CHAPTER THREE

B cell responses to the gut microbiota

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Abstract

Most antibody produced by humans originates from mucosal B cell responses. The rules, mechanisms, and outcomes of this process are distinct from B cell responses to infection. Within the context of the intestine, we discuss the induction of follicular B cell responses by microbiota, the development and maintenance of mucosal antibody-secreting cells,

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and the unusual impacts of mucosal antibody on commensal bacteria. Much remains to be learned about the interplay between B cells and the microbiota, but past and present work hints at a complex, nuanced relationship that may be critical to the way the mammalian gut fosters a beneficial microbial ecosystem.

1. Introduction

Adaptive immune responses have been historically framed around the concept of pathogenesis. Invasive pathogens provoke a response that includes high-affinity antibodies that neutralize, kill, or otherwise clear the pathogen from the host. This framework generates a negative feedback loop that reduces pathogen burden, subsequently resolving the cognate adaptive immune response itself (Fig. 1A). However, and critically, most bacteria sensed by the immune system are benign. In mammals, these interactions occur most frequently in the intestine, which hosts one of the richest bacterial ecosystems on Earth. These commensal bacteria, referred to collectively as gut microbiota, are fundamental to the function of the intestine in nutrient absorption and can impact the overall health of animals. In the 1990's, it was discovered that a substantial fraction of these bacteria are coated with immunoglobulin A (IgA) in healthy mice and humans (Limburg & Mesander, 1996; Shroff, Meslin, & Cebra, 1995). IgA is the most abundantly produced antibody isotype in the human body, and the

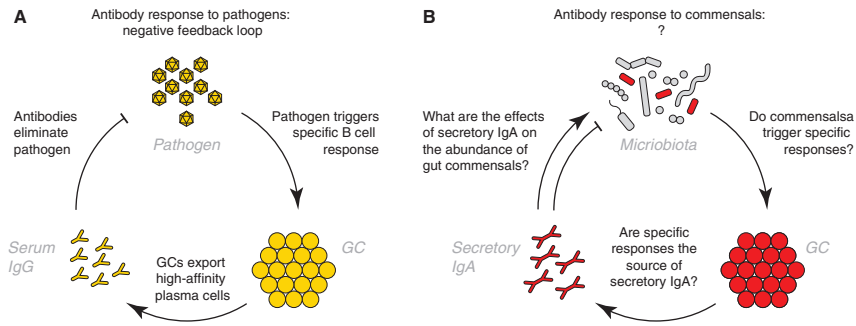

Fig. 1 Antibody feedback loops in disease and homeostasis. (A) A negative-feedback loop characterizes the cartoon version of the antibody response to pathogens. Infection generates B cell responses that involve affinity maturation in GCs, culminating in secretion of high-affinity IgG-type antibodies into serum. Serum antibody then leads to a decrease in pathogen load, which eventually contributes to the cessation of the immune response. (B) The nature of feedback loops in the antibody response to gut commensals is much less clear. Questions still remain as to whether (i) commensals induce secretion of specific, affinity-matured IgA antibodies into the gut lumen; and (ii) what the effects of these antibodies (both positive and negative) are on the abundance and biology of the commensals that triggered them.

majority is secreted into the lumen of the intestine where it can interact with the gut microbiota. However, IgA-coated bacteria are not cleared from the gut, potentially breaking the paradigm of the negative feedback loop in adaptive immunity. In fact, the effects of IgA in the gut appear to be much subtler than in the negative feedback loop paradigm for pathogens (Fig. 1B).

The generation of high-affinity antibodies typically depends on germinal centers (GCs), specialized structures containing rapidly proliferating B cells that actively mutate their immunoglobulin (*Ig*) genes using the enzyme activation-induced cytidine deaminase (AID); $CD4^+$ T follicular helper (Tfh) cells that provide proliferative signals to high-affinity B cells; and stromal follicular dendritic (FDC) cells that retain antigen on their surfaces in a form that B cells can recognize (Victora & Nussenzweig, 2022). These cellular partners operate in concert to produce a Darwinian evolutionary process that progressively increases the affinity of antibodies. Though GCs have been primarily studied in secondary lymphoid organs following immunization with model antigens, GC-like structures can form in response to more complex antigens and are frequently observed in non-lymphoid sites in autoimmunity and cancer. In contrast to model antigen-induced GCs these are often chronic, presumably due to antigen persistence. While experimentally defining these antigens is challenging, clonal expansion and somatic hypermutation are used in these settings as hallmarks of GC responses even if affinity maturation towards a defined antigen cannot be unequivocally demonstrated. This situation is mirrored by the presence of steady-state GCs at mucosal sites, particularly in the Peyer's patches and mesenteric lymph nodes of the intestine.

In this review, we discuss the differences between antibody and GC responses to pathogens and commensals and highlight emerging evidence implicating commensals in driving affinity maturation. We explore potential alternatives to the negative feedback loop of infection that may better describe the commensal-specific antibody response (Fig. 1). We also discuss the output of gut GCs, including IgA^+ plasma cells and soluble IgA, and their effects on shaping the form and function of the microbiota.

2. Commensal bacteria induce germinal centers in the gut

2.1 Evidence for germinal centers in the gut: Histology, somatic hypermutation, affinity maturation

The unusual nature of B cell responses in the gut was known prior to the discovery of IgA-coated microbiota. Peyer's patches are arranged along the small intestinal epithelium, where lymphocytes display distinct antigenic

specificities and activation thresholds adapted to the unique antigenic environment of the gut. Early studies on Peyer's patch lymphocytes in rabbits found that they had a higher activation threshold but were more responsive to exogenous antigens *in vitro* compared to those from the spleen, which the authors proposed as evidence for the gut as a differentiation site for tissue-adapted immune cells parallel to those in the secondary lymphoid organs (Henry, Faulk, Kuhn, Yoffey, & Fudenberg, 1970). This was supported by adoptive transfer experiments in which cells from Peyer's patches more efficiently repopulated the gut compared to cells from popliteal lymph nodes (Craig & Cebra, 1971). Beyond preferential tissue homing, Peyer's patch B cells were also recognized to have unique cell-intrinsic features, particularly in their preference for the IgA isotype (Cebra, George, & Schrader, 1991; Craig & Cebra, 1971). This IgA bias may be due to gut microenvironment conditioning, as B cells in *ex vivo* Peyer's patch cultures preferentially switched to IgA following immunization (Cebra et al., 1991) and retained their IgA preference even when repopulating the spleen (Craig & Cebra, 1971). The importance of these tissue-specific adaptations has been reinforced by studies of pathogenic microbes such as *Salmonella Typhimurium* and rotavirus, where germinal centers in Peyer's patches and mesenteric lymph nodes generate affinity-matured, antigen-specific IgA.

In contrast to lymphoid organs, GCs are chronically present in the gut. The presence of GC phenotype of B cells in Peyer's patches was first described in the early 80s by PNA staining of tissue sections and cell suspensions (Butcher et al., 1982). In contrast to GC B cells from lymphoid tissues, Peyer's patch GC B cells predominantly expressed IgA and, based on allelic exclusion and expression of Cα switch transcripts, these were proposed to be the primary source of IgA^+ plasma cells in the gut (Butcher et al., 1982; Cebra et al., 1991; Weinstein, Schweitzer, Cebra-Thomas, & Cebra, 1991). Light-sheet imaging of steady-state Peyer's patches in mice expressing the tdTomato fluorophore under control of an *Aicda*-driven recombinase revealed an average of five discrete GCs per Peyer's patch, located immediately below the subepithelial dome (Biram et al., 2019). Formation of these GCs was dependent on the chemokine CCR6, which is expressed on B cells as well as other immune cells and is key for organization of mucosal lymphoid tissues *via* CCL20 (Biram et al., 2019).

