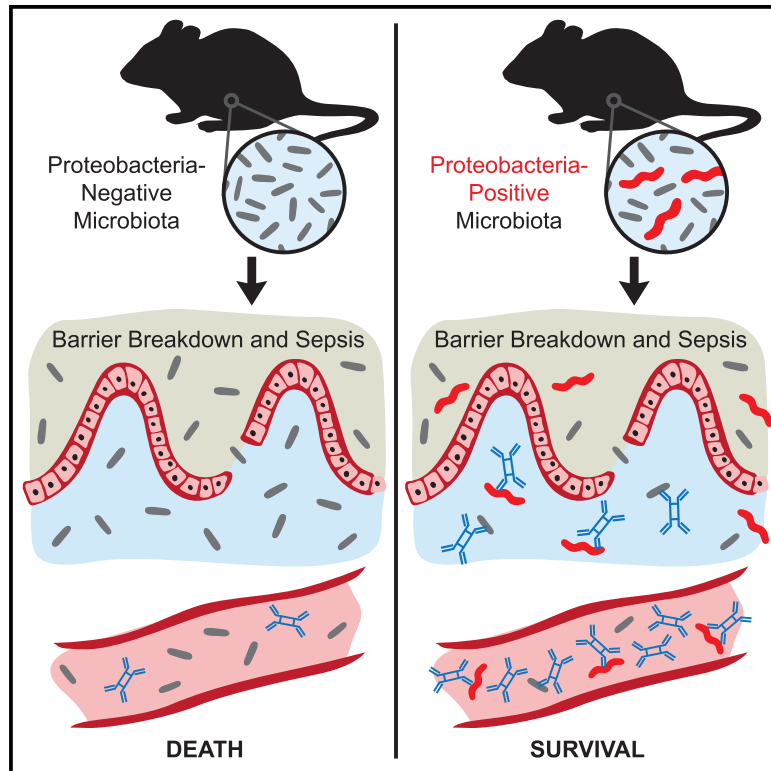


Cell Host & Microbe

Commensal Microbes Induce Serum IgA Responses that Protect against Polymicrobial Sepsis

Graphical Abstract



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In Brief

Wilmore et al. demonstrate a role for serum IgA in protection against polymicrobial sepsis. Induction of protective concentrations of T cell-dependent serum IgA requires colonization of the gut with a complex microbiota that includes bacteria in the phylum Proteobacteria.

Highlights

- Serum IgA concentrations depend on the composition of the gut microbiota
- Cohousing transfers Proteobacteria to naive mice, resulting in increased serum IgA
- T cell-dependent serum IgA is induced in response to select bacteria in the gut
- Serum IgA protects against lethal sepsis following intestinal barrier disruption



Commensal Microbes Induce Serum IgA Responses that Protect against Polymicrobial Sepsis

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SUMMARY

Serum immunoglobulin A (IgA) antibodies are readily detected in mice and people, but the mechanisms underlying the induction of serum IgA and its role in host protection remain uncertain. We report that select commensal bacteria induce several facets of systemic IgA-mediated immunity. Exposing conventional mice to a unique but natural microflora that included several members of the Proteobacteria phylum led to T cell-dependent increases in serum IgA levels and the induction of large numbers of IgA-secreting plasma cells in the bone marrow. The resulting serum IgA bound to a restricted collection of bacterial taxa, and antigen-specific serum IgA antibodies were readily induced after intestinal colonization with the commensal bacterium *Helicobacter muridarum*. Finally, movement to a Proteobacteria-rich microbiota led to serum IgA-mediated resistance to polymicrobial sepsis. We conclude that commensal microbes overtly influence the serum IgA repertoire, resulting in constitutive protection against bacterial sepsis.

INTRODUCTION

For many years, it was widely accepted that mucosal immune responses are physically and functionally separate from processes regulating systemic immunity. With regard to humoral responses, antigenic invasion of mucosal tissues was shown to result in effective local antibody responses, with little or no contribution to serum antibody concentrations (reviewed in Macpherson et al., 2008; Tomasi and Bienenstock, 1968). This viewpoint is supported further by experiments showing that serum IgA antibodies fail to bind to commensal bacterial antigens (Macpherson et al., 2000, 2012). Reciprocally, immunization via non-mucosal routes typically results in systemic humoral immunity without inducing protection at mucosal sites. Taken at face value, these studies would appear to establish a clear dichotomy between mucosal and systemic responses.

