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Peyer's patches: Organizing B cell responses at the intestinal frontier

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Summary

Secondary lymphoid tissues share the important function of bringing together antigens and rare antigen-specific lymphocytes to foster induction of adaptive immune responses. Peyer's patches (PPs) are unique compared to other secondary lymphoid tissues in their continual exposure to an enormous diversity of microbiome- and food-derived antigens and in the types of pathogens they encounter. Antigens are delivered to PPs by specialized microfold (M) epithelial cells and they may be captured and presented by resident dendritic cells (DCs). In accord with their state of chronic microbial antigen exposure, PPs exhibit continual germinal center (GC) activity. These GCs contribute to the generation of B cells and plasma cells producing somatically mutated gut antigen-specific IgA antibodies, but have also been suggested to support antigen-nonspecific diversification of the B cell repertoire. Here we review current understanding of how PPs foster B cell encounters with antigen, how they favor isotype switching to the secretory IgA isotype, and how their GC responses may uniquely contribute to mucosal immunity.

Keywords

B Cells; Dendritic Cells; Antibodies; Cell Trafficking; Repertoire Development; Mucosa

Introduction

Intestinal antibody has a crucial role in neutralization of orally invading pathogens, including viruses such as poliovirus and rotavirus and bacteria such as *Cholera* and *Salmonella* (1, 2). Recent advances in studying the intestinal microbiome have revealed critical influences of mucosal antibody on the host-commensal symbiosis (3). Given these broad ranging functions it is perhaps not surprising that IgA, the major mucosal immunoglobulin (Ig) isotype, is the most abundantly produced antibody in the body (4, 5). IgA is secreted in a dimeric form by plasma cells that are distributed throughout the small intestinal, and to a lesser extent large intestinal, lamina propria (LP) and it is transported into the intestinal lumen by the epithelial polymeric IgA receptor (polyIgR). Intestinal IgA producing cells can arise from a number of origins, including from B cells within mesenteric

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lymph nodes (LNs), spleen and intestinal isolated lymphoid follicles (ILFs), but Peyer's patches (PPs) are the major source.

Peyer's patches were named after Johann Conrad Peyer who described them in 1673 as 'elevated areas composed of lymph nodules in the mucous membrane of the small intestine', though they had been reported in earlier studies (reviewed in (6)). Distributed along the length of the small intestine, they number 100–200 in humans and 6–12 in mice (6, 7). PPs are organized into three major regions: a series of large B cell follicles; the overlying follicle associated epithelium (FAE) and associated sub-epithelial dome (SED) that lies between the follicles and the FAE, and; the small T cell zones that are situated adjacent to the B cell follicles (Fig. 1). A special property of the FAE is the presence of modified epithelial cells termed M cells that bind many luminal antigen types and transcytose them to the SED. As well as containing blood vessels, PPs have a rich content of lymphatic vessels that are used as lymphocyte and plasma cell exit portals.

PPs are continually exposed to mucosally-derived antigens and their follicles almost universally contain preformed germinal centers (GCs), sites of Ig gene somatic hypermutation (SHM) and B cell selection (8). The importance of the microbiome in promoting these responses is demonstrated by the much smaller tissue size and absence of GCs in PPs from germ-free mice (9–11). Many of the B cells present within PPs of conventionally housed animals have undergone isotype switching from IgM to IgA, and PPs give rise to IgA^+ plasma cells, in most cases carrying somatic mutations in their Ig-genes, that home selectively to the intestinal LP.

While the importance of PPs in mucosal immunity is well appreciated, the specialized mechanisms by which these structures support the induction of B cell responses is less understood than for LNs and spleen. This is a significant dearth of knowledge given the important role of PP-derived IgA in host defense and in shaping properties of the microbiome. Here we review current understanding of PP development and organization, and we discuss how B cell antigen encounter may take place in these organs. We then consider what is currently known about the induction of IgA switching in PPs and discuss the properties and functions of PP GCs, including their possible role in antigen non-specific antibody diversification. Finally, we summarize properties of PP-derived IgA^+ memory and plasma cell responses.

Peyer's patch development and lymphocyte trafficking

Fetal and neonatal PP development

Peyer's patch development in mice begins around embryonic day 12.5–13.5 with the appearance of hematopoietic cells in the gut (12, 13). Human PP development also begins quite early in gestation and the reader is referred to Heel et al., (6) for a detailed description of this ontogeny. The first evidence of cellular organization during mouse PP induction is the appearance at E15.5–16.5 of $VCAM1^+$ spots distributed at intervals along the anti-mesenteric side of the intestine. Induction of these PP anlagen is promoted by two types of hematopoietic cells, $cKit^+CD11c^+Ret^+$ lymphoid tissue initiator (LTin) cells and $ID2^+ROR\gamma t^+IL7R\alpha^+$ lymphoid tissue inducer (LTi) cells, also known as type-3 innate

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lymphoid cells (ILC3). A current model posits that RET signaling initiates PP formation by promoting VCAM1-dependent adhesion of LTin cells and their signaling to lymphoid tissue organizer (LTo) stromal cells to induce increased adhesion molecule (VCAM1, ICAM1, MADCAM1) and chemokine (CXCL13, CCL19, CCL21) expression. This then causes accumulation of the IL7-dependent LTi cells that trigger via LTβR signaling further expression of adhesion molecules and chemokines (12, 14). Recent work has found that a fetal Id2-dependent $Arg1^+$ ILC precursor that expressed a transitional developmental phenotype (ftILCP) is the first appearing ILC type in the intestine (15). These cells are able to give rise to ILC3s as well as some other ILC types, and they accumulate at the earliest sites of cell clustering during PP development in a LTα1β2- and RORγt-dependent manner (15). These ILC precursor cells are suggested to act as a local source of LTi cells during PP development. Retinoic acid controls LTi cell maturation, at least in part by promoting RORγt expression and retinoic acid receptor or vitamin A deficiency is associated with defects in PP development (16). In contrast to LTi cells, the fILCP cells do not express CXCR5 and CCR7, leaving it unclear how their clustering is promoted.

Lymphocytes begin arriving in the nascent mouse PPs in the first few days after birth (17) and B cells make an important contribution to the growth and maturation of PPs (10). LTi cells also have a role in promoting organization of postnatal PPs, most likely by LTα1β2 signaling to stromal cells, and when LTi cell function is disrupted in the first days after birth, PPs fail to develop organized follicles and interfollicular T zones (17). This organizing activity of LTi cells is associated with a switch from CXCR5 expression and localization in $CXCL13^+$ nascent follicles, to CXCR4 expression and positioning in nascent interfollicular T zones (17). TGFβ signaling promotes induction of CXCR4 on PP LTi cells, but the signals downregulating CXCR5 remain unclear.

While PPs and LNs share the requirement of LTα1β2 for their development, a second TNF family cytokine that is essential for LN development, TNFSF11 (RANKL, TRANCE), is not necessary for the early steps of PP development (13). However, TNFSF11-deficient mice do show a reduced size of PP (18). *In situ* staining revealed TNFSF11 in low amounts at E17.5, but more strongly at birth, in an area beneath the FAE of the developing PP (19, 20). Induction of M cells critically depends on TNFSF11 and PP M cells are severely reduced in TNFSF11-deficient or blocked mice (10, 21). TNFSFR11 is expressed by epithelial cells and the $TNFSF11^+$ stromal cells below the FAE may be involved in triggering M cell differentiation. How B cells contribute to M cell maturation is unclear, though this does not appear to require TNF or LT-expression by the B cells, factors that are important for follicular stromal cell development (10, 22). Microbial stimuli can also increase the number of M cells and consistent with this, M cell deficiency in TNFSF11-deficient mice is less pronounced toward the distal end of the small intestine where the bacterial load is heavier. In accord with the specialized antigen-transport function of M cells (discussed below), TNFSF11 deficient mice have fewer GCs in their PP follicles and lower levels of fecal IgA (21).

