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Microbial Induction of B and T Cell Areas in Rabbit Appendix

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Abstract

Gut-associated lymphoid tissue (GALT) development requires interaction with the intestinal microbiota. Because murine secondary lymphoid tissue development is driven by positive feedback interactions between B cells and stromal cells, we used *in situ* hybridization to determine whether intestinal commensals influence such interactions during rabbit appendix development. The features of positive feedback interactions we examined (CXCL13 mRNA expression, B cell accumulation and FDC differentiation) increased during early follicle development, but stalled in the absence of intestinal commensals. These features were reinitiated by commensals that stimulated follicle development and intrafollicular B cell proliferation. Our results suggest that rabbit appendix follicles develop in two phases: an initial phase of B cell recruitment to nascent follicles, possibly through positive feedback interactions, and a subsequent phase of intrafollicular B cell proliferation stimulated by intestinal commensals. In addition, we found that intestinal commensals stimulate appendix CCL21 mRNA expression and T cell area formation.

Keywords

Chemokines; B cell follicles; B cell proliferation; Gut-associated lymphoid tissue (GALT); Intestinal commensals; Intestinal microbiota; Rabbit appendix; T cell areas

1. Introduction

The strategy rabbits use to generate a large B cell population expressing diverse antibody specificities differs significantly from that used by mice and humans. While B-lymphopoiesis continues throughout life in mice and humans, most B-lymphopoiesis in rabbits occurs early in ontogeny, decreasing by over 99% within a few months after birth [1]. Adult rabbit B cells are therefore presumably long-lived and/or self-renewing. In further contrast, rabbit B cells utilize a restricted number of different V, D and J gene segments during VDJ gene rearrangement, thereby generating a limited neonatal antibody repertoire. After leaving the bone marrow, newly developed B cells seed gut-associated lymphoid tissues (GALT), particularly the appendix and sacculus rotundus, where they proliferate and concomitantly diversify their V(D)J genes through gene conversion [2,3] and somatic hyper-point mutation [4,5]. These developmental events greatly expand the B cell population and extensively

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diversify the primary antibody repertoire to provide immune protection against foreign antigens.

The intestinal microbiota plays a crucial role in these developmental processes in rabbit GALT. This was first appreciated when Perey and Good [6] demonstrated that preventing microbial colonization of the appendix, through cecal ligation at birth, arrested appendix follicle development. Subsequently, rabbits raised under germ-free conditions were found to have markedly underdeveloped GALT and poor antibody responses to antigenic challenges [7,8]. Recent studies have shown that rabbit appendix development and VDJ gene diversification are induced by select members of the intestinal microbiota [9,10]. In one study, the intestinal microbiota was experimentally altered by hand raising sterilely derived rabbit pups under non-germ-free conditions, but without contact with normal rabbits and their microbiota [9]. GALT was underdeveloped and 70% of the IgM VDJ genes from peripheral blood B cells were undiversified in these rabbits, suggesting that GALT development and VDJ gene diversification were not dependent simply on the presence, but on select members, of the intestinal microbiota. This was confirmed by experiments in which individual intestinal isolates were introduced into rabbit appendices rendered germ-free by microsurgical ligation at birth [10]. Although most of the bacterial isolates tested did not induce appendix follicle development or VDJ gene diversification, the combination of *Bacteroides fragilis* and *Bacillus subtilis* consistently induced both of these processes. These studies demonstrate that select intestinal commensals play an essential role in rabbit appendix development and primary antibody repertoire diversification.

The mechanism by which intestinal bacteria induce rabbit appendix development is not known. The formation of B and T cell areas in murine secondary lymphoid tissues is directed by regional expression of the chemokines CXCL13 and CCL19/CCL21, respectively [11,12]. Furthermore, B cell follicle development in murine secondary lymphoid tissues is driven by chemokine-mediated positive feedback interactions between B cells and stromal cells [13, 14]. Stromal cell expression of the B cell chemoattractant CXCL13 primes these interactions by attracting an initial cluster of B cells expressing the CXCL13 receptor, CXCR5, to the nascent follicle [12,14–16]. B cells are a major source of membrane lymphotoxin ($LT\alpha_1\beta_2$) and tumor necrosis factor (TNF), cytokines that promote follicular dendritic cell (FDC) development through engagement of their receptors, the lymphotoxin β receptor ($LT\beta R$) and TNF receptor 1 (TNFR1), respectively [17–23]. In addition, $LT\alpha_1\beta_2$ upregulates CXCL13 expression in stromal cells and FDCs, which attracts additional B cells to the developing follicle [14]. A positive feedback loop is thereby established that drives follicle development by promoting B cell recruitment and the formation of FDC networks. Positive feedback interactions appear to be of central importance in the development of the spleen, lymph nodes, and Peyer's patches in mice [13]. The development of T cell areas, although less clearly understood, is dependent on stromal expression of the chemokines CCL19 and CCL21, which attract $CCR7^+$ T cells and dendritic cells [11,24].

To gain insight into the mechanism by which intestinal commensals induce rabbit appendix development, we examined B and T cell area formation in normal and manipulated appendix. Our results suggest that appendix follicles develop in two phases: an initial phase, possibly driven by positive feedback interactions, during which B cells are recruited to the nascent follicle, and a subsequent phase driven by intrafollicular B cell proliferation stimulated by intestinal commensals. We also found that intestinal commensals stimulate, with varying effectiveness among species, appendix CCL21 mRNA expression and T cell area development.