Recent observations by several groups, however, highlight the capacity of mucosal immune interactions to affect systemic immunity. For instance, colonization of mice with segmented filamentous bacteria (SFB) facilitates the development of auto-immune arthritis associated with the induction of T_H17 cells and germinal centers (GCs) (Ivanov et al., 2009; Lecuyer et al., 2014; Talham et al., 1999; Wu et al., 2010; Yang et al., 2014). Additionally, immunoglobulin A (IgA) antibodies specific for phosphorylcholine, a common cell wall constituent of many commensal bacteria, are readily observed in sera from common inbred mice (Morahan et al., 1983), and recent work shows that antigen-specific IgA-secreting bone marrow (BM) plasma cells are induced after mucosal immunization with the relatively pro-inflammatory antigen cholera toxin (Lemke et al., 2016). Furthermore, two recent reports revealed that commensal microbes induce serum IgG responses with the capacity to block bacterial infection and modulate mucosal T cell populations (Koch et al., 2016; Zeng et al., 2016). However, whereas it is clear that IgA synthesis plays a critical role in establishing intestinal homeostasis (Fagarasan et al., 2002; Wei et al., 2011), the generation and regulation of serum IgA responses and their role in protective immunity remain largely undefined.

Current models hold that B cells located in the marginal zone (MZ) of the spleen, together with B1 B cells, rapidly generate IgM antibodies against blood-borne bacteria in sepsis and related scenarios (Martin et al., 2001; Pillai et al., 2005). This idea is consistent with the uniquely rapid kinetics with which MZ B cells generate plasma cells in response to toll-like receptor ligands such as lipopolysaccharide (Oliver et al., 1997), and data showing that splenectomy increases susceptibility to bacterial infections including septicemia (Thai et al., 2016). An additional layer of protection may be provided by serum IgG antibodies that result from mucosal B cell responses to the bacterial microbiota in the gut (Koch et al., 2016; Zeng et al., 2016), although the role of such responses in bacterial sepsis has not been tested. Here we report that serum IgA antibodies provide a unique and constitutive protective barrier against polymicrobial sepsis. Our results further show that modulations in the microbial composition of the gut result in heightened serum IgA concentrations that coincide with colonization of the BM by large numbers of IgA-secreting plasma cells and marked changes in the serum IgA repertoire. Such antibodies are induced by a wide variety of bacterial taxa, but are generally enriched with members of the Proteobacteria phylum. Altogether our results demonstrate



that commensal microbes can have a substantial impact on the composition of the serum IgA repertoire and the BM plasma cell pool, resulting in protection against widespread and lethal bacterial invasion.

RESULTS

Commensal Microbes Drive the Generation of Serum IgA and IgA-Secreting BM Plasma Cells

Recent work indicates that shifts in the gastrointestinal (GI) microbiota can affect the generation of effector and regulatory T cells (Atarashi et al., 2013; Ivanov et al., 2009). Perhaps most notably, the introduction of SFB into mice reared at Jackson Laboratories (JAX-SPF) through cohousing with mice reared at Taconic Farms leads to the induction of T_H17 cells in the lamina propria of the small intestine (siLP) (Ivanov et al., 2008, 2009). In early experiments, we found that serum IgA concentrations were substantially higher in C57BL/6 (B6) mice reared in our SPF colony (hereafter termed PENN-SPF mice) compared with age-matched JAX-SPF B6 mice (Figure 1A). To explore this issue, we cohoused female age-matched B6 JAX-SPF and PENN-SPF adults, a strategy that led to increases in serum IgA levels in mice originally housed under JAX-SPF conditions (Figure 1A). Notably, enzyme-linked immunospot (ELISpot) analyses revealed that PENN-SPF and JAX-SPF mice possess very similar frequencies of IgA-secreting plasma cells in the siLP (Figure 1B), suggesting that an alternative source of plasma cells accounted for increased serum IgA levels. Hence, we next quantified IgA-secreting plasma cells in the BM of isolated and cohoused JAX-SPF and PENN-SPF B6 adults. Whereas we detected few, if any, IgA-secreting plasma cells in JAX-SPF B6 adults, PENN-SPF B6 adults and JAX-SPF B6 adults that had been cohoused with PENN-SPF mice for 10 weeks possessed large numbers of BM IgA-secreting cells (Figure 1C).

