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IgA Function in Relation to the Intestinal Microbiota

Andrew J. Macpherson,[1,2] Bahtiyar Yilmaz,[1,2] Julien P. Limenitakis,[1,2] and Stephanie C. Ganal-Vonarburg[1,2]

[1]Maurice Müller Laboratories (Department of Biomedical Research), University of Bern, 3008 Bern, Switzerland

[2]University Clinic of Visceral Surgery and Medicine, Inselspital, 3010 Bern, Switzerland; email: andrew.macpherson@insel.ch

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IgA, microbiota, antibody binding, intestinal inflammation, childhood enteropathy, early-life immunity, immunoglobulin repertoire

Abstract

IgA is the dominant immunoglobulin isotype produced in mammals, largely secreted across the intestinal mucosal surface. Although induction of IgA has been a hallmark feature of microbiota colonization following colonization in germ-free animals, until recently appreciation of the function of IgA in host-microbial mutualism has depended mainly on indirect evidence of alterations in microbiota composition or penetration of microbes in the absence of somatic mutations in IgA (or compensatory IgM). Highly parallel sequencing techniques that enable high-resolution analysis of either microbial consortia or IgA sequence diversity are now giving us new perspectives on selective targeting of microbial taxa and the trajectory of IgA diversification according to induction mechanisms, between different individuals and over time. The prospects are to link the range of diversified IgA clonotypes to specific antigenic functions in modulating the microbiota composition, position and metabolism to ensure host mutualism.

1. IgA FUNCTIONS

IgA is the isotype that constitutes the bulk of immunoglobulin produced in mammals. Most IgA is secreted across mucous membranes, especially in the (small) intestine. In considering the role of IgA in relation to the intestinal microbiota, a useful starting point is the diversity of functions of IgA at the mammalian intestinal surface. These include neutralizing toxins and viruses, blocking excessive live bacterial adherence or translocation, clearing unwanted macromolecular structures at the epithelial surface, and directed sampling of luminal antigen (1–6). Lack of IgA against a specific bacterial epitope has been shown to increase transcriptional evidence of innate immune system activation in the intestine (7). Secreted antibodies in maternal milk shield the young mammal from premature stimulation of its own mucosal immune system and have long-term effects on the composition of its acquired microbiota (8–10). These heterogeneous functions have likely evolved in parallel with the diversity of taxa in the intestinal microbiota. In other words, the classical focus on investigating antibody function mainly in terms of neutralization of pathogen invasiveness or toxigenicity (11) is being widened to include those functional aspects of the interactions of the host with microbes that are being contained as nonpathogens.

The challenge to widen the focus from direct pathogen neutralization to other functional activities lies in the experimental readouts themselves. Measuring changes in the severe phenotype that a single pathogen can exert on its host as a function of antibody induction or specificity is a rather cleaner experimental system than the diversity of compensated functions that manipulate the microbiota or its molecular interactions with its host. For example, evaluating the consequences of secreted antibodies that affect metabolism of benign microbes that exist in the luminal contents may be far more straightforward than determining the long-term consequences for the host. Yet it was the host that generated these antibodies in the first place, and the long-range consequences of microbial metabolism (7) are intimately linked with host metabolism (7, 12). Making functional interpretations is therefore a question of being able to expand the dimensionality of the readouts, both in depth and over time.

Science generally is advanced by a continuum of hard work with established technologies and computational methods and the introduction of game-changing new techniques. To understand IgA function, we have built on classical studies of IgA in relation to cholera toxin as an immunogen itself (13) or as an adjuvant capable of inducing mucosal responses to other antigens (14), where IgA against cholera toxin could be related to protection from its secretory effects on the intestine (4, 15). Cholera toxin was instrumental in revealing the recirculation of IgA lymphocytes through the lymph and the bloodstream after local induction in intestinal secondary lymphoid structures, antibody neutralization at the mucosal surface, the T cell dependence of this arm of the mucosal immune system, and the existence of mucosal immune memory (reviewed in 16). Animal experimentation with a limited defined microbiota (gnotobiotic technology generating isobiotic animals) with century-old origins has been combined with inbreeding and germline genetic manipulation to produce designed isogenic animals: This approach allows in vivo host functions to be studied in the context of a defined limited microbiota (17). Omics have been game-changing technologies for the role of IgA in host microbial mutualism, because (together with the parallel development of computational methods) they allow unprecedented insight into a diverse microbiota and fantastic depth of analysis of the IgA host repertoire. Omics do more than power analysis of animal models, because they enable assessment of the multidimensional complexity of the role of IgA in human-microbial mutualism and disease.

This review focuses primarily on IgA function in relation to the nonpathogenic intestinal microbiota rather than giving an overview of mechanisms of T-dependent or -independent class switch recombination in the intestinal mucosa, for which the reader is referred to recent reviews

(18–21). The quality of the IgA response clearly has an influence on IgA function, so evidence that different pathways of IgA induction have a functional influence on host-microbial mutualism are developed later in the review. We also address how the emerging insights into the detailed IgA repertoire can potentially help resolve the range of IgA-based host-microbial interactions.

2. IgA FUNCTIONAL REDUNDANCY

Redundant is a rotten word, because it makes you think you are useless.

—Billy Connolly

Selective IgA deficiency is the commonest diagnosed immunodeficiency in Caucasians, with a prevalence of approximately 1:600 in measured Iceland, as reported in 2013 (22). Although it has generally been thought to have a rather weak phenotype, affected individuals have an increased incidence of respiratory infections, allergy, and autoimmunity (23). There are also case reports suggesting that immunosuppression or other immunodeficiencies can expose a stronger phenotype (24, 25): The frequency of selective IgA deficiency makes it hard to exclude epidemiologically chance co-occurrence of other (rarer) conditions. Studies now in progress will report the effect of selective IgA deficiency on the human microbiota. In contrast, common variable immunodeficiency (where all immunoglobulin isotypes are deficient) is strongly associated with opportunistic infections, and gastrointestinal complications are common (even in patients without evidence of infection) (26). This suggests that other isotypes can compensate for selective IgA deficiency, especially IgM, which like IgA is transported across the epithelium into the intestinal lumen by the polymeric immunoglobulin receptor (pIgR) (27).

In animal models, isolated IgA deficiency also has a mild phenotype, even with experimental respiratory infections (28), although there are compensatory increases of mucosal and serum levels of IgG and IgM (29). The relevance of compensation can be experimentally determined in mice, since lymphonodular hyperplasia (similar to that seen in humans with common variable immunodeficiency) and an expansion of the microbiota are seen in strains with either total deficiency of activation-induced cytidine deaminase [$AID^{-/-}$ abolishing both isotype class switching and somatic hypermutation (SHM)] or selective AID^{g23s} polymorphism deficiency (which allows class switch recombination but abolishes somatic mutation) (30, 31). These results show that in the absence of IgA, IgM can be sufficient to contain an intestinal microbiota, provided SHM can diversify the repertoire appropriately. The importance of secretion of IgM and/or IgA into the intestinal lumen via the pIgR is shown by a protein-losing enteropathy and compensatory reprogramming of intestinal epithelial gene expression, including increased transcripts for antimicrobial peptides in pIgR-deficient mice (32–34). Protection from mucosal inflammation is achieved by backpack hybridoma secretion of IgA (7) or restoration of IgA levels through B cell adoptive transfer in RAG-hypomorphic mice (35). IgA per se can therefore be compensated by IgM and is thus potentially redundant, although in its function as the dominant secretory immunoglobulin of the intestine it is certainly not useless.

3. IgA MICROBIAL SELECTIVITY

Hier hast du nun eine schöne Auswahl und wahrlich, eine Gelegenheit mit mehr als Einem Schopfe.

