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IgA B CELL RESPONSES TO GUT MUCOSAL ANTIGENS: DO WE KNOW IT ALL?

Topic Editor
Nils Y. Lycke

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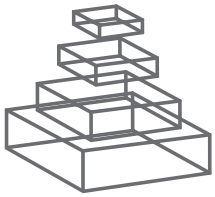

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IgA B CELL RESPONSES TO GUT MUCOSAL ANTIGENS: DO WE KNOW IT ALL?

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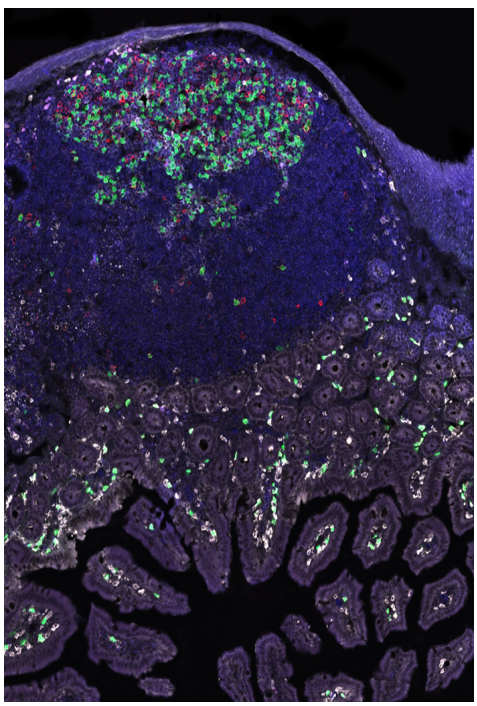

Peyer's patch from a wildtype mouse after transfer of naive NP-specific GFP$^+$ B cells and oral immunizations with NP-hapten conjugated to cholera toxin (NP-CT). Gut IgA plasma cells (white) B cells with B220 (blue), NP-specific plasma cell,s GFP (green) and germinal center B cells, GL7 (red)

Despite its central role, IgA antibody formation in the gut immune system is still incompletely understood. In humans, several grams of IgA are produced each day, and this together with mucins and other soluble components constitutes the first line of defense of the gut mucosal barrier. Plasma cells, at the effector site in the mucosal membrane, form IgA dimers, which are actively transported through the epithelium into the gut lumen as secretory IgA. New IgA plasma cells are constantly generated in the gut through class switch recombination (CSR) of antigen-activated naïve IgM B cells, a process that has been found to greatly depend on gut microbiota. Most IgA CSR in the gut occurs in the gut associated lymphoid tissues (GALT), which consist of Peyer's patches (PP), intestinal lymphoid follicles (ILF) and mesenteric lymph nodes (MLN). In addition, colon patches, apart from ILFs, have been ascribed an inductive role for mucosal IgA responses following intra-rectal immunizations. An alternative, but quite controversial, site for IgA CSR is the non-organized lamina propria (LP) of primarily the large intestine. Evidence in support of IgA CSR in the LP is still scarce and several investigators have recently failed to confirm earlier observations.

Two main developmental pathways have been described for the generation of IgA plasma cells in the gut LP; a T-cell dependent (TD) and a T-cell independent (TI) pathway. Much interest has focused on the development of IgA antibodies against the microflora of the gut. To what extent the small and large intestine reflect the same or different IgA responses has been poorly studied. Considering that the microbiota is hosted mainly in the large intestine, it is surprising that IgA plasma cells are more abundant in the small intestinal LP. Furthermore, strong oral immunogens like cholera toxin are well known to stimulate not only gut mucosal IgA , but also very substantial serum IgG responses. To what extent these responses emanate from the same inductive sites or not, has been poorly investigated.

This Research Topic aims at bringing together recent research that can shed light on some basic principles of how IgA B cell responses are induced and regulated. In particular, we hope to achieve a better concensus as to the role of different inductive sites that have been proposed to be involved in gut IgA B cell responses against the gut microbiota (TI) as opposed to those that are engaged in responding to food antigens and vaccines (TD). Are they the same or different? Does IgA class switch recombination and somatic hypermutation in the GALT require germinal centers and can these be shared by B cells activated by bacteria as well as food or vaccine antigens?

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EDITORIAL
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IgA B cell responses to gut mucosal antigens: do we know it all?

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Keywords: IgA, intestinal, germinal centers, Peyer's patches, gut lamina propria

In this Research Topic of Frontiers in Mucosal Immunology we have focused on gut IgA B cell responses. We have invited world leading researchers in the field to contribute with articles that give the most recent up-date on our current understanding of this interesting and complex protective system. Because a majority of all human pathogens access the body via the mucosal membranes we need an effective innate and adaptive immune system to protect against infectious diseases at these sites. Secretory IgA (SIgA) antibody formation is a hallmark of this protective barrier function (1). However, it does not only play a role for protection against pathogens, it also serves as a key element in establishing stable homeostasis between the host and the microbiota of the mucosal membranes (2). For example, the gut IgA system develops after weaning in early life and critically influences the symbiosis between the host and the commensal bacteria (3, 4). Recent data suggest that each individual accumulates highly mutated gut IgA gene sequences over time and that the development of long-term memory B cells is a cardinal feature of mucosal IgA immunity (5). This appears to apply not only to B cells responding to T cell-dependent (TD) antigens, but, in fact, may also apply to B cells responding to T cell-independent (TI) antigens derived from the microbiota (6). To explain the high level of mutations in gut IgA gene sequences in adults it has been proposed that the IgA B cell inductive sites, primarily the Peyer's patches (PP), play a major role in allowing extensive cell division and, hence, promoting multiple mutations (7). Since mutations are acquired in germinal centers and these are formed as a consequence of T-B cell cooperation following antigen stimulation it has become increasingly important to understand the fine detail of how germinal centers in the PP are regulated. A prime question is whether germinal centers in PP also could host B cells that respond to TI-antigens, which could then promote cell division and allow for the accumulation of multiple mutations in differentiating B cells. Recent findings suggest that activated gut B cells can re-utilize already established germinal centers in PP and in this way gut SIgA responses can be synchronized and oligoclonal, securing a high quality of the protective SIgA antibodies in the lamina propria (8). Based on these observations the function of follicular helper T cells (T_{FH}) in PP then appear to be quite unique compared to that found in peripheral lymph nodes. In fact, failure to develop appropriate T_{FH} (PD-1 deficiency) in PP have been found to drastically affect the composition of the microbiota, arguing in favor of that IgA B cell differentiation directly impacts on gut homeostasis and the presence of certain bacterial species (9). Mice that lack the ability to mutate IgA B cell responses have been shown to host bacterial overgrowth in the gut and have a dysfunctional mucosal barrier in the absence of IgA affinity maturation (10). Importantly, erroneous activation of the mucosal immune system can result in chronic inflammation and development of autoimmunity or allergy. Thus, non-responsiveness or immune tolerance, is critical and a special feature of the regulatory mechanisms that prevail at mucosal immune inductive sites (11). Hence the mucosal immune system must maintain a finely tuned balance between tolerance and responsiveness to mucosal antigens, including TI-antigens derived from the microbiota. As already discussed, this does not mean that the gut SIgA system is ignorant about the microbiota, but rather the contrary, that there is a balance in the adaptive immune response to establish homeostasis and avoid inflammatory reactions.

A series of review articles, which broadly address the different aspects of SIgA and how gut intestinal B cell responses are generated and regulated, is presented in this Research Topic. The first article by Knoop and Newberry gives an overview of the gut mucosal IgA-inductive sites and discusses the role of the various inductive tissues; PP, isolated lymphoid follicles (ILF), and colon patches for normal SIgA homeostasis and what happens in immune dysfunction. The second review paper by Kunisawa and Kiyono focuses on the PP as inductive site for SIgA responses and describes how commensal bacteria can stimulate SIgA, which then can promote increased up-take of bacteria into the PP tissue and also how this mechanism can establish intra-PP habitation of *Alcaligenes* bacteria, i.e., what are the consequences of having an indigenous commensal in PP. Slack, Balmer, Fritz, and Hapfelmeier then describes in the third review the functional flexibility of intestinal IgA formation that is also key to understanding how symbiosis with the microbiota can be maintained in the gut. This is also a theme entertained by Corthésy as he describes the multi-faceted functions of SIgA in the fourth review paper. Special attention is given to the innate properties of SIgA as an anti-inflammatory regulator, showing that not only Fab-specific antigen-recognition contributes to gut homeostasis and protection against pathogens, but also that SIgA exerts an effect on epithelial cell integrity and the mucosal barrier functions. The fifth review paper in this Research Topic explores the different findings of enhanced IgA plasma cell activity in gut lamina propria of patients with celiac disease, the prototypic gut inflammatory condition that results from a dysfunctional immune response to dietary gluten. Especially, Mesin, Sollid, and Di Niro describes

long-term plasma cell production of autoantibodies in the lamina propria against transglutaminase 2, which may constitute as many as 4–24% of the total IgA plasma cell population in celiac lesions. Spencer, Klavinskis, and Fraser continuous on this line of human immunobiology of the SIgA system and discuss burning questions relating to how to understand its organization and regulation in health and disease. In particular, comparisons are made between what we know about human SIgA immune responses and the information we have gained from experimental rodent models. The next review article by Lycke and Bemark addresses how we can explain the fact that SIgA immune responses to TD-antigens in the gut are oligoclonal and appear to be synchronized along the entire small and large intestine. The role of PP in this context is highlighted and regulatory aspects of PP germinal centers are presented in a challenging theory. Finally, the contribution by Conner and Blutt describes various functional aspects of gut SIgA responses against gastrointestinal virus infections and discusses what we can do to harness the protective properties of SIgA to achieve better mucosal vaccines.

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Isolated lymphoid follicles are dynamic reservoirs for the induction of intestinal IgA

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IgA is one of the most important molecules in the regulation of intestinal homeostasis. Peyer's patches have been traditionally recognized as sites for the induction of intestinal IgA responses, however more recent studies demonstrate that isolated lymphoid follicles (ILFs) can perform this function as well. ILF development is dynamic, changing in response to the luminal microbial burden, suggesting that ILFs play an important role providing an expandable reservoir of compensatory IgA inductive sites. However, in situations of immune dysfunction, ILFs can over-develop in response to uncontrollable enteric flora, resulting in ILF hyperplasia. The ability of ILFs to expand and respond to help control the enteric flora makes this dynamic reservoir an important arm of IgA inductive sites in intestinal immunity.

Keywords: isolated lymphoid follicles, IgA, intestine, gut-associated lymphoid tissue

INTRODUCTION

IgA is the most abundantly produced antibody, representing 70% of antibody production (Macpherson and Uhr, 2004; Macpherson et al., 2008). The majority of IgA is secreted at mucosal surfaces, and plays a major role in immune homeostasis by protecting these surfaces from bacterial or viral attack. Accordingly, there is significant interest in how and where IgA production is initiated and how this defense mechanism can be enhanced to meet ongoing challenges. In this review we will focus on the role of isolated lymphoid follicles (ILFs) as sites initiating IgA production, highlighting the dynamic nature of ILFs IgA production and the role of environmental stimuli in this process.

MUCOSAL LYMPHOID TISSUES IN THE GASTROINTESTINAL TRACT AS SITES FOR THE INITIATION OF IgA PRODUCTION

It has long been appreciated that the gastrointestinal tract contains well developed lymphoid tissues with a unique environment specialized for IgA production (Fagarasan, 2006; Macpherson, 2006). Instead of mounting a strong systemic Th1, Th2, or IgG response, a role of these lymphoid tissues is to promote homeostatic responses against luminal antigens by inducing IgA, which is secreted into the lumen. Within the mucosal lymphoid tissues of the intestinal tract, made up of Peyer's patches (PPs), ILFs, and Colonic Patches, TGFβ1 is crucial for IgA production and expressed by several cell types promoting the majority of gut B cells to differentiate into IgA producing plasma cells (Coffman et al., 1989). Mucosal lymphoid tissue dendritic cells (DCs) express the TNF superfamily members a proliferation inducing ligand (APRIL) and B cell activation factor of the TNF family (BAFF) which promote IgA production in B cells expressing the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and the B cell maturation antigen (BCMA), the receptors for APRIL and BAFF (Fink and Frøkiær, 2008; Massacand et al., 2008; Tsuji et al., 2008). T cells found near the B cells follicles in the intestinal tract express yet another TNF superfamily member, CD40L, which promotes class-switch recombination (CSR) in B cells (Fagarasan et al., 2010). In normal SPF housed mice, the continual induction of IgA producing B cells is evident by the presence of $GL7^+$ germinal centers within PP follicles. Activation-induced cytidine deaminase (AID), necessary for both CSR and somatic hypermutation (SHM), is also present in PP B cells. Currently it is thought PPs utilize mostly T cell dependent routes for IgA production as germinal center formation in PPs is dependent on both CD40–CD40L signaling from T cells (Bergqvist et al., 2006) and the presence of T cells (Tsuji et al., 2008). However mice lacking CD40, CD40L, or T cells still have near normal levels of IgA. This IgA may arise from B-1 B cells and have lower specificity and altered functionality. Still, it is evident there are many compensatory mechanisms for IgA production in the intestine.

The best recognized of the IgA inducing lymphoid tissues are PPs, which are scattered along the anti-mesenteric border throughout the length of the small intestine (Schuurman et al., 1994). The murine colon contains a PP equivalents located in the cecum, or the cecal patch (Owen et al., 1991), and multi-follicle structures throughout the colon, or colonic patches (O'Leary and Sweeney, 1986). Depending on the strain of mouse, there are usually 7–10 PPs in the small intestine, each with multiple follicles containing naïve B-2 B cells (Husband and Gowans, 1978). PPs also contain $CD4^+$ $TCR\beta^+$ T cells in between the follicles in T cell zones and different classes of CD11c+ DCs (Iwasaki and Kelsall, 2000). PPs sample luminal antigens through specialized antigen transporting cells known as Microfold cells, or M cells (Gebert et al., 1996). Follicular DCs (FDCs), within the B-cell follicles present the antigen in a T cell dependent manner to B cells, which undergo activation and class-switch from IgM to IgA (Garside et al., 2004).

More recently it was appreciated that the small intestine and colon contained an additional type of lymphoid tissue, the solitary intestinal lymphoid tissues (SILT; Hamada et al., 2002; Lorenz et al., 2003; Pabst et al., 2006). In contrast to PPs, which remain fully developed throughout life, SILT are a spectrum of lymphoid

tissues ranging from nascent cryptopatches (CPs) to fully developed mature ILFs (mILFs; Lorenz et al., 2003; Pabst et al., 2006). CPs, aggregates of approximately 1000 cells, are composed of RORγt$^+$ lymphoid tissue inducer (LTi) cells and lymphoid tissue organizer (LTo) cells found around the crypts of the small intestine (Kanamori et al., 1996). By secreting lymphocyte attracting chemokines, CPs are able to develop into ILFs in response to signals originating from the commensal enteric flora (Pabst et al., 2006). Small immature ILFs contain a mixture of T cells and B cells and through further recruitment of B cells develop into mILFs, which have an overlying follicle-associated epithelium (FAE) containing M cells and B cell follicles with germinal centers (Lorenz et al., 2003). Approximately 1000 SILT are scattered throughout the murine small intestine, and in most situations CPs greatly outnumber ILFs. Current evidence shows that *de novo* development of CPs does not continually occur throughout adulthood (Velaga et al., 2009): as needed, a CP may develop further into an ILF (Taylor et al., 2004), a process that does continue throughout adulthood. In mice aged 2 years, all classes of SILT except CPs, were increased (McDonald et al., 2011), indicating the continual development of ILFs as a site for IgA induction when needed.

Isolated lymphoid follicles were described relatively recently in humans (Moghaddami et al., 1998) and in mice (Hamada et al., 2002) as a small intestinal villi containing a single B cell follicle, but were immediately suggested to be a compensatory mechanism for humoral responses, able to support IgA responses. Similar to PPs, ILFs contain mostly CD19$^+$ B-2 B cells that have yet to undergo CSR (Lorenz and Newberry, 2004). CD4$^+$TCRβ$^+$ T cells and MHCII$^+$ CD11c$^+$ antigen presenting cells make up approximately 25% of the ILFs (Hamada et al., 2002; Lorenz and Newberry, 2004). M cells are found on the FAE of the ILFs, indicating antigen introduction to the follicle also occurs via M cells (Lorenz et al., 2003).

The observation that mice lacking PPs could still produce and secrete IgA in response to luminal antigen led many to believe IgA CSR could occur outside of organized lymphoid tissues (Hamilton et al., 1981; Yamamoto et al., 2000). However following the identification of ILFs, these observations could be reinterpreted to support a role for ILFs in IgA production.

ILFs ARE A DYNAMIC RESERVOIR OF IgA INDUCTIVE SITES

Though CPs and ILFs require many of the same molecules necessary for PP development, signals from the diet and the enteric flora are required for the development of ILFs, but not PPs. PPs are embryonically imprinted, developing around day 14 of gestation, while SILT is only present postnatally, appearing in the first few weeks of life (Kanamori et al., 1996; McDonald et al., 2010). The initial aggregation of LTi cells in both PPs and CPs require lymphotoxin signaling as neither PPs nor any SILT are found in LTα$^{-/-}$ or LTβR$^{-/-}$ mice (DeTogni et al., 1994; Taylor et al., 2004). However recent work has shown a requirement for the expression of the aryl hydrocarbon receptor (AHR) in the differentiation of LTi cells postnatally, but not during embryogenesis (Kiss et al., 2011; Lee et al., 2011). Knocking out AHR, expressed on RORγt$^+$ LTi cells, unexpectedly resulted in the absence of any CPs or ILFs, while the lymph nodes and PPs were undisturbed (Lee et al., 2011). Keeping mice on diets free of AHR ligands reproduced the lack of CPs and ILFs, while mice fed a diet including indole-3-carbinol, an AHR ligand found in *Brassicaceae* plants (such as broccoli or cauliflower) developed normal numbers of intestinal lymphoid aggregates (Kiss et al., 2011). Thus diet initiates one of the early steps in ILF development revealing an unappreciated role for nutrition in IgA induction (**Figure 1**).

After the initial development of a CP, the constituent cells secrete chemokines to recruit lymphocytes to the aggregate. As future IgA induction sites, the recruitment of B cells to the SILT is an important step in the development from a CP to an ILF. LTo cells/stromal cells and CD11c$^+$ cells within CPs and/or ILFs express CXCL13, a B cell attractant (Gunn et al., 1998; Ansel et al., 2000; Honda et al., 2001; Cupedo et al., 2004; van de Pavert et al., 2009; McDonald et al., 2010). Knocking out the CXCL13 or its receptor CXCR5 results in normal numbers of CPs, but an absence of mILFs (Velaga et al., 2009; McDonald et al., 2010). Alternatively, SILT can be pushed from CPs to ILFs by increasing the local concentration of CXCL13 through transgenic expression by gut epithelial cells (Marchesi et al., 2009), resulting in an increase in ILFs, but not the absolute number of intestinal aggregates.

Mature ILFs could form as a result of the expansion of a small number of B cells present within the SILT and/or due to the recruitment of B cells from the systemic pool. Work with the AID$^{-/-}$ mice, which have an expanded ILFs compartment, suggested B cells within SILT expand concurrently with ILF development (Fagarasan et al., 2002). However, analysis of individual ILFs from wild type mice demonstrated the ILFs contained a population of polyclonal B cells which reflected the systemic B cell pool (Wang et al., 2006). Further work with naïve mice revealed diverse variable heavy chain usage in ILFs, again reflecting the systemic population of B cells (McDonald et al., 2011). While evidence exists to support both pathways, recruitment of systemic polyclonal B cell population would give ILFs a greater potential to respond to a wide range of antigens.

Along with changes to the cellular components of the ILFs, changes outside the lymphoid structures must occur during the development of the ILFs. The eventual mILF will have moved from the crypt into a villus, displacing the original lamina propria cells. The epithelium overlying the ILF must change from the villus epithelium to FAE, which lacks goblet cells and contains M cells (Lorenz and Newberry, 2004). While this process is not well understood, the cytokine receptor activator of NF-κB ligand (RANKL) appears to be a strong candidate promoting this transition. RANKL is a TNF super family cytokine that is expressed to varying degrees in all GALT (Taylor et al., 2007). RANKL can be found on the stromal cells throughout CPs, but in PPs and ILFs, RANKL staining is confined to the subepithelial dome, the area right beneath the FAE (Taylor et al., 2007). Though initial development of CPs is not dependent on RANKL, there is a significant decrease of CPs in RANKL$^{-/-}$ mice (Knoop et al., 2011). Furthermore RANKL$^{-/-}$ mice fail to develop ILFs due to a lack of CXCL13 expression within CPs (Knoop et al., 2011). As the ILF develops, RANKL expression becomes restricted to the subepithelial dome inducing the development of M cells and FAE from what was once villus epithelium (Knoop et al., 2009) demonstrating multiple roles for RANKL in the development of GALT.

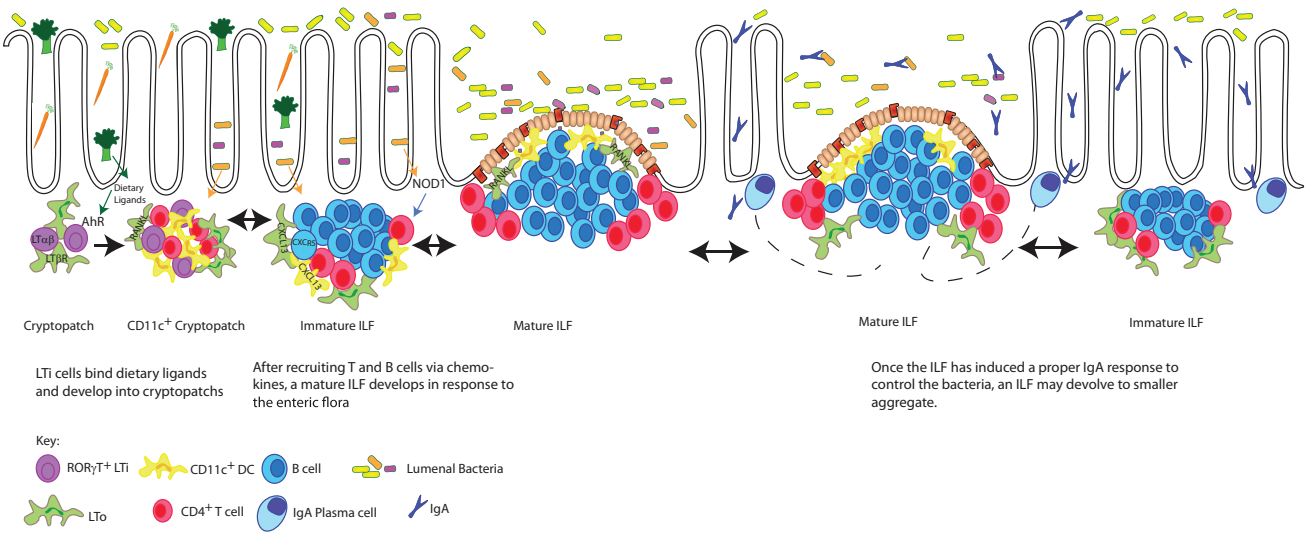

FIGURE 1 | Dietary ligands drive the development of CPs, which expand into mature ILFs in response to bacterial stimuli. The development of CPs requires dietary signals delivered through the aryl hydrocarbon receptor (AHR) expressed by RORγt+ LTi cells. LTi cells in turn express lymphotoxin (LTα1β2) and transmit a signal to lymphotoxin beta receptor (LTβR) expressing LTo (stromal) cells inducing the production of a chemokines resulting in cryptopatch formation. In response to further lymphotoxin signals and enteric flora, chemokines, including CXCL13, recruit lymphocytes to transform CPs into immature ILFs and subsequently mature ILFs. The transition of an immature ILF to a mature ILF is dependent upon further lymphotoxin and RANKL signals driving the development of germinal centers and the development of an FAE. Mature ILFs promote naïve B cells to become IgA plasmablasts responding to luminal antigens delivered via the FAE. These plasmablasts are believed to migrate to the lamina propria by yet undescribed mechanisms to become plasma cells producing IgA specific for luminal antigens and by extension regulating the enteric flora. When challenges from the enteric flora are controlled, mature ILFs can regress to their precursor stages. The pathways presented in this figure are restricted to those discussed in this review.

The transition of CP to mILFs can be driven by changes in the enteric flora. While CPs, immature ILFs, and PPs are found in germfree mice (Kanamori et al., 1996), mILFs are rare and increase in response to conventionalization with normal flora (Lorenz et al., 2003). One mechanism appears to be bacterial stimulation of NOD1, an intracellular pattern recognition receptor found in the epithelium, which has been shown to lead to ILF development (Bouskra et al., 2008). Thus two key signals for ILF development are enteric flora and diet, neither of which are required for the development of PPs. It seems likely the purpose of SILT is to adapt to the ever-changing enteric flora and diet of an individual, producing IgA in response to new environmental cues (**Figure 1**).

Evidence of the adaptability of SILT can be seen in mouse models where PP development is blocked by disrupting lymphotoxin signaling during the embryonic stage; development of PPs is completely blocked and cannot be rescued during adulthood (Rennert et al., 1996; Lorenz et al., 2003). In this model, ILF development still occurs unperturbed throughout adulthood. Furthermore ILF development is increased 10-fold, expanding in response to the lack of PPs (Lorenz et al., 2003). The plasticity of ILF development, seen in the ability of ILFs to expand as needed, aptly compensates for PPs, which are fixed in number and position.

ILF's ROLE IN IgA PRODUCTION

There is little doubt that ILFs are able to induce IgA responses. Early studies on ILFs showed the B cells within the follicles are mostly $B220^+$ $CD19^+CD23^+IgM^{low}IgD^{high}$ cells and relatively few are IgA^+ (Hamada et al., 2002), representing a population of B cells poised to undergo activation, CSR, and SHM. Most work showing ILFs contribute to IgA production was completed in mouse models, which lack PPs, yet are still able to develop ILFs. Indeed, $LT\alpha^{-/-}$ mice, which lack any organized lymphoid structures, including PPs and ILFs, make only a small amount of IgA, presumably by B-1 B cells (Thurnheer et al., 2003). $LT\alpha^{-/-}$ mice reconstituted with C57Bl/6 bone marrow develop ILFs, which restore IgA production in the intestine (Lorenz and Newberry, 2004; Hashizume et al., 2007). Similar results were seen with $ROR\gamma t^{-/-}$ mice, which lack PPs and ILFs: when reconstituted with $ROR\gamma t^+$ bone marrow ILFs develop and IgA production becomes restored (Tsuji et al., 2008).

LTβR blockade by the injection LTβR-Ig fusion protein on or before day 17 of gestation prevents PP development (Rennert et al., 1996) and has been used as a model to infer the role of MLNs in IgA production (Yamamoto et al., 2000). Following *in utero* LTβR blockade, these mice produce normal levels of IgA and make specific IgA against OVA and cholera toxin (Yamamoto et al., 2000). While these studies attributed this IgA production to the MLN, subsequent work revealed that gestational LTβR-Ig treatment resulted in enhanced ILFs development (Lorenz et al., 2003), and in retrospect ILFs could also contribute to the IgA production in these mice.

To be a useful compensatory mechanism for IgA, an ILF should induce high-affinity IgA specific against antigen epitopes. The specificity of IgA produced in ILFs was called into question when specific IgA was not seen in ILFs against tetanus toxoid following immunization (Hashizume et al., 2008). However other

studies provide evidence for ILF generated antigen-specific IgA in response to multiple antigens. Upon oral immunization with the T cell dependent antigen sheep red blood cells, ILFs produced antigen-specific IgA at levels equivalent to PPs (Lorenz and Newberry, 2004). Moreover mice possessing mILFs, but lacking PPs and other secondary lymphoid tissues were found to have antigen-specific IgA in their feces following infection with *Salmonella typhimurium* (Lorenz and Newberry, 2004). Furthermore, analysis of the B cell repertoire in ILFs demonstrated ILFs contain a pool of B cells similar to the repertoire seen in PPs and spleen (McDonald et al., 2011). Collectively, the literature suggests ILFs have the potential to respond to a wide array of antigens and mount specific responses against enteric bacteria.

Some findings suggest that while PPs are the major source of T cell dependent responses, ILFs contribute a compensatory mechanism by housing T cell independent responses (Tsuji et al., 2008). A T cell independent mechanism for IgA production has been described in TCR$\beta^{-/-}\delta^{-/-}$mice, which produce IgA specific for commensal bacteria, and can respond to changes in the gut flora (Macpherson et al., 2000). The T cell independent pathway for IgA has been shown to be dependent on APRIL and suggested to be quite important for the production of IgA specific for commensal bacteria (Castigli et al., 2004). Further work with the TCR$\beta^{-/-}\delta^{-/-}$ mice suggested ILFs, but not PP, can house T cell independent IgA responses (Tsuji et al., 2008). PPs from these mice formed defective GC and failed to activate AID, while ILFs contained AID^{+} B cells undergoing CSR to IgA (Tsuji et al., 2008).

During T cell dependent IgA production, B cells become activated by the presentation of antigen from FDCs and by CD40 signals from T cells (MacLennan, 1994). This leads to upregulation of AID, which is responsible for both SHM and CSR (Longerich et al., 2006). TGFβ1, expressed by several cell types in the gut, promotes CSR to IgA (Fagarasan et al., 2010). In the absence of T cells, DCs from ILFs, but not PPs appear to be sufficient for IgA production from B cells (Fagarasan et al., 2010). The DCs are thought to use APRIL, BAFF, and TGFβ1 to promote IgA production. Interestingly, it was found RORγt^{+} LTi cells are indispensible for the T cell independent production of IgA in this model (Tsuji et al., 2008). The combination of LTi cells, DCs, and naïve B cells are rarely found in the LP outside of ILFs, suggesting this model of T cell independent IgA production can only be induced in organized lymphoid tissue.

Other mouse models indicate that in addition to ILFs, PPs can support T cell independent means of IgA induction. CD40$^{-/-}$ mice were shown to have only rare IgA CSR events in small intestine PPs, though AID was activated (Bergqvist et al., 2006). CD40$^{-/-}$ mice still maintained high levels of IgA, and it was later shown IgA CSR can occur in PPs, ILFs, and colonic patches (Bergqvist et al., 2010). This work concluded though CSR was found in some ILFs, the majority of IgA CSR in the absence of T cells occurs in the PPs. The role for T cells seems to be more important for SHM as the IgA in CD40$^{-/-}$ mice showed few mutations in the variable regions (Bergqvist et al., 2010).

Technology has moved past merely looking for the presence or absence of specific IgA using ELISAs. Now with the genetic analysis options available, the ability to track specific clones of B cells and closely watch the variation in the IgA repertoire during infections can better answer how the majority of IgA is produced in the gut (Spencer et al., 2009). Indeed such genetic analysis of the clonal relationship between B cells and IgA Plasma cells is already being incorporated into reports (Bergqvist et al., 2010) better showing when SHM and CSR has occurred. Not only will this help clarify the field, but also help focus mucosal vaccine work in the attempts to target better IgA responses for protection against gut pathogens.

ILF HYPERPLASIA AS A SIGN OF IMMUNE DYSFUNCTION

As described in the above sections, multiple studies support that ILFs develop in response to changes or imbalances in the luminal microbial community and function to promote IgA production. Furthermore IgA produced at mucosal surfaces can in turn alter or control the luminal microbial community returning it to homeostasis (Cerutti and Rescigno, 2008). Together these observations suggest that ineffective IgA production would be unable to control the luminal microbiota and result in expansion of ILFs, and thus prolonged ILF hyperplasia could be viewed as a sign of IgA dysfunction.

Though ILF hyperplasia has been known to occur in humans for some time (Webster, 1973), the first mouse model that included ILF hyperplasia was the AID$^{-/-}$ mice (Fagarasan et al., 2002). Since AID is required for both CSR and SHM, these mice can only produce low-affinity IgM. In the absence of high-affinity IgA, the enteric flora quickly becomes altered, with a 100-fold expansion in anaerobic bacteria (Fagarasan et al., 2002). Anaerobes are notable for their opportunistic tendencies to quickly expand without proper IgA control (Suzuki et al., 2004; Ohashi et al., 2010). Intriguingly, AID$^{-/-}$ mice develop massive ILF hyperplasia in response to the increases in the enteric flora (Fagarasan et al., 2002). Similarly, mice with a mutated form of AID that allows for CSR but not SHM still have expanded flora due to low-affinity IgA that is unable to control bacterial growth (Wei et al., 2011) and with the expanded flora, these mice also develop ILF hyperplasia.

The ability for ILFs to rescue an inferior IgA response was seen in recent studies investigating chemokine receptor 10 (CCR10) expression by plasma cells (Hu et al., 2011). IgA^{+} plasma cells are unable to migrate to the intestine in CCR10-KO/EGFP-knock-in mice, yet the mice maintain normal levels of fecal IgA and have no significant increase in luminal flora due to an increase in IgA producing plasma cells in ILFs. While evidence to support that a large population of IgA producing plasma cells resides in the ILFs in the setting of CCR10 sufficiency is lacking and the migration and fate of ILF generated IgA+ plasmablasts is largely unknown, it is clear in this model that ILF development is increased in response to the commensals, and that IgA is induced within ILFs to control the enteric flora. Furthermore, the number of ILFs decreased upon antibiotic treatment (Hu et al., 2011), illustrating how ILF may devolve once they are no longer needed.

Immune dysfunction does not have to be caused by a genetic mutation; immunosenescence in aged individuals is seen as a dysfunction in protective immunity and can affect gastrointestinal immunity as well as systemic immunity (Schmucker and Daniels, 1986; Schmucker et al., 1996). Mice aged 2 years show many signs of immune dysfunction in the gut (McDonald et al., 2011). There is a threefold increase in all classes of ILFs and these ILFs contain an aberrant cellular population with decreased numbers of B cells and

increased numbers of T cells, specifically $CD4^+$ $CD8\alpha\alpha^+$ T cells (McDonald et al., 2011). Along with the greatly elevated amounts of IgA, purportedly from the ILFs, these signs all point to immune dysfunction. Since aged rats have been shown to have increased numbers of anaerobic bacteria (Maczulak et al., 1989). A similar expansion could occur in aged mice when immune dysfunction prevents proper control of the bacteria and drive the development of the ILFs in aged mice.

Clinically an increase of ILFs in the small intestine has long been seen as a symptom of immune dysfunction (Bastlein et al., 1988). In children, a common endoscopic finding is the large number of tiny lymphoid nodules located in the terminal ileum and colon referred to as lymphonodular hyperplasia (LNH), or nodular lymphoid hyperplasia (Laufer and deSa, 1978). It was first described as an age-related finding in children of minimal significance, but further work noted a close association between LNH and the development of food allergies. It was shown that children with LNH were two to three times more likely to have non-IgE mediated allergies to food such as milk or cereal (Kokkonen et al., 1999) including high levels of IgG and IgA against milk proteins (Kokkonen et al., 2002), increased numbers of γδ T cells associated with the lymphoid follicles (Kokkonen et al., 2000), and increased CD4+ T cells with decreased T_{h1} cytokines (Bellanti et al., 2003). LNH was not found to be associated with Crohn's disease or NOD2/CARD15 mutations raising speculation that LNH is not related to inflammatory disease, but may be a response to the microbial environment (Shaoul et al., 2006) as children with LNH showed a significant increase in number of bacteria adherent to the intestinal mucosa layer (Conte et al., 2006). Collectively, LNH appears not to be the cause of the food allergies, but rather a symptom of an expanded flora during immune dysfunction.