In addition to Peyer's patches, constitutive GCs can also be found in mesenteric lymph nodes (mLN), which span the intestinal tract and can be categorized depending on whether they drain the duodenum, jejunum, ileum, or colon (Esterházy et al., 2019). *De novo* GCs can also be observed

within the intestinal tissues, either in the form of cryptopatch-dependent isolated lymphoid follicles or tertiary lymphoid structures. Spontaneous GCs can also be observed in the isolated lymphoid follicles of mice treated with blocking antibodies to the lymphotoxin β receptor which lack Peyer's patches, and in tertiary lymphoid structures in RORγt-deficient mice, which lack both Peyer's patches and isolated lymphoid follicles (Lécuyer et al., 2014; Lindner et al., 2012).

Beyond histology, the study of gut GC function has been facilitated by the use of genetically modified mice and by advances in sequencing technologies, both of which furthered our understanding of somatic hypermutation (SHM) and clonal selection in this setting. Early studies unexpectedly found that *Rag1*$^{-/-}$ mice reconstituted with B cells from the hen egg lysozyme-specific MD-4 B cell receptor (BCR) transgenic line and T cells from the OVA-specific DO11.10 T cell receptor transgenic line could form GCs in Peyer's patches in the absence of either antigen (Bemark et al., 2000). Nevertheless, these GC B cells showed near-normal distribution and number of SHM within the V_κ region of the transgene (Bemark et al., 2000). Similar conclusions were made in a BCR knockout system in which B cells expressed the Epstein-Barr virus protein LMP2A in place of the endogenous D_H regions as a surrogate for active BCR signaling. These mice, although unable to mount prototypical GC responses following SRBC or NP-CGG immunization, nevertheless showed normal GCs in Peyer's patches and mesenteric lymph nodes (Casola et al., 2004). Sorted Peyer's patch GC B cells expressed *Aicda* and had comparable SHM levels to those from wild-type mice, demonstrating that *de facto* gut GCs can form in a BCR-independent fashion (Casola et al., 2004). A comprehensive assessment of SHM in gut GCs made use of an *in vivo* passenger allele system, in which allelically excluded passenger test sequences can be used to track SHM in the absence of B cell selection (Yeap et al., 2015). Peyer's patch GC B cells in naïve mice showed elevated SHM frequencies but similar AID hotspot profiles compared to immunized splenic GC B cells, suggestive of tissue-specific, antigen-independent SHM as a diversification mechanism (Yeap et al., 2015).

Alongside SHM, clonal expansion can be used as a surrogate for antigen-specific responses in GCs. In a comprehensive analysis of small intestinal IgA V_H sequences obtained in the absence of any immunization, high SHM burdens were common among highly expanded clones, including public clones shared between mice (Lindner et al., 2012). SHM was dependent on the presence of T cells and microbiota, and although germ-free mice showed comparable IgA diversity to that of SPF mice, SHM levels were lower even

following recolonization. Over the course of aging, SHM increased in expanded clones but was accompanied by recruitment of new, low frequency clones, which was suggested to represent two parallel mechanisms of diversification (Lindner et al., 2012). Human IgA^+ plasma cells from the lamina propria were found to exhibit high SHM loads and pronounced clonal expansion, with 4% of IgA^+ and 11% IgG^+ B cells belonging to expanded clones (Benckert et al., 2011). Expression of these BCR sequences as monoclonal antibodies revealed that, while a subset of these were polyreactive and/or self-reactive, others were able to bind commensal bacteria. Different commensals were preferentially bound by antibody isotypes, with *E. coli* limited to IgG, *E. cloacae* limited to IgA, and *M. morganii* bound by both isotypes. The vast number of gut IgA^+ sequences did not permit a comprehensive assessment of binding profiles, and as such no clonal relatedness between antibodies with specificity for the same microbe was observed nor were any correlations between SHM levels, isotype usage, and affinity or polyreactivity observed (Benckert et al., 2011). Nevertheless, antigen-specificity to commensal bacteria was observed with signs of canonical GC function.

Whether or not affinity maturation towards gut commensals indeed takes place in gut GCs has long been a topic of debate. Our assessment of steady-state gut GCs using *Brainbow* muti-color fate-mapping revealed the kinetics of clonal expansion based on color dominance following labeling, with mLN GCs progressing faster than those in Peyer's patches (Nowosad et al., 2020). This clonal selection was detectable despite the rapid turnover of B cell clones, which showed an estimated half-life of 2 weeks in SPF mice. Sequencing of single-color GCs in Brainbow fate-mapped mesenteric lymph nodes enriched for highly expanded clones with multiple B cells descending from a single SHM variant, indicative of clonal burst-style affinity maturation. In an earlier study, sequencing of full IgH and IgL variable regions of Peyer's patch GCs in SPF mice revealed public expanded clonotypes, with 10 V_H segments enriched compared to splenic naïve B cells (Chen et al., 2020). These included public clonotypes shared between mice and SHM profiles consistent with positive selection. In both studies, synthesis of Igs sequenced from expanded clones as monoclonal antibodies showed binding to fecal bacteria and/or antigens which was decreased by reversion of somatic mutations to their germline sequence, consistent with antigen-specific affinity maturation (Chen et al., 2020; Nowosad et al., 2020). These findings in the mouse were paralleled by a study of human IgA^+ and IgG^+ lamina propria plasma cells, which were found to bind commensal antigens when expressed

as monoclonal antibodies (Kabbert et al., 2020). Germline reversion abolished binding but did not render the antibodies polyreactive. In fact, germline-reverted polyreactive antibodies lost their polyreactivity, suggestive of SHM-driven affinity maturation to single or multiple commensal antigens (Kabbert et al., 2020).

Affinity maturation of GC B cells is dependent on T cell help, and the number of Tfh within a GC regulates the stringency of selection (Victora & Nussenzweig, 2022). PD-1-deficient mice showed altered gut GCs, with lower SHM, altered clonotype usage, and reduced bacterial IgA coating (Kawamoto et al., 2012). However, this effect was not found to be B cell-intrinsic; instead, these phenotypes were attributed to an increase in Tfh number leading to excess of B cell help and decreased stringency of selection. Adoptive transfer of PD-1-deficient Peyer's patch Tfh into T cell-deficient hosts resulted in increased GC size but decreased IgA^+ plasma cells in the lamina propria (Kawamoto et al., 2012). In connection with this, Tfh cells efficiently induced GC reactions in Peyer's patches and IgA synthesis in the gut (Tsuji et al., 2009). In SAP-deficient mice, which show a partial loss of Tfh function, the number of steady-state gut GCs was unaffected, although these were smaller in size (Biram et al., 2020). IgA class-switching within the GC compartment was normal, but clonal diversity, SHM, and affinity maturation (as assessed by the ratio of non-synonymous-to-synonymous mutations) were impaired. However, expanded clones showed evidence of clonal bursting (Biram et al., 2020). Alongside the observation that steady-state gut GCs can form in a BCR-independent fashion, these findings suggest that gut GC B cells may have a lower threshold for induction of SHM as a diversification mechanism.

2.2 Germinal centers following bacterial colonization

Though much of the literature has used colonization of germ-free mice to study gut GC responses to commensals, recent findings have expanded on the observation that germ-free animals contain GCs in mLNs and Peyer's patches, initially made decades ago (Pollard, 1967), to show that these display clear evidence of clonal selection. Compared to SPF mice, germ-free mice showed a skewed GC response, with elevated frequencies of GC B cells in jejunal and ileal mLNs and lower frequencies in duodenal and colonic mLNs and Peyer's patches (Nowosad et al., 2020). Furthermore, germ-free gut GCs showed an isotype bias away from IgG2a and IgA towards IgG1. Unexpectedly, Brainbow fate-mapping showed that

germ-free mesenteric lymph node GCs had accelerated clonal selection, with evidence of strong clonal bursting. The rapid expansion of these BCRs was unlikely to be random, given the recurrent, public use of V_H1–47 and V_H1–12 clonotypes and the scarcity of these in naïve B cells (Nowosad et al., 2020). Enrichment of these exact same clonotypes had previously been shown to occur in germ-free Peyer's patches in an earlier study, and estimation of the generation probability of these recurrent CDR3 sequences demonstrated that these were generated more frequently than would be expected in the naïve B cell repertoire (Chen et al., 2020). The antigenic specificity of these unusual clonotypes remains undetermined (they showed no reactivity to food, bacteria, or tissue (Nowosad et al., 2020)), precluding a strict analysis of affinity maturation. However, sequence-level analysis showed accumulation of convergent somatic mutations across multiple mice, suggestive of antigen-driven selection.