—Friedrich Nietzsche, *Also sprach Zarathustra: Ein Buch für Alle und Keine*

Given the extensive alpha and beta diversity of the mammalian intestinal microbiota, it is unsurprising that there is selectivity in the taxa that are targeted by IgA. This is seen in both mice and

humans and has both public and private characteristics; that is, the same taxa may be selectively targeted across different individuals, although there is variability in the individual responses (10, 36–40).

Some of this selectivity relates to the immune geography of IgA induction and secretion. IgA is dominantly produced in the small intestine, and mouse experiments show that specific responses (through both T cell–dependent pathways and T cell–independent pathways) are selectively directed mainly against small intestinal microbes (38). This is likely to relate to the necessity for the small intestine to be protected from proinflammatory exposure of the small intestinal mucosa to microbial molecules in the face of the thin layer of small intestinal mucus, in contrast to the thick mucous barrier in the large intestine (41). For example, segmented filamentous bacteria (SFB; "*Candidatus Arthromitus*") have a preferential existence in the lower small intestine of rodents, where their extensive auxotrophy can be substituted by undigested macronutrients (42). This taxon has long been noted to preferentially induce IgA (43), which is capable of controlling colonization density (44). Selectivity can also depend on the quality of IgA, according to the mechanism of induction (45), discussed below.

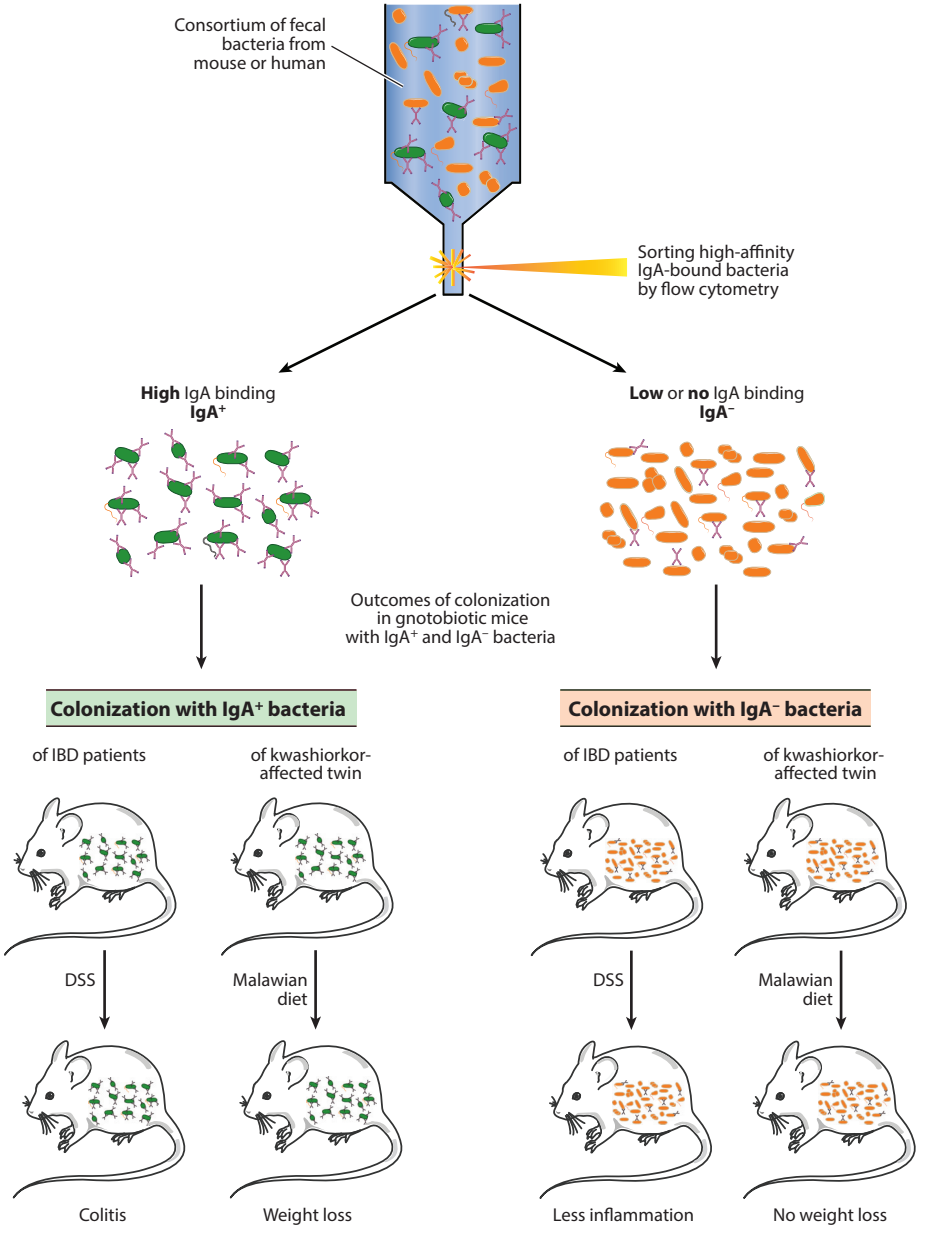
IgA is certainly useful as an extremely effective marker of potentially inflammatory taxa within the microbiota (36). The experimental method IgA-Seq exploits differences in IgA binding between different microbiota, as seen by fluorescence intensity shift of IgA-bound microbes in flow cytometry (**Figure 1**). Those microbes in the high-binding gate and in the low-binding gate are then isolated (either through flow cytometric sorting or through magnetic-activated cell sorting [MACS]), and these different fractions are analyzed for the proportions of different taxa that they contain through highly parallel sequencing that exploits polymorphism in the microbial 16S ribosomal gene loci. This approach has allowed not only identification of those taxa preferentially bound by IgA in vivo in mice and humans but also indirect assessment of their functional significance.

In one colony of C57BL/6 specific-pathogen-free mice, 4 genera were significantly enriched in the IgA^+ consortia: an unclassified genus of *S24–7* from the order *Bacteroidales*, *Lactobacillus*, SFB, and an unclassified genus of the *Erysipelotrichaceae* family (**Figure 2**) (36). In contrast, 23 taxa were significantly enriched in the IgA^- consortia, including *Anaerostipes*, *Adlercreutzia*, 2 genera of the *Ruminococcaceae* family, 2 undetermined genera of the *Lachnospiraceae* family, *Rikenellaceae* gen., *Bacteroides*, *Dehalobacterium*, *Clostridium*, unclassified bacteria, *Mogibacteriaceae* gen., *Coprococcus*, *Oscillospira*, 2 undetermined genera of the *Clostridiales* order, *Bilophila*, *Dorea*, *Mucispirillum*, *Lachnospiraceae* gen., *Sutterella*, *Ruminococcus* (*Lachnospiraceae* family), and *Ruminococcus* (*Ruminococcaceae* family) (**Figure 2**).

Induction of IgA responses to proinflammatory taxa could be demonstrated by first generating dysbiosis and intestinal inflammation through cohousing C57BL/6 wild-type mice with a dysbiotic strain deficient for the apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC; the adaptor protein that activates caspase 1 to generate IL-1β and IL-18 in the inflammasome effector pathway). Highly IgA^+–coated taxa from the cecal microbiota of these C57BL/6 mice after induction of dysbiosis contained an unclassified genus of *Prevotellaceae*, *Helicobacter* sp. flexispira, and SFB: The *Prevotellaceae* genus is the defining proinflammatory taxon in the ASC-deficient donor strain, and all three have proinflammatory potential in different animal models (36).