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Lymphocyte entry, transit and exit

Lymphocytes enter PPs from the bloodstream via HEVs (23). B cells are more frequent in PPs than in LNs and this reflects at least in part the specialized properties of PP HEVs. Like in LNs, many PP HEVs are situated in T cell-rich interfollicular zones. However, PPs also have HEVs located within follicles. PP HEVs have high expression of MADCAM1, the ligand for the α4β7 integrin. This integrin is more abundant on naive B cells than on naïve T cells and is one factor that contributes to the more efficient entry of B cells than T cells to PPs (23). Chemokine mediated attachment to PP HEVs involves CCL21 and CXCL12 as in LNs, but additionally the HEV within PP follicles display CXCL13 and selectively support the attachment and entry of B cells (24).

The organization of PPs into follicles and T zone is mediated by CXCR5-CXCL13 and CCR7-CCL21, respectively (25). The oxysterol receptor, EBI2, allows B cells to distinguish between the outer and center follicle in lymphoid tissues, though the significance of this guidance information in PPs is not known beyond the importance of EBI2 downegulation in fostering GC B cell clustering at the follicle center (26–28). Sphingosine-1-phosphate receptor-2 (S1PR2) induction also contributes to GC B cell clustering (29). The factors promoting positioning of DCs in the SED region of the PPs are not well defined. Although CCR6 was implicated in this process and the CCR6 ligand, CCL20, is made abundantly by FAE (30, 31), subsequent studies (32) including our own recent work (in preparation) have not provided support for this requirement. M cell-derived CCL9 may have a role, though deficiency in the CCL9 receptor CCR1 did not affect PP DC distribution (32, 33), making it likely that additional organizing cues will be involved. The FAE also produces CXCL16 and this may have a role in recruiting CXCR6-expressing activated or effector T cells to the SED (34).

B and T cell egress from PPs shows a similar dependence on sphingosine-1-phosphate receptor-1 (S1PR1) as egress from LNs and ablation of sphingosine kinase-1 (Sphk1) from lymphatic endothelial cells in Sphk2−/− mice causes a block in cell appearance in PP lymphatic sinuses (35, 36). Using mice expressing photoconvertible fluorescent proteins and newly developed PP photoconversion procedures, the transit time of B cells through PPs ($t_{1/2}$~10hrs) was found to be shorter than through LNs ($t_{1/2}$~16hrs) (37–39). This might in part reflect a distinct action of CXCR4 in guiding cell movements in PPs versus LNs. The CXCR4 ligand, CXCL12, is abundantly produced by stromal cells in the vicinity of subserosal lymphatic vessels and guides B cells to this peri-lymphatic location (38). This presumably then facilitates their encounter with lymphatic vessels, allowing S1PR1 signaling to trigger egress. Expression of the CXCR5 ligand, CXCL13, by contrast, is restricted to follicular regions and consistent with follicles being separated from lymphatic sinuses, CXCR5 promotes PP retention of B cells (38). The significance of transiting PPs more rapidly than LNs is unclear but might relate both to the more rapid access of entering cells to follicles, and the more ubiquitous presence of antigen perhaps limiting the scan time that is needed.

As in LNs, the lymphocyte activation marker CD69, a negative regulator of S1PR1 (40), can inhibit lymphocyte egress from PPs (39). Thus CD69 upregulation on B and T lymphocyte might cause their retention in an inflamed PP, promoting local cell accumulation and

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increased chance of antigen-specific lymphocytes being present and encountering the stimulus antigen. Elegant recent work has shown that additional mechanisms control cell egress from PPs. *Salmonella* infection caused egress shutdown and hypertrophy of PPs independently of type I IFNR and CD69. This egress block was persistent over multiple days and did not involve decreased CXCR4 expression (39). Further work will be needed to define the mechanism, but one possibility might involve regulation of local lymphatic flow (41). The chronic nature of this egress blockade suggests it may have functions beyond promoting antigen encounter, perhaps ensuring appropriate local maturation of effector or memory cells, but possibly also helping restrict spread of an invading pathogen or its toxins.

B cell antigen encounter

What we know in lymph nodes

B cell antigen encounter in LNs has been a topic of investigation for many years and a number of pathways have been defined. Lymph-borne antigens enter LNs via afferent lymphatics that connect with the subcapsular sinus (SCS) at locations overlying follicles. Small soluble antigens (~70kd or a few nm in hydrodynamic radius) can gain free access to the follicle or travel in via stromal cell-ensheathed collagen fibers (conduits) for direct B cell encounter (42, 43). Larger antigens do not have free access to follicles due to the filtering function of the lymphatic cells lining the SCS. A range of particle types as well as opsonized antigens can become bound and displayed by specialized ($CD169^+$) SCS macrophages that extend between the sinus and underlying follicle (43). Cognate B cells can capture antigens from the tail processes of these macrophages. Alternatively, noncognate B cells pickup opsonized complexes using complement receptors and deliver them to FDCs for longer-term display (44). In some cases, antigens may be transported into interfollicular regions by migratory DCs and then become displayed for B cell antigen encounter (45). Some lymph-borne antigens elude capture by SCS macrophages but instead become bound by sinus-exposed DCs and these cells may deliver antigens to B cells (46). There is also evidence that B cells migrate through the macrophage-rich LN medullary regions and they may become exposed to antigen in this location (47). Finally, many B cells are expected to encounter antigens on FDCs since these cells serve as long-term reservoirs of opsonized antigens (22, 48, 49). The existence of multiple modes of antigen capture and display in LNs suggests that multiple mechanisms may exist to foster B cell antigen encounter within PPs where this process has been less studied.

M cells and antigen uptake in PPs

Antigen entry to PPs occurs predominantly via M cells (Fig. 2). These specialized epithelial cells can associate with and transcytose a range of antigen types including various bacteria, viruses, fungi, cholera toxin, horseradish peroxidase, immune complexes, prions, and inert particles (latex beads) (50–52). Their ability to interact with particulate antigens is promoted by a low glycocalyx covering, by their having short and irregular microvilli and a flattened surface and by being located in pits. However, the receptor systems allowing the breadth of antigen uptake are not clear. M cells do not express the polyIgR but are somehow able to bind IgA, though not other Ig isotypes, and IgA coating enhances the efficiency with which various antigens are captured and transcytosed (52–54). M cell-expressed Dectin-1 has been

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implicated in transcytosis of glycosylated sIgA-antigen complexes (55), though it was not required for uptake of IgA coated bacteria (53). M cells express glycoprotein-2 (GP2), recognizing FimH (of type I pili) that is present on a subset of commensal and pathogenic enterobacteria (*E.coli, S.typhimurium*) and deficiency in FimH or GP2 leads to defects in bacterial transcytosis (56). They also have unique surface glycosylation, allowing their detection by specific lectins (51, 52) and these sugars may facilitate binding of some microbes. Their expression of glycolipid GM1 supports binding of cholera toxin (50).

On their basolateral side, M cells have large cell-containing pockets (51, 52). A range of cell types have been reported in M cell pockets, including IgD^+ and IgD^- B cells, T cells, DCs and macrophages (6, 57–60), though the small size of the pockets and irregular distribution of M cells in the FAE has made it difficult to obtain quantitative data on the associated cell types. Stimulation by peptidoglycan or LPS increases the uptake of antigens by M cells (61). These stimuli also increase DC association with the FAE, including the density of DCs that associate with M cells and project processes across the FAE, and this may augment or modify antigen uptake.