Although gut GC responses were initially investigated in the context of pathogenic bacterial or viral infection, it was soon appreciated that oral administration of commensal bacteria in germ-free mice could induce GC responses of equal magnitude. Oral immunization with the commensal *Morganella morganii* in germ-free hosts resulted in Peyer's patch enlargement and an increase in GC B cells after immunization (Logan, George, Weinstein, & Cebra, 1990). *Ex vivo* culture of Peyer's patch GC B cells produced *M. morganii*-specific IgA antibodies with affinities comparable to those of antibodies induced by reovirus infection (Logan et al., 1990; London, Rubin, & Cebra, 1987). *M. morganii* immunization also generated IgA^+ plasma cells and memory B cells following the induction of GCs, and the induction of bacterial-specific IgA coincided with the rescue of systemic bacterial spread (Shroff et al., 1995). Since these findings, this phenomenon has been reproduced with bacteria representing the most prominent phyla in the microbiome, in which monocolonization of germ-free mice induces IgA responses specific to that commensal (Yang et al., 2022). These IgA responses had sufficient breadth to cross-react to different strains of the same bacterial species, even when immunized with a more complex defined microbiome, but were dependent on $CD4^+$ T cells (Yang et al., 2022).

In a now classic study, development of a reversible colonization system using an *E. coli* auxotroph allowed for a time-resolved analysis of GC induction following monocolonization (Hapfelmeier et al., 2010). In this system, intestinal bacterial load peaked 9 h after oral gavage and was restored to germ-free levels by 72 h, resulting in the formation of GCs in Peyer's patches and mesenteric lymph nodes 2 weeks after colonization. Upon withdrawal

of bacteria, GCs declined to germ-free levels within 2 weeks, although IgA^+ plasma cells and IgA antibodies persisted for 16 weeks. However, subsequent colonization with other commensals resulted in attrition of *E. coli*-specific IgA, indicative of continuous adaptation of the B cell response to the gut microbiota (Hapfelmeier et al., 2010). In this system, the route of administration affected the magnitude and quality of the response, with oral immunization of germ-free mice generating a predominantly IgA^+ and systemic immunization generating an $IgG2b^+$ response (Li et al., 2020). B cell responses also differed in their activation threshold, CDR3 repertoire and diversification, and choice of bacterial antigens targeted (Li et al., 2020).

However, the full extent to which mucosal antibody responses depend on induced GCs remains a matter of debate, and may vary with bacterial species, diversity, and load. Intestinal IgA against *Enterobacter cloacae* could be detected in SPF and recolonized germ-free mice and were absent in mice lacking Peyer's patches or mesenteric lymph nodes, though IgA titers were found to be independent of GC organization (Macpherson et al., 2000). Oral immunization with engineered *E. cloacae* expressing a model antigen could also induce specific intestinal IgA in wild-type or T cell-deficient SPF hosts, indicating that even in the presence of a complete microbiota, novel commensal-associated antigens can induce antibody responses (Macpherson et al., 2000). However, repeated conditioning of SPF mice with live *E. cloacae* induced both mucosal and serum IgA in a T cell-dependent manner, suggesting a requirement for GCs upon either repeated exposure or dependent on the antigenic load (Macpherson & Uhr, 2004).

Not all gut bacteria are equally stimulatory, and divergent microbiomes between mouse strains account for differences in IgA class switching and production (Moon et al., 2015). These different properties can even vary within one species of bacteria, as different strains of *Bacteroides ovatus* induce varying IgA responses (Yang et al., 2020). Comparison of Peyer's patch GC responses following monocolonization of germ-free hosts with *E. coli* and segmented filamentous bacteria (SFB) showed species-specific differences in the kinetics and magnitude of GC induction (Lécuyer et al., 2014). SFB-induced GCs peaked 3 weeks after colonization, followed by induction of IgA^+ plasma cells and intestinal IgA. Despite the lower frequency of Peyer's patch GC B cells in *E. coli*-colonized mice, *E. coli* stimulated higher numbers of specific IgA^+ plasma cells. Both species induced SHM over time, with higher levels of mutations and larger clonal expansions observed with *E. coli*. However, only SFB was able to induce IgA^+ plasma cells in Peyer's patch and isolated lymphoid follicle-deficient mice, which was associated

with the formation of GC-containing tertiary lymphoid structures as a compensatory mechanism (Lécuyer et al., 2014).

The development of defined, minimal microbial consortia has allowed for a better understanding of GC induction to microbiota of graded complexities. Colonization of germ-free mice with Oligo-MM^{12}, a consortium of 12 bacterial strains representing members of all major phyla in the murine gut that can be stably transmitted across generations (Brugiroux et al., 2016), partially reverted the GC alterations seen in germ-free mice, with increased GC responses in distal Peyer's patches and increased IgG2b isotype usage (Nowosad et al., 2020). In these mice, the rate of clonal selection in mLNs was intermediate between germ-free and SPF, suggesting that selection scales with the complexity of commensal antigens. As with SPF mice, Oligo-MM^{12} mesenteric lymph nodes showed evidence of clonal bursting and affinity maturation towards bacterial antigens. The presence of public clonotypes negatively correlated with the complexity of the gut microbiota, with the germ-free-associated public V_H1–47 and V_H1–12 clonotypes rarely found in SPF mice but present consistently in Oligo-MM^{12} colonized animals, despite similar total intestinal bacterial loads in both settings (Nowosad et al., 2020). Likewise, germ-free mice gavaged with SFB or SPF microbiota, V_H1–47 and V_H1–12 usage remained enriched though these were masked by other dominant clonotypes (Chen et al., 2020). A potential explanation for this phenomenon is that the wide antigenic diversity of a full microbiota is needed to induce a B cell response that is sufficiently broad to completely outcompete the germ-free public clonotype response.

Nevertheless, it remains unclear how representative colonization of germ-free mice with commensal strains is of mice (or humans) colonized from birth. Acute colonization of adult germ-free mice with SPF microbiota by co-housing resulted in a serum IgG$^+$ response comparable in magnitude to systemic immunization, which was attributed to systemic bacteremia (Macpherson et al., 2000). Nevertheless, the offspring of monocolonized germ-free mice, which were colonized since birth, still displayed a specific IgA$^+$ response that was stable into adulthood (Yang et al., 2022). While no direct comparison was made in this study of the magnitude, specificity, and clonotype usage of the gut B cell response in the acutely colonized F0 and natally colonized F1 generations, the magnitude of gut IgA$^+$ binding was comparable (Yang et al., 2022). How representative B cell responses are in colonized germ-free mice may depend on the extent of temporary bacteremia specific to the commensal species in question, as well as the age of the host, although this has yet to be explored.

The gut microbiome is not limited to bacteria and includes an array of fungi and viruses. As with bacteria, fecal fungi are coated in IgA, and colonization of germ-free or altered Schaedler flora (ASF) mice with *Candida albicans* induces Peyer's patch GCs (Doron et al., 2021; Ost, 2021). These GC responses preceded the development of IgA^+ plasma cells and mucosal IgA antibodies, and mucosal IgA to either *C. albicans* or *C. glabrata* were significantly reduced in T cell-deficient mice (Ost, 2021). Although serum IgA antibodies to bacteriophages have been described naturally and following bacteriophage therapy, the relevance of mucosal GC responses to these remain to be fully investigated (Dedrick et al., 2021; Hodyra-Stefaniak et al., 2020).