Turning to humans with chronic intestinal inflammation, 35 species were selectively IgA^+ coated in patients with inflammatory bowel disease (Crohn disease and ulcerative colitis) compared with controls, including 2 *Bacteroides* species, 4 *Lactobacillus* species, unclassified *Pediococcus*, *Weissella* spp., 2 *Clostridiales* species, unclassified *Ruminococcaceae*, *Acidaminococcus* spp., *Veillonella* spp., *Anaerostipes* spp., *Blautia* spp., 2 *Roseburia* species, *Veillonella dispar*, *Haemophilus*

parainfluenzae, *Allobaculum* spp., *Eubacterium dolichum*, *Rikenellaceae* spp., *Ruminococcus* spp., and *Eggerthella lenta* (**Figure 2**). These were also shown to have inflammatory potential: When IgA$^+$ or IgA$^-$ taxa from patients with inflammatory bowel disease were used to colonize germ-free mice, animals colonized with the IgA$^+$ taxa were more susceptible to subsequent induction of dextran sodium sulfate colitis compared with those colonized with the IgA$^-$ taxa (36). In a separate study, IgA-coated *Escherichia coli* strains bearing adherent-invasive virulence factor genes were isolated



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

A FACS-based approach for sorting high-affinity IgA-bound bacteria and colonization of gnotobiotic mice. IgA-binding bacterial consortia can be purified from mouse or human fecal samples using flow cytometric or magnetic bead sorting techniques. The specific bacterial taxa highly coated with IgA are classified as IgA^+ bacteria, whereas the specific bacterial taxa with little or no IgA are classified as IgA^- bacteria. IgA^+ bacteria and IgA^- bacteria are reintroduced into gnotobiotic animals for further characterization of the role of these consortia in the host. Palm et al. (36) exploit these different approaches to identify colitogenic bacteria isolated from patients with inflammatory bowel disease (IBD). IgA^+ consortia from IBD patients increase susceptibility to DSS-induced colitis, whereas IgA^- consortia from IBD patients cause less inflammation in colonized mice. Kau et al. (37) identified bacterial targets of IgA responses in gnotobiotic mice colonized with fecal microbiota from twins discordant for kwashiorkor. IgA^+ consortia from twins discordant for kwashiorkor were transferred to mice, resulting in increased weight loss after DSS-induced colitis, while control transfer of IgA^- consortia showed minimal inflammation. This indicated that the species present within the IgA^+ consortia harbor pathogenic potential and exacerbate disease. See **Figure 2** for taxonomically distinct bacteria in gnotobiotic mice colonized with IgA^+ and IgA^- bacteria.

from patients with Crohn disease complicated by arthritis: These strains exacerbated disease when transferred into the IL-10-deficient mouse model of colitis or the K/BxN model of arthritis (46).

The implication that IgA is selectively induced to mitigate the proinflammatory potential of a taxon is supported by experiments with a backpack hybridoma secreting monoclonal IgA to a capsular polysaccharide of *Bacteroides thetaiotaomicron* in monocolonized mice (7).

4. IgA BINDING AS AN INFORMATIVE PARAMETER FOR ENVIRONMENTAL ENTEROPATHY

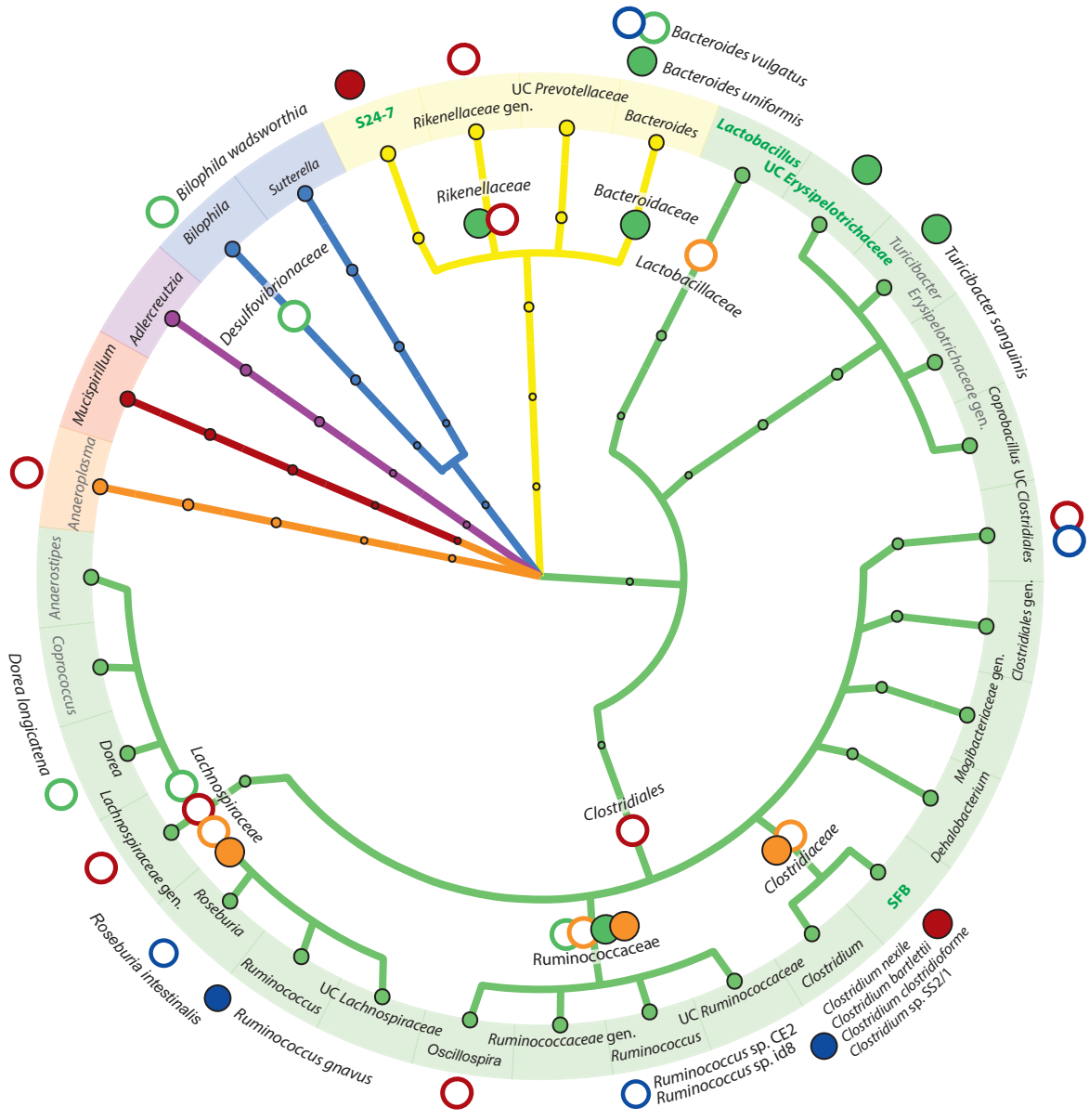
Grief fills up the room of my absent child.

—Shakespeare, *King John*

A similar approach of using preferential IgA binding to different intestinal microbes has been used to identify those taxa that are generating the intestinal inflammation characteristic of environmental enteropathy (**Figure 2**). This rather innocent name is emblematic of one of the greatest health problems of our age. Over 3 million children globally die annually before the age of 5 years (47). The medical causes include low birth weight, sudden infant death syndrome, malnutrition, and infectious diseases. This is only the tip of the iceberg, as 200 million children annually do not reach their developmental potential (48).

Malnutrition is especially important as a cause of death in those aged less than 2 years and stunts the physical growth and cognitive development of survivors; for girls, it also limits the ability to deliver and raise healthy children later in life (49). In addition to associations between abnormally dense intestinal microbial consortia, low-grade intestinal inflammation, and poor intestinal function (reviewed in 50), there is direct evidence that dysbiosis of the intestinal microbiota in early life and consequent intestinal dysfunction are important factors causing infant malnutrition (37, 51).