We also used flow cytometry to confirm and extend these results. Notably, Blanc et al. (2016) recently showed that viable mouse IgM- and IgA-secreting CD138⁺ BM plasma cells are readily identified via surface IgM and IgA expression. With this approach, we also detected few IgA-secreting plasma cells in the BM of JAX-SPF mice (Figure 1D). By sharp contrast, the majority of BM plasma cells in PENN-SPF mice were surface IgA⁺, and these results were recapitulated in JAX-SPF mice after cohousing with PENN-SPF mice for 10 weeks (Figure 1D). Cohousing of JAX-SPF and PENN-SPF adults also led to increased frequencies of IgA⁺ GC B cells in the former mice (Figure 1E). Notably, these results did not correlate strictly with the induction of T_H17 cells in JAX-SPF mice; although, as reported, the siLP of adults purchased from Taconic Farms contained large numbers of T_H17 cells (data not shown) (Ivanov et al., 2008), we observed only modest increases in numbers of BM IgA⁺ plasma cells in mice reared at Taconic Farms (Figure S1). To determine whether antibiotic treatment is sufficient to block the increase in IgA⁺ BM plasma cells seen following cohousing, we placed PENN-SPF mice on an antibiotic cocktail containing vancomycin, neomycin, ampicillin, and metronidazole (VNAM) for 2 weeks prior to cohousing. The VNAM-treated PENN-SPF mice did not induce significant levels of IgA⁺ BM plasma cells in JAX-SPF mice (Figure 1F). Additionally, separate cohorts of JAX-SPF mice were given an oral gavage of fecal slurries pre-

pared from PENN-SPF mice and then half of these mice were placed on VNAM. The VNAM-treated JAX-SPF mice that received the fecal transfer had levels of IgA⁺ BM plasma cells similar to those of untreated JAX-SPF mice, while fecal transfer alone led to significantly increased levels of IgA⁺ BM plasma cells (Figure 1G). Together these data suggest that unique bacterial taxa in PENN-SPF mice induce events that enhance serum IgA levels and production of IgA-secreting plasma cells.

Bacterial Taxa Driving Serum IgA

To correlate specific bacterial taxa with systemic IgA responses, we performed 16S ribosomal gene sequencing of stool samples from JAX- and PENN-SPF mice, as well as JAX-SPF mice that were cohoused with PENN-SPF B6 mice for 4 weeks. Consistent with past work showing that the microbiota between B6 mice housed under disparate conditions is normalized by cohousing within 17 days (Ivanov et al., 2008), within 4 weeks the intestinal microbiota in JAX-SPF mice was reshaped substantially (Figure 2A). Most notably, members of the Proteobacteria phylum were readily observed in PENN-SPF and cohoused JAX mice only (Figures 2B and 2C). Included were small but significant numbers of *Helicobacter* species, shown previously to be highly enriched among IgA-bound GI bacteria (Palm et al., 2014). Furthermore, unlike in JAX-SPF mice, using fluorescence *in situ* hybridization (FISH) analyses we easily detected bacteria in the siLP of PENN-SPF and in JAX-SPF mice that were first cohoused with PENN-SPF adults (Figure 2D).

To test directly whether colonization of the gut by a commensal bacterium facilitates the induction of serum IgA and BM IgA-secreting plasma cells, we delivered the Proteobacteria member *Helicobacter muridarum* to the GI tract of JAX-SPF B6 mice by oral gavage. *H. muridarum* was selected because it was isolated from healthy rodents and is considered a commensal bacterium (Lee et al., 1992). As shown in Figures 3A and 3B, 4 weeks after treatment with this strategy we easily detected serum antibodies specific for *H. muridarum*, but not for the irrelevant skin commensal *Staphylococcus epidermidis*. Additionally, we readily quantified IgA⁺ *H. muridarum*-specific BM and siLP plasma cells in colonized mice, but not in control JAX-SPF mice kept in isolation in our colony (Figure 3C). Therefore, colonization of the GI tract by the commensal bacterium *H. muridarum* induces the genesis of microbe-specific serum IgA and IgA-secreting BM plasma cells.

