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Secretory IgA Enhances Gut B Cells Priming and Systemic IgG Responses Towards Commensals

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by
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Dedicated to my parents, Dr. Zhenwen Zhou and Mei Su.

ABSTRACT OF THE DISSERTATION

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Professor Chyi-Song Hsieh, Chair

IgA is the primary antibody response at mucosal surfaces and is reported to inhibit adaptive immune responses against gut bacteria. Here, we utilize an *in vitro* system to expand and screen IgA memory B cells for their ability to recognize gut bacteria in the context of secretory IgA (sIgA) deficiency in polymeric Ig receptor (*Pigr*$^{-/-}$) mice. Contrary to the prevailing hypothesis that IgA provides an immune exclusionary function, we found that mice lacking sIgA showed decreased anti-bacterial IgA specificities as assessed using flow cytometry. IgA B cell responses against certain taxa such as those of order *Bacteriodales* showed greater dependence on sIgA. Notably, sIgA also facilitated the generation of anti-bacterial IgG B cells, which provided increased resistance to intraperitoneal infection by commensal bacteria. Together, these data suggest that sIgA can facilitate the immune priming of gut commensals to increase anti-bacterial IgA and IgG and enhance immunity.

Chapter 1: Introduction

The mammalian gastro-intestinal tract is colonized by trillions of microbes, many of which perform symbiotic functions for the benefit of both the host and commensal. However, in certain circumstances, colonization of the gut by pathogenic species and virulent penetration into the host has been shown to result in pathology (Coburn et al., 2007; Thomson et al., 2011). Thus, in order to maintain homeostasis, various host mechanisms have evolved to interact with the commensal bacterial population. One layer of protection is thought to be provided by adaptive immunity via the secretion of immunoglobulins (Ig) by B cells. While various Ig isotypes are produced in the mucosa, secretory IgA (sIgA) is the primary antibody that enters the lumen. Recent studies demonstrate the multi-faceted approach in which sIgA can affect the host-microbe interaction, oftentimes in the context of pathogenic infection. What is less clear, is the role of sIgA responses during homeostatic conditions. In this thesis, we analyze the impact of sIgA deficiency on the gut immune system, demonstrate the ability of sIgA to enhance the priming of both IgA and IgG memory B cell responses towards commensals, and show the consequences of sIgA deficiency during septic challenge by commensal bacteria.

Activation of IgA B cells in the gut.

The gut IgA B cell compartment develops from naïve B cells of both B1 and B2 origin. Naïve B-1 cells are identified as IgM^{high} IgD^{low} and $CD5^{+}$, while B-2 cells are IgM^{low} IgD^{high} and $CD5^{-}$. Functionally speaking, B-1 B cells are thought to be more “innate-like”, and do not

require direct T cell help for activation or class switch; as such, they do not normally show signs of affinity maturation (Pabst et al., 2013). B-1 B cells also do not populate follicles such as the Peyer's patches (PP) or mesenteric lymph nodes (MLN) but rather reside in lamina propria or peritoneum (Jiang et al., 2004). On the other hand, B-2 B cells represent the more canonical adaptive immune response and are thought to be mostly T cell dependent for activation. These B-2 cells comprise the larger portion of the gut B cell response, and traffic to immune follicles such as the PP, cecal patch (cP), or isolated lymphoid follicles (ILF), which are collectively referred to as the gut associated lymphoid tissues (GALT) (Lycke and Bemark, 2017). The remainder of this review will focus on the development of this population of B cells.

The GALT is lined with various specialized cell types that aid in activation of naïve B cells (Macpherson et al., 2008). One such group is the follicular associated epithelium (FAE) which contains various specialized epithelial cells contributing to B cell activation by allowing the controlled influx of lumen antigens. In particular, microfold cells (M cells) are thought to have multiple mechanisms for antigen delivery including vesicle mediated bulk transcytosis, formation of transcellular pores, and various receptor mediated mechanisms of antigen capture and transcytosis (Mabbott et al., 2013). Indeed, *RANKL*$^{-/-}$ *or RANK*$^{-/-}$ mice lacking M cells show greatly reduced levels of IgA as well as decreased coating of lumen bacteria by sIgA, thus demonstrating the importance of M cells for gut B cell activation (Rios et al., 2016). The intestinal epithelium also contains goblet cells which are able to form goblet associated passages capable of delivering soluble antigens to CD103^{+} dendritic cells (DCs). These CD103^{+} DCs are potent inducers of conventional IgA B cells (McDole et al., 2012). In certain instances such as stimulation with a virulent antigen, CD103^{+} DCs have been shown to extend trans-epithelial processes for direct antigen sampling (Farache et al., 2013; Knoop et al., 2013). Suffice to say,

there are numerous other mechanisms in which gut antigens can be presented to B cells, and this section is not meant to be an exhaustive list of all such mechanisms.

Upon antigen encounter and DC stimulation under the FAE, activated B cells upregulate CCR6 and home towards its ligand CCL20, which is produced in the subepithelial dome (SED) (Reboldi et al., 2016). Within the SED, TGF-β signaling activates the transcription factor CBFα2 which binds to the Igα germline promoter, resulting in class switch to IgA in an activation-induced cytidine deaminase (AID) dependent manner (Shi and Stavnezer, 1998). The deletion of TGF-β receptor TβRII in B cells results in IgA deficiency, increase in IgG3 class switch, and the expansion of B-1 cells (Cazac and Roes, 2000). Recent work has shown that lymphotoxin-α1β2 (LTα1β2) dependent DCs express a large amount of the integrin αvβ8 (Itgb8), which is responsible to cleaving TGF-β into its active form. The loss of either LTα1β2 or DC specific deletion of Itgb8 results in a significant loss of IgA B cell activation (Reboldi et al., 2016). While the role of TGF-β on IgA B cell activation is clear, the actual source of TGF-β is less defined. It seems likely there are multiple cells producing TGF-β including B cells themselves (Gros et al., 2014), T_{regs}, innate lymphoid cells (ILCs), and DCs (Li et al., 2005). While TGF-β is a strong inducer of IgA B cells, various other regulating molecules such as IL-5, APRIL, iNOS/TNF, and retinoic acid have shown an ability to modulate IgA activation or production through direct and indirect mechanisms (Hiroi et al., 1999; Litinskiy et al., 2002; Tezuka et al., 2007; Villablanca et al., 2011).

Apart from the cytokine signals of the SED, the GALT also contains B cell germinal centers (GCs) used for B cell affinity maturation and expansion. Of the distributed gut lymphoid tissues, the PP is the dominant site for IgA B cell activation (Reboldi et al., 2016); unlike systemic GCs which form following infection or immunization, the PP contains perpetual GCs as

a result of the presence of food and microbial antigens (Macpherson et al., 2008). Within the GC dark zone, antigen activated B cells undergo cellular expansion and somatic hypermutation (SHM). SHM results in the accumulation of randomized mutations on the heavy and light chains of the B cell receptor (BCR) and subsequent selection is a process known as affinity maturation, resulting in the production of antibodies with stronger affinity towards its antigen (Rajewsky, 1996). After a yet to be defined signal, these B cells migrate into the light zone where they compete for survival signals from follicular helper T cells (T_{FH}) based on the affinity of its BCR (Victora et al., 2010). The primary survival signal from T_{FH} cells towards activated B cells is the interaction of CD40L to CD40, and the disruption of this signaling axis results in the termination of the GC reaction (Bry et al., 2006). However, it should be noted that the lack of CD40L does not completely abrogate IgA B cell responses. T-independent IgA B cell activation in $CD40L^{-/-}$ mice has been observed in the peritoneal cavity and PP, albeit with impaired protective function against common oral challenges such as cholera toxin (Bergqvist et al., 2014).

The GC light zone also contains a network of non-hematopoietic follicular dendritic cells (FDCs), which contribute to IgA B cell affinity maturation (Suzuki et al., 2010). FDCs present antigen to B cells in the light zone by trapping free soluble, complement fixed, or immune complexed antigens on their cell surface. It is through competition for these antigens that B cell affinity maturation is thought to progress (Heesters et al., 2014). In the case of the PP, IgA complexes can be seen along the FDC network, suggesting a feedback role for IgA in priming *de novo* IgA responses (Zhang et al., 2016).

B cells undergoing the GC reaction can undergo multiple rounds of SHM and selection before exiting the GC and becoming mature IgA^{+} memory B cells (MBCs) or IgA plasma cells (PC); this fate decision is often thought to be a result of BCR signaling strength (Bemark et al.,

2016; Biram et al., 2019). These cells will then recirculate through the systemic lymphatics before draining into the thoracic duct and eventually reseeding the small and large intestines based on CCR9, CCR10 and α4β7 integrin signals (Hieshima et al., 2014; Kuklin et al., 2001; Pabst et al., 2004). Here, a subset of CD80/PDL-2 double negative memory IgA B cells are able to re-enter the GC reaction upon reencounter with its cognate antigen (Zuccarino-Catania et al., 2014), while the IgA plasma cell population represent a temporally stable population of antibody secreting cells (Hapfelmeier et al., 2010; Quiding et al., 1991).

B cells and T cells in the MLN

While the focus of the previous section has been the activation of IgA B cells in the GALT, another site of IgA B cell induction is the MLN. While the MLNs do not have direct contact with the microbial contents of the lumen, $CD103^+$ DCs do traffic there and have been shown to activate IgA B cell responses (Pabst, 2012). However, the functional difference between IgAs generated at the GALT vs. the MLN are unclear, and some studies debate the requirement of the MLN for IgA mediated immunity (Hahn et al., 2010).

It should be noted however, that the MLNs are a location for T cell activation towards gut antigens, and in particular the activation of *Foxp3*$^+$ regulatory T cells (T_{reg}). For example, *Helicobacter* species have been shown to be a strong inducer of T_{regs} in the colon draining MLN (Chai et al., 2017; Nutsch et al., 2016). Other groups have shown a strong IgA induction capability by $CD25^+$ T_{regs} using the flagellin specific CBir1 transgenic T cell model (Cong et al., 2009). Finally, a group of T_{regs} may seed the formation of follicular regulatory cells (T_{FR}). Their function may be to limit the number of T_{FH} cells in the GC, as to limit the B:T cell stoichiometry

resulting in greater competition for T cell help. Indeed, the deletion of T_{FR} cells has been shown to adversely impact B cell affinity maturation (Kawamoto et al., 2014).

Similarly, studies with *Rorγt*$^{+}$ T_{H17} polarized CBir1 transgenic T cells also show an effect on the production of IgA, and depletion of IL-17 receptor (IL-17R$^{-/-}$) results in a decrease in intestinal sIgA levels (Cao et al., 2012). Furthermore, studies show a large migration of T_{H17} into GCs of the diffuse lamina propria after SFB colonization, (Lécuyer et al., 2014). And there is some evidence using cell fate mapping techniques demonstrating the conversion of T_{H17} cells into T_{FH} cells (Hirota et al., 2013). However, whether these T cell subsets directly aid IgA B cell activation or first convert to *Bcl6*$^{+}$ T_{FH} cells first is currently being studied (Crotty, 2011; Shulman et al., 2014). Thus, while the MLN may not be the principle site of IgA B cell induction, it affects IgA B cells through the development of T cell subsets which interact with B cells.

IgA is transported into the gut lumen by polymeric Ig receptor

While the maturation of naïve B cells into IgA memory and plasma cell populations results in the generation of IgA antibody, this is not the final step before IgA can interact with the gut microbiome. That is because IgA secreted by plasma cells in the gut are primarily IgA dimers (dIgA) linked by the J-chain (Tomasi et al., 1965), which by themselves do not diffuse into the lumen. However, upon secretion into the lamina propria, the transmembrane glycoprotein polymeric Ig receptor (pIgR) expressed on the basolateral side of the gut epithelium covalently binds to the J chain of dIgA (Brandtzaeg and Prydz, 1984) and transports it to the

apical surface, whereupon it is cleaved (Norderhaug et al., 1999). An 80 kDa portion of pIgR known as the secretory component (SC) stays attached to dIgA, forming sIgA. pIgR is also capable of binding to IgM pentamers, also via the J chain. Through this mechanism, IgM is also able to be secreted into the gut lumen, albeit at a lower rate than sIgA (Brandtzaeg and Prydz, 1984). Hence, the transporter pIgR is crucial for allowing IgA interaction with the gut microbiome in the lumen.

Because pIgR is cleaved following transcytosis of IgA, there is a 1:1 stoichiometry between the production of pIgR and sIgA (Kaetzel, 2005). This implies the control of pIgR expression is tightly correlated with how much sIgA is released into the gut, and the control of *Pigr* gene expression based on environmental stimuli will alter the levels of sIgA. Indeed, examination of the intronic and exon 1 regions of the *Pigr* gene reveals several cytokine reactive promoter sites. Briefly, STAT1 and NFκB are two of the primary immune related *Pigr* promoter binding proteins that have been discovered (Johansen and Kaetzel, 2011). Both *in vitro* and *in vivo experiments* have shown an upregulation of pIgR production after stimulation with IFNγ, IL-4, IL-17, TNFα, and various TLR ligands such as LPS (Johansen and Brandtzaeg, 2004). Thus, it is thought that various infections and inflammatory events can modulate pIgR expression and the subsequent release of sIgA into the lumen.

The functional role of sIgA in the gut

Upon secretion into the gut lumen, sIgA has been observed to have a myriad of effector functions on the gut microbiome. Unlike IgG and the systemic humoral response, sIgA is not thought to mediate a sterilizing immune system such as directing cell killing or complement

fixation. Instead, sIgA helps to shape the gut flora through a combination of receptor blockade, steric hinderance, immune exclusion, modulating host-microbe interactions, and immune surveillance (Mantis et al., 2011).

The first sIgA effector mechanism reviewed here is the ability to block pathogenic virulence factors by steric hinderance. One of the most well known examples of this is the sIgA mediated protection of the host from *Vibrio cholerae* via the blocking of cholera toxin (CT). Here, sIgA blockade of CT's B subunit protein prevented its interaction with the host epithelial GM1 toxin receptor, and effectively blocks the onset of diarrhea common to cholera infections. Specifically, the generation of IgA hybridomas from the PP of CT orally vaccinated mice binds to CT's B subunit and not the host GM1 receptor, thus showing the mechanism of IgA action is through blockade of virulence factors (Apter et al., 1993). Another example of sIgA's ability to neutralize virulence factors comes from a study of *Clostridium difficile*, whose toxin A and B results in enterocolitis in humans and mice. In particular, the authors show that sIgA is more effective at neutralizing both toxins than an IgG antibody sharing the same variable domain (Stubbe et al., 2000), thereby reducing the pathology associated with *C. difficile* colonization.

The ability for sIgA to neutralize pathogenic virulence factors is not limited to bacteria. sIgA has also been studied in the context of viral infection and toxin ingestion. For example, during infection with enteric reovirus, several sIgA clones have been isolated which blocks reovirus type 1 Lang from binding to the host epithelium. Specifically, sIgAs have been identified which bind to the reovirus adhesin fiber σ1, thereby blocking entry and pathogenesis (Helander et al., 2004). In an example of blockade against toxins, sIgAs neutralizing ricin have been described. Following oral immunization of ricin, sIgAs binding to both the ricin enzymatic and lectin binding domain were isolated. These antibodies were able to protect host cells from

cytotoxicity when co-administered with ricin toxin (Mantis et al., 2006). From these examples, we see that sIgA is able to bind a large range of noxious particles for the protection of the host.

Secondly, sIgA can also mediate host defense by a process broadly known as immune exclusion. By enchaining or agglutinating bacteria, sIgA synergizes with the mucus layer of the gut and peristalsis to prevent host penetration of potentially infectious bacteria (Deplancke and Gaskins, 2001). One of the best known examples of this occurs during *Salmonella enterica* serovar Typhimurium infection. Here, studies have shown that sIgA targets the LPS O antigen of the bacterial outer member resulting in distortion of the membrane and loss of motility (Michetti et al., 1992). sIgA has also been shown to bind to the flagella of *S. enterica* to further reduce its motility, thereby preventing its egress deep into the host mucosa (Forbes et al., 2008). Furthermore, sIgA can enchain *S. enterica* at the earlier stages of colonization thereby preventing it's growth (Moor et al., 2017). In studies of other pathogenic bacteria, sIgA is observed to entrap *Shigella flexneri* in the mucus layer, resulting in its excretion through peristalsis. This effector function of sIgA is due to both its antigen binding domain as well as the SC, likely due to the SC's ability to interact with the mucin network (Boullier et al., 2009; McGuckin et al., 2011). Through its interactions with the mucus layer, IgA has a defined role in preventing bacterial penetration into the host.

Interestingly, immune exclusion does not seem to be the only way in which sIgA controls the *Shigella* pathology. In studies where rabbits were administered *Shigella* precoated with sIgA or *Shigella* alone, the authors found the addition of sIgA dampened the hosts' inflammatory response by lowering the expression of TNFα, IL-6, and IFNγ (Boullier et al., 2009). The dampening of host responses is due to sIgA binding of the outer member O-antigen, which causes the down regulated the expression of *Shigella*'s type 3 secretion system; a virulence factor

typically used for pathogenic entry into the intestinal epithelial cells (Forbes et al., 2011) and a potent immune stimulator. Thus, by interacting with *Shigella*, sIgA is observed to modulate bacterial protein expression, although the exact mechanism for the downregulation of the type 3 secretion system is unknown.

Another example of IgA induced expression change was observed in mice mono-colonized with *Bacteroides thetaiotaomicron (B. theta)*. In the study by Peterson and colleagues, an IgA was isolated that was specific to capsular polysaccharide synthesis 4 (CPS4). The exposure of *B theta* to this IgA resulted in the selection of bacteria with lower expression of CPS4, which had lower survival fitness in the mucosa (Peterson et al., 2007b). By altering *B. theta*'s competitive fitness, Peterson and colleagues described a dampening of host inflammatory pathways including inducible nitric oxygen species and IFNγ. Thus, IgA exerts a selective pressure on a population of *B. theta* resulting in less host inflammation.

The ability for IgA to modulate the host immune system is not limited to local cytokine secretion, nor is it limited to gut IgA. Work by Koch and colleagues examining the role of maternal IgA and IgGs find mouse pups weened without either had a stronger T cell response later in life (Koch et al., 2016). Furthermore, the introduction of IgA and IgG in the breast milk reduced the number of T_{FH} cells in the developing neonate, resulting in fewer T-dependent IgAs later in life. However, the impact of this observation in adult mice is still not clearly understood. The concept of quorum sensing by the host and microbe to facilitate homeostasis is interesting, and it is clear IgA can play a major role. The full complexities of these interactions are an area of active investigation.

