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

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Abstract

Intrinsic Notch signaling in intestinal epithelial cells restricts secretory cell differentiation. In gut-associated lymphoid tissue (GALT), stromal cells located beneath the follicle-associated epithelium (FAE) abundantly express the Notch ligand delta-like 1 (Dll1). Here, we show that mice lacking Rbpj—a gene encoding a transcription factor implicated in Notch signaling—in intestinal epithelial cells have defective GALT maturation. This defect can be attributed to the expansion of goblet cells, which leads to the down-regulation of CCL20 in FAE. These data demonstrate that epithelial Notch signaling maintained by stromal cells contributes to the full maturation of GALT by restricting secretory cell differentiation in FAE.

Keywords follicle-associated epithelium; gut-associated lymphoid tissues; intestinal epithelial cells; Notch signaling

Subject Categories Development & Differentiation; Immunology; Signal Transduction

DOI 10.15252/embr.2014238942 | Received 22 April 2014 | Revised 2 October 2014 | Accepted 7 October 2014 | Published online 5 November 2014

EMBO Reports (2014) 15: 1297–1304

Introduction

Peyer’s patches (PPs) develop in the sterile environment of the fetus. This process necessitates bidirectional signals among the hematopoietic cells, stromal cells, and intestinal epithelial cells (IECs). At embryonic day 15.5 (E15.5) in mice, lymphotixin α1β2 (LTα1β2)-expressing lymphoid tissue inducer (LTi) cells interact with the surrounding lymphotixin β receptor (LTβR)-expressing lymphoid tissue organizer (LTo) cells, leading to the induction of chemokines and adhesion molecules involved in the recruitment and organization of lymphocytes [1]. Furthermore, in LTo cells, LTβR signaling up-regulates expression of interleukin-7 and receptor activator of nuclear factor-κB ligand (RANKL), a tumor necrosis factor superfamily member, which increases LTα1β2 expression in newly arriving LTi cells [2]. This positive feedback loop promotes maturation of the PP anlagen at the late embryonic stage. In addition, LTα1β2-expressing LTi cells transduce epithelial LTβR signaling at E17.5 to induce CCL20 in follicle-associated epithelium (FAE) that harbors microfold/membranous cells [3]. FAE-derived CCL20 can be assumed to play a significant role in the later stages of PP development, given that the absence of CCR6 results in fewer lymphocytes in PPs without affecting the number of PPs in the intestine [4–6]. This fact suggests the importance of FAE development in the full maturation of PPs. However, little is known about the molecular mechanisms controlling the development and homeostasis of FAE during PP organogenesis.

Notch signaling pathway is an evolutionary conserved mechanism that regulates cell fate decision and development in the various types of cells. Notch ligands are transmembrane proteins termed Jagged (Jag1 and Jag2) and Delta-like (Dll1, 3 and 4). After the ligand binding to Notch receptors on the cell surface of adjacent cells, Notch intracellular domain (NICD) translocates into the nucleus and forms a complex with recombination signal-binding protein for immunoglobulin κ J region (RBP-J). In IECs, the NICD/RBP-J complex activates Notch target genes such as Hes1, which represses the expression of Atoh1, a responsible transcription factor...
for the differentiation of secretory cell lineages [7]. Thus, epithelial Notch activation inhibits the differentiation of secretory cell lineages and plays a critical role in deciding the fate of intestinal epithelial progenitor cells between the secretory and absorptive lineages.

A previous study identified stromal cells expressing the Notch ligand Dll1 beneath FAE of PPs in adult mice [8], although their biological significance was not then elucidated. Here, we show that epithelial Notch signaling contributes not only to the maintenance of the integrity and homeostasis of the FAE but also to the full maturation of gut-associated lymphoid tissue (GALT). Our data demonstrate the biological importance of epithelial–stromal interactions in the development of the gut immune system.

Figure 1. Detection of Delta-like 1 (Dll1)-expressing cells during Peyer's patch (PP) development.

A, B PP sections from C57BL/6 mice at adult stage (A) and embryonic day 18.5 (E18.5) (B) were stained for Dll1 (red) together with VCAM1, ICAM1, desmin, or CXCL13 (green). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). Dotted lines show the apical edge of the follicle-associated epithelium (FAE). Scale bars represent 100 μm. Data are representative of two independent experiments with similar results.
Results and Discussion

Detection of Dll1-expressing LTo cells beneath FAE in PPs

To investigate the role of Notch signaling in GALT formation, we first analyzed the distribution of the Notch ligand Dll1 in PPs using Dll1-lacZ reporter mice [9]. Dll1 was expressed in proximity to the FAE of PPs in adult mice (Supplementary Fig S1A). A previous study showed that these Dll1+ cells express mucosal addressin cell adhesion molecule 1 (MadCAM1), a stromal marker [8]. In addition, we found that the Dll1+ cells beneath the FAE were positive for vascular cell adhesion molecule 1 (VCAM1) and sometimes positive for intercellular adhesion molecule 1 (ICAM1) but not desmin (Fig 1A). Flow cytometry showed that nearly all CD45+/CD11b+ stromal cells expressed VCAM1, and approximately 10% of these coexpressed ICAM1 (Supplementary Fig S1B). Thus, it is VCAM1+ and VCAM1+ICAM1+ cells in the subepithelial dome region that send signals to the FAE via Dll1.