Lymphoid hyperplasia can also be found in adults, though more rarely than in children (Misra et al., 1998; Carroccio et al., 2009). It is also found alongside food allergies in adults (Krauss et al., 2010) but is generally associated with genetic immune disorders such as common variable immune deficiency (CVID; Webster, 1973; Scharenberg et al., 1993). CVID patients have reduced ability to undergo SHM and as a result have low concentrations of IgG and IgA. LHN develops in about 20% of CVID (Bastlein et al., 1988) and those patients have increased intestinal IgM compared to CVID patients without LHN, consistent with ILFs contributing to the antibody response in these individuals (Webster et al., 1977). Treatment is rarely recommended to stop the development of the follicles, differentiating these lymphoid follicles from more problematic follicles that develop in other intestinal inflammatory diseases. The LHN associated with CVID likely develops in a manner similar to the ILF hyperplasia seen in the $AID^{-/-}$ mice; the lymphoid follicles develop not because of the CVID but due to continual signals from the expanded microflora able to grow uncontrollably without the reins of IgA. Until the flora is controlled, usually by means of antibiotic treatment, the follicles will continue to develop in an attempt to fight the perceived threat to the intestines.

CONCLUSION

In this review we have illustrated how ILFs develop in response to diet and commensals and how ILFs can compensate for inadequate IgA responses. Though their relative contribution to IgA production has yet to be enumerated compared to PPs, it is clear that induction of IgA responses is a function of an ILF. Further work still needs to examine the specific antigenic targets of the IgA that comes from ILFs, but one would expect most of the targets come from the enteric flora since the development of ILFs is highly responsive to the billions to bacteria that reside in the gut lumen.

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Alcaligenes is commensal bacteria habituating in the gut-associated lymphoid tissue for the regulation of intestinal IgA responses

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Secretory-immunoglobulin A (S-IgA) plays an important role in immunological defense in the intestine. It has been known for a long time that microbial stimulation is required for the development and maintenance of intestinal IgA production. Recent advances in genomic technology have made it possible to detect uncultivable commensal bacteria in the intestine and identify key bacteria in the regulation of innate and acquired mucosal immune responses. In this review, we focus on the immunological function of Peyer's patches (PPs), a major gut-associated lymphoid tissue, in the induction of intestinal IgA responses and the unique immunological interaction of PPs with commensal bacteria, especially *Alcaligenes*, a unique indigenous bacteria habituating inside PPs.

Keywords: Peyer's patch, IgA, commensal bacteria

INTRODUCTION

Secretory-immunoglobulin A (S-IgA) is predominantly observed in the intestine where it participates in immune defense (Mestecky et al., 2005; Brandtzaeg, 2010). S-IgA inhibits adherence of pathogens to host epithelial cells in the intestinal lumen and neutralizes pathogenic toxins by binding to the toxins' biologically active sites. Based on the immunological importance of S-IgA in immunosurveillance in the intestine, the development of oral vaccines has focused on the induction of antigen-specific S-IgA responses (Kunisawa et al., 2007). In addition to the immunosurveillance in the intestine, S-IgA antibody contributes to the establishment of beneficial gut commensal microbiota and thus dysfunction of S-IgA formation resulted in the alteration of normal bacterial flora (e.g., the reduction of Lactobacillus and increase of segmented filamentous bacteria, SFB; Suzuki et al., 2004).

Peyer's patches (PPs) are major gut-associated lymphoid tissue (GALT) where intestinal IgA responses are initiated and regulated by unique immunological crosstalk via cytokines [e.g., interleukin-4 (IL-4), IL-6, IL-21, and transforming growth factor-β (TGF-β)] and cell–cell interactions (e.g., via CD40/CD40 ligand interactions) among dendritic, T, and B cells (Kunisawa et al., 2008; Fagarasan et al., 2010). Thus, oral delivery of antigens to PPs is considered an important strategy for the effective induction of antigen-specific intestinal IgA responses (Kunisawa et al., 2011).

In addition to host-derived factors, microbial stimulation is also required for the maximum production of S-IgA in the intestine (Cebra et al., 2005). Indeed, germ-free (GF) mice have decreased intestinal IgA responses with immature structure of GALT when compared with mice housed under SPF or conventional conditions (Weinstein and Cebra, 1991). Although it was reported that some commensal bacteria [e.g., SFB and altered Schaedler flora (ASF), a combined eight culturable bacteria] and bacterial products (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Talham et al., 1999; Butler et al., 2005), it is obscure which bacteria is involved in this process indigenously. Because predominant commensal bacteria in the intestine is uncultivable, it was difficult to determine by culture-based method which bacteria regulated specific immune responses. However, recent advances in the genomic analysis allowed us to identify the uncultivable bacteria, which revealed key bacteria in the regulation of specific immune responses (Ivanov et al., 2009; Atarashi et al., 2011) as well as the development of immune diseases (Chow et al., 2010; Hill and Artis, 2010). Using genomic and immunological methods, we recently found that the microbial community inside PPs is different from those on the epithelium of PPs or in the intestinal lumen (Obata et al., 2010).

In this review, we discuss initially the immunological features of PPs in the induction and regulation of intestinal IgA responses. In the later part, we focus on the unique cross-communication between PPs and habitat commensal bacteria, *Alcaligenes*, a unique indigenous bacteria habituating inside PPs and regulating dendritic cells (DCs) for the efficient production of intestinal IgA.

IMMUNOLOGICAL FEATURES OF PEYER'S PATCHES

In the intestine, GALT comprise several different, organized lymphoid structures (Spencer et al., 2009; Fagarasan et al., 2010). Among them, PPs are the largest and most well-characterized sites

for the initiation of intestinal IgA responses, especially responses to T cell-dependent antigens (Kunisawa et al., 2008; Fagarasan et al., 2010). There are generally 8–10 PPs in the mouse small intestine and hundreds in the human small intestine. Each PP is composed of several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as the interfollicular region (**Figure 1**).

Inside PPs, antigen-sampling M cells located in the follicle-associated epithelium transport luminal antigens to DCs situated in the subepithelium region (Neutra et al., 2001), which then form clusters with T-, B-, and stromal cells in the germinal centers and promote μ-to-α-class-switch recombination of B cells with the help of cytokines such as IL-4, IL-21, and TGF-β (Fagarasan et al., 2010). Upon immunoglobulin class-switching from μ to α, IgA-committed B cells (IgA^+ B cells) begin to express type 1 sphingosine-1-phosphate receptor, CCR9, and α4β7 integrin, allowing them to depart from the PPs and subsequently traffic to the intestinal lamina propria (Mora et al., 2006; Gohda et al., 2008). In the intestinal lamina propria, they further differentiate into IgA-secreting plasma cells under the influence of terminal differentiation factors (e.g., IL-6; Cerutti et al., 2011). DCs play a key role in these processes. For instance, nitric oxide, TGF-β, APRIL, and BAFF produced by TNF-α/iNOS-producing DCs (Tip-DCs) promotes IgA production (Tezuka et al., 2007). Also, DCs in the PPs metabolite vitamin A and produce retinoic acid, which induces the expression of gut-homing receptors (CCR9, and α4β7 integrin) on activated B and T cells (Iwata et al., 2004; Mora et al., 2006). Retinoic acid also induces the preferential differentiation into regulatory T (Treg) cells (Hall et al., 2011), and some of Treg cells differentiated into follicular helper T cells to promote IgA production in the PPs (Tsuji et al., 2009).

The identification of the molecular pathway of PP organogenesis allowed the establishment of PP-deficient mice through the loss of any part of this pathway (Nishikawa et al., 2003). Notably, disruption of the PP organogenesis pathway by blockade of tissue genesis cytokine receptor signaling [IL-7R and/or lymphotoxin-β receptor (LTβR)] during a limited fetus time period results in the selective loss of PPs without affecting other lymphoid tissue organogenesis (Yoshida et al., 1999). Experiments with PP-deficient mice showed that the dependency on PPs in the induction of antigen-specific IgA responses depends on the form of the antigen. For instance, the PP-deficient mice failed to develop antigen-specific IgA responses against orally administered antigens in particle form, but retained their ability to respond to soluble forms of antigens (Yamamoto et al., 2000; Kunisawa et al., 2002). It was also reported that lamina propria DCs are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PPs is required for the induction of intestinal IgA production (Martinoli et al., 2007). This is consistent with another finding that DCs in the PPs are responsible for intestinal IgA production (Fleeton et al., 2004). Therefore, PPs are considered to be one of the major sites for the initiation of intestinal antigen-specific IgA responses.

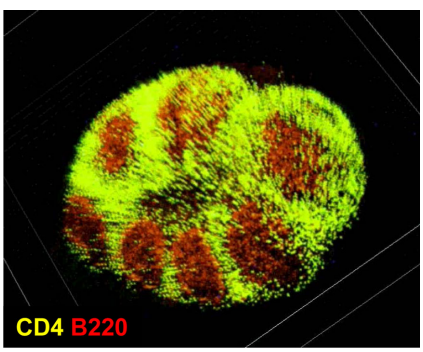

FIGURE 1 | Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and carboxy-SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's patches was observed at the whole-tissue level by using macro-confocal microscopy.

EFFECT OF MICROBIAL STIMULATION ON THE PRODUCTION OF INTESTINAL IgA

It is well known that microbial stimulation is required for the full production of S-IgA in the intestine. Indeed, GF mice have decreased intestinal IgA responses when compared with mice housed under SPF or conventional conditions (Cebra et al., 2005). Studies using mono-associated GF mice with SFB have demonstrated that only a minor proportion of the total intestinal IgA is reactive to mono-associated bacteria (Talham et al., 1999). In addition, bacterial products produced by commonly expressed on commensal bacteria (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Butler et al., 2005). In contrast, a recent study using reversible colonization of GF mice with genetically engineered *E. coli* showed that intestinal IgA induced in those mice bound to parent strain but not other bacteria (Hapfelmeier et al., 2010). Therefore, it remains unclear whether intestinal IgA responses induced by commensal bacteria is mediated by polyclonal stimulation and/or by B cell receptors specific for microbial antigens.

As one mechanism of impaired IgA production of GF mice, it was reported that GF mice have structurally immature GALT (e.g., PPs and ILFs) when compared with SPF mice (Weinstein and Cebra, 1991; Hamada et al., 2002). In the PPs, several key pathways for the IgA production require microbial stimulation. For example, Tip-DCs enhance the IgA production by producing nitric oxide, TGF-β, APRIL, and BAFF, which requires microbial stimulation through innate receptors (Tezuka et al., 2007). Indeed, the number of Tip-DCs was much reduced in the intestine of GF and MyD88-deficient mice (Tezuka et al., 2007). Another cell involved the microbe-dependent IgA production is non-hematopoietic follicular DCs (FDCs). It was reported that microbial stimulation of FDCs resulted in expressing chemokine CXCL13, BAFF, and TGF-β for the germinal center formation and B cell class-switching from IgM to IgA (Suzuki et al., 2010).

ALCALIGENES IS A UNIQUE INDIGENOUS BACTERIA INSIDE PPs

Recent advances in genomic technology make it possible to detect commensal bacteria in the intestine, allowing identification of key bacteria involved in the regulation of specific immune responses. For example, SFB was identified as commensal bacteria inducing

Th17 cells (Ivanov et al., 2009), whereas colonic regulatory T cells were induced by *Clostridium* clusters IV and XIV (Atarashi et al., 2011). These commensal bacteria localize at the surface of intestinal epithelium, but we supposed that the immunological crosstalk between host and commensal bacteria might establish in the regulation of intestinal IgA responses in the GALT. In this issue, we analyzed the composition of the microbial community inside PPs and identified *Alcaligenes* as a major commensal bacteria uniquely locating inside PPs (Obata et al., 2010).

By using the 16S rRNA clone library method, SFB are the predominant commensal bacteria co-habitat on FAE of PPs as like small intestinal epithelium. Although the FAE consisted with antigen-sampling M cells, SFB was not found inside of PPs. Instead, *Alcaligenes* are predominant bacteria inside PPs. The result obtained by the 16S rRNA analysis was further confirmed by fluorescence *in situ* hybridization (FISH) method and thus *Alcaligenes* are present exclusively inside PPs, not on the FAE of PPs, and intestinal villous epithelium and intestinal lamina propria (**Figure 2**). Of note, the preferential presence of *Alcaligenes* was observed not only in mouse but also in monkey and human (Obata et al., 2010). One of interesting but unresolved questions is the species specificity of *Alcaligenes*. We are now investigating whether *Alcaligenes* isolated from human or monkey colonize in the PPs to promote IgA production when they are orally fed to GF mice. Inside PPs, a proportion of the *Alcaligenes* seemed to be alive in mice. The presence and growth of *Alcaligenes* were detected in the PPs of GF mice after adoptive transfer of PP homogenates containing *Alcaligenes* from SPF mice. These findings suggest that *Alcaligenes* are indigenous bacteria ubiquitously living inside the PPs of various mammalian species.

ANTIBODY-MEDIATED RECIPROCAL INTERACTION BETWEEN *ALCALIGENES* AND THE HOST IMMUNE SYSTEM

As mentioned above, M cells located on the FAE of PPs transport luminal bacteria into DCs locating at the subepithelial region of FAE (Neutra et al., 2001). 16S rRNA clone library methods consistently revealed that DCs in the PPs predominantly contain *Alcaligenes*, whereas these bacteria are rarely detected in DCs isolated from other lymphoid tissues (e.g., spleen and mesenteric lymph nodes; Obata et al., 2010). We examined the immunological effects of *Alcaligenes* on DCs and found that the production of IgA-enhancing cytokines such as IL-6, TGF-β, and BAFF was increased when DCs isolated from the PPs of GF mice were stimulated with *Alcaligenes* (Obata et al., 2010). Several lines of evidence have revealed that immunological functions of DCs are different between intestinal and other lymphoid tissues (reviewed in Rescigno, 2010), we are now investigating whether immune stimulatory functions of *Alcaligenes* is specific for the PP DCs or not.

In agreement with the uptake of *Alcaligenes* and subsequent production of IgA-enhancing cytokines by DCs, *Alcaligenes*-specific IgA-forming cells were frequently observed in PPs, and consequent IgA production was noted in the intestinal lumen of SPF mice, but not GF mice (Obata et al., 2010). Although biological role of *Alcaligenes*-specific IgA antibody remains to be elucidated, the antibody might be involved in the creation of intratissue co-habitation of *Alcaligenes* in PPs. To this end, the number of *Alcaligenes* inside PPs is decreased in B cell-deficient CBA/N xid and IgA-deficient mice compared with wild-type mice (Obata et al., 2010). Therefore, it is interesting to suggest that *Alcaligenes*-specific IgA antibody mediates the uptake and presence of *Alcaligenes* in the PPs. Since M cells express IgA receptors

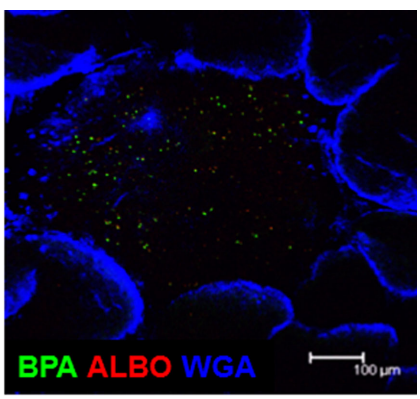

FIGURE 2 | Microarchitecture of murine Peyer's patches. Whole-mount fluorescence *in situ* hybridization was performed to visually analyze the presence of *Alcaligenes* inside PPs. Both BPA and ALBO34a were used as specific probes for *Alcaligenes*. Wheat germ agglutinin (WGA), an *N*-acetylglucosamine-specific lectin, was used to detect epithelial cells. Scale bar indicates 100 μm.

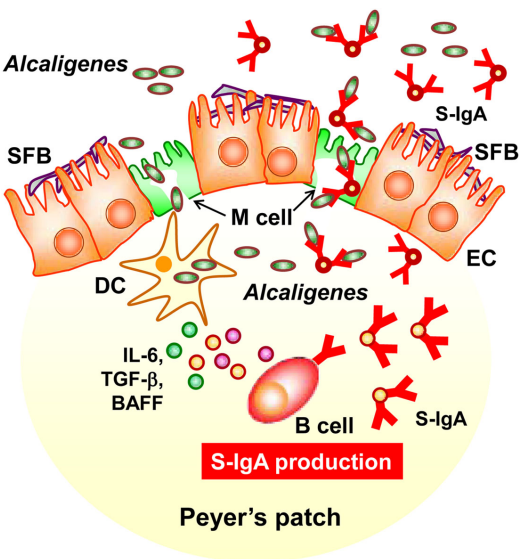

FIGURE 3 | *Alcaligenes* mediates symbiotic communication inside Peyer's patches. On the follicle-associated epithelium of PPs, segmented filamentous bacteria (SFB) is predominantly observed. In contrast, *Alcaligenes* specifically localizes inside Peyer's patches, where some are taken up by dendritic cells (DCs). Stimulation by *Alcaligenes* prompts the DCs to produce IgA-enhancing cytokines [e.g., interleukin-6 (IL-6), transforming growth factor-β (TGF-β)], and B cell activating factor (BAFF), which enhance the intestinal IgA response. The intestinal IgA includes *Alcaligenes*-specific IgA, which might mediate the preferential uptake and presence of *Alcaligenes* in the PPs. The uptake is presumably mediated by M cells.

(Mantis et al., 2002), one possibility is that *Alcaligenes* coated with the *Alcaligenes*-specific antibody are taken up into PPs through M cells. Further, the antigen-specific IgA coating on *Alcaligenes* might be beneficial for the bacteria to create the co-habitation niche since IgA antibody has been shown to non-inflammatory antibody (Mestecky et al., 2005).

CONCLUSION

In this review, we discussed a new concept of symbiotic communication in PPs that is mediated by commensal bacteria-specific IgA antibody. *Alcaligenes*-specific antibodies may mediate the uptake and the presence of *Alcaligenes* in the PPs, and the co-habitation of *Alcaligenes* within the PPs is one of the key factors to promote the intestinal IgA production by enhancing the production of IgA-enhancing cytokines from DCs (**Figure 3**). We still have various questions regarding this co-habitation of *Alcaligenes* in the PPs. For example, it remains unclear whether the presence of *Alcaligenes* inside of PPs is physiologically beneficial or harmful for the host immune system. In this issue, we are now addressing the microbial community in the PPs of mice and human patients suffering from intestinal immune diseases (e.g., intestinal inflammation and allergy). The biological roles of intra-tissue habitation of *Alcaligenes* in the PPs in the appropriate regulation of mucosal immune responses need to be elucidated. The current goal is to elucidate the mechanisms behind the co-habitation of *Alcaligenes* within PPs, and the exact contribution of *Alcaligenes* to educate and guide mucosal immunocompetent cells especially DCs in the PPs for the development, maturation and maintenance of the appropriate host immune system. These studies will provide novel molecular and cellular mechanisms of symbiotic communication with commensal bacteria in the regulation of host immunity.

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Functional flexibility of intestinal IgA – broadening the fine line

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Intestinal bacteria outnumber our own human cells in conditions of both health and disease. It has long been recognized that secretory antibody, particularly IgA, is produced in response to these microbes and hypothesized that this must play an important role in defining the relationship between a host and its intestinal microbes. However, the exact role of IgA and the mechanisms by which IgA can act are only beginning to be understood. In this review we attempt to unravel the complex interaction between so-called "natural," "primitive" (T-cell-independent), and "classical" IgA responses, the nature of the intestinal microbiota/intestinal pathogens and the highly flexible dynamic homeostasis of the mucosal immune system. Such an analysis reveals that low-affinity IgA is sufficient to protect the host from excess mucosal immune activation induced by harmless commensal microbes. However, affinity-maturation of "classical" IgA is essential to provide protection from more invasive commensal species such as segmented filamentous bacteria and from true pathogens such as *Salmonella typhimurium*. Thus a correlation is revealed between "sophistication" of the IgA response and aggressiveness of the challenge. A second emerging theme is that more-invasive species take advantage of host inflammatory mechanisms to more successfully compete with the resident microbiota. In many cases, the function of IgA may be to limit such inflammatory responses, either directly by coagulating or inhibiting virulence of bacteria before they can interact with the host or by modulating immune signaling induced by host recognition. Therefore IgA appears to provide an added layer of robustness in the intestinal ecosystem, promoting "commensal-like" behavior of its residents.

Keywords: mucosal immunology, IgA, microbiota, mucosal infection, natural antibody, adaptive immunity, innate immunity

INTRODUCTION

Immunity at our mucosal surfaces at first appears to tread a very fine line between excessive pathological responsiveness to the abundant commensal microbes, and insufficient responsiveness to potentially pathogenic microbes. In reality, the composition of the microbiota varies with circadian rhythm (Caporaso et al., 2011), eating (Kiss et al., 2011; Li et al., 2011), chemical toxins (Veldhoen et al., 2008), etc. Further, a broad range of immunodeficiencies (Vaishnava et al., 2008; Slack et al., 2009) are known to be compatible with the formation of host–commensal mutualism at least with a specific-pathogen-free microbiota. Thus it is revealed that the host–microbiota relationship is a dynamic homeostasis that is both remarkably robust and mechanistically flexible. The observed inflammation and induction of high-affinity adaptive immune responses during pathogenic infection can be seen as a (preferably temporary) deviation of the bacterial–host relationship sharply away from its usual homeostatic set-point, with the corresponding decrease in function of the intestine that accompanies any such deviation.

The study of genetically manipulated mice has revealed many factors that contribute to the robustness of this homeostasis [including mucus production (Van der Sluis et al., 2006; Johansson et al., 2008), antimicrobial peptide production (Vaishnava et al., 2008, 2011), innate immunity (Slack et al., 2009)]. This robustness may also explain how the intestinal immune system is able to accommodate a wide range of microbes that have evolved a wide variety of intestinal lifestyles ranging from symbiotic to opportunistic pathogenic/"pathobiotic" (Chow et al., 2011).

For many years, the role of intestinal IgA in host–commensal, and host–pathogen, interactions has been actively pursued. The mild phenotype of IgA-deficiency both in mice and in humans [approx. 1 in 600 Caucasians fail to secrete measurable quantities of IgA into the intestine without any clear association to clinical disease (Hammarström et al., 2000)] may be easily explained by the necessary robustness of the intestinal immune system. Nevertheless, IgA is normally produced at a rate of 5 g/day, making it the most abundantly produced antibody isotype, and a considerable metabolic burden. This hints toward a strong evolutionary selective pressure for its maintenance. The purpose of this review is to discuss recently published progress in our understanding of the role and mechanisms of action of secretory IgA in mucosal homeostasis, mucosal vaccination, and responses to infection.

MICROBIOTA-DRIVEN IgA – IS IT "NATURAL"?

"PRIMITIVE" AND "CLASSICAL" IgA

Before we consider the function of IgA, it is first pertinent to discuss one major aspect of IgA biology: many IgA responses are considered to be "primitive" (Macpherson et al., 2000), but the exact meaning of this is easily confused with "natural" and/or "innate."

Evidence of secretory antibodies can be seen in the genome of species as diverse as cartilaginous fish, amphibians, and mammals. Intriguingly, teleost fish, which branched from the mammalian and cartilaginous fish lineages during the Triassic period, produce only variants of IgM with no evidence of intestinal secretion (Tort et al., 2003). This suggests that mammalian IgA arose later than the divergence of teleosts and the rest of the Gnathostomata species, and is therefore considerably less ancient than the adaptive immune system itself.

Nevertheless, although "classical" adaptive immunity generates high-affinity antibodies via T-cell driven somatic hypermutation (**Table 1**), T-cell-deficient mice can still produce so-called "primitive" IgA (**Table 1**) of a sufficient affinity/avidity to bind commensal bacterial antigens on a western blot or ELISA. This suggests that commensal-induced IgA does not absolutely require classical T-cell help for its generation (Macpherson et al., 2000, 2001). The term "primitive" here refers to the seemingly simpler induction mechanism not involving T-cells. It is intriguing to note that the breadth of epitopes, particularly of carbohydrate epitopes never produced by mammalian cells, is much broader in bacteria than in commonly studied viruses such as LCMV (which necessarily rely upon host protein and carbohydrate synthesis machinery). This potentially allows for evolutionary selection of antibody V, D, and J segments capable of forming high-affinity interactions with common bacterial antigens even in germ-line conformation. Indeed, (monoclonal) IgA isolated from monocolonized mice commonly displays no evidence of somatic hypermutation (Bos et al., 1996; Hapfelmeier et al., 2010; Wei et al., 2011; Lindner et al., 2012) but nevertheless can be of sufficient affinity to bind to the bacterial surface of the colonizing species in a flow cytometry stain (where the typical affinity minimum cut-off is considered to be around 10^9 L/mol (Macey, 2007) as opposed to much lower affinity cut-offs that can be detected by Western blot or "dirty-plate" ELISA).

The above observations all support the idea that IgA can be produced in a "primitive" fashion, independently from T-cell-mediated immunity. However, it should be noted that bacterial binding by IgA from T-cell-deficient mice is observed when total IgA concentrations are normalized between wild-type and T-cell-deficient mice, but total intestinal IgA levels are lower in T-cell-deficient mice than in wild-type mice, depending on the microbiota (Macpherson et al., 2000). Further, when T-cell-deficient or CD4 T-cell-depleted mice are challenged intragastrically with either a commensal or a pathogen, we cannot detect IgA of sufficient affinity to be measured by flow cytometry staining of the bacterial surface, even when total IgA concentrations are normalized (Slack et al., 2009; Slack and Hapfelmeier, unpublished observations). As well as T-cells, organized lymphoid structures in the intestine are required for a substantial proportion of IgA^+ plasma cell generation as production of these cells is severely impaired in mice lacking RORγt which fail to develop any organized lymphoid structures in the intestine (Eberl and Littman, 2004), lymphotoxin-alpha deficient mice which lack all organized lymphoid structures in the intestine (Kang et al., 2002; Newberry et al., 2002), and RORγt-deficient mice reconstituted with bone marrow from lymphotoxin-alpha deficient mice which fail to generate follicular dendritic cells in the Peyer's patches (PP; Tsuji et al., 2008). Further, germinal centers are always histologically observed in the mucosa-associated lymphoid tissues of SPF or recently bacterially colonized animals (Talham et al., 1999; Hapfelmeier et al., 2010). Therefore, although "primitive" bacterially induced IgA can be observed in the absence of "classical" T-cell-dependent antibody responses, the generation of abundant and high-affinity IgA still appears to require CD4 T-cell help and organized lymphoid structures.

"NATURAL" AND "INNATE" IgA

How these responses relate to what are defined as "natural" and "innate" antibody responses is also intriguing. Classically, "natural" responses are defined as pre-existing antibody responses that an animal produces in the absence of microbial or antigenic stimulation. Until recently, all antibodies in the serum and intestine of an unvaccinated uninfected SPF animal would be considered "natural." However, at least 90% of the IgA in the intestine is induced by the microbiota (to be further discussed below, see section The Role of of IgA in Host–Commensal Mutualism), suggesting this term can only be classically applied to the antibody responses found in germ-free mice (**Table 1**). Germ-free animals produce reduced but still significant amounts of intestinal IgA. Recent next-generation sequencing of the IgA repertoire indicates that this "natural" repertoire contains a similar distribution of low frequency and high frequency plasma cell clones to that in SPF mice (Lindner et al., 2012). However, intestinal IgA from germ-free mice does not contain sufficient levels of bacterial-antigen-specific clones to bind bacterial antigens on western blots, even when total IgA concentrations are normalized (Macpherson et al., 2000, 2001).

In the fields of virology, antibody affinities of greater than 10^8 L/mol are required to neutralize infection with extensively characterized viruses such as VSV (Bachmann et al., 1997). Such antibody responses are only induced after extensive somatic hypermutation during germinal center reactions, and in this situation, the distinction between natural antibody responses and induced antibody responses is simple to make. For anti-bacterial antibodies, it is almost impossible to define a bacterial equivalent of "neutralizing" antibody unless the response is bacterial toxin-specific, adding considerably to the complexity of discussion.

Thus, both "primitive" T-independent, and "classical" adaptive T-dependent IgA responses appear to operate in parallel in the intestine to provide both extremely rapid, and slower but higher-affinity responses to bacteria, as appropriate. How "natural" the IgA response is in both situations remains to be fully explored, but next-generation-sequencing-based approaches to the IgA repertoire are beginning to provide clear answers to this: there is evidence for the microbiota-induced rapid expansion of a near-germ-line "natural" IgA repertoire (pre-existent in newborns and germ-free animals) combined with the subsequent

Table 1 | Key features of "natural," "primitive," and "classical" intestinal IgA.

IgA type	Induced by	Microbial affinity	T-cell-dependent	Somatic hypermutation	Delay of onset	Selected reference
Natural	?	Negligible	No	None	0 (pre-exists)	Macpherson et al. (2001), Lindner et al. (2012), Harris et al. (2006)
Primitive	Microbes	Low–moderate	No	None	Fast (<14 days)	Macpherson et al. (2000, 2001), Lindner et al. (2012), Bos et al. (1996)
Classical	Microbes (predominantly pathobionts, pathogens)	High	Yes	High (time/age-dependent)	Slow (>14 days)	Wei et al. (2011), Lindner et al. (2012), Bos et al. (1996)

gradual diversification of this repertoire by T-dependent somatic hypermutation in the murine intestine (Wei et al., 2011; Lindner et al., 2012). The relative contributions of the different types of responses to the various functions of IgA are now starting to be unraveled and will be discussed below.

FUNCTIONS OF INTESTINAL IgA

The commensal microbiota of a free-living mammal contains a range of species from those which barely interact with the host, via organisms that form truly mutualistic relationships (*Bacteroides thetaiotaomicron*, *Lactococcus lactis*) through to well known opportunistic pathogens (*Staphylococcus aureus*, *Clostridium difficile*) and "pathobionts" [e.g., "segmented filamentous bacteria" (SFB); Chow et al., 2011]. It should be noted that all such terms are an attempt to draw lines onto the continuous spectrum of bacterial behaviors that depend not only on the bacterial species, but also on the host. Indeed bacteria that have been defined as true commensals or "pathobionts" in wild-type mice can begin to display behavior of true opportunistic pathogens in immunocompromised mouse strains (Fagarasan et al., 2002; Elinav et al., 2011; Wei et al., 2011; Henao-Mejia et al., 2012) further indicating the flexibility of these definitions. A useful distinction is thus that commensalism represents a state of homeostasis that can be maintained long-term, whereas pathogenic infection represents a major deviation from this homeostasis. The function of IgA in both of these situations will be discussed below.

THE ROLE OF IgA IN HOST–COMMENSAL MUTUALISM

It is well established that the majority (approx. >90%) of intestinal IgA production is microbiota-driven, since germ-free animals contain around 10-fold reduced numbers of mucosal IgA-producing cells (Crabbé et al., 1970; Moreau et al., 1978). This state is readily reversed by the re-association of such animals with a normal microbiota (Crabbé et al., 1970; Moreau et al., 1978). In line with this it was shown that most intestinal bacterial cells are IgA-coated *in situ* (van der Waaij et al., 1996), but it has also been noted that not all IgA that is induced by a specific commensal microbe has detectable reactivity to the inducing microbe (Bry et al., 1996; Talham et al., 1999; see also Stoel et al., 2005).

Compensatory immune reactions reveal IgA-mediated modulation of immune function

The study of severely innate immune deficient mice has shown that both innate and adaptive immunity are critically important for intestinal containment also of commensal bacteria (even the completely avirulent laboratory model organism *E. coli* K-12). Indeed, considerable functional complementarity of innate and adaptive immunity can compensate for partial immune deficiency (Slack et al., 2009). This flexibility almost certainly also underlies the lack of overt pathology in $IgA^{-/-}$ or even completely antibody-deficient animals (Shulzhenko et al., 2011). It also suggests that the functionality of IgA in host–commensal interaction is best measured indirectly by measuring upregulation of other, compensatory commensal-induced mucosal immune mechanisms. This has been successfully observed in a number of recent publications, which can be roughly divided into those showing an effect of non-specific IgA, those showing an effect of total IgA and those showing an effect of high-affinity IgA.

The work published by Petersen et al. (Peterson et al., 2007), demonstrates that a monoclonal antibody with high-affinity for the surface of *Bacteroides thetaiotaomicron* can specifically reduce intestinal innate immune activation in a *B. thetaiotaomicron*-monocolonized hybridoma back-pack RAG1-deficient (therefore T and B cell-deficient) mouse model. Increased innate immune activation is also observed in the epithelium of completely antibody-deficient mice (Shulzhenko et al., 2011). Previous studies have also reported spontaneous pathology and small intestinal bacterial overgrowth, predominantly of the "pathobiont" SFB, in AID-deficient mice, which have a defect in somatic hypermutation (Fagarasan et al., 2002; Suzuki et al., 2004; Wei et al., 2011), suggesting that affinity-matured IgA responses are necessary for the control of this species. In contrast to these studies on high-affinity antibodies, quasi-monoclonal IgA from Qm mice, and also the "natural" IgA from germ-free mice, was shown to be sufficient to prevent premature priming of antibody responses against the commensal microbiota in neonatal mice, despite presumably negligible affinity of this IgA for the intestinal microbiota (Harris et al., 2006). Elson and co-workers report another indirect IgA effect in host–commensal mutualism by showing that IgA-deficiency leads to increased commensal-driven generation of intestinal $FoxP3^{+}$ regulatory T-cells (Cong et al., 2009), although no distinction could be made between low-affinity and high-affinity IgA responses in this system.

Taken in parallel with the absence of intestinal inflammation in IgA or antibody-deficient mice in many animal facilities, this suggests that requirements of the IgA system for maintaining homeostasis are highly dependent on the nature of the intestinal

microbiota. In the majority of SPF facilities, compensation by other immune mechanisms appears to prevent the appearance of clinically overt disease. Whilst affinity-matured antibodies appear to be necessary to protect against immune activation caused by bacteria like SFB, non-specific IgA such as in the QM mice, is sufficient to prevent early immune activation by a less invasive microbiota (**Figure 1**).

Mechanisms of IgA-mediated immune modulation

The mechanism by which IgA can minimize induction of innate or adaptive immunity in the intestine remains to be definitely proven. This may be achieved by decreasing bacterial penetration of the intestinal epithelial barrier and minimizing access of bacterial antigen to gut associated lymphoid tissues (GALT), or by alteration of immune signaling induced by recognition of IgA-coated bacteria in the epithelium and lamina propria.

The first of these mechanisms could be called "innate immune exclusion", in analogy of the classical term of "immune exclusion" (i.e., the effect of IgA in downregulating its own induction by excluding the cognate antigen from accessing IgA inductive sites [Stokes et al., 1975]). A prominent example of this is the monocolonization of germ-free animals with a strongly IgA-inducing commensal bacterium like *Morganella morganii*. Despite the persistent nature of bacterial intestinal colonization, this leads to a contained IgA response with only transient germinal center reactions (Shroff et al., 1995).