Complexities of the subepithelial dome (SED)

Once antigens have been delivered into the PP they seem to rapidly disperse through the SED (Fig. 2). The cells involved in this process are not clear but it might be anticipated based on the LN work that different cell types in the SED contribute to the display of different antigen types. SED contain a high density of DCs (Figs. 1 and 2), particularly $CD11b^+CD8^-$ DCs and $CD11b^-CD8^-$ DN DCs (62), lower densities of B cells and macrophages as well as a scattering of T cells and $ROR\gamma t^+$ ILCs ((63) and unpubl. obs.). $CX3CR1^+$ DCs have been reported associated with the FAE (60). It seems possible that DCs help deliver certain antigens from the SED into the adjacent follicles and it is notable that PP follicles have a higher density of $CD11c^+$ cells than usually observed in LN and splenic follicles ((64) and unpubl. obs.). However, as pre-GC and memory B cells in PPs can express CD11c (63, 65) more work needs to be done to quantitate the density of DCs in PP follicles and to establish whether they act as antigen transport cells. A central role for SED DCs in capturing transcytosed antigens has been observed in a number of studies. More than 90% of translocated *E.coli* were captured by DCs (56). $CD11b^+$ $lysozyme^+$ DCs were involved in the uptake of *Salmonella* and dead epithelial cells including M cells (66). SIgA coated *Shigella flexneri* was bound and internalized by $CD11b^+$ DCs (54). Reo virus-containing apoptotic epithelial cell fragments were detected within DN DCs (67) and apoptotic epithelial cells were detected within $OX41^-$ DCs in rat PPs (68). *Candida albicans* and β-glucan particles that reached the SED were selectively associated with $langerin^+$ DCs (69). Translocated prion-containing particles were taken up by $ferritin^+$ cells in the SED that were suggested to be macrophages (70). In some cases, DCs bearing transcytosed antigen were reported to move from the SED to T cell areas after exposure to maturation stimuli such as cholera toxin or live *Salmonella* (52). As well as presenting MHC-peptide complexes to T cells, these DCs might make antigen available for B cell encounter at the follicle-T zone interface.

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Although IgD^+ B cells have been observed in association with M cell pockets (51), whether sufficient numbers of naïve B cells travel to the SED and interact with M cells to provide a chance of encounter with specific antigen is not known, but given the rarity of antigen-specific B cells in the naïve compartment, this seems unlikely. Moreover, whether the pocket-associated IgD^+ cells are all naïve or not is unclear. Our recent findings have shown that CCR6 function is critical for B cell access to the SED and likely to M cell pockets, and CCR6 function was upregulated on activated B cells but was minimal on naïve B cells (unpubl. obs.). However, there are cells with properties of memory B cells (such as being IgA^+) in the SED (58). These cells are likely to have been selected for recognition of antigens of the type that enter into the SED and antigen pass-off from M cell to memory B cell seems possible. Direct encounter between transcytosed antigen (horseradish peroxidase) and pocket-lymphocytes has been observed in electron microscopy studies (71), but the type of lymphocyte and the antigen's subsequent fate are unknown from these experiments.

Reaching the follicles

Molecules below ~70kD in size are able to access LNs via conduits and molecules the size of hen egg lysozyme (~10kD) may gain direct access to the LN follicle (72, 73). One study showed that larger complexes could become proteolyzed by the time of arrival in the LN subcapsular sinus, thereby allowing their direct access to the follicle (74). It seems likely that a range of small molecules or fragments of digested larger molecules will be delivered by M cells into the SED to then gain direct access to the underlying follicle by diffusion. Whether conduits travel between the SED and follicle has not been determined.

PP GCs contain well-developed FDC networks that are orientated toward the SED. FDCs retain and display antigens using complement and Fc receptors. As well as their role in GCs, FDCs in LN primary follicles display antigen for encounter by recirculating naïve B cells (48, 49); whether FDCs are an important site of antigen encounter by naïve B cells in PPs is not known but seems likely. Yet, how antigens reach these specialized antigen-presenting cells in PPs and the requirements for their retention and display are poorly defined. In LNs many complex antigens enter the SCS pre-coated in C3b due to activation of the complement cascade. This allows their complement receptor-mediated capture by non-cognate follicular B cells and delivery to FDCs (44). It is unlikely that antigens are coated in complement in the intestinal lumen and currently there is little evidence indicating that antigens become complement coated after they arrive in the SED, though this possibility merits further analysis. Complement components C1q, C4b and C3b have been detected on the surface of PP FDCs (75–77) though whether this reflects deposition of complement-coated mucosally-derived versus circulating antigens has not been established.

An antibody against an epithelial marker stained PP FDC in mice, leading to the suggestion that epithelial cell-derived exosomes may be deposited on FDCs as a mechanism of antigen display (70). In a possibly related observation, epithelial cell-derived carbonic anhydrase positive membrane particles were observed within follicles in sheep ileal PPs (78). Given that C1q can bind apoptotic membranes (79), it seems possible that complement is involved in FDC display of epithelial cell-derived vesicles. FDCs in PPs express the IgA- and IgM-binding Fcα/μR, though whether it contributes to immune complex retention is not known

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(80); contrary to this notion, absence of the receptor was associated with prolonged retention of certain antigens on splenic FDCs (81). PP FDCs abundantly express MADCAM1 (82). A recent study from our group on LN responses showed that integrins are not critical for GC responses to model protein antigens, but the work did reveal a reduced ability of integrin-deficient B cells to participate in chronic GC responses in MLNs (83) making it possible that integrin ligands on FDCs contribute to the efficiency of antigen acquisition by PP B cells.

We have recently observed that B cell access to the SED is strongly CCR6 dependent, and *in vitro* experiments suggest that BCR-activated but not naïve B cells respond to the CCR6 ligand, CCL20 (in preparation). Whether stimuli other than BCR engagement, for example ligands for pattern recognition receptors, can promote this migration has yet to be determined, but if it were the case, this might be a mechanism promoting B cell antigen encounter. EBI2 has a prominent ability to favor naïve B cell positioning in the outer follicle in a location abutting the SED (26, 28) making it important to assess whether EBI2 expression augments PP B cell antigen encounter (Fig. 2).

IgA isotype switching

Key requirements in PPs

Seminal work in rabbits established PPs as important follicular inductive sites for intestinal IgA production (84). The molecular factors that guide follicular B cells to differentiate to IgA^+ cells are both intrinsic and extrinsic. Expression of activation-induced cytidine deaminase (AID) is required in B cells since this enzyme promotes class switch recombination (CSR) and affinity maturation through somatic hypermutation (SHM) (85). Environmental cues present in PPs, including cytokines, regulate the ability of B cell to undergo CSR, the most critical being transforming growth factor β (TGFβ) (Fig. 3). TGFβ induces germline Ig α-transcripts in B cells, initiating IgA CSR, and its deficiency, as well as the deficiency for TGFβ receptor II (TGFβRII) on B cells, virtually abrogates the production of IgA^+ B cells (86–88). A key source of TGFβ is the B cells themselves (89), though T cells, FDCs and DCs may also produce this cytokine in the PP (4). TGFβ is made in a latent form and PP FDCs have been suggested to promote its activation through production of matrix metalloproteases, but this has not yet been demonstrated *in vivo* (90). In other studies, αvβ8 integrins on DCs are required for TGFβ activation during intestinal regulatory T cell responses (91). Further work is needed to establish the mechanisms responsible for TGFβ activation during IgA switching in PPs.