2.3 Antigen acquisition and selection

A key factor in determining which commensal-derived antigens are seen by the humoral immune system is the macro- and micro-anatomical positioning of different bacterial species within the intestinal tract and the routes available for these antigens to reach B cells. In the steady-state gut, commensal bacteria are physically separated from underlying B cells by the surface epithelium. Thus, initiation of commensal-dependent GC responses is dependent on continuous sampling and transport of bacterial antigen to B cells. This can occur through several pathways, including leakage of antigen through the epithelium, trans-epithelial dendritic cell sampling, goblet cell-associated antigen passages, and M cell transcytosis (Knoop, Miller, & Newberry, 2013; Kulkarni & Newberry, 2019). The relative role of these different pathways in contributing to steady state B cell responses to gut bacteria awaits further study, but evidence outlined below indicates a special role for the M cell.

M cells are rare, unusual epithelial cells that are found in abundance on the dome-shaped epithelium covering Peyer's patches (often referred to as follicle-associated epithelium). They are distinguished from other intestinal epithelial cells by their disordered microvilli, recessed apical surface, and large basolateral pocket that often houses a B cell (Dillon & Lo, 2019). A unique transcytosis process is capable of moving whole bacteria from the apical to the basolateral side of the epithelium. This feature separates M cells from the other mechanisms of macromolecular transport (Kulkarni & Newberry, 2019) that may supply antigens for follicular B cell responses to microbiota. By taking up whole bacterial cells or vesicles, M cells deliver structurally-intact samples of bacteria from the lumen. Early studies found that M cells can deliver pathogens to underlying phagocytic myeloid cells

which initiate adaptive immune responses (Bye, Allan, & Trier, 1984; Fujimura, 1986). M cells are perhaps best known for their role as a portal of entry for invasive *S. enterica,* which pass through the M cell to antigen-presenting cells and then block lysosomal fusion through type three secretion system effector molecules. This "fault" of the M cell has historically overshadowed its function in the steady state immune response to gut bacteria. Multiple mouse models with impaired M cell development show dramatic delays in IgA induction and coating of microbiota (Kimura et al., 2019; Rios et al., 2015), demonstrating the importance of this route of antigen uptake. While M cells can transport any particle of appropriate size, including beads, they also express receptors that target specific bacteria. Glycoprotein 2 is an M cell-specific surface receptor that binds fimbriae-expressing species like *E. coli* to initiate the generation of antigen-specific IgA (Hase et al., 2009). The extent to which M cell preferences for specific bacteria impacts the IgA response to the microbiota is unknown. At the same time, certain bacteria are more likely to colonize the inner mucus and intestinal surface (Donaldson, Lee, & Mazmanian, 2015), making them more likely to be captured by M cells.

One pathway downstream of M cell uptake involves dendritic cells as antigen presenters. In SPF mice challenged intragastrically with *E. cloacae*, live bacteria could be recovered from mesenteric lymph nodes 60 h after immunization, but not the spleen or other lymphoid tissues (Macpherson & Uhr, 2004). These were found to reside within dendritic cells (DCs) but not macrophages, and were found using isolated intestinal loop experiments to be specifically taken up at the mucosa rather than bacterial penetration into the mLN. Furthermore, DC uptake was observed exclusively in Peyer's patches but not in the lamina propria, implicating M cells in antigen transport. Bacteria-loaded DCs could stimulate IgA production by mesenteric lymph node B cells *ex vivo*, which was further increased in co-culture with $CD4^+$ T cells (Macpherson & Uhr, 2004).

No matter the route of antigen acquisition, B cells in the gut encounter an astounding number of potential antigens from a wide range of commensal species, and the range of antigenic specificities is further diversified by the increased kinetics and turnover rate of gut GCs. Canonical affinity maturation is directed against a single antigenic epitope, but with the quantity and diversity of antigens in the gut, these may impose a selective pressure for more complex evolutionary traits, such as polyreactivity or cross-species reactivity to common microbial epitopes. Though many studies have focused on monocolonization of germ-free mice, the rate of clonal selection

scales with the complexity of the microbiota, and the extent of affinity maturation may be greater in monocolonized mice due to decreased competition between bacterial antigens. Nevertheless, we and others have demonstrated that affinity maturation in gut GCs can indeed occur in SPF mice (Chen et al., 2020; Nowosad et al., 2020), suggesting that diversification and affinity maturation are parallel processes required to ensure gut homeostasis.

3. IgA plasma cell export and maintenance in the gut

Peyer's patches were first proposed to be the potential sources of IgA-producing cells in the gut in the 1970s' (Craig & Cebra, 1971). Most evidence indicates that B cells switch from IgM to IgA prior to entering the GC reaction (Reboldi et al., 2016; Roco et al., 2019). The chronic presence of GCs in the gut suggests constant export of plasma cell precursors. Upon exiting the GC, these precursors travel through lymphatics into the bloodstream, subsequently homing to the intestine (Husband & Gowans, 1978). The rules determining plasma cell induction and replacement in the GALT have not been fully elucidated. Over 3/4 of plasma cells in the human gut lamina propria are IgA^+ (Brandtzaeg & Johansen, 2005), and this fraction increases to virtually 100% in mice (Macpherson, Hunziker, McCoy, & Lamarre, 2001). This is contrasted with the much lower prevalence of IgA^+ GC B cells in the inductive sites, in particular mLNs (Nowosad et al., 2020), implying isotype-dependent differences in plasma cell differentiation and homing to effector compartments.

3.1 IgA class-switching

After contacting antigen, mature B cells can undergo class-switch recombination, replacing the $C\mu/\delta$ gene that encodes for the IgM and IgD constant regions with those encoding any of the downstream (ɣ, ε, α) isotypes, with consequent changes of the effector function of the immunoglobulin (Yu & Lieber, 2019). Class-switching towards IgA in B cells is regulated by a multitude of factors and cell populations within the complex environment of the GALT.

IgA class-switching can occur through either T cell-dependent or T cell-independent mechanisms (Cerutti, 2008). T cell-dependent class-switching to IgA requires CD40 interaction with CD40 ligand, resulting in activation of nuclear factor κB (NF-κB) (Cerutti, 2008). Although isotype switching itself most likely occurs prior to GC formation (Lycke & Bemark, 2017), T cell-dependent IgA responses are often amplified by clonal

expansion within GC reactions. Accordingly, IgA^+ plasma cell induction also depends on PD-1 and IL-21 from Tfh cells. Tfh cells from PD-1-deficient mice are less competent in inducing IgA plasma cells and display higher IgA plasma cell turnover and an altered microbiome (Kawamoto et al., 2012). T cell-independent IgA class-switching has long been known to occur in B cells stimulated *in vitro* by TLR4-ligands and TGF-β (Coffman, Lebman, & Shrader, 1989). IgA is also induced by activation of TACI by its ligands APRIL and BAFF (Grasset et al., 2020). APRIL on its own is capable of inducing IgA class-switching in B cells *in vitro* (Castigli et al., 2004). Likewise, signaling by 25-hydroxycholesterol in gut GC B cells induces rapid IgA^+ plasma cell differentiation (Trindade et al., 2021).

Class-switching to IgA may also be aided by direct interaction between B cells and DCs. DCs have been shown to induce IgA switching in B cells *in vitro*, which was most efficient when DCs were extracted from Peyer's patches and was boosted by the addition of retinoic acid (RA), IL-5, and IL-6 (Mora et al., 2006; Sato et al., 2003). *In vivo*, DC-B cell interactions in the subepithelial dome were shown to play a role in IgA class switching through activation of TGF-β, which could be enhanced by either LTβR or retinoic acid (RA) stimulation *in vitro* (Albright et al., 2019; Reboldi et al., 2016)). In contrast, knocking down iNOS from DCs impaired their ability to induce IgA^+ B cells. The multiple pathways found to induce class-switching to IgA raise the possibility that the choice of whether to proceed *via* T-dependent or T-independent pathways may depend on the exact circumstance of antigenic exposure. How the major inductive sites (mLN and Peyer's patches) differ from each other and which types of antigens are preferentially seen by different pathways are not yet clear.

