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Microbial and host cell interactions stimulate rabbit B cells to diversify the primary Ab repertoire in GALT. B cells at the base of appendix follicles begin proliferating and diversifying their V-(D)-J genes around 1 wk of age, ~5 d after B cells first begin entering appendix follicles. To gain insight into the microbial and host cell interactions that stimulate B cells to diversify the primary Ab repertoire, we analyzed B cell trafficking within follicles during the first week of life. We visualized B cells, as well as chemokines that mediate B cell homing in lymphoid tissues, by in situ hybridization, and we examined B cell chemokine receptor expression by flow cytometry. We found that B cells were activated and began downregulating their BCRs well before a detectable B cell proliferative region appeared at the follicle base. The proliferative region was similar to germinal center dark zones, in that it exhibited elevated CXCL12 mRNA expression, and B cells that upregulated CXCR4 mRNA in response to signals acquired from selected intestinal commensals localized in this region. Our results suggest that after entering appendix follicles, B cells home sequentially to the follicle-associated epithelium, the follicular dendritic cell network, the B cell/T cell boundary, and, ultimately, the base of the follicle, where they enter a proliferative program and diversify the primary Ab repertoire. The Journal of Immunology, 2014, 193: 000–000.

Rabbits, similar to some other vertebrates (1–3), generate a diverse primary Ab repertoire through a different strategy than that used by mice and humans (4). Rabbits generate an initial Ab repertoire that is limited by preferential use of the 3’-most V region gene segment of the Ig H chain locus during V-D-J gene rearrangement in the bone marrow (5). The initial Ab repertoire is subsequently diversified in GALT through somatic hypermutation and somatic gene conversion (6, 7). B cells begin immigrating into the appendix, the largest site of rabbit GALT, ~2 d after birth and continue seeding appendix follicles for 1–2 wk in a manner regulated, at least in part, by the expression of peripheral lymph node addressin on appendix high endothelial venules (HEVs) (8). At ~1 wk of age, appendix follicles enter a second phase of development, characterized by extensive B cell proliferation and consequent expansion of the follicles (8, 9). During this proliferative phase, B cells upregulate activation-induced cytokine deaminase (AID) and mutate their V-(D)-J genes through somatic gene conversion and somatic hypermutation, thus generating a highly diverse primary Ab repertoire that fills the periphery by 6 wk of age (6, 7, 10). V-(D)-J gene diversification in GALT is an Ag-independent process, dependent on signals derived from select intestinal commensal bacteria that stimulate polyclonal B cell proliferation (11, 12).

V-(D)-J gene diversification begins ~5 d after B cells first begin entering appendix follicles (12), indicating that the follicle microenvironment rapidly develops the ability to promote and support Ab repertoire diversification. Analysis of B cell intrafollicular trafficking during the first week of life therefore provides an opportunity to identify the cell–cell and cell–microbial interactions that stimulate and support Ab repertoire diversification. Toward this end, we sought to identify the intrafollicular sites B cells home to after entering follicles and ultimately localizing at the follicle base to proliferate and diversify their V-(D)-J genes. Trafficking of immune cells within GALT is largely directed by the homeostatic chemokines CCL20, CXCL13, CCL19, CCL21, and CXCL12. In mouse Peyer’s patches (PPs), CCL20 is selectively expressed by the follicle-associated epithelium (FAE) and mediates homing of immune cells expressing its receptor, CCR6 (13). The FAE contains M cells, which serve as portals through which bacterial cells and food Ags from the intestinal lumen gain entry into the follicle (14). A network of follicular dendritic cells (FDCs), extending throughout PP follicles, highly expresses CXCL13, which attracts immune cells expressing its receptor, CXCR5 (15–17). Homing to the T cell areas flanking the follicles is mediated by two chemokines, CCL19 and CCL21, which share a common receptor, CCR7 (18, 19). CXCL12 is essential for the polarization of germinal centers (GCs) into light and dark zones (20) and is most highly expressed in the GC dark zone, where it mediates homing of centroblasts expressing its receptor, CXCR4.

To gain insight into the microbial and host cell interactions that stimulate rabbit B cells to proliferate and diversify their V-(D)-J genes in GALT, we analyzed homeostatic chemokine expression in the rabbit appendix. Furthermore, we visualized B cell migration during early follicle colonization and examined B cell chemokine receptor expression levels during the first week of life. Our results suggest that B cells home sequentially to four major intrafollicular sites, likely engaging in required microbial and/or host cell

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Abbreviations used in this article: AID, activation-induced cytokine deaminase; Cpx, IgM C region locus; FAE, follicle-associated epithelium; FDC, follicular dendritic cell; GC, germinal center; HEV, high endothelial venule; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; IC, immune complex; ISH, in situ hybridization; MFI, mean fluorescence intensity; PP, Peyer’s patch; SED, subepithelial dome.