Although it is currently unclear whether the development of environmental enteropathy is a consequence of vertical transmission of organisms from the mother (at birth or during lactation) or horizontal transmission from the environment, malnutrition and its consequences of stunted growth and impaired cognitive development cannot therefore be attributed solely to food insecurity; early-life microbial colonization is critical (52). Mortality from severe acute malnutrition in infants can be significantly limited and recovery accelerated by the administration of antibiotics to supplement therapeutic food (53). Increased expression of IgA was shown in the 1980s



Groups

- Kau (IgA$^+$)
- Kau (IgA$^-$)
- Bunker (IgA$^+$)
- Bunker (IgA$^-$)
- Rogier (in the presence of sIgA)
- Rogier (in the absence of sIgA)
- Planer (IgA$^+$ in mouse/human)
- Planer (IgA$^-$ in mouse/human)

Phylum

- *Actinobacteria*
- *Bacteroidetes*
- *Firmicutes*
- *Proteobacteria*
- *Tenericutes*
- *Deferribacteres*

(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

The phylogenetic tree of common IgA-targeting strains. The phylogenetic tree showing mouse fecal bacterial taxa that are significantly coated with IgA was generated from Palm et al. (36). Taxa in green are significantly IgA^+ consortia, whereas gray ones have some degree of difference between groups; however, the data are not significant. These taxa were then compared with other studies to reveal multiple concordant significant differences [although they do not capture differences between Palm et al. and other studies such as *Enterobacteriaceae* in Kau et al. (37)]. Commensal bacteria targeted by IgA at several taxonomic ranks are depicted on the phylogenetic tree of life using Kau et al. (37; labeled open and closed green circles), Bunker et al. (38; labeled open and closed red circles), and Rogier et al. (10; labeled open and closed orange circles). The Kau (37) and Bunker (38) studies identify IgA^+- and IgA^--coated bacteria, whereas Rogier et al. (10) report the gut microbiota profile of pups in the presence and absence of sIgA. Planer et al. (39) exploit the development of gut mucosal IgA responses in healthy twin pairs and recapitulate the results in young gnotobiotic mice colonized with fecal microbiota of twin pairs and fed a sequence of human diets. Abbreviations: gen., classified as a distinct but unassigned genus in the Greengenes reference database (**http://greengenes.secondgenome.com**); sIgA, secreted IgA; UC, unclassified in Greengenes.

(54), although it has only recently been recognized that those taxa within the microbiota that are proinflammatory can be discriminated according to their IgA^+-binding status (37).

The evidence for this immunomodulatory causality combines immune and gnotobiotic technology. When fecal microbiota samples from infant twins discordant for malnutrition were transferred into germ-free mice, the sample from the malnourished infant caused intestinal inflammation and dysfunction, whereas the transfer from the healthy twin was less harmful (37, 51). More generally, this provides a means of identifying the IgA^+-binding status of those taxa that are responsible for the enteropathy (37) (**Figures 1** and **2**). The experimental approach was to humanize germ-free mice (fed a macronutrient- and micronutrient-deficient diet designed to reflect the nutrition of the donor Malawian subjects) with the microbiota from one of a pair of twins suffering from kwashiorkor. This xenobiotic reconstitution of mice allowed the identification of mouse IgA^+-bound taxa. These were notably enriched for *Enterobacteriaceae*, *Brachyspiraceae*, *Erysipelotrichaceae*, *Actinomycetaceae*, and *Rikenellaceae*—the IgA^+ enrichment of these taxa was considerably reduced if the mice were fed a nutritionally complete diet. In the same experiment, germ-free mice humanized with the microbiota of the healthy twin showed IgA^+ enrichment of *Erysipelotrichaceae*, *Verrucomicrobiaceae* and *Bacteroidaceae* (37). Whereas the Palm et al. (36) study described above did not find significant enrichment of IgA coating of the *Enterobacteriaceae* family, this was a prominent finding in the context of malnutrition (37). Viladomiu et al. (46) also showed selective enrichment of IgA-coated adherent-invasive *E. coli* pathotypes in patients with Crohn disease who had arthritis complications.

The proinflammatory relevance of the IgA^+-enriched taxa in this nutritional context was demonstrated by isolating and transferring them from mice colonized with the microbiota of the kwashiorkor twin or from the healthy twin into fresh germ-free mice on the Malawian diet. Whereas the IgA^+ taxa from the kwashiorkor twin proved lethal due to intestinal inflammation and dysfunction in the new germ-free murine recipients, IgA^+ taxa from the healthy twin were well tolerated. The effect could be abrogated by feeding a nutritionally rich standard chow diet to the recipients of the kwashiorkor IgA^+ taxa, linking proinflammatory consortia that develop in the setting of childhood malnutrition and the host IgA response (37).

5. EARLY-LIFE IMMUNITY

For in every adult there dwells the child that was, and in every child there lies the adult that will be.

—John Connolly, *The Book of Lost Things* (55)

Logically it should follow from the possibility of using IgA binding as a marker for potentially proinflammatory taxa in pathogenic conditions that IgA is important in establishing the early-life

balance of intestinal microbes with their host. IgA is the predominant immunoglobulin secreted in milk (56), and there is evidence that the specificities include those corresponding to the recent immune experience of the mother (57–60). One can broadly divide the effects of milk immunoglobulins on the nonpathogenic microbiota into protection of the neonate and their influence on the incoming microbiota.

5.1. Protection of the Neonatal Mucosa

Secreted milk immunoglobulins are an important component of the milk, but milk contains other antimicrobial proteins (such as lactoferrin, xanthine oxidoreductase and lysozyme) and leukocytes. It can be argued that the mammary gland evolution has been driven by both a necessity for innate immune protection of the offspring as well as nutrition (61). To dissect out the effects of adaptive immunity, particularly antibody secretion, experiments with immunodeficient mice can be arranged so that either the dam or sire (father) is immunodeficient through a homozygous versus wild-type cross. In each case the pups are heterozygous and immune competent (**Figure 3**). In experiments using severe combined immunodeficient (*scid*) mice, Cebra's group showed that maternal immunodeficiency resulted in early induction of intestinal IgA in the *scid*/+ offspring at postnatal day 16–17, whereas in the control experiment where the mother was fully immune competent, endogenous IgA in the pups was only induced after weaning (8). These results were later extended with a similar experimental design to mice that were selectively deficient in all immunoglobulin isotypes because of a deletion of J segments at the Ig heavy chain locus: in this case, the effect was formally shown to be driven by the intestinal microbiota by its absence when the entire experiment was carried out under germ-free conditions (9). Unlike humans, where maternofetal IgG transfer is largely complete via the transplacental route at birth, neonatal mice continue to absorb maternal IgG from the milk via the duodenum until approximately postnatal day 12 (62, 63). Nevertheless, parenteral IgG supplementation of pups did not prevent early induction of IgA when they were nursed by a J_H-deficient dam (a mouse in which the J segments on the immunoglobulin heavy chain gene loci have been deleted are deficient in B cells), positioning the protective immunoglobulin effect at the intestinal mucosal surface (9). As with the protective effects of endogenously secreted IgM that can substitute for IgA in adult mice and humans, strain combination experiments with the strain selectively deficient for IgA showed that this also was possible in maternal milk (A.J. Macpherson, unpublished data).