2. Materials and Methods

2.1 Immunofluorescence analysis of thin tissue sections

Tissues were harvested and frozen in TBS embedding medium (Triangle Biomedical Sciences, Durham, NC) in 2-methylbutane cooled in liquid nitrogen. Tissue sections (7 μ m) were cut with a cryostat microtome, fixed in cold acetone, and blocked with goat serum. Sections were stained with the following rabbit-specific or cross-reactive primary antibodies: biotinylated 367, a mouse anti-rabbit C μ mAb (BD Biosciences, Palo Alto, CA) [25]; mouse anti-rabbit CD4 mAb (KEN-4; BD Biosciences); mouse anti-rabbit CD8 mAb (12C7; Serotec Inc., Raleigh, NC); Ki-67 mAb for identification of proliferating cells (Pharmingen, San Diego, CA) [26]. PE-conjugated streptavidin (Invitrogen, Carlsbad, CA) and Cy2-conjugated goat anti-mouse F(ab) (BD Biosciences) were used as secondary reagents. Appropriate isotype-matched antibodies were used as negative controls. LigApx rabbit tissues were generously provided by Dr. Katherine L. Knight (Department of Microbiology and Immunology, Loyola University Chicago).

2.2 Riboprobe preparation

A 500–700 bp PCR product from each gene of interest was cloned into the pGEM-T vector (Promega, Madison, WI). Sense and anti-sense *in vitro* transcription templates were generated by PCR amplification from each plasmid, using the appropriate gene-specific forward or reverse primer with a plasmid-specific primer containing either the T7 or SP6 RNA polymerase promoter (respectively, T7 Univ: 5'-agtgaattgtaatacgaactactataggg-3' and SP6 Univ: 5'-cgccaagctatttaggtgactatagaatac-3'). PCR products were purified with Montage PCR centrifugal filters (Millipore Corp, Bedford, MA) and 200 ng purified PCR product was used as template in *in vitro* transcription reactions containing RNA labeling mix with digoxigenin-UTP (or with fluorescein-UTP for 2 color *in situ* hybridization) (Roche, Mannheim, Germany) and T7 or SP6 RNA polymerase (Fisher Scientific, Pittsburgh, PA). Plasmid template was digested with RNase-free DNase I (Fermentas Inc., Hanover, MD) and the reactions were stopped by addition of 0.2M EDTA. RNA transcript integrity and concentration was assessed by electrophoresis on denaturing 5% polyacrylamide gels. GenBank accession numbers for nucleotide sequences used to prepare riboprobes: CXCL13 (EU407474), C μ (J00666; nucleotides 473–1041), CR1 (EU407475), CXCR5 (EU407476), LT β (EU407477), LT α (M60340; nucleotides 1168–1243; 1327–1433; 1631–2041), CD3 (NM 001082001; nucleotides 50–588), and CCL21 (EU407478).

2.3 In situ hybridization

Tissue sections (10–12 μ m) were fixed in 4% paraformaldehyde and washed in ice-cold PBS. Sections were pre-equilibrated in 0.2N HCl, permeabilized with pepsin (in 0.2N HCl) (Roche, Mannheim, Germany) and washed in ice-cold PBS. Sections were pre-equilibrated in 0.1M triethanolamine and acetylated in 0.25% acetic anhydride (Sigma, St. Louis, MO) in 0.1M triethanolamine. Sections were washed in PBS, equilibrated in 5X SSC, and prehybridized (mRNA *in situ* hybridization solution, DAKO Corp., Carpinteria, CA), 70°C, 2 hr. Sections were hybridized with 10–30 ng/ml digoxigenin-labeled riboprobe, 70°C, 20 hr, in a humidified container (anti-sense riboprobes were used to detect specific mRNAs; sense riboprobes served as negative controls). Hybridized sections were stringently washed, blocked with 10% normal rabbit serum and incubated with HRP-conjugated anti-digoxigenin F(ab') (DAKO). Sections were washed in TBST, incubated in biotinyl tyramide (DAKO Genpoint), washed in TBST, blocked (as above), and incubated with AP-conjugated anti-biotin F(ab') (DAKO). Sections were washed in TBST, developed with BCIP/NBT (Vector Labs, Burlingame, CA), mounted with Supermount aqueous mounting medium (BioGenex, San Ramon, CA) and cover slipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ). Sections were

photographed with a Leica DM IRB microscope (Leica Microsystems, Brannockburn, IL) using a MagnaFire 2.1C digital camera system (Optonics, Goleta, CA).

Two-color *in situ* hybridization was performed as above, except tissue sections were hybridized with both digoxigenin- and fluorescein-labeled riboprobes. The fluorescein-labeled riboprobe was detected with HRP-conjugated anti-FITC F(ab') (DAKO) and Cy3-tyramide (Perkin-Elmer Life Sciences, Boston, MA). HRP activity was exhausted by incubation in 3% H₂O₂ for 1 hr. The digoxigenin-labeled riboprobe was detected with HRP-conjugated anti-digoxigenin F(ab'), followed by biotinyl tyramide amplification, AP-conjugated anti-biotin F(ab') and ELF 97 substrate (Molecular Probes, Eugene, OR) or AlexaFluor 488-conjugated anti-biotin IgG1 (Molecular Probes). Sections were photographed with a Zeiss AxioSkop FISH microscope using MacProbe 4.1 software.