Recent progress in this field has led to a more detailed consideration of intestinal mucus in such an exclusion mechanism. The viscous mucous layer covering the intestinal epithelium physically impedes bacterial contact with the epithelium and concentrates secreted antimicrobials that kill mucus-invading bacteria, without affecting the commensal habitat as a whole. If the viscous mucus layer is missing, the micro-compartmentalized nature of the mucosal barrier literally collapses. This can precipitate excessive defense reactions toward commensal bacteria and chronic inflammatory disease, as can be observed in Muc2-deficient mice (Van der Sluis et al., 2006; Johansson et al., 2008). It has recently been demonstrated that the antimicrobial peptide RegIIIγ is concentrated in the mucus layer and deficiency for this peptide results in heavy bacterial colonization of the mucus layer (Vaishnava et al., 2011). Histologically it is also clear that IgA is concentrated in the intestinal mucus, and similar experiments

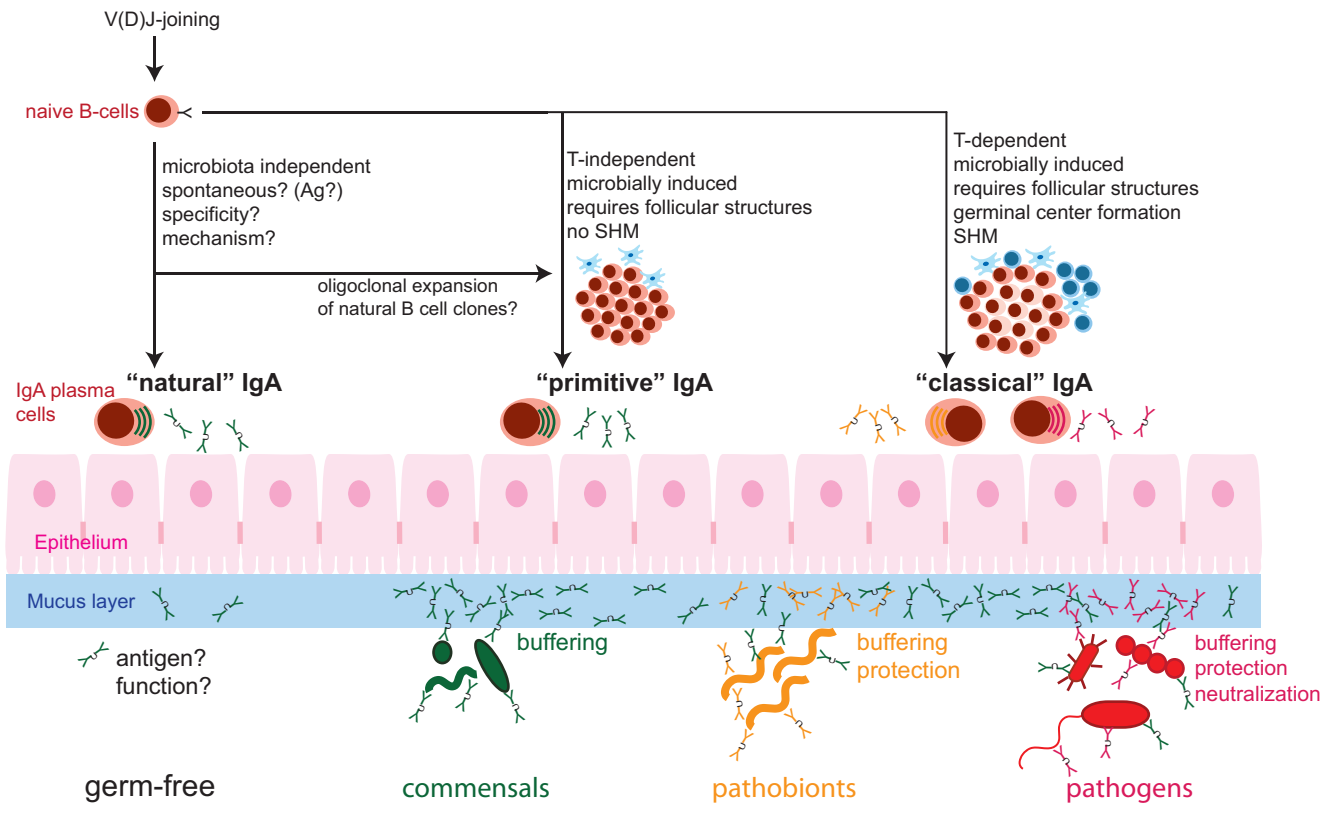

FIGURE 1 | Schematic of the composite nature of the mucosal IgA system, consisting of "natural" (microbiota-independent), "primitive" (microbobially induced, T-cell independent), and "classical" (microbe/pathogen-induced, helper T-cell-dependent) IgA responses. Whether there is a specific immunogen inducing "natural" IgA is unknown. "Natural" IgA can be sufficient for controlling benign commensal bacteria. Commensal microbes induce "primitive" T-cell independent IgA with is sufficient for the management of commensal bacteria (Green), mainly by exclusion from the mucus layer and the resulting "buffering" of mucosal responses. The more invasive pathobionts (orange) and infection with pathogenic microbes (red) further induce "classical" T-cell-dependent IgA of high-affinity that is important for the control of these types of microorganisms via efficient mucosal exclusion and neutralization. SHM, somatic hypermutation.

examining mucus colonization in IgA-deficient and antibody-deficient animals would reveal whether IgA contributes to the sterility of the intestinal mucus.

A second possible mechanism is that recognition of IgA-coated bacteria induces altered signaling when compared to recognition of bacteria alone. Many different proteins have been suggested to bind IgA, in addition to the PolyIg receptor that mediates its transport across epithelia. These include the Fcα/μR (Shibuya et al., 2000), CD89 (reviewed in (Bakema and van Egmond, 2011)), asialoglycoprotein receptors (Stockert et al., 1982), transferrin receptors (CD71; Moura et al., 2001), and secretory component receptors (Lamkhioued et al., 1995). In humans the IgA receptor CD89 is expressed on myeloid cells (although absent from intestinal macrophages) and can signal in either an activatory or inhibitory manner depending on the degree of cross-linking (Reviewed in (Bakema and van Egmond, 2011)). Although mice lack a direct homolog of CD89, transgenic mice expressing human CD89 on myeloid cells and passively transferred with human IgA specific for mycobacterium tuberculosis were protected from infection (Balu et al., 2011). IgA can be shown to bind to the surface of murine macrophages, suggesting that a functional homolog of CD89 exists, but as yet the responsible receptor complex has not been identified (Reljic, 2006). Until further characterization of IgA receptors in the common murine models has been achieved, signaling induced by IgA in different contexts will remain hard to define.

A third proposed mechanism of action of IgA appears paradoxical to the first. It has been observed that IgA also plays a role in increasing the translocation of bacteria across PP follicle associated epithelia, where IgA-coated bacteria bind to the luminal side and are transcytosed *into* the PP (Kadaoui and Corthésy, 2007; reviewed in Corthésy, 2007, 2009). However, it is still not fully understood whether the IgA-coated antigen then feeds into a positive or negative feedback loop of antigen presentation for the regulation of bacterial-specific antibody responses. It is important to keep this bacterial-uptake mechanism in mind, as a common measure of mucosal containment defects is to quantify the load of live bacteria present in the PPs and MLNs. An absence of phenotype in IgA-deficient mice by this method may represent a composite of decreased active transport into the PP and increased passive translocation, masking the effect. This also suggests that the concept of IgA-mediated exclusion of bacteria from the lamina propria is over-simplified and the immune consequences of recognition of IgA-coated bacteria need to be fully investigated.

An entirely different mechanism by which IgA may function is a possible direct effect of IgA on commensal bacterial colonization of the intestine (such as by influencing growth rate, survival, bacterial responses, etc.). Despite enormous technical innovation and experimental creativity, the attempts to demonstrate effects of immune functions on microbiota composition have been a challenge and so far have only found subtle effects of mucosal immunity on the commensal bacterial fitness (Peterson et al., 2007; Goodman et al., 2009). Supporting functional data for the impact of bacteria-binding IgA on commensal bacterial fitness come from the Peterson at al. study (Peterson et al., 2007) where it could be demonstrated that phase variation of surface polysaccharide that leads to escape from IgA recognition confers a selective advantage for intestinal colonization. Interestingly, it is known that inflammatory responses, such as increased antimicrobial peptide production and upregulation of siderophore-sequestering proteins, negatively impact on commensal bacteria. Therefore beneficial effects of IgA on commensal colonization may also be indirect via modulation of the activation state of the mucosal immune system.

In conclusion, the immune set-points and anatomy of the mucosal barrier together with IgA may function primarily in robustly "protecting commensals from themselves," to avoid unnecessary inflammatory reactions where the collateral damage would always outweigh the benefits and likely lead to a chronic state of exaggerated inflammation as in inflammatory bowel diseases. IgA is therefore a typical example of a mucosal immune specialization with the potential to actively exclude bacteria from host tissues without any concomitant induction of acute inflammation, thus broadening the window of immune system and physical barrier function compatible with stable host–microbiota homeostasis.

FUNCTIONS OF IgA IN BACTERIAL PATHOGEN DEFENSE

In considering the literature on the role of IgA in pathogen defense, it is also crucial to make the distinction between low-affinity pre-existing IgA responses found in animals never previously exposed to the pathogen, and high-affinity IgA responses formed during vaccination or by previous exposure to the pathogen.

Can "Natural" IgA protect against pathogens?

In contrast to our knowledge on the role of low-affinity IgA responses in host–commensal mutualism, only limited data supports a role of low-affinity antibodies in protecting from acute infection with bona fide obligate pathogens. Indeed PolyIg receptor (pIgR)-deficient mice, which fail to transport any polymeric immunoglobulin into the intestinal lumen, are equally as susceptible as wild-type mice in the typhoid model of *Salmonella typhimurium* infection (Uren et al., 2005), although pIgR-deficient mice have a reduced colonization resistance toward *S. typhimurium* (Wijburg et al., 2006). However, this may in part be a consequence of the mild non-specific protein losing enteropathy phenotype of this mouse strain in some hygiene conditions (Johansen et al., 1999). Our own data in the streptomycin-pretreatment model of non-typhoidal salmonellosis further confirm these observations (E. Slack and W.-D. Hardt, unpublished data). Similarly, naive IgA-knock-out mice show comparable susceptibility to Influenza infection, to naïve wild-type controls (Mbawuike et al., 1999). Importantly, given the recent progress in our understanding of the role of the microbiota in disease (Stecher and Hardt, 2011), it would be valuable for the field to revisit these observations in gnotobiotic settings. Further, the presence of possible masking of natural IgA effects by compensatory production of other isotypes, of increased innate immunity and increased innate lymphoid cell and T-cell function requires full exploration. However, although antibody-mediated-compensation for innate immunodeficiency in MyD88-deficient mice is sufficient to protect these animals from some commensal bacterial species (Slack et al., 2009), this was shown to be insufficient to protect from *Salmonella* infection (Ko et al., 2009).

High-affinity "classical" IgA induced by pathogens

Induced IgA responses with measurable affinity for particular pathogens have been extensively investigated in the context of mucosal vaccination. Early experiments with cholera toxin vaccination demonstrated strong induction of IgA after gastric administration of the toxin (Svennerholm et al., 1978; Fuhrman and Cebra, 1981). Svennerholm et al. further showed a strong correlation between the titer of IgA against cholera toxin and protection from murine cholera infection. Later, "back-pack" IgA-producing hybridoma experiments and adoptive transfer of monoclonal dimeric IgA revealed that *Vibrio cholerae* LPS-specific IgA was protective against murine cholera whereas anti-toxin IgA alone provided very limited protection (Winner et al., 1991; Apter et al., 1993), although the relative affinities of the IgA clones employed were not assessed. It could also be demonstrated that adoptive transfer with a monoclonal dimeric IgA specific for the outer membrane of *Helicobacter felis* could protect germ-free mice from *Helicobacter* infection (Czinn et al., 1993). Endt et al. (2010) recently elucidated that high-affinity surface (predominantly LPS O-antigen)-binding IgA protects from colitis upon re-infection with *S. typhimurium* in the non-typhoidal salmonellosis model. This appears to be mediated by antibody-mediated bacterial coagulation and "luminal trapping," hindering innate immune recognition and bacterial delivery of proinflammatory effector molecules. It was recently found that pathogen-binding IgA can also protect from pathogens by direct modulation of bacterial motility (Forbes et al., 2008) or virulence organelles, such as type III secretion systems. For example, a monoclonal *Shigella flexneri* LPS-O-antigen specific IgA antibody when bound to the bacterial surface specifically inhibits the *S. flexneri* type III secretion system that is required for epithelial invasion and induction of pathology (Forbes et al., 2011). In analogy to the virus-neutralizing antibodies that directly protect from virus infection, these antibodies could be termed "bacteria-neutralizing." Taken together, these studies indicate that high-affinity IgA responses can provide protection against a number of gastrointestinal pathogens in the stomach and intestines.

Mechanisms of IgA "immune diplomacy" – dampening inflammation wherever it can be avoided

Although counterintuitive at first, an important protective mechanism of high-affinity secretory IgA appears to be also the dampening of bactericidal immune responses provoked by certain mucosal pathogens, for the benefit of the commensal microbiota and for intestinal homeostasis. Seminal work by Stecher et al. and others (Lupp et al., 2007; Stecher et al., 2007; Ackermann et al., 2008; Brown et al., 2008) introduced the concept that mucosal pathogens such as *Salmonella* and *Citrobacter* cause pathology not with the primary goal of colonizing extra-intestinal sites (which may eventually kill the host), but rather to modify the intestinal microenvironment to improve their competitive edge. Commensal species are continuously selected by conditions found within the non-inflamed intestine; i.e., competition for space and resources and resistance to constitutive antimicrobial activity. The majority of mechanisms required to resist full-blown host inflammatory immune responses are energy-demanding and therefore often absent from commensal species. On the other hand, obligate pathogens require such resistance mechanisms to survive and propagate. Although it at first seems counterintuitive, for many intestinal bacterial pathogens induction of inflammation actually provides them with a significant advantage over the commensal microbiota, that are more susceptible to induced immunity. The role of intestinal IgA may therefore be two-fold: Firstly directly inhibiting adhesion, effacement and invasion by intestinal pathogens and secondly, limiting the induction of inflammation by such pathogens. This hypothesis is nicely supported by the observation that LPS O-antigen-specific IgA can prevent *Salmonella* from interacting with the intestinal epithelium, thus preventing induction of inflammation and allowing the commensal microbiota to re-grow (Endt et al., 2010). The specific role of anti-bacterial IgA in dampening the bacterial elicitation of inflammatory responses has also been worked out for *Shigella* [in a rabbit ligated ileal loop infection model (Boullier et al., 2009)] coming to similar conclusions.

TNFα/iNOS-producing IgA plasma cells – more than just IgA producers

A recent finding now indicates that IgA-producing cells in the intestine may have crucial immune functions beyond IgA production. Local intestinal production of nitric oxide catalyzed by the inducible nitric oxide synthase (iNOS) is important for class switch recombination to IgA and generation of small intestinal IgA plasma cells (Tezuka et al., 2007). A recent study identified the IgA plasma cells themselves as a dominant iNOS and TNFα-producing cell population of the intestinal lamina propria, and B-lineage-specific TNFα/iNOS deficiency led to a an intestinal IgA-deficiency equivalent to a full TNFα/iNOS double knockout (Fritz et al., 2012). Intriguingly, the generation of these TNFα/iNOS-producing IgA plasma cells required microbial exposure, since the baseline lamina propria IgA plasma cells of germ-free animals consisted only of TNFα/iNOS-negative cells (Fritz et al., 2012). Colonization and infection experiments using mice with a B cell specific TNFα/iNOS deficiency further suggested that B-lineage-derived TNFα/iNOS contributed to the control of *Citrobacter rodentium* infection and intestinal colonization with the pathobiont SFB (Fritz et al., 2012). These observations document a novel immune regulatory function of IgA plasma cells and suggest that intestinal microbes induce plasma cells with a more "myeloid" cell-like phenotype.

The challenge of inducing protective IgA by oral vaccination

A major challenge in mucosal immunology currently is the development of vaccines that protect against mucosal infections such as typhoidal and non-typhoidal salmonellosis, cholera, shigellosis, enterotoxigenic *E. coli*, and tuberculosis. Currently many vaccines tested against these organisms show low efficacy when compared to vaccination against viruses (measles, influenza, rubella). In part, this reflects the complexity of the pathogens, although novel vaccines against papilloma viruses and *Varicella zoster* combat organisms approaching bacterial levels of complexity (recently reviewed in Stanley, 2008; Abendroth et al., 2010). Intracellular bacterial pathogens appear to be particularly challenging for the immune system, often residing within macrophages or dendritic cells which they modify to best evade immune detection (Bedoui et al., 2010). However, a vaccine that induces a long-lasting high-affinity IgA response can potentially inhibit the initial infection step before bacteria become resident intracellular pathogens. This poses two

challenges for vaccine developers: Firstly, to induce IgA that has a sufficiently high-affinity for a sufficient number of relevant strains. Secondly, to produce a long-lasting IgA response safely.

Recent experiments using reversible colonization of germ-free mice indicate that both of these aims are harder to achieve in the mucosal system than in systemic IgG-based vaccination (Hapfelmeier et al., 2010). Firstly, the mucosal immune system is rather insensitive to bacteria. Whereas as few as 10^3 bacteria given systemically can induce a robust IgG response, over 10^9 live bacteria need to be delivered orally in order to see a measurable IgA response in the intestine (Hapfelmeier et al., 2010). Therefore any oral vaccination needs to introduce very high numbers of the vaccine strain into the system, either by using a live strain that can colonize to over this density, or by giving extremely high numbers of non-replicating bacteria. Alternatively, methods need to be employed that deliver sufficient quantities of antigen across the mucosal epithelium, or deliver strong enough adjuvanticity that these numbers can be reduced. Killed bacteria seem to be at least 100-fold less effective at inducing IgA responses than live bacteria, presumably due to their inactivity and the digestion of dead bacteria during transit through the stomach and intestine (Macpherson and Uhr, 2004; Hapfelmeier et al., 2010). To make matters worse, there is no clear prime-boost effect of intestinal immunization. Rather with each oral dose of bacteria there is a step-wise increase in specific IgA production that is independent of the interval between doses. A further challenge is that in the normally colonized intestine, the rate of attrition of the IgA repertoire is extremely high (Hapfelmeier et al., 2010). Thus it seems that any successful IgA-inducing vaccine must contain high numbers of bacteria, in a format that survives passage through the stomach, and which provides persistent antigen at a high enough concentration to avoid attrition of the response. Intriguingly, older works on the immune response to cholera toxin in the intestine demonstrate robust prime-boost effects of the IgA response and the presence of memory B cells up to 16 weeks post vaccination, suggesting that the nature of the antigen or the adjuvant may be critical in determining longevity of the IgA response (Pierce, 1978).

Remarkably, it could be demonstrated that intestinal infection with virulent *Salmonella* produces more and higher-affinity IgA than an equally densely colonizing avirulent *Salmonella* mutant, and only the IgA induced by virulent bacteria conferred robust protection from colitis upon re-challenge (Martinoli et al., 2007; Endt et al., 2010). More needs to be learned about the underlying qualitative and quantitative differences between pathogen and non-pathogen-induced mucosal immunity to understand this difference. Presumably, like pathogenic and commensal bacteria, also virulent and avirulent variants of a pathogen necessitate different degrees and kinds of immune protection. This is clearly relevant for the design of mucosal vaccines intended to be protective against mucosal infections: it is absolutely possible that the optimization of completely attenuated bacterial strains as live vaccines turns out to be counterproductive. Such a strategy could lead to agents that potently induce anti-commensal immunity but not the intended anti-pathogen immune protection.

IgA-independent protective mucosal immunity

Importantly, whilst IgA can be sufficient for protection from pathogen infection, it can be demonstrated that immunity induced by exposure of mice to a live mucosal vaccine or pathogen is highly redundant and in many cases IgA is dispensable. This has been shown both for *Influenza* virus infection (Mbawuike et al., 1999), and for typhoidal salmonellosis (Uren et al., 2005). In the non-typhoidal model of salmonellosis, Endt et al. (2010) demonstrated robust protection from colitis by specific secretory IgA but an absence of sterilizing immunity – indeed clearance of *Salmonella* from the gut lumen critically required re-growth of the microbiota, rather than active immune mechanisms. It has further been observed in humans that there is no correlation between any anti-typhoid antibody titer measured in serum or saliva and rate of recovery from typhoid (Dham and Thompson, 1982). Further, specifically induced CD4 effector T-cells can confer antibody-independent protection from mucosal infection. This was shown in an intranasal *Klebsiella pneumoniae* infection model, where Th17 cells conferred cross-protection between multiple serotypes of the same pathogen, independent of antibodies (Chen et al., 2011).

CONCLUSION

The intestinal IgA system acts at the interface between body and one the densest bacterial consortia known. The challenge of bacterial containment and discrimination between non-pathogens and pathogens has shaped the evolution of an intestinal immune system that robustly maintains intestinal health due to highly redundant and functionally flexible barrier functions. The current literature suggests that IgA adds robustness to host–microbiota homeostasis in the face of this flexibility, by acting as an immunological "buffer": i.e., that combines protection from invasion by pathogenic microbes with suppression of excessive inflammation induced by non-pathogenic commensal microbes. The affinity of microbe-IgA interaction required to achieve this buffering appears to be highly tailored to the invasiveness of the microbial species in question. How exactly IgA functions in each situation remains only patchily understood with mechanisms proposed that directly affect the microorganism or that modulate host immune signaling. Inducing long-lived, effective mucosal vaccination will require an in-depth understanding of the interaction of different specificities and affinities of IgA with the pathogen in question, as well as the effects of pre-existing IgA and continuous repertoire re-shaping. We propose that great advances in this field will come from a new approach, made possible by modern technologies, aiming to examine the functioning of IgA within the mucosal ecosystem as a whole, simultaneously assessing the effects on microbiota structure and mucosal activation, and subsequently dissecting the crucial relationship between these two entities.

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Multi-faceted functions of secretory IgA at mucosal surfaces

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Secretory IgA (SIgA) plays an important role in the protection and homeostatic regulation of intestinal, respiratory, and urogenital mucosal epithelia separating the outside environment from the inside of the body. This primary function of SIgA is referred to as immune exclusion, a process that limits the access of numerous microorganisms and mucosal antigens to these thin and vulnerable mucosal barriers. SIgA has been shown to be involved in avoiding opportunistic pathogens to enter and disseminate in the systemic compartment, as well as tightly controlling the necessary symbiotic relationship existing between commensals and the host. Clearance by peristalsis appears thus as one of the numerous mechanisms whereby SIgA fulfills its function at mucosal surfaces. Sampling of antigen-SIgA complexes by microfold (M) cells, intimate contact occurring with Peyer's patch dendritic cells (DC), down-regulation of inflammatory processes, modulation of epithelial, and DC responsiveness are some of the recently identified processes to which the contribution of SIgA has been underscored. This review aims at presenting, with emphasis at the biochemical level, how the molecular complexity of SIgA can serve these multiple and non-redundant modes of action.

Keywords: secretory IgA, mucosal homeostasis, antibody, epithelium, infectious agents, commensal bacteria

INTRODUCTION

Secretory IgA (SIgA) is the principal immunoglobulin (Ig) on mucosal surfaces of humans and many other mammals. Globally, more IgA is produced than all other Ig isotypes combined. Due to its particular biosynthetic pathway relying on production by plasma cells in the lamina propria and poly Ig receptor (pIgR)-mediated secretion by epithelial cells overlying mucosal surfaces, SIgA displays a very different molecular form as compared to IgA antibodies found in the circulation and tissues. SIgA operates in an ever-changing environment whose function is to physically separate the inside of the body that needs to remain sterile from the outside world rich in antigenic stimuli including those present in air, liquid, and food. In the gastro-intestinal tract, a further challenge for host-defending SIgA is to discriminate between symbiotic harmless commensal bacteria and periodic invading, potentially life-threatening microorganisms. The complexity of mechanisms involved is far from being fully understood. From a more global immune surveillance's point of view, the mucosal immune system, including SIgA, must constantly monitor the environment and maintain a balance between tolerance to the normal microbiota and immunity to microbial pathogens while the systemic immune system is designed to vigorously react to any foreign antigen or microbe. Given the intrinsic fragile nature of the gut and airway mucosal barriers ensured by a single layer of epithelial cells, the contribution of SIgA in maintaining homeostasis appears essential. This is reflected by the growing evidence of the role of maternal milk SIgA from early in life in the process of epithelial maturation. However, it is fair to mention that polymeric IgM actively transported across epithelia by pIgR (just like polymeric IgA), as well as IgG transuding from plasma into local secretions, can also participate in protection of the intestine and the respiratory tract (1–4).

As this will become apparent when discussing the structure-function relationship, the various molecular forms of the antibody are highly glycosylated comprising sugar-derived residues in each constituent polypeptide. With respect to pIgA glycosylation, both human IgA1 and IgA2 subclasses have two conserved N-glycan sites on each heavy chain. Moreover, IgA2 preferentially found in secretions, harbors one or two additional N-glycans present on the Cα1 domain. IgA1 is the only subclass with O-carbohydrates in the hinge region. Mice have one class of IgA which is structurally similar to human IgA2 in terms of polypeptide assembly and glycosylation. In comparison with monomeric serum IgA, additional biochemical features found in SIgA include the joining (J) chain and secretory component (SC) (5), a polypeptide comprising the extracellular portion of the precursor pIgR that transports polymeric IgA across epithelial cells, a process also known as transcytosis (6) (**Figure 1**, pathway 1). The J chain, upon covalent binding to two IgA monomers, triggers dimerization (possibly, yet less commonly, oligomerization of higher magnitude) during biosynthesis in mucosal IgA-secreting plasma cells that are abundant in the lamina propria underlying the epithelium. With only one N-moiety, the J chain is the least glycosylated peptide constituent of SIgA. Incorporation of J chain within polymeric IgA (and IgM pentamers) is essential for selective recognition of the two antibody isotypes by membrane bound pIgR or purified free SC from colostrum or from recombinant origin. Carbohydrate residues represent up to 20% of the SC molecular mass, with seven

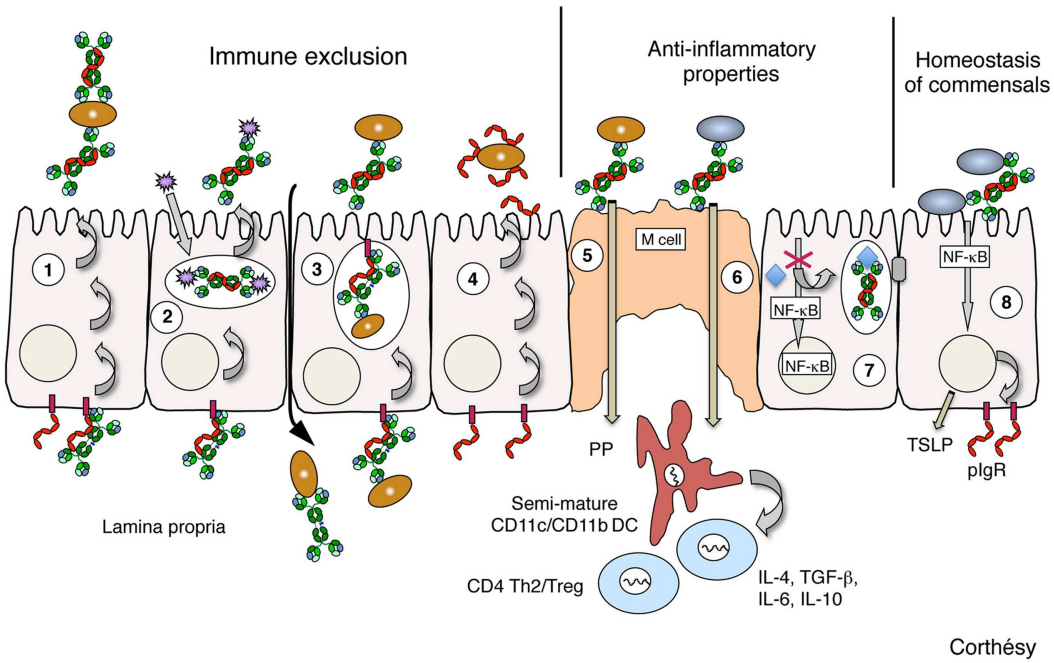

FIGURE 1 | Schematic representation of the identified levels by which polymeric IgA, SIgA, or SC may contribute to protection of mucosal surfaces, as defined in various *in vivo* and *in vitro* models. (1) Polymeric IgA produced by local plasma cells in the lamina propria is transported across epithelial cells (a process referred to as transcytosis) by the polymeric Ig receptor (pIgR), and released in luminal secretions in the form of SIgA performing immune exclusion via interaction with environmental antigens (bacteria, viruses, toxins, etc). (2) Polymeric IgA on their way to pIgR-mediated secretions can intercept incoming viruses intracellularly, and excrete them in the form of non-virulent immune complexes. (3) Polymeric IgA may neutralize in the lamina propria invading infectious agents that have penetrated through breaches occurring in the inflamed epithelium; subsequent transport by pIgR will favor clearance of immune complexes. (4) Via glycans abundantly found on its surface, free SC released in secretions neutralizes pathogen-derived products, and contributes to protection of epithelial surfaces as well; this property is conserved when SC is bound to polymeric IgA in SIgA. (5) Sampling of SIgA by M cells in Peyer's patches (PP) leads to specific targeting of the antibody to dendritic cells (DC) in the subepithelial dome region. In the form of immune complexes with noxious antigens, presentation to naïve T cells in the PP and draining mesenteric lymph nodes (not drawn) results in the onset of attenuated, Th2-biased mucosal immune responses with concomitant quenching of inflammatory circuits. (6) Remarkably, the same SIgA-mediated retro-transport is achieved with commensal bacteria, leading to the shaping of the mucosal immune system toward a non-inflammatory, tolerogenic pattern that takes place through the induction of regulatory T cells. (7) Neutralization of Gram-negative bacterial lipopolysaccharide (LPS) in apical recycling endosomes by transcytosing polymeric IgA abrogates NF-κB-mediated activation of pro-inflammatory gene products, thus preserving the epithelial barrier's integrity. (8) Cross-talk between the probiotic bacteria and the intestinal mucosa is enhanced by SIgA, with various consequences extending from increased expression of epithelial pIgR and tight junction proteins to production of thymic stromal lymphopoietin (TSLP) involved in priming of mucosal DCs. Brown ellipses depict pathogen bacteria; gray ellipses depict commensal bacteria; purple spiky spheres depict virus; polymeric IgA are drawn in green; Free secretory component and polymeric Ig receptor (pIgR) are drawn in red; TSLP, thymic stromal lymphopoietin.

sites of N-glycosylation identified (7). The function of SC in SIgA is manifold (see below), and may justify of why it is released in association with polymeric IgA from its precursor pIgR synthesized by epithelial cells after having ensured single transcytosis.

PROTECTIVE OPERATIVE MECHANISMS RELEVANT TO SIgA FUNCTION

Immune exclusion is the primary mechanism by which SIgA blocks microorganisms and toxins from attaching to mucosal target epithelial cells, thereby preventing surface damage, colonization, and subsequent massive invasion (8). In the context of the gut, immune exclusion is defined as the ability of SIgA, through its recognition of multiple antigenic epitopes on the surface of viruses and bacteria as well as proteins, to cross-link these various antigens in the intestinal lumen and consequently delay or abolish their intrinsic potential to adhere to and/or penetrate the epithelium (**Figure 1**, pathway 1). Such a consensual mode of action of SIgA against bacterial, viral, and parasitic mucosal pathogens, as well as toxins and possibly food allergens, has been defined via compelling evidence from animal models, *in vitro* models and human epidemiological studies.

IgA has been used in humans for passive protection or therapeutic intervention at mucosal surfaces (9–17), yet with different degrees of success, possibly because the complete SIgA molecule was not used. In the intestine of mice, passive oral delivery of specific IgA antibodies also protected against bacterial infections including *Salmonella typhimurium* (18, 19), *Vibrio cholera* (20), *Shigella flexneri* (21), and *Helicobacter pylori* (22). Monoclonal IgA antibodies directed against respiratory syncytial virus applied passively to the nasopharyngeal mucosa of mice subsequently prevented initial infection and pneumonia (23). Similar observations as to the crucial role of passively instilled IgA in

preventing viral infection has been documented for influenza virus (24) and reovirus (25, 26). Intravenous injection of similar virus-neutralizing doses of anti-influenza polymeric IgA mAb, but not monomeric IgA, protected mice against viral infection due to transport into nasal secretions (27). Antigen-specific IgA antibodies produced by an IgA-secreting hybridoma clone implanted in the back of mice (backpack technique) were shown to provide efficient protection against *Vibrio cholerae* (28) and rotavirus (29) following pIgR-mediated transport into secretions. These studies with monoclonal antibodies demonstrated that immunologically naive animals could be protected using IgA as the sole immune agent. While the levels of protection observed in these various experimental settings were generally good, it is important to keep in mind that under natural conditions, the mucosal immune response would be polyclonal, and therefore more effective. In this respect, passive administration of colostrum rich in specific and non-specific SIgA has been shown to protect against gastrointestinal and airway infections (30, 31). In support of these numerous studies underscoring the protective function of SIgA of defined specificities, genetically modified mice unable to produce IgA, J chain, or pIgR all presented deficiencies in their capacity to fight against mucosal infectious agents (32–36).

The use of epithelial cell lines grown as polarized monolayers mimicking the mucosal barrier found in the gut and airways has proven a valuable tool to demonstrate the properties of immune exclusion exerted by IgA/SIgA toward pathogens and toxins *in vitro*. In such models, the antibody acted by blocking binding of cholera toxin (37), *C. difficile* toxin A (38), and ricin (39), thus preventing subsequent damage including fluid loss, cytotoxicity, and intoxication of exposed epithelial cells. Interference with attachment to epithelial cells and blocking of transmission of HIV from epithelial cells to peripheral blood mononuclear cells used as viral target was confirmed as a valid mechanism of action of HIV gp120-specific IgA (40). The crucial implication of the IgA isotype antibody in the process was further exemplified by the demonstration that SIgA, but not IgG, isolated from seropositive patients prevented HIV entry (41, 42). Along the same line, adhesion of enteropathogenic *Escherichia coli* strains capable of targeting epithelial cells could be inhibited by SIgA (43). When tested, polymeric IgA turned out to be systematically superior in maintaining cell integrity as compared to monomeric IgA or IgG of the specificity, indicating that the highest avidity associated with polymeric antibodies was important in the process of neutralization, possibly by favoring agglutination (38, 44).

Such an *in vitro* model has further shown its value by underscoring the ability of transcytosed SIgA to neutralize invading influenza, Sendaï, or rotaviruses intracellularly (45–48) (**Figure 1**, pathway 2). During their journey to the apical surface, specific polymeric IgA antibodies colocalized with viral hemagglutinin, neuraminidase, or surface viral proteins within the apical recycling endosomes, thus preventing intracellular replication or assembly, eventually resulting in reduced viral titers in the supernatant and cell lysates. Apical to basolateral transcytosis of HIV isolates across polarized epithelial cell monolayers demonstrated that HIV dissemination was blocked by polymeric IgA directed against the glycoprotein (gp)41 envelope protein, thus excluding the virus from spreading to the lamina propria (49). As for other viruses, intracellular neutralization took place inside the apical recycling endosome, and SIgA-based immune complexes were selectively recycled to the apical, lumen-like surface of the polarized monolayer. Intracellular neutralization with transcytosing IgA directed against HIV gp120, but not IgG with identical Fv domains, was accompanied by inhibition of viral replication inside epithelial cells (50). Interestingly, neutralization was dependent on the concentration of polymeric IgA added in the basolateral, serosal-like compartment, and reached a plateau which corresponded to the SIgA content in human secretions, i.e., about 100 μg/ml (51). The excretory function of SIgA appears as another plausible mechanism that contributes to microbial elimination at mucosal surfaces: when soluble polymeric IgA-based immune complexes were added to the basolateral compartment of polarized monolayers of epithelial cells expressing pIgR, the complexes were transported intact to the apical side (52). Capture of an antigen by polymeric IgA present in the lamina propria and subsequent secretion by intestinal crypt cells expressing high amount of basolateral pIgR was further demonstrated *in vivo* (53) (**Figure 1**, pathway 3).

