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Microbial induction of B and T cell areas in rabbit appendix

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Summary
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.

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

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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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 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.

Materials and methods

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 μm) 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 $C\mu$ 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).

Riboprobe preparation

A 500–700 bp PCR product from each gene of interest was cloned into the pGEM-T vector (Promega, Madison, WI) (Table 1). 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′-agtgaattgtaatacgactcactataggg-3′ and SP6 Univ: 5′-cgccaagctatttaggtgacactatagaatac-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