2.4 LT β R-Ig and TNFR1-Ig Adenovirus Production

The extracellular regions of LT β R and TNFR1 were cloned from rabbit appendix cDNA (LT β R PCR primers: RbLTBR KpnI f: 5'-ggtaccagtggccaccatgcgccg-3', RbLTBR BamHI r: 5'-ggatcccagcacggtcctggcaacgg-3'; TNFR1 PCR primers: RbTNFR1 KpnI f: 5'-ggtaccagcccaaatggggagtgagag-3', RbTNFR1 BamHI r: 5'-ggatcccagcactgtggcgccga-3'). PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and the fidelity of the nucleotide sequences was determined using an automated ABI Prism 310 sequencer with Big Dye-labeled terminators (Perkin-Elmer Applied Biosystems, Foster City, CA). The cloned DNA was excised with KpnI and BamHI restriction endonucleases, gel-purified and ligated into the pCDNA 3.1 vector, similarly restriction digested, into which cDNA encoding the Fc region of rabbit IgG was previously cloned (kindly provided by Dr. D. A. Dichek, Univ. of Washington School of Medicine, Seattle, WA). The nucleotide sequences were determined, as above, to ensure in-frame fusion of the LT β R and TNFR1 nucleotide sequences with that encoding the rabbit IgG Fc region. The fusion constructs were then cloned into the pShuttle-CMV AdEasy transfer vector (Qbiogene, Carlsbad, CA), and viral stocks were prepared following the manufacturer's instructions. Briefly, the cloned constructs were linearized with PmeI and transformed into BJ5183-Ad competent cells (Stratagene, La Jolla, CA). Recombinants were selected on kanamycin plates, amplified and transformed into DH10B competent cells. DNA from transformants was linearized with PacI and transfected into QBI-293A cells (Qbiogene). Virus was extracted from viral plaques by freeze thawing and lysates were used to infect QBI-293A cells for primary propagation and large-scale amplification. Viral bands were isolated on cesium chloride gradients, dialyzed, titered using the Tissue Culture Infectious Dose 50 (TCID₅₀) method (Qbiogene) and stored at -80°C.

2.5 Administration of recombinant adenovirus

In 0.5 ml PBS, 10⁹ LT β R-Ig and/or TNFR1-Ig recombinant viral particles were injected intraperitoneally into newborn rabbit pups and the rabbits were sacrificed 1 week or 3 weeks later for tissue collection. In one experiment, rabbits were reinfected at weekly intervals to ensure that the fusion protein titers remained high throughout a 3-week period. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Loyola University Chicago.

2.6 Western Blot Analysis

Ig fusion proteins were immunoprecipitated from sera with protein G beads. Samples were electrophoresed on a 10% resolving SDS-PAGE gel and transferred to a nitrocellulose membrane. Proteins were detected with a mouse anti-rabbit IgG mAb (C101-359; BD Biosciences), followed by HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA) and ECL detection reagent (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's instructions. Serum concentrations were

determined by comparison of bands to those of standards of known concentration using ImageQuant TL v2005 software (Amersham Biosciences).

Results

3.1 B cell follicle formation during early rabbit appendix development

Rabbit appendix is composed of numerous large B cell follicles and interfollicular T cell areas, which are sites of CXCL13 and CCL21 mRNA expression, respectively (Fig. 1, A and B). We examined B cell follicle formation during neonatal rabbit appendix development by using *in situ* hybridization to search for evidence of positive feedback interactions between B cells and stromal cells/FDCs. Of the genes examined (CXCL13, C μ , CR1 and LT β), only CXCL13 mRNA was expressed at birth, and this expression was confined to the appendix domes (Fig. 1E, and data not shown). We first detected B-lymphocytes entering the appendix domes on postnatal day 2, with increasing numbers accumulating over subsequent days (Fig. 2, top row). We also found that CXCL13 mRNA expression expanded concomitantly with the influx of B-lymphocytes, and remained confined to the developing follicles (Fig. 2, middle row).

We used a complement receptor 1 (CR1, or CD35) riboprobe to detect follicular dendritic cells (FDCs), because FDCs express high levels of CD35 [27]. The CR1 riboprobe detected cells that first appeared within developing follicles on postnatal day 3 (Fig. 2, bottom row), consistent temporally with positive feedback-driven FDC differentiation. We confirmed that our CR1 riboprobe did not detect B-lymphocytes, which express CR1 at low levels, by two-color *in situ* hybridization of 5-day-old rabbit appendix with C μ and CR1 riboprobes. We found that the C μ and CR1 riboprobes detected distinct, closely apposed, cell populations, characteristic in appearance to that of B-lymphocyte:FDC clusters (Figure 3). Furthermore, unlike cells detected with the C μ riboprobe, cells detected with the CR1 riboprobe were only seen within domes, suggesting they developed *in situ*, as expected for FDC differentiation.

By 1 week of age, well-developed appendix follicles contained numerous B cells, robust CXCL13 mRNA expression and well defined FDC clusters, as well as cells (presumably B cells) expressing CXCR5, LT β and LT α mRNA, molecules critical for B-lymphocyte participation in positive feedback interactions (Figure 4 and data not shown). We conclude that the temporal pattern of initial stromal CXCL13 expression, subsequent B cell accumulation associated with increasing CXCL13 expression, and concurrent appearance of FDCs that we observed during postnatal appendix development is identical to that observed during positive feedback-mediated follicle development, in which B cells, attracted by stromal CXCL13 expression, stimulate increased stromal CXCL13 expression and FDC differentiation through signals mediated by membrane-associated LT $\alpha_1\beta_2$ and TNF.