From these various modes of action, one can conclude that multiple levels of SIgA-mediated protection fulfill complementary functions in order (1) to create a barrier at mucosal surfaces, (2) to eliminate within epithelial cells, or (3) to keep noxious microorganisms away from the body's internal compartments. Although not tackled in this review, one has to keep in mind that a variety of other back-up mechanisms involving systemic IgA and FcαR1-bearing cellular partners are available to ensure efficacious protection against a myriad of pathogenic antigens (54, 55).

In human, correlation between resistance to infection and high specific SIgA titers was described in several studies dealing with immunity to *Vibrio cholera* infection. The presence of LPS-specific SIgA, as determined by antibodies measured in feces by ELISA, and in ELISPOT assays detecting antibody-secreting cells, allowed establishment of a strong association between SIgA and reduced level of infection (56, 57). Correlation between a strong mucosal IgA response and protection against influenza virus was also documented in vaccinated mice (58). Humans suffering from IgA deficiency (IgAD) exhibit increased frequency of upper respiratory and gastrointestinal tract infections (59, 60), yet the consequences are not always profound, mostly because compensatory adaptive or innate mechanisms such as the substitution of SIgM for SIgA take over (61). Moreover, assessment of the true contribution of IgA is complicated by the fact that the lack of IgA is rarely absolute and may be accompanied by deficiencies in other isotypes. The association of certain major histocompatibility complex haplotypes (62) and mutations in transmembrane activator and calcium-modulating cyclophilin ligand interactor (63) in patients with IgAD may further contribute to confusion when it comes to assigning a direct and unique role to IgA in the prevention of gastrointestinal diseases (64). In this respect, IgA-knock-out mice appear to display the same alterations in the expression of other isotypes and defects in immune responses (65, 66), thus making it difficult to draw unambiguous conclusions.

MULTIPLE FACETS OF THE FUNCTIONALITY OF SC IN SIgA ANTIBODIES

Free SC is composed of five Ig-like domains folded as compact ellipsoids stabilized internally by several disulfide bridges. Overall, the molecule displays a J-shape with all seven glycosylation sites

exposed on the same surface, away from the binding site for polymeric IgA (67). The specific and stable interaction of SC with polymeric IgA in SIgA involves basically all domains, with domain 1 serving as the original anchoring site for polymeric IgA, and domains 2 and 3 spatially constraining domain 5 to ensure formation of a productive disulfide bridge with one Cα2 domain of one monomer in polymeric IgA (68, 69). Three dimensional analyses of human SIgA1 and SIgA2 subclasses shows SC wrapping domains Cα2 and Cα3 of polymeric IgA compactly, a feature that may be essential to the remarkable stability of the antibody (70). While exposure to intestinal proteases of polymeric IgA leads to rapid degradation into Fab and F(ab′)2 fragments, cleavage sites within domains Cα2 and Cα3 are masked in the presence of bound SC, resulting in a close to 24-h delay in enzymatic clipping (71, 72). Cross-protection takes place, as bound SC remains unaffected, in contrast to free SC, which is rapidly and totally degraded, to an extent similar to control IgG antibodies. Stability of SIgA is also increased upon binding of antigens of various size and nature: hierarchy shows the best protection toward proteases following interaction with a bacterium, then with a virus, and finally with a protein toxin (73). Such intrinsic properties makes SIgA well-suited to survive the hostile environment that prevails in the gut, and allowing to fulfill its protective function.

Another characteristics of bound SC in SIgA is its ability to confer hydrophilic properties to the Fc fragment of the antibody via the seven surface-exposed N-linked oligosaccharides equipped with terminal sialic acid residues. It is thought that this pattern is important for interaction with mucus, and therefore proper location of the antibody in the close proximity to the mucosal surfaces it is supposed to protect. SIgA-based immune complexes tethered within the mucus layer overlying the epithelium further limits diffusion in the luminal environment and aids their clearance from the gut via peristalsis (74). SIgA anchoring in mucus may account for the observation that the outer mucus layer is the preferential habitat for the microbiota in the colon (75). However, the identification of CD71 as a SIgA receptor on the apical surface of intestinal epithelial cells (IECs) grown in Ussing chambers (76), together with the fact that SIgA binds commensal bacteria via SC (77) suggests that a more dynamic situation occurs in this part of the gut. In a mouse model of lung infection by *Shigella flexneri*, mucus-mediated anchoring of SIgA was found to be instrumental to guarantee neutralization of the bacterium preventing entry into the tissue (78); polymeric IgA mostly found in the lumen of the nasal cavity and bronchi was inefficient at protecting the mice. Similarly, removal of carbohydrate chains of SC within SIgA molecules abolished anchoring to mucus and associated protective function (79). SC can thus be seen as an essential constituent of SIgA, in that it ensures both sustained stability and proper localization in the mucosal environment, two features instrumental to the optimal function of the antibody.

In SC, N-glycans contribute to approximately 20% of the molecular weight of the protein, and endow SIgA with further binding sites for bacterial lectin-like adhesins, in addition to the four Fab domains (80). For example, SC interacts directly with a surface protein of *Streptococcus pneumoniae*, choline binding protein A (CbpA), a bacterial factor involved in colonization of the nasopharynx of rats (81). Binding was dependent on amino acid sequences present in domains 3 and 4 of SC (82) and on a highly conserved hexapeptide motif within CbpA (83). It was proposed that such an association will serve to preclude contact with epithelial cells, yet a report using the unencapsulated *S. pneumoniae* strain Rx6 had described facilitated invasion of pIgR expressing Detroit 562 cells (84). The innate-like properties of free SC in the defense against mucosal pathogens was further demonstrated in the case of *Clostridium difficile* toxin A and enteropathogenic *Escherichia coli* intimin (85) (**Figure 1**, pathway 4); neutralization of the bacterial products by free SC or non-specific SIgA prevented infection of target epithelial cells via interaction with sialic and galactose residues displayed on the surface of SC. Denaturation of the free SC polypeptide scaffold brings sugar moieties in a conformation no longer able to interact with the bacterial epitopes, arguing for the possibility that finely tuned spatial disposition is important for specific recognition by complex carbohydrates.

The importance of the glycosylated nature of SC and SIgA in exerting their protective function can be further illustrated as for instance in the case of sialyloligosaccharides preventing epithelial adhesion of *Escherichia coli* through type I fimbrial lectin (86, 87). SC in SIgA recovered from human colostrum was described to inhibit adhesion of *Helicobacter pylori* to human gastric surface mucous cells in a fucose-dependent manner (88). Carbohydrate side chains in SIgA serve as a docking site for ricin toxin; human SIgA with no Fab-dependent specificity for ricin reduced attachment to the apical surface of epithelial cell lines in culture and to the luminal surfaces of human intestinal villi via SC and the IgA heavy chain (89). Although not a universal mechanism, these many examples identify free and bound SC as microbial scavengers contributing to the anti-pathogenic arsenal that protects the body epithelial surfaces.

INTERFERING EFFECTS OF SIgA ON FITNESS OF INFECTIOUS BACTERIA

Blocking of interaction with epithelial cells possibly through agglutination of mucosal microorganisms may not be the only mechanism by which SIgA exerts its protective function. Recent evidence argues for a more direct effect on the bacterial viability or pathogenicity, as for example by perturbation of the bioenergetic machinery, impact on motility, disruption of virulence factors involved in bacterial entry (90). For example, in the presence of sub-agglutinating amounts of IgA specific for the O-antigen of LPS (Sal4 mAb), the capacity of *Salmonella typhimurium* to invade epithelial cell monolayers was reduced by a factor of 20 (91). In support of this observation, Fab fragments derived from the same IgA, although unable to trigger agglutination, blocked entry as efficiently as the whole antibody molecule. In addition, treatment with Sal4 led to a complete paralysis of the bacterium within 15 min, again independently of agglutination (91). These data are consistent with the idea that IgA-mediated interference with motility and entry accounts for the protective function of Sal4 in the case of *Salmonella* invasion. Further studies revealed that Sal4 treatment impaired T3SS-mediated translocon formation and attenuated the delivery of tagged effector proteins into target epithelial cells (92). Changes in surface ultrastructure, alterations in outer membrane permeability, a partial reduction in membrane energetics and intracellular ATP levels were all detected upon association

of Sal4 IgA with *Salmonella*, a series of features that can render the bacterium avirulent. This occurs by triggering a cyclic dimeric guanosine monophosphate-dependent signaling pathway through YeaJ, a proposed inner membrane-localized diguanylate cyclase and a known regulator of cellulose biosynthesis. For the bacterium, this results in loss of motility due to exopolysaccharide production and biofilm formation (93). From an antibody point of view, IgA possesses the ability to convert *S. typhimurium* from an invasive, motile status to a non-motile, avirulent condition via direct impact on several metabolic pathways. A similar inhibitory mechanism occurs upon binding of a murine monoclonal IgA (IgAC5) to the O-antigen of *Shigella flexneri* serotype 5a (94): transient impairment (45–60 min) of the T3SS, which is necessary for bacterial entry into IECs is coincident with a partial reduction in the bacterial membrane potential and a decrease in intracellular ATP levels.

THE ROLE OF SIgA IN CONTROLLING EPITHELIAL TRANSPORT

An extension of the function of SIgA at mucosal surfaces is the importance of immune exclusion for the protection of the host against excessive antigenic challenge from environmental macromolecules. IgAD subjects with IgE-mediated atopic disease had increased allergen penetration through mucosal membranes and formation of circulating immune complexes (95, 96) initially suggested that SIgA had a role in controlling absorption of food antigens and in reducing susceptibility to atopic allergies. Experiments performed in mouse models of airway allergy supported the finding that antigen-specific SIgA suppresses features associated with inflammation and asthma (97–100). The importance of IgA in the process was further illustrated in the gut by the finding that mice sensitized with bovine lactoglobulin had much lower frequencies of IgA-producing cells in Peyer's patches, as well as reduced fecal SIgA when compared to mice actively tolerized with the same protein (101). The production of saliva antigen-specific SIgA was consistently enhanced in a mouse model of allergic asthma in which sublingual vaccination triggered protection against subsequent challenge (102). However, allergen-specific SIgA is not always increased in successfully tolerized animals, and can even be present in large amounts in sensitized ones without conferring protection (103). Oral tolerance can be induced in pIgR knock-out mice lacking SIgA, with protection against systemic hypersensitivity ensured via compensatory Treg function (104). This series of contradictory results in allergy and inflammatory diseases adds to the continuing debate about the protective role of SIgA in these deleterious processes. Moreover, the importance of SIgA against allergic diseases remains unclear with respect to recent clinical studies. Patients with IgAD displayed increased risk of food hypersensitivity at the age of 4 years (105), whereas in another cohort, IgAD did not show any correlation with food allergy (106). Further studies are required to clarify the importance of SIgA in the maintenance of local tolerance, and eventually the integrity of the intestinal barrier.

In addition to play an essential role in immune exclusion, SIgA, in contrast to IgM and IgG, exhibits the striking ability to adhere selectively to the apical membrane of M cells overlying mouse and human Peyer's patches (107, 108). Subsequent limited transport across the epithelium resulted in the presence of small amounts of SIgA in the M cell pocket and in processes that extend in the basal lamina (109). To date, an M cell-specific receptor ensuring controlled retro-transcytosis of SIgA has not yet been identified, although one can speculate that it needs to display particular properties (low expression, binding activity in the presence of a co-receptor, recognition of altered molecular forms of SIgA) to avoid overwhelming entry of the large excess of SIgA in the intestinal lumen. *In vivo* uptake of SIgA delivered into mouse ligated ileal loop containing a Peyer's patch resulted in specific targeting to, and internalization by dendritic cells (DC) in the subepithelial dome region (110). *Ex vivo*, only $CD11c^{+}CD11b^{+}$ DC isolated from Peyer's patches and draining mesenteric lymph nodes showed selective binding and internalization mimicking the *in vivo* situation (111) (**Figure 1**, pathway 5). Interestingly, in mucosal tissues, such DC are poor producers of IL-12 but potent inducers of IL-10 secreting T cells (112) and IgA production from naïve B cells (113). DC-SIGN was recently identified as a possible candidate for SIgA recognition by mouse DC (114), while the existence of CD89 and CD71 (transferrin receptor) has been documented on maturing human DC (115). In support of these complementary mechanisms, modulation of DC function with inhibition of IL-12 production by IgA has been recently described (116).

Such observations led to the obvious question of the immunological relevance of the transport of SIgA molecules across the M cell and its subsequent association with DC. When administered orally in the presence of the mucosal adjuvant cholera toxin (117), genetically engineered SIgA carrying a foreign epitope from *Shigella flexneri* invasin B triggered the production of both salivary and systemic antibodies specific for the bacterial antigen (118). To further assess the nature of the mucosal immune response following re-entry of SIgA across the intestinal mucosa, mice were immunized orally with heterologous SIgA consisting of mouse polymeric IgA and human SC in the absence of any adjuvant. Engineered SIgA triggered production of human SC-specific antibodies and mixed Th1/Th2 type responses, preserved or induced IL-10 and TGF-β expression in MLN, and migration and maturation of DC along the Peyer's patch-MLN-spleen axis (119) (**Figure 1**, pathway 5). By comparison with human SC adjuvantized with cholera toxin, it turned out that SIgA induced low degrees of activation in a non-inflammatory context favorable to preserve local homeostasis of the gastro-intestinal tract. Neutralization of *Shigella flexneri* by SIgA led to local suppression of pro-inflammatory circuits leading to gut tissue damages, a feature resulting form the stability of the immune complex in the harsh intestinal environment (120) (**Figure 1**, pathway 7).

An intriguing possibility in the context of SIgA-based immune complexes would be that these latter contribute to local immunomodulation, or early in life, to educate the mucosal immune system toward a tolerogenic profile. In support of this, milk antibodies, and in particular SIgA, prevents neonatal responsiveness against commensal bacteria (121). In this respect, timely provision of a set of maternal antibodies fitting the newborn gut microbiota primarily represented by a hand-over from the mother (at least after "classical" vaginal delivery) may justify from such regulatory mechanisms. It makes a sense to speculate that maternal milk SIgA antibodies passing across the epithelium

direct associated antigens to DC, and shapes the gastro-intestinal immune system both in terms of defense or tolerization during initial exposure to non-self antigenic structures. Based on the evidence of SIgA re-entry into Peyer's patch, a broad interpretation of the data would suggest that SIgA-coated, neutralized bacteria could prime the immune system of naïve individuals within a whole population in the absence of global infection.

THE ROLE OF SIgA IN REGULATING THE MICROBIOTA

More recently, SIgA has been identified as a necessary partner in maintaining the fragile balance between the triad composed of the microbiota, the IECs lining the gastro-intestinal tract and the underlying mucosal immune system. The homeostatic control taking place at gut mucosal surfaces is essential to keep billions of colonizing, and at first sight potentially harmful microorganisms in order to ensure optimal symbiosis with the host. Indeed, any potential dysfunctions can lead to the development of pathologies such as inflammatory bowel diseases (122), or affect processes of extraction of energy and digestion of otherwise unavailable sources of nutrients such as the final degradation of carbohydrates. Commensal bacteria have been directly associated with the proper development of gut-associated lymphoid tissues such as isolated lymphoid follicles (123) or with the secretion of normal levels of SIgA (124) with unknown specificity called "natural" SIgA (125). It appears that the IgA repertoire is restricted to a minimum considering the enormous varieties of antigens encountered at mucosal surfaces (126), arguing in favor of the presence of polyspecific, low affinity antibodies in intestinal secretions (127, 128). This notion was challenged by a recent study using high-throughput sequencing to investigate the shaping of the IgA repertoire (129). Analysis of more than one million V_H sequences revealed that the IgA repertoire comprised both highly expanded and low frequency clones which both contributed to high diversity, a phenomenon amplified with aging due to hypermutation. Similar to mice IgA sequences, human VH sequences carry numerous somatic hypermutation (130). Whether this process relies on the reutilization of germinal centers in multiple Peyer's patches as recently identified (131) is in need of further investigation. Programed cell death protein 1 knock-out mice that have elevated numbers of Peyer's patch Treg cells exhibit changes in the binding capacity of their SIgA, which in turn affects the nature of the commensal bacteria (132). The fact that commensal bacteria are naturally coated by SIgA in feces of humans and mice strongly suggests that this interaction is necessary to maintain a steady-state commensal colonization. Mice expressing an activation-induced cytidine deaminase hypomorph (which disrupts somatic hypermutation but still supports class switch recombination) display changes in the composition of their microbiota (133). Together, this suggests that SIgA keeps the microbiota at bay using both Fab-dependent adaptive and glycan-mediated innate immune interactions.

By using free SC and non-specific SIgA (purified from hybridoma cell lines and colostrum) serving as substitutes of natural mucosal antibodies, the molecular basis pertaining to the interaction between SIgA and intestinal resident bacteria, i.e., *Lactobacillus*, *Bifidobacteria*, *Escherichia coli*, and *Bacteroides* strains, was identified as the many glycans residues carried by SC (77). While the interaction with Gram-positive bacteria indicated the essential role of carbohydrates in the process, binding to Gram-negative bacteria was preserved whatever the molecular form of protein partner used, suggesting the involvement of different binding motifs. Poor or absent association between Gram-positive bacteria and control IgG identified the critical role of sugar moieties in SC in selective binding of the highly diverse microbiota by the whole SIgA protein.

Recognition of commensal bacteria by IECs has been recognized to play a fundamental role in mucosal homeostasis by promoting for instance cytokine release, cell expansion, and reinforcement of the barrier integrity (134–136). Further, commensal strains coated by SIgA can potentiate the responsiveness of reconstituted IEC monolayers *in vitro* (137) (**Figure 1**, pathway 8). Unexpectedly, association with SIgA increased the bacterial anchoring at the apical surface of IECs, resulting in the reinforcement of the barrier integrity through increased phosphorylation of tight junction proteins promoting cell-to-cell contact. In addition, secretion of pro-inflammatory cytokines/chemokines by IECs was quenched, while expression of pIgR was promoted. As pIgR is involved in transcytosis of SIgA from the basolateral to the apical pole of IECs one can conclude that commensal bacteria complexed with SIgA generate a positive feedback on pIgR expression, leading to more receptors being available for active SIgA transcytosis. This phenomenon could account for the sustained SIgA secretion resulting from commensal colonization as observed previously (138). This contributes to further defining the function of SIgA in keeping commensal bacteria at bay through a delicate balance combining appropriate neutralization and proper sensing by the IECs. Whether the presence of the transferrin receptor (CD71) capable of binding SIgA at the apical pole of IECs (76) is involved in governing binding of SIgA-commensal bacteria complexes remains to be determined. Early in life, the role of maternal SIgA may be considered of primordial importance in limiting a potential inflammation induced by primary colonization in the gut of newborns. The presence of SIgA could contribute to the initial sensing of the newly implanted microbiota and allow proper development of the immune system under non-inflammatory conditions. Such a mechanism might be relevant to the understanding of inflammatory bowel disease which is, among others, associated with deregulated inflammatory responses to intestinal bacteria (139).

While data reported above shed light on the role of SIgA in mucosal monitoring of commensals by IECs, they do not say much on how the communication with partners of the underlying immune system is established. Limited uptake of bacteria including a *Lactobacillus* and a *Bacteroides* occurs through sampling by M cells found in intestinal Peyer's patches, and regulated entry can be promoted upon association with non-specific SIgA (140) (**Figure 1**, pathway 6). The almost absent transepithelial passage observed in germ-free mice having barely detectable gut SIgA can be compensated for by administration of pre-formed SIgA-bacteria complexes. Commensal bacteria given alone get coated with endogenous SIgA within 3 h, strongly suggesting that association takes place under steady-state conditions anytime, and hence participates in keeping the large majority of bacteria in the intestinal lumen. The role of SIgA in shaping the gut microbial community composition may arise from its ability to suppress expression of certain bacterial epitopes (141), and therefore favor the fitness of one species or genus over others. Selective

SIgA-mediated targeting of bacteria is restricted to the tolerogenic $CD11c^{+}CD11b^{+}CD8^{-}$ DC subset and macrophages located in the subepithelial dome region of Peyer's patches, indicating that the host is not ignorant of its resident commensals (140). Upon coating of commensal bacteria, natural and/or specific SIgA largely maintains luminal compartmentalization of the microbiota, while occasionally permitting rare translocation events necessary to control the continuous dialog between the host's immune system and its resident symbionts. Commensal bacteria associated with local DC in the subepithelial dome region do not penetrate further than the draining mesenteric lymph nodes, resulting in the confinement of immune induction against the microbiota to the mucosa (142, 143). Making the systemic immune system relatively ignorant of these organisms at this stage would permit adequate stimulation in the case of sepsis. While transport of SIgA alone or in complex with protein antigens or bacteria through Peyer's patch M cells is well established, it remains to be determined whether other transepithelial pathways including for example M cells in isolated lymphoid follicles (144), lamina propria DC snorkeling dendrites across the tight epithelium (145), Peyer's patch DC extending dendrites around M cells (146), or Goblet cell-mediated passage (147) can account for selective sampling and targeting of cells regulating intestinal immune responses.

CONCLUSION

Mucosal surfaces at the interface between the external world and the inside of the body are the primary sites of continuous challenge with potentially infectious agents, commensal bacteria, and foreign proteins. Maintenance of the integrity and selective function of these delicate epithelia implies that tightly controlled homeostasis is ensured anytime. As a consequence, depending on the nature of the stimulus, very different immunoregulatory mechanisms have to be duly activated. A prominent effector in this network, SIgA plays a crucial role in the essential communication occurring between the host's mucosal environment and the proper sensing of harmless inhabitants or noxious pathogens/antigens (**Figure 1**). To fulfill this demanding multi-task function, SIgA displays several properties that extend from classical immune exclusion and permanent checking of the microbiota to local immunomodulation via intricate contacts with microorganisms, epithelial cells including enterocytes and M cells, and DC in the mucosal associated lymphoid tissue. It must be emphasized that biochemical features associated with SIgA, such as stability in an aggressive medium, anchoring in mucus, heavy glycosylation, Fab-independent recognition of antigens, transcytosis and retro-transcytosis across the intestinal epithelium all contribute to allow the antibody to perform optimally in the particular environment of mucosal surfaces.

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The intestinal B-cell response in celiac disease

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The function of intestinal immunity is to provide protection toward pathogens while preserving the composition of the microflora and tolerance to orally fed nutrients. This is achieved via a number of tightly regulated mechanisms including production of IgA antibodies by intestinal plasma cells. Celiac disease is a common gut disorder caused by a dysfunctional immune regulation as signified, among other features, by a massive intestinal IgA autoantibody response. Here we review the current knowledge of this B-cell response and how it is induced, and we discuss key questions to be addressed in future research.

Keywords: celiac disease, autoantibodies, mucosal immunity, intestinal mucosa, B cells

The immune system has evolved multiple strategies to maintain intestinal homeostasis. A unique feature of intestinal immunity is the ability to provide protection toward pathogens while preserving the number and composition of the commensal bacteria in a state of mutualism (Hooper and Macpherson, 2010). Secretory IgA is considered to be one of the crucial immune effector mechanisms in maintaining homeostasis at mucosal surfaces (Brandtzaeg et al., 1999; Strugnell and Wijburg, 2010). Therefore, it is not surprising that the mucosal immune compartment is tightly regulated. In this review, we aim at summarizing and discussing the basic concepts of intestinal immunobiology in the context of a prevalent disorder, celiac disease. The study of this condition provides unique understanding of how an intestinal IgA response is induced and reshaped from the healthy to the affected intestinal state, as well as it pinpoints areas of scarce knowledge and poses key questions for future research.

FEATURES OF INTESTINAL IMMUNITY

THE INTESTINAL IMMUNE SYSTEM

The generation of secretory IgA is confined to intestinal lymphoid microenvironments that are composed of the inductive and the effector tissue compartments (Brandtzaeg and Pabst, 2004). The inductive compartment of the intestinal immune system consists of gut-associated lymphoid tissue (GALT) and the regional lymph nodes, whereas the effector compartment consists of the lamina propria (LP) and surface epithelia. Together, these form the largest effector organ of humoral immunity, containing at least 80% of the body immunoglobulin (Ig)-producing cells (Brandtzaeg and Johansen, 2005).

GUT-ASSOCIATED INDUCTIVE LYMPHOID TISSUE

The GALT is the main site for the induction of mucosal IgA B cells. The GALT is comprised of aggregated lymphoid follicles, termed Peyer's patches and isolated lymphoid follicles. Gut-associated lymphoid follicles are organized structures covered with a specialized follicle-associated epithelium that contains microfold cells (M cells; Neutra, 1999). In the canonical pathway, antigens from the gut lumen are internalized and delivered to subepithelial dendritic cells (DCs) via M cells or receptor-mediated endocytosis by epithelial cells (Neutra et al., 2001). Antigen-loaded DCs migrate from the subepithelial dome into the perifollicular T cell-rich area, where they can induce a response of helper T cells (Rimoldi et al., 2005). B cells become activated by the presentation of antigens from follicular DCs (Harwood and Batista, 2010) and by CD40-mediated signals from antigen-primed helper T cells (Elgueta et al., 2009). Gut-associated lymphoid follicles are, therefore, characterized by germinal centers (GC) that promote antigen-specific interaction between T and B cells, an essential mechanism for B cell differentiation and diversification. The GALT further drives intestinal IgA production by providing cytokines with IgA-inducing functions, including transforming growth factor-β (TGF-β; Gonnella et al., 1998; Stavnezer and Kang, 2009), retinoic acid (RA; Mora et al., 2006), IL-6 (Sato et al., 2003), and inducible nitric oxide synthase (iNOS; Tezuka et al., 2007). These events lead to up-regulation of the gene encoding for the enzyme activation-induced deaminase (AID), which is central to both class-switch recombination and somatic hypermutation of Ig genes (Muramatsu et al., 2000). This T cell-dependent pathway usually results in generation of plasma cells (PCs) producing intestinal IgA antibodies with high rates of somatic hypermutation (SHM), as well as memory B cells (Bemark et al., 2012). CD40-mediated signal from T cells is crucial to GALT GC initiation (Bergqvist et al., 2006), although it is possible that human intestinal GC-associated B cell responses are not exclusively dependent on cognate T cell/B cell interactions (Spencer et al., 2012). Studies have indicated that

GCs can appear in Peyer's patches and mesenteric lymph nodes without the need for the classic T cell/B cell interaction based on BCR specificity (Casola et al., 2004). Accordingly, SHM can take place as an antigen-independent process without being necessarily linked to affinity maturation (Reynaud et al., 1995; Casola et al., 2004). B cells that are activated in this way are thought to depend on "bystander" T cell help in the form of cytokines such as IL-5, IL-6, and IL-10 in order to induce an IgA response (Jiang et al., 2004).

T cell-independent intestinal IgA responses have been reported in both humans (Ferrari et al., 2001; Levesque et al., 2009) and mice (Guy-Grand et al., 1975; Mombaerts et al., 1994). In mice a T cell-independent, antigen-driven pathway in response to highly conserved microbial antigens recognized by Toll-like receptors (TLRs; Cerutti, 2008) generates a primitive intestinal IgA antibody repertoire to commensal bacteria (Macpherson et al., 2000; Bergqvist et al., 2006, 2010). TLR-triggered class switching to IgA is mediated by BAFF and APRIL expression in DCs, monocytes, macrophages, granulocytes, and intestinal epithelial cells (Fayette et al., 1997; Litinskiy et al., 2002; Cerutti et al., 2011). T cell-independent intestinal IgA responses in mice originate from peritoneal and intestinal LP B-1 cells as well as conventional follicular B-2 cells (Fagarasan et al., 2010). Thus, it has been suggested that the intestinal LP may act as a potential site for T cell-independent IgA induction. A lack of consensus, as well as a lack of homology between murine and human models (Gibbons and Spencer, 2011) as the human equivalent of mouse B-1 cells is still not well described (Descatoire et al., 2011), does however exist when it comes to the LP being a site for IgA class switching and diversification (Fagarasan and Honjo, 2000; Macpherson et al., 2000; Fagarasan et al., 2001; Shikina et al., 2004; Boursier et al., 2005; Crouch et al., 2007; He et al., 2007; Barone et al., 2009). Although T cell-independent responses are biologically possible and certainly relevant in mice, in humans their contribution to the intestinal IgA repertoire remains uncertain.

GUT-ASSOCIATED EFFECTOR LYMPHOID TISSUE

The intestinal LP acts as the effector compartment of the humoral mucosal immune response, and contains predominantly terminally differentiated PCs (Farstad et al., 2000). These are characterized by the expression of the markers CD138 and CD27, whereas levels of CD19 and CD45 are more heterogeneous; the lack of expression of the Ki-67 marker indicates that these cells are not blasting and should therefore be referred to as PCs rather than as plasmablasts (Di Niro et al., 2010). Human intestinal PCs preferentially produce IgA dimers (80%) and IgM pentamers (20%), whereas a small fraction produces IgG (Brandtzaeg et al., 1999; Brandtzaeg and Johansen, 2005). The amount of Ig produced by intestinal PCs is massive, being estimated at 3 g/day in adults (Brandtzaeg and Johansen, 2005). Dimeric IgA is formed of two monomeric IgA units joined by a bridging J chain that is recognized by the polymeric Ig receptor (pIgR), an antibody transporter expressed on the basolateral surface of epithelial cells (Brandtzaeg and Prydz, 1984; Johansen et al., 2001). Upon interaction with pIgR, dimeric IgA is translocated to the surface of epithelial cells as secretory IgA where it exerts its function of immune exclusion, intracellular neutralization, and antigen excretion (Cerutti and Rescigno, 2008; Strugnell and Wijburg, 2010). The intestinal LP in addition to PCs contains a variety of other cell types, including macrophages, DCs, and neutrophils which in association with epithelial cells may play a crucial role in creating and maintaining the niche for PC survival (Benson et al., 2008).

Antigen-specific IgA antibodies generated upon immunization can be identified only locally (Ogra and Karzon, 1969) or both systemically and locally (Crabbe et al., 1969) depending on the immunization strategy. These observations, together with the fact that IgA reactive to commensal bacteria is exclusively present in gut secretions (Macpherson et al., 2000), support the notion of mucosal immunity as an independent compartment. Rotavirus-specific memory B cells were shown to have different antibody repertoires (Weitkamp et al., 2003) than effector mucosal PCs (Di Niro et al., 2010), which raises some questions about the nature of the interplay between these two compartments.

THE INTESTINAL IgA REPERTOIRE

In principle, both unmutated IgA antibodies with broad reactivity to self and non-self antigens and somatically mutated antigen-specific IgA antibodies could contribute to the intestinal PC repertoire. There are several observations suggesting that the latter pathway may be dominating. Barone et al. (2011) reported results supporting GC origin of human intestinal IgA-producing PCs. Cloning and sequencing of Ig variable region genes of IgA PCs from LP of human small intestine revealed uniformly high degrees of SHM and high ratios of replacement to silent (R/S) mutations in complementarity determining regions (CDR), which argues in favor of antigen-mediated selection (Dunn-Walters et al., 1997; Boursier et al., 1999). Our group isolated human intestinal PCs specific to rotavirus and cloned antibody genes, observing high numbers of mutations (17 mutations per sequence on average, with an R/S of 2.3, in the VH only; Di Niro et al., 2010). Similarly, Benckert et al. (2011) cloned hundreds of human monoclonal antibodies (hmAbs) from IgA and IgG intestinal plasmablasts from the ileum of healthy donors. Regardless of their reactivity, the antibodies had many mutations, averaging 23 in the VH. The majority of the antibodies were specific for foreign or self antigens whereas 25% were polyreactive. Only 7% of IgA displayed cross-reactivity with diverse bacterial strains. To note, the polyreactive antibodies also had high degree of SHM suggesting that antibody polyreactivity of gut plasmablasts may be acquired by somatic mutations. Selection of somatically mutated variants of polyreactive antibodies may simultaneously act as a parallel mechanism in defining and contributing to antigen-specific immune response against foreign antigens. Revision of light chains expressed by IgA PCs is another distinct feature of human intestinal PCs and it is confined to gene rearrangements at the lambda loci (Su et al., 2008). This has been suggested as a beneficial mechanism in order to diversify the intestinal IgA repertoire and to remove non-functional or autoreactive antibodies.

Contrary to initial studies suggesting that the intestinal IgA repertoire is oligoclonal and of low diversity (Dunn-Walters et al., 1997; Holtmeier et al., 2000; Yuvaraj et al., 2009), Lindner et al. (2012) using a high-throughput sequencing method, recently demonstrated that the intestinal IgA population in mice is highly polyclonal. The repertoire is comprised of both highly expanded

and low frequency clones, and with age new clones are introduced. Notably, expanded clones with previously selected specificities repopulated the small intestine after PC depletion and showed similar SHM frequencies, hence indicating the presence of a functional intestinal IgA memory compartment. A polyclonal and highly diverse IgA repertoire would parallel the broad range of intestinal antigens to which the intestinal mucosa is constantly exposed, although it also appears that the repertoire continuously adapts to the current composition of the microflora (Hapfelmeier et al., 2010). The high-throughput analysis of the intestinal IgA repertoire performed by Lindner et al. (2012) also suggested that clonal expansion is likely to occur predominantly in the periphery rather than locally in the LP, which has been a subject of debate in previous studies (Yuvaraj et al., 2009).

Taken together, these studies strongly indicate that the great majority of gut IgA antibodies develop from antigen-specific B cell responses, which evolve by acquisition of somatic mutations to confer effectiveness and high specificity.

LONGEVITY OF INTESTINAL IgA PLASMA CELLS

Another matter of debate has been whether human intestinal IgA PCs can provide long-term humoral immunity. Evidence for a long-lived, commensal-specific IgA response was observed when germ-free mice were reversibly colonized by bacteria (Hapfelmeier et al., 2010). In absence of competition from newly generated cells, IgA PCs were shown to have a half-life of at least 16 weeks. The dynamic of such IgA response, however, reflected the contents of the intestinal lumen, suggesting that the number of long-lived PC niches is limited. As a consequence, in presence of competition such as that deriving from the continuously evolving microflora, "older" PCs – despite their long-lived potential – are constantly displaced by new ones, which are generated in response to the most recent stimuli. In agreement with these observations, we have demonstrated that the human small intestine harbors a population of non-proliferating PCs that are maintained by the local supportive microenvironment for long-term survival (Mesin et al., 2011). Moreover, an inflammatory microenvironment may enhance the niche capacity, resulting in more robust PC responses (Radbruch et al., 2006).

MUCOSALLY INDUCED TOLERANCE

The homeostatic role of the intestinal immune system is to provide suppressed immune responses as to generate mucosally induced tolerance. Such tolerance can be directed toward orally administered antigens or toward gut bacteria. Thus, there are two layers of intestinal anti-inflammatory homeostatic mechanisms: immune exclusion of commensal bacteria by secretory antigen-specific IgA and immune suppression to avoid hypersensitivity to innocuous food antigens. These two mechanisms of mucosally induced tolerance appear to act independently in order to attenuate a broad range of immune responses (Weiner et al., 2011; Pabst and Mowat, 2012). The lack of such homeostatic tolerance results in intestinal immune pathology. Active proinflammatory immune responses directed toward the gut microbiota, inducing imbalance in IgA and IgG repertoires, are associate with the development of inflammatory bowel disease, such as Crohn's disease and ulcerative colitis (Baklien and Brandtzaeg, 1976; Macpherson et al., 1996). In celiac disease (CD) there is an active proinflammatory immune response to cereal gluten antigens.

