Niche-specific MHC II and PD-L1 regulate CD4⁺CD8αα⁺ intraepithelial lymphocyte differentiation

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Conventional CD4⁺ T cells are differentiated into CD4⁺CD8αα⁺ intraepithelial lymphocytes (IELs) in the intestine; however, the roles of intestinal epithelial cells (IECs) are poorly understood. Here, we showed that IECs expressed MHC class II (MHC II) and CD4-lineage transcription factor, T helper 2 domain with cosignals from IECs constitutes niche adaptation signals to develop tissue-resident CD4⁺CD8αα⁺ IELs. Therefore, IEC-specific deletion of MHC II and PD-L1 hindered the development of CD4⁺CD8αα⁺ IELs. Intracellularly, PD-1 signals supported the acquisition of CD8αα by down-regulating the CD4-lineage transcription factor, T helper–inducing POZ/Krüppel-like factor (ThPOK), via the Src homology 2 domain–containing tyrosine phosphatase (SHP) pathway. Our results demonstrate that noncanonical antigen presentation with cosignals from IECs constitutes niche adaptation signals to develop tissue-resident CD4⁺CD8αα⁺ IELs.

Introduction
Intestinal intraepithelial lymphocytes (IELs) are a heterogeneous T cell population residing in the gut epithelium, consisting of diverse subpopulations classified by their origin and surface markers (McDonald et al., 2018). Among the subpopulations of IELs, there are phenotypically and functionally distinct subsets of CD4⁺ T cells that coexpress CD4 and CD8αα, CD4⁺CD8αα⁺ double-positive (DP) IELs, distinguishing them from other conventional CD4⁺ T helper subsets or regulatory T cells in the periphery (Cheroutre and Husain, 2013; Faria et al., 2017; Reis et al., 2014). A subsequent study reported that T-box expressed in T cells (T-bet) is a critical upstream regulator of DP IEL differentiation, inducing Runx3 and suppressing ThPOK expression, and T-bet–inducing cytokines in the intestinal milieu such as IFN-γ preferentially promote DP IEL differentiation in the presence of gut microenvironmental cues, including TGF-β and retinoic acid (RA; Reis et al., 2014).

Studies using germ-free (GF) mice show that the gut microbiota is crucial for DP IEL development (Mucida et al., 2013; Sujino et al., 2016), and the commensal Lactobacillus reuteri with derivatives of dietary tryptophan was identified to promote the reprogramming of CD4⁺ T cells into DP IELs (Cervantes-Barragan et al., 2017). However, the detailed process that initiates TCR stimulation upon encounter of antigens, including commensal microbes, in the gut epithelium, where CD4⁺ T cells lose their ThPOK expression to convert to DP IELs, has not been clearly understood.

Analysis of TCR use by CD4⁺ single-positive (SP) and DP IELs suggests that clonal selection precedes DP IEL development

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(Cervantes-Barragan et al., 2017; Wojciech et al., 2018). This implies that MHC class II (MHC II)-mediated antigen presentation is required in the small intestine for TCR engagement of DP IELs, emphasizing the role of professional APCs expressing MHC II or other antigen presentation machinery in the intestine. However, many reports have indicated that MHC II is expressed in intestinal epithelial cells (IECs; Kambayashi and Lauffer, 2014; Londei et al., 1984; Skoskiewicz et al., 1985). Furthermore, IECs regulate CD4+ T cells in the intestine through their MHC II (Biton et al., 2018; Koyama et al., 2019). These results led us to hypothesize that, given the unique niche for DP IELs, namely the intracellular region between IECs, MHC II antigen presentation mainly occurs in IECs for driving DP IEL differentiation.

Here, we report the evidence that IECs are key regulators that drive the differentiation of DP IELs in the intestine. We show that IECs provide TCR stimulation and coreceptor signal via their expression of MHC II and programmed death–ligand 1 (PD-L1), respectively, in a microbiota- and IFN-γ-dependent manner. Additionally, programmed cell death protein 1 (PD-1)/PD-L1 signaling promotes down-regulation of ThPOK expression, thus turning on the lineage redirection process required for DP IEL differentiation.

Results and discussion

To address the role of IECs as atypical APCs during DP IEL differentiation, we first analyzed the expression of MHC II on IECs from the proximal to the distal part of the small intestine. We isolated IECs from the duodenum, jejunum, and ileum of naive mice and analyzed MHC II expression. Notably, MHC II expression on IECs was gradually increased from the duodenum to the ileum (Fig. 1 A), RNA-sequencing (RNA-seq) analysis of sorted IECs from each part of the small intestine also showed significant increases in gene expression associated with antigen processing and presentation via MHC II in the ileum (Fig. S1, A and B; and Fig. 1, B and C). Gene set enrichment analysis (GSEA) also revealed enriched expression of genes related to MHC II synthesis and antigen presentation via MHC II in the ileum compared with that in the duodenum (Fig. 1 D). Moreover, the frequency of DP IELs increased from the duodenum to the ileum (Fig. 1 E), resulting in a positive correlation between epithelial MHC II expression levels and DP IEL frequency (Fig. 1 F). This correlation was stronger at the ileum, where the abundance and diversity of microbiota are higher than in other segments (Fig. 1 F; Mowat and Agace, 2014). Interestingly, we also observed differential expression patterns of genes, including response to IFN-γ, cytokines, and the oxidation–reduction process, although the role of those genes alternatively expressed in each segment of the small intestine in DP IEL development has never been explored. The expression of Ifng1 and Ifng2 was comparable among all regions of the small intestine (Fig. S1, C–E).