3.2 Follicle development in the absence of intestinal microbiota

To determine the role of intestinal commensals during appendix follicle development, we assessed CXCL13 expression, B cell recruitment and FDC differentiation (as identified by *in situ* hybridization with a CR1 riboprobe) in appendix rendered germ-free by ligation at birth to prevent bacterial colonization, while the vasculature was undisturbed (LigApx rabbits) [10] (samples generously provided by Dr. Katherine L. Knight). In germ-free appendix, follicles do not develop beyond the accumulation of small B cell clusters in the domes (Figure 5A, top left). We found, however, considerable CXCL13 mRNA expression and FDC differentiation in the appendix domes of LigApx rabbits (Figure 5A, top right and bottom left, respectively). The extent of FDC differentiation appeared comparable to that observed in normal appendix of 1-week-old rabbits (compare Figure 5A, bottom left, with Figure 4, top left). These results suggest that positive feedback interactions were initiated in germ-free

appendix. If so, these interactions apparently stall in the absence of intestinal commensals, because follicles fail to develop beyond a rudimentary stage in germ-free appendix.

3.3 Stimulation of follicle development by intestinal commensals

To understand how intestinal bacteria stimulate follicle expansion, we examined CXCL13 mRNA expression, B cell clusters and FDC differentiation in follicles induced by intestinal commensals in LigApx rabbit appendix. Rhee et al. (2004) demonstrated that co-introducing *Bacteroides fragilis* and *Bacillus subtilis* isolates into LigApx rabbit appendix induced robust follicle expansion [10]. Together, these commensals induced intrafollicular B cell proliferation (Fig. 5B, bottom right), which did not occur in the absence of intestinal commensals (Figure 5A, bottom right). We found that follicle expansion induced by *B. fragilis* plus *B. subtilis* was accompanied by an increase in the size of B cell and FDC clusters, as well as increased CXCL13 mRNA expression (Figure 5B, top left, bottom left and data not shown), demonstrating that these features of positive feedback interactions, in addition to intrafollicular B cell proliferation, were stimulated by signals derived from *B. fragilis* plus *B. subtilis*.

3.4 Impact of LT β R and TNFR1 signaling blockade on rabbit appendix development

During positive feedback-mediated follicle development in murine secondary lymphoid tissues, B cells deliver critical signals, via surface LT $\alpha_1\beta_2$ and TNF, through the lymphotoxin β receptor (LT β R) and TNF receptor 1 (TNFR1), respectively, expressed by stromal cells and FDCs [19–22]. To determine whether positive feedback interactions drive appendix follicle development, we blocked these signals with soluble LT β R-Ig and/or TNFR1-Ig fusion proteins introduced into newborn rabbits via recombinant adenovirus. Littermate controls were infected with adenovirus expressing only the Ig portion of the fusion proteins, or with no adenovirus. The rabbits were sacrificed 1 or 3 weeks later for tissue analysis. We confirmed the presence of fusion proteins in peripheral blood by western blot analysis of sera (Figure 6A). We first assessed the impact of the Ig fusion proteins on spleen development, which in mice is highly dependent on both LT β R and TNFR1 signaling [28]. Both the LT β R-Ig (Figure 6B, first and second rows, right) and TNFR1-Ig (data not shown) fusion proteins markedly disrupted splenic T and B cell area formation at 1 and 3 weeks of age. Furthermore, each of the fusion proteins strikingly reduced both CXCL13 and CCL21 mRNA expression in the spleen at 3 weeks of age (LT β R-Ig results shown in Figure 6B, third and fourth rows, right). These results demonstrate that, as in the mouse, both LT β R and TNFR1 signaling are required for B and T cell area formation in the rabbit spleen.

The LT β R-Ig fusion protein, alone or with the TNFR1-Ig fusion protein, hindered follicle formation during the first week of appendix development (Figure 6C, top right). By visual inspection, the follicles were markedly smaller than those of control littermates and contained reduced numbers of B cells, demonstrating an important role for LT β R signaling during early follicle development. Surprisingly, however, appendix follicles were normally developed by 3 weeks of age, even when both fusion proteins were introduced weekly (Fig. 6C, bottom right). These results suggest that, in marked contrast to the spleen, neither LT β R nor TNFR1 signaling is critical for appendix development after the first week of life and that some mechanism other than positive feedback interaction drives follicle development at this time. We cannot, however, rule out the possibility that the spleen is much more sensitive to disruption of LT β R and TNFR1 signaling and that serum levels of the fusion proteins were insufficient to impede appendix development after the first week of life. Taken together, our results suggest that rabbit appendix follicles develop in two phases. During an initial phase, comprising approximately the first week of life, peripheral B cells are recruited to nascent follicles, possibly through positive feedback interactions and without a requirement for intestinal commensals. In the second phase, follicle expansion is driven by intrafollicular B cell proliferation stimulated by intestinal commensals.

3.5 T cell area formation during early rabbit appendix development

Large T cell areas are located in the interfollicular regions of the rabbit appendix. To study T cell area development, we examined CCL21 expression and T cell localization, by *in situ* hybridization, during early appendix development. We found that CCL21 mRNA was first expressed approximately 2–3 days after birth, in the basolateral region of the appendix, well below B cell clusters forming in appendix domes (Figure 7B and data not shown; some CCL21 mRNA expression was detectable by day 2 with the more sensitive BCIP staining). CCL21 mRNA expression increased and expanded rapidly over subsequent days, first contacting, and then surrounding, the growing B cell follicles (Figure 7C and D). As shown in Figure 7E, immigrating T cells localized to the region of CCL21 expression. The characteristic interfollicular localization of T cell areas, therefore, results from B cell follicles expanding through a previously uninterrupted region of CCL21 mRNA expression.