Consistent with the TGFβ requirement, intestinal DCs express inducible-nitric-oxide-synthase (iNOS) and this augments IgA CSR in PPs through a mechanism suggested to involve upregulation of TGFβRII on B cells (92). Although *in vitro* studies indicate NO can promote TGFβRII mRNA expression by naïve B cells in a PI3K and AKT-dependent manner, whether NO acts directly on naïve B cells *in vivo* or indirectly through changes in other cells remains undefined. PP DCs and epithelial cells are a source of a proliferation inducing ligand (APRIL) and mice deficient in APRIL and some humans with mutations in the receptor for APRIL (TACI), are characterized by reduced IgA production (93, 94). B cell-activating factor of the TNF family (BAFF) can be expressed by DCs, epithelial cells and FDCs and can augment IgA CSR *in vitro* (90, 95, 96), *In vivo* BAFF overexpression

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leads to an elevated commensal specific IgA response (97). However, BAFF and BAFF-R deficient mice show normal or mildly reduced IgA (98, 99), and patients with deletion of the BAFF-R gene have normal serum IgA (100). More work is needed to determine whether antigen-specific intestinal IgA levels are altered by BAFF deficiency. DCs also produce retinoic acid, which can promote IgA CSR and gut-homing receptor expression on B cells (101). Recent *in vivo* work indicates that intrinsic retinoic acid receptor signaling in B cells is essential to induce α4β7, but may be less critical for IgA CSR (102). Instead, retinoic acid may cause PP DCs to augment IgA induction by other mechanisms (95, 103)(unpubl. obs.).

The above observations converge on the view that DCs have a direct role in promoting B cell IgA CSR. This raises questions about the compartment where PP B cells are subjected to the influences of DCs, and a likely location is the DC-rich SED. As already noted, we have recently found that activated B cells upregulate CCR6 and efficiently migrate into the SED prior to entering the GC response (manuscript in preparation). Our data suggest that such movement is important for promoting interactions with SED DCs and inducing productive IgA CSR. Consistent with switching being initiated outside GCs, PP B cells with a $GL7^{int}$ activated phenotype had high amounts of Cα switch circles (104). However, whether switching at this stage is restricted to T-independent IgA production or can play a role in T-dependent IgA responses is still unknown.

The extent to which T cells directly promote IgA CSR in PPs remains an active area of investigation. T cell-deficient mice show about a 75% reduction in intestinal IgA establishing that the majority of IgA – at least in specific pathogen free mice – is T-dependent (TD), but also indicating there is a substantial amount of T-independent (TI) IgA (105–107). T cells likely promote IgA production through two mechanisms: by contributing signals that induce IgA CSR, and by promoting expansion of B cells that have already completed CSR. *In vitro* studies have established that CD40L promotes AID expression and switching to all Ig isotypes, and that it can cooperate with other signals to favor switching to IgA (108). Somewhat surprisingly, however, $CD40^{-/-}$ mice had almost normal intestinal IgA levels (108). It seems likely that the CD40L-CD40 contribution to IgA CSR will vary with the type of antigen driving the response since *in vitro* studies indicate that some innate signals, such as through TLR4, can substitute for CD40 to promote AID expression and CSR (109). Consistent with this view, CD40-deficient mice were severely compromised in their ability to mount an antigen-specific IgA response upon oral immunization with cholera toxin, a protein antigen (104, 108). However, it still remains unclear whether CD40 acts during the initial induction of isotype switching or during the expansion of cells that have already received inductive signals. The critical role of T cells and CD40 during the expansion phase is highlighted by the absence of PP GCs in TCR- and CD40-deficient mice (110–112). Evidence that PP GCs make a major contribution to the expansion of gut IgA^+ cells comes from the findings that 85% or more of IgA encoding antibody sequences in intestinal plasma cells are somatically mutated, often heavily (105–107). Beyond expression of CD40L, PP T cells may influence IgA CSR through IL21 production. IL-21, produced by Tfh cells and $CXCR5^+$ Th17 cells (113, 114) synergizes with TGFβ to drive mouse and human B cells toward IgA production (115, 116). In summary, while it seems almost certain that T cells will contribute to the inductive phase of IgA CSR, a lot more work is needed to

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understand the temporal order in which the signals that promote IgA switching are received during B cell activation, proliferation and differentiation in PPs.

T-independent switching

As noted above, the lack of T cells or CD40 leads to only a partial reduction in IgA, establishing that T cell independent (TI) IgA responses contribute to the total intestinal IgA level. Past and recent studies in T cell-deficient mice have shown TI-IgA responses to target preferentially commensal bacteria, with both B2 and B1b cells contributing to the response (111, 112). These data are consistent with the idea that microbe-derived signals can substitute for CD40 signals to promote IgA responses. Indeed, IgA was produced against largely the same constellation of commensals in wild-type and T cell-deficient mice, with the exception that a small number of epithelium-attaching commensals evaded the TI-response (112), However, given that the majority of IgA antibodies, including those against commensals (106), are somatically hypermutated, it seems likely that T cells are normally involved in the induction of antibody responses towards many commensals. In support of this view, mice harboring a mutant form of AID, AID^{G23S}, that supports CSR but not SHM had normal amounts of IgA but greatly reduced V-region mutation and this led to GC hyperplasia and bacterial expansion in the small intestine (117). In another informative study, mice that lacked the inhibitory co-receptor programmed cell death-1 (PD-1) gene generated an excess of abnormal Tfh cells and IgA with reduced affinity maturation and commensal binding capacity, and this was associated with alterations of gut microbial homeostasis (118). Taken together, the studies to date indicate that TI responses are capable of generating IgA that binds many commensals, but AID-dependent and Tfh-dependent responses make important contributions to the amount and quality of the commensal-specific IgA. A caveat with all the studies examining TI IgA responses is that they have been performed in mice unable to mount TD responses. It will be crucial in future work to determine the relative contribution of TD vs TI IgA responses to gut bacteria and other antigens in animals able to mount both types of response.

IgA switching outside the PP

Although PPs are the main inductive sites for IgA in the intestine, mice with reduced or absent PPs show a variable but not absent number of IgA plasma cells in their gut LP, indicating that other sites in the intestine besides PP can sustain IgA CSR. Isolated lymphoid follicles (ILFs) are single clusters of B cells, surrounded by many DCs and a few T cells, located in the terminal ileum and colon. ILF formation, similarly to PP formation, depends on $ROR\gamma t^{+}$ LTi cells and on their interaction with stromal cells through the $LT\alpha 1\beta 2$-$LT\beta R$ axis. However, ILF formation occurs after birth, and their maturation can be promoted by recognition of peptidoglycan derived from commensal bacteria through NOD1 (11, 119). Mice deficient for the transcription factor $ROR\gamma t$ lack ILFs as well as LNs and PPs. Upon irradiation and reconstitution with wild-type BM, $ROR\gamma t$-deficient mice show an increased frequency of IgA plasma cells in the LP. Since ILFs, but not PPs, can be generated in these chimeras, this result suggests that ILFs can play a role as an IgA inductive site. Similarly, mice lacking both $\alpha\beta$ and $\gamma\delta$ T cells, show an increase in AID induction and IgA CSR in ILFs compared to WT mice, accounting for the intestinal IgA level present in the animals (4). These observations suggest that while ILFs normally make only a small contribution to

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IgA production in mice housed in specific pathogen free animals facilities, physiological conditions likely exist where these structures make more substantial contributions to the IgA response.

In the absence of all gut-associated lymphoid tissue (GALT), IgA plasma cells in the LP can still be detected, suggesting IgA CSR may also be able to occur directly in the LP. Two sets of observations support this hypothesis: first, the expression of AID has been observed by in situ staining and the use of reporter mice (96, 120, 121); second, Ig Cα germline and Ig α circle transcripts has been detected in B cells isolated from LP (122). However, other studies failed to detect CSR in the LP (108, 123); this inconsistency might depend on the age, mouse strain, and commensal community.