To clarify the role of epithelial MHC II in the differentiation of DP IELs in vivo, we generated mice with a specific deletion of MHC II in IECs (MHC II−IECs; Fig. S1 G). Interestingly, we found a significant decrease of DP IEL frequencies in the small intestine of MHC II−IECs compared with MHC II+/+ control (Fig. 1 G), while no significant change was detected in the other IEL subsets (Fig. S1 H). To further confirm the requirement for epithelial MHC II in DP IEL development, we analyzed immunofluorescence images of ileal tissues from MHC II+/+ and MHC II−IECs mice. Consistently, DP IELs were dramatically decreased in MHC II−IECs mice compared with those in MHC II+/+ mice (Fig. 1 H). Most DP IELs were in contact with the basolateral surface of the epithelial layer, where MHC II was highly expressed (Fig. 1 H). Thus, MHC II expression on IECs is required for the generation of DP IELs in the small intestine.

IFN-γ is a strong inducer of MHC II expression in nonhematopoietic cells, including IECs (Kambayashi and Lauffer, 2014; Koyama et al., 2019; Thelemann et al., 2014). As expected, MHC II expression on IECs was not observed in IFN-γ receptor–deficient mice (IFN-γR−/−; Fig. 2 A). Almost complete suppression of DP IEL development was observed in IFN-γR−/− mice, and decreased expression of epithelial MHC II and frequencies of DP IELs was detected in mice receiving IFN-γ-neutralizing antibodies, indicating a causal relationship between IFN-γ–mediated MHC II expression on IECs and DP IEL differentiation (Fig. 2, A and B). Nonetheless, a previous report showed that IFN-γ is required for the differentiation of DP IELs by inducing the transcription factor T-bet, suggesting a direct role of IFN-γ in the functional maturation of DP IELs (Reis et al., 2014). Therefore, to elucidate the roles of IFN-γ in T cells or IECs for DP IEL differentiation, we generated bone marrow (BM) chimeras lacking IFN-γR expression in either nonhematopoietic or hematopoietic cells. Notably, IFN-γ directly controlled the expression of MHC II on IECs (IFN-γR−/+ → IFN-γR−/− chimera), and the absence of IFN-γR signaling in both hematopoietic and nonhematopoietic compartments (IFN-γR−/+ → IFN-γR−/−) prohibited DP IEL development (Fig. 2 C). However, DP IELs were dramatically decreased in mice having IFN-γR deficiency in either only hematopoietic (IFN-γR−/+ → IFN-γR−/+) or only nonhematopoietic cells (IFN-γR−/+ → IFN-γR−/−; Fig. 2 C). These results suggest that intact IFN-γR signaling in both hematopoietic and nonhematopoietic cells, including CD4+ IELs and IECs, respectively, is necessary for DP IEL differentiation. Interestingly, distinct regional differences in MHC II expression and DP IEL frequency between the proximal versus distal parts of the small intestine, as observed in the normal mice, were not detected in each BM chimera mouse (Fig. 2 C). It is speculated that the immune reconstitution in the irradiated lymphopenic recipients may trigger the changes in gut microenvironment toward inflammatory conditions (Min, 2018), presumably by producing more IFN-γ than at the steady state, which results in the disappearance of regional differences in MHC II expression and DP IEL frequency along the small intestine.

To directly assess whether MHC II+ IECs acted as APCs for DP IEL differentiation, we generated the small intestine organoids from MHC II+/+ mice and cocultured these organoids with CD4+ T cells. We stimulated organoids with recombinant IFN-γ for up-regulation of MHC II (Fig. 2, D and E) and pulsed them with OVA peptide (Fig. 2 F). OVA-specific CD4+ T cells (OT-II) were preactivated with anti-CD3ε/CD28 and then cocultured with IFN-γ–stimulated organoids in the presence of TGF-β and RA, both of which induce DP IELs.
Figure 1. MHC II expression on IECs is required for DP IEL differentiation. (A) Representative plots (left) and MHC expression (right; mean ± SEM) on IECs (CD45.2−EpCAM+ gated) in each small intestine segment, duodenum (d), jejunum (j), and ileum (i) of C57BL/6 mice (n = 6). Expression level is shown as mean ± SEM. (B) Antigen processing and presentation of exogenous peptide antigen via MHC II. (C) Heatmap of gene expression in C57BL/6 mice showing significant upregulation of MHC II and PD-L1 on IECs regulating CD4+CD8α+ IELs (https://doi.org/10.1084/jem.20201665). (D) MHC II synthesis and antigen presentation via MHC II. (E) Percent CD8α+ in CD4+ T cells in each small intestine segment. (F) Scatter plots showing the relationship between MHC II expression and CD8α+ in CD4+ T cells in each small intestine segment. (G) Comparison of MHC II expression in IECs and non-IEC cells. (H) Confocal imaging showing MHC II expression in CD4+CD8α+ IELs.
IELs in vitro (Reis et al., 2013). TCR engagement by cognate antigen presentation through MHC II on IECs increased the differentiation of DP IELs, which was achieved with TGF-β and RA (Fig. 2 G). Collectively, these results demonstrate that IECs are atypical APCs for CD4+ T cells in the epithelium and that in situ differentiation of DP IELs prefers a specific anatomical location, namely the ileum of the small intestine, where IFN-γ-mediated MHC II induction prevails.