3.2 Plasma cells

Regardless of the mode of IgA induction, the majority of IgA^+ plasma cells in the lamina propria appear to be GC-derived under homeostatic conditions. Although IgA^+ plasma cells can still be found in the absence of T cell help or GCs (Bunker et al., 2015; Grasset et al., 2020), they are reduced in number. Furthermore, multiple studies have shown that in fully immunocompetent hosts, gut plasma cells carry a mutational load consistent with prior affinity maturation within the GC. A study of IgA^+ and IgM^+ plasma cells from human ileal and jejunal tissue samples concluded that the average mutation rate of V_H genes was 8.5% and 9.0% for IgM^+ and IgA^+ cells, respectively (Fischer & Küppers, 1998). This was confirmed by a study in

humans that revealed substantial SHM loads in the light chains of steady-state lamina propria plasma cells (Barone et al., 2011). Likewise, deep sequencing of IgAs from the mouse gut showed that SHM loads increased as mice aged (Lindner et al., 2012). Thus, the preserved presence of IgA^+ plasma cells in mice carrying various T cell deficiencies (Bunker et al., 2015; Kruglov et al., 2013; Macpherson et al., 2000) may indicate the presence of compensatory mechanisms that come into play in the absence of a fully-fledged T cell-dependent IgA^+ response. Still, the question remains as to why there is such redundancy and what the benefits are of GC-dependent IgA plasma cell generation in homeostatic conditions.

How much each inductive site (*e.g.*, Peyer's patches or mLN) contributes to the gut plasma cell population is also not clear. Earlier studies proposed that Peyer's patches were the predominant source of gut plasma cells, demonstrating through *Ig* sequencing that Peyer's patch GC B cells and ileal IgA^+ plasma cells were clonally related (Dunn-Walters, Isaacson, & Spencer, 1997). Outside of steady state, immunization of mLN-resected rats with cholera toxin showed increased IgA responses, suggesting that mLN are not required for specific IgA induction, and may even play a suppressive role in this process (Hahn, Thiessen, Pabst, Buettner, & Bode, 2010). In contrast, rotavirus infection has been shown to induce specific IgA responses in the mLN (Blutt, Miller, Salmon, Metzger, & Conner, 2012). Overall, the relative contribution of Peyer's patch and mLN inductive compartments to the intestinal IgA^+ plasma cell pool in homeostasis and disease remain unclear.

3.3 Migration of gut-associated plasma cells

Upon exiting Peyer's patch GCs, IgA^+ plasmablasts enter lymphatic vessels and reach the thoracic duct, through which they enter systemic blood circulation and subsequently home to either the intestine or the bone marrow. An early study in rats showed that upon cholera toxin immunization, detectable IgA^+ plasma cells appeared in thoracic duct lymph by 15 days, but were not detected in the lamina propria until 4 days later (Pierce & Gowans, 1975). These studies suggested an intrinsic ability of B cells activated in the gut to home back to the intestine, even though they must rely on a systemic pathway to find their way there. Plasmablast homing to the gut is thought to be mediated by expression of the α4β7 integrin and of the CCR9 and CCR10 chemokine receptors (Lycke & Bemark, 2017). α4β7 binds mucosal vascular address in cell adhesion molecule (MAdCAM), expressed by venular endothelial cells located mainly in the lamina propria

and other mucosal lymphoid tissues (Shyjan, Bertagnolli, Kenney, & Briskin, 1996), whereas CCR9 and CCR10 respond to ligands (CCL25 and CCL28 respectively) selectively expressed by epithelial cells of the small intestine and colon (Hieshima et al., 2004; Lindner et al., 2012; Pabst et al., 2004). Gut-derived IgA^+ plasma cells have also been shown to seed the bone marrow (Lemke et al., 2016), raising the question of whether GALT-derived IgA^+ plasmablasts "choose" between bone marrow and lamina propria homing, potentially downstream of signals delivered while their precursors are still in the GC. Previous studies have shown that, whereas IgA^+ plasma cells in the intestinal lamina propria had a mutational load that was normally distributed, those in the bone marrow showed a bimodal distribution, which could suggest selective export of distinct populations of plasma cells into each niche (Barone et al., 2011). Differences in gut-derived IgA^+ plasma cell seeding between a steady-state and mucosal infection/immunization settings also need to be clarified. More recently, gut-derived IgA^+ plasma cells have also been observed in anatomic sites other than the ones discussed above, including the central nervous system. For an in-depth review on these non-classical PC niches, see Gommerman 2021 (Isho, Florescu, Wang, & Gommerman, 2021).

3.4 Plasma cell longevity

Once IgA^+ plasma cells home the gut, how long do they stay, and what is their turnover rate? A study using carbon-14 dating of lamina propria IgA^+ plasma cell genomic DNA in humans, using the fact that atmospheric carbon-14 peaked during the cold war and then fell abruptly with nuclear test banning, estimated the median half-life of these cells at 22 years (Landsverk et al., 2017). Cholera toxin immunization and rotavirus infection experiments have been used to study the longevity of lamina propria IgA plasma cells in mice. IgA titers against rotavirus remained constant up to 14 months post-infection (McNeal & Ward, 1995). Likewise, long-lived specific IgA^+ plasma cells in the bone marrow were found at 9 months after cholera toxin immunization (Lemke et al., 2016). Survival of lamina propria plasma cells is promoted by the cytokines APRIL and IL-6. Cultures using these cytokines were able to keep non-proliferating plasma cells alive for over 4 weeks (Mesin, Niro, Thompson, Lundin, & Sollid, 2011).

Although the longevity of non-proliferative plasma cells has been clearly demonstrated, there is also evidence for a contribution of continuous memory B cell activation to maintaining steady-state IgA levels. Previous work has shown that plasma cell clones outlasted their "intended"

microbiome, given that clones were retained over time even after disruption of the microbiota by either antibiotic treatment or infection (Lindner et al., 2015). These same clones acquired more somatic mutations, indicating either continuous GC residence or re-entry of memory B cells into GCs (a pathway that is rarely used in the systemic response (Mesin et al., 2020)) as possible mechanisms. As with gut-associated GC formation, much of what is known about the cellular dynamics of gut-derived plasma cells is based on immunization and infection experiments. Development of tools allowing for plasma cell tracking in the steady-state will likely be needed if we are to better understand the dynamics of the IgA^+ plasma cell response to the microbiota.

3.5 Secretory IgA (sIgA)

IgA has several unique aspects compared to other immunoglobulins. In humans, serum IgA is present mainly as a monomer, whereas in mucosal surfaces the majority of IgA immunoglobulins are secreted by local plasma cells as dimers, consisting of two monomers linked by a joining (J-)chain. Dimeric sIgA secreted in the lamina propria is transported to the gut lumen *via* the polymeric immunoglobulin receptor (pIgR) expressed by gut epithelial cells. Ultracentrifugation experiments done in the 1960s showed that secreted IgA differed from serum dimeric IgA (South, Cooper, Wollheim, Hong, & Good, 1966; Tomasi & Zigelbaum, 1963). Further work showed that secreted mucosal IgA contained an additional "secretory component" consisting of a portion of pIgR that was be cleaved of upon transcitosis of dimeric IgA to the mucosal surface (Brandtzaeg & Prydz, 1984). The secretory component is thought to protect IgA dimers from proteolytic cleavage in the harsh environment in the mucosa. Although polymeric IgM has been shown to have similar affinity for pIgR, dimeric IgA more efficiently undergoes transcytosis across the epithelial membrane (Natvig, Johansen, Nordeng, Haraldsen, & Brandtzaeg, 1997).