5.2. Antibody Influence on the Incoming Microbiota

Milk composition has a profound influence on early microbiota development in human and animal-model studies. These microbiota effects are also certainly not limited to secreted antibodies (10, 37), as other milk proteins such as lactoferrin (64) and milk oligosaccharides (65, 66) influence the specifics of the microbial consortia that populate the intestine early in life. In some cases, the mechanisms can be inferred from the metabolic capability of certain taxa, for instance to metabolize particular saccharides (64, 67), or the potential of antibodies to neutralize pathogens (68). Nevertheless, experiments with pIgR-deficient mice have shown that polymeric immunoglobulins (IgA and IgM) that are actively secreted via this transport mechanism can shape the long-term intestinal microbiota that is acquired by pups (10). These effects, which are particularly manifest in increased representation of *Proteobacteria* and *Firmicutes*, were present not only in the weanling mice nursed by pIgR-deficient dams, but also in these mice after they became adults. Just as pIgR-deficient adults show compensatory reprogramming of epithelial gene expression—an effect that is driven by the microbiota (33, 34)—so too do neonates that are being nursed by a pIgR-deficient

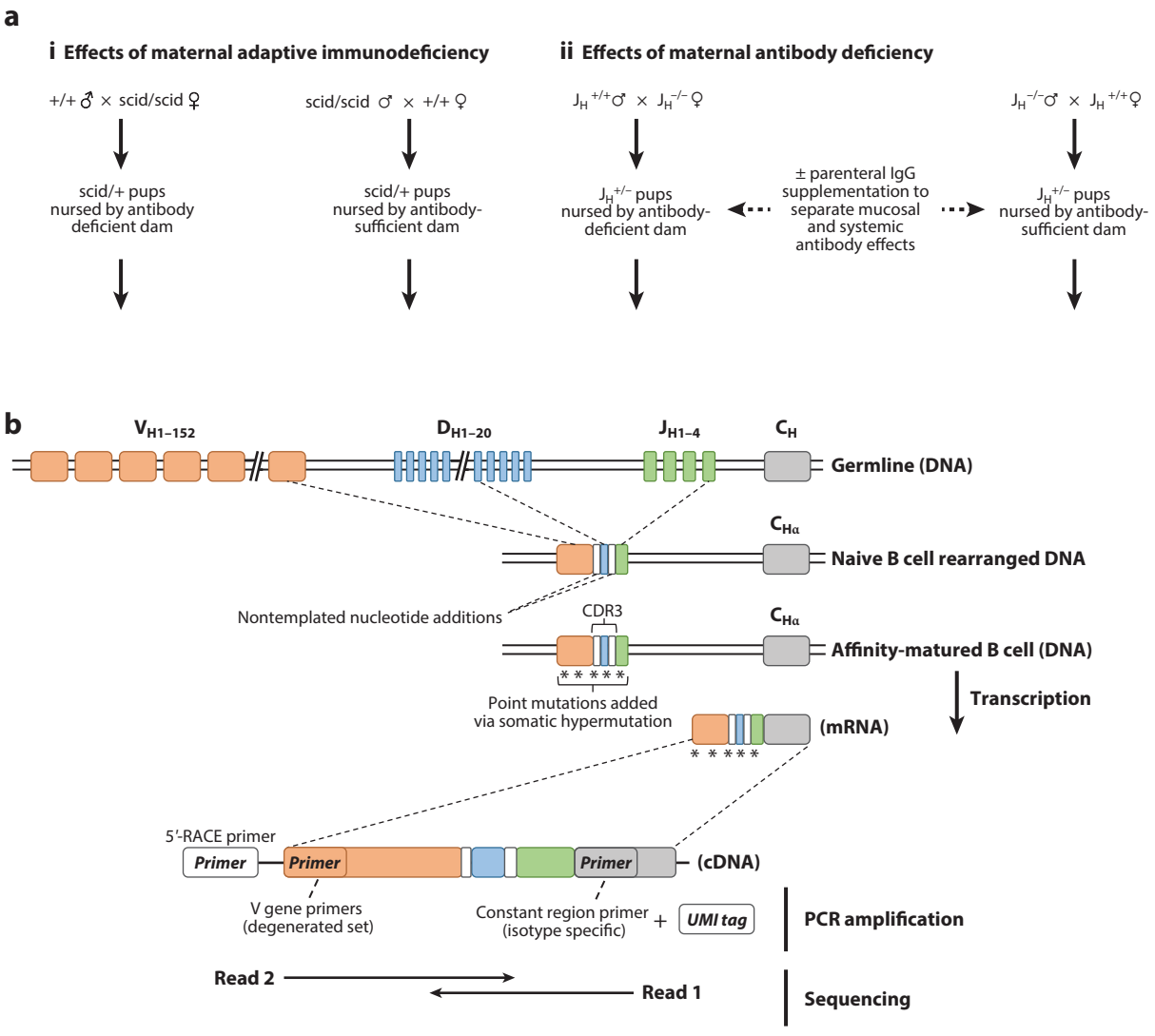


Figure 3

(*a*) Murine experimental models of maternal antibody deficiencies. The effect of maternal antibodies on the offspring can be studied by using immunodeficient (scid) or antibody-deficient ($J_H^{-/-}$) mice. (*i*) Wild-type (+/+) males are crossed with immunodeficient (scid/scid) females to obtain antibody-sufficient (scid/+) pups that are nursed by antibody-deficient (scid/scid) dams. As controls, scid/scid males are crossed with wild-type females to obtain antibody-sufficient (scid/+) pups that are nursed by antibody-sufficient (+/+) dams. (*ii*) $J_H^{+/+}$ males are crossed with $J_H^{-/-}$ females to obtain antibody-sufficient ($J_H^{+/-}$) pups that are nursed by antibody-deficient ($J_H^{-/-}$) dams. As controls, $J_H^{-/-}$ males are crossed with $J_H^{+/+}$ females to obtain antibody-sufficient ($J_H^{+/-}$) pups that are nursed by antibody-sufficient ($J_H^{+/+}$) dams. (*b*) Principles of IgH amplification for repertoire sequencing (RepSeq), and schematic steps toward the generation of antibody RepSeq data. The germline IgH locus is shown (V, D, and J segment numbers are representative of the mouse genome locus), followed by the successive diversification processes, first in naive B cells via DNA segment rearrangement and nontemplated nucleotide additions at the D-J and V-D junctions, then in affinity-matured B cells via somatic hypermutation. The 5′-RACE experimental protocol starting from RNA molecules follows. The PCR amplicon is generated using a degenerate set of primers at the 5′ end to probe the diversity of V genes, and an isotype-specific primer at the 3′ end. A unique molecular identifier (UMI) tag can be added to the primer to mark PCR products uniquely for PCR error correction.

dam (10). In other words, the protective effects of actively secreted polymeric immunoglobulins are able not only to protect the early-life mucosa in the fragile postnatal mouse before development of its own innate and adaptive immune mechanisms, including prevention of intestinal microbes penetrating the epithelial defenses to translocate to the mesenteric lymph nodes (9), but also to shape the long-term composition of the microbial consortia that successively populate the early-life intestine (10). Another likely example of this is the case of SFB, which temporarily undergo an exaggerated bloom in the intestines of *scid*/+ mice nursed by a *scid*-deficient dam (44), and colonization is related to the IgA levels in maternal milk and IgA that is endogenously produced after weaning (69). Age-discriminatory bacterial taxa defining a program of consortial assembly and maturation across healthy twin pairs and a distinctive pattern of progression of IgA responses have also been shown in humans (39) (**Figure 2**).

These experiments collectively support the classical view that IgA (or IgM) provides mucosal protection, whereas passively acquired IgG provides maternal immune memory against pathogens. However, it was recently shown that there is T cell–independent and Toll-like receptor dependent induction of maternal IgG_{2b} and IgG_3 against members of the intestinal microbiota (58). This result was obtained by flow cytometric analysis of taxa in the intestinal microbiota bound by serum IgG in a setup similar to those used for the IgA-Seq experiments described above, but comparing IgG binding from colonized and germ-free mice. Uptake of this microbiota-specific IgG by pups had the functional effect of limiting early mucosal follicular helper CD4 T cell responses. In a different approach, our group has used a reversible colonization system to show that the maternal microbiota per se causes extensive reprogramming of epithelial gene expression in her pups (70). In these experiments, the pregnant dam was only transiently exposed to live *E. coli*—she became germ-free again 48 h later because the test microbe lacked the ability to synthesize bacterial specific amino acids, which were unavailable in the sterile mammalian host. Therefore, the pups were delivered germ-free to a germ-free dam. Maturation of the innate immune system (including type 3 innate lymphoid cells and intestinal mononuclear cell populations) and transcriptional reprogramming of the neonatal intestinal mucosa depended on the transfer of microbial metabolites from dam to pup via the placenta and milk. These effects were greatly amplified by uptake of maternal IgG by the pups, and functionally complemented specific IgA in protecting the early-life intestinal mucosa from penetration of bacteria given as test doses into the pups' intestine (70).