Because MHC II+ IECs support the cognate stimulation of CD4+ IELs as APCs, we hypothesized that they might express other coreceptors capable of regulating T cells in concert with MHC II. These coreceptors would be expected to be linked to IFN-γR signaling in IECs, which is critical for the MHC II expression on IECs. Therefore, we explored IFN-γ-dependent changes in gene expression in IECs by RNA-seq analysis of intestinal organoids to screen for candidate molecules that could provide co-signals to support DP IEL differentiation in the intestinal epithelium. Surprisingly, we identified Cd274, the gene encoding PD-L1, as the gene showing the greatest fold increase among the T cell coreceptor ligands following IFN-γ treatment in the organoids (Fig. S2, A and B). A heatmap of differentially expressed genes (DEGs) and a volcano plot revealed that expression was significantly increased along with MHC II and PD-L1 expression on IECs (Fig. S1 F). The data shown are representative of six independent experiments. (F) Correlation analysis between epithelial MHC II expression and DP IEL frequency by linear regression fit in each intestinal segment (n = 29). Analysis was performed on pooled data of six independent experiments. R, correlation coefficient; P, significance of the slope. (G) DP IEL frequency in MHC II−/− and MHC II+/+ IEC mice (n = 13–14). The data shown are pooled from five independent experiments. (H) Immunofluorescence images (left and middle) and quantification (right) of DP IELs in ilea of MHC II−/− and MHC II+/+ IECs. Yellow arrowheads represent DP IELs overlapping CD4 (red) and CD8α (green). Scale bar, 20 µm or 10 µm (for inset). 7 or 13 villi from 2 mice per group were imaged and quantified. *, P < 0.05; **, P < 0.01; ***, P < 0.001. One-way ANOVA with Tukey’s post hoc test (A and E) or unpaired Student’s t-test (G and H). FSC-A, forward scatter area.
Figure 2. MHC II expression on IECs for DP IEL differentiation is IFN-γ dependent. (A and B) MHC II expression on IECs (left; mean ± SEM) and DP IEL frequency (right; mean ± SEM) in IFN-γR+/+ and IFN-γR−/− mice (n = 3; A) and in WT mice injected with isotype or IFN-γ-neutralizing antibodies (anti-IFN-γ; n = 7; B). The data shown are representative of three (A) or two (B) independent experiments. (C) MHC II expression on IECs (left; mean ± SEM) and DP IEL frequency (right; mean ± SEM) in each BM chimera (n = 8–14). The data shown are pooled from two independent experiments. (D) Representative plots (left)
These data suggest that T cell-intrinsic PD-1 signaling is required for DP IEL differentiation. Next, we investigated the molecular mechanisms through which PD-1 signaling modulates the transcriptional reprogramming of CD4+ IELs. We transferred splenic T cells from ThPOK-GFP reporter mice to RAG-1−/− mice administered anti–PD-1 antibodies during the reconstitution period (Fig. 5 D). These results showed that loss of ThPOK and DP IEL development was inhibited by anti–PD-1 treatment (Fig. 5, E and F). Interestingly, the expression of Runx3 was unchanged by anti–PD-1 (Fig. 5 F). PD-L1-mediated down-regulation of ThPOK expression was confirmed in vitro by ligation of CD4+ T cells with PD-L1-coated beads (Fig. 5 G). The inhibition of Src homology 2 domain–containing tyrosine phosphatase (SHP), the canonical PD-1 signaling pathway in T cells, reversed the PD-L1–induced suppression of ThPOK and CD8αα acquisition (Fig. 5, H and I). Taken together, these results suggest that PD-1 signaling through the canonical SHP pathway in CD4+ IELs suppresses ThPOK expression, leading to enhancement of DP IEL differentiation.

Tissue-resident T cells receive signals from tissues for their adaptation in a specific niche (Faria et al., 2017; Mueller and Mackay, 2016). Universal antigen presentation to MHC I in most nucleated cells results in CD8+ T cell responses to cognate antigen presented from the parenchyma of tissues, including epithelial cells (Allez et al., 2002). However, the direct antigenic stimulation of CD4+ T cells by epithelial cells is less likely due to the restriction of MHC II expression in professional APCs (Kambayashi and Laufer, 2014). In this study, we found that MHC II–mediated antigen presentation in IECs was required for the differentiation of CD4+CD8αα DP IELs. Interestingly, this epithelial MHC II–mediated regulation of CD4 IELs occurred in a specific anatomical region, namely the distal part of the small intestine. This suggests that there may be a connection with the bacterial burden of the gut microbiota, which is an essential component for MHC II expression in IECs and DP IEL development (Cervantes-Barragan et al., 2017; Koyama et al., 2019; Sujino et al., 2016). The role of TCR engagement for the development of DP IELs has recently been suggested by a study by Bilate et al., in which the researchers found that TCR signaling is required for the differentiation of SP IELs to DP IELs but not for the maintenance of DP IELs (Bilate et al., 2020). Furthermore, the inducible deletion of MHC II in IECs down-regulated the development of DP IELs. Thus, those results consistently indicate a crucial role of MHC II–mediated antigen presentation in IECs for the DP IEL differentiation. However, additional studies are required to address whether MHC II on IECs indeed presents microbiota-derived antigens or whether clones of CD4 IELs responding to microbiota antigen are differentiated into DP IELs.