3.6 Impact of intestinal microbiota on appendix T cell area formation

We examined CCL21 expression and T cell distribution in LigApx rabbit tissues to determine whether T cell area formation is influenced by the intestinal microbiota. In the absence of intestinal microbiota, CCL21 mRNA expression was almost completely confined to small regions at the basolateral margin of the B cell clusters (Figure 8A and G). Some CCL21 mRNA expression was also seen beneath the follicle-associated epithelium (FAE), as well as occasionally spanning the regions between adjacent B cell clusters, suggesting the initiation of T cell area formation. Few T cells, however, accumulated in these regions of CCL21 mRNA expression (Figure 8M). These features were not significantly altered by the introduction of an *E. coli* isolate into germ-free appendix (Figure 8B, H and N). The introduction of *Clostridium subterminale* (Figure 8C, I and O) or *Enterobacter faecalis* (Figure 8D, J and P) isolates into germ-free appendix, however, induced nearly continuous lateral CCL21 mRNA expression, as well as interfollicular regions of T cell accumulation. More robust continuous lateral CCL21 mRNA expression, as well as well defined interfollicular T cell area formation, was induced by introduction of a *Bacteroides fragilis* isolate (Figure 8E, K and Q). These features were most pronounced following co-introduction of *Bacteroides fragilis* and *Bacillus subtilis* isolates, where the lateral continuity of CCL21 mRNA expression was interrupted by robust follicle expansion (Figure 8F, L and R). These results demonstrate that signals derived from members of the intestinal microbiota induce appendix CCL21 mRNA expression and T cell area formation, and that commensals differ in their ability to induce these developmental processes.

4. Discussion

In the rabbit, the appendix functions as an important site for expansion of the B cell population and diversification of the primary antibody repertoire [3,25]. Early in postnatal life, newly developed B cells emigrate from the bone marrow and migrate to GALT, of which the appendix is a major site [29,30]. Within GALT, B cells proliferate extensively and diversify their V(D)J genes through somatic gene conversion and somatic hyper-point mutation [2–5]. These developmental processes are driven by select members of the intestinal microbiota [9,10]. B cell proliferation and V(D)J gene diversification, for example, do not occur in appendices rendered germ-free through surgical manipulation, and are stimulated by subsequent inoculation with only a few of the bacterial isolates that have been tested [9,10]. The mechanisms by which intestinal commensals stimulate these developmental processes are not currently understood. In the present study, we sought to gain insight into these mechanisms by examining the impact of intestinal commensals on positive feedback interactions between B cells and stromal cells, as well as on T cell area formation, in the rabbit appendix.

In mice, follicle development in secondary lymphoid tissues is driven by chemokine-mediated positive feedback interactions between B cells and stromal cells [13,14]. Stromal cells initiate these interactions by expressing the B cell chemoattractant CXCL13, which attracts an initial cluster of B cells to the nascent follicle [14]. B cells in turn upregulate stromal cell CXCL13 expression, and stimulate FDC differentiation, through membrane-expressed lymphotoxin $\alpha_1\beta_2$ (LT $\alpha_1\beta_2$) and tumor necrosis factor (TNF). The resultant positive feedback loop drives follicle development by promoting B cell recruitment and the formation of FDC networks. We reasoned that similar feedback loop interactions likely also contribute to follicle development in rabbit appendix and, if so, intestinal commensals must somehow influence these interactions. To test this hypothesis, we used *in situ* hybridization and immunohistochemistry to search for evidence of positive feedback interactions during follicle development in normal and manipulated appendices.

Our results suggest that positive feedback interactions drive early follicle development. We found, for example, that stromal cells in appendix domes express CXCL13 at birth, before the arrival of the first B cells. This early CXCL13 expression is reminiscent of murine stromal cells priming positive feedback interactions in nascent follicles by expressing a basal level of CXCL13 to attract an initial cluster of B cells [14]. Consistent with this interpretation, we found that B cells began immigrating into the domes on postnatal day 2. Over postnatal days 3 through 5, additional B cell immigration coincided with increasing CXCL13 expression within the appendix domes and, by postnatal day 3, the appearance of FDC clusters. The temporal progression of these events is consistent with that observed during positive feedback loop-driven follicle development. In addition, developing follicles contained cells (presumably B cells) expressing CXCR5, LT α and LT β mRNA, molecules critical for positive feedback interactions. Finally, LT β R-Ig and TNFR1-Ig fusion proteins markedly hindered early follicle development.