To gain a more global picture of the commensal bacterial taxa that are able to induce serum IgA responses, we sought to identify intestinal bacteria bound by serum IgA among complex bacterial intestinal communities. To this end, we extended recently described approaches for identifying IgA- or IgG-bound bacteria whereby Ig-bound and unbound bacteria are purified before being subjected to 16S rDNA gene sequencing (Bunker et al., 2015; Koch et al., 2016; Palm et al., 2014). First, we evaluated the degree to which serum IgA from various mice bound to fecal bacteria. To avoid the complication associated with bacterial binding by endogenous IgA, we derived bacterial samples from adult *B6.RAG1*^{-/-} mice that were first cohoused with B6 mice in our colony. As expected, because cohoused mice are naturally coprophagic (Ebino et al., 1988), 16S data from stool samples indicated similar representation of bacterial taxa in cohoused PENN-SPF B6 and *B6.RAG1*^{-/-} mice, especially relative

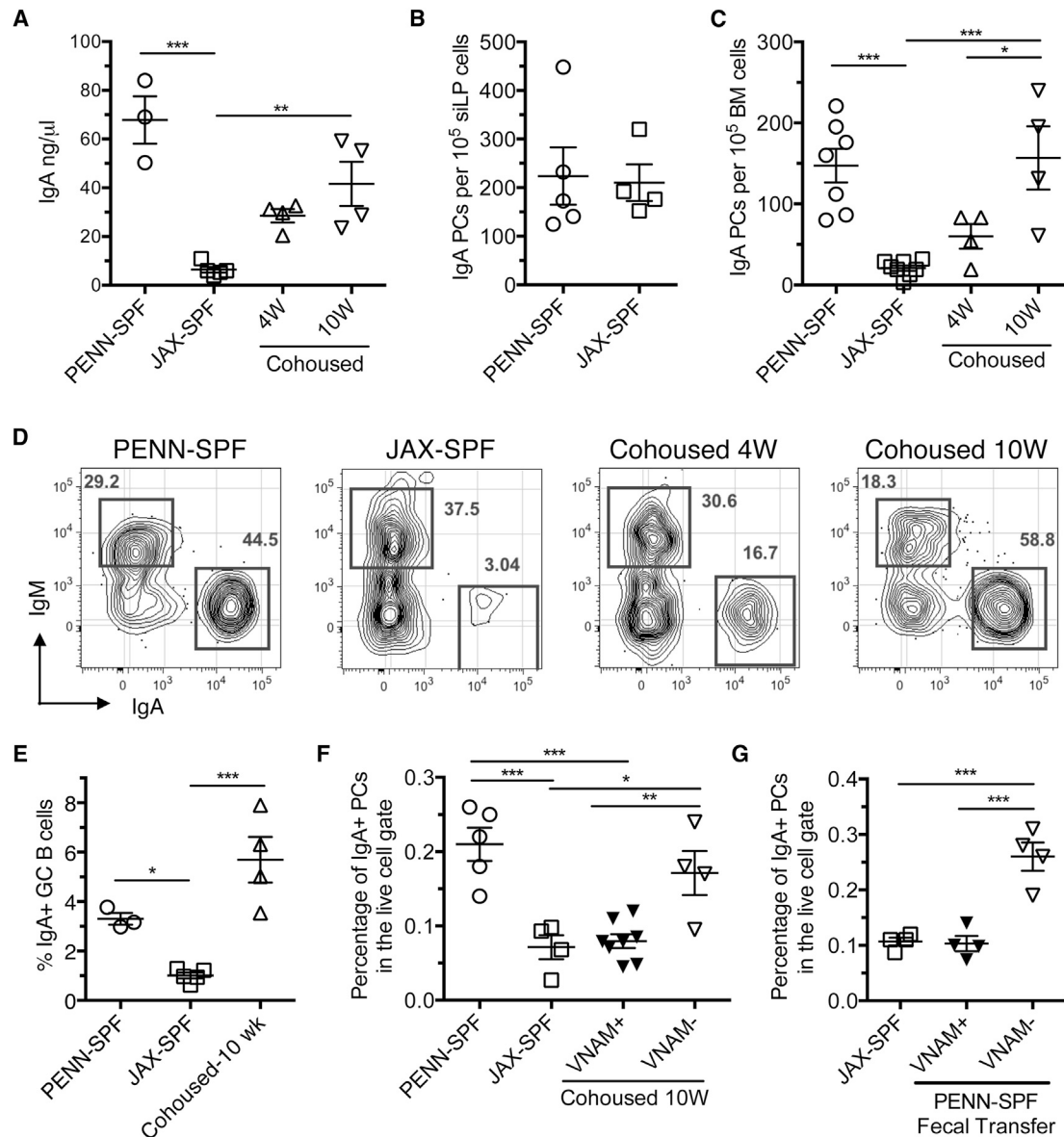


Figure 1. Serum IgA Concentration and BM IgA⁺ Plasma Cell Numbers Are Dependent on Housing Conditions

(A) Serum ELISpot performed to determine the concentration of IgA from PENN-SPF, JAX-SPF, and cohoused mice, with 3–5 mice per group.

(B) Cells from siLP of PENN-SPF or JAX-SPF B6 adults were plated onto ELISpot plates precoated with anti-Ig(H+L) and spots were developed using IgA-specific antibodies.