Unique properties of PP GC responses

A signature feature of PP follicles is the presence of GC responses in the absence of immunization or infection by pathogens. These chronic responses are a consequence of the continual exposure to microbiome-derived molecules as they are largely absent in germ-free mice (9). Like GCs in other lymphoid tissues, PP GCs are organized into light and dark zones. The LZ contains a well-developed FDC network and the DZ contains a network of $CXCL12^{+}$ reticular cells (CRCs) involved in attracting $CXCR4^{hi}$ GC B cells to this compartment. CRCs in PPs form a network that typically occupies the entire DZ, a distribution that is more extensive than found in LN and spleen GCs (124, 125). This may indicate that the chronic PP GC DZ requires more vigorous stromal support than is needed for the transient GCs forming in other lymphoid tissues.

Minimal antigen-recognition requirements for PP GCs

PP GC responses can be induced by a range of antigen types including commensals, pathogens and pathogen products. As discussed above, PP GC responses are strongly T cell- and CD40-dependent, like GC at other sites, and most studies indicate they have a conventional role in generating B cells with improved affinity for the inducing antigens while avoiding generating autoreactive B cells (126). The properties of conventional GC responses have been reviewed elsewhere (8). However, a small number of studies have pointed to the possibility that under some circumstances the antigen-recognition requirements for inducing and sustaining PP GCs responses may be less stringent than in other tissues.

A study of the requirements for GC B cell SHM made the surprising finding that RAG1-deficient mice carrying Ig and TCR transgenes contained GCs even without immunization with the cognate antigen (127). The GC B cells in these mice demonstrated SHM (127). Further support for the possibility that cognate antigen recognition may not be essential for PP GC formation emerged in a striking study in 2004 showing the presence of PP GCs in mice carrying the EBV LMP2A protein-encoding gene in place of their endogenous Ig heavy chain gene. LMP2A contains immunoreceptor tyrosine-based activation (ITAM) motifs and causes constitutive BCR pathway signaling when expressed in B cells (128). This signaling property might explain how the B cells become activated to enter GC responses, but leaves the puzzle of how the cells receive T cell help. What antigen(s) are being

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presented to what T cell type(s)? Could non-classical antigen recognition, such as by γδT cells, CD1d-restricted natural killer (NK) T cells or MR1-restricted mucosal-associated invariant T (MAIT) cells be involved? Consistent with an ability of cells other than CD4 T cells to have a role, MHC class II-deficient mice retain small PP GC responses (129). Or are LMP2A-driven GCs T cell independent? Similarly to the Bemark *et al.*, study (127), these workers showed that mice carrying knocked in Ig heavy chains and transgenic light chains exhibited PP GC responses in the absence of immunization with cognate antigen, though this was not tested on a RAG-deficient background (128, 129). A more recent study found that mice carrying a knocked in hapten-specific heavy chain contained PP GC B cells that had undergone extensive SHM and this was again taken to favor the conclusion that a program of GC hypermutation can operate in PPs to promote Ig diversification (130). While the concept of PPs contributing to repertoire diversification is an intriguing idea, especially given the prominence of this process in a number of other species as discussed below, more work needs to be done to rigorously test this possibility in mice. At this point, putting the LMP2A transgenic mice aside as a special case, it seems most likely that BCR antigen binding is required for PP GC responses and that Tfh cells support most of these responses, but low affinity interactions with continually and abundantly available microbiome-derived antigens may be sufficient to drive them (131) (Fig. 4). This type of response might be especially prominent in contexts where the starting B cell clonal diversity is low or absent, as in the case of Ig transgenic mice, since the lack of commensal-reactive Ig in the gut may lead to a high microbial antigen burden in the PP.

PPs as sites of antibody diversification in other species

A function for intestinal lymphoid tissue GC-like structures in the generation of B cell repertoire diversity has been established in rabbits, sheep, cattle and chicken (132–134). Newly generated B cells migrate to GALT that includes the appendix, PPs and sacculus rotundus (a type of ileal PP) in rabbits, a very large (100–200 cm long) ileal PP in sheep and cattle, and the Bursa of Fabricius in chicken. In rabbits and sheep, the Ig diversification involves SHM and intestinal microbiota are required (though in sheep the diversification begins in the sterile embryonic environment). This diversification mechanism may be particularly important in these species because they generate only limited B cell repertoire diversity during early B cell development; for example, 80–90% of the Ig heavy chain rearrangements in the rabbit use the 3′ most IGVH gene (133). The B cell proliferation underlying intestinal tissue-associated diversification is thought to be driven in an antigen non-specific manner (polyclonally) and based on the minimal presence of T cells in rabbit ileal PP's and a study in embryonically thymectomized sheep, the diversification process may not require conventional T cell help (132, 133). TLRs have been suggested to be major drivers in some species, though involvement of a BCR superantigen has also been invoked (133). In rabbits, the diversification activity is developmentally timed to populate the periphery with diversified B cells at 3 weeks (weaning), when maternal Ab is waning (135); in sheep the ileal PP involutes starting from 3–4 months of life (whereas the jejunal PPs that have a typical secondary lymphoid tissue character, are maintained) (132). The findings suggesting mice may be able to mount PP GC responses in the absence of strong antigen recognition makes it attractive to think that related processes contribute to repertoire diversification in a broader range of species, possibly including mice and humans. Indeed,

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some antigen-independent SHM-based diversification of the human B cell repertoire has been suggested to occur in the first few years of life (136), though whether this is truly antigen-independent has been debated (137, 138) and the location where it takes place remains to be determined. The finding of RAG gene expression in neonatal mouse LP B cells and Ig locus rearrangement in this compartment (139) adds to the notion that mice share some features of gut-based B cell repertoire diversification with other species. More work is needed on the neonatal intestinal immune system to rigorously explore to what extent and in what form GALT-based antibody diversification contributes to B cell repertoire diversity in humans.

Exchange of cells across PP GCs

A recent IgA lineage tree analysis has suggested that B cell exchange between PP GCs plays a role in establishing synchronized oligoclonal antigen-specific responses along the length of the mouse small intestine (126, 140). Consistent with these data, next generation sequencing of IgA genes revealed very similar repertoire representation in different regions of the small intestine (131). The nature of the B cells exchanging between GCs via circulation is not fully defined, though an ability of transferred $GL7^+$ B cells to selectively invade existing PP GCs was demonstrated (140). It seems unlikely that the exchanging cells are GC cells given their intrinsic propensity for apoptosis and inability to survive *in vivo* outside the GC niche (141). Instead, the circulatory cells maybe newly generated memory B cells that retain measurable GL7. In an experiment tracking the movements of endogenous PP B cells using photoconversion, significant memory B cell exchange took place between PPs over a 3 day period (131). Presumably the exchanging cells are programmed to favor homing back to mucosal sites, perhaps by having high $\alpha4\beta7$ integrin expression. The unique presence of $CXCL13^+$ HEVs within PP follicles (24) might also facilitate homing of $\alpha4\beta7^{hi}$ $CXCR5^+$ $CCR7^-$ B cells between PPs. Whatever the mechanism, this type of clonal exchange may ensure that the same clones are expanded in GCs along the length of the small intestine and give rise to a sufficiently large number of plasma cells to seed the entire LP, possibly enhancing the quality of the antigen-specific IgA response in a manner that augments host-commensal symbiosis or anti-pathogen responses. The observation that following haptenated-cholera toxin immunization up to 15% of plasma cells in the small intestine can be antigen-specific (140) highlights the large scale of the response.

At present it is hard to reconcile how conventional affinity-maturing GC responses and chronic, diversifying GC responses could co-occur in PPs (Fig. 4). In this regard it is interesting to consider recent findings suggesting that GC responses in LNs have an early phase that favors the generation of a diversity of low affinity cells and a late phase that generates affinity-matured cells ((142) and Shlomchik et al., in press). Perhaps the chronic commensal microbe-driven GC responses that are continually present in PP GCs share features with the early GC phase of LN responses, supporting diversification with limited affinity maturation. When a strong conventional-type response is provoked by a newly arriving T-dependent antigen it may ‘take over’ the existing GCs and lead to selection of high affinity plasma cells and memory B cells. Once this response has been successful and the antigen is diminished, the chronic commensal-driven GC response might re-occupy the structure and continue to generate a diversity of commensal-reactive B cells. Recent deep

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sequencing analysis of individual mice across a period of months revealed that expanded and hypermutated clones were a typical feature of the intestinal IgA repertoire, providing evidence that the diversification mechanism may principally act on memory B cells (131). The authors speculate that use of a limited set of B cell clones and diversifying them through SHM might help sustain stable symbiotic host-microbe interactions.