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interactions at each site, prior to initiating diversification of the primary Ab repertoire.

Materials and Methods

Rabbits with germ-free ligated appendix

The rabbits used were from the colony maintained by Dr. Katherine L. Knight in the Comparative Medicine Facility at Loyola University Medical Center. For germ-free ligated appendix results presented in Figs. 2 and 3, the appendix lumen was surgically ligated at the cecal–appendix junction within 24 h after birth to prevent colonization of the appendix by microbiota, as reported previously (21). The appendices were confirmed as sterile at the time of euthanasia by cultivating appendix washes under aerobic and anaerobic conditions. For germ-free and gnotobiotic ligated appendix results presented in Fig. 5, frozen tissue blocks from a previously reported study (11) were used. All procedures were performed in compliance with an experimental protocol approved by the Loyola University Medical Center Institutional Animal Care and Use Committee.

Immunofluorescence and immunohistochemical 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. For rabbit IgM, 67 double immunofluorescence staining, tissue sections (7 μm) were cut on a cryostat microtome, fixed in cold acetone, and blocked with normal goat serum. Sections were stained with the following rabbit-specific or cross-reactive primary Abs: biotinylated mouse anti-rabbit IgM mAb (clone 367; BD Biosciences, Palo Alto, CA) (22); and Ki-67, an anti-human mAb, for identification of proliferating cells (BD PharMingen, San Diego, CA) (23). Cy3-conjugated streptavidin (Invi- trogen, Carlsbad, CA) and DyLight 488–conjugated goat anti-rabbit mAb (BD Biosciences) were used as secondary reagents. Sections were counterstained with Hoechst 33342. Appropriate isotype-matched Abs were used as negative controls. For CCR6 immunohistochemical staining, 10-μm tissue sections were fixed in PIPES/formaldehyde (1.7 vol PIPES/I vol 10% formaldehyde), followed by inactivation of endogenous alkaline phosphatase activity with BioTaq endonuclease and alkaline phosphatase blocking solution (Vector Laboratories, Burlingame, CA). Sections were stained with goat anti-human CCR6 polyclonal Ab (Thermo Fisher Scientific, Rockford, IL), followed with biotinylated donkey anti-goat IgG (Rockland Immunochemicals, Gilbertsville, PA) and alkaline phosphatase–conjugated mouse anti-biotin (Jackson ImmunoResearch Laboratories, West Grove, PA) as secondary and tertiary Abs, respectively. Sections were developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT (Vector Laboratories). Appropriate isotype-matched Abs were used as negative controls. All sections were photographed with a Leica DM IRB microscope (Leica Microsystems, Brannockburn, IL) using a MagnaFire 2.1C digital camera system (Optronics, Goleta, CA).

Riboprobe preparation

Regions of mRNAs of interest were RT-PCR–amplified from rabbit appendix cDNA and cloned. The nucleotide sequences were confirmed by DNA sequence analysis. Sense and antisense in vitro transcription templates were generated by PCR amplification from each plasmid using gene-specific forward or reverse primers with a plasmid-specific primer containing either the T7 or SP6 RNA polymerase promoter (T7 universal primer, 5′-ATCGTCTCGGAGCCAGGAAAC-3′; rabbit CXCR4 reverse, 5′-GTTGTCGGAATCGACAGAACAAC-3′; rabbit CCL20 forward 2, 5′-CC- CAGACGTCAGGACGAT-3′; rabbit CCL20 reverse 2, 5′-GTTTCT- TCCTAAATAGTTCACACATTGC-3′; rabbit CCL19 forward 5′-CACCACTACGAC-3′; rabbit CCL19 reverse 5′-GCAT- TGCAGTCTGGTGGAGGAGAAG-3′; rabbit CXCR5 forward 2, 5′- ATGCATTCGACAAGATGCATTCTCA-3′; rabbit CXCR5 reverse 2, 5′-GCATGGGGTTGAGGCAGCAGT-3′; rabbit CXCR5 3′ RACE forward 2, 5′-GTCGCGGAGCCAGGAAAC-3′; rabbit CXCR5 reverse 3, 5′-ACCGGGTCTACCGATGCGTGGT-3′; CXCR5 B cells were detected by simultaneous hybridization of two ~500-nt riboprobes complementary to different regions of the CXCR5 mRNA. Primers used to prepare all other riboprobes have been reported previously: CXCL13, Cμ, CCL21 (9), and AID (12). Primers specific for rabbit hypoxanthine-guanine phosphoribosyltransferase (HGPRT quantitative PCR forward, 5′-AGCCACAGGGTGTGATTAGTG-3′; reverse 5′-CATCACATCTCG- AGACCACTCTTCCAG-3′) were used to assess cDNA quality in laser capture microdissection RT-PCR experiments.