We subsequently examined Dll1 expression during PP development at the late embryonic stage. As with a previous study [10], Dll1+ cells were abundant in the PP anlagen at E18.5, and some were in contact with FAE (Supplementary Fig S1C). These cells were identified as LTo cells by their expression of CXCL13, which is important for PP development [11] (Fig 1B).

Role of epithelial Notch signaling in integrity of the FAE and maturation of GALT

The expression of the Notch ligand in the subepithelial dome regions raised the possibility that LTo cell-mediated activation of Notch signaling may contribute to the organization of the FAE. To directly address this possibility, we analyzed the FAE of mice with an IEC-specific knockout of the gene coding for RBP-J (RBP-JIEC-KO mice). We have previously reported a marked increase in all three types of secretory epithelial cells in the intestine of RBP-JIEC-KO mice [12]. The same proved true for FAE. Although there were very few goblet cells in the FAE of control mice without RBP-J deficiency, RBP-JIEC-KO mice showed abnormal expansion of goblet cells (Fig 2A–D). This observation was further confirmed by up-regulation of genes associated with goblet cells (Fig 2E). This observation was further confirmed by up-regulation of genes associated with goblet cells (Fig 2E), indicating that Notch signaling is essential for the restriction of goblet cell differentiation in FAE.

Unexpectedly, the number of lymphoid follicles per PP was significantly reduced in RBP-JIEC-KO mice compared with controls (Fig 3A and B), although the total number of PPs in the small intestine was comparable between the two groups (Fig 3B).
Figure 3. Characterization of gut-associated lymphoid tissue (GALT) in RBP-JIEC-KO mice.

A Peyer’s patch (PP) tissues were analyzed for lymphoid follicle formation. Asterisks indicate lymphoid follicles. Data are representative of three independent experiments. Scale bars represent 1 mm.

B Surface area, the number of PPs, follicles per PP, and total number of PP lymphocytes were quantified. Values are presented as the mean ± standard deviation (n = 3–4). **P < 0.01, as calculated with the Student’s t-test. n.s.: not significantly different.

C Immunofluorescence staining of the PP tissues from RBP-JIEC-KO mice and control littermates was performed using monoclonal antibodies against CD3e (red) and B220 (green) and polyclonal antibodies against CCL21 or CXCL13 (green). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (blue). Data are representative of three independent experiments. Scale bars represent 0.2 mm.

D, E Whole-mount staining of small intestines from 10- to 14-week-old RBP-JIEC-KO and control mice was performed using a monoclonal antibody against B220. Representative macroscopic views of the serosal side of stained small intestines from RBP-JIEC-KO and control mice are shown (D). Scale bars represent 2 mm. The numbers of immature and mature ILFs in the small intestine were quantified (E). Values are presented as the mean ± standard deviation (n = 3). *P < 0.05, as calculated with the Student’s t-test. n.s.: not significantly different.

Source data are available online for this figure.
Likewise, the total number of PP cells in RBP-JIEC-KO mice was decreased by almost half compared with controls (Fig 3B). Normal PP microstructure [13] and the composition of the PP immune cell population were maintained in the absence of RBP-J. Notably, the frequency of germinal-center B cells was significantly increased in RBP-JIEC-KO mice (Supplementary Fig S2), which might reflect a compensatory reaction to the reduction in lymphoid follicles resulting from RBP-J deficiency. Collectively, these results suggest that IEC-intrinsic Notch signaling is important for the maturation of PPs but does not affect the composition of the PP immune cell population.

Isolated lymphoid follicles (ILFs), another constituent of GALT, function as an inductive site for mucosal immune responses [14]. ILFs are postnatally generated from cryptopatches through two steps. First, immature ILFs are induced by the action of LTαβ-expressing LTi cells; they then undergo further maturation in response to colonization by commensal microbiota [16]. Mature ILFs consist of a single B-cell follicle with a germinal center and FAE. The absence of PPs results in a compensatory increase in the number of mature ILFs [17]. We therefore analyzed the development of ILFs in RBP-JIEC-KO mice. The number of mature ILFs was significantly reduced in RBP-JIEC-KO mice, whereas the number of immature ILFs remained unchanged (Fig 3D and E). Thus, epithelial Notch signaling appears to be indispensable for the maturation of PPs and ILFs.

