A, Lerner H, Kato T, Farber DL. 2016. Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. *Nat Med* 22: 72-7
39. Cook L, Munier CML, Seddiki N, van Bockel D, Ontiveros N, Hardy MY, Gillies JK, Levings MK, Reid HH, Petersen J, Rossjohn J, Anderson RP, Zaunders JJ, Tye-Din JA, Kelleher AD. 2017. Circulating gluten-specific FOXP3(+)CD39(+) regulatory T cells have impaired suppressive function in patients with celiac disease. *J Allergy Clin Immunol* 140: 1592-603 e8
40. Zanzi D, Stefanile R, Santagata S, Iaffaldano L, Iaquinto G, Giardullo N, Lania G, Vigliano I, Vera AR, Ferrara K, Auricchio S, Troncone R, Mazzarella G. 2011. IL-15 interferes with suppressive activity of intestinal regulatory T cells expanded in Celiac disease. *Am J Gastroenterol* 106: 1308-17

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Fig. 1

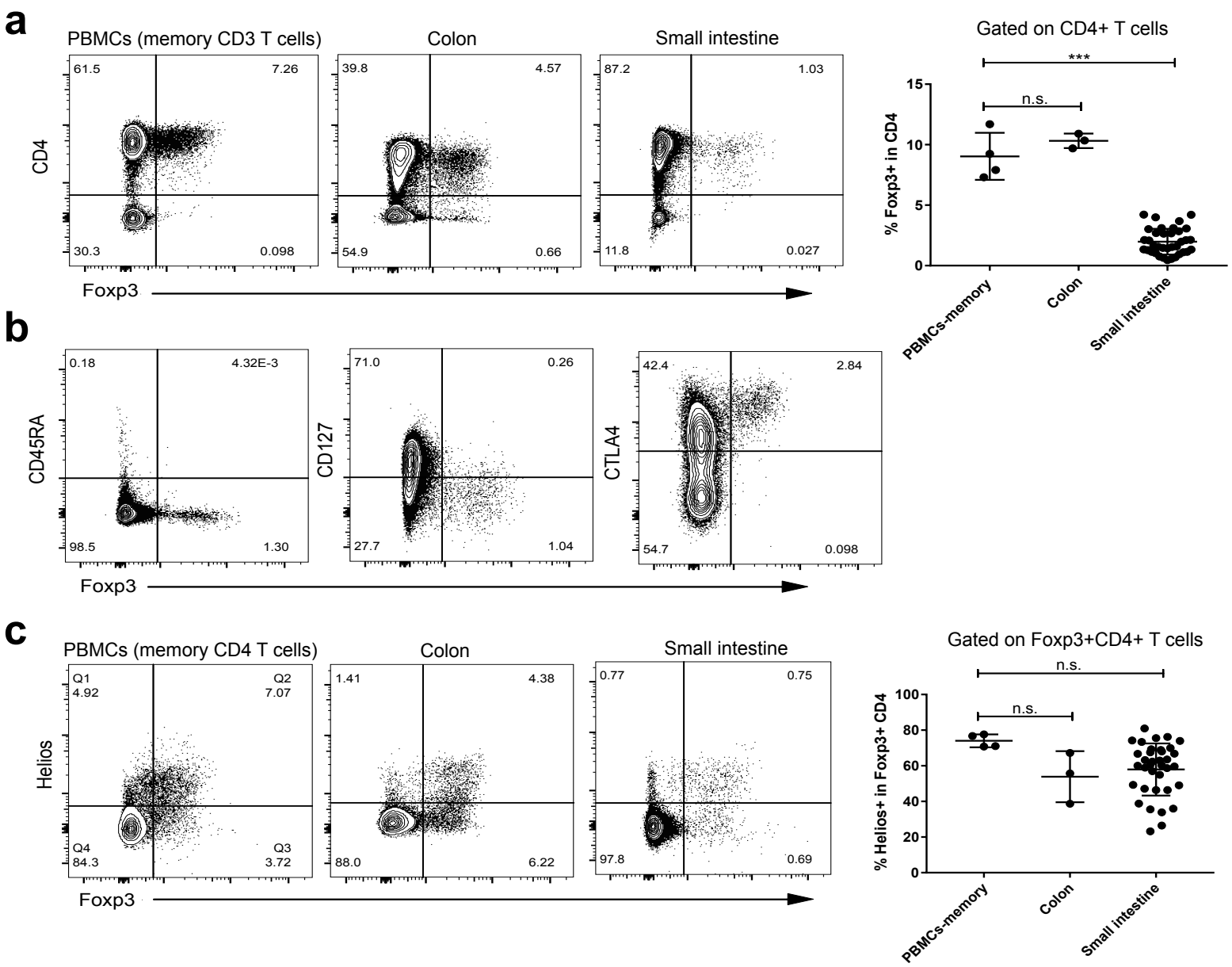

Fig. 2

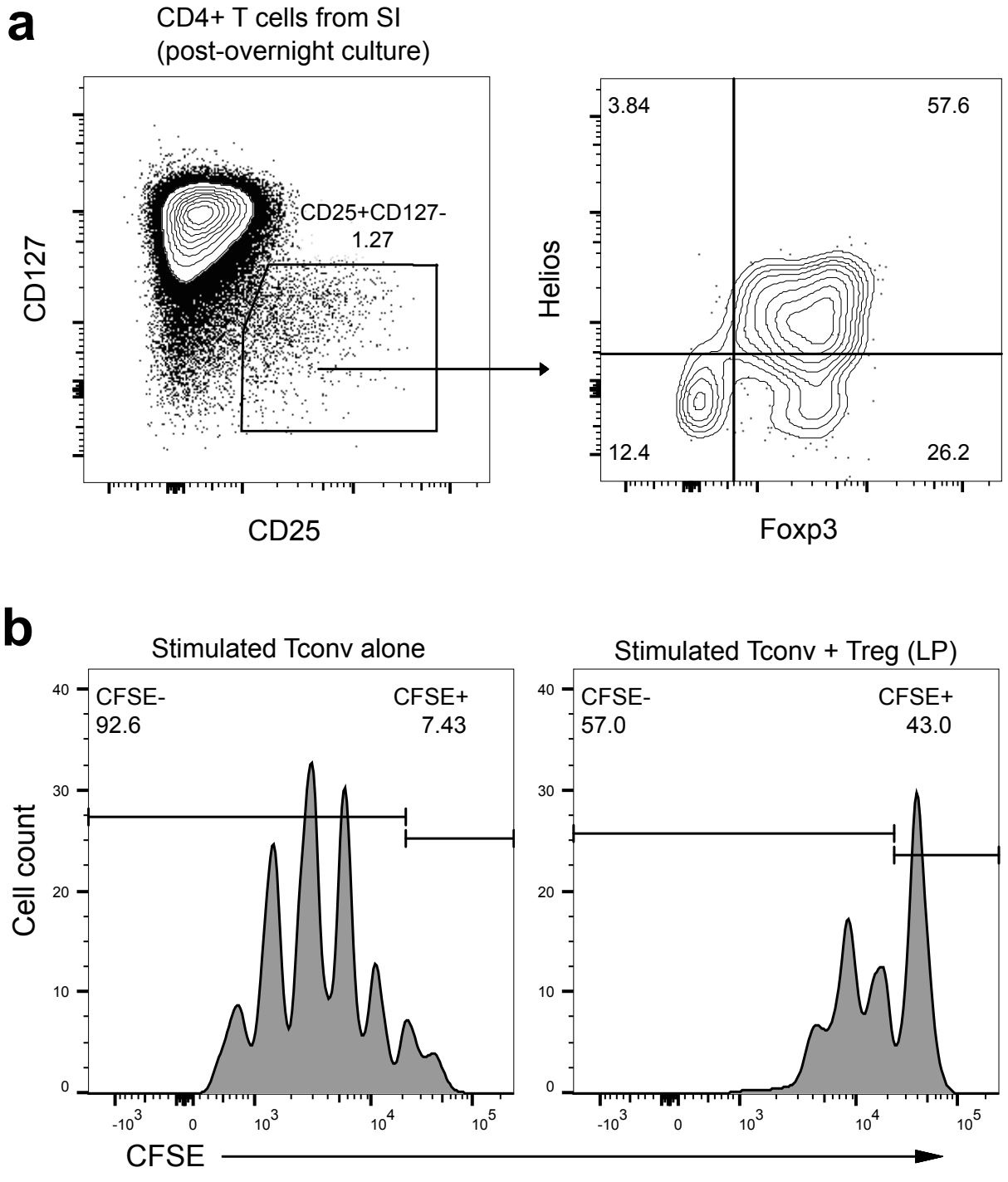

Fig. 3

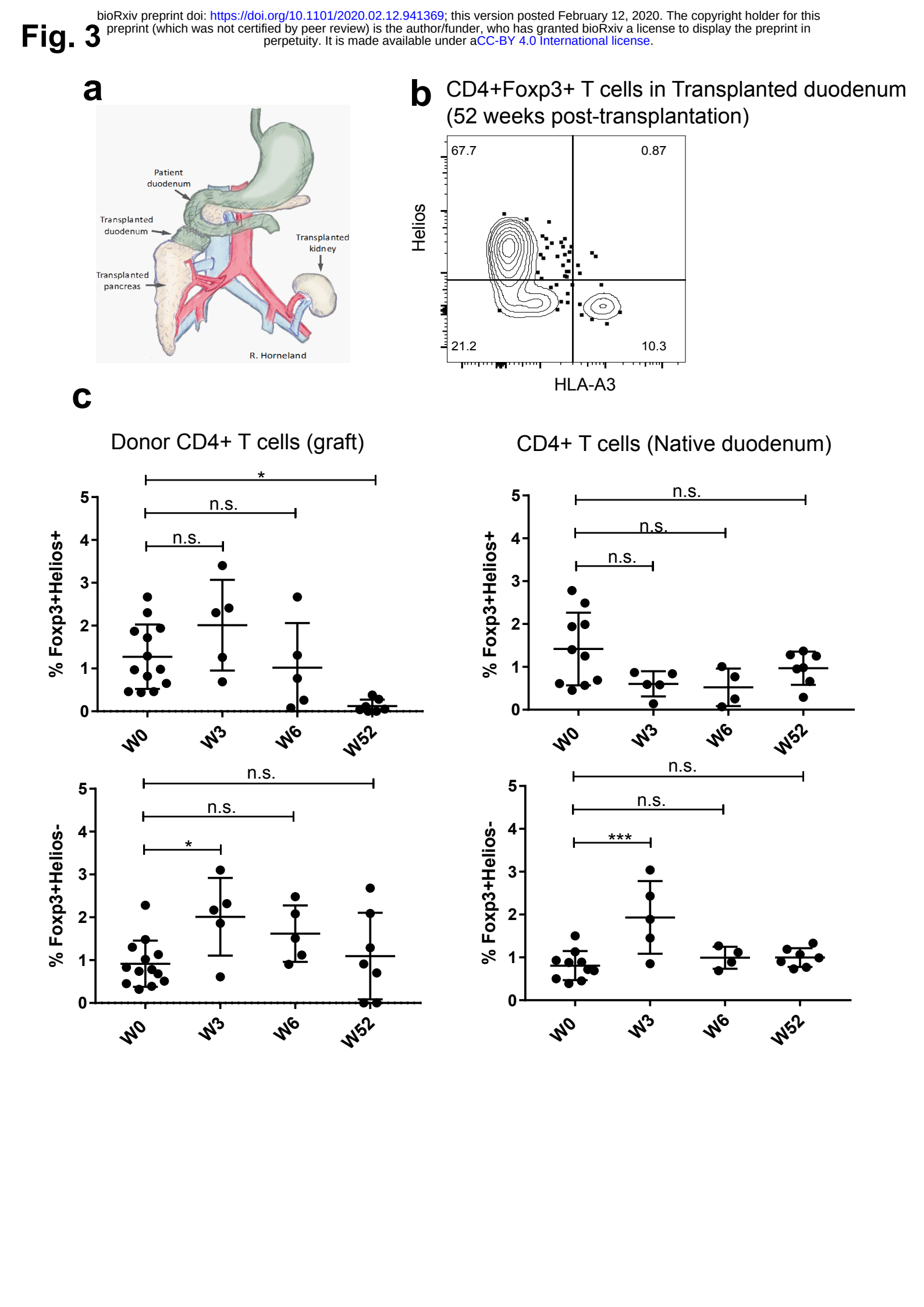

Fig. 4

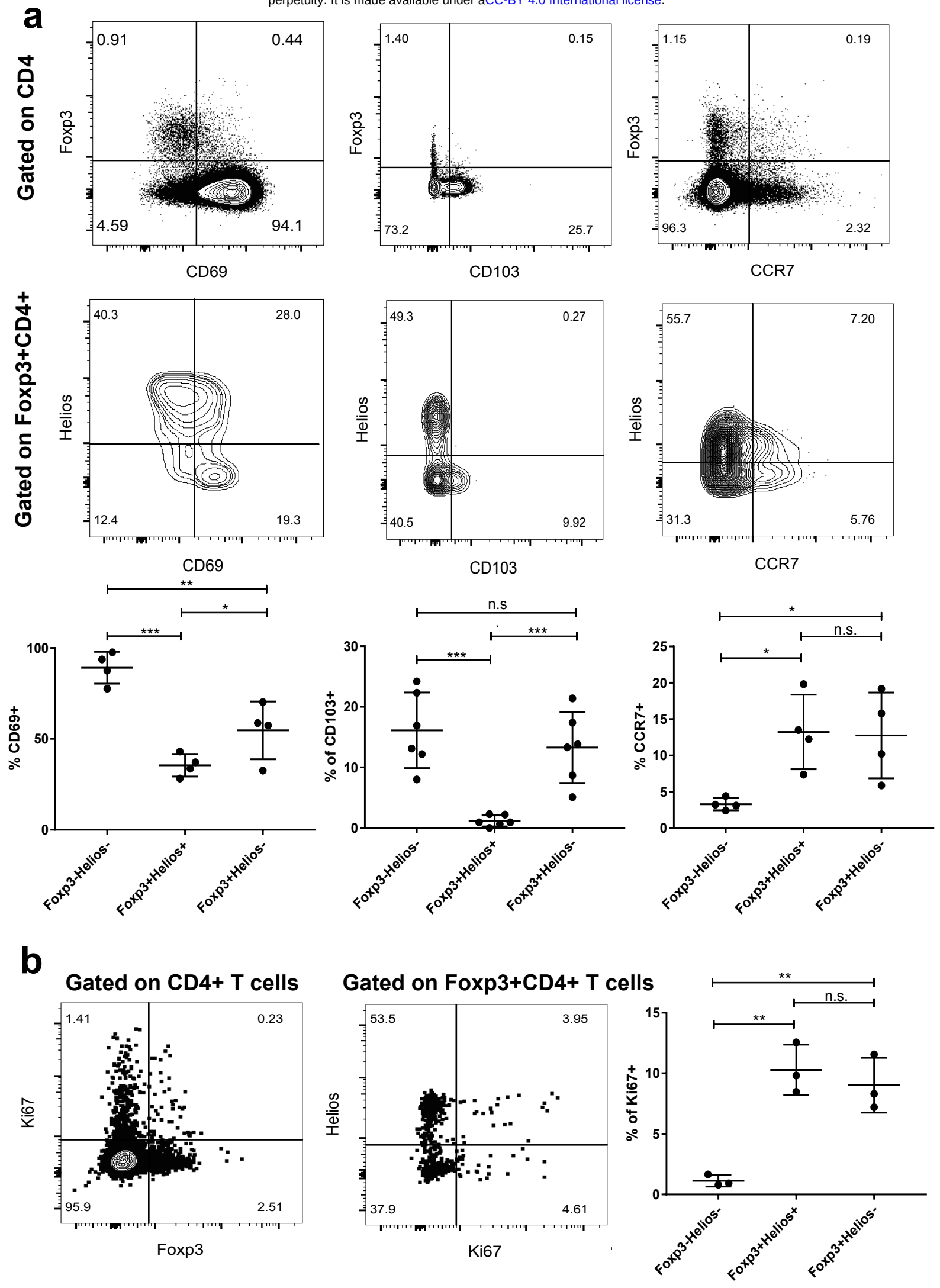

Fig. 5