Notably, we found that PD-1, a T cell co-inhibitory receptor, was also involved in DP IEL differentiation. Thus, as atypical APCs, IECs induced signals in CD4+ T cells from the TCR and PD-1 co-receptor by up-regulating MHC II and PD-L1. PD-1 signaling blocks T cell activation signals induced by the TCR and CD28 costimulatory molecule (Hui et al., 2017; Sun et al., 2018). Interestingly, the expression of Nur77, a downstream molecule of TCR signaling, was inversely correlated with the acquisition of CD8αα, suggesting that the dampening of TCR signaling precedes DP IEL differentiation (Bilate et al., 2020). Considering that T cell-intrinsic PD-1 signaling is required for DP IEL differentiation (Fig. 5 C), we are tempted to speculate that TCR signaling is actively down-regulated by PD-1–PD-L1 interaction during DP IEL development. Undoubtedly, microbiota- and IFN-γ–dependent PD-L1 expression in IECs contributes to the ligation of PD-1 in CD4 IELs; however, the provision of PD-L1 from other cells is also possible. Therefore, during DP IEL development, clonal selection of CD4 IELs may occur via MHC II on IECs, although the differentiation of selected precursors into DP IELs may be achieved by PD-1 signaling, which down-regulates ThPOK expression via the canonical SHP pathway. We hypothesize that MHC II–PD-L1 IELs likely regulate DP IEL differentiation as a single niche, providing TCR engagement with cognate antigen and co-signaling with PD-L1 simultaneously. However, some IELs are motile by covering a large number of IECs rather than remaining in a fixed position (Hoytema van Konijnenburg et al., 2017). Therefore, we could not rule out the possibility that CD4+ IELs receive alternate cues from MHC II SP IECs and then move to DP IECs.

The pivotal roles of immune–epithelium communication in the intestine have been described (Peterson and Artis, 2014). Our study indicates that the regional specialization of IECs with altered gene expression contributes to their interplay with tissue-resident immune cells, adjusted to the physiological conditions in different anatomical locations. In addition to the intestines, MHC II expression has also been observed in other epithelial cells in the lungs and skin (Gereke et al., 2009; Tamoutounour et al., 2019; Wosen et al., 2018). Given the enormous surface areas of these tissues, epithelial cells may contribute to the regulation of tissue-resident CD4+ T cells as atypical APCs.

Materials and methods

Animal procedures

All animal experiments were performed in accordance with animal guidelines and approved by the institutional animal care and use committee of the Pohang University of Science and Technology (POSTECH; approval numbers POSTECH-2015-0065,
POSTECH-2017-0005, POSTECH-2018-0032, and POSTECH-2020-0035). All mice were on a C57BL/6 background and maintained in an SPF or GF animal facility at POSTECH. MHC IIΔIEC mice or PD-L1ΔIEC mice were generated by crossing H2-Ab1fl/fl (013181; The Jackson Laboratory) or PD-L1fl/fl (generated by Cyagen Co.) with Villin-cre (021504; The Jackson Laboratory) mice, respectively. Several mouse strains were generously provided: PD-L1−/− mice by Dr. Sang-Nae Cho (Yonsei University, Seoul, Republic of Korea), PD-L1−/− mice by Dr. Lieping Chen (Yale University, New Haven, CT), IFN-γR−/−/− mice by Dr. Inhak Choi, Inje University, Busan, Republic of Korea (originally generated by Dr. Lieping Chen, Yale University, New Haven, CT), and ThPOK-GFP mice by Dr. Daniel Mucida (The Rockefeller University, New York, NY). Mice were used at 8–16 wk of age for all experiments. Littermates or WT mice maintained by the same vivaria were used as control animals.

To generate BM chimera mice, BM cells were acquired from leg bones of donor mice by flushing with RPMI 1640 containing 5% newborn calf serum (NCS). After filtering with 40-µm mesh, 2×10^6 BM cells were transferred into lethally irradiated

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Figure 3. IFN-γ–inducible PD-L1 expression on IECs is important for DP IEL differentiation. (A and B) DEG heatmap (A) and volcano plot (B) of IFN-γ–treated and untreated (control) small intestine organoids (n = 2 per group). In the volcano plot, the x axis shows the fold change in gene expression between two groups; the y axis shows statistical significance (negative log10 of q value). Genes with significant fold differences after IFN-γ treatment are depicted in blue or red. (C) PD-L1 expression on small intestine organoids (7AAD−CD45−EpCAM− gated) after IFN-γ treatment is shown as mean fluorescence intensity (MFI; n = 2). The data shown are representative of two independent experiments. (D and E) PD-L1 expression on IECs in IFN-γR+/+ and IFN-γR−/− mice (n = 3; D) and on IECs in mice administered injections with isotype or anti–IFN-γ antibodies (n = 7; E). The data shown are representative of (D) or pooled from (E) two independent experiments. (F–H) Frequencies of DP IELs in PD-L1+/+ and PD-L1−/− mice (n = 5; F), in splenic T cell–reconstituted RAG−/− recipients that received injections with isotype or PD-L1–blocking antibody (anti–PD-L1) during the reconstitution period (n = 5–6; G), and in PD-L1fl/fl and PD-L1ΔIEC mice (n = 12; H). The data shown are pooled from two (F) or four (H) independent experiments or are representative of two independent experiments (G). *, P < 0.05; **, P < 0.01; ***, P < 0.001. One-way ANOVA with Tukey’s post hoc test (C) and unpaired Student’s t test (D–H). d, duodenum; i, ileum; j, jejunum. Results (C–H) are expressed as mean ± SEM.
recipient mice by i.v. injection. Donor and recipient cells were distinguished with congenic markers (CD45.1, CD45.1.2, and CD45.2). After an 8 wk reconstitution period, mice were sacrificed for subsequent analysis.