We unexpectedly found evidence that these initial events (increased stromal cell CXCL13 mRNA expression, B cell recruitment and FDC differentiation) also occurred in germ-free appendices. In these appendices, however, follicles did not develop beyond an early, rudimentary stage. Taken together, these results suggest that follicle development is initiated by positive feedback interactions, without a requirement for signals derived from intestinal commensals. These results further suggest that this initial development is the first of two phases of follicle development, the second phase requiring microbial signals and thus not occurring in germ-free appendices. We suggest that the first phase of follicle development occurs during approximately the first week of life, waning shortly thereafter due to declining numbers of immigrating B cells to sustain ongoing positive feedback interactions. This view is supported by a recent study suggesting that B cells developmentally seed the rabbit appendix during the first week of life [31]. Sinha et al. (2006) reported evidence suggesting that B cell immigration into the neonatal rabbit appendix occurs during a short early time period delimited by the regulated expression of peripheral lymph node addressin (PNAd) on high endothelial venules (HEVs) [31]. These investigators demonstrated that PNAd⁺ HEVs increase sharply in total area during the first week of rabbit appendix development and decline sharply in total area shortly thereafter. These authors also found, in adoptive transfer experiments, that 100 times more B cells were recruited into the appendices of 1-week-old, than 6-week-old, rabbits. We therefore suggest that follicle development stalled in germ-free appendices because a developmentally regulated decline in B cell immigration stalled positive feedback interactions, and the absence of microbial signals prevented commencement of the second phase of follicle development.

Although the microbial signals that initiate the second phase of follicle development are not known, they likely drive follicle expansion by stimulating extensive intrafollicular B cell proliferation. The presence of large numbers of proliferating B cells in rabbit appendix follicles

has long been noted [32]. Furthermore, Rhee et al. (2004) reported that follicle development induced in germ-free appendices after the introduction of bacterial isolates was accompanied by intrafollicular B cell proliferation [10]. It is tempting to speculate that B cells produced during intrafollicular proliferation, induced by microbial signals, drive positive feedback interactions within developing follicles after B cell immigration wanes. All of the parameters of positive feedback interactions we examined (CXCL13 expression, B cell accumulation and FDC differentiation), for example, increased during proliferative follicle expansion induced by introducing *B. fragilis* + *B. subtilis* into germ-free appendix. The results of our LT β R-Ig and TNFR1-Ig fusion protein experiments, however, do not appear to support this idea.

We introduced soluble LT β R-Ig and TNFR1-Ig fusion proteins into newborn rabbits via an adenovirus system to test directly, by blocking LT β R and TNFR1 signaling, whether positive feedback interactions drive appendix follicle development. We were surprised to find that, although both fusion proteins dramatically reduced CXCL13 and CCL21 mRNA expression and B and T cell area formation in the spleen, appendix follicles developed normally by 3 weeks of age. The LT β R-Ig fusion protein did, however, significantly hinder appendix follicle development during the first week of life, suggesting, together with our *in situ* hybridization results, that positive feedback interactions play an important role during the first phase of follicle development. It is not clear why the fusion proteins had no impact on the second phase of appendix follicle development. It is possible that the spleen is much more sensitive to, or received higher local concentrations of, the fusion proteins and that serum levels of the fusion proteins were not sufficient to interrupt the proliferative phase of appendix follicle development. It is also possible that novel or redundant signals might drive positive feedback interactions during the second stage of follicle development. Alternatively, the proliferative phase of follicle development might be driven by some other mechanism. Although positive feedback interactions drive follicle development in murine secondary lymphoid tissues, for example, germinal centers develop independently of CXCL13 [14]. Similarly, despite lacking lymph nodes and Peyer's patches, LT α -deficient mice occasionally develop rudimentary mesenteric lymph nodes [33]. Nasal-associated lymphoid tissues (NALT) also develop independently of LT α [34,35]. Whatever mechanism drives proliferative expansion of follicles in rabbit appendix, it clearly requires signals derived from the intestinal microbiota, and intestinal commensals differ in their ability to deliver these signals.

The cellular and molecular mechanisms controlling T cell area formation in secondary lymphoid tissues are not as well understood as those controlling B cell follicle development. Splenic T cell area formation in the mouse is directed by regional stromal cell CCL19 and CCL21 expression that is dependent on both LT β R and TNFR1 signaling [28]. Similarly, we found that both signals are required for CCL21 expression and T cell area formation in the rabbit spleen. As we observed with follicle development, however, normal T cell areas formed, by 3 weeks of age, when both signals were presumably blocked with Ig fusion proteins. In addition, we unexpectedly found that, also like follicle development, CCL21 mRNA expression and T cell area formation are stimulated by intestinal commensals and that commensals differ in their ability to induce or maintain these developmental events.

By examining early postnatal appendix development, we found that the characteristic interfollicular organization of T cell areas in adult appendix results from an interesting developmental process. Unlike CXCL13, CCL21 was not expressed in newborn appendix. Appendix T cell area formation was initiated by the onset of CCL21 expression, 2–3 days after birth, in the basolateral region of the appendix. The region of CCL21 mRNA expression, initially localized well below B cell clusters forming in the domes, expanded markedly over subsequent days, contacting and surrounding the growing follicles, which simultaneously distended downward through it. T cell areas ultimately occupy their characteristic interfollicular location following complete distension of the expanding follicles through the

region of CCL21 expression. Furthermore, we found that this developmental process, like follicle formation, is influenced differentially by intestinal commensals. In sterile appendix, CCL21 mRNA expression was largely confined to the basolateral margins of domes, suggesting a possible role for B cells in its induction or maintenance. Nonetheless, few T cells localized to these regions. It is possible that these regions did not express CCL21 protein or that T cell expression of the CCL21 receptor, CCR7, requires signals from intestinal commensals. While appendix colonization with *E. coli* resulted in little, if any, additional CCL21 expression, both *E. faecalis* and *C. subterminale* induced nearly continuous CCL21 expression throughout the appendix, as well as localized regions of T cell accumulation. *B. fragilis*, especially in combination with *B. subtilis*, stimulated robust CCL21 expression and well-formed T cell areas. In the rabbit appendix, therefore, the development of T cell areas, like that of follicles, requires signals derived from the intestinal microbiota, and intestinal commensals differ in their ability to supply these signals.