In situ hybridization

In situ hybridization (ISH) was performed as previously described (12). Briefly, 10-μm tissue sections were fixed in 4% paraformaldehyde, permeabilized with pepsin, acetylated in 0.25% acetic anhydride (Sigma-Aldrich, St. Louis, MO) in 0.1 M triethanolamine, equilibrated in 5× SSC, and prehybridized (mRNA ISH solution; Dako, Carpinteria, CA) with 250 μg/ml torula yeast tRNA at 70°C for 2 h. Sections were hybridi- zed with 30 ng/ml riboprobe at 70°C for 20 h in a humidified con- tainer. Hybridized sections were stringently washed, blocked with 10% normal rabbit serum, and incubated with HRP-conjugated anti-digoxigenin or anti-FITC Fab (1:5000; Dako) at 4°C for 18 h. Sections were washed in TBST, incubated in biotinyl tyramide (GenPoint; Dako), washed in TBST, and incubated with alkaline phosphatase–conjugated anti-biotin Fab (1:3000; Dako) at 25°C for 2 h. Sections were washed in TBST, developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT (Vector Laboratories), and photographed with a Leica DM IRB microscope (Leica Microsystems) using a MagnaFire 2.1C digital camera system (Optronics).

Flow cytometric analysis of appendix B cell chemokine receptor expression

B cells were freed from appendix tissue by gentle scraping between two microscope slides and subsequent passage through a strainer. Cells were washed and cultured in RPMI 1640 medium/10% FCS for 20 min to allow the chemokine receptor cycling increase receptor expression. B cells were washed and stained with PE-Cy7–conjugated anti-CXCR4 (clone 12G5; R&D Systems, Minneapolis, MN), PE-Cy5–conjugated anti-CXCR6 (clone 11A9; BD Biosciences), PE-conjugated anti-CR2 (clone 150503; R&D Systems), allopurinol–conjugated anti-CXCR5 (clone 51505; R&D Systems), and biotinylated mouse anti-rabbit IgM (clone 367; BD Bio- sciences). All anti-chemokine receptor reagents were labeled with LYNX Rapid AB conjugation reagents (AbD Serotec, Oxford, U.K.). Cells were then stained with DyLight 488–conjugated streptavidin ( Molecular Probes/ Life Technologies, Grand Island, NY), followed by staining with Live/Dead fixable aqua dead cell stain (Invitrogen). Cells were analyzed on a FACS- Canto II (BD Biosciences) and data were analyzed with FlowJo software, version 7.5 (Tree Star, Ashland, OR). From the lymphocyte gate in a side light scatter versus forward light scatter plot, we identified live B cells as IgM+, Live/Dead -aqua, Percentage positive B cells and mean fluorescence intensity (MFI) were determined for each chemokine receptor on live B cells.

Laser capture microdissection and CXCL12 RT-PCR

Tissue sections (10 μm) were placed on RNase-free slides, dehydrated in graded ethanol, cleared in xylene, stained with cresyl violet (Ambion/Life Technologies, Grand Island, NY), and dehydrated in graded ethanols and two washes in xylene. Laser capture microdissection was performed with an Arcturus XT laser capture microdissection instrument (Arcturus Engineering, Mountain View, CA) using CapSure HS caps (Arcturus MDs Analytical Technologies, Sunnyvale, CA). The cap film, with micro- dissected tissue, was immediately placed in TRIzol reagent (Invitrogen). RNA was isolated and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). The cDNA was used as template for RT-PCR using JumpStart ReadyToGo PCR beads (Satga-Aldrich, St. Louis, MO) (hot start, 94°C, 3 min followed by 40 cycles of 94°C, 30 s; 56°C, 45 s; 72°C, 1 min; and a final extension, 72°C, 5 min). Rabbit CXCL12 and HGPRT (control for cDNA quality) PCR primers are listed above. One-tenth vol- ume of each PCR product was electrophoresed on a 5% PAGE gel, stained with ethidium bromide, and scanned on a Typhoon imager (GE Healthcare Biosciences, Pittsburgh, PA).