CELIAC DISEASE

Celiac disease is a common intestinal disorder affecting 1% of the population in Europe and the US, although only a fraction of patients is readily diagnosed due to the highly variable clinical presentation of the disease (Green, 2005). CD can be considered a food intolerance to wheat gluten (consisting of the gliadin and glutenin subcomponents) and related proteins from rye and barley. In genetically predisposed individuals, gluten ingestion can cause an inflammatory reaction in the upper small intestine which gives tissue damage leading to villous atrophy (Sollid, 2002). The lesion and inflammatory changes disappear after weeks or months when patients for treatment purpose commence a gluten-free diet (GFD). The inflammatory reaction appears to be driven by activation of Th1-like $CD4^+$ T cells (see **Box 1**) that recognize gluten peptides post-translationally modified by the enzyme transglutaminase 2 (TG2; Molberg et al., 1998; van de Wal et al., 1998). What initiates this "aggressive" T cell response and lack of oral tolerance to gluten is not known. It has been suggested that some part of gluten may have innate properties or that infections may play a role (reviewed in Jabri and Sollid, 2009). In steady-state conditions, the maintenance of intestinal homeostasis is initiated by intestinal DCs that are affected by enterocyte-derived factors, such as retinoic acid and TGF-β, conferring tolerogenic properties on the DCs. Tolerogenic DCs educate the intestinal immune system to respond in a non-inflammatory manner to orally administered proteins by the induction of regulatory T (Treg) cells. An alteration of the intestinal environment, as observed in CD, characterized by a high level of inflammatory cytokines such as IL-15 and IFNα, may affect the acquisition of the tolerogenic phenotype of intestinal DCs. This will prevent the induction of Treg cells, further promoting the differentiation of proinflammatory T cells (Jabri and Sollid, 2009). Interestingly, beside the strong gluten-specific T cell response, CD presents autoimmune features, most notably the production of autoantibodies. These antibodies are primarily directed against TG2 (Dieterich et al., 1997), but antibodies specific for other autoantigens like actin, collagen and others have also been described (Alaedini and Green, 2008). Whereas the T cell response to gluten has been thoroughly characterized and is relatively well understood, significantly less is known about the B cell responses in CD.

TG2 has several biological functions which include transamidation (cross-linking) and deamidation, and is involved in many physiological processes (Lorand and Graham, 2003). TG2 is present in large amounts in the gut LP, in particular in a subepithelial layer. It is hardly coincidental that TG2 is also the target of the autoantibodies in CD. Studies based on *in situ* detection with immunofluorescence (Korponay-Szabo et al., 2004) and phage display antibody libraries (Marzari et al., 2001) suggested that anti-TG2 antibodies are produced locally in the small intestine, and recently we were able to visualize the intestinal PCs producing such antibodies (Di Niro et al., 2012). In the following, we will describe the current knowledge and the future directions in the study of the intestinal B cell response in CD.

BOX 1 | Immunopathogenesis of CD.

CD is a multifactorial disease with a complex interplay between genetic and environmental factors eventually leading to chronic inflammation (see Abadie et al., 2011; Meresse et al., 2012 for review and references therein). Of these factors, the most significant genetic component is HLA; 90% of celiac patients carry a variant of DQ2 termed DQ2.5, whereas most of the remaining patients carry DQ8. The HLA association has been extensively investigated, and the study of lesion-derived T cell lines and T cell clones has allowed a detailed description of gluten T cell epitopes. Several epitopes exist and some epitopes are more frequently recognized than others (reviewed in Sollid et al., 2012). An important feature of both the DQ2.5 and DQ8 molecules is their preference for binding of negatively charged amino acid residues (i.e., glutamate or aspartate) in certain binding pockets (P4, P6, and P7 for DQ2.5; P1 and P9 for DQ8). Gluten proteins have very few negatively charged residues, however they carry a high amount of glutamine and proline residues. Interestingly, glutamine can be deamidated to glutamate by the enzyme TG2, and TG2-modified gluten peptides show strong immunogenicity. This suggests that, under particular circumstances, deamidation happens *in vivo*, leading to the formation of post-translationally modified gluten peptides that are suitable for presentation by DQ2.5 or DQ8 molecules. The gluten T cell epitopes are furthermore hallmarked by the presence of multiple proline residues, and this is particularly so for epitopes presented by DQ2.5. Proline, in addition to influencing MHC binding, exerts a force in the selection of T cell epitopes at two additional levels. First, peptides rich in proline are resistant to proteolysis, and proline-rich gluten peptides survive gastrointestinal digestion allowing them to reach the LP where they can by loaded on HLA-DQ molecules expressed by antigen-presenting cells. Second, proline guides the specificity of TG2 so that glutamine residues in the motif glutamine-X-proline are targeted. Notably, the peptides harboring T cell epitopes in a complex gluten digest are the preferred TG2 substrates. How these forces work can be visualized by looking at the α2-gliadin, a representative α-gliadin. Upon treatment with gastric and pancreatic endopeptidases a relatively large fragment, the 33-mer LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (residues 57 to 89), survives digestion. Due to its length this peptide is resistant to digestion by small intestinal brush-border membrane ectopeptidases as well. Three glutamine residues within the 33-mer are deamidated by TG2. This deamidated 33-mer harbors six overlapping T cell epitopes and is an extremely potent antigen. Upon recognition of deamidated gluten peptides, the $CD4^+$ T cells become activated and start producing cytokines including interferon-γ and interleukin-21. The priming of the gluten-specific $CD4^+$ T cells likely takes place in GALT or mesenteric lymph nodes and the primed cells seed via the blood into the LP as effector cells. These T cells may be important for forming PC survival niches in the LP.

THE INTESTINAL B CELL RESPONSE IN CD

The celiac lesion is characterized by considerable expansion of the PC population (Douglas et al., 1970; Soltoft, 1970) and enhanced local immunoglobulin secretion (Lancaster-Smith et al., 1974; Wood et al., 1987). In addition, there are IgA deposits at the epithelial basement membrane of the small intestine (Shiner and Ballard, 1972; Korponay-Szabo et al., 2004) which can be observed without overt histological changes (Salmi et al., 2006a). The plasmacytosis (increased median PCs per mucosal tissue unit of 2.1, 3.8, and 2.9-fold for IgA, IgM, and IgG respectively; Baklien et al., 1977; Scott et al., 1980) may relate to bolstering of a PC survival niche. Local plasmacytosis in CD appears to be homeostatic with an unaltered immunoglobulin isotype distribution and marked preponderance of IgA PCs (Brandtzaeg, 2006). Notably, the duodenal IgA PC population in active CD maintains mucosal phenotype by J-chain expression and consists of a higher proportion of the IgA2 subclass than in the normal duodenal mucosa (Kett et al., 1990). Upon dietary gluten restriction, intestinal PC numbers are reduced (Holmes et al., 1973).

ANTI-GLIADIN AND ANTI-TG2 ANTIBODIES

Early experiments performed by ELISA, ELISpot, and immunofluorescence indicated local intestinal secretion of anti-gliadin antibodies (Stern and Dietrich, 1982; Ciclitira et al., 1986; Labrooy et al., 1986; Lycke et al., 1989). These studies suggested that gliadin-specific PCs account for 1–2, 10 and 5–10% of total IgA, IgM, and IgG PCs, respectively, in the small intestine of CD patients. Anti-gliadin IgA and IgG antibodies are detected in sera of untreated CD patients and can be harnessed as a diagnostic tool. These antibodies disappear after commencement of a GFD (Savilahti et al., 1983; Kilander et al., 1987), and they rise again when gluten is reintroduced into the diet (Koninckx et al., 1984). Thus, their level seems to mirror the immune reaction triggered by gluten in the intestine and, further, their decrease is related to a clinical improvement of the intestinal mucosa (Mayer et al., 1989; Valletta et al., 1990). IgA gliadin-specific B cells have been detected in peripheral blood of CD patients (Hansson et al., 1997; Sblattero et al., 2000); these possibly are circulating IgA plasmablasts homing to the LP.

Celiac disease patients also develop autoreactive antibodies originally identified as targeting connective tissue constituents, in particular the endomysium (Chorzelski et al., 1983). The enzyme TG2 was identified as the major endomysial autoantigen (Dieterich et al., 1997). In the diagnostic workup of CD, assessment of serum of anti-TG2 autoantibodies has become an important tool for the diagnosis particularly in children where new recommendations allow the diagnosis to be made without histological examination of small intestinal biopsies (Husby et al., 2012). Similarly to anti-gliadin antibodies, the production of anti-TG2 antibodies is dependent on dietary gluten exposure (Dieterich et al., 1998; Sulkanen et al., 1998). Anti-TG2 antibody titers have been shown to correlate with abnormal small intestine histopathology (Tursi et al., 2003). While serum anti-gliadin antibodies have a significant IgA2 component, only a minor portion of serum IgA antibodies reactive to the endomysium were found to belong to this subclass (Osman et al., 1996).

Recently, we demonstrated that TG2-specific PCs can be visualized by immunofluorescence of tissue sections (**Figure 1**) and by flow cytometry of single-cell suspensions from duodenal biopsy specimens (Di Niro et al., 2012). To note, TG2-specific PCs comprise 4–24% of the total IgA PC population in the celiac lesion. This massive accumulation of TG2-specific PCs is further supported by the notion that IgA intestinal antibody deposits target the same antigen in the extracellular matrix and the endothelium of the small blood vessels (Korponay-Szabo et al., 2004), thus reflecting an abundant local antibody production. Notably, TG2-targeted IgA intestinal deposits are present at all stages of

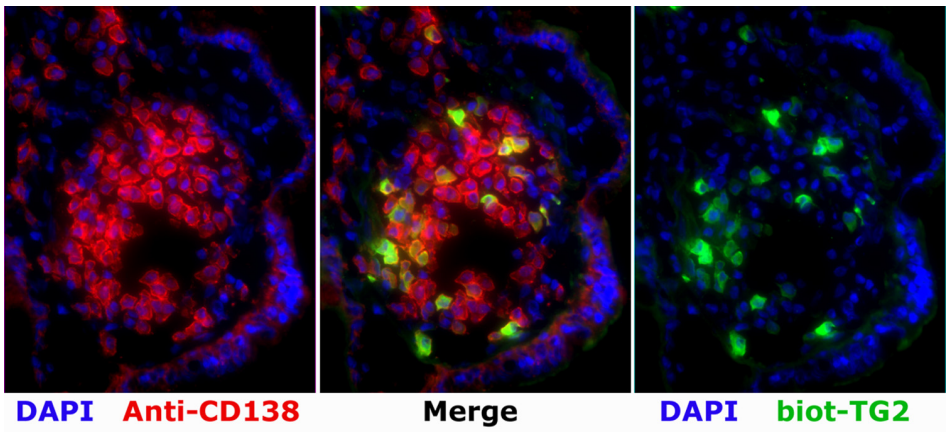

FIGURE 1 | A patch with high frequency of TG2-specific PCs as revealed by immunofluorescence analysis on a cryosection of the duodenal mucosa of a patient with active CD. Staining performed with biotinylated TG2 (biot-TG2, followed by fluorescent streptavidin, *green*), a plasma cell marker (anti-CD138, *red*), and a nuclei stain (DAPI, *blue*).

CD, including early developing CD (prior to villous atrophy; Kaukinen et al., 2005; Paparo et al., 2005; Tosco et al., 2008) as well as the advanced lesion stage in rare seronegative patients (Salmi et al., 2006b).

CHARACTERISTICS OF THE ANTI-TG2 ANTIBODY REPERTOIRE

In our recent, thorough dissection of the antibody (Ab) repertoire of the intestinal autoimmune response to TG2, we found that despite extensive class switch to IgA, antibody genes had a very limited amount of somatic mutations (Di Niro et al., 2012). From the analysis of 60 sequences of heavy chain variable region genes cloned from single intestinal PCs of CD patients, we observed on average less than half the number of mutations found in the rest of the intestinal PC compartment. Interestingly, anti-TG2 antibodies with heavy chain encoded by the VH5 gene, which accounted for 44% of the response, were significantly less mutated than those encoded by other genes, including several that were entirely germline encoded. A trend of low mutation in VH5 genes in the gut was observed before (Boursier et al., 1999), but the reason for this and for the preferential recruitment of cells expressing antibodies encoded by VH5 in the autoimmune repertoire is not known. It may be related to structural properties of the VH5 region and/or to the fewer mutational hotspots in the VH5–51 gene. Worth noting is that a number of VH5 antibodies with different specificity did not bind TG2 to any extent, thus showing that the anti-TG2 reactivity is CDR-encoded and not depending on unspecific binding of the VH5 framework regions (Di Niro et al., 2012).

This significantly differs from all other intestinal human antibody repertoires described to date, as presented and discussed above, in which antibody genes with such a limited number of mutations were seldom observed. This represents a unique feature of the anti-TG2 response and may therefore provide clues into its generation.

GENERATION OF TG2-SPECIFIC B CELLS

A schematization of the current knowledge and hypotheses for the generation of the intestinal TG2-specific B cell response is shown in **Figure 2**. The scheme assumes a T cell-dependent mechanism for generation of the response. Does instead the observed phenotype, with scarce SHM, indicate a T cell-independent one? Indeed, despite numerous efforts, to date there is no convincing evidence for the existence of TG2-specific T cells. However, the clinical observation of the strict HLA-dependent appearance of TG2-specific antibodies speaks against this model. In a population based cohort study, Bjorck et al. (2010) compared the presence of TG2-specific antibodies in children with or without the DQ2 or DQ8 HLA-risk alleles, and found that 73 of 1620 (4.5%) individuals with HLA-risk alleles were positive for anti-TG2 antibodies, versus none of 1815 subjects without HLA-risk alleles. This strongly suggests T cell involvement. How to reconcile these data with the inability to identify TG2-specific T cells? Once again, clinical observations provide clues: anti-TG2 antibodies rapidly disappear when gluten is removed from the diet (Dieterich et al., 1998; Sulkanen et al., 1998). The regulation of the B cell response by gluten intake is the foundation of the "hapten-carrier-like model" proposed several years ago (Sollid et al., 1997). In this model, intestinal gluten-specific T cells provide help to TG2-specific B cells. This is possible because of the enzymatic activity of TG2. The enzyme can form covalently linked complexes between itself and gluten peptides (Fleckenstein et al., 2004), which then can act as "hapten-carrier-like" complexes. Upon withdrawal of gluten from the diet, the T cell help will cease and anti-TG2 antibodies will disappear. Although this model has not been formally demonstrated *in vivo*, we recently provided *in vitro* evidence that TG2-specific B cells indeed can present gluten peptides to gluten-reactive T cells when offered TG2–gluten peptide complexes (Di Niro et al., 2012).

Based on strong clinical evidence, T cells thus appear to be involved in the generation of the anti-TG2 B cell response. In what is regarded as the "canonical" response, T and B cells cooperate in the GC, where B cells not only class-switch, but also accumulate mutations so that clones with increased affinity are selected (affinity maturation) in a process that ultimately generates memory B cells and long-lived PCs. In particular, B cell memory is "designed"

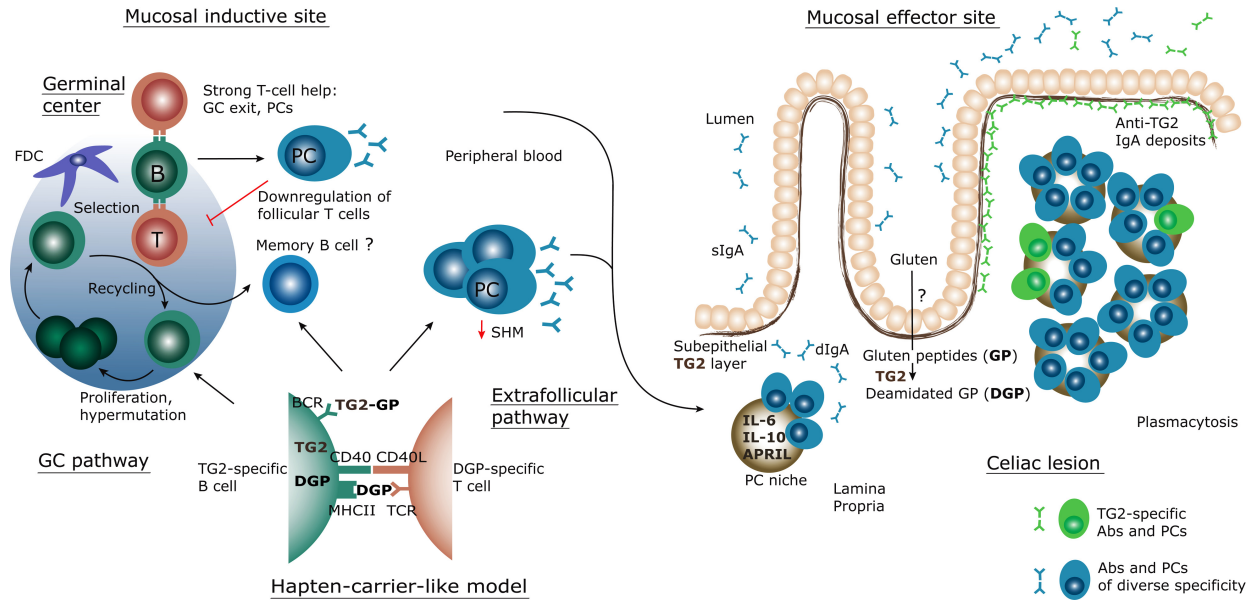

FIGURE 2 | Schematic representation of the inductive and effector sites for the mucosal B-cell response in CD. Through yet unknown mechanisms, gluten peptides (GP) cross the epithelium and become deamidated gluten peptides (DGP) by the action of transglutaminase 2 (TG2). These peptides are then transported, possibly by dendritic cells, to local lymphoid tissue structures where a strong, proinflammatory T cell response takes place. Some native peptides may reach the lymphoid tissue and may become targeted by TG2 there. In lymphoid tissues TG2-specific B cells likely interact with gluten-specific T cells, according to the hapten-carrier-like model described in the text which builds on the ability of TG2 to form TG2-GP/DGP complexes. The B cell response generated follows either a canonical germinal center (GC) pathway or an extrafollicular pathway, resulting in massive plasmablast generation. The plasmablasts migrate via the blood to the lamina propria, where they mature as plasma cells (PC), secrete antibodies (Abs) and survive for extended time in what is called a PC survival niche. In active CD, this results in plasmacytosis and sustained secretion of IgA dimers (dIgA) – as well as IgM pentamers – which are transported across the epithelium to the lumen as secretory IgA (sIgA). TG2-specific antibodies produced at this site are also deposited on the subepithelial layer of TG2. BCR, B cell receptor; FDC, follicular dendritic cell; TCR, T cell receptor.

to respond more rapidly to a secondary antigen challenge, and repeated stimulation leads to the generation of a switched response composed of high affinity clones which have acquired many mutations. In humans, this is clear in the cases of HIV, influenza, and rotavirus infections (Wrammert et al., 2008; Scheid et al., 2009; Di Niro et al., 2010).

A possible scenario to explain the phenotype of the anti-TG2 response is that it is generated at extrafollicular sites, thus bypassing GC formation (MacLennan et al., 2003) but nonetheless maintaining the requirement for T cell involvement. In mice, this has been observed in more than one context, such as *Salmonella* infection (Cunningham et al., 2007) and, interestingly, the autoimmune RF response (William et al., 2002). Contrary to what commonly thought, AID can be expressed at EF sites (Marshall et al., 2011), and in the autoimmune context SHM has clearly been shown to vigorously take place at this site (William et al., 2002). The intestinal environment may play a role in this process. AID-dependent class switching to IgA at the LP level has been shown in mice (Fagarasan et al., 2001) and studies in humans suggested that proliferation, class-switching, and SHM could take place in the intestinal LP (He et al., 2007; Yuvaraj et al., 2009). As discussed above, a study of mice based on analysis of IgA sequences obtained by high-throughput sequencing suggested instead that extra-mucosal expansion is followed by massive seeding in the LP, of which clonal relatedness is a consequence (Lindner et al., 2012). Although still being a matter of major debate, it cannot be excluded that the TG2-specific response expands and evolves in the LP, and that B cells do not enter GCs at all. If they do, the scarce number of mutations suggests that they undergo limited rounds of selection, possibly a single one, in the GC. The scope of the GC is to ultimately produce memory cells and long-lived PCs. Does this happen in the case of the anti-TG2 response? For both cell populations evidence is limited and further studies are required. Upon gluten removal the TG2-specific IgA serum titer decreases within months (Maki et al., 1998), suggesting that the long-lived PC compartment is limited. Although finally differentiated to a PC phenotype, IgA PCs in the small intestine are only relatively long-lived, with a lifespan of some months (Mesin et al., 2011); the microenvironment is likely to play a major role. Similarly, we have only partial evidence for the existence of a TG2-specific, IgA memory compartment in CD, which appears to be more substantial in GFD-treated rather than active CD patients. It has to be noted that small amounts of memory B cells seem to be produced also in the absence of GCs (Toyama et al., 2002; Foote and Kearney, 2009).

POSSIBLE REGULATION MECHANISMS OF THE ANTI-TG2 IMMUNE RESPONSE

Whether cells are or not generated in GCs, undoubtedly specific factors limit GC activity. To note, the diagnosis of CD (and thus specimen sampling) often occurs many months or years after the appearance of the symptoms. Moreover, the anti-TG2 response

precedes symptoms and intestinal damage. All the patients from whom we obtained intestinal specimens were adults (Di Niro et al., 2012); the anti-TG2 response had likely been present for several months or years. In this time frame, according to the canonical model of immune response, GCs should be formed, memory generated, upon secondary stimulation memory cells preferentially and rapidly reactivated, resulting in extensive affinity maturation and accumulation of mutations. What inhibits this? Our current knowledge only allows speculation. Several different, and not mutually exclusive, scenarios can be envisaged.

(i) Feedback mechanisms. These could happen both at the antibody and the antigen level, as well as at the cellular level. The extent of the anti-TG2 response (10% of intestinal PCs, on average) and the consequent massive antibody production may provide a negative feedback that inhibits GC activity. This could be seen as a self-regulating mechanism of the immune system. With regard to antigen availability, chronic stimulation (i.e., continuous gluten ingestion) may be different than repeated antigen challenge (i.e., seasonal flu infections). Another feedback mechanism may result from antigen-specific, isotype-switched PCs that before homing to the LP act as potent antigen presenting cells. PC-controlled antigen presentation was shown to suppress the development and function of antigen-specific follicular helper T cells (Pelletier et al., 2010). Such function of PCs would create bidirectional regulation of adaptive immunity by providing a negative cognate regulatory mechanism which may serve as a functional sensor of PC production that can control ongoing GC B cell responses.

(ii) Affinity. The binding strength of the anti-TG2 antibodies may be a factor. Even when a panel of mutated anti-TG2 hmAbs was reverted to their germline counterparts, strong binding to TG2 was observed (Di Niro et al., 2012). This is atypical as in the case of anti-flu antibodies removal of mutations gave a dramatic loss in affinity (Di Niro et al., 2012). It has been previously shown in mice that high affinity may favor EF T cell-dependent PC responses (Chan et al., 2009). Alternatively, it may be that inside GCs it is affinity that regulates the fate of B cells – i.e., whether to become a memory B cell or PC – and that high affinity favors the latter. In both scenarios, this would result in generation of a scarce memory compartment and, irrespective of the specific mechanism, to continuous activation of naïve B cells thus explaining scarce SHM. As an alternative explanation, it has been shown that the initial affinity of the B cell receptor (BCR) is inversely correlated with accumulation of mutations in GC T cell-dependent antibody responses *in vivo* (Shih et al., 2002). This effect was due to GC selection, as both high and low affinity B cells had the same frequency of mutations in non-coding sequences.

(iii) T cell control. T cells may represent an important regulator. It has been shown that a robust and efficient T cell response increases the magnitude of the PC response while preventing GC recycling and memory cell differentiation (Fujihashi et al., 1991; Bolduc et al., 2010; Heiser et al., 2011). Furthermore, it has been suggested that the decision to become a PC upon receiving T cell help is antigen dose-dependent (Victora and Nussenzweig, 2012). If the help to TG2-specific B cells indeed is provided by gliadin-specific T cells, the amount of antigen and strength of the T cell response would be compatible with a limited GC reaction.

(iv) Nature and location of the response. The nature (autoimmune) and the location (intestine) differ from any other B cell responses that have been characterized in humans, and hence models for comparison are not easily available. In mice, peritoneal reservoirs of B1 cells significantly contribute to intestinal responses (Kroese et al., 1995) – often via T cell-independent mechanisms, that do not efficiently form GCs (Toellner et al., 2002). Whether similar mechanisms take place in humans is not fully understood (Griffin and Rothstein, 2012). Could instead the unique features of the anti-TG2 response relate to the self nature of the antigenic target? Does the immune system sense the antigen as self and redirects the response toward an EF one? Germ-line encoded autoreactivity has been described, for instance in RA (Victor et al., 1991), but does not seem to be the general rule. In mice, there are notable examples of autoimmune responses developing at EF sites – the response to RF being such one situation (William et al., 2002). Important insights will come from the analysis of the repertoire of the gluten-specific B cell response. Would gluten-specific PCs show SHM at the same level as TG2-specific PCs, or would they have high degrees of SHM as generally seen in intestinal IgA PCs? Together with the investigation of the CD-specific B cell memory, this is one of the most interesting immunological aspects toward which research should focus.

(v) Structural features. As mentioned above, anti-TG2 antibodies have high affinity even when germ- line encoded. What confers such high affinity and, in particular, what favors VH5 selection over other V regions is not known. On the surface of a B cell, BCR cross-linking is one potent mechanism for activation. We recently showed that, *in vitro*, TG2 can mediate covalent cross-linking of IgD (and, to a minor extent, IgM, but not of IgA or IgG) antibodies, providing a hypothetical model where continuous activation of naïve B cells is favored over IgA-switched memory cells, thus explaining lack of accumulated mutations (Di Niro et al., 2012). Similarly, it is conceivable that the VH5 dominance is based on a similar mechanism.

To address these questions, we need to know more about how antibodies bind to TG2. The hmAbs that we have cloned from intestinal IgA PCs represent a unique tool to better understand their interaction with TG2. We are currently investigating the epitopes recognized by the autoantibodies, as well as their ability to bind TG2 in its different forms (i.e., free vs bound to fibronectin, open vs close conformation, GTP vs Ca^{2+}-bound, etc). Ultimately, fundamental insights will derive from efforts directed toward the generation of crystal structures of TG2-hmAb complexes.

TG2-SPECIFIC B CELLS AS ANTIGEN PRESENTING CELLS AMPLIFYING THE ANTI-GLUTEN T CELL RESPONSE

B cells can program $CD4^+$ T cell responses (reviewed in Barr et al., 2012). This is so, much because B cells and T cells interact in an antigen-specific manner. By BCR-mediated uptake and concentration of antigen, B cells serve as potent antigen-presenting cells for T cells (Lanzavecchia, 1985). This mechanism is essential for B cells to receive cognate T cell help, but notably it has also direct consequences for the T cells. B cell-mediated antigen presentation leads to proliferation and clonal expansion of antigen-specific

T cells (Crawford et al., 2006). If TG2-specific B cells are able to load deamidated gluten peptides for presentation to gluten-specific T cells *in vivo* (Sollid et al., 1997), this would likely result in expansion of gluten-specific T cell clones. These gluten-specific T cells would then be able to interact both with TG2-specific B cells as well as with B cells specific for deamidated gluten peptides. Collectively, these events will support the antibody responses to TG2 and deamidated gluten peptides and importantly lead to an amplification of the anti-gluten T cell response. In this scenario, B cells would be at the center stage of the immunopathogenesis of CD, and could therefore be a potential target for therapy even if CD is considered primarily a T cell-mediated disease.

PATHOGENIC ROLE AND INTERACTION OF AUTOANTIBODIES WITH INTESTINAL STRUCTURES

Since the discovery of autoantibodies in CD, and subsequently the identification of TG2 as the main target, there has been speculation about whether the antibodies themselves are pathogenic. Most research has focused on the effects of antibodies on enzymatic activity, with discordant results (Esposito et al., 2002; Dieterich et al., 2003; Di Niro et al., 2012). A reason for this could be the variety of assays and experimental conditions used to assess TG2 activity. Although in some cases weak inhibition has been reported, as of now there is no compelling evidence that the effects of autoantibodies on TG2 play a major role, either in the pathogenesis or in relation to the clinical features of CD. The great majority of the hmAbs from our newly generated panel neither inhibited nor enhanced TG2 activity, consistent with a central role for TG2 in the enzymatic deamidation of gluten peptides.

Among other proposed effects, anti-TG2 antibodies could contribute to the formation of the lesion by inhibiting angiogenesis (Myrsky et al., 2008) as well as by interfering with the differentiation of epithelial cells (Halttunen and Maki, 1999). Effector function of antibodies could contribute to the tissue damage seen in CD. Complement-dependent inflammation has been observed in the CD lesion (Halstensen et al., 1992). Unlike IgA, IgM does fix the complement, and TG2-specific IgM are indeed produced in patients, especially in those with IgA deficiency (Borrelli et al., 2010; Di Niro et al., 2012).

Celiac disease patients with active disease have increased transport of gliadin peptides across the epithelium (Schumann et al., 2008), and IgA antibodies have also been suggested to have a role in such transport (Rauhavirta et al., 2011). In the intestinal mucosa TG2 binds fibronectin, forming a sub-epithelial layer, and in patients IgA/IgM deposits are observed at this location. Moreover, IgA is found on the brush border. Matysiak-Budnik et al. (2008) have shown that gliadin peptides can be retro-transcytosed as IgA–gliadin complexes via the transferrin receptor, which is abnormally expressed at the apical surface of enterocytes in active CD. TG2 localized at the apical side of the epithelium may as well play a role in this mechanism (Lebreton et al., 2012). In the LP, intact immunostimulatory gliadin peptides might act by triggering a local immune response and promoting inflammation. We hypothesize that dimeric anti-TG2 IgA play a role in this mechanism. Anti-TG2 antibodies could detach TG2 from fibronectin and the complexes could be transported across the epithelium, where sampling of gliadin peptides by TG2 itself or by anti-gliadin antibodies could take place. We have preliminary evidence that a fraction of anti-TG2 antibodies can in fact compete for the fibronectin binding site.

In conclusion, as of now the evidence for a role of anti-TG2 antibodies is scarce, and it derives from *in vitro* or cell culture systems; when anti-TG2 antibodies were expressed *in vivo* in mice (Di Niro et al., 2008), no obvious effect was seen. This highlights the need for the generation of an animal model of CD.

ANIMAL MODELS OF CD

Several animal models have been developed that try to recapitulate CD, however none of them entirely succeeded reproducing the complex mechanisms causing this disease (reviewed in Marietta and Murray, 2012). Expression of anti-TG2 autoantibodies *in vivo* by means of adeno-associated virus-based gene transfer led to lifetime production of such antibodies, analogous to what observed in patients, but no clinical features were associated (Di Niro et al., 2008). Some signs of disease were obtained when pre-sensitized $CD4^+$ T cells were transferred in Rag-deficient mice, inducing weight loss and duodenitis (Freitag et al., 2009); however also this system was not able to recapitulate the majority of the immune features observed in CD. Interestingly, Jabri's group has described a humanized HLA-DQ8-transgenic mouse model (DePaolo et al., 2011), characterized by over-expression of IL-15 in the LP. When gliadin-fed, these animals develop IFN-γ-producing anti-gliadin T cells, anti-gliadin and anti-TG2 antibodies, and intraepithelial lymphocytosis. Despite lacking the hallmark of villous atrophy, this model does resemble early stages of CD. Mice are not the only species where CD models are investigated, as a screening of macaques also led to identification of animals with signs and symptoms of CD (Bethune et al., 2008); the usefulness of such model remains to be evaluated. In general, efforts are being made toward the generation of an animal model of CD, which will greatly facilitate research.

THE MICROFLORA AND ITS IMPACT ON INTESTINAL IMMUNITY

Genetic factors may account only for about half of the risk to develop CD thus leaving an important role for the environment in the pathogenesis. Gluten exposure obviously is critical, but environmental factors outside of gluten may be implicated as well. Such factors could be pathogenic infectious agents or commensal bacteria. Recently, it has become clear that the gut microflora profoundly influence intestinal immunity (Kau et al., 2011). There is a complex interplay between intestinal immunity and the populations of commensal bacteria, and these two components regulate each other. Not only does the microflora regulate several aspects of the innate and adaptive immunity, as well as of several metabolic pathways, but it has also been shown that dysregulated immunity (for instance as a consequence of experimental manipulation of molecules such as PD-1 and AID; Wei et al., 2011; Kawamoto et al., 2012) results in skewed gut microbial communities, and this in turn may have detrimental effects. In future, it will be of major importance to understand how the microbial communities contribute to the intestinal immune response in a context such as CD where both the T and the B cell intestinal populations seem altered as compared to healthy individuals.

CONCLUSION

In the last two decades we have learnt a lot about CD and its interplay with intestinal immunity. Among the most remarkable discoveries, TG2 has been identified as the main autoantigen of CD, and its role in creating potent T cell epitopes has been unraveled. We have made huge steps forward in understanding the role of HLA genes, and many non-HLA susceptibility genes have been identified. Some limited progress has also been made in understanding the role of innate immunity factors in CD. Recently, the knowledge of the intestinal B cell response in CD has significantly improved. We have learnt that anti-TG2 antibodies form deposits in the small intestine, a number of highly sensitive serological tests based on serum antibodies have been developed, monoclonal antibodies have been isolated by phage display and single cell cloning, and the cells producing these antibodies have been visualized, characterized and enumerated. However, many important features of CD, in particular related to the intestinal environment in which the disease takes place, remain to be tackled. These include characterization of the anti-gliadin B-cell response, the IgG anti-TG2 repertoire, identification of the memory compartment, and others. A better understanding of human intestinal immunobiology is needed to address these questions.

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The human intestinal IgA response; burning questions

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The title of this special topic invites us to identify areas in the field of IgA biology that are uncertain or in need of clarification. The inductive phase of the human intestinal IgA response has been a controversial area for some years. Therefore, to structure this review, we have identified key questions that are debated in this field. We have provided explanations of the origins of the uncertainties and have provided our own reasoned answers to the questions we pose.

Keywords: human, IgA, gut-associated lymphoid tissue

Secretion of a huge quantity of IgA with a diverse antigen-binding repertoire is essential for the maintenance of intestinal homeostasis and the prevention of mucosal and systemic infection (Conley and Delacroix, 1987; Brandtzaeg et al., 1999; Wei et al., 2011). Most of this IgA is secreted by the vast plasma cell population, which is located beneath the intestinal epithelium throughout the gastrointestinal tract.

Many of the questions that continue to perplex those working in the field of mucosal B cell biology, and confuse those outside, relate to the biology of intestinal B lineage cells prior to terminal differentiation into plasma cells. Problems in providing secure answers derive from inherent difficulties in studying the human intestinal immune system. Difficulties extend beyond the obvious restrictions to sampling tissue, and the existence of some basic species differences (Gibbons and Spencer, 2011). Some difficulties are exemplified by the earliest experiments that defined this field. For example, Gowans and Knight (1964) showed that the thoracic duct lymph of rats contains a continuous flow of immunoblasts without the need for prior immunization (Gowans and Knight, 1964). These gut-derived activated cells were destined for the blood. Adoptive transfer of labeled cells demonstrated that thoracic duct immunoblasts were pre-programmed to home back to the intestine by selective extravasation through the capillary network. This demonstrated not only that the intestinal immune system is a distinct entity, but also that understanding the immunology of the IgA response would require an appreciation of immune physiology and anatomy because multiple anatomically separated sites and structures were involved, connected by lymph and the blood. It is particularly difficult to study such a system definitively in humans.