For adoptive transfer experiments, T cells were purified from the spleens of ThPOK-GFP reporter mice with magnetic-activated cell sorting (Miltenyi Biotec) according to the manufacturer’s protocol. 5 × 10^6 purified cells were transferred i.v. to RAG-1−/− hosts that were subsequently administered isotype control or neutralizing antibodies against PD-1 or PD-L1 (anti–PD-1, BE0273; isotype for anti–PD-1, BE0089; anti–PD-L1, BE0101; isotype for anti–PD-L1, BE0090; all from Bio X Cell) i.p. for 4 wk (100 µg twice per week). For IFN-γ blockade, mice were administered i.p. injections with control or IFN-γ-neutralizing antibodies (anti–IFN-γ, BE0054; isotype for anti–IFN-γ, BE0088; all from Bio X Cell) for 2 wk (100 µg twice per week).

To deplete the gut microbiota, an antibiotic cocktail consisting of 0.5 g/liter of vancomycin, 1.0 g/liter of ampicillin, 1.0 g/liter of neomycin, and 1 g/liter of metronidazole, which were all purchased from Sigma-Aldrich (vancomycin, V1130; ampicillin, A0166; neomycin, N6386; metronidazole, M1547), was administered to SPF C57BL/6 mice in drinking water for 4 wk.

**Antibodies and flow cytometry**

Single-cell suspensions were preincubated with antismouse CD16/32 for 15 min on ice before surface marker staining to block Fc receptors and then subsequently stained with the following fluorophore-conjugated antibodies according to the manufacturer’s recommended concentrations for 15 min on ice. Antibodies were purchased from eBioscience (anti–CD4, 48-0042-82; anti–CD8α, 25-0081-82; anti–CD45.2, 11-0454-81; anti–PD-L1, 46-5982-82; anti–MHC class II, 48-5321-82 or 47-
Figure 5. **PD-1 signaling–mediated ThPOK suppression induces DP IEL generation.** (A) PD-1 expression on each IEL subset (left) and separation of TCRαβ+CD8α IELs depending on the expression of PD-1 and CD8α (right) in C57BL/6 mice. (B) The frequency of DP IELs in PD-1+/+ and PD-1−/− mice (n = 3; mean ± SEM). The data shown are representative of two independent experiments. (C) Generation of DP IELs in RAG−/− recipients transferred with a 1:1 mixture of PD-1+/+ and PD-1−/− splenic T cells. The frequency of DP IELs from the same recipient are connected by a line (n = 8). The data shown are representative of three independent experiments. (D–F) Experimental scheme (D), representative plots (left) and frequency (right; mean ± SEM) of DP IELs (E), and expression of ThPOK or Runx3 (F; mean ± SEM) in RAG−/− recipients administered injections with isotype or PD-1 receptor–blocking antibody (anti–PD-1) during the reconstitution period (all gated on TCRβ+CD4+ T cells; n = 7). The data shown are pooled from two independent experiments. (G) The frequency of ThPOKhi cells (mean ± SEM) after 3 d in in vitro culture of CD4+ T cells in the presence of TGF-β, RA, and epoxy beads covalently coated with different amounts of PD-L1 protein. The percentage of PD-L1− coated beads represent the amount of PD-L1 protein over total protein coated on beads (n = 4). The data shown are pooled from two independent experiments. (H and I) Representative plots (H and I, left) and quantification of CD8α induction (H, right; mean ± SEM) and...
In vitro CD4+ T cell cultures

CD4+ T cells were purified from splenocytes by magnetic-activated cell sorting according to the manufacturer’s protocol. Purified cells were cultured for 3 d in 96-well plates precoated with 5 µg/ml of anti-CD3ε and 2.5 µg/ml of soluble anti-CD28 (anti-CD3ε, 40-0031-M001; anti-CD28, 30-0285-US050; Tonbo Biosciences). For induction of CD4+CD8αα+ cells, CD4+ T cells were stimulated with 10 nM RA (R2625; Sigma-Aldrich) during the culture period.

Immunofluorescence staining and image quantification

Freshly isolated mouse ileal tissues were opened and washed with clean PBS three times. Tissues were fixed in 4% paraformaldehyde at 4°C overnight and were washed with clean PBS. Samples were frozen in optimum cutting temperature compound block of ileal tissues by using a Leica CM1850 cryostat. Each section was incubated with a blocking solution of 1% BSA with 0.5% Triton X-100. Tissues were fixed in 4% paraformaldehyde for 1 h at 4°C to isolate crypts. Isolated crypts were filtered through a 70-µm cell strainer and were centrifuged at 1,500 rpm for 10 min at 4°C. The cell pellet was resuspended, layered to a 40%/70% Percoll gradient, and centrifuged at 1,500 rpm for 10 min at 4°C. The tissue suspension was pelleted by centrifugation at 4°C and 900 g for 5 min. Approximately 500 crypts were washed with 20 µl of Matrigel (356231; BD Biosciences) and plated in 48-well plates. After polymerization of Matrigel, we added 300 µl of culture media (12634-010; Invitrogen) containing penicillin-streptomycin (15140-122), GlutaMAX (35050-061), GlutaMAX (35050-061), Hepes (15630-080; all from Gibco), N-2 supplement (17502-048), B-27 supplement (17504-044; all from Invitrogen), N-acetyl-L-cysteine (A9165; Sigma-Aldrich), epidermal growth factor (315-09-1000), R-spondin 1 (120-38-1200), and Noggin (250-38-100; all from PeproTech). Fresh media were replaced every 2–3 d, and organoids were passaged every week with a 1:3 split ratio. For coculture with T cells, organoids were stimulated in the presence of 5 ng/ml of IFN-γ (315-05; PeproTech) and 30 µg/ml of OVA323–339 peptide (synthesized by Peptron) for 24 h. CD4+ T cells isolated from OT-II mice were stimulated for 3 d in 96-well plates coated with anti-CD3ε and soluble anti-CD28. 105 stimulated CD4+ T cells and roughly 50–100 organoids were seeded together into round-bottom 96-well plates, with 10 nM of RA and 2 ng/ml of TGF-β. Cells were analyzed after 24 h of coculture.