PP-derived plasma cells and memory B cells

IgA plasma cell homing

IgA plasma cells derived from the small and large intestines have different Ig repertoires, indicating that they likely arise from different sources (143). Plasma cells differentiating in PPs are marked by induction of α4β7 and CCR9, molecules that foster homing to the small intestinal LP since MADCAM1 is selective to mucosal post capillary venules and the CCR9 ligand CCL25 (TECK) is made broadly by small intestinal epithelium (144). An important factor promoting induction of these mucosal homing molecules is retinoic acid, derived from PP DCs and epithelial cells (144–146). In mesenteric LNs, lymphoid stromal cells as well as DCs are a source of retinoic acid (147, 148) and it seems possible that stromal cells will also produce retinoic acid in PPs. Retinoic acid may cooperate with TGFβ1 signaling in promoting CCR9 and α4β7 expression (149). Egress of plasma cells from spleen and LNs into blood involves S1PR1 (150) and a study that employed the S1PR1 modulating drug, FTY720, provided evidence that this is also the case for PPs (151).

The ligand for CCR10, CCL28 (MEC) is made by epithelium in both the small and large intestine and many LP B cells and plasma cells express CCR10 (152, 153). Although CCR10 deficiency does not affect IgA levels under homeostatic conditions, this was found to reflect increased IgA^+ cell production by ILFs (152). These compensatory IgA^+ cells carried reduced frequencies of somatic mutations in their Ig genes, consistent with a reduced contribution by cells derived from GC-bearing lymphoid tissues. The factors favoring CCR10 induction are not as well defined as for CCR9. IL21 and TGFβ may cooperate to promote CCR10 expression in human plasma cells (116). In mice, CCR10 induction occurred strongly on activated B cells in the caecal patch, and induction could be promoted by caecal patch-derived DCs (154). The mouse caecal patch has a similar organization to PPs, but cells from the caecal patch are able to travel to both the small intestine and large intestine whereas cells from the PP are less efficient at homing to the large intestine (154). Consistent with these findings, removal of the caecal patch by appendectomy reduced the ability to generate colonic but not small intestinal IgA plasma cells. Whether higher CCR10 expression could account for the difference in large intestine homing efficiency needs further investigation though it is notable that the impact of CCR10-deficiency on transferred cell homing appeared greater for large intestine than small intestine (152).

Plasma cell trophic support

Once they arrive in the LP, plasma cells are supported by multiple factors, the most studied being APRIL and IL-6. Depletion experiments have established a role for eosinophils in plasma cell maintenance (155). Eosinophils are a source of IL-1β and have multiple effects in the gut, including on the microbiome and on PP development, and may augment IgA

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responses in several ways (156, 157). The key cellular sources of APRIL in the intestine are not defined but production by epithelial cells, and production or presentation by DCs, seems likely to be important (92, 158, 159). Although some studies have suggested a role for IL-6, possibly from eosinophils, this contribution may be redundant with other factors since mice deficient for IL-6 show normal mucosal IgA levels in steady state and after cholera toxin immunization (157). Initial investigation into the kinetics of LP plasma cells in the small intestine of mice estimated their half-life to be 5 days, with a maximum persistence of 6–8 weeks (160). Those data suggested that LP plasma cells are short-lived, a finding that is in striking contrast with the half-life of BM plasma cells, which can survive over 1 year in mice (161). However, this discrepancy might be due to technical factors: for example, the radioactive labeling was performed only at an early time in the animal's life. There is evidence that early LP plasma cells are mainly derived by TI-response and they get replaced later in life with plasma cells derived by TD-response (131, 162). Thus it is possible that the short life span observed was a function of their TI origin and replacement by plasma cells arising from GC responses. In addition, at least three recent studies suggest the existence of long-lived IgA plasma cells in the gut. First, when germ-free mice are colonized in a reversible way, IgA plasma cells specific for the given commensal persisted in the gut over 100 days, even after the animals were returned to a germ-free state (163). Second, adult mice maintained a stable clonal composition of their IgA repertoire over time even after antibiotic treatment (131). Third, oral immunization with CT generates IgA plasma cells in the gut which were maintained for at least 9 months (159). Gut plasma cells are also quite radioresistant (164), but can be eliminated by treatment with proteasome inhibitors (143). Such depletion is short lasting, since the plasma cell niche is completely repopulated 7 days after treatment. While these findings begin to provide a consensus in establishing intestinal plasma cells can be long-lived, more work is needed to define the intrinsic factors influencing plasma cell lifespan and the stromal cell types acting to provide trophic support for these cells in the LP.

IgA$^+$ memory B cells

Mucosal memory B cells are generated by oral immunization and contribute to intestinal protection in humans (165, 166) and mice (167–169). Less is known about memory B cell regulation against commensal organisms. Pioneering investigation of the regulation of B cell memory was performed using a strain of *E.coli*, which allowed reversible intestinal colonization (163). When germ-free animal were reversibly colonized, an *E.coli* specific IgA response was mounted in the first 2 weeks and persisted for over 17 weeks. However, when animals with commensal flora were reversibly colonized, an *E.coli* specific IgA response was mounted in the first 2 weeks similarly to the germ-free animals, but it became attenuated over time. This result led to the speculation that intestinal IgA$^+$ memory B cells were regulated differently from the memory B cells in peripheral tissue, since the dominant bacterial species currently present could shape the IgA response. Recent data have shown that the IgA$^+$ memory B cell repertoire mirrors the intestinal IgA plasma cell repertoire and they both show similar frequencies of somatic mutations (131). The IgA repertoire of memory B cells in PPs and spleen showed clonal relationships, suggesting recirculation between these sites. Moreover, the IgA repertoire and mutation rate in lactating mammary

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glands largely mirrored that in the gut, indicating that the plasma cells in mammary glands likely arise from circulating memory B cells originating from gut immune responses (131).

IgA^+ memory B cells are characterized by high expression of IL-17Rc and IL-22Rα2, suggesting a specific ability to respond to the cytokines IL-17 and IL-22, both abundant in intestinal tissues (170). TGFβ signaling molecules of the Smad family (Smad2, Smad3 and Smad4) and the TGFβ-associated adaptor Daxx are highly expressed in IgA^+ memory B cells (170). RORα, a transcription factor acting downstream of TGFβ and IL-17 signaling, is also highly expressed and interfering with its expression affects survival and BCR expression in IgA^+ memory B cells (170). IgA^+ memory B cells express integrin α4β7 and likely also CCR9 and CCR10 (152), which contribute to their gut-tropism. Based on developmental requirements, signaling propensity, repertoire and tissue-homing potential, it can be concluded that IgA^+ memory B cells generated in PPs are distinct from memory B cell generated in other peripheral lymphoid organs. Further investigations will be needed to elucidate the mechanisms that concur to generate and maintain IgA^+ memory B cells.