Finally, sIgA has one more function that at first glance may seem contradictory to everything we've described to this point: its ability to bring antigens into the lamina propria. Work from Mantis, Corthesy and colleagues have shown sIgA complexed antigens being selectively acquired by M cells (Mantis et al., 2002). Microscopy studies show fluorescently labelled biotin conjugated to sIgA, but not IgG or IgM, cluster to the apical surface of M cells. Furthermore, vesicles within the M cells also showed the presence of fluorescently labelled sIgA-biotin complex. Another study visualized this IgA mediated uptake process using mice infected with mouse mammary tumor virus (MMTV) in the presence of an IgA hybridoma backpack specific to MMTV (Weltzin et al., 1989). The study noted MMTV glycoprotein gp52 conjugated with sIgA was found in systemic circulation in the liver and bile. To further support the idea of IgA mediated antigen uptake, Rochereau and colleagues demonstrated orally administered HIV P24 protein conjugated to IgA, but not IgG, enhanced antigen trafficking and improved systemic B cell responses to P24 (Rochereau et al., 2013).

In order to facilitate reverse transcytosis of antigens, sIgA has been shown to undergo conformational changes after binding to antigens. This conformational change improved sIgA binding to receptors expressed on the apical surface of M cells (Duc et al., 2010). Further studies found Dectin-1 expressed on M cells are responsible for the binding of sIgA complexes (Rochereau et al., 2013). These studies showed that Dectin-1 binds to the Cα1 domain and various glycoproteins of sIgA. After Dectin-1 binding and reverse transcytosis, the sIgA-antigen complex is released into the SED, where $CD11c^+$ $CD11b^+$ DCs expressing the DC-SIGN receptor appear to be the primary recipient of these new antigens (Baumann et al., 2010). Thus, the life of the sIgA molecule appears to come full circle, delivering antigens from the lumen into the SED, with the potential to activate a new round of IgA B cell selection.

Summary

The maturation of naïve B cells into memory and plasma IgA B cells is a complex process. Multiple different physiological locations, cellular subsets, and cellular signals are involved from the initial antigen encounter, the GC reaction, and the secretion of IgA; each of which can be modulated based on microbial colonization or inflammatory conditions. It should not come as a surprise then, that the factors affecting the formation of IgA B cell responses remains an open question. What bacterial antigens do these B cells see at steady state? What drives the cross reactive IgA activation versus a more traditional adaptive response? These are some of the questions actively being pursued at the moment.

While the question of IgA B cell activation is complex, so too is the question of sIgA function in the lumen. Many of the studies highlighted in the previous section utilized model antigens in gnotobiotic mice, oral vaccinations, or pathogenic infections. The function of sIgA during homeostasis against a fully complex microbiome is not clearly defined. The paradoxical role of sIgA immune exclusion and sIgA immune surveillance is a problem worth addressing. Are the two functions truly mutually exclusive, or is it dependent on the target bacteria? Addressing these questions and gaining a better understanding of IgA function has the potential to unlock many therapeutic innovations, ranging from oral vaccine strategy, to a better understanding of food allergen responses, to dealing with inflammatory bowel disease.

Chapter 2: sIgA enhances the priming of gut B cells

2.1 Abstract

Previous studies show mixed results on sIgA's impact on priming the gut adaptive immune response. Depending on the model antigen used, sIgA sometimes blocks T and B cell activation, and other times help stimulate the immune response. Here, we utilize an *in vitro* culturing system to expand gut B cells and test the effects sIgA has on the gut adaptive immune system under homeostatic conditions with an unaltered microbiome. Interestingly, we see that the loss of sIgA in *Pigr*$^{-/-}$ mice results in lowered IgA MBC responses towards commensals. This loss of reactivity was not seen across all bacteria, but in a specific subset.

2.2 Introduction

The mammalian gut is colonized by trillions of microbes which are segregated from the host to maintain homeostasis. A number of mechanisms exist to control host:microbial interactions, including mucin and antimicrobial peptides at the mucosal surface (Brogden, 2005; Hancock et al., 2016; Pelaseyed et al., 2014). Another layer of protection is thought to be provided by adaptive immunity via the secretion of immunoglobulins (Ig) by B cells (Macpherson et al., 2008; Pabst, 2012). While various Ig isotypes are produced in the mucosa, IgA is the primary antibody that enters the lumen. Dimeric IgA, but not IgG, produced by B cells is transported from the lamina propria into the gut lumen by the polymeric Ig receptor (pIgR), whereupon pIgR is cleaved, leaving the secretory component attached to dimeric IgA forming secretory IgA (sIgA) (Mantis et al., 2011). Thus, host IgA responses are thought to be important for maintaining homeostasis to gut bacteria (Koch et al., 2016; Macpherson et al., 2008a; Pabst, 2012).

The role of sIgA has been extensively studied during infection. Examples include the ability of sIgA to enchain enteropathogens such as *Salmonella typhimurium* to facilitate bacterial clearance (Moor et al., 2017), and to neutralize cholera toxin during *Vibrio cholerae* infection (Apter et al., 1993). Secretory IgA (sIgA) has also been implicated as a mechanism for the uptake of bacterial antigens (Brown et al., 2013; Mantis et al., 2002) and facilitate immunosurveillance of pathogens such as *Shigella flexneri* (Kadaoui and Corthésy, 2007). Thus, sIgA is thought to provide an immunologic barrier during infection.

However, the role of sIgA responses to gut commensal bacteria under homeostatic conditions is less clear. Previous reports suggest that, similar to its role against pathogens, sIgA provides immune exclusion by limiting commensal bacterial antigen access to the adaptive immune system (Cong et al., 2009; Peterson et al., 2007a). For example, presentation of orally administered flagellin antigen was increased to CBir1 TCR transgenic cells in PigR-deficient mice (Cong et al., 2009), which lack the ability to transport IgA into the intestinal lumen. However, two recent studies suggested that IgA could facilitate bacterial growth or colonization. One study of *Bacteroides thetaiotaomicron* showed that metabolic utilization of IgA was required for optimal colonization (Nakajima et al., 2018). A study of *Bacteroides fragilis* found that host sIgA was used to gain access to certain gut niches to allow colonization (Donaldson et al., 2018). These data suggest that the interaction of host immunity and IgA with gut bacteria is complex and may occur in a bacteria-specific manner. Thus, while it is clear that host immune responses generate sIgA against gut bacteria during homeostasis (Pabst, 2012; Palm et al., 2014), it remains unknown whether sIgA facilitates or inhibits the priming of gut B cell responses to bacteria.

Here, we studied the role of sIgA in shaping the anti-commensal Ig repertoire during homeostasis. We use the polymeric Ig receptor (*Pigr*) knockout mouse, which lack the receptor required for transporting IgA and IgM into the intestinal lumen, but otherwise do not show overt signs of inflammation (Johansen et al., 1999). To efficiently screen for anti-bacterial IgA and IgG specificities, we developed an *in vitro* system to expand small numbers of memory B cells to collect their secreted Ig. Unexpectedly, we found that sIgA deficient mice show decreased IgA reactivity towards certain members of the microbiota, suggesting that sIgA facilitated B cell priming to gut bacteria.

2.3 Materials and Methods

Ethics Statement

Animal experiments were performed in specific pathogen free facilities in accordance with the guidelines of the Institutional Animal Care and Use Committee at Washington University.

Mice

Pigr$^{-/-}$ mice (Johansen et al., 1999) were obtained from H.W. Virgin, and bred *Pigr*$^{+/-}$ males to *Pigr*$^{-/-}$ females. CT2 (Nutsch et al., 2016), CT6 (Lathrop et al., 2011), and OTII (Jax #004194) TCR transgenic mice have been previously described. *Rag1*$^{-/-}$ (Jax #002216) and *Foxp3*$^{\text{IRESGFP}}$ (Jax #006769) were obtained from Jackson Labs.

T cell analysis and adoptive transfer

Naïve TCR transgenic T cells from the mesenteric, axillary, and inguinal lymph nodes, and spleens, were sorted using a FACAria IIu (Becton Dickenson) based on CD4^{+} CD25^{-} CD44^{lo} CD62L^{hi} expression. Cells were labelled with Cell Trace Violet (Invitrogen) and 2x10^{5} T cells were retro-orbitally injected into 7-8 week old CD45.2 host mice anesthetized with isoflurane. After 1 week, the CD45.1^{+} CD45.2^{-} CD4^{+} transferred T cells were analyzed by flow cytometry from the MLNs. Activated T cells were gated on CD62L^{lo} CD44^{hi}, Foxp3 expression was

determined by GFP, and cell proliferation was gated on CTV^{lo} populations. Oral ovalbumin (50mg) was fed via gastric gavage 24 and 48 hours after adoptive transfer of OTII cells.

B cell analysis and culture

Single cell suspensions of ileal PP, colonic lamina propria, or MLN were analyzed by flow cytometry. Memory B cell populations were identified as $B220^{+}$ IgD^{-} $CD138^{-}$ $GL7^{-}$ and IgA or IgG^{+}. ASCs were identified as $B220^{lo}$ IgD^{-} $GL7^{-}$ and IgA^{+} or IgG^{+}. MBCs were expanded *in vitro* via coculture with NIH-3T3 cells expressing BAFF and CD40L as described (Purtha et al., 2011). Briefly, MBCs (1-1600/well) were sorted into mitomycin-c treated BAFF and CD40L expressing 3T3 cells and cultured in 96 well plates and stimulated with IL2, 6, 10, LPS, Pokeweed mitogen, and CpG DNA. After 7 days, supernatant was harvested and IgA/IgG quantified by a standard sandwich ELISA using plate-bound anti-mouse IgK capture (eBioscience), biotinylated anti-mouse IgA or anti-mouse IgG (BD), and HRP Streptavidin (Biolegend). TMB detection was used and quenched with 1M H_2SO_4; detection was performed at A450. Purified mouse IgA or IgG was used to generate standard curves.

Bacteria FACS and sorting

3 week old *Rag1*$^{-/-}$ mice were orally gavaged with 200 µL 0.5 g/L vancomycin, 1g /L neomycin, ampicillin, and metronidazole twice over 48 hours. 72 hours after the first gavage, *Rag1*$^{-/-}$ mice were gavaged with total intestinal lumen preparations from *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice.

2 weeks after fecal transfer, intestinal lumen contents and colonic mucus scrapings from *Rag1*$^{-/-}$ mice were collected and frozen in 40% glycerol stocks at -80° C to generate the fecal repository.

For bacteria FACS, fecal stocks from *Rag1*$^{-/-}$ mice were treated with 5mM L-acetyl cysteine for 10 minutes to disrupt the mucous, washed with PBS, and filtered through 70µm nylon mesh. 20µL were stained with 150µL of Ig containing supernatant in a 96 well U bottom plate on ice for 1 hour. Ig-stained intestinal samples were then washed twice with PBS and stained with 1mM DAPI, Dylight anti-mouse IgA, PE anti-mouse IgG, and a FITC isotype control for 1 hour on ice. After washing, flow cytometry and sorting of bacteria was performed on a BD FACSAria IIu. Ig^+ fractions were sorted directly into a 96 well PCR plate containing 25µL of H_2O.

Bacteria 16S rDNA sequencing

Bacterial 16S rDNA was amplified using Bioline Ranger polymerase and barcoded primers for the V4 region of the 16S rDNA (Caporaso et al., 2011) and sequenced on the Illumina MiSEq platform (250 bp paired end reads). OTU picking was performed using UPARSE (usearch v.9.0.2132)(Edgar, 2013) and taxonomy assigned via uclust method (QIIME v1.9)(Caporaso et al., 2010) using Greengenes 13.8 database.

Statistical Analysis

Graphpad Prism v8, R v3.5.2, R Studio v1.1.447, and Qiime v1.9 were used for statistical and graphical analysis. Student's t test, Mann-Whitney U test, two-way repeated measures ANOVA, Fisher's exact test (Prism v8 and R package Stats v3.6.0), and PERMANOVA (R

package Vegan v2.5-4) were used for between subject analyses. Benjamini-Hochberg FDR correction (R package Stats v3.6.0) was to correct for multiple comparisons containing normally distributed P values and Storey Q-value correction (R package qvalue v2.14.1) was used to adjust for multiple comparisons where P values were left skewed. Outlier detection for high IgA reactivity utilized the R packages Extremevalues (v2.3.2) and Fitdistrplus (v1.0-14). Heatmaps were generated using R package Heatmap3 (v1.1.16); OTU and wells distance matrices and clustering were done using the R package Vegan (v2.5-4). Statistical significance key: * : $p < 0.05$, ** : $p<0.001$, *** : $p<0.0001$, **** : $p<0.00001$.

2.4 Results

sIgA does not affect T cell responses to colonic Helicobacter species

Previous reports suggested that anti-commensal IgA could inhibit T cell responses to gut bacteria (Cong et al., 2009; Peterson et al., 2007a). Recently, we identified *Helicobacter typhlonius* and *apodemus* as bacterial stimulators (Chai et al., 2017) of peripheral Treg cell development of TCR transgenic lines CT2 and CT6 (Lathrop et al., 2011; Nutsch et al., 2016). *Helicobacter* spp. have also been reported to induce IgA responses *in vivo* (Arnold et al., 2016). We therefore asked if activation of these bacterial-reactive T cells was inhibited by sIgA. In contrast to CBir1 T cells (Cong et al., 2009), the proliferation and Treg development of transferred naïve CT2 and CT6 cells were not affected by loss of sIgA in $Pigr^{-/-}$ mice (**Fig. 2.1.A-C**), which were generated in this study by breeding $Pigr^{+/-}$ males with $Pigr^{-/-}$ dams. Consistent with the data from transferred TCR transgenic cells, we also did not observe wholesale changes in the frequencies of polyclonal effector or regulatory T cells in the Peyer's patches, mesenteric lymph nodes (MLN), or colon lamina propria (cLP) in $Pigr^{-/-}$ compared with $Pigr^{+/-}$ mice (**Fig. 2.2.A-B**). To assess whether $Pigr^{-/-}$ mice exhibited alterations in the presentation of soluble oral antigens in unimmunized mice (Johansen et al., 1999), we examined OTII responses to oral ovalbumin. Like CT2 and CT6, T cell activation of transferred naïve OTII cells was unaffected by PigR-deficiency (**Fig 2.1.D**). Thus, these data suggest that for these commensal and food antigens, sIgA does not limit T cell activation in the gut.

sIgA deficient mice show lower IgA reactivity to commensals.

We then asked whether sIgA limits the B cell response to gut commensals. Similar to the polyclonal T cell populations (**Fig. 2.2**), no significant differences in the IgA^+ memory B cells (MBC), naïve B cells, or germinal center B cells were seen in the small intestinal Peyer's Patches (PP), MLN, and cLP from 7 week old *Pigr*$^{-/-}$ and *Pigr*$^{+/-}$ control littermates (**Fig. 2.3.A-B**). A small increase was found in the antibody secreting cells (ASC) population within the MLN of *Pigr*$^{-/-}$ mice. Overall, the B cell population, like the T cell population, was not markedly affected by the absence of sIgA in the gut.

Although we did not observe marked changes in the B cell population, the absence of PigR could still alter the frequency of anti-bacterial B cells. As *Pigr*$^{-/-}$ mice do not secrete sIgA into the lumen (Johansen et al., 1999), this precludes the approach of using flow cytometry for assessing IgA specificity for fecal bacterial (**Fig. 2.4.A**) (Kau et al., 2015; Palm et al., 2014). An alternative approach has been to clone from individual IgAs single cells and re-express them to test specificity (Tiller et al., 2009). However, this is a time intensive approach and cannot be easily used to analyze the antigen specificities of B cell populations between mice of different genotypes as only a hundred or so BCR clones can be sampled. Instead of BCR cloning, *ex vivo* culture systems have been used to obtain IgA from ASCs that could then be used to label fecal bacteria. However, individual ASCs do not make sufficient IgA in culture (Cocco et al., 2012) to assess antigen reactivity at clonal or near-clonal cell numbers.

Because of these issues, we utilized an *ex vivo* B cell culture system to study the bacterial specificity of IgA produced by MBCs. We chose to study MBCs from the ileal PP, as the PP are the primary site for IgA B cell activation in the GALT (Reboldi et al., 2016). Furthermore, the

ileum contains the highest number of PPs, and IgA B cells from the ileum also appear to be the most mature compared to those found in the duodenum or jejunum (Biram et al., 2019). IgA^+ MBCs were sorted onto NIH-3T3 cells expressing B cell growth factors (Purtha et al., 2011) and expanded *in vitro* to secrete IgA antibodies. The antibodies in the supernatant were then used to stain IgA-free intestinal bacteria and detected by flow cytometry using a fluorescent anti-IgA antibody (**Fig. 2.5.A**). Intestinal bacterial preparations of the small and large intestines were generated by antibiotic treatment followed by oral gavage of $Rag1^{-/-}$ mice with fecal matter combined from $Pigr^{+/-}$ and $Pigr^{-/-}$ mice. One caveat of this approach is that the microbial proportions in the $Rag1^{-/-}$ hosts are not identical to that of the donors, although most of the constituents are present in the repository (**Fig. 2.5.B**). Another issue is that bacteria present at low frequency in the repository may be difficult to identify via FACS purification. Finally, flow cytometry will not detect anti-bacterial IgA that are reactive to soluble antigens or smaller particles such as outer membrane vesicles (Hickey et al., 2015). However, one advantage of an *in vivo* source of bacteria is that they are more likely to express the appropriate array of antigens, which may not occur with *in vitro* culture (Hickey et al., 2015). With these considerations in mind, we reasoned that this *ex vivo* approach would allow us to directly compare the bacterial reactive repertoire of PP IgA+ MBCs that develop in the presence or absence of sIgA.