ThPOK [I; middle; mean ± SEM] or Runx3 [I; right] expression in CD4+ T cells cultured for 3 d in the presence of TGF-β, RA, plate-coated PD-L1, and SHP inhibitor (SHPi; n = 5–13). The data shown are pooled from two or four independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. Unpaired (B, E, and F) or paired (C) Student’s t test and one-way ANOVA with Tukey’s post hoc test (G–I). d, duodenum; i, ileum; j, jejunum.
Automated quantification was performed using ImageJ software provided by the National Institutes of Health. For the quantification of epithelial expression of MHC II and PD-L1, the area that stained positive for MHC II and PD-L1 was measured over the area that stained positive for EpCAM, and the percentage of area occupancy was calculated. To quantify the number of DP IELs, circularly shaped cells that stained positive for both CD4 and CD8α were automatically counted per villus.

RNA-seq analysis
Total RNA was extracted from FACS-sorted epithelial cells (CD45−EpCAM+α) from the small intestines or intestinal organoids. cDNA was synthesized with a QuantiTect Reverse Transcription Kit (205311; QIAGEN). The library for RNA-seq analysis was generated using the TruSeq RNA Sample Prep Kit version 2 or the TruSeq Stranded Total RNA Sample Prep Gold Kit and sequenced on a HiSeq 4000 system. For the analysis of IECs, expression data were normalized as a log$_{2}$-transformed fragments per kilobase of transcript per million mapped reads value. DEGs were defined as genes of mouse replicate number 1 with fold change ≥ 2 between duodenum and ileum and fold change ≥ 1.5 between duodenum and jejunum. Visualization of enriched Gene Ontology (GO) terms in DEGs was performed as previously described (Bonnot et al., 2019). For the analysis of epithelial cells from organoids, raw sequencing data were quantified with kallisto (mm10 was used for the mouse reference genome), and the R library sleuth and Enhanced-Volcano were used for data processing and plotting. To draw a DEG heatmap, expression data were normalized as log$_{2}$-transformed (transcripts per million +1) values, and genes with fold changes greater than or equal to one between the average of nontreated and IFN-γ–treated organoids were used. DAVID (version 6.7) and the GO resource were used for GO analysis. GSEA was performed using GSEA software (version 4.0) provided by the Broad Institute.

Statistics
Statistical analysis was performed using GraphPad Prism software, and the tests used are indicated in the figure legends.

Data availability
The RNA-seq data of IECs sorted from three segments of the small intestine and untreated or IFN-γ–treated intestinal organoids have been deposited in the ArrayExpress archive and are available under accession numbers E-MTAB-9744 and E-MTAB-9756, respectively.

Online supplemental material
Fig. S1 relates to Fig. 1 and shows RNA-seq analysis of IECs sorted from each small intestine section, the gating scheme for DP IELs, and epithelial MHC II expression or IEL subsets in MHC II$^{−}$IECs mice. Fig. S2 relates to Fig. 3 and shows enriched GO terms of DEGs or fold increases in gene expression of T cell coreceptor ligands between untreated and IFN-γ–treated organoids. Fig. S3 relates to Fig. 3 and shows the expression of PD-L1 and/or MHC II on IECs in C57BL/6, PD-L1$^{−}$IECs, MHC II$^{−}$IECs, or GF mice.

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Author contributions: S.-W. Lee and Y. Park conceived and supervised the study and wrote the manuscript. S. Moon and Y. Park performed experiments, analyzed data, and wrote the manuscript. S. Hyeon, Y.-M. Kim, J.-H. Kim, H. Kim, and S. Park performed the mouse experiments. S. Park, K.-J. Lee, Y.-M. Kim, B.-K. Koo, and S.-J. Ha helped with data analysis. B.-K. Koo provided expertise for the organoid culture system. All authors reviewed and provided edits of the manuscript.