(C) BM cells from PENN-SPF, JAX-SPF, or JAX-SPF cohoused with PENN-SPF for 4 or 10 weeks were plated onto ELISpot plates as in (B) and detected with IgA-specific antibodies.

(D) BM samples from the groups indicated in (C) were stained with the indicated antibodies and 2 × 10⁶ events collected on an LSR2 flow cytometer.

(E) Frequencies of Peyer's patch GC B cells (CD19⁺ IgD⁺ CD38⁺ PNA⁺) in the indicated mice. Mean and SEM are shown for 3–5 mice/group.

(F) Percentages of IgA⁺ plasma cells within the live cell gate of the BM were determined for mice cohoused with PENN-SPF mice pretreated with VNAM or JAX-SPF, PENN-SPF, and cohoused controls.

(G) JAX-SPF mice were gavaged with fecal slurry from PENN-SPF and the treated with VNAM or maintained as controls. Displayed are percentages of IgA⁺ plasma cells within the live cell gate of the BM. Mean and SE are indicated for at least three mice per group.

*p < 0.05, **p < 0.01, ***p < 0.001 determined by one-way ANOVA with Tukey's post-test or t test where appropriate. Data are representative of three independent experiments.

to B6 JAX-SPF mice (Figures S2A and S2B). For flow cytometric analyses of bacterial communities, we used the fluorescent dyes SytoBC and DAPI, which together allow viable bacteria to be identified as SytoBC⁺ DAPI^{low} cells (Figure 4A, leftmost panel)

(Ben-Amor et al., 2005). As shown, whereas sera from JAX-SPF B6 mice stained only small numbers of bacteria, serum from PENN-SPF or previously cohoused JAX-SPF B6 mice stained markedly increased numbers of viable bacteria from