6. THE QUALITY OF THE IgA RESPONSE AS DETERMINED BY MECHANISMS OF IgA INDUCTION

Non refert quam multos sed quam bonos habeas.

—Lucius Annaeus Seneca, *Epistulae Morales ad Lucilium*

IgA is induced mainly in the secondary lymphoid structures of the intestine [Peyer patches (71)], although evidence from mice that lack Peyer patches shows that other sites can also sustain IgA class switch recombination (21) (**Figure 4**). Although T cell–dependent responses in germinal centers provide the means for affinity maturation through interactions with antigens that are presented on follicular dendritic cells (72) (**Figure 4**), there is evidence that class switch recombination occurs in the subepithelial dome through interactions between activated B cells and dendritic cells within the dome expressing TGF-β (73). Innate lymphoid cells in the Peyer patches promote lymphotoxin beta receptor (LTβR)-dependent maintenance of dendritic cells (73). Abrogation of these interactions in the subepithelial dome through blocking CCR6-dependent B cell recruitment reduced the intestinal IgA response. Intestinal dendritic cells therefore can determine B cell fate, positioned precisely for sampling of luminal intestinal antigens, including taxa of the microbiota

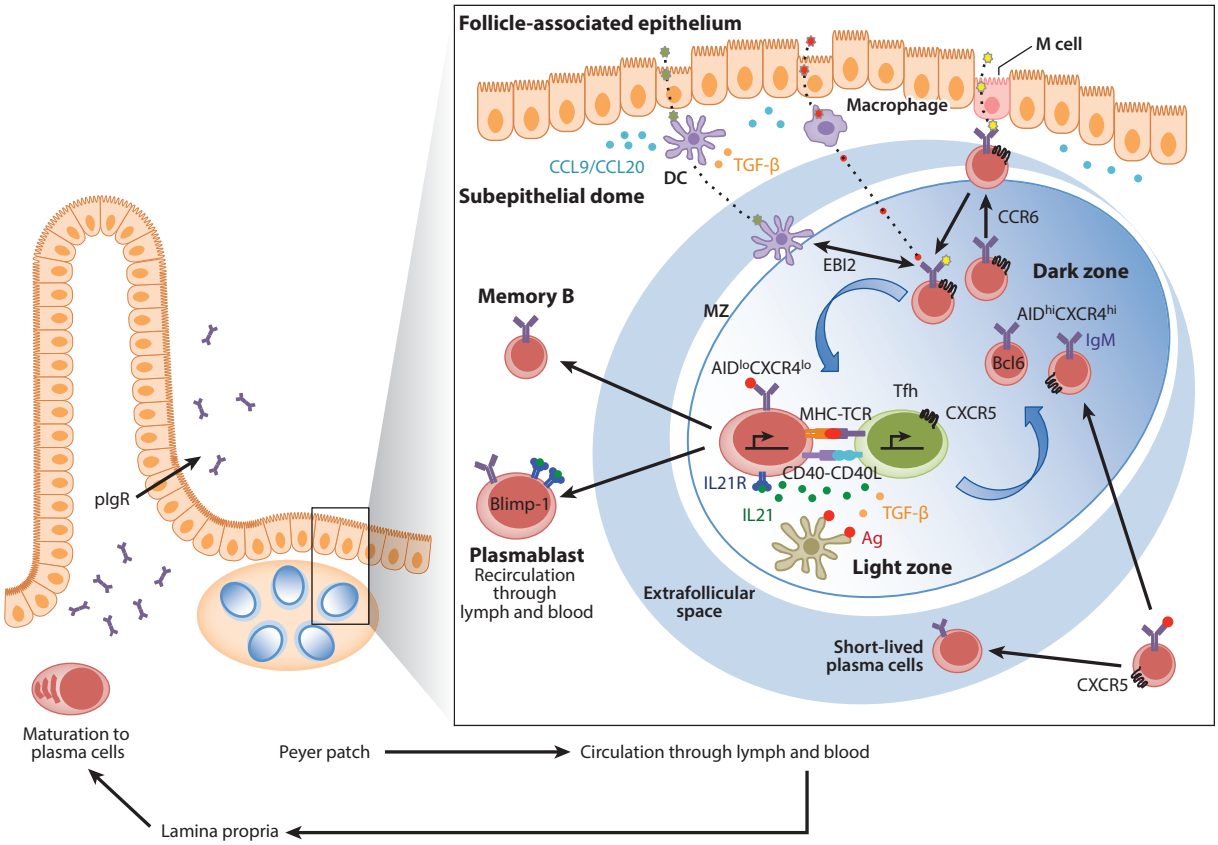


Figure 4

Induction of IgA in intestinal Peyer patches. Activated B cells may enter GCs within the intestinal Peyer patches via engagement of the chemokine receptor CXCR5, where they undergo T cell–dependent activation in the light zone and CSR and SHM in the dark zone. GC Tfh cells in the light zone mediate affinity maturation for antigens selected and presented by FDCs. Interaction between B cells and Tfh cells involves class II MHC:T cell receptor interactions as well as CD40:CD40L engagement. Tfh cells further produce IL-21 that is sensed by the IL21R on the follicular B cells. B cells subsequently upregulate AID required for CSR and SHM in the dark zone. Recirculation between the light zone and the dark zone can occur several times. IgA CSR can also happen through events taking place in the subepithelial dome. Antigens entering the intestine through M cells are taken up and presented by DCs to B cells at the interface to the follicles. Migration of B cells to the follicle border is mediated through activation of the oxysterol receptor EBI2 on B cells. TGF-β production by the DCs supports IgA CSR. In addition, antigens entering through M cells can be phagocytosed and processed by subepithelial macrophages, allowing them to diffuse freely into the germinal centers where they can be recognized by B cells. Last, follicular B cells can migrate to the epithelium through CCR6:CCL20 interaction, where they take up antigens directly beneath M cells. Activated and affinity-matured B cells leave the GC as plasmablasts or memory B cells. The plasmablasts circulate through lymph and blood back to the lamina propria, where they mature into fully functional IgA-producing plasma cells. Dimeric IgA is then transcytosed through the epithelium into the gut lumen via the polymeric immunoglobulin receptor (pIgR). Abbreviations: AID, activation-induced deaminase; DC, dendritic cell; FDC, follicular dendritic cell; GC, germinal center; IL21R, IL-21 receptor; MZ, marginal zone; SHM, somatic hypermutation; Tfh, T follicular helper.

(3). These results are consistent with the observations that although CD40-deficient mice lack germinal centers in the Peyer patches, IgA levels are near normal (74, 75). The CD40 pathway is not the only means of stimulating expression of AID and its functions of class switch recombination and SHM: the dendritic and mononuclear cell soluble ligands APRIL (a proliferation-inducing ligand) and BAFF (B cell–activating factor) engage the receptor TACI (transmembrane activator

and cyclophilin ligand interactor) on B cells, which binds MyD88 to support rapid extrafollicular T cell–independent AID induction (76; see also 77 for a recent review on the downstream B cell signaling pathways). Strain combination experiments that abrogate APRIL or TACI signaling also reduce the overall expression of IgA, IgG, and IgE (78, 79), and these B cell–intrinsic pathways likely also allow the T-independent generation of IgA in isolated lymphoid follicles (80).