Role of epithelial Notch signaling in compartmentalization of lymphoid follicles during PP development

We further explored the role of epithelial Notch signaling in organogenesis of PPs in RBP-JIEC-KO mice. PP anlagen are defined as discrete regions expressing VCAM1 [5]. Whole-mount immunostaining for VCAM1 showed normal formation of PP anlagen in RBP-JIEC-KO mice at E17.5 (Fig 4A); however, follicles showed impaired compartmentalization at the neonatal stage (Fig 4B). These observations suggest that epithelial Notch signaling contributes to the compartmentalization of follicles during the later stages of PP development. Thus, the observed reduction in the number of lymphoid follicles in PPs of adult RBP-JIEC-KO mice may be due to a defect in the compartmentalization of lymphoid follicles at the late embryonic stage.

Role of epithelial Notch signaling in FAE-specific chemokine expression and FAE cell proliferation

A reduced number of lymphoid follicles in PPs has also been observed in CCR6-deficient mice [6,18]. CCR6 is expressed by the immune cells in PPs, including B cells and subsets of innate lymphoid cells, dendritic cells, and T cells [19]. The ligand for CCR6, CCL20, is specifically expressed by FAE from E17.5 onward over the course of lymphoid follicle compartmentalization [4,5]. LTαβ-expressing LTi cells in the PP anlagen induce CCL20 expression in FAE by activating LTβR signaling in epithelial cells at the late embryonic stage [4]. Therefore, CCL20 appears to play an important role in the maturation and compartmentalization of lymphoid follicles by recruiting CCR6-expressing immune cells at this stage. The CCL20-CCR6 axis is also important for ILF maturation [20]. We therefore analyzed the expression of CCL20 in PP FAE of RBP-JIEC-KO mice. Immunostaining of PPs showed remarkably attenuated expression of CCL20 in FAE in RBP-JIEC-KO mice (Fig 4C), along with expansion of goblet cells completely devoid of CCL20 expression (Fig 4D). The lack of CCL20 expression in goblet cells might be due to the lower expression of LTβR in secretory epithelial cells than in absorptive enterocytes, although further investigation is required to prove this possibility. Taken together, these observations imply that Notch-dependent suppression of goblet cell differentiation ensures constitutive CCL20 expression, which is a prerequisite for maturation of PP follicles.

To assess the efficiency of CCL20 induction in villus epithelium, we treated RBP-JIEC-KO mice with LTβR-agonistic antibodies. We observed significantly lower induction of CCL20 in RBP-JIEC-KO mice than in control mice (Supplementary Fig S3A), supporting the notion that goblet cell hyperplasia results in reduction of CCL20 expression. Indeed, goblet cell hyperplasia coincided with impaired formation of FAE and lymphoid follicles at both neonatal and adult stages (Supplementary Fig S3B and C). In contrast, RBP-J knockdown in T84 human absorptive epithelial cells did not interfere with LTαβ-dependent CCL20 induction (Supplementary Fig S3D). Therefore, Notch signaling appears dispensable for the intracellular signaling pathway regulating CCL20 expression, although it is required for the restriction of goblet cell expansion in FAE. Notch-dependent inhibition of goblet cell differentiation seems to be a prerequisite for full FAE maturation.

Partial rescue of PP compartmentalization defect by deletion of Atoh1 in RBP-JIEC-KO mice

Impaired GALT maturation associated with goblet cell hyperplasia in RBP-JIEC-KO mice may result from aberrant activation of the Atoh1 transcription factor due to lack of RBP-J-dependent Hes1 expression. To test this hypothesis, we compared PP formation between Atoh1-deficient and Atoh1-sufficient RBP-JIEC-KO mice (Atoh1+/RBP-JIEC-KO and Atoh1+/+/RBP-JIEC-KO, respectively). The defect in the compartmentalization of PP follicles in Atoh1−/−/RBP-JIEC-KO mice was at least partly improved in Atoh1+/RBP-JIEC-DKO and Atoh1+/+/RBP-JIEC-DKO mice (Fig 4E). We confirmed that Atoh1+/RBP-JIEC-DKO mice were devoid of goblet cells and died around weaning age (Fig 4E), consistent with a previous report on Atoh1−/− mice [21]. We therefore analyzed lymphoid follicle formation in PPs at 2 weeks of age. Consistent with the observation at neonatal stage, deletion of the Atoh1 gene at least partially rescued the reduction of lymphoid follicles in RBP-JIEC-KO mice (Fig 4F). These results confirmed that epithelial Notch signaling-dependent repression of Atoh1 contributes to the maturation of PPs.