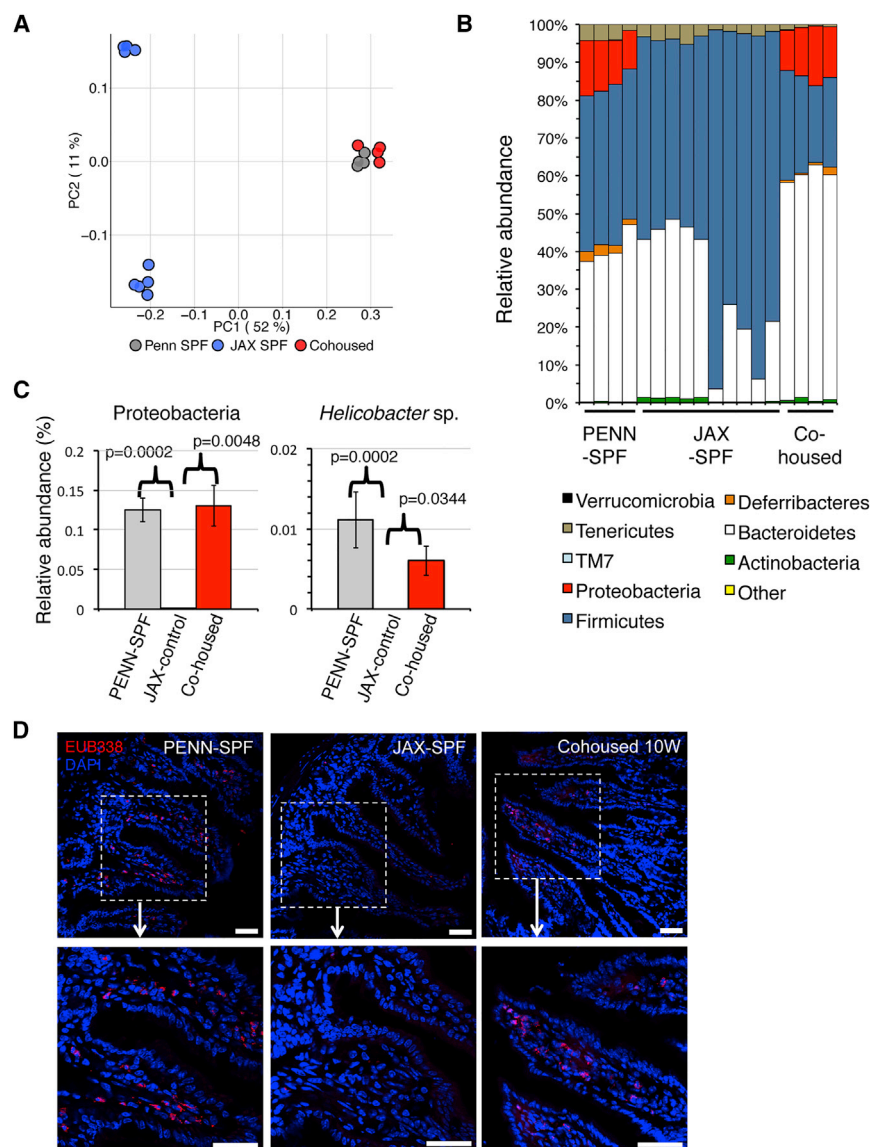


Figure 2. 16S Sequencing of JAX-SPF Mice Demonstrates a Lack of Commensal Bacteria the Phylum Proteobacteria

(A–C) Stool samples from JAX-SPF, Penn-SPF, and cohoused mice were subjected to 16S V4 rDNA gene sequencing.

(A) Principal coordinate analysis illustrating degree of variance for the indicated mice within each group.

(B) Relative abundance of each bacterial phylum for each sample.

(C) Comparison of JAX-SPF, Penn-SPF, and cohoused JAX-SPF mice for relative abundance of the phylum Proteobacteria and the genus *Helicobacter*.

Data are means and SE for each group.

(D) Confocal images of FISH analyses performed on sections of the small intestine from JAX-SPF, 10-week cohoused, or Penn-SPF B6 adults. 16S probe EUB338 staining is shown in red, with nuclear stain (DAPI) in blue. Dashed boxes outline enlarged areas displayed below. Images are representative of two different areas of the intestine from four mice from each group. Scale bars, 40 μ m.

p Values were calculated using one-way ANOVA with Tukey's post-test. Experiments contain at least four mice per group and are representative of two independent experiments.

IgA⁺ fraction. Taxa achieving a score of 10 or more are considered substantially enriched among IgA-bound bacteria (Palm et al., 2014).

16S sequence data for serum IgA⁺ and IgA[−] bacteria derived from stool samples or the small intestine revealed that members of the *Burkholderia*, *Sphingomonas*, and *Bacillus* genera were each highly enriched among serum IgA-bound bacteria from either source, with enrichment scores for the small intestine of 2,546, 80, and 15, respectively

(Figures 4B–4D). In addition, consistent with the notion that IgA responses target bacteria in the small intestine, we also observed significant enrichment of two additional bacterial taxa among IgA⁺ bacteria in the small intestine, members of the genus *Gallionella*, and unidentified members of the Burkholderiaceae family. It should also be noted that SFB, *Bacteroides*, and Betaproteobacteria were enriched in the IgA-bound fraction in stool and small intestinal samples at levels ranging from 3 to 9. Finally, IgA-bound bacteria in stool samples from B6 mice were enriched significantly for members of the *Burkholderia* and *Sphingomonas* genera, both members of the Proteobacteria phylum (Figure S4). We conclude that select members of the bacterial microbiota induce the production of systemic IgA responses.