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References
Allez, M., J. Brimnes, I. Dotan, and L. Mayer. 2002. Expansion of CD8$^+$ T cells with regulatory function after interaction with intestinal epithelial cells. Gastroenterology. 123:1516–1526. https://doi.org/10.1053/gast.2002.36588
Bilate, A.M., M. London, T.B.R. Castro, L. Mesin, J. Bortolatto, S. Kongthong, A. Harnagel, G.D. Victorica, and D. Mucida. 2020. T cell receptor is required for differentiation, but not maintenance, of intestinal CD4$^+$ intraepithelial lymphocytes. Immunity. 53:1001–1014.e20. https://doi.org/10.1016/j.immuni.2020.09.003
Biton, M., A.L. Haber, N. Rogel, S. Beyaz, A. Schnell, O. Ashenberg, C.W. Su, C. Smillie, K. Shekhar, et al. 2018. T helper cell cytokines modulate intestinal stem cell renewal and differentiation. Cell. 175: 1307–1320.e22. https://doi.org/10.1016/j.cell.2018.10.008
Bonnot, T., M.B. Gillard, and D.H. Nagel. 2019. A simple protocol for informative visualization of enriched Gene Ontology terms. Bio-IOI. e3429.
Cervantes-Barragan, L., J.N. Chai, M.D. Tianero, B. Di Luccia, P.P. Ahern, J. Merriman, V.S. Cortez, M.G. Caparon, M.S. Donia, S. Gillfillan, et al. 2017. Lactobacillus reuteri induces gut intraepithelial CD4$^+$CD8α$^+$ T cells. Science. 357:806–810. https://doi.org/10.1126/science.aah5825
Cheroutre, H., and M.M. Husain. 2013. CD4 CTL: living up to the challenge. Semin. Immunol. 25:273–281. https://doi.org/10.1016/j.smim.2013.10.022
Faria, A.M.C., B.S. Reis, and D. Mucida. 2017. Tissue adaptation: Implications for gut immunity and tolerance. J. Exp. Med. 214:1211–1226. https://doi.org/10.1084/jem.20162014
Gereke, M., S. Jung, J. Buer, and D. Bruder. 2009. Alveolar type II epithelial cells present antigen to CD4$^+$ T cells and induce FoxP3$^+$ regulatory T cells. Am. J. Respir. Crit. Care Med. 179:344–355. https://doi.org/10.1164/rccm.200804-592OC
Hoytema van Konijnenburg, D.P., B.S. Reis, V.A. Pedicord, J. Farache, G.D. Victorica, and D. Mucida. 2017. Intestinal epithelial and intraepithelial T cell crosstalk mediates a dynamic response to infection. Cell. 171: 783–794.e13. https://doi.org/10.1016/j.cell.2017.08.046
Hui, E., J. Cheung, J. Zhu, X. Su, M.J. Taylor, H.A. Wallweber, D.K. Sasmal, J. Huang, J.M. Kim, I. Mellman, et al. 2017. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science. 355: 1428–1433. https://doi.org/10.1126/science.aaf2922
Kambayashi, T., and T.M. Lauber. 2014. Atypical MHC class II expressing antigen-presenting cells: can anything replace a dendritic cell? Nat. Rev. Immunol. 14:719–730. https://doi.org/10.1038/nri3754
Koyama, M., P. Mukhopadhyay, I.S. Schuster, A.S. Henden, J. Hülsdunker, A. Varelias, M. Vetizou, R.D. Kuns, J.R. Robb, P. Zhang, et al. 2019. MHC class II antigen presentation by the intestinal epithelium initiates graft-versus-host disease and is influenced by the microbiota. Immunity. 51: 885–898.e7. https://doi.org/10.1016/j.immuni.2019.08.011

Londel, M., J.R. Lamb, G.F. Bottazzo, and M. Feldmann. 1984. Epithelial cells expressing aberrant MHC class II determinants can present antigen to cloned human T cells. Nature. 312:639–641. https://doi.org/10.1038/312639a0

McDonald, B.D., B. Jabri, and A. Bendelac. 2018. Diverse developmental pathways of intestinal intraepithelial lymphocytes. Nat. Rev. Immunol. 18:514–525. https://doi.org/10.1038/s41577-018-0013-7

Min, B. 2018. Spontaneous T cell proliferation: a physiologic process to create and maintain homeostatic balance and diversity of the immune system. Front. Immunol. 9:547. https://doi.org/10.3389/fimmu.2018.00547

Movat, A.M., and W.W. Agace. 2014. Regional specialization within the intestinal immune system. Nat. Rev. Immunol. 14:667–685. https://doi.org/10.1038/nri3738

Mucida, D., Y. Park, G. Kim, O. Turovskaya, I. Scott, M. Kronenberg, and H. Cheroutre. 2007. Reciprocal Th17 and regulatory T cell differentiation mediated by retinoic acid. Science. 317:256–260. https://doi.org/10.1126/science.1145697

Mucida, D., M.M. Hussein, S. Muroi, F. van Wijk, R. Shinnakasu, Y. Naoe, B.S. Reis, Y. Huang, F. Lambolez, M. Doherty, et al. 2013. Transcriptional reprogramming of mature CD4+ helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. Nat. Immunol. 14:281–289. https://doi.org/10.1038/ni.2523

Mueller, S.N., and L.K. Mackay. 2016. Tissue-resident memory T cells: local specialists in immune defence. Nat. Rev. Immunol. 16:79–89. https://doi.org/10.1038/nri.2015.3

Peterson, L.W., and D. Artis. 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat. Rev. Immunol. 14: 141–153. https://doi.org/10.1038/ni.3608

Reis, B.S., A. Rogoz, F.A. Costa-Pinto, I. Tanuchi, and D. Mucida. 2013. Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4+ T cell immunity. Nat. Immunol. 14:271–280. https://doi.org/10.1038/ni.2518

Reis, B.S., D.P. Hoytema van Konijenburg, S.I. Grivennikov, and D. Mucida. 2014. Transcription factor T-bet regulates intraepithelial lymphocyte functional maturation. Immunity. 41:244–256. https://doi.org/10.1016/j.immuni.2014.06.017

Sato, T., R.G. Vries, H.J. Snippert, M. van de Watering, N. Barker, D.E. Stange, J.H. van Es, A. Abo, P. Kujala, P.J. Peters, et al. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature. 459:262–265. https://doi.org/10.1038/nature07935