Understanding current perception of the intestinal B cell response initially requires appreciation of the anatomical and microanatomical sites involved, and the associated terminology (Brandtzaeg and Pabst, 2004; Brandtzaeg, 2009). The intestinal immune system can be divided into organized gut-associated lymphoid tissue (GALT), the lamina propria, the intraepithelial compartment, and the regional lymph nodes.

Gut-associated lymphoid tissue (**Figure 1**) is organized lymphoid tissue with discrete B and T cell zones, that is, by definition intimately associated with a specialized follicle associated epithelium (FAE). The "microfold" or "M" cells of the FAE selectively sample particulate antigen from the gut lumen and transport it to the underlying lymphoid tissue (Chabot et al., 2006) forming a discriminatory gatekeeper function, but also a connection with the luminal microbiota. The Peyer's patches are aggregates of GALT that are most concentrated in the terminal ileum. Isolated follicles of GALT as will be discussed below are also located throughout the small and large intestines.

In contrast, the lamina propria comprises diffuse connective tissue with a lymphoid component that includes largely effector cells; both cytokine-producing T cells and plasma cells (Farstad et al., 2000). The vast majority of B cells in lamina propria are CD19+, CD20−, and these cells are almost certainly the extravasated immunoblasts en route to terminal differentiation to plasma cells. The lamina propria is sited below an epithelial barrier, which in contrast to the FAE is secured by tight junctions and covered by a layer of mucins and microbicidal molecules on the luminal aspect (Turner, 2000).

The intraepithelial lymphoid compartment (other than the FAE associated lymphocytes) does not include many B cells, and is not relevant to this review. The mesenteric lymph nodes on the other hand contain and abundance of active B cells and the scale of their involvement in human intestinal immunity is not known (**Figure 2**).

A major goal in human intestinal immunity must be to deduce how the IgA response can be harnessed for mucosal vaccination. Understanding the rules that govern the generation and dissemination of the antigen-specific plasma cells in the intestine would move us considerably further toward that goal, though there are still many areas that lack clarity. **Table 1** comprises a list of current

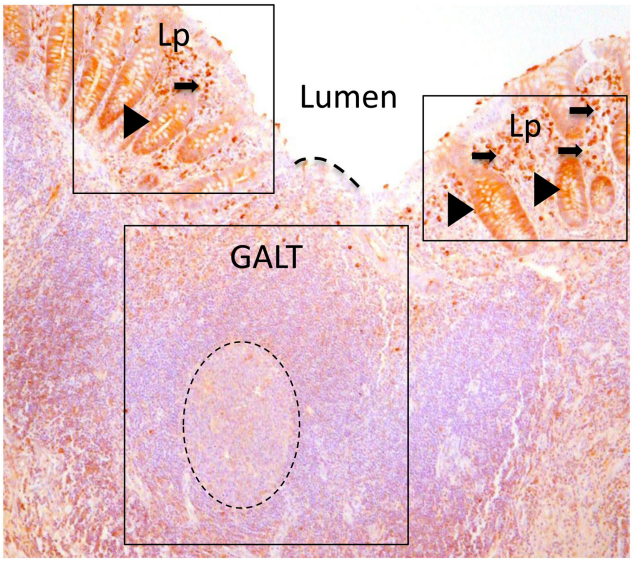

FIGURE 1 | Paraffin sections of human appendix stained to detect IgA (brown). Gut-associated lymphoid tissue (GALT) is boxed. GALT is comprised mostly of B cells and contains a prominent germinal center of dividing B cells (dotted circle). The epithelial barrier between the GALT and the intestinal lumen, marked with a dotted line, is the FAE. Lamina propria (Lp) is boxed either side of GALT. The lamina propria includes abundant effector cells including plasma cells (arrowed) Unlike the epithelium adjacent to GALT, crypt epithelium of the lamina propria contains IgA that is in the process of being transported to the intestinal lumen (arrow heads).

questions that relate to the human intestinal immune response that might be answered unambiguously in the near future. The questions are accompanied by our own simple best answers. The final column is our measure of faith in our answer. Our reasoned responses to the questions and rationale behind the answers comprise the text of this review.

DO THE EVENTS THAT INITIATE THE HUMAN IgA RESPONSE OCCUR EXCLUSIVELY IN GUT-ASSOCIATED LYMPHOID TISSUE?

It is clear from simple histological observations that GALT is an inductive site for intestinal B cell responses. The Peyer's patches invariably have prominent germinal centers of dividing B cells. Dividing cells are also apparent in the large marginal zone that forms the outermost B cell area of GALT (Boursier et al., 2005). Suggestion that lamina propria might be an inductive site followed a high profile study of cell suspensions prepared from the intestines of mice with the Peyer's patches removed (Fagarasan et al., 2001). This work identified that the enzyme activation-induced cytidine deaminase (AID), the essential catalyst of class switch recombination and somatic hypermutation, was present in the isolates. Several years later, it was proved that GALT is required for the IgA response, and that the features of the inductive phase of the IgA response ascribed to lamina propria cells was in fact a consequence of contamination of the isolates by cells from the organized GALT in isolated lymphoid follicles (ILF). The isolated follicles are not macroscopically visible, and are developmentally

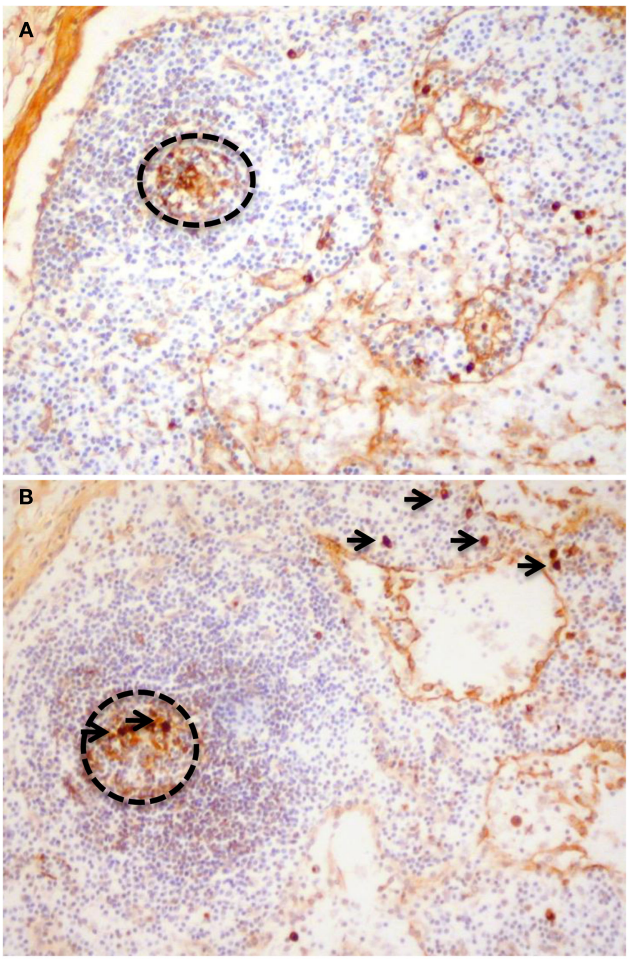

FIGURE 2 | Paraffin sections stained to detect (A) IgA1 and (B) IgA2. Cells with cytoplasmic immunoglobulin of both subclasses can be observed in germinal centers (dotted circles) demonstrating that the mesenteric nodes could be classified as a non-GALT site of induction for IgA responses. The presence of cells with cytoplasmic IgA2 in mesenteric nodes (arrows) also demonstrates that switching to IgA2 is not only a feature of terminal differentiation in the colonic lamina propria.

independent of the Peyer's patches (Tsuji et al., 2008; Lindner et al., 2012). Meanwhile the concept of lamina propria as an inductive site had gathered momentum and has been documented in reviews (Cerutti, 2008a; Suzuki and Fagarasan, 2009).

It has been suggested that IgA class switch recombination to IgA2 can occur in human colonic lamina propria, driven by the IgA switch factor a proliferation-inducing ligand (APRIL) produced by epithelia in response to TLR5 ligation by colonic luminal bacteria (He et al., 2007). Although this idea provides an elegant explanation for the relatively higher frequency of plasma cells synthesizing the IgA2 subclass in colon (Kett et al., 1986; Kett and Brandtzaeg, 1987), it has been contested by groups that give full consideration to the lymphoid microenvironments in intestine. The study of both mouse and human lamina propria cells that were sampled from tissue sections *in situ*, or isolated plasma cells did not detect the expression of AID in lamina propria of large or

Table 1 | Outstanding questions in human IgA immunobiology.

Question	Authors' best guess answer	Degree of certainty in answer (%)
Do the events that initiate the human IgA response, occur exclusively in gut-associated lymphoid tissue?	Yes	90
Is there an equivalent of murine cryptopatch precursor of ILFs in humans?	No	95
Can gut-associated lymphoid tissue be acquired in response to bacterial antigens in humans?	Yes	100
Are there T cell dependent and T cell independent routes to IgA production in man?	Yes	95
Are intestinal T cell dependent IgA responses, necessarily dependent on conventional cognate interactions in humans?	No	60
Do innate receptors have any role in driving the human mucosal IgA response?	Yes	100
Might germinal centers be involved in both T cell dependent and T cell independent IgA responses in humans?	Yes	95
Are there differences in the induction of IgA responses that seed the small bowel and the colon?	Yes	70
Is the human intestinal IgA response antigen specific?	Yes	100
Is light chain drifting a feature of the IgA response?	Yes	100
Is there local proliferation of plasma cell precursors in the human intestinal lamina propria?	No	90
Are human intestinal IgA plasma cells long lived?	Yes	90

small intestine (Boursier et al., 2005; Bergqvist et al., 2006, 2010; Di Niro et al., 2012). Class switch recombination also requires cell division; a process that has not been observed in lamina propria cells directly (Boursier et al., 2005; see Is there Local Proliferation of Plasma Cell Precursors in the Human Intestinal Lamina Propria?). Cells in germinal centers of mesenteric lymph nodes can express IgA2, again, consistent with class switch to IgA2 prior to homing to the lamina propria environment (**Figure 2**).

It was suggested that the lamina propria B cell response may be initiated by antigens sampled by dendritic cells (DC) that extend processes through the epithelium (Rescigno et al., 2001; Niess et al., 2005). It was also suggested that the lamina propria B cell response in mice might involve the B1 lineage (Kroese et al., 1989). However, it is now apparent that the lamina propria in mice and humans may not be so different, with most having features exclusively of effector niches. The DCs that take up antigen from the lumen do not mediate antigen presentation in the lamina propria, but transfer antigen to DCs that mediate their function in the mesenteric lymph nodes (Pabst et al., 2007; Pabst and Mowat, 2012).

The exclusivity of GALT as an inductive site is not correct if the contribution of the mesenteric lymph node is considered, hence the lack of certainty in **Table 1**. GALT is defined in part by its relationship to the intestinal epithelium; so mesenteric lymph node is therefore potentially a non-GALT inductive site (Brandtzaeg and Pabst, 2004). Human mesenteric nodes are immunologically active structures containing germinal centers that could represent initiation or amplification of human intestinal B cell responses (**Figure 2**). It would most accurate to say that the human IgA response is generated in organized lymphoid tissue, and that the lamina propria is not an inductive site.

IS THERE AN EQUIVALENT OF MURINE CRYPTOPATCH PRECURSOR OF ILFs IN HUMANS?

Cryptopatches of mice are small isolated clusters of lymphoid progenitors located around the base of the small intestinal crypts (Kanamori et al., 1996). Engagement of epithelial NOD1 innate receptors by bacteria from the intestinal lumen initiates the development of ILF from cryptopatch precursors (Bouskra et al., 2008). GALT acquired in this way in mice exists as single isolated follicles that are invisible to the naked eye, rather than the macroscopically visible clusters of lymphoid tissue that comprise murine Peyer's patches.

One problem in addressing the question of whether humans have cryptopatches or even a direct equivalent of ILF is that GALT in humans, in general, is either invisible or just visible to the naked eye. GALT can be visualized from the luminal aspect in humans by injecting dye into the mucosa, by fixation or by using magnification (Cornes, 1965). GALT in normal human small intestine can exist as large clusters, small clusters or isolated follicles in the small bowel and each of these categories appears to be structurally and developmentally equivalent (**Figure 3**), and not necessarily to be associated differently with the microbiota in the intestinal lumen.

In their search for cryptopatches, Moghaddami et al. (1998) described lymphocyte filled villi; clusters of lymphocytes within a villus structure. The significance of these structures, that are not rare, remains unknown. The current consensus is that humans do not have cryptopatches (Moghaddami et al., 1998; Brandtzaeg, 2010), but it is possible that they exist during a small window in time just after birth, though they are still to be discovered.

CAN GUT-ASSOCIATED LYMPHOID TISSUE BE ACQUIRED IN RESPONSE TO BACTERIAL ANTIGENS IN HUMANS?

Probably the clearest example of acquisition of GALT in humans in response to bacteria is the acquisition of GALT in the stomach in response to the gastric bacterial pathogen *Helicobacter pylori* (Wotherspoon et al., 1991). Normal stomach is rich in mucus and acid and is generally sterile in health (Williams, 1992; Mobley, 1996). The lamina propria underlying the gastric epithelium contains a sparse infiltrate of lymphoid and myeloid cells and there is no GALT. *H. pylori* is able to colonize the stomach by neutralizing the acidic microenvironment by the production of

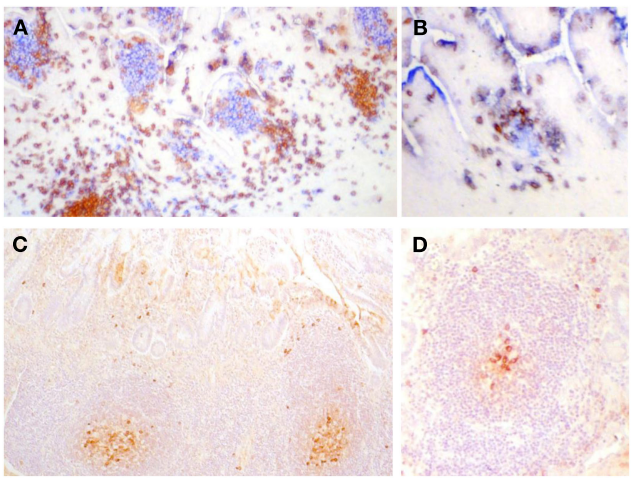

FIGURE 3 | (A,B) Frozen sections of human fetal intestine of 22 weeks of gestation. **(A)** Is stained with CD3 (T cells in brown) and CD20 (B cells in blue). **(B)** Is stained for CD3 (T cells in brown) and CD5 (B cells in blue since human fetal B cells are CD5+). Follicles in clusters in **(A)** or as ILF in **(B)** are the same in terms of the cellular composition and stage of development. Paraffin sections of human intestine at 4 days old stained for cytoplasmic IgM. **(C)** Illustrates a cluster of follicles in a Peyer's patch and **(D)** illustrates a single follicle that could be classified as an ILF. In **(C,D)**, cytoplasmic IgM+ cells can be identified in the germinal center and on the periphery of the follicle and they appear very similar. There was very little IgA apparent in sections of either in stained serial sections. These images demonstrate that in human ileum there is no apparent developmental difference between follicles in clusters and isolated lymphoid follicles.

ammonia. Infection with *H. pylori* is relatively common and the gastritis associated with *H. pylori* infection invariably includes the acquisition of GALT.

Helicobacter pylori infection may be a causative contributor to gastric malignancy including lymphoma of mucosa-associated lymphoid tissue (MALT; Wotherspoon et al., 1993). Therefore pathologists are acutely aware of the changes in the gastric mucosa and the lymphoid cells it contains in response to *H. pylori* infection. If cryptopatches were involved in the development of acquired ILFs in human stomach in response to bacteria, they would almost certainly have been noticed. However, murine stomach is not necessarily equivalent to murine intestine in the biology of cryptopatches (Ishikawa et al., 1999) and this does not reflect on the issue of human cryptopatches as a whole, which remains unclear.

ARE THERE T CELL DEPENDENT AND T CELL INDEPENDENT ROUTES TO IgA PRODUCTION IN MAN?

The identification of T cell independent contributions to the human intestinal IgA response would not have been possible without examples in human disease phenotypes. For example, the subset of patients with hyper-IgM syndrome who have mutations in CD40 have intestinal IgA responses despite the inability to recruit cognate T cell help through CD40/CD40L interaction. This is also true in CD40−/− mice that have IgA plasma cells (Ferrari et al., 2001; Bergqvist et al., 2006, 2010; He et al., 2007; Cerutti et al., 2011). Consistent with a lack of germinal center formation however, for which cognate T cell interaction remains a prerequisite, there are no mutations in the immunoglobulin heavy chain variable region genes (IGHV; Bergqvist et al., 2006, 2010). A further example demonstrating that the IgA response is not necessarily T cell dependent in humans is the presence of an IgA response in individuals with severe T cell depletion or loss of germinal centers as a consequence of HIV infection (Levesque et al., 2009), though this response may be reduced, especially in the IgA2 subclass (Schneider et al., 1996; He et al., 2007).

Dendritic cells can produce APRIL that can support class switch recombination to IgA in the absence of T cell derived CD40 ligation (Fayette et al., 1997; Litinskiy et al., 2002; Cerutti, 2008b). Despite its name, APRIL is not specifically a B cell activating factor and its function as a switch factor in models of APRIL class switch function generally include additional elements such as an initiator of cell division and cytokine, for example IL4 (Hardenberg et al., 2007). In contrast, T cell dependent class switch to IgA involves ligation of CD40 on B cells by T cells expressing CD40L and binding of the cytokine TGFβ to its receptor on B cells (McIntyre et al., 1995; Zan et al., 1998).

It has been suggested, based on mouse models, that T cell dependent IgA responses are initiated in Peyer's patches, whereas T cell independent IgA responses occur in ILF (Suzuki and Fagarasan, 2009), though this may not be exclusive, particularly in the context of infection with viruses expressing highly repetitive antigenic determinants on their envelopes or capsids. The early murine immune response to rotavirus infection includes a T-independent IgA response (Franco and Greenberg, 1997; VanCott et al., 2001; Blutt et al., 2008), driven by a B cell/DC axis in Peyer's patches (Blutt and Conner, 2010). In humans, functional differences between Peyer's patches and ILFs are not yet apparent; both contain APRIL-secreting cells and cells expressing the receptors for APRIL; TACI; and BCMA (Barone et al., 2009). The colonic isolated follicles (lymphoglandular complexes; O'Leary and Sweeney, 1986) may have large, small, or undetectable germinal centers, and it is possible that colonic GALT may be different to GALT in the small bowel by having less dependence on cognate B cell/T cell interactions and germinal center formation (Garside et al., 1998). If confirmed, this may suggest a new avenue for mucosal vaccine design, facilitating induction of high affinity IgA+ B cells independent of strict cognate B cell/T cell signaling. Currently, enteric vaccine designs are largely focused on more conventional approaches to harness T-dependent B cell pathways with the goal of stimulating IgA memory B cell responses, as illustrated by the live attenuated *Shigella* vaccines progressing through the clinic (Simon et al., 2011a). Sub-unit enteric vaccines can contain the outer membrane polysaccharide moiety of lipopolysaccharide (LPS; O-Ag), a major protective antigen of many Enterobacteria (Svenson et al., 1979; Rasolofo-Razanamparany et al., 2001; Levine et al., 2007). As such, polysaccharide antigens may purely drive T-independent IgA responses, though these are weakly immunogenic in infants (reviewed in Pollard et al., 2009; Renz et al., 2011). Nonetheless, considerable evidence demonstrates that conjugation of O-Ag haptens to protein carriers successfully drives potent T-dependent responses both in mice (Phalipon et al., 2006; Simon et al., 2011b) and in humans (Passwell et al., 2003).

ARE INTESTINAL T CELL DEPENDENT IgA RESPONSES DEPENDENT ON CONVENTIONAL COGNATE INTERACTIONS IN HUMANS?

Conventional T cell dependent B cell responses that result in the formation of germinal centers initially involve the endocytosis of antigens bound by the B cell receptor and presentation of peptides derived from them to pre-primed T cells (Garside et al., 1998). The firmest evidence for a lack of requirement for such cognate interaction in mucosal responses stems from a study in which the requirement for tonic signaling for B cell survival through the B cell receptor in a murine model was substituted by the latent membrane protein of Epstein Barr virus (EBV; Casola et al., 2004). These mice were unable to endocytose and present antigen, and therefore could not recruit T cell help and germinal centers did not form in the systemic lymphoid tissues. Interestingly however, germinal centers did form in the Peyer's patches and these were T cell dependent and also dependent on the luminal microflora.

Is there any evidence of possible disconnectivity between B cell and T cell specificity that also relates to humans? Human MALT lymphomas are malignancies of mucosal marginal zone B cells. The tumor cells are intimately associated with germinal centers and can enter, divide and differentiate in germinal centers (Isaacson and Spencer, 1987). The immunoglobulin specificities of MALT lymphomas have now been studied by many groups and a dominant reactivity with autoantigens is a consistent feature (Hussell et al., 1993; Greiner et al., 1994; Craig et al., 2010). The development of MALT lymphomas in general is associated with infection of the gastric mucosa with the bacterial pathogen *H. pylori* (Wotherspoon et al., 1993). The T cells have been shown to proliferate in response to the stimulating bacterial strains presented appropriately, but bizarrely there is no evidence that the T cell reactivity and B cell reactivity overlap in a way consistent with cognate interaction (Hussell et al., 1996). It is therefore possible that participation in human intestinal germinal center associated B cell responses is not dependent on cognate interactions. However, the lack of germinal centers in CD40−/− mice demonstrates that CD40 itself remains absolutely central to GALT germinal center initiation (Bergqvist et al., 2006).

DO INNATE RECEPTORS HAVE ANY ROLE IN DRIVING THE HUMAN MUCOSAL IgA RESPONSE?

They almost certainly do, as suggested by reports of increased invasive Salmonella and *Shigella* in patients with inborn errors in the signaling effectors IRAK-4 and MyD88 downstream of the TLRs (Picard et al., 2010). The absence of several enteric viral infections, may simply suggest that RIG-I-like helicases (RLRs) and NOD-like receptors (NLRs) may provide a compensatory role in MyD88 and IRAK-4 deficient individuals. Whether TLR, NLR, or RLRs operate any differently in the intestine compared to in peripheral systemic lymphoid tissues or tonsil is unknown.

The expression of innate TLR receptors by human and mouse B cells is very different, and it is not possible to draw any parallels in this respect. The most notable difference is the lack of TLR4 expression by human B cells and therefore inability to respond to LPS (Bourke et al., 2003; Wagner et al., 2004). In contrast to humans, LPS is the classic TI-1 B cell antigen that has been part of the murine B cell immunologists' toolbox for many years. The ability to respond to LPS by innate B cell subsets is a potentially important property in the gut and enabled identification of a functional link between murine peritoneal B1 and intestinal responses (Murakami et al., 1994).

Probably the most relevant and functionally important B cell innate receptor known in humans is TLR9. Whilst being extremely important to B cell function, is not known to have a specific role at mucosal surfaces. A recent study showed that there appeared to be no difference between the involvement of TLR9 in mucosal versus systemic responses (Barone et al., 2011). Interestingly, human tonsillar B cells are reported to undergo T-independent IgA production through an innate TLR3 BAFF dependent pathway (Xu et al., 2008). Whether intestinal infections with RNA viruses such as rotavirus or treatment of intestinal B cells with dsRNA trigger this TLR3 driven T-independent pathway is currently unknown.

MIGHT GERMINAL CENTERS BE INVOLVED IN BOTH T CELL DEPENDENT AND T CELL INDEPENDENT IgA RESPONSES IN HUMANS?

Although class switch recombination to IgA can be T cell independent or T cell dependent as described above (Zan et al., 1998; Litinskiy et al., 2002; Kaminski and Stavnezer, 2004), high rates of somatic hypermutation appear to be germinal center dependent (Bergqvist et al., 2006). Almost without exception, IgA plasma cells of healthy humans have high frequencies of mutations in IGHV, so that a germinal center independent contribution to the plasma cell population is not apparent (Boursier et al., 1999; Dunn-Walters et al., 2000; Di Niro et al., 2010). It is likely that the boundaries between T cell dependence and independence are blurred *in vivo*. In health, B cells are never truly remote from T cells or their lymphokines. The intestinal immune system is very flexible and the plasticity in the system probably testifies to its importance (Gibbons and Spencer, 2011). However, features that are observed when the system is compromised may not make a major contribution in health when the optimal paths and paths of least resistance would presumably be favored. T cell-independent responses are certainly possible, but it is not possible to gage the extent to which they actually occur in healthy humans.

ARE THERE DIFFERENCES IN THE INDUCTION OF IgA RESPONSES THAT SEED THE SMALL BOWEL AND THE COLON?

The GALT in human colon is different in structure to that in the small intestine. It tends to arise on the serosal side of the muscularis mucosae and to extend through a gap in the muscularis toward the surface epithelium forming a narrow FAE (O'Leary and Sweeney, 1986). The colonic FAE tends not to protrude into the intestinal lumen as FAE does in the small bowel (Spencer et al., 2009). Colonic GALT in humans was reported to tend not to include the large germinal centers observed in small intestinal GALT, though the same paper commented that the frequency of germinal centers in lymphoglandular complexes was more frequent in specimens with malignant growth (O'Leary and Sweeney, 1986).

The plasma cells that home to the colonic mucosa are known to have a greater tendency to migrate toward CCL28 through detection of this colonic epithelium derived chemokine by CCR10 (Kunkel et al., 2000). This contrasts with plasma cells destined for

the small bowel that tend to express CCR9 that mediates movement toward CCL25 (Pan et al., 2000; Morteau et al., 2008). It is not known if these chemokine receptors that determine the site of extravasation of plasma cell precursors are induced differently in different microenvironments, though this would seem likely.

The human intestinal plasma cell population includes many clones of widely disseminated cells along the intestine. Early studies that visualized the spread of human plasma cell clones by microdissection of small numbers of cells identified clonal identity by sequencing IGHV saw that clones of plasma cells could span the small and large intestine, but that it was more common for clonally related groups of plasma cells to be identified in either small bowel or colon (Dunn-Walters et al., 1997; Holtmeier et al., 2000). This is consistent with the distribution of chemokine directed clones as described above. This pattern of plasma cell dissemination has now been elegantly confirmed through deep sequencing of the mouse IgA immunoglobulin repertoire. The elegant piece of work demonstrates the clonal spread of plasma cells, focused but not exclusive to either the small bowel or the large bowel (Lindner et al., 2012).

A difference between the plasma cells in human colon and those in the small intestine is the relatively high frequency of plasma cells secreting the IgA2 rather than IgA1 subclass of IgA. As discussed above (see Do the Events that Initiate the Human IgA Response Occur Exclusively in Gut-Associated Lymphoid Tissue?) it has been suggested that this switch to IgA2 may happen once cells have homed to the lamina propria (He et al., 2007). However, cells switched to IgA2 have been observed in colonic GALT (Barone and Spencer, 2010) and it is therefore possible that the switch to IgA2 and induction of CCR10 expression may both be features of induction of a colonic B cell response. This inductive process might be promoted by the high colonic bacterial load (Kett et al., 1995). It is also possible that bacterial load could induce factors such as CCL28 that recruit effector cells propagated in the colonic GALT.

IS THE HUMAN INTESTINAL IgA RESPONSE ANTIGEN SPECIFIC?

IgA antibodies to polio were identified in intestinal secretions of immunized individuals, but not serum (Ogra and Karzon, 1969). This study was a major contributor to the establishment of mucosal immunology as a discipline. Polyspecific antibodies that bind to multiple antigens were also identified in secretions (Wijburg et al., 2006). However these studies did not allow the analysis of specificity at a single cell level. Since then, two developments have made a huge and precise contribution to our understanding of mucosal B cell specificities. The first is based on the engineered expression of immunoglobulin heavy and light chain sequences that had been expressed by intestinal plasma cells or their precursors (Weitkamp et al., 2005, 2006; Di Niro et al., 2010; Benckert et al., 2011). Such studies have permitted the *in vitro* expression of immunoglobulins and have identified that human intestinal plasma cells have pathogen specificity, but they are also reactive with a range of autoantigens in assays including the binding of antibodies to the HEpC cell line, normally used in the diagnosis of autoimmune diseases. The second method is dependent on the expression of surface immunoglobulins by intestinal plasma cells (Di Niro et al., 2010). This has permitted the quantification and isolation of cells binding fluorescence-tagged rotavirus antigens by flow cytometry, as well as the subsequent analysis of expressed protein following heavy and light chain gene sequencing. Antigen specificity within the human IgA response and persistent production of specific antibodies over time (Mesin et al., 2011) are encouraging observations that imply that effective intestinal vaccination is a totally realistic goal.

IS LIGHT CHAIN DRIFTING A FEATURE OF THE IgA RESPONSE?

Revision of light chains expressed IgA plasma cells is a feature of the human intestinal plasma cell response that is not widely appreciated and the factors regulating it are not known. Biases in gene rearrangements at the lambda loci of IgA plasma cells and their precursors are extreme and do not mirror the rearranged IGL gene profile of IgD+ mature naïve B cells (Su et al., 2008). A recent study into the origin of distinct memory B cell subsets describe a drift from the expected 60:40% κ:λ light chain isotype ratio in humans toward dramatically increased expression of lambda light chains (up to 80% Ig λ+ cells) by IgA+ memory B cells of the gut (Berkowska et al., 2011). This resonates the need for ongoing Ig gene rearrangements within a subset of cells required to continually diversify in response to the complex array of intestinal antigens. Each immunoglobulin heavy chain locus cannot rearrange more than once because the recombination signal sequences that flank the D segments are deleted by the initial rearrangement event. This is not a problem for the IGL rearrangements that do not involve D segments. The kappa locus can be inactivated by the kappa deleting element so that it is not always available for secondary rearrangements. The lambda locus however is not constrained in either of these ways and appears to be able to be sequentially activated and to permit drifting in the rearranged repertoire (Spencer et al., 2009). The detection of RAG genes in human GALT certainly supports this notion, though how this is regulated is unknown (Su et al., 2008).

IS THERE LOCAL PROLIFERATION OF PLASMA CELL PRECURSORS IN THE HUMAN INTESTINAL LAMINA PROPRIA?

Some early studies of rat intestine involved the surgical creation of isolated circles of intestine, so-called Thiry Vella loops. The isolated loops were separated from the rest of the intestine, through which the luminal contents would transit (Husband and Gowans, 1978). If antigen was introduced into the loops there was a tendency for antigen-producing cells to localize there and increase in number. Cells that homed to loops containing no specific challenge disappeared with time. This was interpreted as local proliferation of cells that homed to the lamina propria in response to the challenging antigen. It is now known however that such expansion was likely to have occurred in ILF within the loop. Cell division cannot be detected in isolated lamina propria plasma cells by immunohistochemical methods (Boursier et al., 2005). However, it has been claimed that clonal expansions of plasma cells within intestinal microenvironments is evident by identification of local groups of related IGHV sequences by PCR (Yuvaraj et al., 2009). In this model, the isolated IGHV sequences are identifiers of clones of

cells. Other groups claim that such findings are a consequence of dissemination of large clones of cells throughout the intestine (Boursier et al., 2005).

A recent thorough and detailed analysis of mouse IgA plasma cell repertoire has modeled the distribution of plasma cell clones and asked whether this actual distribution fits a mathematical model that includes rounds of cell division after homing of plasma cell precursors to the lamina propria. This study concluded that the data best fits a model where plasma cell precursors home and differentiate either without or with very little cell division (Lindner et al., 2012). It is possible that cell division occurs during a narrow window of time or that it is very slow over a long time so that it is rarely observed. The expression of surface immunoglobulin by human intestinal plasma cells and their precursors certainly implies that they potentially remain receptive to external stimuli that could initiate cell activation (Di Niro et al., 2010).

ARE HUMAN INTESTINAL IgA PLASMA CELLS LONG LIVED?

A recent study by MacPherson's team using mice colonized by live bacteria with a finite life span (Hapfelmeier et al., 2010) has shown that intestinal plasma cell niches will fill according to current challenges and will be replaced depending on changes in the contents of the intestinal lumen. The question of plasma cell life span in humans was difficult to consider, until recent advances in expression of immunoglobulin variable region genes derived from plasma cells as immunoglobulin proteins. This enabled the detection of plasma cells secreting rotavirus specific antibodies when there was no recent history of rotavirus infection, clearly demonstrating long-term plasma cell survival (Di Niro et al., 2010; Mesin et al., 2011). It remains unknown whether rotavirus infections might be subclinical and undetected, or if antigen might be retained, but nevertheless this insight is important because it shows that clinically relevant specific responses can be maintained.

Gene expression profiles of human intestinal plasma cells show them to have intermediate properties between short- and long-lived plasma cell populations (Medina et al., 2003). A study of whole biopsies in organ culture have identified that intestinal plasma cells may have a longer lifespan *in situ* than in isolation (Mesin et al., 2011). APRIL can support plasma cell survival in bone marrow (Belnoue et al., 2012), and APRIL produced by macrophages and epithelial cells is likely to abundant in lamina propria (Barone et al., 2009). Blocking APRIL binding to its receptor blocks lamina propria plasma cell survival in *in vitro* organ cultures, implying that sustaining long-term plasma cell survival would be dependent on the local supportive microenvironment (Mesin et al., 2011).

CONCLUSION

IgA B cell responses to gut mucosal antigens: Do we know it all? The answer is clearly no. In this review we identified what we considered to be 12 important questions relating to this topic, but we were unable to answer the majority with any certainty. It is also highly unlikely that all researchers in this field would agree fully with our deductions. What is clear however is that the intestinal plasma cell response is geared toward generation of repertoire diversity in response to challenge from the luminal microbiota. Superimposing specificity and memory onto such a system is a major challenge for intestinal vaccine design.

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The role of Peyer's patches in synchronizing gut IgA responses

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Because Peyer's patches (PP) are the main inductive sites for gut IgA responses we have focused this review on what we know about the function of PP germinal centers (GC). The vast majority of IgA gene sequences in the gut lamina propria (LP) are heavily mutated arguing for an origin in GC. Because PP GC formation is dependent on the presence of CD4 T cells, we speculate that all IgA responses in the normal gut are directly or indirectly T cell-dependent (TD). We hypothesize that the CD4 T cell involvement in gut IgA responses against the microbiota is different from that in systemic responses since cognate T–B cell interactions appear not to be required. In the absence of cognate interactions the function of CD4 follicular helper T cells (Tfh) in PP GC is unclear. However, production of IL-21 and IL-6 is more pronounced than in peripheral lymph nodes. Importantly, we discuss how multiple PP are involved in generating specific IgA responses to TD antigens given orally. Recently we found that oral immunization with NP-hapten conjugated to cholera toxin (NP-CT) stimulated a strong highly synchronized, oligoclonal and affinity matured IgA response. This was achieved through re-utilization of GC in multiple PP as GC IgA B cells emigrated into already established GC. Clonally related B cells were present in both inductive and effector lymphoid tissues in the gut and clonal trees involving multiple PP could be constructed in individual mice. Through adoptive transfer of B1-8^{hi} NP-specific B cells we demonstrated that GL7^{+} PP B cells could enter into pre-existing GC in PP, a process that was antigen-dependent but did not to require cognate Tfh interactions. Finally, we discuss the role of PP GC for the generation of memory B cells and long-lived plasma cells in the light of contrasting findings regarding IgA memory development to colonizing commensal bacteria versus that to oral immunization with enteropathogens or TD antigens.