We first assessed the efficiency of culturing IgA^+ MBCs from control $Pigr^{+/-}$ mice i*n vitro*. Approximately 17% of wells seeded at one IgA^+ MBC per well produced IgA detectable by ELISA (**Fig. 2.6.A**). Of the IgA positive wells, 46% bound bacterial particles as detected by flow cytometry, giving an estimated ~8% efficiency per sorted cell for generating bacteria-reactive IgA. We did not observe a correlation between the IgA concentration in the supernatant and the percent IgA bound bacteria, suggesting that the IgA binding is antigen specific (**Fig**

2.6.B). Furthermore, pIgR-deficiency did not lead to a B cell intrinsic IgA production deficit as assessed by IgA ELISAs of the *in vitro* culture supernatants (**Fig. 2.6.C**). As the limiting factor in this analysis was the total number of wells that could be analyzed for IgA-bound bacteria by flow cytometry in a given day, we asked whether increasing the number of MBCs input per well would allow us to sample a larger proportion of the MBC population in *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice. Comparison of IgA produced by MBCs from *Pigr*$^{-/-}$ and *Pigr*$^{+/-}$ mice wells revealed a difference in the frequency of IgA-bound bacteria at 25 cells/well (**Fig. 2.7.A**). The difference between *Pigr*$^{-/-}$ and *Pigr*$^{+/-}$ MBCs was diminished or lost at higher cell numbers (100-1000/well), suggesting that this assay has a saturable component such as the number of IgA-binding sites on bacteria. Twenty-five cells per well would be predicted based on the Poisson distribution to result in approximately 1-3 bacteria reactive IgA B cell clones per well, limiting the ability to accurately count bacterial-specific MBCs present in the population at high clonal frequency as there maybe multiple instances per well. Nonetheless, we reasoned it would be worth this tradeoff to capture a substantially greater fraction of the B cell repertoire by using 25 MBCs/well.

To confirm the difference seen between MBCs from *Pigr*$^{-/-}$ or *Pigr*$^{+/-}$ mice in the cell number titration experiment (**Fig. 2.7.A**), we analyzed supernatants obtained from cultured ileal PP IgA^{+} MBCs sorted from 7 mice per genotype (48 well/mouse; 25 cells/well). As before, the frequency of IgA-bound bacterial particles assessed per well by flow cytometry was decreased on average from *Pigr*$^{-/-}$ vs littermate *Pigr*$^{+/-}$ control MBCs (**Fig. 2.8.A**). In fact, 24% of IgA containing wells from *Pigr*$^{-/-}$ IgA MBCs showed background levels of binding to bacteria (dashed line, Fig. 2.8.A), in contrast with less than 10% of wells from control *Pigr*$^{+/-}$ MBCs. These data suggest that deficiency in sIgA results in decreased generation of bacteria-specific

IgA B cells, which is contrary to the hypothesis that sIgA primarily excludes bacteria from interacting with the adaptive immune system (Cong et al., 2009; Peterson et al., 2007a).

Deficiency in sIgA does not significantly alter the microbiota

One potential explanation for the decrease in IgA B cell reactivity in sIgA deficient mice would be a large shift in the microbiota between *Pigr*$^{-/-}$ and *Pigr*$^{+/-}$ mice, which are weaned as littermates by sex, but not genotype, into the same cage. sIgA may regulate the abundance of specific bacteria species (Fadlallah et al., 2018; Macpherson et al., 2008a) and disrupt the community structure. To address this, we analyzed the microbiota by 16S rDNA sequencing of lumen contents of the small intestine and colon, as well as bacteria that were associated with the mucosa. We did not observe significant differences in the microbiota at the operational taxonomic unit (OTU) or order level taxa between *Pigr*$^{-/-}$, *Pigr*$^{+/-}$, or *Pigr*$^{+/+}$ mice (**Fig. 2.9.A**, PERMANOVA). In addition, principle coordinate analysis (PCoA) of Bray-Curtis dissimilarity distances did not reveal distinct clustering by *Pigr* genotype (**Fig. 2.9.B**). Finally, we did not observe significant differences in alpha-diversity measures between *Pigr*$^{-/-}$, *Pigr*$^{+/-}$, or *Pigr*$^{+/+}$ mice (**Fig. 2.9.C**). In summary, these data from 7 wk old mice do not reveal a marked shift in the microbial community resulting from a deficiency in sIgA, suggesting that the increased bacteria specificity of IgA B cells from PigR-sufficient mice are a result of a differences in microbial priming of the immune system.

sIgA increases IgA B cell reactivity towards specific taxa

The unexpected decrease in bacteria-reactive IgA produced by MBCs from sIgA deficient *Pigr*$^{-/-}$ mice could be due to a loss of IgA reactivity to specific taxa vs "innate" like IgA that are cross-reactive to bacteria from many different taxa (Bunker et al., 2017). To address this, we sorted the IgA-bound bacteria labeled by supernatants from the 25-cell pools and analyzed their taxonomy by 16S rDNA sequencing (Caporaso et al., 2010). Comparison of the IgA-bound bacteria from each well revealed a number of OTUs that were consistently found, mostly from the *Bacteroidales* order (top rows, **Fig. 2.10.A**). There were a number of wells that contain similar levels of IgA by ELISA, but did not show IgA binding by FACS, arguing against the possibility that these taxa express IgA binding proteins or other features that non-specifically bind IgA. To assess whether the results seen with 25 cell/well may be due to multiple bacterial reactive clones per well, we sequenced IgA bound bacteria using IgA generated from wells with 5 MBCs, where we expect only one expanded bacterial reactive clone per well (**Fig. 2.10.B**). Together with the 25 well sort data, these data suggest that there are OTUs found in almost all wells that may represent background in this assay, OTUs that are often found together as a group in a number of wells suggestive of cross-reactive IgA (Bunker et al., 2017), and OTUs found in only a few wells suggesting recognition by lower clonal frequency MBCs. Our data are consistent with the idea that many/most IgA clones exhibit cross-reactivity to multiple species.

We then asked whether there were wells that showed IgA binding to specific taxa above "background" IgA binding, suggestive of the presence of an IgA clone with high reactivity to that OTU. We first looked for OTUs that show a high ratio between the IgA bound frequency divided by its frequency in the total population used for staining. However, this method skewed towards rare bacteria which had large enrichment ratios due to a small denominator (data not

shown). Next, we used non-parametric and parametric methods for outlier detection using the 16S frequency in the top quartile or above 2 standard deviations (SD), respectively, for all wells of both genotypes of a given OTU. While this approach worked for some OTUs, for others it appeared to result in false positives (e.g. s541 for interquartile range (IQR)) or false negatives (e.g. s3846 for 2xSD) (**Fig. 2.11.A, B**). One limitation of these approaches is that they are based on assumptions regarding the distribution of IgA binding frequency. We therefore examined the distribution of data across all wells for each OTU by comparing the skewness and kurtosis (**Fig. 2.11.C**) via a Cullen Frey graph (see Methods), which suggested that the best fit distribution of frequencies was an exponential distribution. This was further supported by examining the Akaike information criterion, where the exponential distribution has the best fit (lowest value) compared with other distributions across all OTUs (**Fig. 2.11.D**). Use of an algorithm for outlier detection fitted to an exponential distribution (**Fig. 2.11.A, E**) allowed us to estimate with greater reliability the frequency of IgA MBCs wells that show what we will call "high reactivity" to specific bacteria taxa. Using this method, we found that the presence of sIgA resulted in a significantly increased frequency of MBCs with high reactivity to a subset of OTUs determined (**Fig. 2.12.A**). In fact, OTUs showing a greater than 4-fold difference in the number of IgA high reactivity wells were preferentially bound by IgA MBCs from *Pigr*$^{+/-}$ compared with *Pigr*$^{-/-}$ mice (**Fig. 2.12.B**, vertical dashed lines).

Because these experiments used multiple breeders and litters over a period of weeks to months, we performed a pairwise comparison between the individual sIgA-deficient *Pigr*$^{-/-}$ mouse and control *Pigr*$^{+/-}$ littermate sorted on each experiment day to limit the effects of litter to litter variation. For each OTU, we subtracted the number of wells with high reactivity IgA from the *Pigr*$^{+/-}$ vs its paired *Pigr*$^{-/-}$ littermate–a positive number indicating a greater number of wells

with high reactivity to that OTU in the *Pigr*$^{+/-}$ mouse. For most OTUs, there were more IgA reactive wells from the sIgA-sufficient mouse (blue, **Fig. 2.12.C**). However, a subset of OTUs show increased numbers of IgA reactive wells from the sIgA-deficient mouse (red, **Fig. 2.12.C**). Taken together, these data suggest that the presence of sIgA generally enhanced the generation of high reactivity IgA to specific taxa.

We next asked if the sIgA dependent IgA were targeted based on higher taxonomic levels, hypothesizing that species of certain taxa may express common antigens. To test this, we summed the number of wells showing high IgA reactivity at a given taxonomic level as a proportion of total wells and observed that members of the *Bacteroidales* order elicit a larger IgA B cell response in *Pigr*$^{+/-}$ mice than in *Pigr*$^{-/-}$ mice (**Fig. 2.12.D**). Finally, we asked whether sIgA affected the anti-IgA coverage of the bacterial species that reside within the gut. We were able to detect wells with high IgA reactivity towards ~60% of the commensal population in *Pigr*$^{-/-}$ mice compared to ~80% in *Pigr*$^{+/-}$ mice (**Fig 2.12.E**). In summary, these data suggest that the presence of sIgA enhanced the frequency of bacterial reactive IgA^{+} MBCs, the proportion of clones with high reactivity to gut bacteria, and the breath of bacterial recognition by high reactivity IgAs.

2.5 Discussion

In this chapter, we show a major role of sIgA is to facilitate B cell responses to commensal bacteria, contrary to the notion that sIgA inhibits immune interaction with gut bacteria. These responses appeared to be taxa specific, as Ig responses to OTUs from the *Bacteroidales* order show greater dependence on sIgA.

Our data suggest that under homeostatic conditions, sIgA is not required for preventing wholesale commensal bacterial entry into the lamina propria, consistent with a recent study (Rogier et al., 2014). Unlike previous studies (Cong et al., 2009; Peterson et al., 2007), we found that T cell responses to *Helicobacter* were not increased in the absence of sIgA. These results may differ because the model systems used previously may not represent homeostatic conditions. For example, CBir1 T cell activation was assessed to exogenous antigen administered orally and not antigen endogenously produced by gut bacteria (Cong et al., 2009). Similarly, decreased T cell responses to *B. theta* was seen in a monocolonized mouse which also had monoclonal production of anti-*B. theta* IgA (Peterson et al., 2007). Another possibility is that *Helicobacter*, which does induce IgA responses in our experience as well as others (Xu et al., 2018), can bypass inhibition by sIgA to gain access to the immune system as it lives in crypts of the colon and cecum (Pedron et al., 2012). sIgA may still play an immune exclusion role for certain taxa, as we observed that some OTUs showed increased IgA^+ MBC responses in $Pigr^{-/-}$ vs. $Pigr^{+/-}$ mice. In addition, the assay of MBC specificities may not be sensitive to ongoing B cell priming which could be diminished by pre-existing IgA. Thus, sIgA may inhibit immune activation to only a subset of luminal antigens.

Instead, our data suggests that an important role of sIgA during homeostasis is to facilitate immunosurveillance. We observed that the frequency of anti-bacterial IgA is increased in the presence of sIgA. Although we do not address how this might occur, a mechanism by which sIgA facilitates antigen uptake from the gut lumen into the lamina propria was suggested to involve Dectin-1 (Rochereau et al., 2013). In addition, sIgA may interact with the gut mucin network (Biesbrock et al., 1991), resulting in adherence of bacterial targets to mucin which may potentially result in antigen uptake. Future studies will be required to understand the mechanism by which sIgA facilitates immune priming, and how it fits amongst the various methods by which commensal bacteria trigger adaptive immune responses in the gut.

OTUs of order *Bacteroidales* were particularly dependent on sIgA for induction of adaptive immunity, although the rationale for this is unclear. One interesting question is whether high reactivity antigen-specific sIgA is necessary for this process. Although further studies are needed, we speculate that the poly-specificity of many IgA (Bunker et al., 2017), which we observed in our studies often binding to *Bacteroidales* members, may facilitate uptake and subsequent generation of high reactivity bacteria-specific IgA.

2.6 Figures

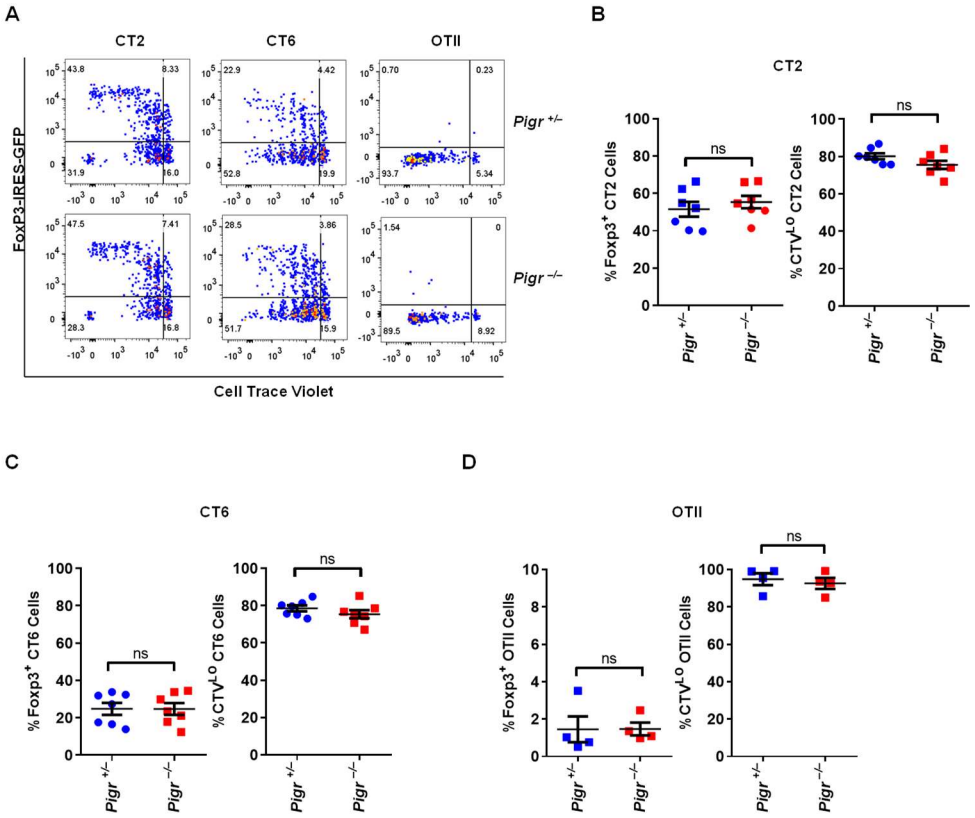


Figure 2.1: *Pigr*$^{-/-}$ mice have normal transgenic T cell activation

(A-D) TCR transgenic responses to gut antigens. TCR transgenic responses to gut antigens. 2x10^5 naïve CD45.1 OTII, CT2 or CT6 transgenic T cells were transferred into CD45.2$^+$ *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ hosts. MLN (OTII) or distal MLN (CT2, CT6) were analyzed 7 days post transfer and donor T cell expression of Foxp3^{GFP} and cellular division (Cell Trace Violet, CTV) was assessed by flow cytometry. Representative FACS plots are shown in (A) and summarized in (B-D) for CT2, CT6, and OTII, respectively. Significance determined by Student's t test. Each dot represents an individual host and combine 2-3 independent experiments.

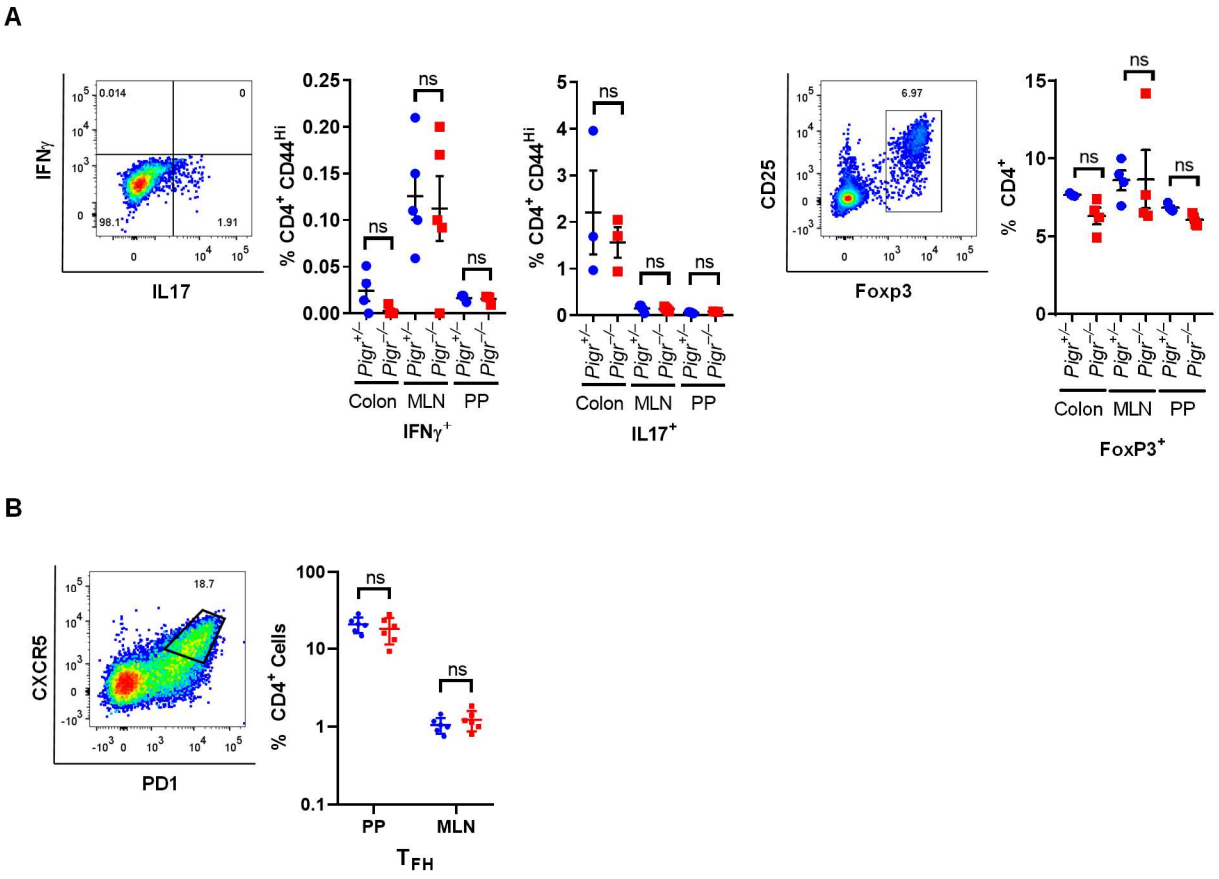


Figure 2.2: *Pigr*$^{-/-}$ mice have normal polyclonal T cell compartments in the gut