Results

Expression of CCL20, CXCL13, and CCL21 mRNA in appendix follicles

We previously showed that B cells begin immigrating into rabbit appendix follicles ~2 d after birth and rapidly colimize the follicles

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over subsequent days (9) (Fig. 1A). In Fig. 1, we provide representative examples of previous results that are foundational to the present studies. We and others also previously showed that at ∼1 wk of age, B cells at the follicle base begin proliferating, up-regulate AID, and begin diversifying their V-(D)-J genes through somatic gene conversion and somatic hypermutation to generate the primary Ab repertoire (6, 7, 10). Upon first entering follicles at 2 d of age, B cells appear to home to the FAE (Fig. 1A, left panel). Because the chemokine CCL20 mediates homing to the FAE in mouse PPs (13), we used ISH to determine whether the FAE in rabbit appendix follicles expresses CCL20. We found that CCL20 mRNA was expressed in the FAE of nascent appendix follicles at birth (Fig. 2B, left panel) and was highly and specifically expressed in the FAE of appendix follicles at 1 wk of age (Fig. 2A, left panel). Furthermore, we found by immunohistochemical staining that CCR6+ cells, likely primarily B cells, localized to the FAE and subepithelial dome (SED) region at 1 wk of age (Fig. 2A, right panel).

The FAE contains M cells that transport particulates and bacterial cells from the appendix lumen into the follicle, and it is thus a major candidate site where B cells might acquire commensal-derived signals required to initiate repertoire diversification. Because CCL20 can function as both a homoeostatic and an inflammatory chemokine, we considered the possibility that commensal bacteria might be required to maintain CCL20 expression in the FAE after microbial colonization of the appendix. Intestinal epithelial CCL20 expression, for example, can be induced by flagellin in a TLR5-dependent manner (24–26). In the absence of commensal-stimulated CCL20 expression, therefore, immune cell trafficking to the FAE that might be required for repertoire diversification would be disrupted. We found, however, that CCL20 mRNA was expressed in the FAE of appendices that had been surgically ligated at birth to prevent microbial colonization (Fig. 2B, right panel). Such appendices develop as germ-free tissue and do not exhibit B cell proliferation or V-(D)-J gene diversification (21). We found that FAE CCL20 mRNA expression persisted in germ-free appendices until at least 7 wk of age (data not shown). CCL20 therefore appears to be constitutively expressed in the FAE of rabbit appendix follicles and, furthermore, is likely available to direct homing of B cells when they first begin entering follicles 2 d after birth.

By 3 d of age, in addition to B cells localized near the FAE, B cells accumulated near the base of nascent follicles (Fig. 1A, right panel). We found that the base of nascent follicles is a site of abundant CXCL13 mRNA expression (Fig. 3A, left panel). During early appendix development, CXCL13+ stromal cells probably develop into a network of FDCs through positive feedback interactions driven by B cell lymphotxin α1β2 and stromal cell CXCL13, as has been described in mouse lymphoid tissues (9, 27). FDCs are first detectable in rabbit appendix follicles by 3 d of age, and extensive CXCL13+ FDC networks develop by 1 wk of age (9) (Fig. 3A, center panel). Furthermore, CXCR5+ cells, likely predominantly B cells, colocalize with the CXCL13+ FDC networks at 1 wk of age, as detected by ISH (Fig. 3A, right panel; compare with Fig. 3A, center panel). Appendix CXCL13 expression also occurs independently of the intestinal microbiota, as CXCL13+ FDCs develop in the follicles of germ-free ligated appendix (9). The accumulation of B cells at the follicle base at 3 d of age suggests that, after homing to the FAE, B cells migrate to CXCL13-expressing stromal cells/FDCs.

In previous studies, we found that CCL21 mRNA was not expressed in the appendix at birth, but was detectable by ISH at 2 d of age and was rapidly upregulated over subsequent days (9). By 1 wk of age, CCL21 mRNA was highly expressed in the T cell area that develops around the basolateral margin of the follicle (Fig. 3B, left panel) (9). CCL19 mRNA was expressed with similar distribution in the T cell area (data not shown). Although T cell areas, as well as CCL19/CCL21 mRNA expression, develop normally in germ-free ligated appendix (Fig. 3, right panel, and data not shown), they gradually decay over time in the continued absence of intestinal commensals (9). Experiments in which
rabbit 1 wk of age (CCL21 riboprobe was used to stain appendix tissue sections from a normal CXCR5+ cells (right). Arrows indicate CXCL13-stained stromal cells/FDCs and CXCR5* cells (A) and CCL21-stained T cell area around the basolateral margin of the follicles (B). All histological stains are representative of three individual experiments. Original magnification $\times 100$.