Differential impact of intestinal commensals on T cell accumulation and CCL21 expression has been reported in mouse Peyer's patches [36]. Maeda et al. (2001) reported that some bacterial species, when mono-associated with germ-free mice, induced high levels of Peyer's patch CCL21 mRNA expression and T cell infiltration, while others did not [36]. In contrast to our results, these authors found that *E. coli* induced high levels of Peyer's patch CCL21 expression and T cell infiltration. Because *E. coli* induced little, if any, CCL21 expression and T cell accumulation in rabbit appendix, these results suggest that the response of GALT to different intestinal commensals might differ between species or anatomical sites. The mechanism by which the intestinal microbiota influences these features of GALT development is not known. This is a potentially important area of study, because CCL21, in addition to facilitating T cell-dendritic cell interactions through its chemoattractant properties, possesses potent stimulatory activities. CCL21, for example, costimulates naïve T cell proliferation and T_H1 polarization, and induces proinflammatory DC differentiation [37,38]. Elucidating the mechanism by which intestinal commensals influence CCL21 expression and T cell area formation in GALT, therefore, might provide insight into the host-microbial relationships underlying, not only rabbit appendix development, but human health and disease.

Our results demonstrate that both T and B cell area formation in the rabbit appendix are dependent on signals delivered by intestinal commensals. Our results further suggest that B cell follicles develop in two phases: an initial phase driven by B cell recruitment, possibly through positive feedback interactions, and a second phase driven by intrafollicular B cell proliferation stimulated by intestinal commensals. While the nature of the commensal-derived signals driving these developmental processes is not known, experimental evidence suggests that B cell proliferation within follicles is not initiated by antigen-specific adaptive immune responses, but by signals that stimulate B cells polyclonally [39–41]. Polyclonal B cell activation could occur through recognition of microbial molecules by pattern-recognition receptors such as Toll-like receptors [42] or through antigen-independent BCR stimulation by a superantigen expressed, or induced, by intestinal commensals [43]. B cells could also be polyclonally stimulated by cytokines or other stimulatory molecules expressed by dendritic cells activated by interaction with intestinal commensals. The dependence of both T and B cell area formation in the rabbit appendix on signals derived from intestinal commensals might indicate that the two processes are inter-dependent, perhaps facilitating cellular interactions necessary to induce proliferative B cell expansion and V(D)J gene diversification. Further exploration of the potential mechanisms by which select intestinal commensals induce B and T cell area formation in rabbit GALT will increase our understanding of the host-microbial interactions that drive these developmental processes.

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Abbreviations

Cμ (or μ)	IgM constant region
CR1	complement receptor 1
FDC	follicular dendritic cell
HEV	high endothelial venule
LigApx	ligated appendix
LT (or LT$\alpha_1\beta_2$)	lymphotoxin
LTβR	lymphotoxin beta receptor
TNFR1	tumor necrosis factor receptor 1
PNAd	peripheral lymph node addressin
TNF	tumor necrosis factor

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Figure 1. CXCL13 and CCL21 mRNA *in situ* hybridization analysis of rabbit appendix sections. Six week appendix sections stained with: *A*, CXCL13 riboprobe; *B*, CCL21 riboprobe. *C*, CXCL13 negative control riboprobe; *D*, CCL21 negative control riboprobe. *E*, Newborn appendix section stained with CXCL13 riboprobe. CXCL13 mRNA expression within an appendix dome is indicated by an arrow. D, dome, F, follicle, T, T cell area. Magnification: 100X; Scale bars: 250 μ m.

Figure 2.

Time course analysis of B cell immigration, CXCL13 mRNA expression and FDC cluster formation in postnatal rabbit appendix. *In situ* hybridization analysis with the following riboprobes: C μ , to detect B cells (arrows) (*top row*); CXCL13 (arrows) (*middle row*); complement receptor 1 (CR1), to detect FDC clusters (arrows) (*bottom row*). Rabbit age is indicated above each column. Appendix lumen located at upper right in all panels. D, dome. Magnification: 200X; Scale bars: 100 μ m.

Figure 3. Two-color RNA *in situ* hybridization analysis of B cell-FDC clusters from appendix of a 5-day-old rabbit. B cells are stained with a C μ riboprobe (red; *left panel*) and FDCs are stained with a CR1 riboprobe (green; *middle panel*). The merged hybridization results are shown in the *right panel*. Magnification: 200X; Scale bars: 100 μ m.

Figure 4.

RNA *in situ* hybridization analysis of appendix sections from 1-week-old rabbits. Riboprobes used for staining are indicated above each column. Lower panels: negative controls.

Magnification: CR1, LT β : 100X; CXCR5, LT α : 200X; Scale bars: 250 μ m (100X), 100 μ m (200X).

A Germ-free appendix

B *B. fragilis*+ *B. subtilis*

Figure 5.