Skoskiewicz, M.J., R.B. Colvin, E.E. Schneeberger, and P.S. Russell. 1985. Widespread and selective induction of major histocompatibility complex-determined antigens in vivo by gamma interferon. J. Exp. Med. 162:1645–1664. https://doi.org/10.1084/jem.162.5.1645

Sujino, T., M. London, D.P. Hoytema van Konijenburg, T. Rendon, T. Buch, H.M. Silva, J.J. Lafaille, B.S. Reis, and D. Mucida. 2016. Tissue adaptation of regulatory and intraepithelial CD4+ T cells controls gut inflammation. Science. 352:1581–1586. https://doi.org/10.1126/science.aaf3892

Sun, C., R. Mezzadra, and T.N. Schumacher. 2018. Regulation and function of the PD-L1 checkpoint. Immunity. 48:434–452. https://doi.org/10.1016/j.immuni.2018.03.014

Tamoutounour, S., S.J. Han, J. Deckers, M.G. Constantinides, C. Hurabielle, O.J. Harrison, N. Bouladoux, J.L. Linehan, V.M. Link, I. Vujkovic-Cvijin, et al. 2019. Keratinocyte-intrinsic MHCII expression controls microbiota-induced Th1 cell responses. Proc. Natl. Acad. Sci. USA. 116: 23643–23652. https://doi.org/10.1073/pnas.1912432116

Thelmann, C., R.O. Eren, M. Coutaz, J. Brasseit, H. Bouzourene, M. Rosa, A. Duval, C. Lavanchy, V. Mack, C. Mueller, et al. 2014. Interferon-γ induces expression of MHC class II on intestinal epithelial cells and protects mice from colitis. PLoS One. 9:e86844. https://doi.org/10.1371/journal.pone.0086844

Wojciech, L., E. Szurek, M. Kuczma, A. Cebula, W.R. Elhefnawy, M. Pietrzak, G. Rempala, and L. Ignatowicz. 2018. Non-canonicaly recruited αβ TCR+ IELs recognize microbial antigens. Sci. Rep. 8:10848. https://doi.org/10.1038/s41598-018-29073-7

Wosen, J.E., D. Mukhopadhyay, C. Macaubas, and E.D. Mellins. 2018. Epithelial MHC class II expression and its role in antigen presentation in the gastrointestinal and respiratory tracts. Front. Immunol. 9:2144. https://doi.org/10.3389/fimmu.2018.02144
Figure S1. MHC II expression on IECs is enriched in the distal small intestine, constituting a favorable niche for differentiation of DP IELs but not other IEL subtypes. (A) Significantly enriched GO terms of DEGs among IECs sorted from duodenum (d), jejunum (j), and ileum (i). DEG analysis was performed using the PANTHER classification system. (B–D) Heatmap of MHC II–related genes (B), cytokines and DEGs annotated in oxidation–reduction process (GO:0055114; C), and DEGs annotated in response to IFN-γ (GO:0034341; D). The same numbers below d, j, and i correspond to the replicates from the same mouse. (E) Expression of Ifngr1 and Ifngr2 from RNA-seq analysis of IECs sorted from d, j, and i (mean ± SEM). (F) Gating scheme of DP IELs. (G) MHC II expression (mean fluorescence intensity [MFI]; mean ± SEM) on IECs in d, j, and i of MHC IIfl/fl and MHC IIΔIEC mice (n = 8). The data shown are pooled from three independent experiments. (H) Frequencies of IEL subsets in MHC IIfl/fl and MHC IIΔIEC mice (n = 5; mean ± SEM). The data shown are pooled from two independent experiments. IEL populations were gated as TCRβ+CD4−CD8α+CD8β+ (TCRβCD4−CD8α+CD8β+), TCRβ+CD4+CD8α− (TCRβCD4+CD8α−; SP IEL), TCRβ+CD4−CD8α+CD8β+ (TCRβCD4−CD8α+CD8β+), or TCRγδ+CD8α+ (TCRγδCD8α+). *, P < 0.05; ***, P < 0.001, ns, not significant. One-way ANOVA with Tukey’s post hoc test (E) or unpaired Student’s t test (G and H). FDR, false discovery rate; FSC-A, forward scatter area; FSC-H, forward scatter height; SSC-A, side scatter area; SSC-H, side scatter height.
Figure S2. Increase of Cd274 expression in the IFN-γ–treated intestinal organoids. (A) Enriched GO terms of DEGs between untreated and IFN-γ–treated organoids. (B) Fold change in gene expression of T cell coreceptor ligands in IFN-γ–treated over untreated organoids. NA, not applicable.
Expression of both PD-L1 and MHC II on IECs is regulated by environmental factors including the microbiota rather than by each other’s expression. (A) Immunofluorescence analysis showing PD-L1 and MHC II expression on IECs in the ileal villi of PD-L1^{fl/fl} or PD-L1^{ΔIEC} mice. Scale bar, 20 µm. Two mice per group were imaged, and the data shown are representative of two independent experiments. IECs and lamina propria cells positive for PD-L1 are indicated by white and orange arrowheads, respectively. (B and C) MHC II expression and PD-L1 expression (mean ± SEM) on IECs in PD-L1^{ΔIEC} (B) and MHC II^{ΔIEC} (C) mice, respectively ($n$ = 4–5). The data shown are pooled from two independent experiments. (D) Representative plots (left) and frequencies (right; mean ± SEM) of IECs expressing both MHC II and PD-L1 (CD45^-EpCAM^+ gated) in each small intestine segment ($n$ = 7). The data shown are pooled from two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. Unpaired Student’s t test (B–D). d, duodenum; i, ileum; j, jejunum.