FIGURE 3. CXCL13 and CCL21 ISH of rabbit appendix. (A) A CXCL13 riboprobe was used to stain appendix tissue sections from normal 3-d-old (left) and 1-wk-old (center) rabbits; two CXCR5 riboprobes, complementary to different regions of the mRNA, were used to stain an appendix tissue section from a normal 1-wk-old rabbit (right). (B) A CCL21 riboprobe was used to stain appendix tissue sections from a normal rabbit 1 wk of age (left) and a germ-free ligated-appendix rabbit 10 d of age (right). The localization of CXCR4 mRNA + B cells at the follicle base, forming a region analogous to the GC dark zone.

CXCL12 mRNA expression in appendix follicles

The localization of CXCR4 mRNA+ B cells at the follicle base suggests that CXCL12 is expressed by stromal cells in this region. We were, however, unable to detect CXCL12 mRNA in follicles by ISH, a finding that was not unexpected because CXCL12 mRNA is expressed at very low levels in GC dark zones (20). We therefore used laser capture microdissection and RT-PCR to assess CXCL12 expression (Fig. 4). Initially, we performed immunohistochemical staining to localize Ki-67+ (proliferating) B cells in the basal follicle regions of appendix tissue from a 1-wk-old rabbit (data not shown). In a serial section from the same appendix, we then microdissected the basal and apical regions from follicles, prepared cDNA, and performed RT-PCR analysis (Fig. 4B). RT-PCR–amplified HGPRT mRNA served as a control for cDNA quality. In each of two such experiments, we obtained CXCL12 PCR product from the basal, but not the apical, region of the follicles (Fig. 4C). These results demonstrate that CXCL12 mRNA expression is polarized at the basal region of the follicle, where it likely contributes to the establishment of a B cell proliferative region. CXCL12 mRNA expression is similarly polarized in GCs, being most highly expressed in the dark zones.

Appendix B cell CXCR4 expression

Around 1 wk of age, B cells at the base of the follicle begin proliferating and diversifying their V-(D)-J genes (12). This partitioning of the follicle into a nonproliferative region and a region in which B cells proliferate and mutate their V-(D)-J genes is reminiscent of light and dark zone formation in GCs (28). We therefore asked whether, as in GCs, the localization of proliferating B cells is mediated by CXCL12 and its receptor CXCR4. As shown in Fig. 4A (right panel), we found that CXCR4 mRNA was specifically expressed by B cells at the follicle base. The localization of CXCR4 mRNA+ B cells corresponds to that of AID mRNA+ B cells that have begun proliferating and diversifying their V-D-J genes (Fig. 1B) (12). These results suggest that activated appendix B cells upregulate CXCR4 and localize at the base of the follicle, where they proliferate and diversify their V-(D)-J genes.

Rabbit appendix B cells are activated in an Ag-independent manner by proliferative signals acquired from select intestinal commensals (11). Because our results suggested that activated B cells upregulate CXCR4 and localize at the follicle base to proliferate and diversify their V-(D)-J genes, we tested whether B cells upregulate CXCR4 in response to commensal-derived signals. We performed CXCR4 ISH on appendixes from normal 3-d-old rabbits (center) and 1-wk-old rabbit (arrow). (B) Basal regions were laser capture microdissected from several follicles (top panel) and a second cap was placed on the tissue section to microdissect the apical regions (bottom panel). (C) CXCL12 and HGPRT (cDNA quality control) analysis of cDNA prepared from basal and apical microdissected tissue. Original magnification in (A) and (B) $\times 100$.

FIGURE 4. CXCR4 ISH and CXCL12 RT-PCR analysis of appendix from a 1-wk-old rabbit. (A) ISH of appendix from 1-wk-old rabbit with a CXCR4 riboprobe (arrow). (B) Laser capture microdissected regions of appendix follicles.
although it is also expressed at lower levels in the light zone (20). Because we found no evidence of CXCL12 mRNA expression in the apical region, CXCL12 expression might be more highly polarized in rabbit appendix follicles than in GCs. Taken together, our results demonstrate that rabbit appendix expresses the homeostatic chemokines CCL20, CXCL13, CCL19/21, and CXCL12, which are known to direct immune cell trafficking in lymphoid tissues of other species. Furthermore, expression of CCL20, CXCL13, and CCL19/21 mRNA is not dependent on intestinal commensals, at least early in life. Our results do, however, implicate commensal-derived signals in CXCR4 upregulation by proliferating B cells, which localize at the follicle base where they diversify their V-(D)-J genes. Collectively, these results suggest that, after entering follicles during early appendix development, B cells home sequentially to the FAE, the FDC network, and, ultimately, the base of the follicle, where they diversify the primary Ab repertoire.

