Supplementary Information

Epithelial-stromal interaction via Notch signaling is essential for the full maturation of gut-associated lymphoid tissues

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Supplementary Methods

Whole mount staining

The whole-mount specimens of PP were fixed with BD Cytofix/Cytoperm (BD Biosciences, CA) followed by incubation with Alexa Fluor 488-conjugated WGA for goblet cell staining and Alexa Fluor 647-conjugated phalloidin (Invitrogen, CA) for F-actin counterstaining. The stained specimens were analyzed using a DM-IRE2 confocal laser scanning microscope and Leica confocal software (Leica Microsystems, Mannheim, Germany).

For immunofluorescent staining of Muc2 and CCL20 on FAE, FAE sheets were isolated as described previously [1], fixed in ice-cold 4% PFA/PBS, and incubated with primary antibodies (anti-Muc2, Santa Cruz biotechnology, TX, sc-15334; anti-CCL20, AF760, R&D Systems MN; Acti-stain 670 phalloidin, Cytoskeleton, CO) in 0.2% saponin/0.2% BSA/PBS for overnight at 4°C. The binding of primary Ab was detected with Alexa Fluor 488-conjugated anti-goat IgG and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch). The stained specimens were mounted in 30% glycerol in PBS, and analyzed using a FV300 confocal laser scanning microscope (Olympus).

To detect ILFs, the small intestinal serosa was removed using filter paper and tweezers while monitoring with a stereoscopic microscope. The specimens were fixed in ice-cooled 2% PFA/PBS for 30 min, and then washed with PBS for 10 min at 4°C. To block endogenous peroxidase activity, the fixed specimens were incubated with 0.3% H2O2 in methanol for 15 min and then rinsed in PBS three times for 10 min each. The specimens were incubated with PBS-MT (1% skim milk and 0.1% Triton X-100 in PBS) for 1 h at 4°C to block non-specific staining, followed by incubation with primary antibodies: anti-mouse/human CD45R/B220 (RA3-6B2, BD Biosciences) and anti-mouse CD106/VCAM-1 (429, BD Biosciences) in PBS-MT overnight at 4°C. After washing four times in PBS-MT at 4°C for 1 h each, specific binding of primary antibodies was detected with HRP-conjugated Goat anti-Rat IgG (BIOSOURCE, CA) overnight. The specimens were treated with PBS-T at RT for 20 min, soaked in PBS-T (0.1% Triton X-100 in PBS) containing 0.08% NiCl2, 0.025–0.03% diaminobenzidine (DAB; Dojin Chemical Co., Kumamoto, Japan) and 0.025% H2O2 for 30 min, followed by treatment with 4% PFA/PBS to terminate enzymatic reaction. Immature and mature ILFs can be classified on the basis of the size of B220 clusters and the presence or absence of FAE. Because of the presence of FAE, mature ILFs can be identified from the luminal side; immature ILFs lacking FAE are not well recognized from villous side of intestine as described earlier [2,3].
To detect PP anlagen, the small intestines from fetal (E17.5) and neonatal mice were stained with anti-mouse CD106/VCAM-1 (BD Biosciences) as described above. In a separate experiment, the neonatal tissues were stained with anti-CD106/VACM-1 and then embedded in paraffin. Paraffin sections were deparaffinized, rehydrated and stained with hematoxylin and Alcian blue to detect goblet cells in the FAE region.

To visualize adult PP lymphoid follicles in the small intestinal wall, the whole-mount specimens of the small intestine was incubated with 3% acetic acid for 15 min at room temperature. The stained specimens were analyzed using a stereoscopic microscope (SZX16, OLYMPUS).

**Histology**

Prefixed small intestinal tissue sections were deparaffinized, rehydrated and stained with either hematoxylin-eosin or Alcian blue-nuclear fast red.

**Immunostaining**

Immunohistochemical detection of CCL20, CCL21, CXCL13, VCAM1, ICAM1, MadCAM1, Desmin and Dll1 was performed as described previously with a minor modification. Briefly, paraffin sections of PP prefixed with Zn-formalin solution (Polysciences, PA) were dehydrated, incubated with Target retrieval solution (DAKO Cytomation, Glostrup, Denmark), treated with 0.5% blocking solution (Roche, Basel, Switzerland) in PBS, and then goat anti-VCAM-1 (AF643), anti-CCL20 (AF760), anti-CCL21 (AF457), anti-CXCL13 (AF470) or sheep anti-Dll1 (AF5026) polyclonal antibodies (all from R&D Systems), rabbit anti-Desmin (ab8592, Abcam, UK), or Armenian Hamster anti-ICAM1 (3E2, BD Bioscience). After the incubation with 3% H2O2, the binding of primary antibodies was detected with biotinylated donkey anti-goat, anti-rat, anti-sheep, or anti-rabbit IgG (Jackson ImmunoResearch, PA) followed by streptavidin-horseradish peroxidase (ABC Elite; Vector Laboratories, CA), and was visualized with 3, 3’-diaminobenzidine (DAKO Cytomation), Fluorescein Tyramide or Cyanine 3 Tyramide (PerkinElmer, MA). The tissue specimens were counterstained with Alcian blue or DAPI.