Immunohistochemical and *in situ* hybridization analysis of appendix sections from 7-week-old LigApx rabbits. *A*, germ-free appendix sections stained with: anti-μ mAb (*top left*); CXCL13 riboprobe (*top right*); CR1 riboprobe (*bottom left*); anti-μ (red) and Ki67 (green) mAbs (*bottom right*). *B*, Sections from ligated appendix into which *B. fragilis* + *B. subtilis* were introduced, stained with: anti-μ mAb (*top panel*); CR1 riboprobe (*bottom left*); anti-μ (red) and Ki67 (green) mAbs (*bottom right*). Appendix sections are from rabbits in which the appendix was ligated at birth and either kept germ-free (*A*) or inoculated with *B. fragilis* and *B. subtilis* at 3 weeks of age (*B*), as described in [10]. All rabbits were sacrificed for tissue analysis at 7 weeks of age. D, dome. Magnification: 100X; Scale bars: 250 μm.

Figure 6.

Western blot analysis of sera (A) and immunohistochemical and RNA *in situ* hybridization analysis of spleen (B) and appendix (C) tissue sections from rabbits treated with soluble LTβR-Ig and TNFR1-Ig fusion proteins. A, Western blot analysis of recombinant viral fusion proteins in sera of treated rabbits (serum concentrations were determined as described in Materials and Methods, and are listed in parentheses). *Lane 1*, 3-week-old uninjected littermate control rabbit; *lanes 2 and 3*, 1-week-old rabbits treated with LTβR-Ig (221 μg/ml) and LTβR-Ig + TNFR1-Ig (243 μg/ml) recombinant viruses, respectively; *lanes 4 and 5*, 3-week-old rabbits treated with LTβR-Ig (204 μg/ml) and LTβR-Ig + TNFR1-Ig (259 μg/ml) recombinant viruses, respectively; expected sizes of the LTβR-Ig and TNFR1-Ig fusion proteins are both 50 kDa. B, Spleen sections from: 1-week-old rabbits stained with anti-μ (red) and anti-CD4 (green) mAbs (*top row*); 3-week-old rabbits stained with anti-μ (red) and anti-CD4 + anti-CD8 (green) mAbs (*second row*), CXCL13 (*third row*) and CCL21 (*bottom row*) riboprobes. C, Appendix sections from: 1-week-old rabbits stained with anti-μ and anti-CD4 + anti-CD8 (green) mAbs (*top row*); 3-week-old rabbits stained with anti-μ (red) and anti-CD4 + anti-CD8 (green) (*bottom row*). Results representative of 3 experiments with 1 rabbit receiving each treatment per experiment. Magnification: 100X; Scale bars: 250 μm.

Figure 7.

In situ hybridization analysis of neonatal rabbit appendix. Two-color *in situ* hybridization with Cμ (red) and CCL21 (green) riboprobes of appendix sections from: A, 2-day-old, B, 3-day-old, C, 4-day-old and D, 1-week-old rabbits. E, appendix section from 1-week-old rabbit stained with CD3 riboprobe. Arrows: B cells (A and B), CCL21 mRNA expression (B, C and D), T cells (E). Magnification: 100X; Scale bars: 250 μm.

Figure 8.

CCL21 and CD3 *in situ* hybridization analysis of appendix sections from 7-week-old LigApx rabbits. Sections from germ-free (*first column*) and ligated appendix inoculated with *E. coli* (*second column*), *Clostridium subterminale* (*third column*), *Enterobacter faecalis* (*fourth column*), *Bacteroides fragilis* (*fifth column*) or *Bacteroides fragilis* + *Bacillus subtilis* (*sixth column*) stained with: CCL21 riboprobe (*top row* (25X), *middle row* (100X)); CD3 riboprobe (*bottom row* (100X)). Appendix sections are from rabbits in which the appendix was ligated at birth and either kept germ-free (*first column*) or inoculated with intestinal commensal isolates at 3 weeks of age (*all other columns*), as described in [10]. All rabbits were sacrificed for tissue analysis at 7 weeks of age; Scale bars: 1 mm (25X), 250 μ m (100X).

Table 1PCR primers used to generate *in vitro* transcription templates for riboprobe synthesis.^{1,2}

Gene	PCR primers	size (bp)
CXCL13	OSRbBLC f: 5'-gcccaaggtgtcctggaggccta-3' RbBLC r2: 5'-cactectcctgatgagtagca-3'	501
Cμ	Cmu f: 5'-gtgagcctgtcatctccaa-3' RbCmu r2: 5'-ctctcggatgatgtcagcat-3'	568
CR1	HuCR1f: 5'-agcagtttccatttcccagtccta-3' HuCR1r: 5'-gacaaacccgggctgacatcta-3'	673
CXCR5	RbCXCR5 RT: 5'-atcgtctgtcacaagatcaacttcta-3' RbCXCR5 r2 – 5'-gcatggggttgaggcagcagt-3'	586
LTβ	HuLTb f: 5'-atgggggactggggct-3' HuLTb r2: 5'-gggtgactgatgtgacgtacac-3'	686
LTα	LTa f: 5'-atgacaccactggagcttcta-3' LTa r – 5'-ctacagagcaaaaggctccaaagaa-3'	593
CD3	RbCD3 epsilon f: 5'-cggtgagcgcccatgag-3' RbCD3 epsilon r: 5'-ggtggaggcctctcttcttc-3'	545
CCL21	HuSLC f2: 5'-gacccaaggcagtgatggag-3' Q ₁ RACE primer [44]): 5'-gaggactcgagctcaagc-3'	721

¹ Rb, rabbit-specific primer; Hu, human-specific primer; f, forward primer; r, reverse primer.² The CCL21 *in vitro* transcription template cDNA was cloned using 3' RACE according to Frohman [44].