Flow cytometric analysis of B cell chemokine receptor expression

To pursue additional evidence for B cell intrafollicular trafficking, we performed flow cytometry analysis to determine B cell expression of the homeostatic chemokines identified above. We used six-color flow cytometry, which allowed gating of live B cells and subsequent expression analysis of the following chemokine receptors (with ligand and site of ligand expression denoted in parentheses): CCR6 (CCL20, FAE), CXCR5 (CXCL13, FDCs), CCR7 (CCL19/21, T cell area), and CXCR4 (CXCL12, dark zone at follicle base). B cells were analyzed from appendices of rabbit kits at 2, 4, 6, and 8 d of age, and MFIs were determined for each chemokine receptor to track changes in receptor expression over time.

Our initial analyses identified two B cell subpopulations that differed in BCR expression levels (Fig. 6A). Whereas surface IgMhi B cells predominated at 2 d of age (median ratio of surface IgMhi/surface IgMlo B cells of 10.4), surface IgMlo B cells accumulated rapidly during the following days, with the median ratio of surface IgMlo/surface IgMhi B cells decreasing to 5.6, 1.6, and 1.3 by days 4, 6, and 8, respectively (Fig. 6B). Surface IgMlo B cells are likely activated, because proliferating appendix B cells downregulate their BCR (Fig. 1B, left). If this is the case, these results indicate that B cell activation begins well before a detectable B cell proliferative region forms at the base of the follicle at ∼7 d of age. To further investigate this possibility, we analyzed chemokine receptor expression on both IgMhi and IgMlo B cells.

Consistent with our ISH results suggesting that B cells first home to the FAE upon entering follicles (Fig. 1A, left panel), we found that both the percentage of CCR6+ B cells and their MFIs were highest at day 2 and decreased gradually over subsequent days (Fig. 7A). The decrease in percentage CCR6+ B cells was statistically significant between days 2 and 6 for IgMlo B cells, as was the decrease in MFI between day 2 and all subsequent days. Because B cells continuously seed the appendix during the first 1–2 wk of life (8), our post–day 2 results are somewhat conservative, representing B cells at various stages of migration into the appendix and within follicles. By comparison with IgMhi B cells, IgMlo B cells exhibited lower CCR6 expression (MFI) at days 2 and 4 and lower percentages of CCR6+ cells at days 6 and 8. Although these results are consistent with IgMlo B cells being activated and destined to localize at the base of the follicle, they did not achieve statistical significance. Taken together, the temporal expression pattern of B cell CCR6 observed in these experiments, the homing of the first B cell immigrants at 2 d of age to the FAE (Fig. 1A, left panel), which specifically expresses CCL20 (Fig. 2), and the localization of CCR6+ cells in the FAE and SED region of the follicle (Fig. 2A, right) strongly suggest that B cells first home to the FAE upon entering follicles.

In mouse PPs, the CXCR5 ligand, CXCL13, is selectively displayed on follicular HEVs and plays an important role in B cell entry into the follicle (29). Consistent with a similarly important role for CXCL13 during B cell entry into rabbit appendix follicles,
we found that CXCR5 was most highly expressed by appendix B cells on day 2 (Fig. 7B, bottom). We observed a statistically significant decrease in B cell CXCR5 expression (MFI) by day 4 for both IgM<sup>hi</sup> and IgM<sup>lo</sup> B cells, consistent with homing to the FAE (through CCR6 expression, as discussed above), rather than to CXCL13<sup>+</sup> FDCs, after entry into the follicle through CXCL13<sup>+</sup> follicular HEVs. Also consistent with our results suggesting that B cells home to CXCL13<sup>+</sup> stromal cells/FDCs after leaving the FAE, both the percentage of CXCR5<sup>+</sup> B cells and B cell CXCR5 expression (MFI) gradually increased from days 4–8, as the percentage of CCR6<sup>+</sup> B cells and B cell CCR6 expression (MFI) gradually decreased (compare Fig. 7A and Fig. 7B). The increase in CXCR5 expression (MFI) achieved statistical significance for IgM<sup>lo</sup> B cells between days 4 and 8.