For the detection of CD3ε and B220, PP tissues were fixed with 4%PFA/PBS for 2 h at 4°C followed by the treatment with 30% Sucrose before embedding in OCT compound. Frozen sections were treated with 0.5% blocking solution (Roche, Basel, Switzerland) in PBS, and then Armenian hamster anti-CD3ε (145-2C11) and rat anti-CD45R/B220 (RA3-6B2) (BD Bioscience). The binding of primary antibodies was detected with Alexa Fluor 488-conjugated
anti-rat IgG and Cy3-conjugated anti-Armenian hamster IgG (Jackson ImmunoResearch) followed by counterstaining with DAPI.

**β-galactosidase staining**

Intestines were isolated, immediately flushed with PBS and incubated in ice-cold fixative (4% paraformaldehyde, 1.25mM EGTA, 2mM MgCl₂ in PBS) for 10 min on ice. After washing with PBS the samples were embedded in OCT compound. Frozen sections were incubated in the β-galactosidase substrate [5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆·3H₂O, 2 mM MgCl₂, 0.02% NP40, and 1 mg/ml X-gal in 0.1 M phosphate buffer, pH 8.0] in the dark overnight at 37°C. The specimens were washed twice in PBS, mounted on microscope slides and analyzed using a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

**Image analysis**

Confocal images were acquired by photon counting mode of a FV1000 confocal microscopy system with FV10-ASW software (Olympus) and LSM 710 NLO (Carl Zeiss, Oberkochen, Germany). Muc2-positive cells per FAE area (µm²) were calculated using ImageJ software.

**Flow cytometric analysis**

The following monoclonal antibodies (mAbs) conjugated with FITC, PE, PE-Cy7, APC, APC-eFluor780, Pacific Blue, or V500 were purchased from BD Bioscience, eBioscience or Biolegend: anti-mouse CD3ε (clone: 145-2C11), CD4 (GK1.5), CD44 (IM7), CD45R/B220 (RA3-6B2), CD62L (MEL-14), CD95 (Jo2), GL7 (GL-7). The stained samples were analyzed using a FACS CantoII (BD Bioscience) and the obtained raw data were further analyzed using FlowJo software version 9.4.10 (Tomy Digital Biology, Tokyo, Japan).

**Preparation of lymphocytes**

A mononuclear cell fraction including lymphocytes and stromal cells was prepared as described previously[4]. Briefly, PPs and the small intestine were minced and dissociated with RPMI1640 medium containing 20 mM HEPES (pH 7.4), 2% FCS, 0.5 mg/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan), 250 U/ml dispase (BD Biosciences) and 0.5 mg/ml DNase I (Roche) at 37°C for 30 min. The single-cell suspensions were subsequently pooled in RPMI 1640 medium supplemented with 2% FBS, and were subjected to Percoll gradient separation to isolate the PP lymphocytes.
Analysis of LTβR-dependent expression of CCL20 in epithelial cells

RBP-JIEC-KO mice and control littermates were injected intraperitoneally with an agonistic antibody specific for mouse LTβR (ACH6), kindly provided by Jeffery L. Browning. 6h later, epithelial cells were isolated from the small intestine of these mice. Total RNA was isolated from the epithelial cells using the RNeasy mini kit (QIAGEN, Hilden, Germany) and was subjected to reverse transcription using the ReverTra Ace kit (TOYOBO, Osaka, Japan) following the manufacturer’s instructions. The cDNA samples were amplified with the SYBR premix Ex Taq (TAKARA BIO, Shiga, Japan) and the primer sets specific for Ccl20 and Actb genes. The sequences of the primer sets are available upon request. Target gene expression was assessed by a comparative cycling threshold (CT) method according to the manufacturer’s protocol (Thermal Cycler Dice Real Time System, TAKARA BIO). The value of Ccl20 was normalized to that of Actb.

In vitro culture experiment

Stable knockdown of Rbpj gene in T84 cells was performed using MISSION lentiviral transduction particles (SIGMA-ALDRICH), following the manufacturer’s instructions. Rbpj knockdown and control T84 cells were grown to be a confluent monolayer for 11 days on transwell filters (6-mm diameter, 3um pore, Corning, NY), and then stimulated with 100 ng/ml recombinant human LTαβ2 protein (R&D system) for 3 h. Total RNA from the cells was isolated using the TRIzol reagent (Life Technologies MD), and was subjected to reverse transcription using the ReverTra Ace kit (TOYOBO, Osaka, Japan) following the manufacturer’s instructions. The cDNA samples were amplified with the SYBR premix Ex Taq (TAKARA BIO, Shiga, Japan) and the primer sets specific for Ccl20 and Gapdh genes. The sequences of the primer sets are available upon request. Target gene expression was assessed by a comparative cycling threshold (CT) method according to the manufacturer’s protocol (Thermal Cycler Dice Real Time System, TAKARA BIO). The value of Ccl20 was normalized to that of Gapdh.