Keywords: gut IgA, cholera toxin, germinal center re-utilization, Peyer's patches, B cells, germinal centers

INTRODUCTION

To protect us against infections and to maintain homeostasis with the microbiota the gut relies on secreted IgA antibodies (Russel and Kilian, 2005). Given that the vast majority of B cells in the gut immune system are activated and undergo class-switch recombination (CSR) from IgM to IgA in the Peyer's patch (PP) we need to expand our knowledge about the function of PP and especially its germinal centers (GC; Macpherson et al., 2008). Whereas, comparative studies in germ-free and wild-type mice have demonstrated that a large proportion of IgA plasma cells in the gut lamina propria (LP) are activated by antigens or molecular patterns associated with the microbiota, little is known as to whether these responses should be considered T cell-independent (TI) or T cell-dependent (TD; Fagarasan et al., 2010; Hapfelmeier et al., 2010; Bemark et al., 2012). For example, we have documented in CD40-deficient mice that, irrespective of a complete absence of T–B cell cognate interactions, these mice exhibit near normal levels of IgA plasma cells in their gut LP (Bergqvist et al., 2006). Hence, while lacking GC the CD40-deficient mice still promote IgA CSR in the PP, but gut IgA plasma cells in the LP have not undergone somatic hypermutation (SHM), in accordance with a TI differentiation pathway (Bergqvist et al., 2010). In contrast, gut LP IgA gene sequences revealed substantial SHM in wild-type mice, suggesting that they, indeed, had an origin in GC in the PP and appear to have been under the influence of CD4 T cells (Bergqvist et al., 2010; Bemark et al., 2012). Other recent studies in both humans and mice have also clearly documented that IgA gene sequences in general are highly mutated (Barone et al., 2011; Benckert et al., 2011; Lindner et al., 2012). This raises the question as to what the nature of the CD4 T cell influence could be (**Figure 1**).

Our findings in CD40-deficient mice argue that while cognate interactions are not required for B cells recognizing microbiota-associated TI-type antigens to undergo IgA CSR, the formation of GC is critical for SHM and affinity maturation. Thus, in this sense, these IgA B cell responses in the normal gut are dependent on GC and, therefore, indirectly dependent on CD4 T cells. However, this dependence is most likely not a cognate interaction. This interpretation finds support in a study by Casola et al. (2004) showing that B cells carrying antigen non-recognizing surrogate Ig receptors can establish GC in the PP, a process which was found to rely on non-cognate T cells and the presence of the microbiota. Thus, the microbiota could promote GC formation in PP independently of B cell antigen-recognition and CD4 T cells supported this process. This finding argues that tonic non-antigen

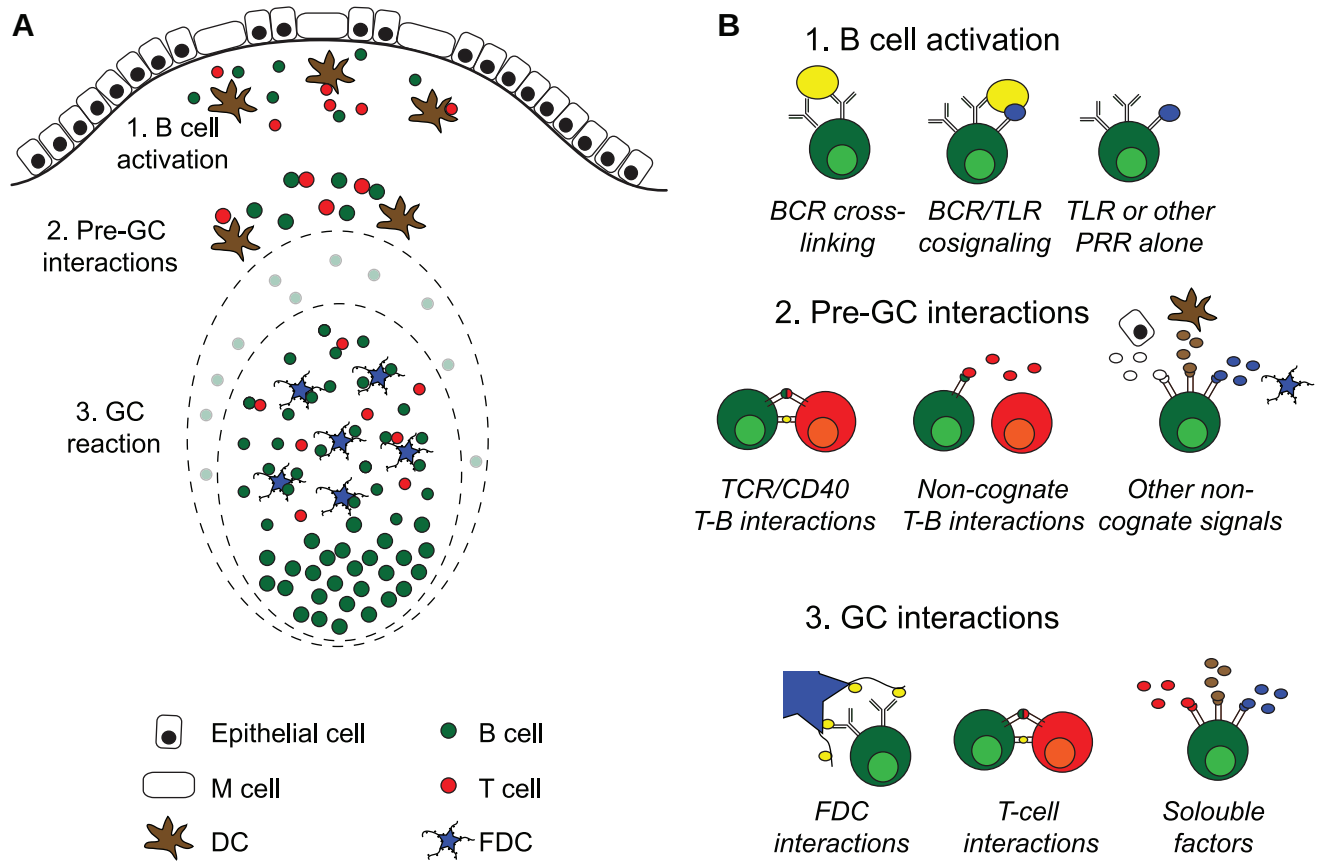

FIGURE 1 | Activation of B cells and germinal center formation in Peyer's patches. (A) Intestinal T cell-dependent antigens enter the gut via the oral route or are locally produced by the gut microbiota. Peyer's patches (PP) are covered with a specialized follicle-associated epithelium hosting M cells that can transport antigen into the subepithelial dome. Here, B cells and DC encounter antigen, leading to B and T cell activation. After interactions with cells and soluble factors outside of the germinal centers (GC), B cells enter into preformed GC where they proliferate and expand before seeding the gut with IgA producing plasma cells. **(B)** Systemic T cell-dependent responses strictly rely on two signals, antigen cross-linking and TCR/CD40 mediated cognate interactions with antigen-specific T cells, before *de novo* GC formation take place. After that, the cells are dependent on direct interaction with follicular dendritic cells (FDC) and T cells in addition to soluble factors in the GC milieu for class switch recombination and somatic hypermutation followed by affinity maturation. GC are continuously present in PP, and antigen-specific B cells enter into preformed GC rather than to form new ones. PP GC can form in the absence of antigen-specific cross-linking, and although T cells are needed for their formation, the role of cognate interactions with T cells is unclear. This suggests that signals from pattern-recognition receptors (PRR), i.e., toll receptors (TLR), can substitute and/or collaborate with BCR cross-linking during B cell activation, and that non-cognate interactions with T cells and/or soluble factors may substitute for CD40 signals. Somatic hypermutation of antibody genes is a feature shared between systemic and PP GC. However, whereas antigen-specific B cells can affinity mature in both, we do not know if the cellular interactions that influence PP GC are the same as those governing GC at systemic sites.

mediated B cell receptor (BCR) signals are sufficient for GC formation in PP. Possibly, alternative activation pathways, such as TLR signaling, are sufficient to drive B cell activation and GC formation in PP (Casola et al., 2004). However, if this is a major pathway in wild-type immunoproficient mice, it would lead to a massive presence of polyclonal IgA plasma cells in the LP, a notion that is not supported by gene sequencing data. Rather this data indicate an accumulation of highly mutated oligonal IgA repertoires with increasing age (Lindner et al., 2012).

If cognate T–B cell interactions are not required, what is then the role of the CD4 T cell? An elegant study by Wei et al. (2011) demonstrated that failing to accumulate appropriate SHM was associated with dysfunctional IgA antibodies, which lead to bacterial overgrowth and translocation, resulting in hyperplasia of GC in PP and ILF. Hence, unmutated, presumably low affinity, IgA antibodies against the microbiota are less effective in protecting the host as the mucosal barrier is perturbed. Taken together, it, thus, appears that functional, anti-inflammatory gut IgA-responses against the microbiota are critically dependent on CD4 T cell influences and, therefore, are not archetypical TI-responses.

The fact that we normally associate GC with the development of long-lived plasma cells and memory B cells raises the question if memory development occurs against antigens associated with the microbiota. This notion was recently challenged by a study that demonstrated a lack of recall IgA responses against bacteria that had transiently colonized the gut intestine of germ-free mice (Hapfelmeier et al., 2010). Although long-lived plasma cells developed it appeared that IgA memory B cells could not develop against the bacterial antigens in these mice. Considering that the generation of long-lived plasma cells and memory B cells are the

result of differentiation in GC and that the normal mouse PP continuously exhibit GC this observation is unexpected (Tangye and Tarlinton, 2009). We have observed a significant number of GC also in germ-free mice (Bergqvist et al., unpublished observation). But we do not know to which extent antigen-specific IgA B cells against the bacteria in the transiently colonized mice were generated in PP GC, albeit $GL7^+$ B cells were observed (Hapfelmeier et al., 2010). Conversely, ample documentation shows that oral immunization with TD-antigens generate IgA memory B cells. For example, oral immunization in mice with cholera toxin (CT) effectively stimulates long-lived plasma cells in the LP and memory B cells that protect against CT-challenge even 1.5 years later (Lycke and Holmgren, 1987). Likewise, studies on gut immunity to rotavirus have clearly documented priming of a specific IgA memory B cell response, which protected against rotavirus challenge through a mechanism that appeared to depend on the frequency of rotavirus-specific memory B cells in the GALT (Moser and Offit, 2001). Thus, evidence both in support of and opposing gut IgA B cell memory development has been published. If GC and CD4 T cells indeed are involved in responses against the microbiota then a natural question must be how is it possible for the GC reaction to differentially support memory B cell development against TD-antigens but not to antigens belonging to the microbiota?

The present review will address these fundamental aspects of PP function and discuss recent findings that help shed light on the questions raised in the introduction. It is an attempt to discuss the role of GC in PP in the light of what we currently know about mutations in antigen-specific gut IgA responses or based on observations using IgV_H-chain gene sequencing of gut IgA. Special focus will be given to how synchronization of IgA B cell responses in the GALT may occur, as we have recently published a study using the well characterized hapten (4-hydroxy-3-nitrophenyl)acetyl(NP) conjugated to CT for studies aimed at dissecting the different processes that critically are needed for a strong synchronized gut IgA response (Bergqvist et al., 2012).

GERMINAL CENTERS AND THEIR ROLE IN ANTIGEN-SPECIFIC RESPONSES

The formation of GC during immune responses plays a crucial role for the development of class-switched antibodies and the formation of B cell memory (Tangye and Tarlinton, 2009). Following antigen-recognition, B cells in the follicle will form GC together with follicular dendritic cells (FDC), which are key elements in the reaction (Schwickert et al., 2007). As the GC reaction progresses SHM of the BCR randomly generates relatively higher affinity variants from which plasma cells and memory B cells are selected (Berek et al., 1991). B cells expand in the dark zone and later undergo positive selection in the light zone, interacting with antigen presented as immune complexes on the FDC (Allen et al., 2007; Victora et al., 2010). Humans as well as mice lacking CD40–CD40L interactions fail to develop GC and characteristically present with hyper-IgM syndrome, i.e., high levels of IgM but little switched antibodies in serum, no TD antigen responses and lack of switched memory B cells (Kawabe et al., 1994; Xu et al., 1994; Weller et al., 2001). CD40 and CD40L interactions occur at the border between the B and T cell areas, where these cells meet after antigen-specific activation (Garside et al., 1998). Importantly, this interaction not only seems to be required for GC formation but also promotes GC maintenance, as blockade of CD40 disrupted existing GC (Han et al., 1995).

The constitutive GC formation that is observed in PP and tonsils, and to a lesser extent, in mesenteric lymph nodes (MLN) may be driven through signals other than those critically important for GC reactions in systemic lymphoid tissues. We have demonstrated that no GC develop in PP of CD40-deficient mice, but how BCR signaling or other receptor interactions critically influence PP GC formation remains to be further investigated (**Figure 1**). As aforementioned, GC in PP have been observed independently of BCR-recognition and hence the role of TLR signals may be critical in PP for GC development and for maintaining normal homeostatic function of the gut IgA immune system (Casola et al., 2004). To what extent such BCR-independent induction of gut IgA is the source for "natural IgA" that has been claimed to be part of the gut mucosal barrier function is still poorly understood (Kramer and Cebra, 1995; Macpherson et al., 2000). Recent progress in IgA gene sequence analysis using next generation sequencing techniques will most probably help answer some of the confusions around the concept of low-affinity binding "natural IgA," encoded by poorly mutated near germ-line sequences (Lindner et al., 2012).

PP IS THE MAIN IgA B CELL INDUCTIVE SITE IN THE GALT

Whereas most investigators agree that PP are the main inductive sites for gut IgA responses, irrespective of if they are elicited by antigens that belong to the microbiota or more classical TD antigens, a particular problem with the current view on the function of PP is how we can explain their role in driving protective gut LP IgA responses. IgA antibodies must be of sufficient quality to effectively protect against, e.g., pathogens or toxins (Russel and Kilian, 2005). Depending on variability between mouse strains, the average small intestine has between 8 and 12 PP preferentially located to the proximal and distal parts of the small intestine (Macpherson et al., 2008). In addition, the colon has patches that contain large aggregates of lymphocytes that in many respects appear to mimic the PP in their function (Dohi et al., 1999; Bergqvist et al., 2010). Antigen-activated PP B cells leave the tissue and migrate via the draining lymph to the MLN and then continue via ductus thoracicus to the blood from where they home back to the intestine (Macpherson et al., 2008). A consequence of this model is that in mice 10–12 PP would actively be delivering B cell blasts and plasma cells to the gut LP, and that these would represent diverse unique B cell clones with varying affinities. The protective efficacy of gut IgA, therefore, could vary greatly along the gut intestine, and in humans that often have more than 200 PP, this notion would be even more difficult to reconcile with an effective protective IgA response (Cornes, 1965). However, if we instead predict that the system is synchronized between PP and that only high affinity IgA cells are selected to dominate in the gut LP, it is easier to explain how gut IgA antibodies can effectively protect. One possibility could be that synchronization occurs in the MLN, but a recent study failed to ascribe an essential role for MLN in developing strong gut antitoxin IgA responses (Hahn et al., 2010). Thus, presently we largely lack convincing data to explain if and how synchronization of the gut LP IgA response can be achieved. Therefore, we recently directly addressed this question in a study

where we focused on gut antigen-specific IgA B cell responses to the NP-hapten after oral immunization with NP-CT, a TD antigen (Bergqvist et al., 2012).

MULTIPLE PP GERMINAL CENTERS SYNCHRONIZE AND EXERT QUALITY CONTROL OF GUT IgA ANTIBODY RESPONSES

The main finding of our study was the observation that extensive clonal lineage trees of NP-specific IgA B cells and plasma cells could be identified both at inductive and effector sites of the gut immune system following oral immunization (Bergqvist et al., 2012). In particular, when analyzing NP-specific IgA sequences that used the V_H*186.2* V region, we observed that clonally related B cells were found not only within single PP and the gut LP, but that the same clone was present in multiple PP in the same mouse, suggesting that the expansion of these clones was synchronized and a consequence of an antigen-dependent selection process (**Figure 2**). Repeated oral immunizations resulted in enhanced antibody affinity and we could follow the acquisition of a particular mutation in the CDR1 region, resulting in a 10-fold enhanced affinity of anti-NP IgA antibodies, with increased number of oral immunizations (Bergqvist et al., 2012). Remarkably, the frequency of anti-NP IgA cells with the affinity-enhancing V_H*186.2* W33 to L33 mutation in the CDR1 region increased more rapidly in the PP than in the gut LP. After the second dose of oral NP-CT only 20% of gut LP IgA carried the mutation compared to 60% in PP, while a third oral immunization resulted in that 60% of the NP-specific IgA cell clones exhibiting high affinity maturation also in the LP. Thus, the gut immune system effectively selected for higher affinity with repeated oral immunizations, and a small number of anti-NP IgA clones dominated the response after three oral immunizations. In addition, we observed that clonally related IgA cells were distributed to the LP of both the small and large intestine, albeit the frequency of related clones was higher in the small intestine than between the small and large intestine, suggesting that there was some compartmentalization of the IgA response. This notion finds support in the work by Lindner et al. (2012), where clones distinctively clustered separately to the small or large intestine.

The extensive lineage trees and the oligoclonal domination of anti-NP IgA cells after repeated oral immunizations indicated that the gut IgA response was not only strongly regulated but also synchronized between PP. This synchronization must be achieved through simultaneous antigen-driven selection in multiple PP, which suggested that antigen-activated NP-specific IgA B cells from one PP were distributed to GC of multiple PP. Hence, we hypothesized that following the priming immunization, $GL7^+$ NP-specific B cells could leave the GC in one PP and migrate to already established GC in other PP. This was indeed supported by experiments that followed the distribution of NP-specific B1-8^{hi} GFP B cells in an adoptive transfer model, which demonstrated that NP-specific B cells first expanded in proximal PPs after a priming immunization while after a third oral immunization these cells were equally frequent also in distal PP. Furthermore, when we transferred $GL7^+$ B cells isolated from PP after one oral priming immunization, these could migrate into GC in multiple PP of the recipient mouse provided NP-CT had been administered prior to the transfer (**Figure 2**). Thus, B cells from GC of one PP can migrate into already established GC in multiple PP – attesting to the notion that synchronization of the gut IgA response occurs through reutilization of already established GC in multiple PP. These data for the first time provide evidence that there is synchronization of gut IgA responses, and that these responses are dependent on the repeated exposure to antigen and effective distribution of antigen-activated PP B cells into multiple PP.

REQUIREMENTS FOR REUTILIZATION OF GERMINAL CENTERS IN PP

These findings pose a number of questions as to what is required for the PP synchronization system to operate? Firstly, we can conclude that the migration of NP-specific B cells into already established GC required the presence of antigen, as no invasion of NP-specific B cells was observed in mice orally immunized with CT only (Bergqvist et al., 2012). The finding that newly activated B cells can reutilize already existing GC has previously been elegantly shown by Schwickert et al. (2009) and excellently discussed by Or-Guil et al. (2007). Consistent with our results, Schwickert et al. (2009) demonstrated that sequential administration of two different antigens lead to efficient invasion of existing GC in draining lymph nodes by heterologous high affinity B cells.

However, a prerequisite for an effective reutilization system in the gut is that early GC emigrants, and not only newly activated B cells, can invade already established GC in PP (Tarlinton, 2006; Or-Guil et al., 2007). To test the ability of activated B cells to do this, we transferred $GL7^+$ PP GC B cells 7–9 days after an oral priming immunization (Bergqvist et al., 2012). In this case, antigen was still required for invasion but the presence of CD4 T cell help appeared not to be critical as antigen administration only 24 h prior to transfer of $GL7^+$ B1-8^{hi} GFP B cells was sufficient, a time frame that excludes the generation of effective CD4 T cell help. Thus, for localization of GC-expanded $GL7^+$ PP B cells into already existing heterologous GC in multiple other PP presence of antigen but not cognate interactions with CD4 T cells was essential. This contrasted to the previous studies by Schwickert et al. (2009) that indicated that the reutilization required that B cell epitopes/antigens shared the same carrier protein, suggesting that cognate interaction with shared CD4 T cell help was critical. Hence, $GL7^+$ B cells have different requirements than newly activated B cells, or, alternatively, PP GC are unique and different from GC in lymph nodes and the spleen with regard to CD4 T cell involvement. Reutilization of PP GC could, for example, be dependent on non-cognate functions of CD4 T cells as found with the localization of LMP2A-expressing B cells to PP GC (Casola et al., 2004). One important factor that could influence this pattern is IL-21 (Pistoia and Cocco, 2009). Whereas the formation of GC requires CD40 signals in both PP and other secondary lymphoid tissues, T cell subsets distinct from archetypical $CD4^+$ T cells appear to be present in PP. For example, Seo et al. (2009) demonstrated that IL-21 was more strongly expressed by CD4 T cells in PP than in spleen and that in the context of TGFβ the dominant isotype produced was IgA, whereas IgG2b antibody production was effectively suppressed by IL-21. Whether other aspects than IgA CSR were affected by IL-21 in PP was not further investigated

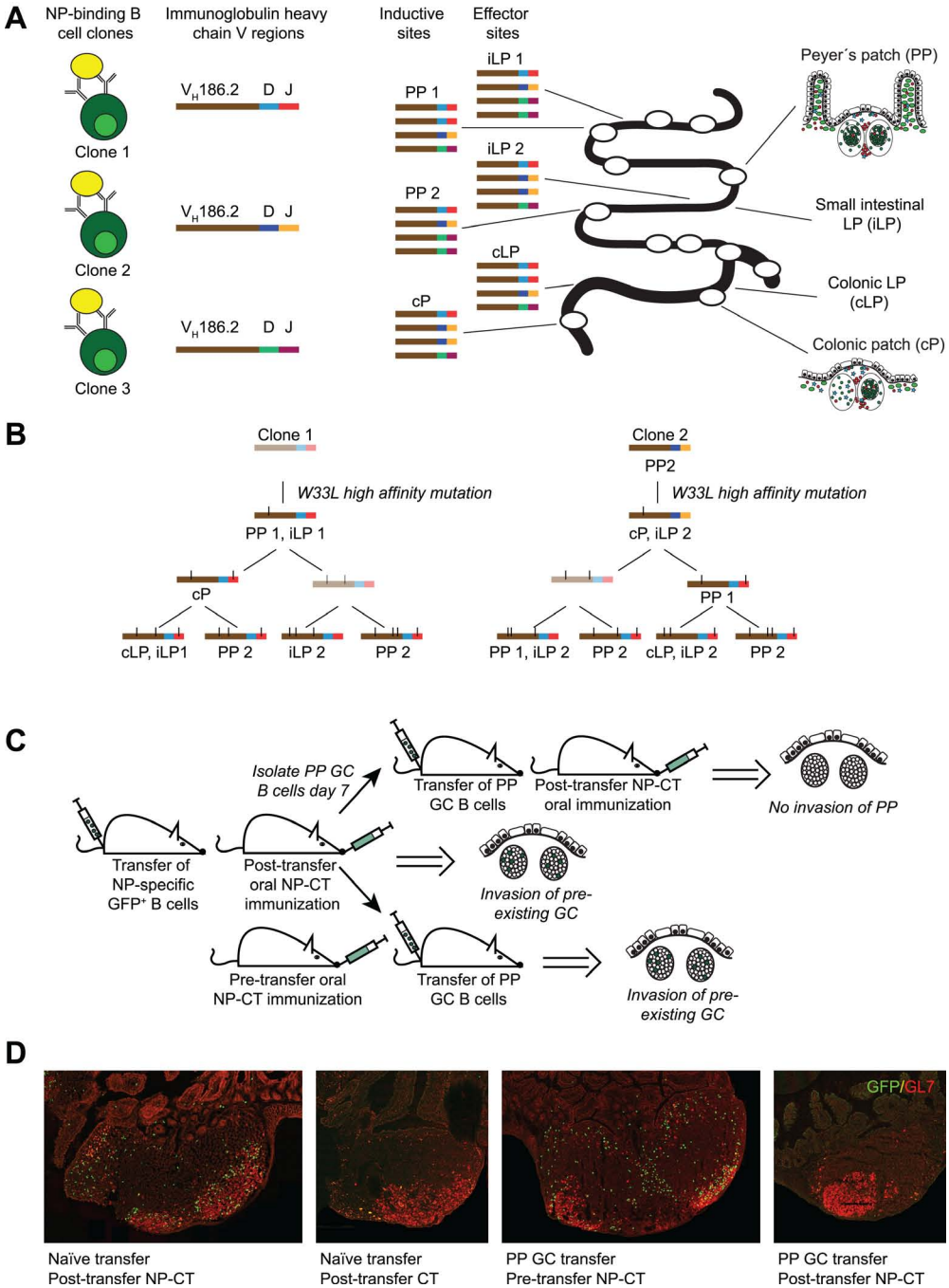

FIGURE 2 | Antigen-dependent invasion of preexisting Peyer's patch (PP) germinal centers as a mechanism to synchronize gut responses. We recently published a study that demonstrated that IgA responses are synchronized through invasion of pre-existing germinal centers in PP (Bergqvist et al., 2012). This conclusion was based on three observations. **(A)** The response to NP was dominated by B cells carrying a specific heavy chain V region (V_H *186.2*). Because antibody sequences differ in the CDR3 region it allows for clonal analysis of the response. Sequencing of V_H *186.2-IgA* genes from wild-type mice orally immunized with NP-CT demonstrated that the response to NP was highly oligoclonal and that B cells carrying identical CDR3 regions were simultaneously present at distinct inductive sites. This suggested that activated B cells from one inductive site, i.e., one PP GC, could distribute to multiple PP as well as seed the lamina propria (LP). **(B)** When clonal trees were constructed, it was evident that one specific mutation (W33L), which is known to increase the affinity 10-fold, appeared early in the response it also was represented at other inductive sites. This way the IgA response was effectively synchronized. **(C,D)** To confirm that PP GC could be invaded by GC B cells, a transfer experiment was conducted using GFP-expressing NP-specific B cells. We found that when naïve splenic B2 cells were transferred into wild-type recipients that were subsequently orally immunized with NP-CT, GFP^+ cells invaded preexisting PP GC. By contrast PP GC B cells could not invade other PP after adoptive transfer into syngeneic naïve recipient mice unless oral NP-CT was given 24 h prior to cell transfer. Thus, antigen, but not T cell help, appeared to be required for migration of PP GC B cells into already existing heterologous GC.

in this study, but Dullaers et al. (2009) found that recombinant TGFβ and IL-21 can drive naïve human B cells toward a mucosal phenotype. It is therefore plausible that IL-21 in PP promotes survival, proliferation, and differentiation of activated B cells in PP GC.

Follicular helper T cells (Tfh) in the PP have not been studied in detail and presently we do not know their role in supporting B cells that reutilize GC and that recognize heterologous antigens (Hashiguchi et al., 2011; Danisch et al., 2012). Clearly, PD-1 is critical for the generation of Tfh in the PP, and PD-1-deficient mice have a reduced ability to develop gut IgA antibodies that recognize the microbiota, resulting in reduced bacterial binding capacity and breaching of the mucosal barrier (Kawamoto et al., 2012). This observation identifies that Tfh in the PP GC are critically needed for functional IgA production, and argues in favor of that also IgA B cells recognizing TI-type of gut antigens, belonging to the microbiota, require CD4 T cell help to acquire SHM and produce protective antibodies. Diverse T cells are present in GALT, including T_H1, T_H2, T_H9, T_H17, T_{reg}, Tr1, T_{FH}, and $CD8^+$ α/β T cells as well as γ/δ cells, and there is considerable functional overlap between these T cells with regard to their ability to support B cell differentiation (Hayday and Gibbons, 2008; Wan, 2010). However, this process may not require cognate interactions, but may rather rely on IL-21 and other factors produced by PP Tfh. Recent data suggest that the origin for Tfh cells in the GALT may be unique in that they could be derived from FoxP3-expressing T_{reg} cells, rather than from T_H1 or T_H2 cells, although both these latter subsets have been found to function as Tfh in PP (Garside et al., 2004; Tsuji et al., 2009). An intriguing possibility may be that activated B cells, migrating into already established PP GC, do not require cognate T cell help, but rather interact with CD40L-expressing PP Tfh in the absence of cognate interactions. This could be a unique function of PP Tfh – to provide contact-dependent CD40L/CD40 signals but not to engage into TCR/MHC interactions. In this case, migrating activated PP B cells would require BCR-recognition and CD40L-expressing Tfh to proliferate in already established PP GC. What the consequences of such CD40- and BCR-driven B cell expansion and differentiation could be, as opposed to a cognate-dependent process in the spleen or lymph nodes, can only be a matter of speculation. Perhaps, the large number SHM in gut IgA cells bear witness of a less stringently regulated system in PP GC where B cells will continue to contract mutations even after high-affinity binding is achieved. Nevertheless, future studies will address this important question and hopefully we will better understand how an ensemble of GC in multiple PP rather than individual GC, critically functions to synchronize protective gut IgA responses and what role Tfh cells have to support the development of functional IgA antibody production when cognate T–B cell interactions are not required.

IgA MEMORY B CELL DEVELOPMENT FOLLOWING ORAL IMMUNIZATION IN THE ABSENCE OR PRESENCE OF GERMINAL CENTERS

The ability of the gut immune system to protect the mucosa against pathogens by long-term production of antigen-specific IgA antibodies is pivotal to mucosal vaccine development. A recent study by Macpherson and colleagues addressed the importance of mucosal memory after mono-colonization of germ-free mice with a commensal bacterial strain dependent on nutrients not present in the mammalian hosts (Hapfelmeier et al., 2010). Using this reversible colonization model, the authors found that repeated bacterial exposures gave rise to increasing specific SIgA titers in an additive rather than a synergistic manner, failing to exhibit a classical prime-boost effect. Interestingly, in the absence of competing antigens, the half-life of the LP plasma cells was very long, whereas in the presence of other bacteria that triggered the formation of new IgA plasma cells, the lifespan of the IgA plasma cells in the gut LP was dramatically reduced (Hapfelmeier et al., 2010). Based on these observations, the authors concluded that memory B cells did not develop, and that, although LP plasma cells had the potential for longevity, they were short-lived in conventionally reared mice.

These conclusions may be relevant for gut B cell responses driven by the microbiota, but they contrast with findings after oral immunizations with TD-antigens, as discussed below. Because GC and SHM play a critical role for both types of IgA responses we believe it will be important to understand if there are fundamentally different regulatory principles that operate in the PP GC depending on the type of antigen that drive the B cell response (Bemark et al., 2012). Whereas memory B cells can form independently of GC, such memory B cells, mostly recognizing TI-antigens, are of a different quality and harbor less mutations and affinity maturation than GC-dependent memory responses (Toyama et al., 2002). To what extent specific anti-bacterial gut IgA responses are more or less mutated still need further investigation, but recent data suggest that they are more mutated and carry GC-dependent features (Lindner et al., 2012). Hence, to understand why commensal bacteria do not stimulate memory B cell development, but TD-antigens, such as CT, do, we will have to predict that memory B cell development in PP is not an intrinsic property of the GC, but rather a quality that is under additional regulatory control. In this context, interactions with Tfh cells may be critical.

Tfh cells have been shown to induce a memory B cell phenotype in GC B cells cultured *in vitro* and can produce IL-21, IL-10, IL-6, and IL-4, which all have been implicated as instructive signals in GC plasma cell and memory B cell differentiation (Casamayor-Palleja et al., 1996; Hashiguchi et al., 2011; Shlomchik and Weisel, 2012). Given that PP Tfh appear to be different from archetypical Tfh in systemic secondary lymphoid tissues, we propose that they may provide instructive signals to B cell memory development even in the absence of cognate interactions. Hence, failure to develop memory B cells, concomitant with a strong induction of long-lived plasma cells, as reported by Hapfelmeier et al. (2010) may be a consequence of what type of, or lack of, Tfh activity that the commensal bacteria induced. In conventionally reared mice this situation may not have occurred as a multitude of commensal species are present in the intestine and potentially can stimulate a broadly functional Tfh population in PP GC. Of note, the composition of the microbiota plays an important role in shaping gut T cell functions as evident in studies showing, for example, that segmented filamentous bacteria stimulate the development of T_H17 cells, which dramatically could influence activation of mucosal B cells (Ivanov et al., 2009; Datta et al., 2010). In addition, we must consider that transiently colonizing germ-free mice with *E. coli* K-12 bacteria most probably will lead

to a TLR-mediated hyper-responsive state, which could facilitate BCR signaling and result in a TI-type of response, lacking memory development. Nevertheless, future studies are much needed to resolve this important and puzzling question.

Humans indeed maintain a sizable proportion of blood B cells that appear to be circulating IgA$^+$ memory cells (Klein et al., 1998; Harris et al., 2009; Tengvall et al., 2010). Furthermore, IgA-producing plasma cells reactive to rotaviral antigens were isolated from the duodenum of 9 out of 10 human adults that had not recently experienced rotavirus, arguing for the presence of long-lived plasma cells in the gut LP (Di Niro et al., 2010). In mice, we know from previous studies that anti-toxin IgA plasma cells can reside in the gut LP more than 6 months after oral immunizations with CT (Lycke and Holmgren, 1987). However, after 12 months specific plasma cells had largely disappeared, but memory B cells in the GALT could easily be triggered by a challenge-immunization with oral CT, giving rise to a vigorous anti-toxin IgA plasma cell response in the gut LP within 3 days upon re-challenge (Lycke and Holmgren, 1987). The duration of gut anti-toxin protection, thus, clearly reflected the ability of long-lived IgA memory B cells to elicit a rapid recall response to a renewed exposure to the antigen.

We have previously demonstrated that memory B cells from the GALT can be adoptively transferred to naïve syngeneic recipient mice, and upon an oral immunization with CT, elicit a strong gut IgA response in the LP (Lycke and Holmgren, 1989). More recently we have observed that these transferable memory B cells are present exclusively among CD80$^+$ B cells from spleen, MLN, and PP even 1 year after oral priming immunizations (Andersson et al., 2007; Bemark et al., 2011; Bemark et al., unpublished observation). Others have demonstrated that adoptively transferred rotavirus-specific memory B cells can effectively clear the infection from rotavirus-infected RAG-deficient mice (Williams et al., 1998). Interestingly, in the latter study splenic memory B cells with the ability to clear rotavirus infection through SIgA production could be identified on the basis $\alpha_4\beta_7$ integrin expression, whereas B cells lacking this integrin did not clear the infection. Taken together, it is clear that mucosal IgA responses against TD-antigens following infection or oral vaccination also effectively stimulate the development of antigen-specific memory B cells and long-lived plasma cells.