In humans, the IgA Fc binds to the IgA Fc receptor (FcαRI), which is expressed on the surface of myeloid lineage cells (Aleyd, Heineke, & Egmond, 2015); mice do not express FcαRI nor do they have a homolog. Although similar to other Fc receptors with two Ig-like ectodomains, it more closely resembles the leukocyte receptor cluster and is located on the same chromosome (Wit, Morton, Capel, & Winkel, 1995). Apart from the two Ig-like ectodomains, FcαRI contains a transmembrane segment and a cytoplasmic tail. It was demonstrated that complexed IgA binds and cross-links FcαRI with higher avidity compared to monomeric and dimeric IgA

(Wines, Sardjono, Trist, Lay, & Hogarth, 2001). Signaling requires FcαRI to associate with dimerized FcR-γ chains, which carry the immunoreceptor tyrosine (ITAM) motifs needed for downstream signaling. While binding of complexed IgA induces pro-inflammatory responses such as NETosis and neutrophil migration (Aleyd et al., 2014), binding of monomeric IgA, in which case FcαRI is not crosslinked, leads to transmission of an inhibitory signal. This results in the formation of intracellular structures appropriately termed "inhibisomes", and the inhibitory signaling is referred to as ITAMi (Bakema, 2011).

The secretory component on sIgA has been shown to decrease affinity for FcαRI through steric hindrance (Spriel, Leusen, Vilé, & Winkel, 2002), revealing an elegant mechanism in which sIgA has a higher threshold for inducing an inflammatory response in the mucosa. This suggests three layers of protection with varying thresholds for inducing a FcαRI-mediated inflammatory response. The sIgA in the lumen has low affinity for FcαRI even when bound to bacteria. Dimeric IgA in the lamina propria can complex with bacteria that break the mucosal barrier and induce a pro-inflammatory response. Monomeric IgA in the serum provides the "last-resort" scenario, where bacteria in circulation are opsonized and eliminated (Bakema, 2011). This sequence is compatible with a negative feedback loop, suggesting IgA's function as one of maintenance; in the steady state, with an intact mucosal barrier, sIgA complexed with bacteria in the lumen does not induce inflammation readily, and unbound monomeric serum IgA is anti-inflammatory.

4. The effect of IgA on the microbiota

IgA binding to commensal bacteria impacts their physiology and colonization capabilities in a variety of ways. This is further complicated by the diversity of animal gut microbiomes, each of which contain hundreds of species of bacteria. Effects of antibody binding vary between bacterial species, likely due to divergent microbial lifestyles. Even the distinction between pathogen and commensal is often blurred in the intestine, and the relationship between IgA responses and pathogenic "potential" is unclear. Whereas the relationship of each type of bacteria with IgA may be as unique as each species, common threads have emerged from the combination of systems-level and reductionist approaches to the study of IgA function in the gut.

Critically, IgA antibodies appear not to be particularly harmful to the normal gut bacteria. This is established by the observation that a substantial portion of the bacterial cells (30–50%) in the feces of healthy humans are

coated with IgA (Limburg & Mesander, 1996). At the same time, gnotobiotic mouse models showed that gut bacteria can establish persistent colonization of the intestine despite invoking a humoral immune response (Shroff et al., 1995). It was therefore suggested that the role of mucosal IgA binding to commensal bacteria was to confine them to the lumen of the gut and prevent invasion of the animal's tissues. Indeed, there is evidence for a barrier function of IgA, but this appears to not be the entire story. One mouse study has indicated that IgA reduces the concentration of microbial metabolites found in other tissues during steady state (Uchimura et al., 2018). In inflammatory conditions where barrier function becomes paramount, pIgR-deficient mice exhibited worse colitis, whereas IgA-deficient mice only mild weight loss, indicating a role for free secretory component or compensatory IgM in maintaining the barrier (Murthy, Dubose, Banas, Coalson, & Arulanandam, 2006). In some cases, IgA may impose the classic negative pressure (Fig. 1A) on bacteria with pathogenic potential, as treatment with a monoclonal IgA that binds proteobacteria improved outcomes in a colitis models (Okai et al., 2016). However, beyond this barrier function, IgA also has a more nuanced role in broadly shaping the microbiota through mechanisms that are not entirely understood (Kubinak & Round, 2016). We discuss some of these roles below.

4.1 The role of specificity

IgA binds all types of gut bacteria, but certain species are more frequently coated in IgA at the cellular level, as assessed by fluorescence-activated cell sorting followed by 16S ribosomal RNA amplicon sequencing. Bacteria coated at a higher frequency in humans with inflammatory bowel disease (Palm et al., 2014) or malnutrition (Kau et al., 2015) can exacerbate disease models when transferred to gnotobiotic mice. However, bacteria more frequently coated in IgA in healthy individuals had the opposite effect, protecting mice from disease (Kau et al., 2015). These frequently coated species also tend to be more abundant in mucus (Kubinak et al., 2015), making them more likely to interact with the host and be bound by IgA by proximity. It is important to note that the sort and sequence method only measures enrichment or de-enrichment of taxa after sorting, so results are dependent on the relative abundance of each individual taxa (Jackson et al., 2021), making interpretation difficult. A detailed explanation of the technical biases and limitations of the sort and sequencing methods has been laid out in a recent review (Box 1 of reference (Huus, Petersen, & Finlay, 2021)).

Polyreactive IgA (Bunker et al., 2017; Shimoda, Inoue, Azuma, & Kanno, 1999) and nonspecific binding mediated by glycans decorating the heavy chain and secretory component (Huus et al., 2020; Mathias & Corthésy, 2011) provide additional ways by which IgA can bind bacteria independently of antigen specificity. In the case of *Bacteroides* species, initial study of IgA from humans binding to isolates from their own gut showed species-specific reactivity to capsular polysaccharides (Zitomersky, Coyne, & Comstock, 2011). Gnotobiotic mouse studies have lent further support to the notion that IgA binds *Bacteroides* in an antigen-specific fashion that distinguishes not just different species but different strains (Donaldson et al., 2018; Porter, Canales, Peterson, & Martens, 2017; Yang et al., 2022). Thus, whereas specificity is not always accounted for in the literature on IgA function, the bulk of the evidence suggests that germinal center-derived, affinity matured B cell responses towards defined antigens are important for the effects of IgA on the microbiota.

4.2 IgA deficiency

One way to understand the impact of IgA on the microbiota is to look at cases of selective IgA deficiency, the most common primary human immunodeficiency. This is clinically defined as having low (20-fold lower than average) serum levels of IgA with normal levels of IgG and IgM. A subset of patients with undetectable IgA may have distinct clinical outcomes from those with low IgA (Moll et al., 2021), which is not accounted for in the epidemiological literature. Nonetheless, selective IgA deficiency is associated with an increased risk of certain mucosal infections and inflammatory diseases (Singh, Chang, & Gershwin, 2014; Yazdani, Azizi, Abolhassani, & Aghamohammadi, 2017; Yel, 2010). Whereas IgA is typically measured in serum, deficiency in this case usually correlates with a defect in sIgA as well. Frequently, levels of secretory IgM are substantially increased, but not to the normal levels of sIgA in the lumen of the gut. The corresponding increase in coating of commensal bacteria with IgM does not fully recapitulate either the binding capacity or specificity of IgA (Catanzaro et al., 2019). Several studies have shown that IgA deficiency is associated with reduced microbial diversity (Catanzaro et al., 2019; Fadlallah et al., 2018; Jørgensen et al., 2019), which has also been observed in cases of combined variable immunodeficiency (Jørgensen et al., 2019). The spatial organization of the microbiota may also be disrupted in IgA deficiency (Fadlallah, Kafsi, et al., 2018). If the role of IgA is primarily that of a barrier or a negative selective pressure, as required for a

negative-feedback loop to form (Fig. 1A), it is counterintuitive for a lack of IgA to result in a decrease in diversity. Furthermore, while IgA-deficient patients do have increased systemic IgG responses to commensals (Fadlallah et al., 2018), there is no evidence that commensal invasion causes pathology in these patients. Collectively, studies of IgA deficiency in humans indicate that the primary function of IgA is in interacting with commensal microbes in a manner that is more nuanced than negative selection.