Immunization

For oral immunization, mice were deprived of food for 2 h and then given a solution of sodium bicarbonate to neutralize gastric acid before oral immunization. Thirty minutes later, the mice were orally immunized by gastric intubation with 1 mg/mouse OVA (SIGMA-ALDRICH, MO) and 10 µg/mouse CT (List Biological Laboratories, CA) as a mucosal adjuvant. This oral
immunization procedure was conducted on days 0, 7 and 14. Fecal samples were obtained at
day 28 after administration of OVA.

Detection of OVA-specific IgA

Ninety-six-well plates were coated with 1 mg/ml OVA (SIGMA-ALDRICH) at 4°C overnight
and blocked with 2% BSA at room temperature for 2 h. Fecal samples were diluted 50-fold in
2% BSA. After the incubation, the plates were washed with 0.2% Tween20/PBS and
incubated with HRP-labeled anti-mouse IgA antibodies (BETHYL Laboratory, TX) for 2 h
at room temperature. TMB (3, 3’, 5, 5’-tetramethylbenzidine) with H2O2 was added for color
development.

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Supplementary figure 1. Detection of Dll1-expressing cells during PP development.
(A and C) PP sections from Dll1-lacZ mice at adult (A) and E18.5 (C) stages were stained with β-galactosidase substrate solution to detect Dll1-expressing cells. Dashed lines show the FAE region. Scale bars represent 200 μm (left panels) or 100 μm (right panels). Data are representative of two independent experiments with similar results. (B) The expression of Dll1 in PP cells from adult mice was analyzed using a flowcytometry. The expression of Dll1 by CD45+ and CD45− PP cells is shown (left, histograms). CD45−Dll1+ cells were further analyzed for VCAM1 and ICAM1 expression (right). Data were representative of two independent experiment with similar results.
Supplementary figure 2. Flow cytometric analysis of the PP cellular composition
(A and B) The cellular composition of PP was analyzed using a flow cytometer. Representative FACS plots are shown (A). The frequency of T/B lymphocytes, CD4+/CD8+ T lymphocytes, naïve (CD44loCD62hi), effector (CD44hiCD62lo) and germinal center B cells was quantified (B). Values are mean ± SD (n = 4). *p < 0.05, as calculated by Student’s t-test. n.s.: not significantly different. Data are representative of two independent experiments with similar results.
Supplementary figure 3. Goblet cell hyperplasia in FAE of neonatal and adult RBP-\( ^{\text{IEC-KO}} \) mice.
(A) RBP-\( ^{\text{IEC-KO}} \) mice and control littermates were injected intraperitoneally with an anti-LTBR agonistic antibody. The small intestinal epithelium was prepared for Q-PCR analysis using a specific primer set for CCL20. Data were normalized to expression of Actb (encoding β-actin). Values are mean ± SD (\( n = 4 \)). *\( p < 0.05 \) (Mann-Whitney U test). n.s.: not significantly different.
(B) Neonatal small intestine was subjected to whole mount immunostaining for VCAM-1 to identify lymphoid follicles. Tissue sections of the stained specimens were further treated with Alcian blue for detection of goblet cells.
(C) Adult PP sections were stained with Alcian blue followed by nuclear fast red counterstaining. Scale bars represent 100 μm. Data are representative of two independent experiments.
(D) Rbpj knockdown and control T84 cells were stimulated with 100 ng/ml recombinant LT\( \alpha_1 \beta_2 \) for 3 hours on transwell filters. Cells were prepared for Q-PCR analysis using a specific primer set for CCL20. Data were normalized to expression of GAPDH. Values are mean ± SD (\( n = 3 \)). **\( p < 0.01 \), n.s.: not significantly different, as calculated by one-way ANOVA test.
**Supplementary figure 4. Diagram model of GALT maturation**

After the induction of the organizing center for PP lymphoid follicles by the interaction between LTi and LTo cells during E15.5, Notch ligand Dll1-expressing LTo cells interact with intestinal epithelial cells during late stage of embryogenesis. Epithelial Notch activation restricts secretory cell differentiation by repressing the expression of Atoh1. After the formation of FAE, LTi cells activate LTβR signaling in epithelial cells, leading to CCL20 expression. This chemokine recruits CCR6+ lymphocytes into the PP anlagen, which induces the compartmentalization of PP lymphoid follicles. Based on this model, epithelial Notch signaling plays a key role in the full maturation of GALT by ensuring interactions among LTi, LTo and epithelial cells.