(A) Frequency of helper T cell populations in the GALT. FACS gating strategy and summarization of IFNγ^{+}, IL17^{+}, or *Foxp3*$^{+}$ T cell populations in the cLP, MLN, or PP of 7-8 week old *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice were assessed by intracellular staining after PMA + Ionomycin stimulation. (B) Frequency of follicular T cells in the GALT. PD1^{+} CXCR5^{+} CD4^{+} cells in the PP or MLN of 7-8 week old *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice were assessed by flow cytometry. Statistical significance determined by Student's T test (n = 6, 6)

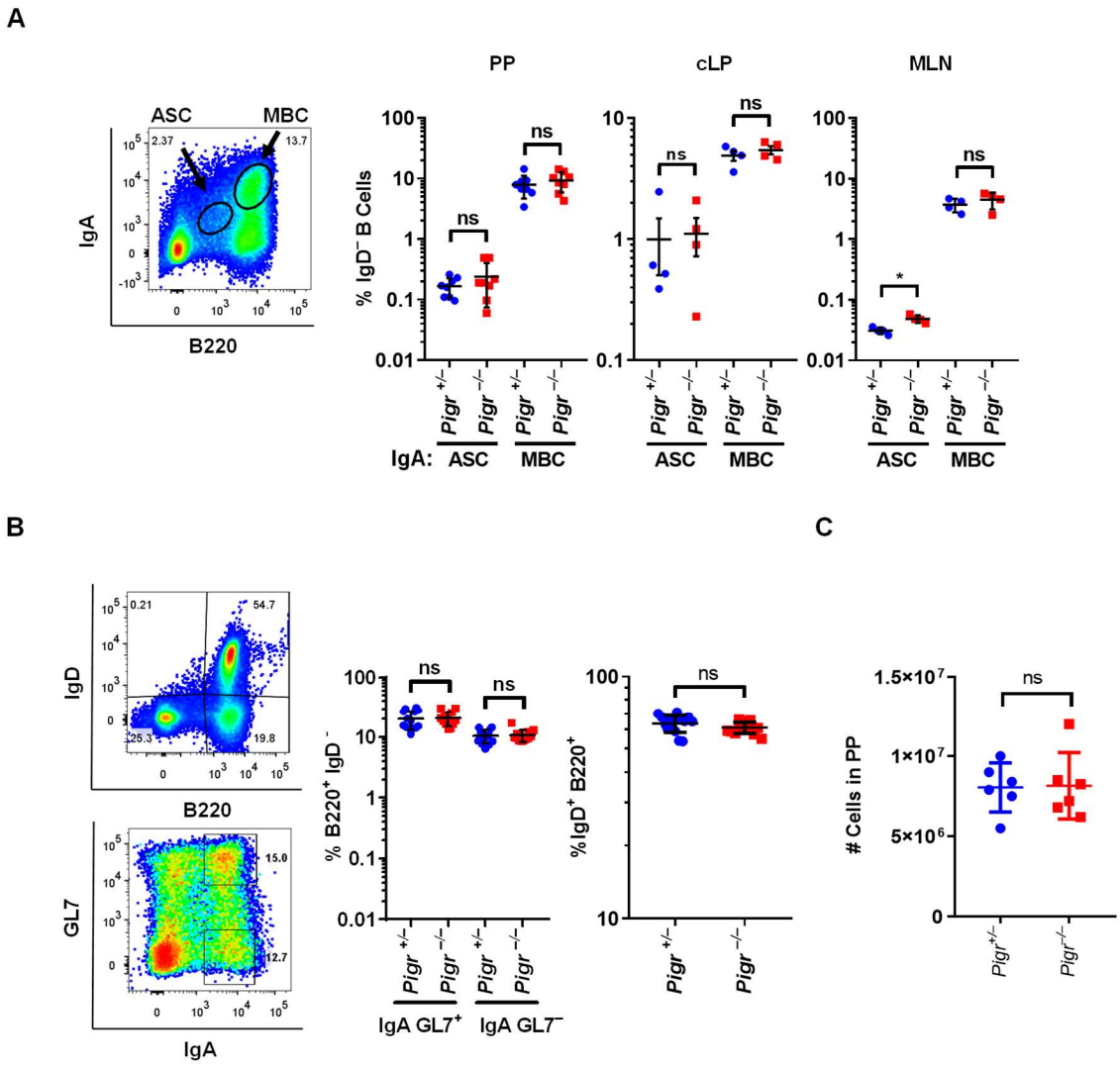


Figure 2.3: *Pigr*$^{-/-}$ mice have normal B cell compartments in the gut

(A) Frequency of B cells in the GALT and MLN. FACS gating strategy for B220^{+} IgA^{+} IgD^{-} MBCs or B220^{lo} IgA^{+} IgD^{-} ASCs is shown on the left. On the right, the frequency of ASCs and MBCs within the IgD^{-} B cell population of 7-8 week old *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ is shown within the Ileal PP, colon lamina propria (cLP) or mesenteric lymph node (MLN). Significance determined by Student's t test. Each dot represents an individual host and combine 2-3 independent experiments. (B) FACS gating strategy for ileal PP B cells is shown on the left. On the right, the

frequency of $B220^{+}$ IgD^{-} $GL7^{+}$ IgA^{+} germinal center cells, or $B220^{+}$ IgD^{-} $GL7^{+}$ IgA^{-} memory B cells of 7-8 week old *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice is shown. Statistical significance determined by Student's t test (n = 15, 14). (C) Total cell counts within the PP. Statistical significance determined by Student's T test (n = 6, 6). Each dot represents data from an individual mouse.

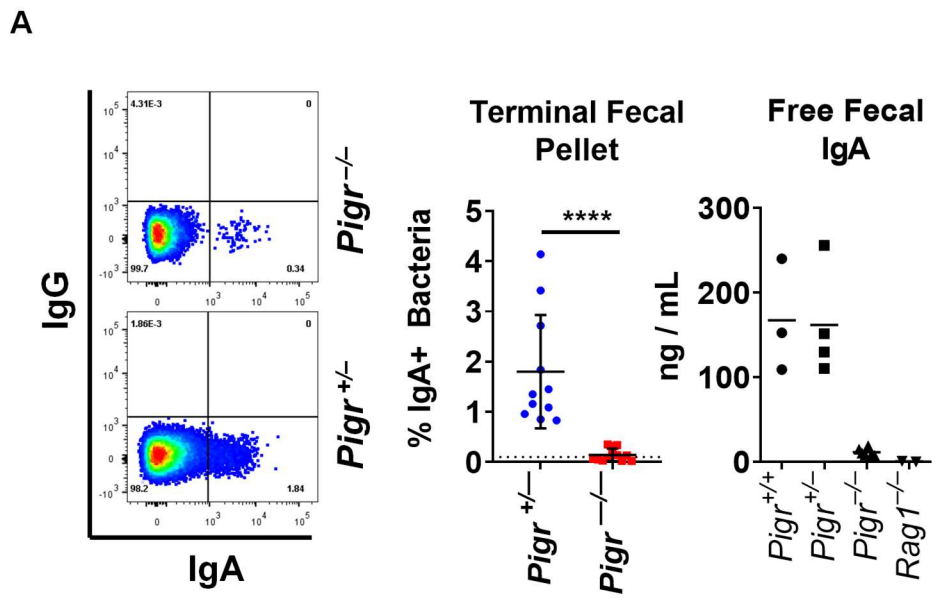


Figure 2.4: *Pigr*$^{-/-}$ mice have reduced sIgA in the gut lumen

(A) Fecal IgA assays of *Pigr*$^{-/-}$ mice. Data shown are (left) FACS gating for IgA bound bacteria of fecal pellets, (middle) summary of IgA bound bacteria from 7-8 week old *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice, and (right) quantification of free IgA detected in fecal matter by ELISA.

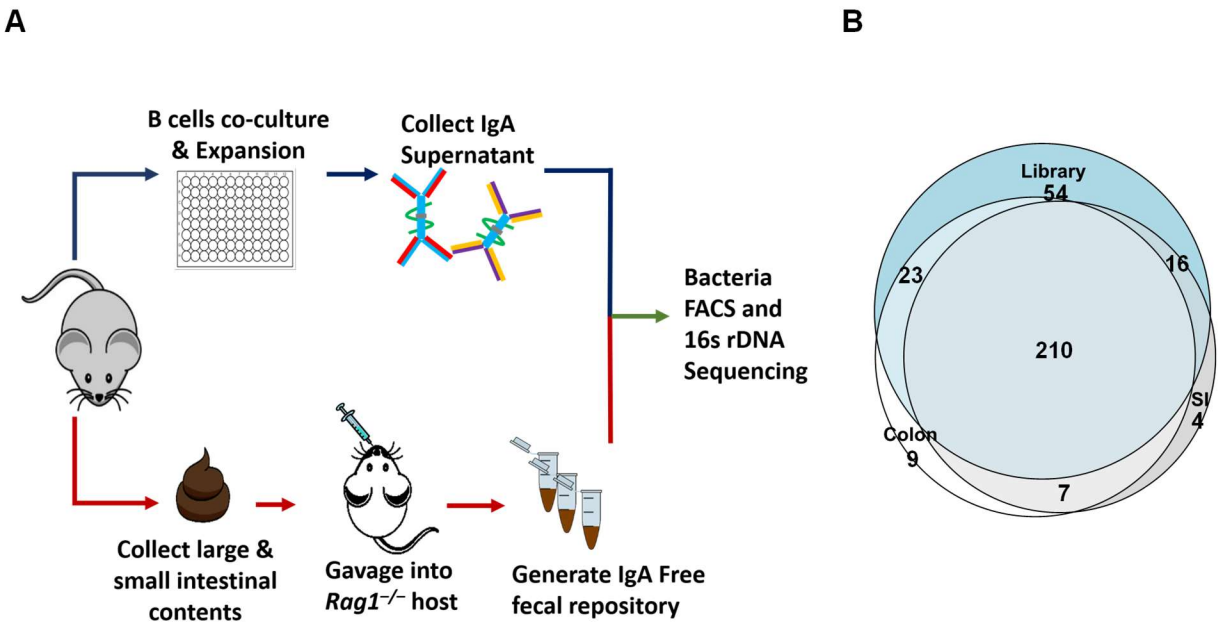
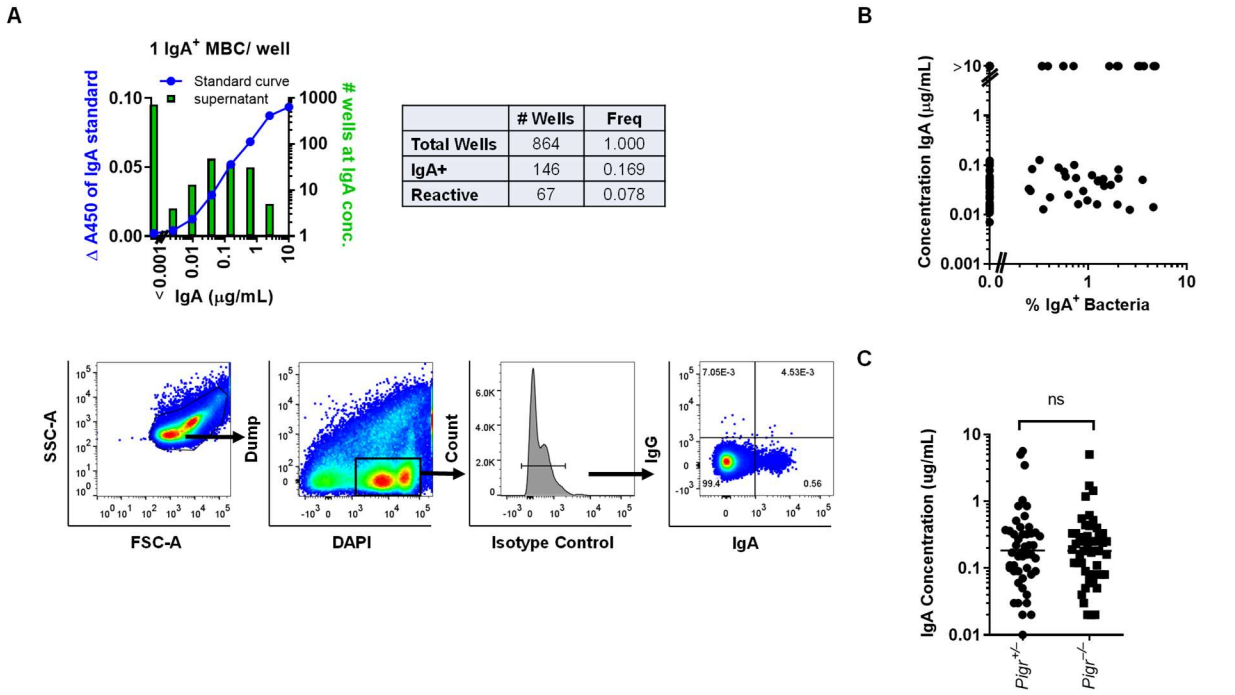


Figure 2.5: Method to detect anti-bacterial IgA specificity in sIgA deficient mice

(A) Experimental protocol for assessing bacterial reactivity. IgA MBCs are expanded *in vitro* via coculture with NIH-3T3 cells expressing BAFF and CD40L. IgA in the supernatant is used to stain intestinal bacteria from *Rag1*$^{-/-}$ mice for bacterial flow cytometry and 16S rDNA sequencing. (B) Bacterial composition of fecal repository vs lumen contents. 16S rDNA identities of the fecal repository was compared to lumen contents from *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ small intestine (SI) or colon lumen contents. Numbers indicate how many OTUs are unique or in the intersection of each source.



	# Wells	Freq
Total Wells	864	1.000
IgA+	146	0.169
Reactive	67	0.078

Figure 2.6: Bacteria reactive IgA MBCs represent a fraction of total sorted B cells

(A) Analysis of clonal expansion efficiency. 864 IgA MBCs from *Pigr*$^{+/-}$ ileal PPs were single cell sorted and expanded via the coculture system as per (Fig. 2.5.A). The number of wells with IgA in a given concentration range determined by ELISA is shown in green bars. The standard curve for IgA ELISA is shown as a blue line. Expanded wells were tested for bacterial reactivity by flow cytometry and summarized in the table. Data are pooled from 3 independent experiments. (B) IgA concentration and bacteria binding. IgA concentration from single cell IgA MBC supernatants after *in vitro* expansion was quantified by ELISA and compared with the frequency of bacteria binding. (C) IgA concentration versus IgA MBC source. IgA concentration from wells containing 25 IgA MBCs from either *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ ileal PPs were determined by ELISA. Statistical significance determined by Student's t test.

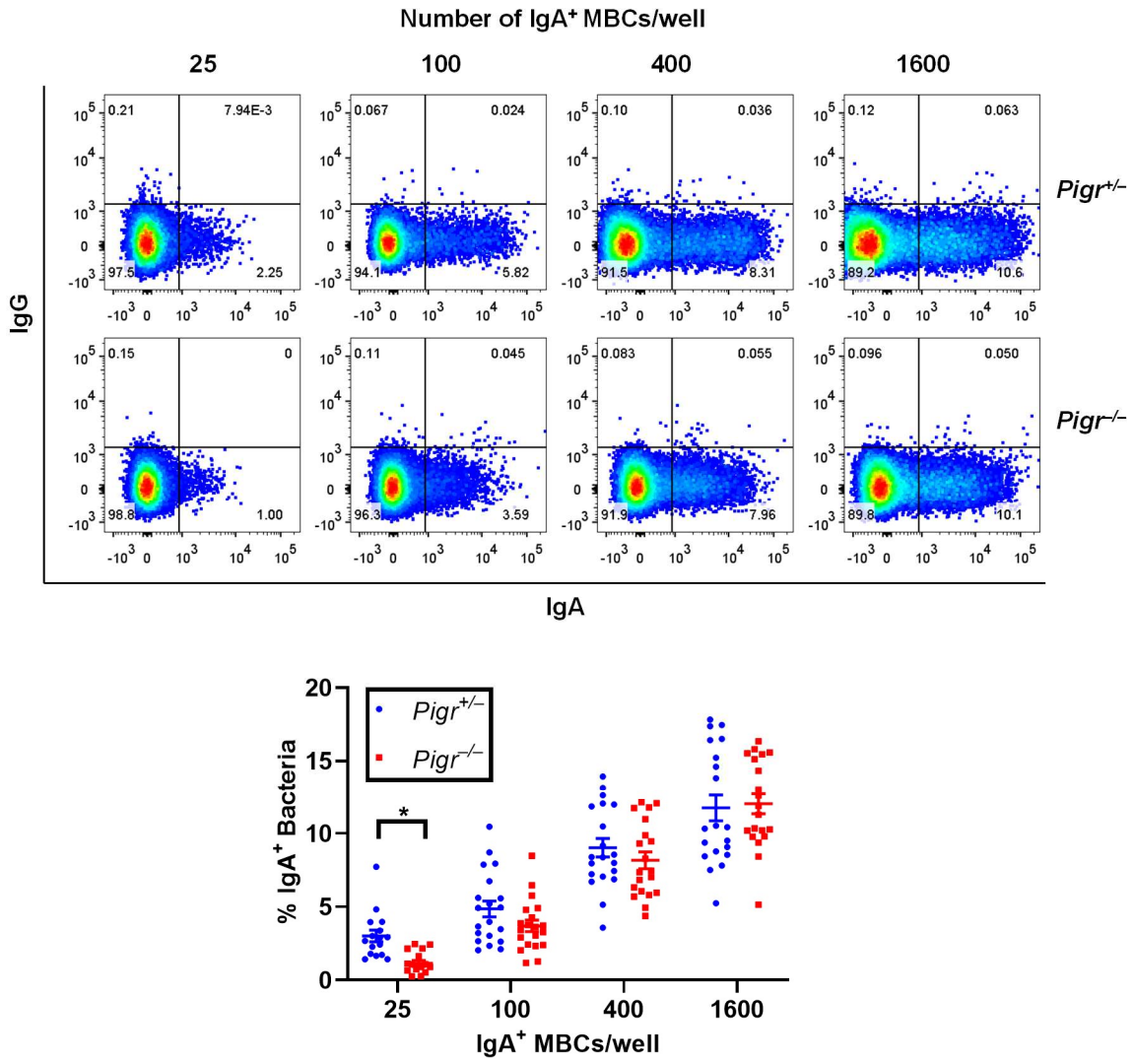


Figure 2.7: Optimizing IgA MBC sort numbers for sampling resolution vs depth

IgA bacterial binding and cell numbers used for *in vitro* culture. IgA MBCs from *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ ileal PPs were titrated from 25-1600 cells sorted per well and tested for bacteria-reactive IgA as per (Fig. 2.5.A). Statistical significance was determined by Student's t test with Storey fdr correction (n = 20 wells per group).