Concluding Remarks

The recent advances in gut microbiome research have highlighted the deep interrelationship between host and commensals and have led to a realization that many factors work together to maintain a healthy symbiosis. That IgA is one crucial factor in this crosstalk has led to renewed interest in understanding how IgA production is induced, maintained and regulated. PPs are a nexus between host and commensal – and between host and gut pathogen – providing a compartment that ensures antigen encounter by rare antigen-specific B cells and helper T cells, and induction of antibody responses. While a lot of advances have been made in understanding the importance of PPs as sites of TI and TD B cell responses, many questions about PPs and IgA B cell biology remain to be explored, as highlighted throughout this review. The mechanisms by which antigens travel from M cells to B cells and the role of various SED DCs in this process are poorly understood. Recent work suggests that some members of the commensal population, possibly those that are more colitogenic, are more heavily coated with IgA (171). Further studies are needed to understand whether these commensals gain greater access to PPs or whether they drive stronger IgA responses by other mechanisms. Host genetic factors strongly influence the magnitude of the IgA response against commensal bacteria and this in turn impacts on the ability to resist pathogen infection (53). Much work needs to be done to decode the key genetic polymorphisms influencing the quality and magnitude of the IgA response. The central role of TGFβ in promoting IgA switching in PPs is well defined but the details of where the TGFβ signal is received and how the magnitude of this signal, and others that promote IgA switching, are regulated are only now coming to light. PPs are a site of continual GC activity and these structures function in a conventional way to support induction of high affinity antibody responses against some gut antigens, but they may also support sustained SHM in a manner that serves more to diversify the repertoire than to improve affinity. How these chronic GC responses are sustained and how such a diversification mechanism operates in the context of maintaining a stable commensal population remain outstanding questions. Finally, IgA deficiency is the most common form of human immunodeficiency (172), yet how this genetic condition impinges on the properties of the microbiome – and thus the

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health of the host – remains an open question. Reciprocally, elevated IgA levels in serum are associated with IgA nephropathy (97, 173) yet the requirements for production of serum versus mucosal IgA are incompletely defined. An increased understanding of the factors controlling IgA^+ memory and plasma cell generation and maintenance will have wide ranging implications for therapies to improve human health.

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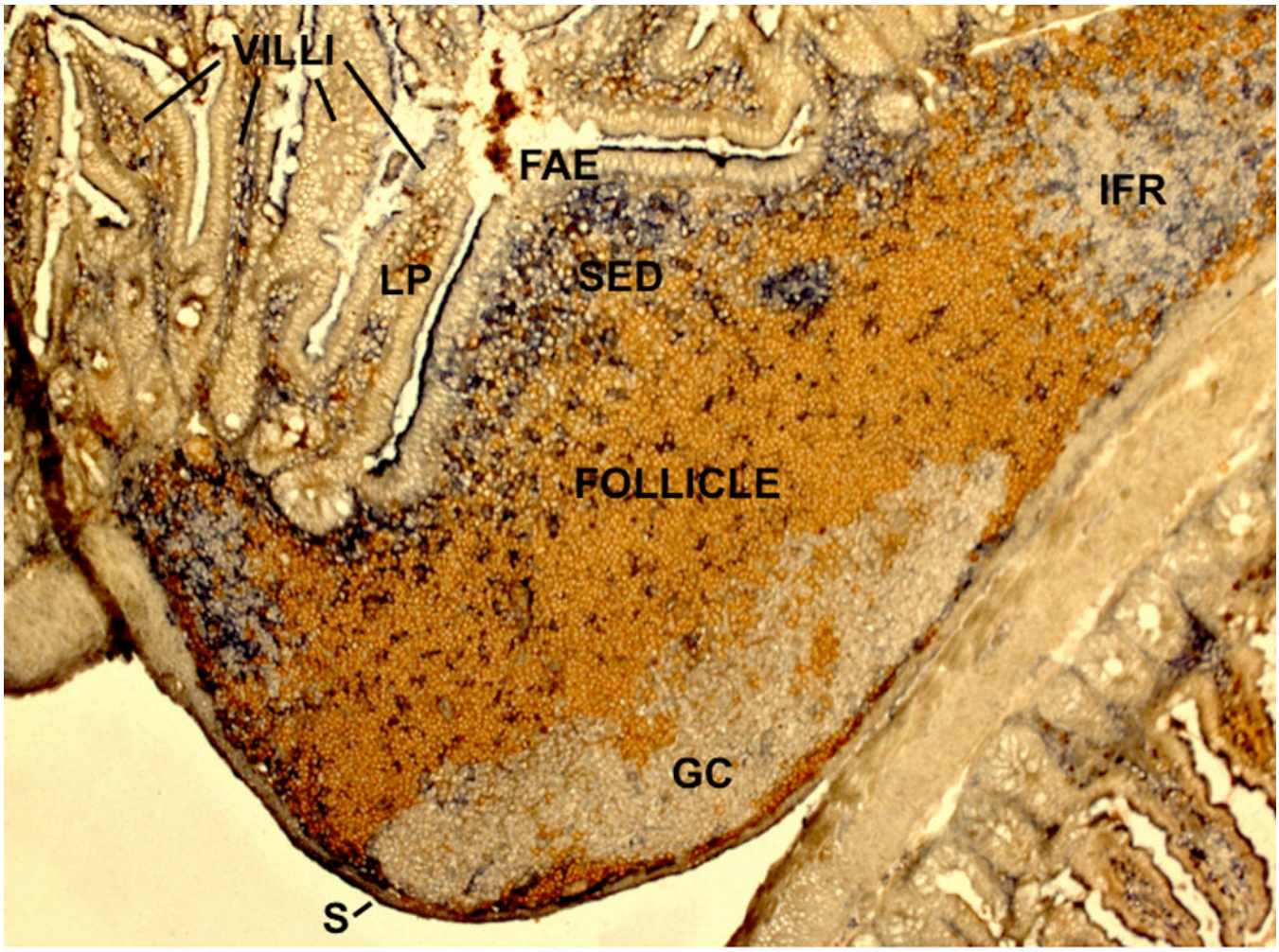


Figure 1. Cross-sectional view of mouse Peyer's patch showing main anatomical compartments

Stained to detect naïve B cells (IgD, brown) and dendritic cells (CD11c, blue). FAE, follicle associated epithelium; SED, subepithelial dome; GC, germinal center; IFR, interfollicular region (T cell zone); LP, lamina propria; S, serosa. During embedding of the tissue for preparation of the 7 μm frozen sections the PP became juxtaposed to another loop of small intestine (bottom right of the image).