Various mouse models of IgA deficiency or secretory antibody deficiency largely support the general observations made in humans. An isotype-specific knockout of the Cα heavy chain was generated more than 20 years ago, which prevents B cells from class-switching to IgA (Harriman et al., 1999). These mice have elevated levels of IgM in both serum and feces, recapitulating what is observed in humans (Catanzaro et al., 2019). AID-deficient mice, which have defects in both class-switch recombination and SHM, were the first model used to assess impacts on the microbiota (Fagarasan et al., 2002). As an alternative model, deletion of pIgR has been used to prevent secretion of IgA and IgM into mucosal surfaces. However, the secretory component of pIgR appears to have functions independent of antibody (Phalipon & Corthésy, 2003). Another model in which mice are mice deficient in the J-chain has similar caveats, since these mice have alterations in memory B cells and dendritic cells that express J-chain for uncharacterized reasons (Källberg & Leanderson, 2006, 2008). Nonetheless, these various mouse models generally agree that IgA has broad effects in shaping the microbiota (Fagarasan et al., 2002; Kawamoto et al., 2012; Kubinak et al., 2015; Shulzhenko et al., 2011; Suzuki et al., 2004) and promoting diversity within the community (Gopalakrishna et al., 2019; Kawamoto et al., 2014; Nagashima et al., 2017). Effects on diversity may not always be revealed in mice due to their limited environmental exposure to microbes in the laboratory.

A limited number of studies have isolated specific effects of IgA deficiency, including increases in proteobacteria and SFB and decreases in the family Bacteroidaceae (Mirpuri et al., 2014; Suzuki et al., 2004). In some cases, the effects even on individual species are complex. In the absence of IgA, SFB loses its spatial confinement to the distal small intestine and spreads to the proximal small intestine. Interestingly, SFB levels in its normal habitat (the terminal ileum) decrease concomitantly (Suzuki et al., 2004). In addition to deficiency, both mice and humans exhibit massive variation in concentrations of sIgA in the gut. Although this has not been studied directly in humans, sIgA variability contributes to shaping the microbiota in the case

of mice (Moon et al., 2015). Different genetic strains of mice also vary in their capacity to produce IgA. Balb/c mice inherently produce much more IgA than C57BL/6 mice and harbor a more diverse microbiome with less *Akkermansia muciniphila* and more *Lactobacillus* (Fransen et al., 2015). How exactly IgA variability and deficiency lead to these microbiome changes is a non-trivial question.

4.3 Mechanistic functions of IgA

Many of the classic mechanisms of antibody function either do not apply to, or are altered in the context of sIgA interactions with commensal bacteria. The lethal edge of IgG is missing as IgA is poor at both complement fixation and opsonization for phagocytosis. Complement and phagocytosis may be relevant in cases of barrier dysfunction when commensals invade tissue, but this is likely mediated by other signals as well. In the case of pathogenesis, antibodies also function through neutralization of toxins or other virulence factors involved in host-interfacing or motility. IgA may be involved in suppressing flagellum-mediated motility by gut bacteria (Cullender et al., 2013), hypha formation by fungi (Doron et al., 2021; Ost, 2021), and other behaviors that are not beneficial to the host. However, the most abundant gut microbes such as species of *Bacteroides* and *Clostridia* appear to be non-motile and do not form other structures that are suppressed by IgA coating. Very little is known about whether commensal gut bacteria have receptors that could bind epithelial cells without causing pathology. IgA neutralization may play a role in regulating such behavior, but more research is required into commensal colonization of the epithelial surface.

Aggregation is a classic effect of antibody that is more likely to be relevant to IgA function in the gut. The dimeric nature of sIgA provides greater aggregation potential. Indeed, IgA binding causes aggregation *in vitro* of *Lactobacillus* (Huus et al., 2020) and *in vivo* of *Salmonella enterica* (Moor et al., 2017) and *Bacteroides fragilis* (Donaldson et al., 2018). Seemingly paradoxically, this effect is associated with a negative effect on colonization by the pathogen *S. enterica* and a positive effect on the commensal *B. fragilis*. This is likely due to differences in the lifestyle of these two microbes. *Salmonella enterica* is a motile pathogen of the intestine that adheres to and invades the epithelium. To do so it must move through the mucus (an environment in which it would not be able to compete in the long term with the commensal microbes) to reach the epithelial surface. By aggregating the bacteria and enchaining daughter cells, IgA may prevent such flagellum-mediated

migration as well as block type III secretion and other virulence mechanisms related to invasion of the epithelial cells (Moor et al., 2017). In contrast, *B. fragilis* is a commensal that thrives within the mucus (Donaldson et al., 2020). By reinforcing microcolonies within the mucus and even on the epithelial surface, the aggregating function may relate to the ability of IgA to anchor *B. fragilis* in its favored spatial niche in the proximal colon (Donaldson et al., 2018). Unconventional functions of antibody, including this anchoring role, are discussed below.

Reductionist studies have revealed that IgA binding somehow regulates the gene expression program and behavior of bacteria in the gut. Daniel Peterson pioneered the investigation of a defined IgA reactivity specific for a commensal gut bacterium, *Bacteroides thetaiotaomicron* (Peterson et al., 2015; Peterson, McNulty, Guruge, & Gordon, 2007). Using genetics in the microbe, the antigen target was identified as one of several capsular polysaccharides that *B. thetaiotaomicron* can dynamically switch on and off to differentially decorate the surface of the cell. Binding by the antibody not only suppressed the expression of its own capsular epitope, but also caused global changes to bacterial gene expression, including regulating the response to oxidative stress (Peterson et al., 2007). The effect of anti-capsule IgA regulating capsular polysaccharide expression was later reproduced in a polyclonal system (Porter et al., 2017). IgA also targets sugar utilization systems of *B. thetaiotoamicron*, regulating microbial metabolic behavior *in vivo* (Joglekar et al., 2019; Nakajima et al., 2018). The mechanism for these IgA-induced gene expression changes remains unknown. A recent study has shown that IgA with varying specificities can impact *E. coli* physiology in specific ways by modulating nutrient intake, bacteriophage receptor availability, bile acid sensitivity, and swimming motility (Rollenske et al., 2021). Plausibly, IgA may have similar impacts on the physiology of *B. thetaiotaomicron*, and the gene expression changes observed reflect feedback mechanisms within the bacterial cell. As in these specific examples, general IgA binding to both protein and sugar antigens appears common, though the relative roles of these different stimuli on inducing follicular B cell responses is unclear.

Given the concentration of IgA and frequency with which intestinal microbes are bound, IgA may be thought of as a normal component of the surface of commensal bacteria. The abundance of IgA is staggering, as milligram quantities are secreted in the human intestine daily. Consequently, bacteria are coated in as many as tens of thousands of molecules of sIgA (Tsuruta et al., 2009). This is within the range of a medium to high abundance outer membrane protein in bacteria (Smith, 1992).

Many commensal species have no other habitat outside of the animal gut; therefore host factors, immune or otherwise, are part of their normal environment. Accordingly, *Bacteroides* species decorate their capsular polysaccharide surfaces using glycans derived from host mucus (Coyne, Reinap, Lee, & Comstock, 2005) and bacteria grown in laboratory culture display a comparatively thin capsule (Donaldson et al., 2018). In this context, IgA may be considered another part of the host-adapted surface of normal gut bacteria. The composition of the protein and sugar surface of a bacterium can broadly impact physiology, and host-derived components of the bacterial surface represent an underappreciated aspect.