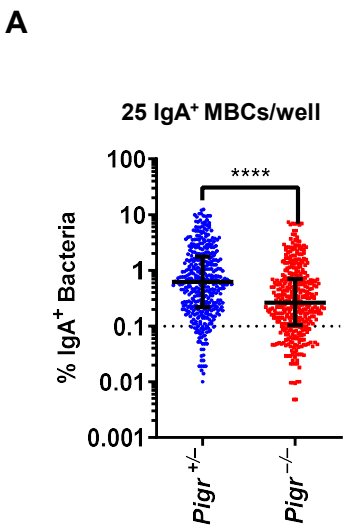
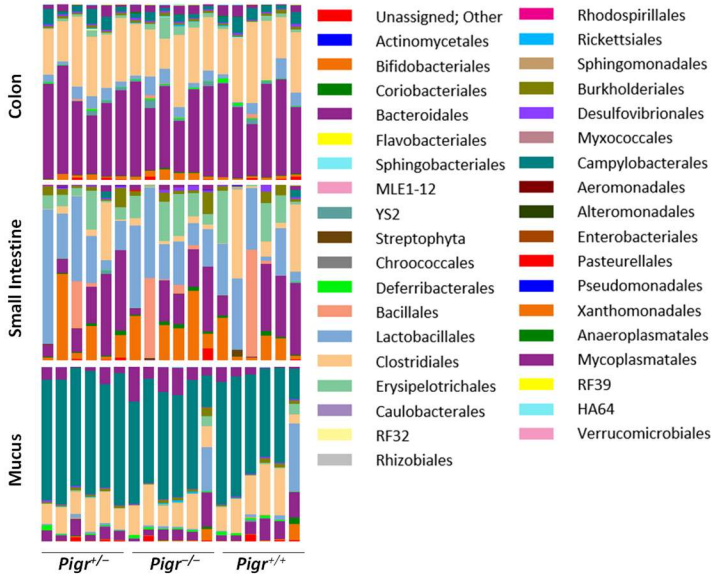


Figure 2.8: Decreased commensal reactivity of IgA MBCs from *Pigr*$^{-/-}$ mice

(A) Role for sIgA for bacterial reactive IgA. IgA MBCs from *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ ileal PPs are sorted (25/well) and assessed for bacterial reactivity as per (Fig. 2.5.A). Percent IgA bound bacteria from each well is shown. Significance determined by Student's t test of logged values. Data are pooled from 7 mice/group, 48 wells/mouse.

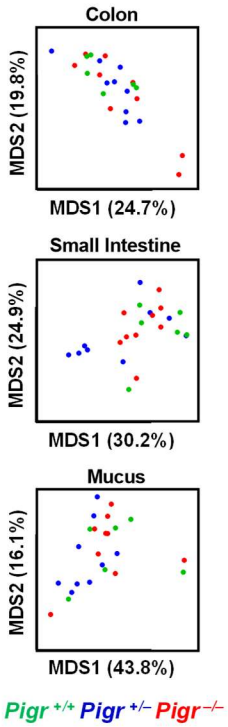
A



PERMANOVA p

	Order	Family	Genus	OTU
Colon	0.788	0.857	0.921	0.807
SI	0.797	0.899	0.909	0.979
Mucus	0.333	0.341	0.407	0.430

B



C

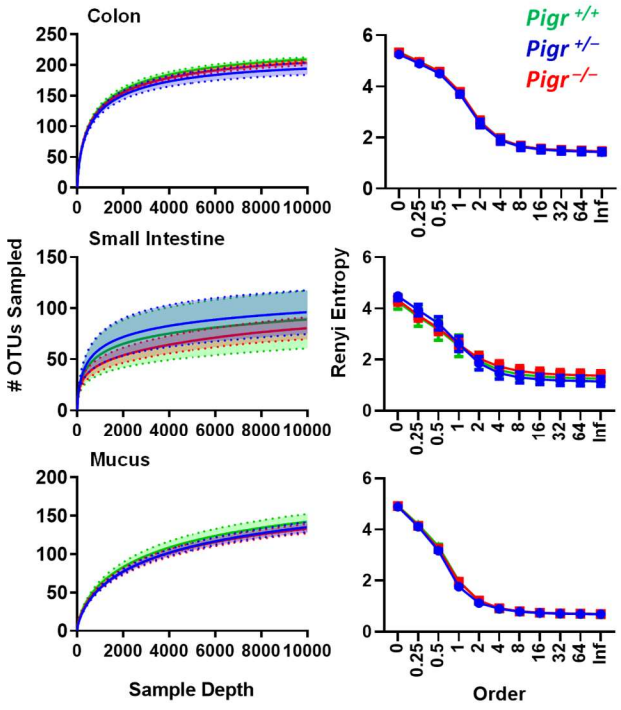


Figure 2.9: sIgA deficiency does not dramatically alter the microbiota

Gut commensal bacteria comparison. (A) Bacterial compositions of *Pigr*$^{+/-}$, *Pigr*$^{-/-}$, or *Pigr*$^{+/+}$ from colon or SI lumen, or colonic mucosal scrapings. Statistical comparison of compositions for various phylogenetic levels done between genotypes for all 3 gut tissues using PERMANOVA. (B) PCoA plots of all 3 genotypes and tissue combinations based on weighted Bray-Curtis dissimiliarty distances. (C) Alpha diversity of bacterial compositions across genotypes and tissues based on species sampling and Renyi entropy.

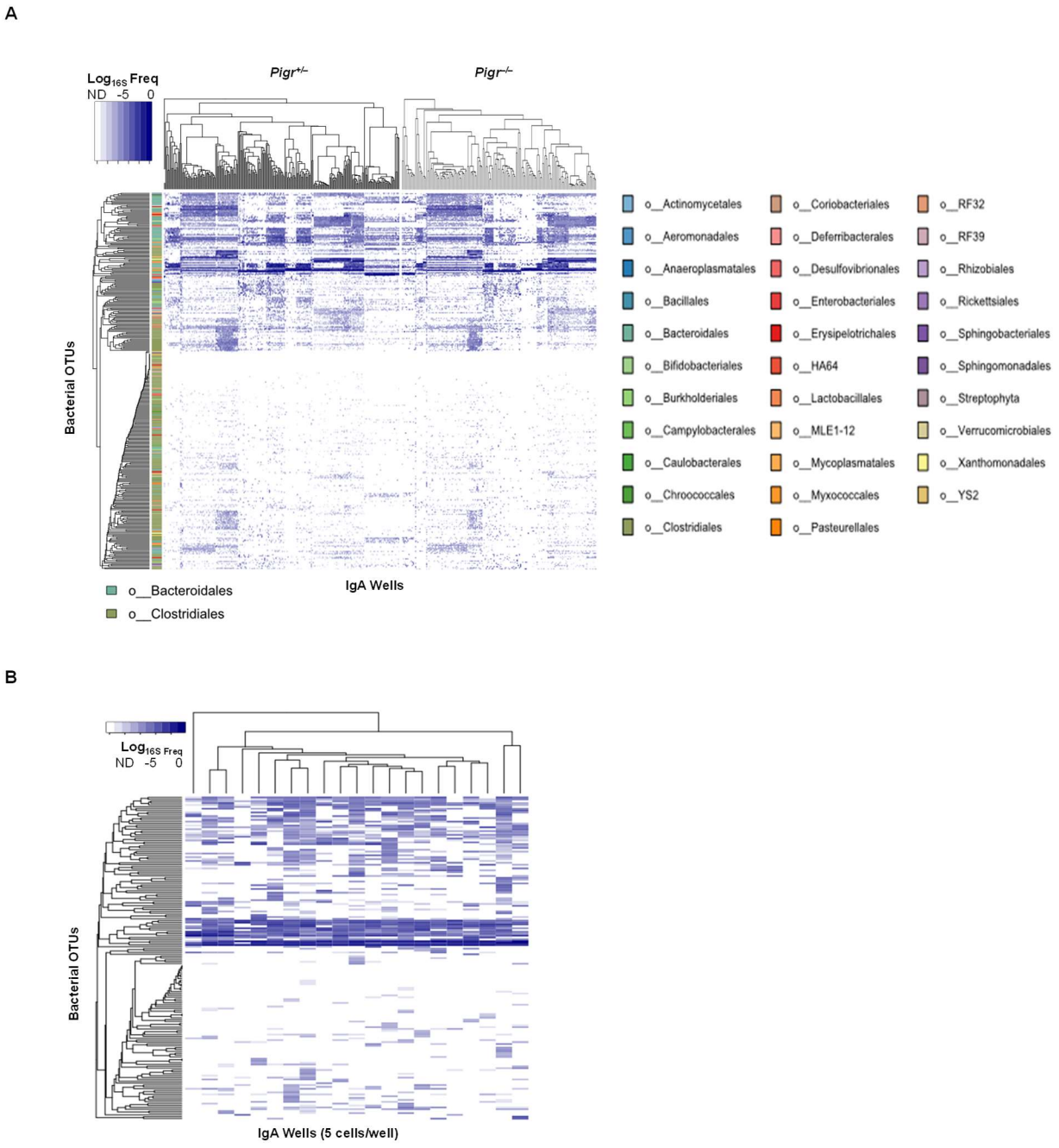


Figure 2.10: 16S of IgA bound bacteria show varying amounts of background binding

(A) OTUs frequencies of IgA-bound bacteria sorted from MBC cultures as per Fig. 2.5.A. Data shown are OTUs in rows and individual wells (25 MBC/well) in columns. Left

color key indicates which phylogenetic order the OTU belongs to. Clustering uses Bray-Curtis dissimilarity distances. (B) Analysis of 16S frequencies of IgA enriched OTUs from wells using lower numbers of IgA MBCs. 5 IgA MBCs are sorted per well and used for sorting bacteria as per (A).

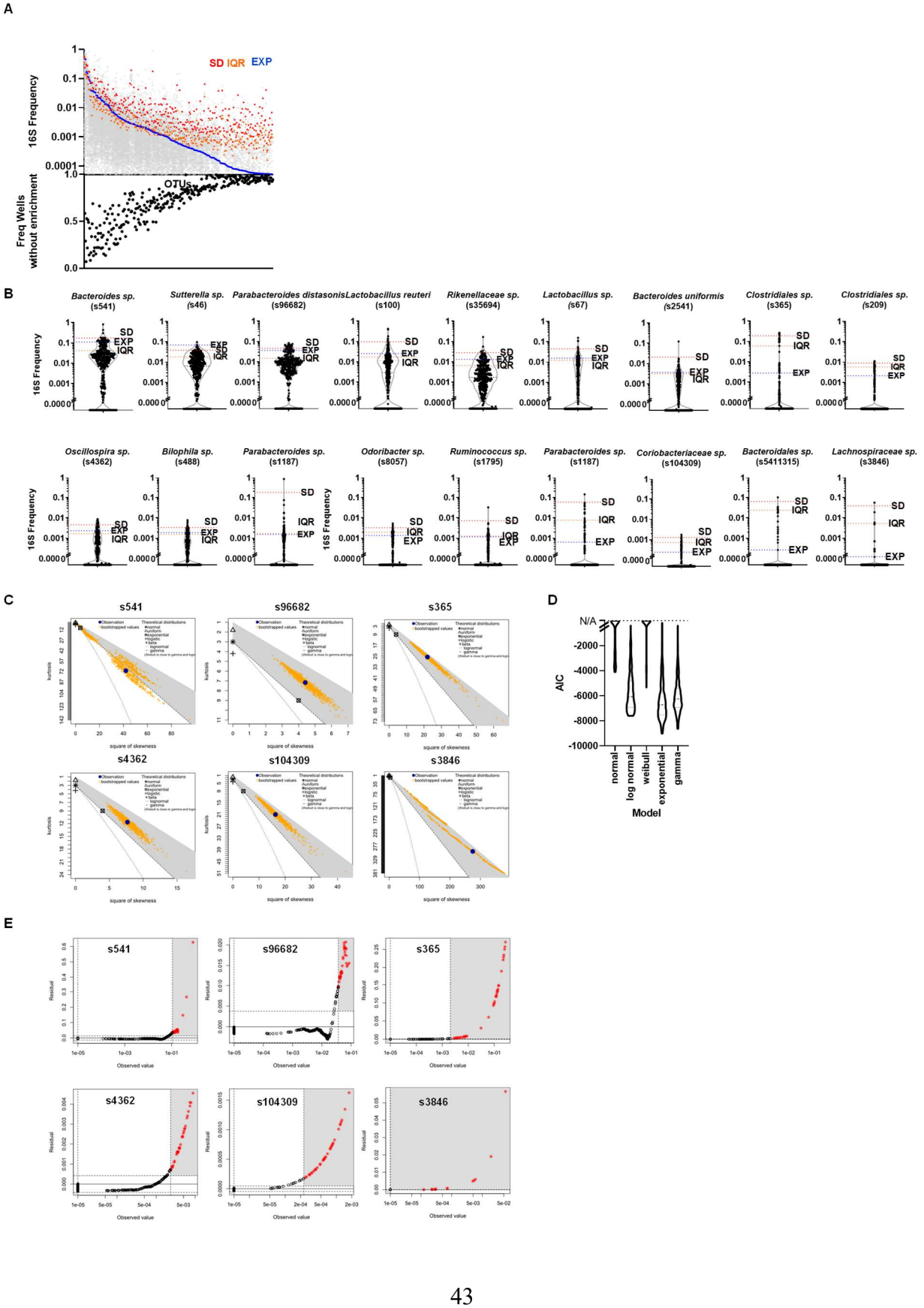

A
16S Frequency
SD IQR EXP
OTUs
Freq Wells without enrichment
B
Bacteroides sp. (s541)
Sutterella sp. (s46)
Parabacteroides distasonis (s96682)
Lactobacillus reuteri (s100)
Rikenellaceae sp. (s35694)
Lactobacillus sp. (s67)
Bacteroides uniformis (s2541)
Clostridiales sp. (s365)
Clostridiales sp. (s209)
Oscillospira sp. (s4362)
Bilophila sp. (s488)
Parabacteroides sp. (s1187)
Odoribacter sp. (s8057)
Ruminococcus sp. (s1795)
Parabacteroides sp. (s1187)
Coriobacteriaceae sp. (s104309)
Bacteroidales sp. (s5411315)
Lachnospiraceae sp. (s3846)
C
s541
s96682
s365
s4362
s104309
s3846
kurtosis
square of skewness
D
AIC
Model
normal
log normal
weibull
exponential
gamma
E
Residual
Observed value

Figure 2.11: Distinguishing high reactive IgA via outlier detection

(A) Comparison of three different outlier detection methods. 16S frequencies of each bacterial OTU across all wells is plotted against the predicted cutoffs for outlier detection based on interquartile range (IQR), 2.5 standard deviations (SD), or outliers based on an exponential distribution (EXP). Bottom panel displays the frequency of wells where the OTU is not detected in the IgA enriched fraction. (B) Individual OTU (every 15^{th}) 16S frequencies from all wells containing 25 IgA MBCs is shown. IQR, SD, EXP cutoffs are plotted. S numbers are an internal designation. (C) Distribution of 16S frequencies for select OTUs are shown by Cullen Fray graphs. Predicted distribution is shown in blue, and bootstrapped values are shown in orange. OTU taxonomic information as per (B). (D) Akaike information criterion of various modelled 16S frequency distributions. The AIC value for the distribution of 16S frequencies are calculated for each OTU. Lower values indicate better fit. (E) Outlier detection of "high reactivity" IgA based on a predicted exponential distribution. Plot shows observed 16S frequencies versus the residuals based on a predicted exponential distribution. Right upper quadrant highlights the outliers in red based on a 0.05 two tailed alpha. OTU taxonomic information as per (B).

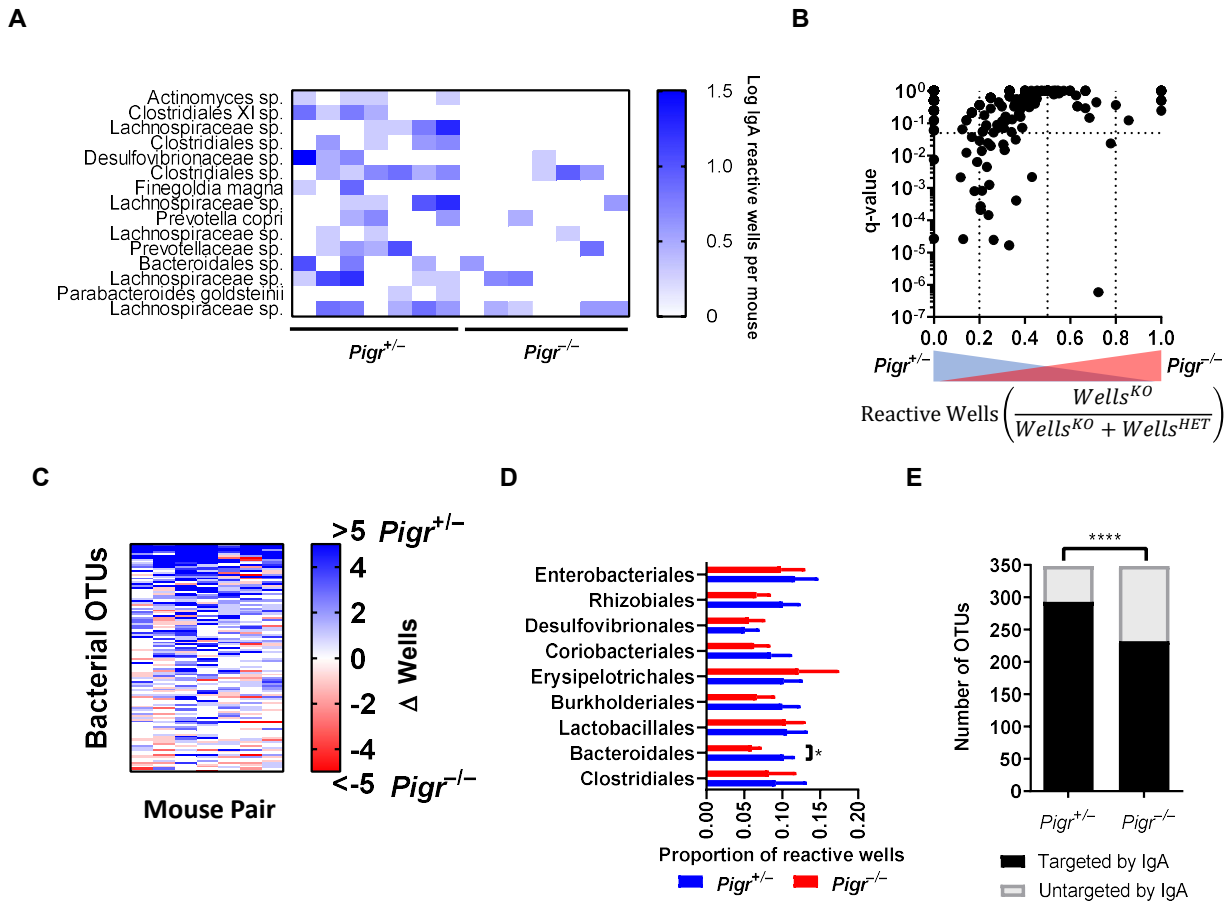


Figure 2.12: MBCs from *Pigr*$^{-/-}$ show decreased IgA reactivity to specific taxa.