Despite the initial occurrence of T-independent class switch recombination to IgA, only about a quarter of total IgA can be generated in the absence of T cells (81): It should also be pointed out that measurements of the presence of T-independent IgA binding to taxa within the microbiota have been carried out in strains deficient in T cell help, so the T independent pathway may be partly compensating for the T-dependent pathway under these conditions (38, 81), and a diversified microbiota cannot be adequately contained solely by T-independent mechanisms (82).

The presence of follicles containing germinal centers is a defining feature of Peyer patches, whose durable formation depends on T cells (21). Follicular helper CD4 T (Tfh) cells, with emblematic CXCR5 expression, sustain germinal center development through expression of CD40 ligand (CD40L) as well as secretion of IL-4 and IL-21. Through CD40L signaling they also guide the selection and survival of B cells, and the upregulation of AID for SHM and affinity maturation of expressed immunoglobulins (20). As described in the previous section, there is evidence that AID-dependent SHM—whether on the IgA or IgM isotype—is important to generate a repertoire that can contain the intestinal microbiota (31). The importance of Tfh cells to establish a diversified intestinal microbiota with host microbial mutualism has been shown in strain combination experiments where $CD3\varepsilon^{-/-}$ T cell–deficient animals with limited microbiota diversity were reconstituted with naive T cells (which reduced microbiota diversity further) or with $Foxp3^{+}$ ± naive T cells, which resulted in increased microbiota diversity. The effect was aligned with germinal center formation from dependence on Bcl6 expression by the transferred cells, and with the quality of IgA induction, as $Foxp3^{+}$ reconstitution allowed the generation of high-IgA^{+}-binding and low-IgA^{+}-binding taxa (45). The Fagarasan group (83) have also shown that appropriate feedback control of the Tfh population through the inhibitory receptor PD-1 is also important to generate an appropriate IgA repertoire for microbiota control.

These observations indirectly promote the relevance of mechanisms aligned with generating high-affinity IgA (or IgM) in controlling host microbial mutualism within the intestine. Certainly, the generation of neutralizing antibodies against cholera toxin (which can be demonstrated in vivo by preventing fluid accumulation in ligated loops of intestine) is predictably T cell dependent (4). Conversely, strain-combination studies that demonstrate IgA binding to intestinal microbes imply both T-dependent and T-independent pathways of induction (38). This suggests a degree of flexibility of mechanisms of IgA induction that depends on the contextual composition of the microbial consortia present and possibly its plasticity over time, as reversible colonization experiments show that specific IgA intestinal plasma cells can be very durable, and that the specific response integrates overall exposure rather than showing the secondary exaggerated responses of classical systemic prime-boost immunization (84). Gut memory B cells can show variable clonal relatedness to long-lived plasma cells (85, 86).

One must be cautious about the assumption that the occurrence of germinal center–mediated pathways in Peyer patches necessarily implies obligatory specific B cell receptor engagement, for either class switch recombination or SHM. In experiments where the B cell receptor function was replaced with Epstein-Barr virus LMP2A protein signaling without antigen-recognition capability, germinal centers were absent from the spleens, but present in the Peyer patches and the mesenteric lymph nodes of D_HLMP2A mice. Despite the nonspecific nature of signaling, both AID induction and SHM of immunoglobulin light chains were found in their Peyer patch follicular B cells (87). These responses that occur in the absence of antigen recognition through the B cell receptor were

substantially reduced by reducing the microbiota density with a broad spectrum of antibiotics in the drinking water (87). In another approach, mice harboring one productive V(D)J allele at the J_H locus and a further nonproductive passenger test sequence on the other IgH locus in a RAG-deficient background showed significantly greater SHM on both the productive and the nonproductive IgH alleles in the Peyer patches compared with the spleen when germinal centers were induced without significant antigen-specific stimulation (88). The high levels of SHM in the Peyer patches and mesenteric lymph nodes may provide a diversified repertoire available for later affinity-based selection. Reboldi & Cyster (21) have suggested that the early phase of germinal center responses in lymph nodes that generate a diversity of low-affinity B cells can be equated to the chronic stimulation in Peyer patches, with a later phase that is antigen specific. Lineage tree analysis suggests that synchronization of IgA oligoclonality between germinal centers of different Peyer patches in the small intestine can be achieved, at least for the T-dependent immunogen 4-hydroxy-3-nitrophenylacetyl conjugated to cholera toxin (89). Such a process may also capture and expand B cells from the diversified repertoire—possibly even those that originally were derived from T cell–independent induction (90). As discussed in the next section, this could be consistent with the oligoclonal nature of the (most frequent clonotypes) in individual mouse small intestinal IgA, but with heterogeneity between different animals (85).

Contextual effects that depend on the exact microbiota present in humans or experimental animals are currently challenging to resolve, given that the genetically outbred human population has substantial interindividual microbiota variation (91–93), and that almost all experimental vivaria with inbred animal models are working with different microbiota compositions (17, 40). In some cases, such as SFB, this variability can be exploited to identify very strong effects, with highly specific T cell responses (94, 95), with the generation of additional tertiary lymphoid structures in the intestine (96). Such contextual effects resulting in differences in lineage plasticity (97) may also explain apparent discrepancies between results that show the importance of Tregs or T cells that have expressed IL-17 as a source of Tfh cells for germinal center–dependent IgA induction (45, 98, 99). Such caveats imply that to understand host microbial mutualism, as it applies to intestinal IgA, we need much better information about the functional relevance of the repertoire in relation to the detailed taxa composition and niches of the microbiota. Agent-based model simulations in silico imply that IgA mediates differential adhesion of gut bacteria (100). This means that in addition to context effects depending on the consortial composition present we should experimentally aim for a higher spatial resolution in sampling the IgA repertoire along the digestive tract.

7. THE IgA REPERTOIRE

Petit à petit, l'oiseau fait son nid.

—French Proverb

One approach to addressing the IgA repertoire is to examine what it can bind as a first step to understanding how it functions. Conveniently for classical immunology, antibodies capable of neutralizing toxins or pathogen uptake are largely of high affinity, so they can be detected in ELISA or FACS-based binding assays after substantial dilution, and the slow off-time allows binding to persist during the necessary washing steps. Nevertheless, such responses can represent the tip of the iceberg of the overall repertoire (11, 101). Binding does not necessarily mean neutralizing function in pathogen studies (102, 103), but the relation between binding and function is even less clear for intestinal IgA, whether directed against pathogens (6) or the microbiota. As shown above, the relevance of IgA binding to taxa within the intestinal microbiota has been indirectly shown by generation of intestinal inflammation after transfer into germ-free mice—in some cases after

the additional provocation of dextran sodium sulfate treatment. Relatively few specific microbial epitopes have been systematically explored using monoclonal IgA, but in vivo hybridoma backpack models suggest that even within a single taxon, depending on the epitope that is targeted, the functional impact of IgA can be to modulate bacterial gene expression or to limit the inflammatory effects of the bacteria on the intestinal mucosa (7, 104).

Considering just binding, there is a component of broad specificity, the extent of which varies between experimental mouse strains (105). Cloning of IgA and IgG antibodies expressed from the human adult lower intestine has shown that at least a quarter of the repertoire is polyreactive through ELISA binding against the classical antigens dsDNA, ssDNA, insulin, and lipopolysaccharide (106) with broad reactivity to a diverse subset of microbiota (107). By subtraction, three-quarters of the repertoire is not polyreactive by this readout, although the specificities are almost completely unknown.