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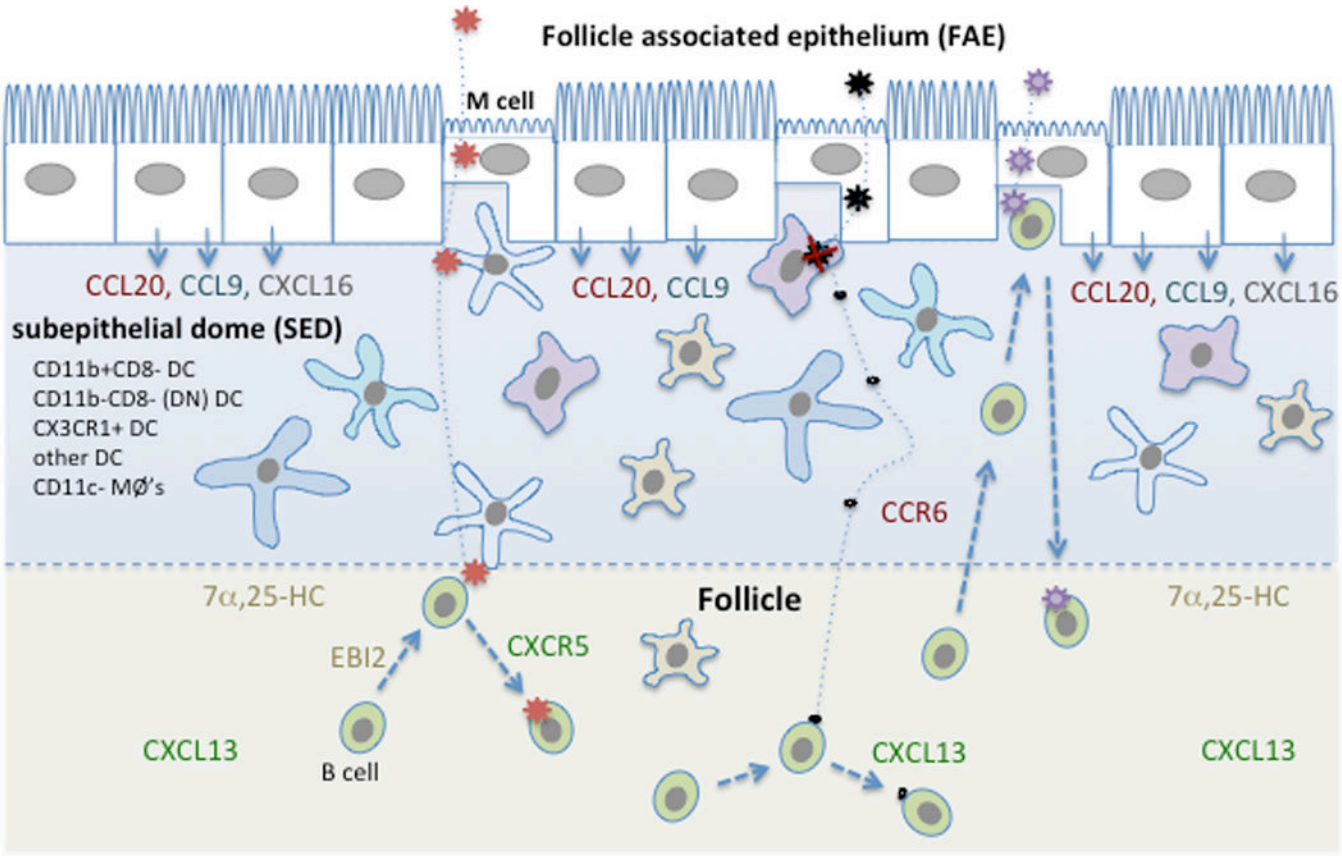


Figure 2. Diagram highlighting cellular complexity of the PP subepithelial dome (SED) and showing several possible mechanisms of B cell antigen exposure

Major myeloid cell types present in the SED are listed and represented by different cell shapes. Distribution of major chemoattractant factors is indicated. Stromal cells, which include the cells that produce the CXCR5 ligand CXCL13 and the EBI2 ligand, 7α,25-HC, are not shown. Three example antigens are shown entering the SED after M cell-mediated transcytosis. The red antigen then travels via DC to reach the interface with the follicle. B cell movement to this interface is favored by EBI2 responding to 7α,25-HC. The black antigen is taken up by a macrophage and largely degraded, but some small fragments travel away passively and reach follicular B cells. The purple antigen is encountered directly by a B cell that is suggested to have accessed the SED and M cell pocket in a CCR6-CCL20 dependent manner, likely a recently activated or memory B cell.

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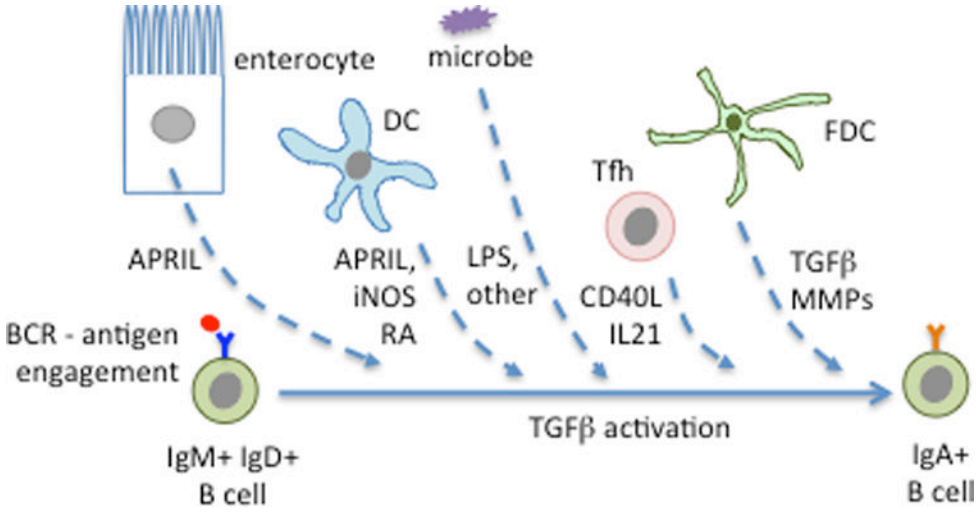


Figure 3. Factors promoting IgA switching in PPs

The highlighted factors are known to be produced by the indicated cell types but in several cases it has not yet been established which is the key cellular source of the factor and whether the action on the B cell is direct or indirect. Microbe-derived ligands for pattern recognition receptors, such as LPS, may also promote switching. See text for further details.

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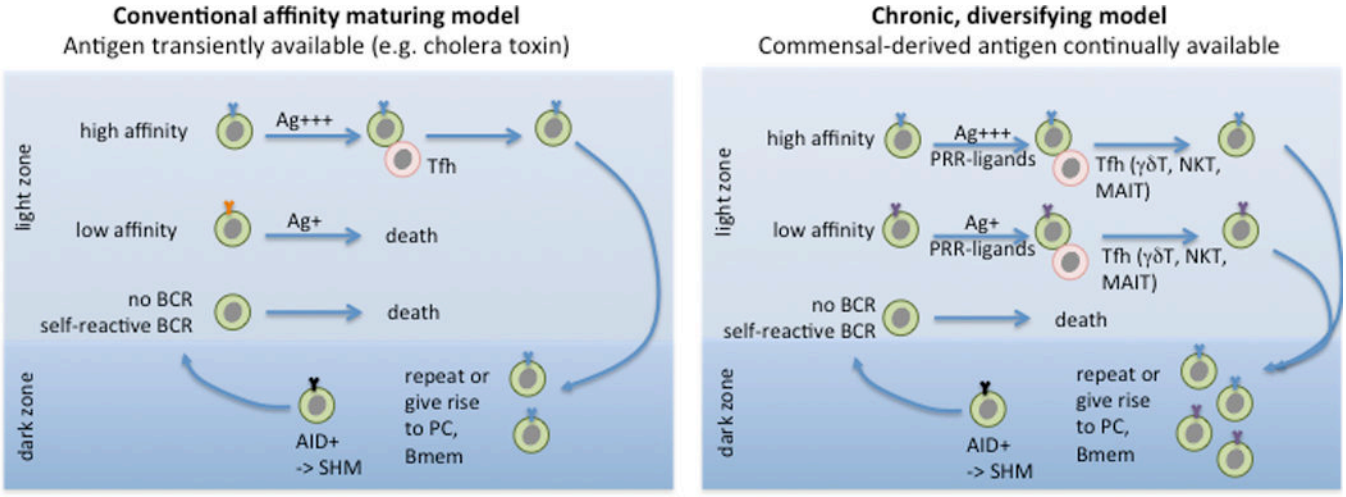


Figure 4. Two models of GC function in PPs

In the conventional model, GC B cells that acquire a high affinity BCR through AID-dependent SHM in the dark zone take up more of the transiently available antigen (Ag) than low affinity cells while in the light zone and win-out in receiving T cell help, leading to their selective survival and continuation on in the GC reaction or emergence as high affinity memory B cells (Bmem) or plasma cells (PC). In the diversifying model, there is continually available antigen, and sufficient T cell help (possibly of a range of types) to support the survival and differentiation of low and high affinity cells. Commensal-derived signals acting via pattern-recognition receptors (PRRs) likely cooperate with T cell-derived signals to support the chronic GC responses.