(A) Analysis of IgA MBC reactivity at the OTU level. OTUs that elicited significantly different IGA MBC responses between *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice as assayed by the 25 cell/well in vitro assay per Fig. 2A is shown. Each column represents the log total number of wells with "high reactivity" for each OTU per mouse. (B) Effect of sIgA on the number of high reactivity anti-bacterial IgA MBCs. The number of sorted wells with and without high reactivity IgA for a given OTU were compared between *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ samples in a 2x2 contingency table using a Fisher's exact test corrected by Storey fdr (fdr level = 0.1, y-axis). A high reactivity IgA well

was determined by outlier detection (Fig. S3C-H). The *Pigr*$^{-/-}$ fraction of all wells with high reactivity IgA for that OTU between genotypes is shown on the x-axis (dotted lines denote 4 fold differences). (C) Normalization of high reactivity IgA MBCs across litters. Differences between the number of high reactivity wells for each OTU were calculated between each *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ littermate pair. OTUs with more wells in the *Pigr*$^{+/-}$ samples are blue; more in *Pigr*$^{-/-}$ samples are red. (D) The role of sIgA on IgA MBC reactivity towards different taxa. The fraction of high reactivity IgA wells for a given OTU clustered by phylogenic order per mouse was compared between *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ genotypes. Statistical significance was determined by Student's t test with Storey fdr method. (E) Number of OTUs recognized by high reactivity anti-bacterial IgA MBCs from *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ mice. The total number of OTUs are from 16S rDNA sequencing of the lumen of the colon and SI of *Rag1*$^{-/-}$ mice. An OTU is considered IgA targeted if found in at least 1 well as an outlier. Statistical significance determined by Fisher's exact test.

Chapter 3: sIgA enhances the IgG responses towards commensals

3.1 Abstract

While IgA is the dominant antibody response in the mucosa, the IgG response also make up a significant portion of total intestinal B cells. In the previous chapter, we showed that sIgA increases the immune priming of IgA MBCs towards commensals. Here, we utilize the established in vitro B cell culture and screening system to probe the gut IgG response. We find that similar to the IgA response, gut IgG specifies towards commensals are degraded in the absence of sIgA. As a result of this loss of IgG specificity, we observe increased morbidity and mortality when sIgA deficient *Pigr*$^{-/-}$ mice are challenged with a sepsis modelling gut barrier breach. Finally, we probe the bacterial specificities of the IgA vs IgG response and find the IgA response to be more cross reactive towards bacterial antigens than the IgG response.

3.2 Introduction

The protective role of IgG B cells in systemic immunity is well established (Bruhns, 2012; Vidarsson et al., 2014a). However, despite comprising a large portion of the gut B cell compartment (Zeng et al., 2016), the role of IgG during homeostasis is unclear. Functionally, IgG has a different role than its counterpart, IgA. Rather than having the live and let live functions associated with IgA (chapter 1), IgG mediate the direct killing of microbes, providing sterilizing immunity. Here, we will briefly review the mechanistic properties of IgG distinct from IgA.

One of the functions of IgG is the ability to bind complement. IgG on the surface of a microbe can recruit C1q, which is able to initiate the classical complement pathway. The activation of complement has two main effects: release of anaphylatoxins (C3a, C5a), and the assembly of the membrane attack complex (C5-C9). The anaphylatoxins act as immune stimulators, causing the release of histamine from mast cells, and inducing oxidative bursts from neutrophils. C3a and C5a also have the ability to stimulate B and T cell expansion and activation through the C3a and C5a receptors (Sarma and Ward, 2011). The membrane attack complex is able to form cytotoxic pores on the surface of microbes, and the ability of IgG to induce hemolysis in bacteria in vitro has been demonstrated (Neuberger and Rajewsky, 1981). It should be noted that not all isotypes of IgG are equivalent in binding C1q: IgG1 and 3 are the primary activators in humans (Vidarsson et al., 2014a), while IgG2a, 2b and IgG3 are the primary activators in mice (Neuberger and Rajewsky, 1981).

A second function of IgG is associated with its activation of various Fcγ receptors expressed on the surface of various immune cells. Fcγ receptor biology is complicated by the

number of receptors expressed (7 identified in humans, 5 in mice), and the number of IgG isotypes expressed (4 in humans, 5 in mice). Like interactions with C1q, not all IgG isotypes will bind to all Fcγ receptors with equal ability; furthermore, Fcγ receptors represent a family of receptors with both activating, inhibitory, cell surface expressed and secreted variants (Bruhns, 2012).

Activating Fcγ receptors (FcγRI, IIIA, and IV in the mouse) are expressed by a range of leukocytes including DCs, monocyte and macrophages, neutrophils, basophils, mast cells, eosinophils, and natural killer (NK) cells (Bruhns, 2012). These Fcγ receptors associate with the FcRγ subunit which contain immunoreceptor tyrosine-based activation motifs (ITAM), while IIA and IIC contains their own ITAM domains. Upon binding to IgG, the clustering of Fcγ receptors results in the phosphorylation of the ITAM motifs by Src family kinases, resulting in the recruitment and activation of the tyrosine kinases Syk and ZAP70 (Billadeau and Leibson, 2008). Activated Syk can then interact with various adaptors depending on cell type to induce calcium signaling and PKC signaling which turns on genes for effector function (Getahun and Cambier, 2015). Depending on the cell expressing the activating Fcγ receptor, the result can be degranulation, phagocytosis, cytokine or iNOS production and antibody-dependent cellular cytotoxicity (ADCC). Therefore, activating Fcγ receptors and complement activation represent effector functions aimed at the destruction of the IgG targeted microbe.

As previously mentioned, the Fcγ receptor family also contains an inhibitory receptor, FcγRIIb. FcγRIIb is primarily expressed by B cells, and signals through the use of an immunoreceptor tyrosine-based inhibitory motif (ITIM). Upon clustering of FcγRIIb, the phosphatase SHP-1 is recruited and mediates downstream effects (D'Ambrosio et al., 1995). FcγRIIb is thought to interact with the BCR complex by dephosphorylating CD19 and blocking

Pi3K signaling (Getahun and Cambier, 2015); the result of this interaction is the inhibition of B cell activation or apoptosis. It is thought that FcγRIIb may play a role in affinity maturation, when follicular DCs are coated with IgG bound antigens. Therefore, only the B cells expressing the highest affinity BCRs could compete against this negative signal (Fournier et al., 2008).

Finally, a third class of IgG receptor is the neonatal Fc receptor (FcRN). Originally described as a receptor responsible for transporting IgG into the mammary glands (Brambell, 2004), FcRN has also been shown to prolong IgG serum half-life by aiding in its recycling from lysosomal degradation (Ghetie et al., 1996). Interestingly, FcRN has also been suggested as a vehicle for transporting IgG across mucus barriers such as the lung and gut, thereby acting as an IgG homologue to pIgR (Spiekermann et al., 2002; Yoshida et al., 2004, 2006). However in our experiments as well as others (Castro-Dopico et al., 2019; Zeng et al., 2016), we do not traditionally see IgG in the gut lumen of healthy mice. A possible explanation for this discrepancy could be the use of the human-FcRN genetic knock-in model used by Yoshida et al. Normally, FcRN is expressed on the gut epithelium in neonatal mice (Bruhns, 2012; Roopenian and Akilesh, 2007), but downregulated in adults. It is possible that the expression of hu-FcRN in the mouse model does not accurately reflect physiological levels of FcRN in the gut and provided a pathway for IgG entry into the lumen that does not normally occur in healthy mice.

Without a bona fide method of transport into the gut lumen in healthy subjects, the study of IgG and its impact on the commensal population has proven difficult. Instead, IgG has primarily been studied during infection or inflammation. IgG has been shown to be important for clearing luminal infections of attaching and effacing bacteria such as *Citrobacter rodentium* (Maaser et al., 2004). There is also evidence that IgG may exacerbate inflammation by binding commensal bacteria in patients with inflammatory bowel disease (Castro-Dopico et al., 2019).

Whether anti-commensal IgGs are routinely generated at homeostasis, and whether these IgGs are regulated by the presence of sIgA, remains unclear.

To address this issue, we adapted our in vitro screening system for testing IgA bacterial specificities in *Pigr*$^{-/-}$ to test IgG bacterial specificities. This allowed us to bypass the lumen secretion issue that typical gut IgG studies face, as well as compare the anti-commensal IgG response in the presence or absence of sIgA. Here, we find that similar to the IgA response, gut IgG specifies towards commensals are less robust in the absence of sIgA. By cataloguing the gut IgG response, we were able to compare it with the gut IgA response and find that IgG is less cross reactive towards gut bacteria than IgA. Finally, we asked if the decrease in IgG responses due to sIgA deficiency results in weaker protection against gut bacterial infection. We reasoned that IgG typically mediates clearance of invading pathogens, and a decreased IgG response would result in susceptibility towards gut bacteria sepsis. Indeed, we find without sIgA, the gut IgG is less able to clear invading bacteria resulting in increased morbidity and mortality in our gut sepsis model.

3.3 Materials and Methods

Ethics Statement

Animal experiments were performed in specific pathogen free facilities in accordance with the guidelines of the Institutional Animal Care and Use Committee at Washington University.

Mice

Pigr$^{-/-}$ mice (Johansen et al., 1999) were obtained from H.W. Virgin, and bred *Pigr*$^{+/-}$ males to *Pigr*$^{-/-}$ females. *Ighm*$^{-/-}$ (μMT) mice were obtained from M. Diamond (Jax #002288). All mice were on the C57BL/6 genetic background.

B cell analysis and culture

See chapter 2.3 *B cell analysis and culture.*

Bacteria FACS and sorting

See chapter 2.3 *Bacteria FACS and sorting.*

Bacteria 16S rDNA sequencing

See chapter 2.3 *Bacteria 16S rDNA sequencing.*

Peritoneal bacterial infection

Luminal contents of small intestines and colon from *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ mice were resuspended in 40% glycerol/PBS and aliquots frozen at -80°C. Frozen stocks were thawed and grown overnight in 5mL of Brain Heart Infusion (BHI) broth (Sigma-Aldrich 53286) at 37°C with shaking. The next day, cultures were diluted 1:100 in 5mL fresh BHI broth and expanded until log-phase growth (OD_{600} of 0.7 – 0.8), spun down, and resuspended in 500µL PBS. 100µL was injected I.P. into *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice. 24 hours after injection, spleens were collected from mice and homogenized in 1 mL PBS using a Roche MagNA lyser (6000 RPM, 1 min). Homogenized spleen was diluted 1:10^2 and 1:10^4 in PBS and plated as 10 x 20µL drops on BHI agar plates, overnight at 37°C. Colonies were then counted for live bacteria before plates were scraped using a bacteria pick into 500 µL sterile PBS. 5 µL of picked bacteria were used for 16S rDNA amplification and sequencing.

Pasteurella pneumotropica infection

Pasteurella pneumotropica was isolated from a single colony on BHI agar plates plated with homogenized spleens from animals infected i.p. with aerobic gut bacterial cultures. Taxonomy was determined by V1-V5 16S Sanger sequencing using universal 10µM 8F/27F (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AGAGTTTGATCMTGGCTCAG-3') and 1391R

(5'-GACGGGCGGTGTGTRCA-3') primers and matched with *P. pneumotropica* 16S rDNA V4 MiSeq sequencing. Stocks of this *P. pneumotropica* isolate were made in 40% glycerol and kept at -80°C. 7-8 week old mice were injected i.p. with ~$2x10^{11}$ CFUs of bacteria in log phase growth in 100 µl PBS. Mice were monitored every 8 hours for disease progression up to 72 hours. A blinded scorer assessed the severity of infection based on a sepsis disease scale from 0-6 (0, healthy; 6, dead) (Powers et al., 2015). Spleens were collected from mice requiring euthanasia (absent righting reflex=score of 4 or 5) or at 72h and homogenized for bacterial CFU counts.

Blood was obtained from 7-8 week old $Pigr^{+/-}$ or $Pigr^{-/-}$ mice by aortic puncture in 100µL heparin and spun down at 3600xG for 5 minutes to collect plasma Ig. Unpurified plasma IgG concentrations were quantified by IgK capture ELISA before being used to stain bacterial isolates (Fig. 5D). Serum IgG was then pooled based on $Pigr^{+/-}$ or $Pigr^{-/-}$ genotype and purified using Pierce Protein A IgG purification kits (ThermoFisher) per manufacturer instructions. 10µg of purified IgG was injected retro-orbitally into $Pigr^{-/-}$mice 2 hours prior to infection with *P. pneumotropica*.

Statistical Analysis

See chapter 2.3 *Statistical analysis.*

Comparisons of linear regressions done by ANCOVA in Prism. Gradient boosted modeling was done using the gbm package in R (v2.15) with 5 fold cross-validation of a bernoulli distribution model generated from a random 75% of wells. This model was tested by

the remaining data. 40-160 iterations were performed for each parameter set. Optimization of n.minobsinnode, bag.fraction, and interaction.depth for area under the curve (AUC) was performed prior to final analysis shown in Fig. 5B. Network analyses was done using Gephi (v0.9.2) using the ForcedAtlas2 model. Comparison of survival curves uses the Mantel-Cox log ranked test on the Kaplan-Meier curves in Prism. Statistical significance key: * : $p < 0.05$, ** : $p<0.001$, *** : $p<0.0001$, **** : $p<0.00001$.

3.4 Results

sIgA enhances the priming of anti-bacterial IgG

Although IgG is not thought of as the traditional B cell response to gut bacteria during homeostasis, IgG B cells represent a substantial fraction of the gut B cell population. We therefore asked whether IgG reactivity to gut bacteria might also be affected by the presence of sIgA using the same *in vitro* coculture system. Like IgA MBCs, IgG B cell frequency was not affected in $Pigr^{-/-}$ mice (**Fig. 3.1.A**), but IgG supernatants from $Pigr^{-/-}$ PP MBCs (25/well) bound to a lower frequency of bacteria compared with those from $Pigr^{+/-}$ MBCs (**Fig. 3.1.B**). 16S rDNA sequencing of IgG bound bacteria revealed that $Pigr^{+/-}$ mice generated more wells with "high reactivity" anti-bacterial IgG than their $Pigr^{-/-}$ littermates (**Fig. 3.2.A, 3.2.B**). At the taxonomic order level, we again see a difference in the number of *Bacteroidales* specific IgG MBC wells between $Pigr^{+/-}$ and $Pigr^{-/-}$ mice (**Fig. 3.2.C**). We also noted that $Pigr^{-/-}$ MBCs had a lower IgG coverage of the commensal population compared to $Pigr^{+/-}$ littermates (**Fig. 3.2.D**). Therefore, similar to priming of anti-bacterial IgA, sIgA increases the priming and activation of IgG MBCs towards gut commensals.

Commensal specific IgGs exhibit less cross-reactivity than IgA

We also asked if there was a correlation between the frequency of high reactivity IgA vs IgG wells for any given OTU. When we plotted the average number of high reactivity IgA vs IgG wells per mouse directed against a particular OTU, we found most OTUs exhibited a strong correlation between IgA and IgG reactivity, with the majority of OTUs falling within a 90%

prediction interval of the linear regression (**Fig. 3.3.A**). However, we noticed a significantly higher number of high reactivity IgG vs. IgA wells in *Pigr*$^{+/-}$ mice than in *Pigr*$^{-/-}$ mice (**Fig. 3.3.A**, blue vs red line, respectively). This further supports the hypothesis that IgA bound bacteria experience enhanced sampling by the immune system and thus provides positive feedback for the induction of IgG responses.

While we observed a number of similarities with IgA, we did note that fewer OTUs were found in each sorted IgG^{+} well compared to the IgA^{+} wells (**Fig. 3.3.B**, **2.10.A**) using very similar fecal repositories (**Fig. 3.3.C**). Consistent with the heatmap impression, IgA wells reacted towards larger segments of the phylogenetic tree than IgG wells (**Fig. 3.4.A**). To independently confirm this idea, we utilized network analysis to visualize the relationships of OTUs that are bound by our IgA or IgG wells. Under this method, we treat each bacterial OTU as a node. Edge weights are determined by calculating the Pearson's Correlation, filtering on edges with $p < 0.05$; thus, a high correlation between two OTUs indicates they are commonly enriched by a well of antibodies. Based on graph theory, nodes will "repel" one another unless they are tethered by an edge; the stronger the edge- the more often two OTUs are enriched by the same wells, the closer in proximity nodes will maintain. We can see in our IgA samples, the OTUs form a large central cluster (**Fig 3.4.B**), meaning these OTUs are often enriched by the same IgAs. This supports the idea there are cross reactive IgAs. Conversely, we see much less OTU clustering from the IgG samples, suggesting IgG is less cross reactive. This is further supported by the average degree measurement of the networks, measuring the average number of edges from each node. IgA OTUs have a much higher degree (51.26) compared to IgG (17.36) (**Fig 3.4.C**). Together, these data support the idea there are more cross reactive IgAs than IgGs against commensal bacteria.

Increased susceptibility to bacterial infection in Pigr$^{-/-}$ mice due to decreased pre-formed IgG

In contrast to IgA, IgG has a well described pathogen neutralization and clearance function. This led us to hypothesize that pre-existing anti-bacterial IgG may act as a safeguard against bacterial entry and sepsis in case of barrier breach. To model this, we asked whether gut bacteria would be more efficiently cleared after i.p. injection into *Pigr*$^{+/-}$ vs $^{-/-}$ hosts. At 24 hours post injection of aerobic luminal bacteria expanded *in vitro*, we sacrificed the mice and assessed the bacterial burden in the spleen. Consistent with our hypothesis, there were significantly higher bacterial CFUs in *Pigr*$^{-/-}$ mice compared to *Pigr*$^{+/-}$ littermates (**Fig. 3.5.A**), suggesting that sIgA sufficient mice are better able to control peritoneal infection by aerobic commensal bacteria.