The generation of memory B cells after oral immunization raises several questions with regard to if, and in that case, how they differ from memory cells that are generated through systemic immunization. This question is not only important from a mechanistic point of view, but may also have important implications for the development of more effective mucosal vaccines. Moreover, if, as discussed above, B cells recognizing antigens from the microbiota enter PP GC, will they give rise to memory B cells and long-lived plasma cells, or are cognate B–T cell interactions needed? Given that the mucosal immune system holds several unique traits compared to the systemic immune system we would argue that it could also apply to the ability to support memory B cell and long-lived plasma cell development. There are indeed data to suggest that oral immunization can be more efficient than systemic immunization for the generation of long-term immune protection, especially against enteropathogenic infections (Lycke, 2012). Whether this is because of the expression of $\alpha_4\beta_7$ integrin on mucosal memory B cells, as suggested by the rotavirus studies, or could be a function of other properties of these memory B cells compared to systemic memory B cells is poorly understood. Moreover, we know that oral immunization stimulate mucosal and systemic memory B cell development, whereas systemic immunization fails to stimulate mucosal memory responses (Lycke, 2012; Bemark et al., unpublished observation). Understanding what the difference is between the immunization routes is critical for vaccine development, in general, and for mucosal vaccine development, in particular. Presently, we lack information about the relationship between systemic memory IgG and mucosal memory IgA cells following oral immunization. Are these memory B cells clonally related and do they emanate from the same or different inductive sites? We are currently addressing these pressing questions using the NP-CT system and the B1-8^{hi} NP-specific IgH knock-in B cell adoptive transfer model described above, with the hope to defining which Tfh functions and molecular pathways that are involved in memory B cell development in the PP GC.

CONCLUSION

The GC in the PP appears to be a unique site for expanding and synchronizing gut IgA B cell responses. We have found that clonally related antigen-specific IgA cells are distributed to both inductive (PP) and effector sites (LP) following oral immunization with a TD antigen, NP-CT. This was achieved through reutilization of already established heterologous GC in multiple PP for the expansion and selection of antigen-primed GL7$^+$ B cells. In adoptive transfer experiments we observed that presence of antigen was critical for the migration of both newly activated and GL7$^+$ B cells into the GC, while cognate Tfh interactions appeared not to be important, at least in the latter case. This raises the question as to the role of Tfh in PP GC. Recent studies in Irf4-deficient mice have clearly demonstrated that Tfh activity is required for GC formation in PP (Bollig et al., 2012). Hence, Tfh cells are clearly involved, but other studies suggest that non-cognate signals may be more important than cognate T–B cell interactions to support IgA B cell differentiation. Because most IgA gene sequences in the gut LP are heavily mutated it is thought that both TD and TI-antigen triggered IgA B cells have passed through PP GC. For example, IgA responses against the microbiota in CD40-deficient mice exhibit few mutations, but in wild-type mice it can be assumed that microbiota-reactive B cells have passed through GC as almost all IgA-producing plasma cells have undergone significant SHM, arguing in favor of a Tfh activity independent of cognate recognition in PP GC. Given that a GC origin appears to be shared by B cells responding to TD and microbial antigens, it would be expected that both types of responses are able to generate long-lived plasma cells and memory B cells. However, a recent study has provided evidence for a failure to develop B cell memory even after repeated transient bacterial colonization in germ-free mice. These conflicting findings point to that PP GC exhibit special conditions that are unique and completely different from those in GC in systemic peripheral lymph nodes. Future detailed studies will hopefully shed light on these questions and help resolve the confusing picture that currently is an impediment for a rational design of effective oral vaccines.

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The gastrointestinal frontier: IgA and viruses

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Viral gastroenteritis is one of the leading causes of diseases that kill ~2.2 million people worldwide each year. IgA is one of the major immune effector products present in the gastrointestinal tract yet its importance in protection against gastrointestinal viral infections has been difficult to prove. In part this has been due to a lack of small and large animal models in which pathogenesis of and immunity to gastrointestinal viral infections is similar to that in humans. Much of what we have learned about the role of IgA in the intestinal immune response has been obtained from experimental animal models of rotavirus infection. Rotavirus-specific intestinal IgA appears to be one of the principle effectors of long term protection against rotavirus infection. Thus, there has been a focus on understanding the immunological pathways through which this virus-specific IgA is induced during infection. In addition, the experimental animal models of rotavirus infection provide excellent systems in which new areas of research on viral-specific intestinal IgA including the long term maintenance of viral-specific IgA.

Keywords: IgA, rotavirus, calicivirus, norovirus, adenovirus, astrovirus, small intestine, gastrointestinal virus

INTRODUCTION

Gastrointestinal infections kill about 2.2 million people each year worldwide (1). In the United States, between 60 and 70 million are affected annually with gastrointestinal diseases (2) and viral gastroenteritis ranks among the top 15 principal discharge diagnoses from hospital admissions (3). Viral infections of the gastrointestinal tract are divided into two broad categories based on whether infection results in disease there (enteropathogenic) or elsewhere (non-enteropathogenic). Classical enteropathogenic viruses actually infect cells that comprise the gastrointestinal system resulting in gastrointestinal disease symptoms such as vomiting, diarrhea, malabsorption, and pain. The majority of viral gastrointestinal illnesses are caused by rotavirus, norovirus, adenovirus, and astrovirus. By contrast, although non-enteropathogenic viruses enter the body via the gastrointestinal tract, they cause mild to no gastrointestinal disease because they are distributed systemically and cause disease in other organ systems. Examples of important human non-enteropathogenic viruses include polio virus, coxsackievirus, echovirus, and hepatitis A virus. Although not a perfect fit in either category, HIV can enter through the lower gastrointestinal tract and can be associated with mild gastrointestinal disease. HIV infects cells of the immune system both in the gastrointestinal tract and systemically and thus its most severe effects are on the immune system. Both enteropathogenic and non-enteropathogenic gastrointestinal viruses induce IgA that functions in protective immunity. This review will focus on enteropathogenic gastrointestinal virus infections highlighting rotavirus, since much has been learned from the experimental animal models. The role that gastrointestinal IgA plays in protective immunity and the mechanisms through which intestinal IgA is induced will be discussed. Emerging areas in IgA research during viral gastrointestinal infections will be explored.

IgA AND IgA DEFICIENCY IN THE GASTROINTESTINAL TRACT

IgA is one of the main effector molecules produced by initiation of immune responses in the gastrointestinal tract. IgA is predominant in the intestinal lumen and it is synthesized in quantities that far exceed any of the other antibodies (4). Despite high levels of IgA in the gastrointestinal tract, its importance in intestinal immunity to pathogens has been difficult to prove. IgA clearly functions in binding to antigens, toxins, foreign proteins, and microorganisms to inhibit penetration of the intestinal epithelium (5–11). Intestinal IgA is also critical for regulation of commensal bacterial populations (12) and in its absence these populations expand, eventually escaping the gastrointestinal tract, resulting in both local and systemic activation of the immune system (12–14). By containing and controlling the microorganism population, IgA prevents their access to the intestinal immune system and thus functions to reduce local inflammation induced by endogenous bacteria (15).

IgA deficiency is the most common primary immunodeficiency although incidence depends on genetic background (16). IgA deficiency ranges from 1:223 to 1:1000 in community studies and from 1:400 to 1:3000 in healthy blood donors (17–19). Selective IgA deficiency is defined by serum levels of IgA below 0.05 g/L (19, 20). Low levels of IgA have been associated with a range of clinical manifestations including increased incidence of gastrointestinal diseases such as giardiasis, malabsorption, lactose intolerance, celiac disease, ulcerative colitis, lymphoid hyperplasia, and malignant proliferation (21–24). Patients with IgA deficiency suffer from an increased incidence of gastrointestinal infections and multiple bouts of diarrhea compared to IgA normal individuals (25–27). Despite these general statements, there are no well controlled studies that address the question of whether or not IgA deficiency predisposes individuals to increased susceptibility to

and recurrence of gastrointestinal viral infections. In fact, it is estimated that 85–90% of IgA-deficient individuals are asymptomatic (25). One explanation might be that individuals with low levels of serum IgA may actually have sufficient secretory IgA at their mucosal surfaces to remain asymptomatic (28, 29). Another might be that other antibody isotypes, in particular IgM, via transport to the mucosal surface, compensates for the loss of IgA (30–32).

IgA AND PROTECTIVE IMMUNITY AGAINST GASTROINTESTINAL VIRAL INFECTIONS

Since IgA is produced in large quantities at mucosal surfaces including the gastrointestinal tract, it has long been presumed that IgA is a critical factor in protection of these surfaces against viral infections. Many studies in humans correlate increases in viral-specific IgA levels at the mucosal surface with either the cessation of virus excretion or protection against infection and disease (33–37). With the lack of an overt clinical profile in IgA-deficient humans, it has been difficult to discern the importance of IgA in the immune response to gastrointestinal viruses. Adding to this difficulty are the relatively few animal models of enteropathogenic or non-enteropathogenic gastrointestinal virus infections in which the pathogenesis and immune response, including IgA induction to the virus, faithfully models infection in humans. There are several reasons for the lack of robust animal models. Several of the common enteropathogenic and non-enteropathogenic viruses only replicate in humans or primates, limiting studies that can be performed to determine IgA importance (38). Other viruses infect non-primate animals but the pathogenesis is dramatically different from infection and disease in humans (38), leading to questions regarding the relevance of conclusions drawn from these models to human health.

Non-enteropathogenic viruses invade the body by either breeching or crossing the epithelium of the gastrointestinal tract (39–43). Although present in the gastrointestinal tract, in most cases these viruses do not infect a significant number of cells but once they have crossed the gastrointestinal tract barrier, disseminate systemically to access secondary target sites of viral replication (42). Because these viruses are able to spread systemically, IgG usually plays a significant role in protective immunity (44–50). However, there is evidence linking mucosal IgA to protective immunity for some of these viruses (**Table 1**).

Poliovirus, a good example of a non-enteropathogenic gastrointestinal virus, induces a secretory IgA response that appears to neutralize the virus and is associated with decreases in virus shedding in stool (51–55). Mucosal IgA correlates with protection against polio infection (56). An intestinal IgA response is also induced with the live replicating oral polio vaccine (OPV) and it is surmised that OPV prevents infection through IgA-mediated viral neutralization in the intestine. Live poliovirus vaccine appears to induce long-lived memory immune responses as elderly people who received this vaccine and still had detectable serum and salivary IgA were resistant to reinfection (56). In addition, IPV vaccination of individuals that were 20–44 years of age and had previously been vaccinated with OPV induced IgA^+ $\alpha4\beta7^+$ antibody secreting cell by 7 days post vaccine but not in individuals of similar age previously vaccinated with IPV, which does not induce intestinal IgA responses. These data are consistent with the induction of memory IgA responses in the intestine. Therefore, whether IgA memory in the intestine to enteric viral pathogens undergoes the continuous adaptation observed with a commensal organism (57) is still an open question. For other non-enteropathogenic viruses, such as hepatitis A, coxsackievirus, and echovirus, much less is known about the relative importance of mucosal IgA in protective immunity (**Table 1**).

Table 1 | Role of IgA in protective from non-enteropathogenic and pathogenic intestinal viral infections.

Virus	IgA			
	Induced by infection (natural/exp)	Correlate of protection		Required for protection
		Humans	Animals	
NON-ENTEROPATHOGENIC				
Poliovirus	Y	Y	?	?
Coxsackievirus	Y	?	?	?
Echovirus	Y	?	?	?
Hepatitis A	Y	?	?	?
Reovirus	Y	?	Y	Y
HIV	Y	Y/N	Y/N	?
ENTEROPATHOGENIC				
Rotavirus	Y	Y	Y	Y
Calicivirus	Y	Y/N	?	?
Adenovirus	Y	?	?	?
Astrovirus	Y	?	?	?

Y, yes; N, no; ?, unknown.

Reovirus, although not causing significant disease, is often used as a model system of non-enteropathogenic infection in mice. In reovirus infection, following binding and transport across M cells in the Peyer's patches, the virus is distributed systemically where it can cause disease (58). It also infects the adjacent intestinal epithelium at the basolateral membrane (59) but is not an important cause of gastrointestinal disease in humans Reovirus infection induces intestinal IgA production and IgA protects against infection when administered orally at the same time as the virus or when secreted from subcutaneous tumors (9, 60). Mice lacking expression of IgA are susceptible to reinfection with reovirus (11) indicating that IgA is an essential component of immune protection. Whether the reovirus model faithfully predicts the role of IgA in immunity to other non-enteropathogenic viruses awaits definitive proof. Protective immunity against most non-enteropathogenic infections has focused on systemic immune responses or the immune response at the site of disease rather than on the mucosal IgA response. The lack of adequate animal models has severely limited insights into the relative importance of mucosal IgA in protective immunity to these viruses.

Enteropathogenic gastrointestinal viral infections are the major cause of diarrhea and vomiting disease in humans worldwide and most induce IgA within the first week after viral exposure. Astroviruses and adenovirus are important causes of acute gastroenteritis primarily in infants and young children as well as

in the elderly and immunocompromised patients (61–68). There is limited evidence that protection from both adenovirus and astrovirus infections correlates with mucosal virus-specific IgA in humans (34, 69, 70). These viruses lack small animal models, in which pathogenesis and immunity is similar to that observed in humans. The number of cases and disease severity of gastroenteritis caused by these two viruses is far less than that caused by calicivirus (71–74).

Two genera of caliciviruses, norovirus and sapovirus cause infectious gastroenteritis (75). Sapoviruses cause gastroenteritis in young children and in long term health care settings but the number of cases is far less than noroviruses (75). Norovirus is becoming the predominant cause of viral diarrhea in all age groups worldwide (76) and is the causative agent of >96% of all outbreaks of non-bacterial gastroenteritis (77). Epidemiological data gathered from human studies suggests a link between mucosal IgA induced either by infection or by non-replicating vaccines and short term protective immunity from norovirus infection (78–80). Elucidation of the role of the gastrointestinal IgA response to norovirus has been limited by the absence of animal models in which human noroviruses replicate or that mimic the course of gastrointestinal infection and disease in humans. Several non-human primate models have been developed with limited success in advancing knowledge of clinical infection and illness arising from norovirus infection (81–84). More has been gleaned from the gnotobiotic pig and calf models which exhibit diarrhea, virus shedding in feces, seroconversion, and immunocytopathic changes in the intestine (85–87). In norovirus infected gnotobiotic pigs, anti-norovirus IgA is detected as early as 6 days following virus exposure and diarrhea and severity moderately correlated with convalescent phase intestinal antibody IgA titers (87). Similarly, both norovirus-specific IgA and IgA secreting cells were present 28 days following norovirus exposure in gnotobiotic calves (86). Unlike norovirus infection in gnotobiotic piglet and calf, murine norovirus infection differs substantially from human norovirus pathogenesis, clinical manifestations, host receptors, and infected cell types (88) but a requirement for B cells and antibody in timely virus clearance and vaccine-induced protection is implicated in the mouse (89, 90). The limitations of these animal model systems, such as the lack of intestinal microbiota and differences in pathogenesis, have precluded elucidation of whether mucosal IgA is likley protective in human infection and disease.

Like norovirus, rotavirus is a major cause of gastroenteritis especially in pediatric populations where the disease is most severe. Rotavirus accounted for nearly half a million deaths in children younger than 5 years old worldwide each year prior to introduction of vaccines (91). However, unlike norovirus, mucosal rotavirus-specific IgA strongly correlates with less severe disease and prevention of rotavirus infection in humans (37, 92–95). The differences in pathogenesis between these two viruses and the lack of good reagents and model systems in which to advance our limited understanding of the pathogenesis and immune response to norovirus infection potentially explain these correlative differences. Rotavirus-specific IgA has been shown to neutralize the virus as well as mediate heterotypic protection (96). Vaccine development strategies primarily focused on utilizing live attenuated strains that replicate in the intestine and have been successful likely because of the induction of mucosal IgA responses. Unlike norovirus, there are excellent animal models of rotavirus infection and disease that range from horses to rodents that mimic human disease. From these models (discussed in more detail in the coming sections below), virus induced IgA has been shown to play an important role in clearance of infection and protection from reinfection.

MECHANISMS OF IgA INDUCTION

Protective IgA responses to gastrointestinal viruses are thought to be comprised of high affinity antibodies that recognize and neutralize the viruses. High affinity IgA producing cells arise from the actions of helper T cells, within the context of the germinal center environment in the gastrointestinal inductive sites, Peyer's patches, isolated lymphoid follicles, and mesenteric lymph nodes (97). These helper T cells signal B cells using such molecules as TGFβ and CD40, to induce class switch recombination and somatic hypermutation resulting in the production of high affinity IgA (98) that is thought to function to neutralize the intestinal virus. Once signaled to become a high affinity IgA^+ B cell, the cell leaves the inductive site germinal center and circulates back to the intestinal lamina propria based on cell surface expression of markers, such as α4β7 (97, 99–101). This process takes at least 7–10 days following initial virus infection in the gastrointestinal tract.

In contrast to the production of high affinity IgA that results from interactions between T and B cells in the germinal center environment, unmutated low affinity IgA can be synthesized very rapidly in the gastrointestinal tract in a T cell independent fashion (102, 103). This low affinity antibody primarily functions to limit penetration of commensal microbes through epithelial cells (104) and most believe that it does not play an important role in limiting pathogens, including gastrointestinal viruses. However, virus-specific intestinal IgA, that is presumably high affinity, develops rapidly and many acute viral infections are resolved prior to the time frame required for generation of germinal center high affinity IgA antibody. Mice that have defects in germinal center formation develop specific intestinal IgA responses, including to viruses (105–107), providing further evidence that germinal center reaction might not be necessary for clearance of infection and production of virus-specific antibody. Therefore, an alternate possibility is that IgA generated through T cell independent pathways develops sufficient affinity to limit viral replication. Rapid T cell-independent virus-specific antibody responses are generated during many acute virus infections, including VSV, influenza, and polio (108–116). These antibodies mediate virus clearance and limit replication and dissemination prior to generation of T cell-dependent IgA (117). Virus-specific IgA can be induced in the absence of $CD4^+$ T cells (110, 118, 119). Mice lacking expression of MHC II (120), CD40 (121), or CD40L (122) can exhibit antibody class-switching and it is thought that molecules such as BAFF and APRIL produced by dendritic and epithelial cells drive class switch recombination and somatic hypermutation in B cells independently of germinal centers (123–127). Emerging evidence implicates a greater role for T cell independent non-germinal center generated IgA in pathogen-specific responses in the intestine.

ROTAVIRUS: A MODEL SYSTEM TO STUDY INTESTINAL IgA INDUCTION

Rotavirus is a highly infectious double-stranded RNA virus that replicates in epithelial cells of the small intestine (128–131). Virus is excreted in the stool and is transmitted from infected individuals by the fecal oral route. Infection, measured by excretion of the virus in stool, lasts on average 3–8 days and is manifested by fever, emesis, and diarrhea. Disease is most severe in the young, the elderly, and the immunocompromised. Rotavirus-specific intestinal IgA is one of the principle effectors of long term immunity based on correlative studies (37, 92–95). Rotavirus is one of the few gastrointestinal viral infections in which the pathogenesis and immune response in experimental animal models closely mimics that of humans. Virtually all naïve individuals and animals are susceptible to rotavirus infection but rotaviruses exhibit some species specificity. All of the animal models of rotavirus infection and disease (horse, cow, sheep, gnotobiotic piglet, rat, and mouse) exhibit the same primary tropism of virus to the small intestinal epithelial cells, excretion of the virus in the stool, kinetics of infection, most severe disease in the young, and induction of rotavirus-specific intestinal IgA that correlates with clearance of infection and protective immunity (129–149). Both humans and animals exhibit widespread systemic distribution of the virus (141, 150). The similarity of pathogenic features of disease and immunity between rotavirus infection in humans and across all animal models is nearly unique among gastrointestinal viral and bacterial infections making the rotavirus experimental animal models excellent systems for understanding human pathogenesis and immunity.

ANIMAL MODELS

The gnotobiotic piglet and the mouse model are the principle models of experimental rotavirus infection. Gnotobiotic piglets exhibit diarrheal disease after infection with multiple porcine and at least one human rotavirus strains and disease severity diminishes with age (136). Protection against human rotavirus infection correlates with both serum and intestinal rotavirus-specific IgA levels and antibody secreting cells in this model (136). Mice of all ages, irrespective of genetic background, are susceptible to murine rotavirus infection (e.g., EC_{wt}, EDIM, and McN) but have limited susceptibility to non-murine strains of rotavirus (132, 151–153). Rotavirus-associated diarrheal disease in mice is age restricted and is observed only up to 2 weeks of age (151). Following inoculation of adult mice with murine strains, rotavirus is detectable in stool by 24–48 h and systemically in the blood between 48 and 72 h (154). Mice resolve infection between 5 and 7 days after viral inoculation concurrent with the detection of stool IgA (151). Rotavirus-specific IgA is the predominant immunoglobulin response in the intestine and IgA titers persist long term. Mice are completely protected from reinfection for the lifetime of the mouse (155).

PASSIVE PROTECTION

Passive protection from rotavirus has been demonstrated in many animal model systems and early studies indicated that it is primarily mediated by presence of antibody in the intestine not in the circulation (156–158). In mice, IgA was shown to be more potent than IgG in protecting pups from rotavirus disease but protection was observed with both isotypes (156). Passive protection is mediated by neutralizing antibody to two rotavirus neutralization antigens VP4 and VP7 (159) but an IgA monoclonal antibody to VP6 administered by backpack also protects through intracellular neutralization (160–162). Passive protection of infants and toddlers from nosocomial infection or during an outbreak in an orphanage has been assessed in several small studies in which children were administered either human gammaglobulin or bovine colostrum from hyperimmunized cows (163–166). Significant protection was observed in some but not all studies. Whether protection was mediated by rotavirus-specific IgA or IgG is not known. A role for IgA in passive protection of children from rotavirus has been suggested based on breast feeding studies but not all studies have supported the protective effects of breast feeding against rotavirus (167–171).

PROTECTION INDUCED BY VACCINES

During the development of both non-replicating and replicating rotavirus vaccine candidates, IgA has been explored as major correlate of protective immunity induced by rotavirus vaccines (139, 153, 172–185). However, demonstration of a conclusive role for vaccine-induced IgA in protective immunity against infection and disease in children has remained elusive (186, 187). In the last 7 years, Rotarix (GSK Biologicals) and RotaTeq (Merck), two live oral rotavirus vaccines were licensed for use and have been show to prevent severe disease and death in children (188–192). Lower levels of vaccine-induced serum IgA titers correlate with higher child mortality (193). In addition, vaccine efficacy and duration of protection could be predicted by serum IgA titers. What is lacking in these analyses is whether the levels of serum IgA induced by the rotavirus vaccines can accurately predict protection from infection. One caveat to determining the role of vaccine-induced IgA in protective immunity may be that in humans, there are differences between serum IgA and IgA present in the intestine, the most significant being that serum IgA is a different isoform than the IgA present at mucosal surfaces. Further studies are necessary to identify whether vaccine-induced serum or mucosal IgA are true effectors of rotavirus protective immunity.

IgA AND ROTAVIRUS

The conclusion that gastrointestinal IgA is critical for resolution of rotavirus infection and in protection from reinfection is drawn mainly from studies in gene knockout mice. Mice lacking B cells exhibit significant delays in clearance of a rotavirus infection (194) and fail to establish protective immunity against a second infection (140). These deficits contrast to the limited to no defects in clearance or protection in mice lacking T cells (118, 195). High levels of protection against rotavirus infection are induced in mice lacking T cells, T cell knockout animals produce ~60% of wild type rotavirus-specific IgA (118). T cell independent antibody plays a role in resolution of infection (196). Mice without IgA exhibit similar delays in viral clearance and in the development of protective immunity (197), leading to the conclusion that IgA is critical. Further conclusions about the importance of mucosal IgA to rotavirus immunity come from studies in mice lacking the ability to transport IgA and IgM from the intestinal lamina propria to the lumen due to the absence of J-chain expression, a protein required for transport. J-chain knockout mice exhibit an almost identical delay

in clearance and absence of protective immunity to that reported in both B cell and IgA knockout mice (179). Together these studies indicate that IgA in the intestinal lumen is a key component in the immune response to rotavirus.

Emerging work has focused on the type and origin of B cells that are required for the IgA response to rotavirus. Adoptive transfer studies where $\alpha4\beta7^{-}$ and $\alpha4\beta7^{+}$ B cells were transferred into mice chronically infected with rotavirus demonstrate that α4β7 expression, which is expressed on B cells that circulate to the intestinal lamina propria, plays an important role in clearance of an ongoing rotavirus infection (198). In addition to α4β7, recruitment of B cells to the intestine also depends on expression of CCR9 and CCR10, receptors for ligands CCL25 and CCL28 which are exclusively expressed in the gastrointestinal tract (199). Circulating B cells that ultimately reach the intestine can originate from several sources including the peritoneal cavity (B1 cells), the Peyer's patches (naïve B cells), and the bone marrow (memory B cells). Data in the gnotobiotic piglet model suggests that bone marrow B cells do not play a significant role in rotavirus immunity (200). Although B1 cells are a major source of intestinal antibody, B1 cells are not critical to the IgA response to rotavirus (201). B cells in the Peyer's patches are currently being studied and hold great potential for understanding fundamentals of induction of the IgA response to rotavirus. Peyer's patches are inductive sites for mucosal IgA responses in the small intestine (202). Rotavirus induces specific IgA antibody in the PP and this precedes appearance of IgA in the lamina propria (101), suggestive that rotavirus-specific antibodies originate in the PP and not the lamina propria. This is supported by studies in mice that lack expression of the TNF family member LTα that do not develop Peyer's patches (203) and these mice are unable to clear rotavirus infection or produce stool rotavirus-specific IgA following virus exposure (204). This is similar to the response seen in B cell, IgA, and J-chain knockout animals (140, 179, 194, 197). Therefore, Peyer's patch B cells appear to be critical for intestinal rotavirus-specific IgA. Analysis of the Peyer's patches of mice within 24–48 h after infection indicates there are large increases in activated B cells (205), which may be driven by type I interferons secreted by dendritic cells (206). This activation is followed by an increase in local production of rotavirus-specific antibody (205). Similar levels of Peyer's patch B cell activation and early antibody production occur in the absence of T cells (205), indicating that the early Peyer's patch B cell response to rotavirus is T cell-independent with minimal T cell activation and inflammatory response induced during infection (205, 207). Since T cells are a mainstay of the germinal center environment, T cell independence suggests the lack of germinal center involvement in the rotavirus induced B cell activation and IgA induction. Mice lacking the classical germinal center molecules CD40 and CD40L produce wild type levels of intestinal rotavirus-specific IgA (107). Indeed, the first rotavirus-specific B cells detected in the Peyer's patch early after infection are extrafollicular B cells (101). Therefore, rotavirus activates Peyer's patch B cells and induces IgA through non-classical T cell independent extra-follicular pathways (**Figure 1**).

Although experimental models of rotavirus infection have been used to characterize the B cell and IgA response to rotavirus, there is still more to learn from these models about the role of IgA in gastrointestinal virus infections. Little is known about signals that direct PP B cells toward extra-follicular growth instead of germinal center formation during rotavirus infection in the mouse. Classically, TGFβ and signaling through CD40 modulate the switch to IgA in the germinal center with the help of cytokines such as IL-10, L-6, IL-4, and IL-2 produced by T cells (103). Consistent with the hypothesis that germinal centers are not playing a large role, IL-6 is not required for rotavirus-specific IgA production or virus immunity (208). It remains to be discerned whether any of the other cytokines linked to IgA induction are critical to or affect mucosal IgA levels to rotavirus. The evidence that rotavirus induces extrafollicular B cells that do not rely on T cell help raises the possibility that other factors are necessary to direct the B cell response. BAFF and APRIL are candidates as well as yet unidentified factors (125, 209). Rotavirus infection in mice lacking BAFF and APRIL expression should determine whether these molecules are critical for the intestinal IgA response to rotavirus. Interestingly, absence of CCR6 results in a 70–80% decrease in rotavirus-specific IgA levels compared to wild type mice (210). CCR6 is critical for localization of dendritic cells to the subepithelial dome of the Peyer's patch (210) raising the question as whether Peyer's patch dendritic cells regulate induction of B cell activation and IgA responses during rotavirus infection. Rotavirus infection activates dendritic cells in the Peyer's patches prior to and concurrent with the activation of the B cells (211) and activation of B cells is dependent on dendritic cells (206). The Peyer's patch activated dendritic cells produced IL-10, IL-12/23, and TNFα and upregulate expression of INFα and INFβ but B cell activation appears to be dependent on type I IFN (206, 211). The dendritic cell derived signaling contributed to the IgA response to rotavirus (206).

USE OF THE ROTAVIRUS ANIMAL MODELS TO OPEN NEW AREAS OF RESEARCH INTO VIRAL-SPECIFIC IgA

Because rotavirus induces a profound intestinal IgA response, the rotavirus model systems are valuable tools in which the plasticity of the IgA repertoire can be explored. Almost nothing is known about how the diversity and specificity of IgA in the gastrointestinal tract is shaped or altered during viral infections. There has been some indication, using methods that involve expansion of single rotavirus-specific circulating B cells, that there is low number of somatic hypermutation in circulating rotavirus-specific IgA^{+} B cells isolated from infants and adults that have previously experienced a rotavirus infection and that VH1–46 is the immunodominant gene segment, except in $CD5^{+}$ B cells in young children (212–214). The availability of more rapid and broad sequencing approaches provides a new methodology to probe and understand the diversity and composition of the intestinal IgA repertoire. Using high throughput sequencing, the IgA producing plasma cell pool in the small intestine was recently demonstrated to contain two subfractions: frequent oligoclonal plasma cells that have low diversity and are present in high numbers and polyclonal plasma cells that have high diversity but are present in low amounts (215). Analysis of over one million Vh sequences extracted from plasma cells in the proximal, middle, and distal portions of the small intestine revealed that there are both highly expanded IgA secreting plasma cells (clonally related) as well as low frequency clones (clonally unrelated). The authors

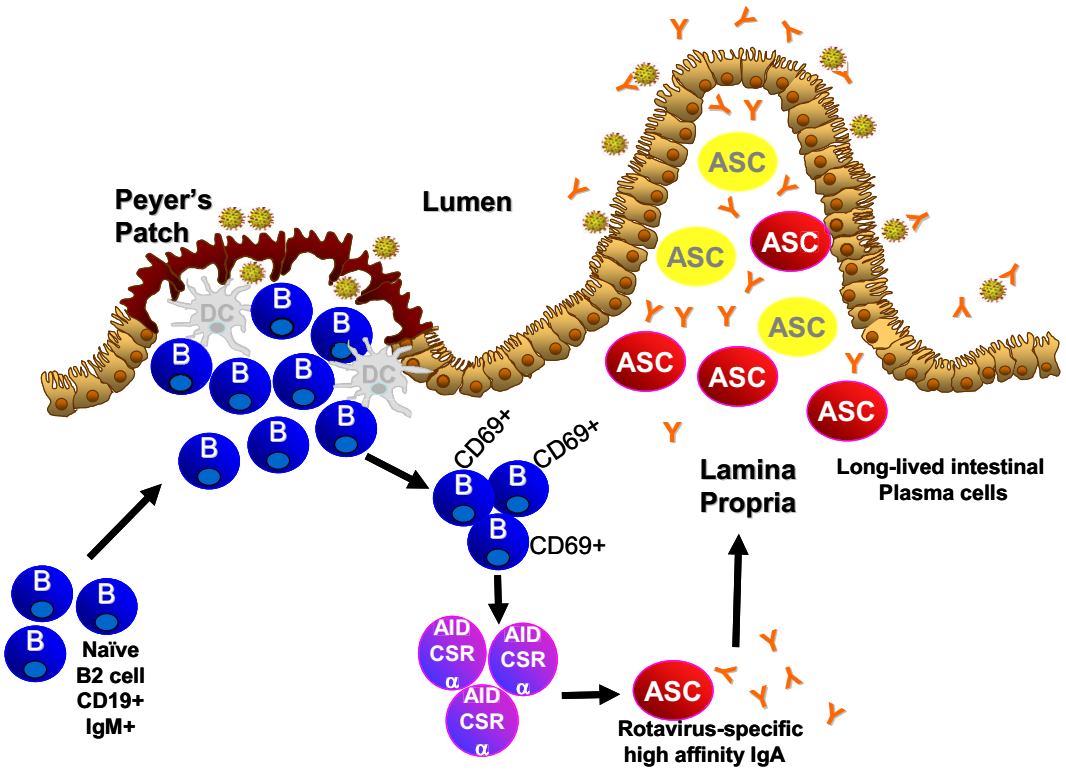

FIGURE 1 | Novel pathway of IgA induction and longevity in the intestine.

concluded that there is more diversity in the IgA repertoire than has previously been demonstrated. It remains to be determined how a highly pathogenic viral infection that induces a substantial IgA response alters the plasma cell composition. In both animals and humans, rotavirus-specific IgA, once induced, can persist for long periods of time (136, 155, 216–220). This persistence suggests that rotavirus infection makes a permanent alteration to the IgA repertoire. Next-generation sequencing is a powerful new tool which has the potential to reveal pivotal insights as to how viral pathogens shape the IgA composition within the gastrointestinal tract.

The persistence of rotavirus-specific IgA in humans and experimental animal models is intriguing and provides the perfect backdrop in which to study the maintenance of viral-specific IgA antibody secreting cells. Plasma cells develop following reactivation of quiescent memory B cells. Antibody secreting cells were thought to be short lived (~2–3 weeks) (221) but this is still being debated. Recently, populations of long-lived plasma cells that produce antibodies for several to many years have been shown to reside in "niches" in the spleen, lymph nodes, and bone marrow (222). The repertoire of these plasma cell niches could be rapidly recalled after temporary depletion indicating the likelihood that these plasma cell niches have a memory component (215). Several characteristics of rotavirus infection in mice indicate that long-lived IgA^+ plasma cells are generated following rotavirus infection and mediate protective immunity. First, infection of naïve mice results in intestinal and fecal rotavirus-specific IgA that stabilizes around 3 weeks after infection and stays constant over the lifetime of the mouse (132, 151). Second, murine rotavirus infection in mice induces lifetime protection against reinfection (132, 151). Upon re-exposure to rotavirus, viral proteins are not detected in the intestinal tract or in the feces (132, 151) and there is no increase in the titers of rotavirus-specific IgA in the feces (132, 151). The lack of discernible induction of antibody titers following re-exposure suggest: (i) that there is no reactivation of rotavirus-specific memory B cells or generation of new antibody secreting cells and (ii) that the antibody produced by the long-lived plasma cells is sufficient to neutralize the viral challenge. In addition, there is no indication that rotavirus replicates or that antigens are maintained chronically in the mouse past the time when it is detectable in stool (220, 223). This suggests that the plasma cells that produce rotavirus-specific antibody do so in the absence of virus. Work in the gnotobiotic piglet indicates that the bone marrow does not house the IgA response to rotavirus but rather the plasma cells are located in the intestinal lamina propria (200). It remains to be determined whether the specific environment of the intestinal lamina propria facilitates the development and maintenance of long-lived IgA secreting plasma cells that have a memory component (**Figure 1**).

SUMMARY

Although IgA is produced in large quantities in the gastrointestinal tract, its importance in the immune response to gastrointestinal viral infections is unclear. In part, this is due to the lack of good

animal models in which the pathogenesis, disease, and immune response induced during gastrointestinal viral infection reflects that which occurs in humans. The animal models of rotavirus infection closely mimic many parameters of infection in humans including a profound induction of rotavirus-specific intestinal IgA that correlates with clearance of infection and protection from reinfection. These animal models a currently being used to investigate and characterize the molecular pathways through which the virus induces the intestinal IgA response and will contribute significantly to our understanding of the important role IgA plays in the defense against intestinal virus infections. The models, combined with new technologies, are positioned to reveal new and exciting information as to the location, diversity, and maintenance of long-lived IgA^+ plasma secreting cells. Furthermore, these studies will facilitate the design and development of future oral vaccines by providing new and more efficient targets to induce protective IgA.

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