Defect in induction of mucosal immune responses in RBP-JIEC-KO mice

Because GALT plays a pivotal role in immunosurveillance at the mucosal surface by inducing antigen-specific immunoglobulin A production [13], we investigated the contribution of epithelial Notch signaling to the mucosal immune response. After oral immunization with ovalbumin (OVA) in the presence of cholera toxin as a mucosal adjuvant, the amount of OVA-specific immunoglobulin A in feces was significantly decreased in the RBP-JIEC-KO mice compared with...
control mice (Fig S5), demonstrating that maturation of GALT supported by epithelial Notch signaling is essential for the efficient induction of antigen-specific mucosal antibody responses.

On the basis of these observations, we propose the following model of the role of epithelial Notch signaling in PP formation (Supplementary Fig S4). First, LTo cells in PP anlagen express Dll1 to inhibit the differentiation of secretory epithelial cells in the proto-FAE region via activation of Notch signaling prior to the compartmentalization of lymphoid follicles. This mechanism facilitates the formation of FAE. Therefore, Dll1⁺ stromal cells function as “FAE organizers” at the late embryonic stage. Considering that the FAE harbors microfold cells and serves as a portal for mucosal antigen...
uptake, the restriction of goblet cell differentiation byDll1+ stromal cells may also contribute to the efficient uptake of mucosal antigen into lymphoid follicles by reducing the mucous layer overlying the FAE. Although further investigation will be required to uncover the mechanism ofDll1 expression by LTo cells in PP anlagen, our finding provides novel mechanistic insight into the involvement ofDll1+ stromal cells and epithelial Notch signaling in the histogenesis of GALT.

Materials and Methods

Detailed descriptions of our methods can be found in the Supplementary Methods.

Animal experiments

Mice carrying a floxed Rbpj allele (RBP-J<sup>F/F</sup>) [22] were obtained from RIKEN BioResource Center (Yokohama, Japan). Atoh1<sup>F/F</sup> mice and Dll1-lacZ reporter mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). To obtain RBP-JIEC-KO and RBP-J-Atoh1IEC-KO mice, we crossed RBP-J<sup>F/F</sup> and RBP-J-Atoh1<sup>F/F</sup> mice with Villin-Cre transgenic mice obtained from The Jackson Laboratory. The whole-mount specimens of PPs were fixed with BD Cytofix/C6 enzyme-linked immunosorbent assay. Values are presented as the mean ± standard deviation (n = 6–9 for day 0, n = 5–6 for day 28). **P < 0.01, as calculated with the Student's t-test. n.s.: not significantly different.

Figure 5. Attenuated antigen-specific mucosal immune response in RBP-JIEC-KO mice.

RBP-J<sup>IEC-KO</sup> and control mice were administered ovalbumin (OVA) orally in the presence of cholera toxin as a mucosal adjuvant. The levels of OVA-specific immunoglobulin A in fecal suspensions were determined at days 0 and 28 by enzyme-linked immunosorbent assay. Values are presented as the mean ± standard deviation (n = 6–9 for day 0, n = 5–6 for day 28). **P < 0.01, as calculated with the Student's t-test. n.s.: not significantly different.

Source data are available online for this figure.

Differences between two groups were analyzed with the Student's t-test. When variances were not homogeneous, the data were analyzed by the Mann–Whitney U-test. Differences among more than two groups were analyzed by one-way analysis of variance followed by the Dunnett’s test.

Acknowledgements

We would like to thank Dr. Tasuku Honjo for providing RBP-J<sup>F/F</sup> mice, Dr. Jeffrey L. Browning for the anti-LTβR antibody, and Dr. Peter D. Burrows for critical reading and English editing of the manuscript. We also thank Dr. Takashi Kanaya, Dr. Daisuke Takahashi, and Ms. Toshi Jinnohara for valuable comments and suggestions.

Funding

This study was supported in part by grants from the Japan Society for the Promotion of Science (24117723 to KH, 24249029 to HO, and 252667 to YO), Japan Science and Technology Agency (KH), RIKEN RCJ Young Chief Investigator program (KH), Uehara Memorial Foundation (KH), Mochida Memorial Foundation for Medical and Pharmaceutical Research (KH), Toray Science Foundation (KH), and Takeda Science Foundation (HO).

Author contributions

YO contributed to study design, acquisition and analysis of a large part of the data, and writing of the manuscript. SK contributed to acquisition and analysis of data. GN, KI, YN, YM, YFur, and YFuj contributed to acquisition of data. ME, MS, and TI contributed to the provision of experimental protocols. KS contributed to the provision of materials. KH contributed to study concept, analysis and interpretation of data, and writing of the manuscript and obtained funding. HO contributed to interpretation of data and critical editing of the manuscript and obtained funding.

Conflict of interest

The authors declare that they have no conflict of interest.

References