Because of its constant production and transport across the epithelial surface, IgA is also a substantial component of the mucus layer that covers the intestine. Specialized epithelial cells called goblet cells secrete mucins: highly glycosylated, linear proteins that dramatically expand to form a gel material in the presence of water. This mucus layer provides an important biophysical barrier to pathogens as well as a habitat for commensal bacteria (Donaldson et al., 2015). A study in the early 1990's first revealed that IgA mediates the interaction between mucus and oral pathogens (Biesbrock, Reddy, & Levine, 1991). As mucus is constantly sloughed off the epithelium and replaced with newly secreted mucins, trapping of pathogens in mucus provides a mechanism of protecting the host tissue. The intestinal mucus contains high relative concentrations of IgA (Rogier, Frantz, Bruno, & Kaetzel, 2014), which may be retained *via* glycan-glycan interactions between mucins and secretory component (Phalipon et al., 2002). Just as bacteria use the complex polysaccharides decorating mucins as carbon and energy sources, plausibly they may take advantage of the similar glycans on the secretory component and the heavy chain of IgA. Interestingly, the types of bacteria that are coated in IgA with a higher frequency are also found in the mucus at a higher frequency (Kubinak et al., 2015; Palm et al., 2014). The direction of causality is unclear: bacteria may be more coated in IgA because they live and grow in mucus. Alternatively, those mucosal bacteria may be the ones that induce specific B cell responses.

The relationship between IgA and mucus inspired the hypothesis that antibody could serve as an anchor for commensal adherence. The idea of a "pro-microbial" function for IgA was first hypothesized nearly 20 years ago (Bollinger et al., 2003) and demonstrated *in vitro* using tissue cultured epithelial cells and *Escherichia coli* (Bollinger et al., 2003). Similar proof-of-principle *in vitro* studies replicated this effect with *Lactobacillus* and *Bifidobacterium* (Huus et al., 2020; Mathias et al., 2010). This anchoring

function might explain the curious observation that the adherent pathogen *Helicobacter pylori* colonizes mice better in the presence of IgA (Akhiani, Stensson, & Schon, 2005). A similar story emerged for another pathogen, *Acinetobacter baumannii* (Ketter et al., 2018). For commensal bacteria, evidence for anchoring function has been seen *in vivo* for several species of *Lactobacillus* (Perruzza et al., 2019) and for *Bacteroides fragilis* (Donaldson et al., 2018). In the case of *B. fragilis*, IgA was shown to increase mucosal colonization, epithelial microcolony formation, and reinforce the stable colonization of an animal by a single strain of the species (Donaldson et al., 2018). Conceptual support for the anchoring theory has also emerged from computational modeling suggesting that anchoring provides stronger ecosystem-level control than negative selection (McLoughlin, Schluter, Rakoff-Nahoum, Smith, & Foster, 2016).

4.4 Closing the loop

The functional impacts of IgA on commensal microbes do not conform to the typical negative feedback loop of B cell responses to pathogens (Fig. 1). When a germinal center reaction to a pathogen results in a neutralizing antibody, pathogen load is decreased, creating a negative feedback loop that limits the adaptive immune response. For commensals, the antibody response does not typically decrease bacterial load. The pro-microbial function of anchoring commensals could in fact create a feed-forward loop. And even without a pro-microbial function, IgA coating may increase uptake by M cells (Mantis et al., 2002; Rol, Favre, Benyacoub, & Corthésy, 2012) which deliver antigen to the local immune follicles such as Peyer's patches in the small intestine. How such feed-forward loops function in homeostasis is unclear, since a strict positive feedback loop would lead to indefinite expansion of any bacterial species capable of inducing growth-promoting antibodies towards itself (Fig. 1B). However, if less strict, this type of interplay between B lymphocytes and the commensal microbiota may strengthen symbiosis through the constancy of immune sensing and response. Most commensal bacteria are incapable of invasion or survival in host tissues; therefore their physical habitat is constrained by the anatomy of the gut. Within the intestine, the diversity of the mammalian microbiota ensures robust nutrient niche competition. Only those bacteria capable of exploiting an available spatial and nutrient niche can stably colonize an animal's gut. Therefore, these ecological factors likely play a greater role in shaping the identity of the microbiota and limiting the growth of individual

species. As such, a positive feedback loop between gut bacteria and B cells would not lead to indefinite increase of a population but rather to stabilization of the ecosystem.

Regardless of the particular effects of IgA on individual species of gut microbes, in a broader sense IgA may maintain a homeostatic relationship between host and microbiota. Defining such homeostasis in the context of the microbiome has proven difficult. At least three models of what a "healthy microbiome" looks like have been proposed based on (i) presence/absence of specific microbes; (ii) spatial organization; and (iii) ecological diversity/stability. The simplest idea of a healthy microbiome is one that is simply devoid of pathogens, which is reflected in the broadly-accepted usage of "specific pathogen-free" laboratory mice as a standard for microbiome quality control. However, there is little evidence that IgA prevents carriage of pathogens or pathobionts (commensals during homeostasis that are also opportunistic pathogens). Plausibly, IgA may be involved in the retention of beneficial microbes, though it is not easy to characterize any particular species as inherently "beneficial" in all contexts. IgA also has a role to play in the spatial organization of the microbiota, which may be an important aspect of homeostasis. With widespread use of 16S rRNA amplicon sequencing to profile microbiomes, the diversity and/or stability of the community has also been associated with homeostasis or health. From the known functions of IgA, it is reasonable to suggest it may have an impact on these ecological dynamics, though longitudinal studies of IgA deficient patients are required to test this in humans.

Why certain species of bacteria are more often coated in IgA remains a debated question. Rather than label different bacterial species found in the gut as pathogens or commensals, one of the pioneers of functional microbiome studies, Rene Dubos, described the microbiota of animals in terms of their ecological relationship with the gut (Dubos, Schaedler, Costello, & Hoet, 1965). The autochthonous microbiota are native to the gut, having stably colonized their host. But at any given time, there are also allochthonous microbes which are transient, originating from food or from upstream regions of the digestive tract. Commensal bacteria that are adherent or able to colonize mucus have an advantage in stable colonization (Donaldson et al., 2015, 2018, 2020; Lee et al., 2013). Coupling this idea with the observation that mucosal bacteria are more likely to be coated in IgA (Kubinak et al., 2015; Palm et al., 2014) suggests the hypothesis that IgA may more frequently coat the autochthonous microbiota. Indeed, a recent study used defined communities in gnotobiotic mice and found that IgA has specificity for the "self" gut

microbiota (Yang et al., 2022). This pattern emerges despite the capacity of the IgA repertoire to bind with broad specificity. In the case of *B. fragilis* anchoring by IgA, this function protects the pioneering strain in an animal and excludes invasive strains (Donaldson et al., 2018). This may underlie the remarkable observation that most people are colonized by a single, dominant strain of *B. fragilis* (Scholz et al., 2016; Yassour et al., 2016) and supports the hypothesis that IgA binds the autochthonous microbiota to the benefit of these microbes. In this sense, IgA binding can be imagined as an "ID card" for gut bacteria: granting access to privileged niches while ensuring that the immune system can track and restrict their behavior.

5. Conclusion

The study of the gut microbiota is not new. Almost two centuries before Darwin's "Origin of Species," Antonie van Leeuwenhoek turned his microscope toward his own body and discovered that we do not live alone. His letters describe a diverse oral and gut microbiome during health, which appeared to change during disease. However, it would turn out that solving problems related to disease, such as with antibiotics, proved a much more tractable endeavor than understanding the complex ecosystem living in our intestines that is integral to our nutrition. In the context of association with animals, bacteria have been largely studied as pathogens, and the field of immunology reflects the historical bias within microbiology. Immunomodulation by commensal species is sometimes viewed as a curiosity or exception, despite the fact that commensal interactions with immune cells are far more common than those of pathogens.

GCs in the mammalian gut are chronic, representing a constant response to the microbiome. IgA-secreting plasma cells are local to the tissue and surprisingly long-lived, providing the largest scale antibody-making enterprise in our bodies. Consistent with this, individual strains of commensal gut bacteria can persist within one human host for decades. The B cell response to the gut microbiota described here is a relatively poorly-understood but major function of the adaptive immune system in recognizing commensal bacteria for what they are: our allies.

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