We found that B cells expressed CCR7 most highly at day 2, with expression levels declining markedly by day 4 and remaining essentially constant through day 8 (Fig. 7C, bottom). The CCR7 ligands CCL19 and CCL21 probably contribute importantly to B cell capture and rolling on HEVs in the appendix, as has been described in mouse PPs (8, 29). Capture and rolling are the first stages of a multistep process by which lymphocytes pass from the blood into lymphoid tissues, followed by integrin-dependent firm adhesion and, ultimately, by lymphocyte migration across the endothelium and into the lymphoid tissue (30, 31). The decrease in CCR7 expression observed after day 2 is consistent with most B cells downregulating CCR7 after migrating into follicles. Sinha and Mage (32) similarly observed markedly decreased B cell CCR7 expression by appendix B cells, as compared with B cells in peripheral blood (32). We were somewhat surprised to find that a high percentage of B cells expressed CCR7 throughout the first week of life (Fig. 7C, top), because Ab repertoire diversification is thought to occur in an Ag-independent manner. Repertoire diversification, however, occurs near the B cell/T cell boundary during early appendix development (9, 12), and our data suggest that B cell CCR7 expression might contribute to this localization.

Both the percentage of CCR7<sup>+</sup> B cells and B cell CCR7 expression (MFI; after day 2) were remarkably consistent during the first 8 d of life (Fig. 7C). This suggests that CCR7 might act as a “default” receptor, mediating B cell homing to the B cell/T cell boundary when activated B cells downregulate both CCR6 and CXCR5 sufficiently. Also, consistently fewer IgM<sup>lo</sup> B cells than IgM<sup>hi</sup> B cells were CCR7<sup>+</sup>, and this difference achieved statistical significance on days 6 and 8 (Fig. 7C, top). This is consistent with IgM<sup>lo</sup> B cells being activated B cells that are downregulating their BCRs and being lost from the flow cytometry B cell gating. Furthermore, gated B cells exhibited both low percentages of CCRX4<sup>+</sup> cells and low CXCR4 levels (MFI) (data not shown), indicating that B cells upregulate CXCR4 after BCR downregulation. CXCR4 upregulation would facilitate interaction with nearby CXCL12<sup>+</sup> stromal cells, which likely provide signals conducive to B cell proliferation and V-(D)-J gene diversification (Fig. 8).

**Discussion**

Based on our results, we propose a model of sequential microbial and host cell interactions that stimulate B cell proliferation and Ab repertoire diversification in rabbit GALT (Fig. 8). Our model in the present study is based on ISH and flow cytometric analyses of four chemokines and their receptors shown in other species to be major determinants of B cell migration in lymphoid tissues. Although our model has not yet been tested by interrupting chemokine–receptor interactions with neutralizing Abs or chemical antagonists, our data suggest that B cells first home to the FAE after entering follicles, probably in response to FAE CCL20 expression. Jasper et al. (33) reported evidence in IgH-transgenic rabbits similarly suggesting that B cells first home to the FAE. These
ICs to FDCs in mouse lymph nodes are dependent on complement. B cell shuttling of ICs and subsequent transfer from the subcapsular sinus of lymph nodes (38) and from the FAE, and subsequently home to the FDC network, which highly expresses the CXCR5 ligand, CXCL13. We speculate that B cells constitutively transport bacteria to CXCL13+ FDCs during this homing event. CXCR5 is downregulated, and CCR7 expression mediates homing to the B cell/T cell boundary in response to the CCR7 ligands CCL19 and CCL21. B cells activated by commensal-derived signals and interaction with FDCs and T cells upregulate CXCR4, downregulate their BCRs, and home to CXCL12+ stromal cells, where they proliferate and diversify the primary Ab repertoire.

B cells likely acquire stimulatory signals from commensal bacterial species spores stimulating Ab repertoire diversification in rabbit GALT. Additionally, blocking BAFF (and APRIL) signaling in neonatal rabbits with a TACI–Ig fusion protein markedly reduced the size and number of proliferating B cell follicles (45). In vitro studies, however, demonstrated that BAFF primarily promoted survival, rather than proliferation, of appendix B cells. Furthermore, B cells themselves were found to express BAFF, as well as BAFF receptor 3, suggesting that B cell BAFF signaling might be primarily autocrine in nature. Interestingly, these studies also showed that intestinal commensals are required for BAFF expression in appendix follicles.