Another approach is to examine the diversity of the repertoire from sequencing the somatically expressed immunoglobulin genes (**Figure 3**). High-throughput methodology (repertoire sequencing, RepSeq) has enabled a far greater depth of analysis of the repertoire than the earlier approach of Sanger sequencing of monoclonal or diverse B cell populations (108–110). The usefulness of the approach to understand how the repertoire develops, and the relative influence of the host genetic background or antigenic drive, depends on experimental and informatics techniques that are informative about inherent sequencing errors and can distinguish V, D, and J segment usage from untemplated N-nucleotide insertions (for a review see 111). The recent development of tools inferring germline genes directly from repertoire sequencing data (112) opens the avenue of studying repertoires in nonmodel organisms as well as accurately taking into account interindividual variation in germline genes in human studies (113). When applied to mouse intestinal IgA, high-throughput sequencing has shown clonal persistence and diversification within an aging individual over time (114), but the individual repertoires are very different even between mice of the same strain kept under the same hygiene conditions (85, 114). The repertoire in the small intestine is distinct, but diversification can be independent of Peyer patch formation (114). These experiments are examining the progressive development of the IgA repertoire in colonized but pathogen-free animals. As technology progresses quickly, from these two landmark publications, future studies relying on deeper sequencing will enable the exploration of a larger extent of IgA repertoire diversity and the extent of SHM in different populations of IgA. Accurate tracking of SHM from RepSeq data is affected by the error-prone nature of PCR-based protocols, so recent efforts have been made to implement unique molecular identifiers (UMIs) to remain true to the original sequence (115) (**Figure 5**). This is a tradeoff with the need to explore the maximum repertoire diversity, as UMIs restrict the maximum number of molecules and therefore clonotypes obtained at a given depth of sequencing. Given the expanded V gene repertoire of mice compared to humans, human and mouse IgA repertoires need to be compared for levels of SHM, in case V repertoires affect the mechanisms in place to generate the necessary diversity (113, 116) (**Figure 5**).

In a complementary approach, high-throughput sequencing has been applied to determine V-gene usage, clonal expansion, clonal diversity, and repertoire size at different stages of B cell development with, or without, systemic immunization using three antigenically different proteins (117). Whereas the IgG repertoire in pre-B and naive B cells is substantially publically determined between individuals with the same host genetic background, the plasma cell repertoire is 40% privately determined (within each individual) by the stimulus of immunization (117). This is a developing area that should be transformative for understanding the specifics of IgA expression: on one hand to distinguish the effects of mucosal exposure to different microbial taxa and on the other to express the induced antibodies in pure form to determine their binding specificities,

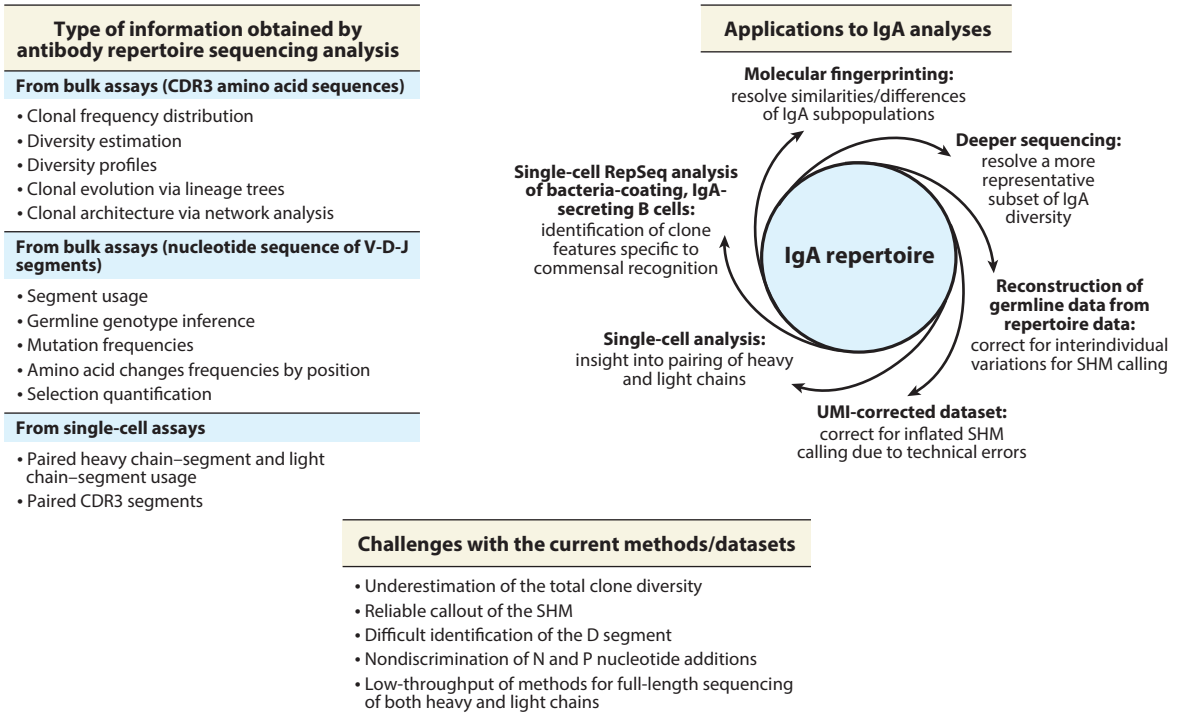


Figure 5

Insights from and challenges of the RepSeq approach to studying immunoglobulin diversity. Considerations for the benefits and limitations of current protocols for antibody RepSeq, including potential applications for IgA analyses. Abbreviations: RepSeq, repertoire sequencing; SHM, somatic hypermutation; UMI, unique molecular identifier.

affinities, and function. Computational approaches are being developed that aim to understand the selection and evolution of immunoglobulin repertoires by probabilistic (117–120) or network-based methods (85, 121, 122). Machine-learning methods can also be used to identify immunological signatures through dissection of the high-dimensional complexity of the data (123–127). These approaches may be combined with the proteomic identification of antibodies, using the RepSeq repertoire to interpret the mass spectrometry data. This has already been performed with libraries of monoclonal antibodies, but it remains challenging for complex repertoires with unknown antigen-binding properties (128). Ongoing efforts to predict antigen identity and affinity from RepSeq data aim to fill the gap. Predictability using extracted features from the antibody sequence data has been achieved toward the identification of public versus private clones across individuals (117) or following single-cell sequencing of cells of known antigen-binding properties toward the identification of receptors associated with a specific antigen (129, 130) (**Figure 5**). Linking the diverse repertoire to the range of antigen binding is a potentially transformative goal.

8. CONCLUSIONS

There is good evidence that despite functional redundancy with mutated IgM, IgA secretion into the intestine is far from useless. IgA binding can be predictive of proinflammatory taxa, and appropriately diversified (somatically mutated) IgA is required to maintain the diversity of taxa in the microbiota. Nevertheless, the somatic mutational process that results in IgA diversity can

potentially proceed in the intestinal secondary lymphoid structures without high-affinity B cell receptor engagement. A major challenge in understanding the role of IgA in host-microbial mutualism is to be able to relate the clonotypes in the IgA repertoire to the epitopes (or range of different epitopes) that bind on different taxa and how far high-affinity germinal center responses are driving the process. The idiosyncrasies of the intestinal IgA repertoires between different individuals need to be explained in terms of the differences in timing and specific taxa that constitute antigen exposure or the potentially overlapping redundancy of different epitope groups. There is also good evidence, albeit from the small number of specific epitopes studied, that bound IgA can modify microbial metabolism and abrogate a mucosal inflammatory response. Understanding the functional relevance of a far wider range of specific clonotypes on the microbial taxa, their consortial formation, and the overall interactions with the host should bring us closer to accurate insight into the detailed functioning of the isotype that constitutes the bulk of immunoglobulin production in mammals.

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