16S rDNA sequencing of the bacterial colonies from the splenic lysate revealed that these bacteria were mostly from the *Enterococcus* and *Streptococcus* genus (**Fig. 3.5.B**). One particular OTU, *Pasteurella pneumotropica*, was preferentially found in *Pigr*$^{-/-}$ but not *Pigr*$^{+/-}$ splenic cultures (**Fig. 3.5.C**). *P. pneumotropica* was rarely identified as a bacterial OTUs recognized by IgA or IgG MBCs in our *in vitro* assay, likely as it was not found or found at very low frequency in our repository and therefore difficult to detect (**Fig. 3.5.D**). To further study the immune response to *P. pneumotropica*, we isolated a pure culture from our colony with the identical V4 16S rDNA sequence (**Fig. 3.5.E**). I.p. injection of only *P. pneumotropica* resulted in increased morbidity and mortality in *Pigr*$^{-/-}$ mice (**Fig. 3.6.A**). Splenic CFU counts were high from mice that were euthanized due to morbidity (**Fig. 3.6.B**), and absent or low in mice alive at the conclusion of the study (72h), consistent with the hypothesis that disease is caused by dissemination of *P. pneumotropica*. Thus, these data show that *Pigr*$^{-/-}$ mice are more susceptible to peritoneal infection by commensal aerobes such as *P. pneumotropica*.

To determine whether B cells were important for protection against *P. pneumotropica*, we i.p. infected B cell deficient μMT (*Ighm*$^{-/-}$) mice. Compared to control *Pigr*$^{+/-}$ mice, μMT mice were highly susceptible to infection, often expiring within 6 hours after injection (**Fig. 3.7.A**). This suggested that B cells were essential for protection in this model. To establish that this protective effect was due to IgG, we purified plasma IgG from either *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice. We found that *Pigr*$^{+/-}$ mice had substantially higher titers of anti-*P. pneumotropica* IgG than *Pigr*$^{-/-}$ mice as assessed using flow cytometry to cultured *P. pneumotropica* (**Fig. 3.7.B**). This appeared to be bacteria specific, as increased binding of *Pigr*$^{+/-}$ IgG was not seen to *E. casseliflavus*, which showed no significant outgrowth in our early screens, or *C. rodentium,* a pathogen not found in our mouse colony (**Fig. 3.7.B**). Finally, we asked if administration of purified IgG could protect *Pigr*$^{-/-}$ mice from *P. pneumotropic* peritoneal infection. Consistent with the *in vitro* IgG binding data, IgG harvested from *Pigr*$^{+/-}$ mice injected i.v. was more protective against *P. pneumotropica* infection that that from *Pigr*$^{-/-}$ mice (**Fig. 3.7.C**). Taken together, we conclude that the presence of sIgA results in the generation of higher serum titers of anti-*P. pneumotropic* IgG protective against peritoneal infection by this commensal aerobe.

3.5 Discussion

In this chapter, we show that sIgA enhances the gut IgG MBC response. Like the effect of sIgA on the gut IgA MBC compartment, the increase in IgG reactivity towards gut bacteria is OTU specific, and particularly increases reactivity towards bacteria belonging to the *Bacteroidales* order. Furthermore, comparing the gut IgA response to the IgG response against bacteria revealed IgA is significantly more cross reactive towards bacteria; being able to bind to OTUs spanning across multiple phylogenetic genuses and families. Lastly, we demonstrate the deficiency of IgG bacterial reactivity in *Pigr*$^{-/-}$ mice results in susceptibility to sepsis when challenged with gut bacteria.

An unexpected result in this study was the observation that IgG responses to gut bacteria are commonly elicited during homeostasis, and that this was in part dependent on sIgA. These data may explain why there is a large population of IgG B cells in the gut generated during homeostasis. We hypothesize that these IgG B cells provide a pre-formed IgG response in case of barrier breach. While anti-bacterial IgG may contribute to pathology with extensive mucosal injury such as that seen in ulcerative colitis (Castro-Dopico et al., 2019), our data, as well as generally accepted dogma, suggests that IgG is typically protective (Vidarsson et al., 2014b).

Consistent with this, we observed that aerobic bacteria such as *P. pneumotropica* introduced intraperitoneal were more lethal in mice without sIgA. However, *P. pneumotropica* may not be the only gut bacteria that is cleared by sIgA dependent IgG; a caveat to our study is the way we cultured the total lumen bacteria before injection (Fig 3.5.A). By culturing on BHI agar plates, aerobic conditions, and 37^{o}, we are selecting for a very particular subset of bacteria, therefore biasing our initial screen. This is probably why we did not see any outgrowth of

Clostridiales members in the spleen, despite their high representation in the gut (Edwards et al., 2013; O'Brien et al., 2071). A change of media, and anaerobic conditions may reveal a further subset of bacteria which *Pigr*$^{-/-}$ are susceptible towards.

One interesting difference between IgG and IgA was their spectrum of binding to bacteria. Both the raw data and computational modelling suggest fewer cross reactive IgGs than IgA. This is consistent with the notion that IgG and IgA memory cells do not arise from an identical pool of naïve B cells. IgA MBCs are formed from both follicular and extra follicular sources, whereas IgG MBCs come from predominantly follicular sources (LeBien and Tedder, 2008). While extra follicular and T independent mechanisms of IgA class switch have been described in isolated lymphoid follicles of the lamina propria (Macpherson et al., 2000), the data for extra follicular generation of IgG B cells is not clearly defined in the gut. Future studies are required to show that polyreactive IgA MBCs arise from a different developmental origin than high reactivity bacterial-specific IgG and IgA MBCs.

3.6 Figures

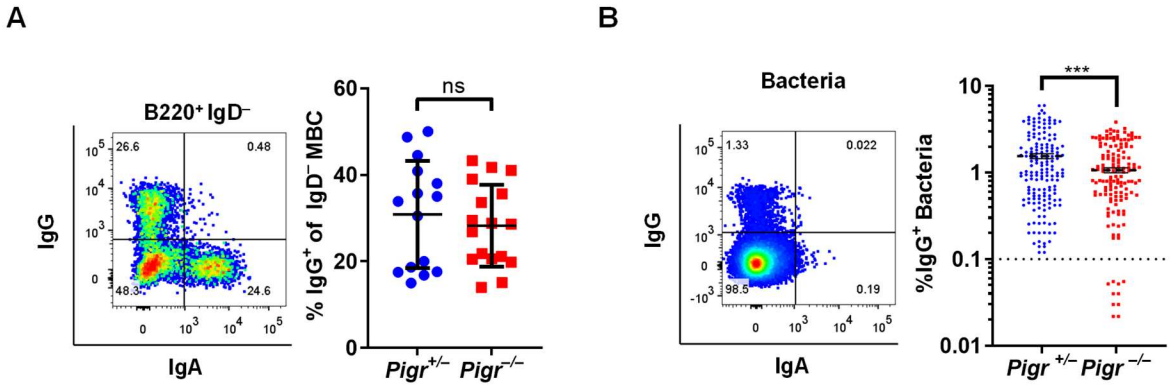


Figure 3.1: Decreased commensal reactivity of IgG MBCs from *Pigr*$^{-/-}$ mice

(A) Frequency of IgG MBCs in the ileal PP. FACS gating of IgD^{-} B220^{+} IgA^{-} IgG^{+} MBC populations on the left and quantified on the right between *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice. Statistical significance determined by Student's t test (n = 15, 17). (B) Role for sIgA in generating bacteria reactive IgG. IgG MBCs from *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ ileal PPs were sorted (25/well) and assessed for bacterial reactivity as per Fig. 2.5.A. Percent IgG bound bacteria from each well is shown. Significance determined by Student's t test of logged values (n = 6 mice/group, 24 wells/mouse).

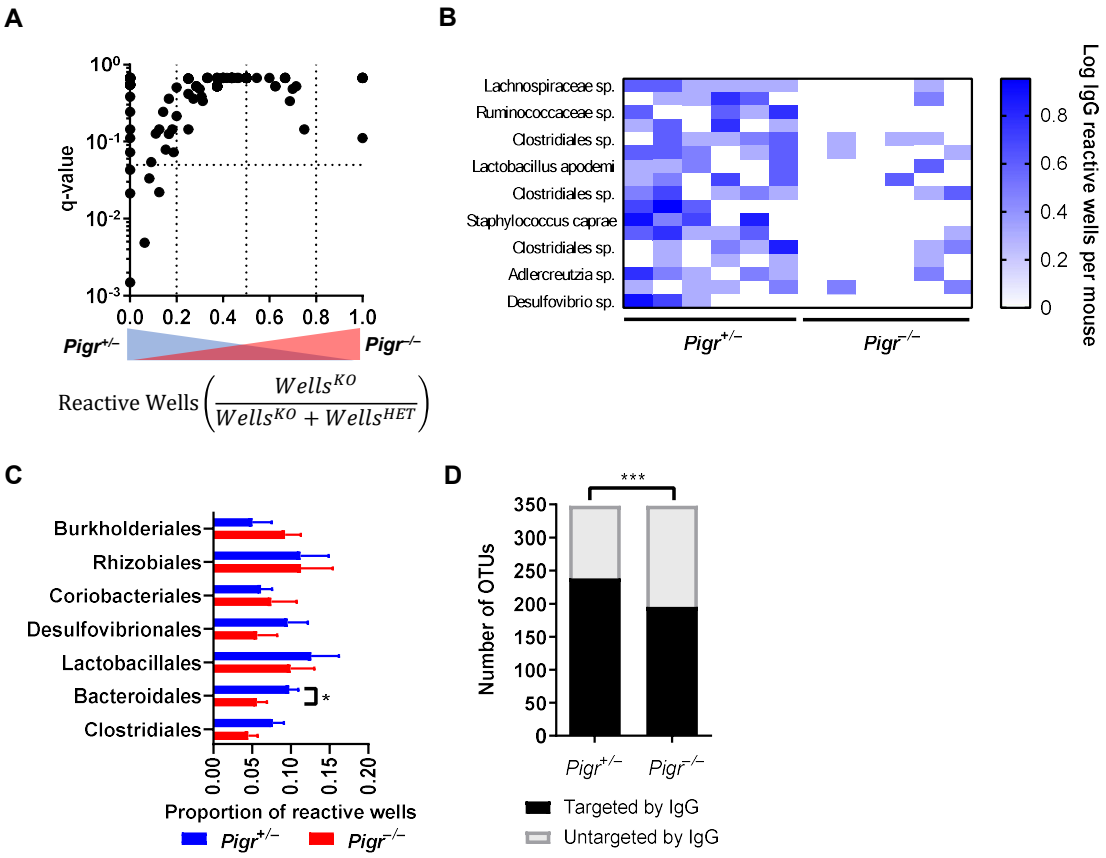


Figure 3.2: IgG MBCs from *Pigr*$^{-/-}$ show decreased reactivity to specific taxa

(A) Effect of sIgA on the number of high reactivity IgG wells. Analysis was performed as per Fig. 2.12.B for outlier detection of 16S rDNA sequencing data from sorted IgG^{+} bacteria using supernatant from *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ IgG MBCs. (B) Analysis of IgG MBC reactivity at the OTU level. OTUs with significantly different numbers of "high reactivity" IgG wells are shown here as per Fig. 2.12.A. (C) The fraction of high reactivity IgG MBC responses towards OTUs of a given order was compared between *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ samples as per Fig. 2.12.D. (D) Number of OTUs recognized by high reactivity IgG MBCs from *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ mice as per Fig 2.12.E.

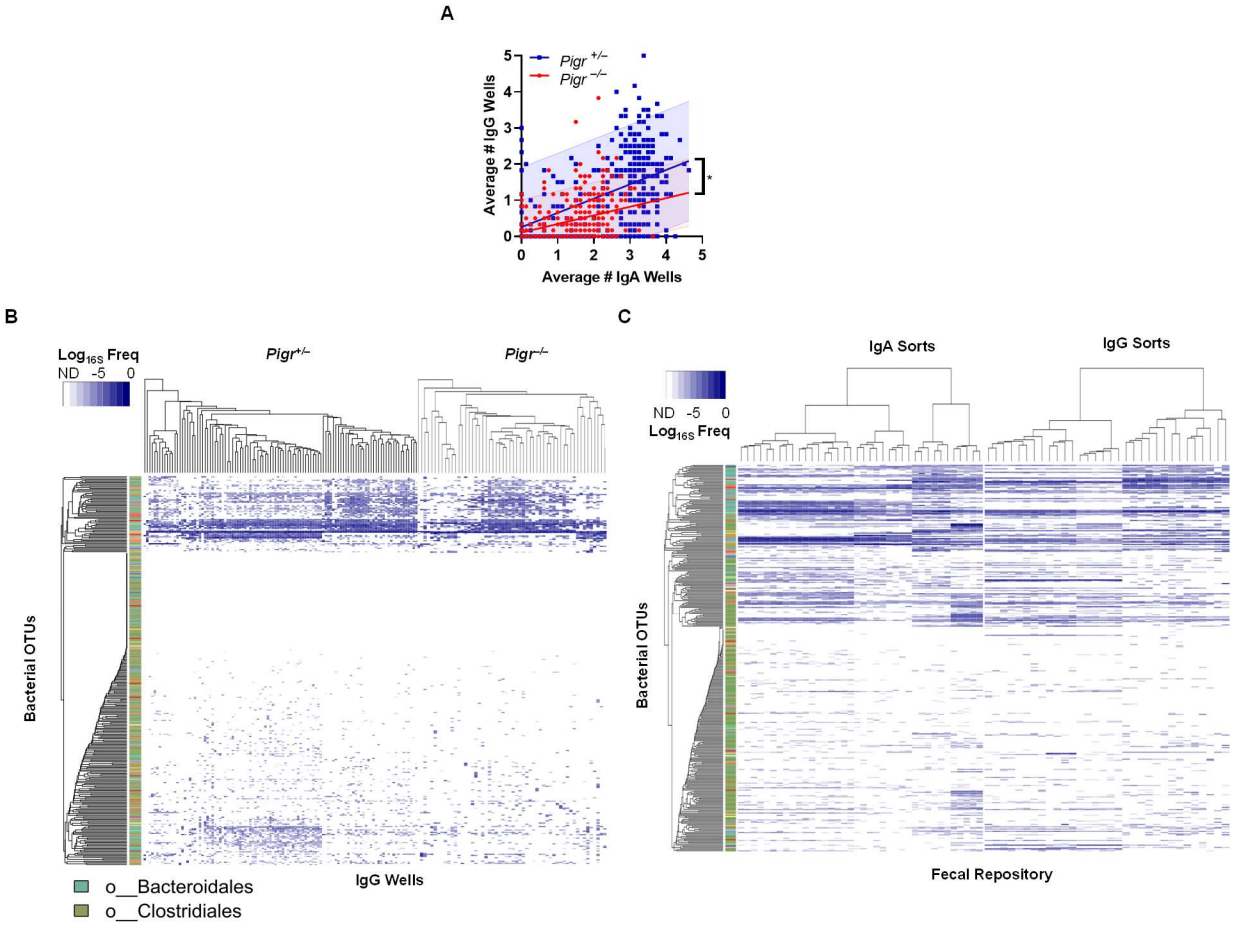
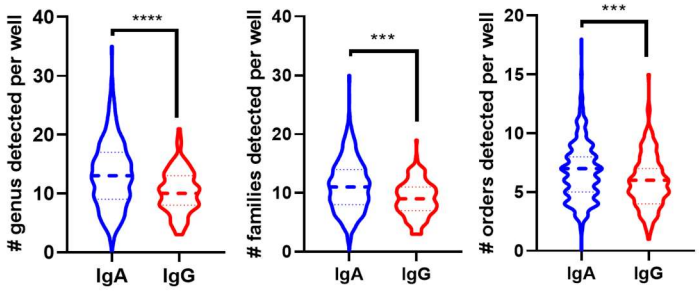


Figure 3.3: Comparison of anti-bacterial reactivity of IgA and IgG MBCs

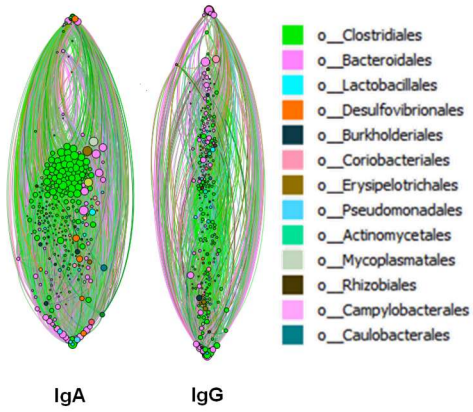
(A) Correlation of IgA MBC and IgG MBC bacterial reactivity. Data shown are the average number of IgA vs IgG wells across all samples that show high reactivity for an OTU by genotype. Linear regression between IgA and IgG shown in solid lines; difference in slopes is significant by ANCOVA. Shaded areas indicate the 90% confidence interval for the best fit line. (B) OTUs frequencies of IgG-bound bacteria sorted from MBC cultures as per Fig. 2.5.A. Rows show individual bacterial OTUs, and columns show individual wells (25 IgG MBC/well). Clustering and color key as per Fig. 2.10.A. (C) Fecal repository composition over multiple experiments. 5-6 samples of total bacteria for each experiment set as per Fig. 2.8.A and 3.1.B

were FACS sorted. 16S rDNA frequencies are shown for the total bacteria content. Clustering and color key as per Fig. 2.10.A use Bray-Curtis distances.