We suggest that, after interacting with FDCs, B cells localize near the T cell area along the basolateral margin of the follicle in response to local CCL19/21 expression. We found that the CCL19/21 receptor, CCR7, was consistently expressed at high levels (MFI) by a large percentage of B cells. CCR7 might therefore act as a “default” receptor, mediating B cell migration to the B cell/T cell boundary after downregulation of CCR6 and CXCR5. Although Ab repertoire diversification in GALT is T cell–independent, in the sense of not being driven by cognate B cell–T cell interactions (34, 41), our results suggest that T cells contribute to the process. This is consistent with the observation that during early follicle development, B cell proliferation occurs in the region of the follicle immediately adjacent to the T cell area (9, 12). T cells likely provide contact-dependent and/or soluble factors that promote B cell proliferation and/or survival during Ab repertoire diversification. Such factors probably provide support to proliferating B cells, as they downregulate the BCR, which is required for B cell viability, and enter a fragile blasting state. Potential T cell factors include cytokines (IL-4, IFN-α, and IFN-γ), CD40L, and Plexin-B1 (46–48). Although CD40–CD40L signaling is required for B cell proliferation in rabbit GALT, as discussed above, activated T cells are not a required source of CD40L (41). The potential roles of other T cell factors in Ab repertoire diversification have not been studied.

Our results demonstrate that CXCR4–CXCL12 interactions contribute to diversification of the primary Ab repertoire. B cells...
activated by microbial and host cell interactions, as described above, downregulate their BCRs and upregulate CXCR4, facilitating interaction with CXCL12+ stromal cells in the basolateral region of the follicle. The appearance of a B cell proliferative region at the follicle base at ~1 wk of age bears a striking resemblance to the formation of the GC dark zone, also a region of B cell proliferation and V(D)J gene mutation (28). Our results implicate an intriguing parallel between GCs and rabbit appendix follicles. Both employ CXCR4 and CXCL12 to establish a region of B cell proliferation and V(D)J gene mutation, but do so toward the accomplishment of differing ends. GC dark zones function as sites of affinity maturation, in which Ag-specific B cells, generated during T cell–dependent immune responses, proliferate and mutate their V(D)J genes through somatic hypermutation. B cells expressing V(D)J genes of higher affinity for Ag are thought to be subsequently selected by competitive acquisition of Ag trapped on FDCs and presentation to T follicular helper cells in the light zone (49). Selected B cells ultimately exit the GC as plasma or memory cells (50). Affinity maturation thus generates plasma cells to combat ongoing infection through secretion of abundant amounts of high-affinity Ab, as well as memory B cells to provide protection against future encounters with the pathogen. In contrast, the B cell proliferative region in rabbit appendix follicles is a site where B cells, polyclonally activated by commensal-derived signals in an Ag-independent manner, proliferate and mutate their V(D)J genes through somatic hypermutation and somatic gene conversion (6, 7). Although the B cells clearly undergo subsequent selection (51, 52), it is not known how selection operates, because follicles generate mature B cells expressing a highly diverse array of Ab specificities. Selected B cells ultimately exit the follicles as mature IgM+ B cells that provide systemic immune protection by establishing the primary Ab repertoire in the periphery.

B cell proliferation, AID mRNA expression, and V(D)J gene diversification are first detectable at the follicle base by ~1 wk of age (12), indicating that the follicle microenvironment rapidly develops the ability to induce and support diversification of the primary Ab repertoire. The rapid accumulation of IgMlo B cells observed in the present study suggests that B cell activation, during which the BCR is downregulated, begins well before a proliferative region forms at the follicle base. Indeed, a considerable fraction of appendix B cells were IgMlo by 4 d of age (Fig. 6B), 2 d after B cells begin seeding appendix follicles. A precisely timed sequence of developmental events initiates and supports Ab repertoire diversification in early rabbit GALT. CCL20 and CXCL13 mRNA are expressed in the FAE and basal stromal cells, respectively, of nascent follicles at birth (Fig. 2, left panel) (9). These chemokines are likely available to direct trafficking when B cells first begin seeding follicles at 2 d of age, when, coincidentally, the appendix lumen is being colonized by intestinal commensals. FDCs begin developing by 3 d of age, and T cell areas develop around the basolateral margin of the follicle by 4 d of age (9). Our results suggest that B cells traffic sequentially to the FAE, the FDC network, and the B cell/T cell boundary prior to localizing at the follicle base to proliferate and begin diversifying the primary Ab repertoire. Determining the nature of the microbial and host cell signals B cells acquire at each site will lead to a more complete understanding of the remarkable host-microbial relationship rabbits rely on to generate a diverse primary Ab repertoire.

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Disclosures

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References


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