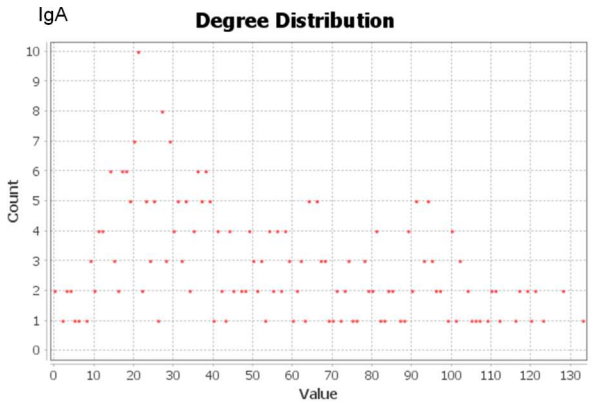
A


genus detected per well
families detected per well
orders detected per well
****
***
***
IgA
IgG

B


o__Clostridiales
o__Bacteroidales
o__Lactobacillales
o__Desulfovibrionales
o__Burkholderiales
o__Coriobacteriales
o__Erysipelotrichales
o__Pseudomonadales
o__Actinomycetales
o__Mycoplasmatales
o__Rhizobiales
o__Campylobacterales
o__Caulobacterales
IgA
IgG

C


IgA
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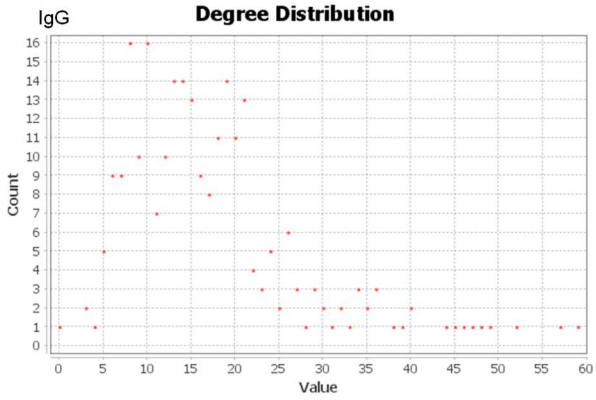
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Figure 3.4: Computational modelling of IgA vs IgG reactivity to bacteria

(A) Taxonomic breadth of IgA and IgG wells towards commensal bacteria. The number of phylogenetic genus, families or orders enriched by each well is shown. Median indicated by horizontal line. Significance between IgA and IgG determined by Mann-Whitney U test. (B) Network analyses of IgA- or IgG-bound bacteria. Each node is a bacterial OTU. Edge distances are calculated via Pearson's correlation for the association of 16S frequencies between the two OTUs across all IgA or IgG samples. Network modelled using Gephi with ForcedAtlas2 parameters. Color key indicates phylogenetic order of the OTU. (C) Network analysis distribution of degrees. Plots show the average number of degrees, or edges, each OTU nodes possess for IgA and IgA wells. Larger degree values indicate a higher likelihood of OTUs being enriched by the same well.

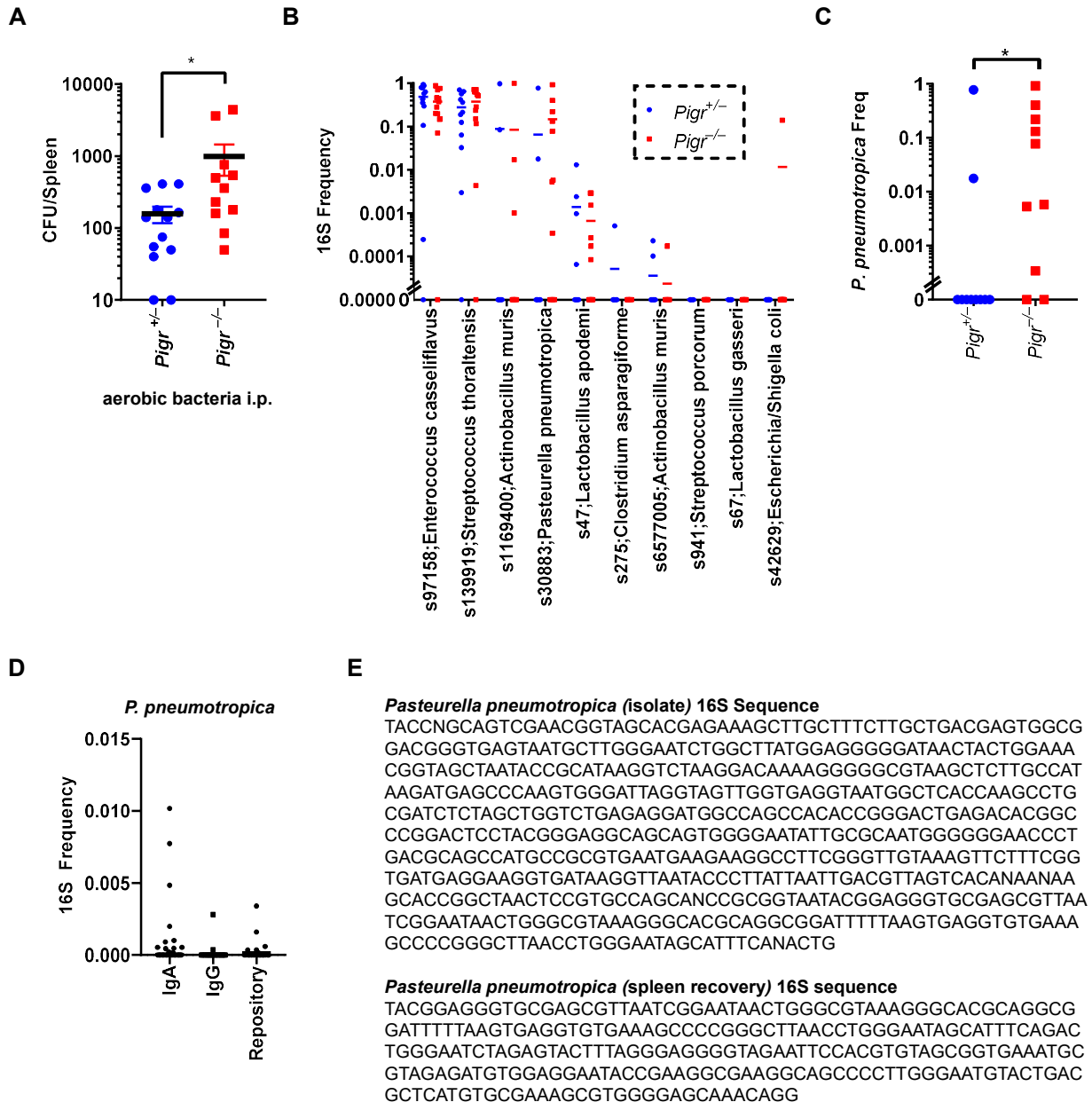

Figure 3.5: *Pigr*$^{-/-}$ mice are more susceptible to IP challenge from aerobic bacteria

(A) Intraperitoneal infection of aerobic gut bacteria. 2x10^{9} CFUs of aerobically cultured commensal bacteria from the gut lumen were injected i.p. into *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice. Splenic

colony counts were obtained 24 hours later on BHI agar plates. Statistical significance determined by Student's t test (n = 13 and 11). (B) The effect of sIgA on splenic bacteria after peritoneal infection.16S frequency of live bacteria cultured on BHI agar from the spleens of 7-8 week old *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ 24 hours post i.p. injection with aerobically grown gut bacteria. (C) Frequency of *P. pneumotropica* 16S sequences from splenic cultures. BHI agar plates from (A) were scraped and sequenced for 16S rDNA. 16S frequencies of *P. pneumotropica* are shown (n = 13 and 11 from 3 independent experiments). Significance determined by Mann-Whitney U test. (D) Frequency of *P. pneumotropica* from the *ex vivo* MBC screens. 16S rDNA frequencies of *P. pneumotropica* in the IgA enriched fractions from Fig. 2.12.B, IgG enriched fractions from Fig. 3.2.A, or the fecal library described in Fig. 2.5.B. (E) 16S rDNA sequence of *P. pneumotropica* 16S rDNA sequence based on V1-V5 sequencing of cultured isolates (top) and 16S rDNA sequence based on V4 sequencing of live bacteria collected from the spleen as per (A) (bottom).

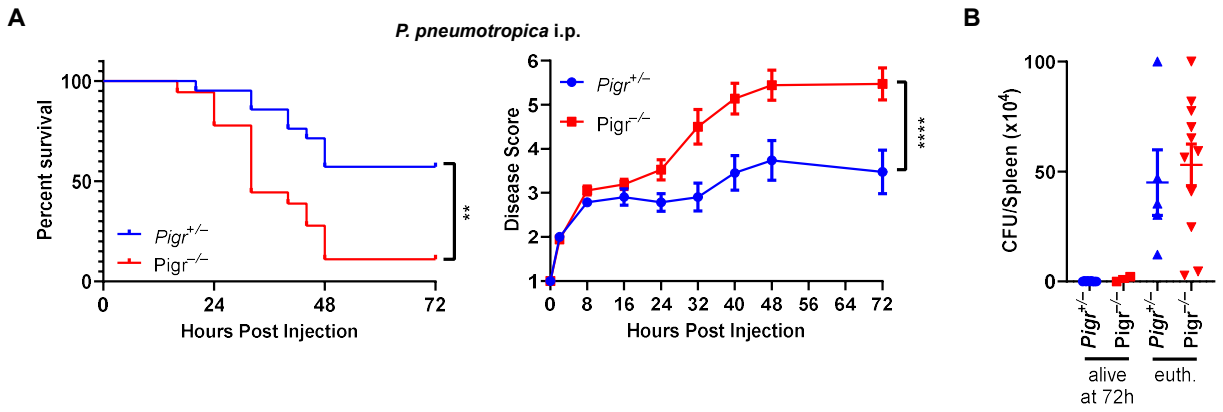

Figure 3.6: *Pigr*$^{-/-}$ mice are more susceptible to IP challenge from *P pneumotropica*

(A) Effect of sIgA on *P. pneumotropica* infection. 2x10^{11} CFUs of *P. pneumotropica* were injected i.p. into *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice. Survival (left) and morbidity/mortality score (right) was monitored for 72 hours. Scoring: 1=Animal is bright and alert; 2=Alert but less active, with spontaneous movement; 3=Huddled with ruffled fur, decreased or no spontaneous movement; 4=Hunched and ruffled, animal does not move when touched, and "righting reflex" absent; 5=Recumbent; 6=Death. Statistical significance determined by log rank test (left) and repeated measures ANOVA (right) (n= 21 and 18 from 3 independent experiments).

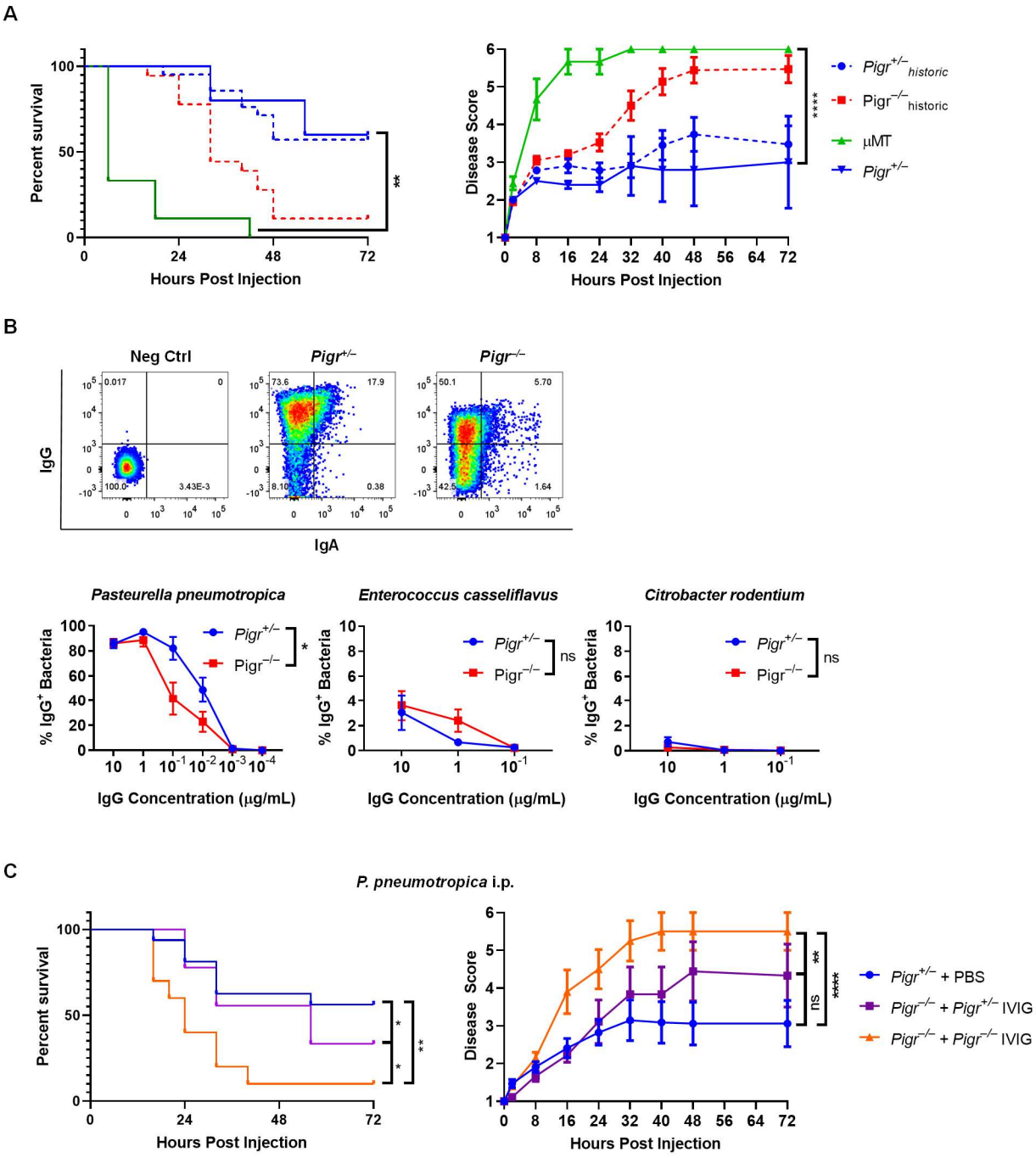
A
Percent survival
Hours Post Injection
Disease Score
Hours Post Injection
Pigr+/– historic
Pigr–/– historic
µMT
Pigr+/–
B
Neg Ctrl
Pigr+/–
Pigr–/–
IgG
IgA
Pasteurella pneumotropica
Enterococcus casseliflavus
Citrobacter rodentium
% IgG+ Bacteria
IgG Concentration (µg/mL)
ns
C
P. pneumotropica i.p.
Percent survival
Hours Post Injection
Disease Score
Hours Post Injection
Pigr+/– + PBS
Pigr–/– + Pigr+/– IVIG
Pigr–/– + Pigr–/– IVIG

Figure 3.7: IgG is protective against IP challenge from *P pneumotropica*

(A) Requirement for B cells on *P. pneumotropica* infection. *P. pneumotropica* was injected IP into 7-8 week old μMT or *Pigr*$^{+/-}$ mice. Disease progress was tracked over 72 hours; historic *Pigr*$^{+/-}$ and *Pigr*$^{-/-}$ data plotted in dotted lines for reference. Left panel shows survival curve. Right panel tracks disease score as per Fig. 3.6.A. (B) Serum IgG reactivity to bacterial isolates. Serum from *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice was tested for IgG reactivity towards *P. pneumotropica, E. casseliflavus,* or *C. rodentium,* by flow cytometry; negative control is no serum added. Statistical significance determined by ANOVA (n = 4 per group). (C) Effect of IgG on *P. pneumotropica* infection. IgG was purified from the serum of *Pigr*$^{+/-}$ or *Pigr*$^{-/-}$ mice and injected i.v. into *Pigr*$^{-/-}$ mice 2 hours prior to i.p. infection with *P. pneumotropica* and assessed as per Fig. 3.6.A (n = 16 *Pigr*$^{+/-}$ + PBS, 9 *Pigr*$^{-/-}$ + *Pigr*$^{+/-}$ IgG, and 10 *Pigr*$^{-/-}$ + *Pigr*$^{-/-}$ IgG over 2 independent experiments).

Chapter 4: Concluding Remarks and Future Directions

sIgA increases gut B cell reactivity towards bacteria

Our data showed that in sIgA deficient *Pigr*$^{-/-}$ mice, the activation of IgA and IgG MBCs towards gut bacteria is impaired (Figures 2.8, 3.1). We hypothesize this loss of reactivity is due to a reduction in the influx of bacterial antigens into the SED and GC caused by the lack of sIgA mediated immune sampling. This defect in IgA and IgG MBC priming effected the immune response against certain bacterial OTUs more than others, suggesting certain species are more susceptible to sIgA mediated antigen uptake. There may be multiple reasons for this: certain gut species may naturally bind more sIgA than others, or they colonize a gut niche that is in closer proximity to the receptors responsible for sIgA retro-transport (Pedron et al., 2012). It would be interesting to see further experiments aimed at understanding the reason behind sIgA dependent immune activation.

On the other hand, some OTUs generated higher IgA and IgG MBC responses in *Pigr*$^{-/-}$ mice. There were slightly higher IgA and IgG reactivities detected towards members of the *Erysipelotrichales* and *Coriobacteriales* orders, respectively (Fig. 2.12.D, 3.2.C). While not statistically significant, this does suggest that sIgA can sometimes play a dominantly immune exclusionary function towards certain OTUs (Deplancke and Gaskins, 2001; Forbes et al., 2008b; Moor et al., 2017). One benefit of our in vitro modelling system was its ability to not only detect the direction of the gut B cell response, but also approximate the magnitude of the response. Thus, we were able to roughly differentiate between OTUs that benefit or are inhibited

by sIgA regarding B cell activation.

Clinical implications of sIgA immunosurveillance

A challenge for understanding the role of IgA is that human patients with IgA deficiency are often undiagnosed and do not exhibit a high degree of immunodeficiency (Cunningham-Rundles, 2001). While this may be in part due to the improved hygiene of modern man, IgA-deficient patients do show increased frequency of recurrent sinopulmonary infections (Cunningham-Rundles, 2001), and increased mortality following liver transplantation (Van Thiel et al., 1992). Although future studies will be required to determine if the sIgA dependent response we observe in the gut are also seen in the upper airway, our results lead us to speculate that these issues may, in part, be due to loss of IgG responses in the absence of IgA.

Our work demonstrating the connection between the gut IgA and IgG response adds to the growing body of literature on how the gut microbiome can affect the systemic immune response. It may be possible to leverage this concept into developing an oral vaccine strategy by inoculating a subject with viral peptides conjugated to IgA. This should in theory generate an IgA as well as IgG response with systemic protection. Indeed, a proof of principle study of this was recently published for sIgA-HIV p24 immunization in mice (Rochereau et al., 2015). In addition, these findings may also have implications in regards to maternal IgA found in breast milk, which we predict will facilitate infant immunity to gut bacteria. Although the effect of maternal IgA and IgG has been interpreted as decreasing immune effector T cell activation presumably via immune exclusion (Koch et al., 2016), we speculate that an alternative possibility

may be that maternal sIgA facilitated education of the anti-commensal response which led to the generation of tolerogenic, rather than pro-inflammatory, T and B cell responses.

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