T Cell-Dependent Nature of Systemic IgA Responses

Recent work suggests that much of the IgA coating of intestinal bacteria occurs via T cell-independent mechanisms (Bunker

B6.RAG1^{−/−} mice (Figure 4A). Of note, the frequency of bacteria bound by serum IgA taken from cohoused JAX-SPF mice was similar to the fraction of bacteria bound by intestinal IgA reported by Palm et al. (2014).

We purified serum IgA-bound bacteria with two rounds of cell sorting before subjecting DNA from these cells (IgA-coated versus uncoated) to 16S sequencing. We term this strategy “serum IgA-seq.” Samples were sorted by gating on SytoBC⁺ DAPI[−] events; however, it should be noted that we observed very similar taxa when we analyzed serum IgA-bound bacteria in the SytoBC⁺ DAPI⁺ fraction (Figures S3A and S3B). Additionally, we analyzed stool and small intestinal bacteria because recent data indicate that bacteria in the latter site preferentially induce IgA responses (Bunker et al., 2015). Finally, to identify specific highly enriched taxa in serum IgA-bound fractions with the resulting 16S sequence data, we exploited the approach of Palm et al. whereby the relative abundance of each taxa in the IgA-bound fraction is divided by its relative abundance in the

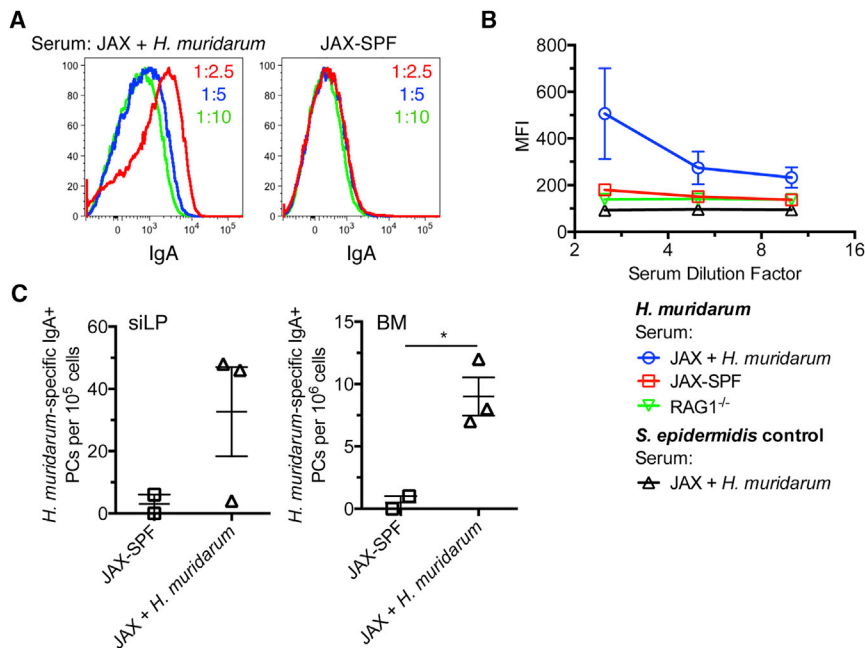


Figure 3. Marrow IgA⁺ Plasma Cells Induced by a Commensal Bacterium

(A) JAX-SPF mice were colonized with *Helicobacter muridarum* by oral gavage 4 weeks previously; cultured *H. muridarum* was stained with sera from JAX-SPF controls housed in isolation or JAX-SPF mice colonized with *H. muridarum* 4 weeks earlier. Bacteria were then stained with PE-anti-IgA and analyzed on an LSR2 flow cytometer.

(B) Graphical representation of data presented in (A) with *B6.RAG1*^{-/-} serum and *Staphylococcus epidermidis* used as negative controls. *S. epidermidis* was stained with serum from JAX-SPF *H. muridarum* mice because it is not found by 16S sequencing in the feces of PENN-SPF or JAX-SPF mice. Data are means and SE for each group.

(C) *H. muridarum*-specific ELISpot analysis performed using siLP and BM cells from (A). Experiments contain at least three *H. muridarum* treated mice per group and are representative of two independent experiments. Symbols represent individual mice with error bars indicating SE. *p < 0.05.

et al., 2015). However, Palm et al. (2014) reported that IgA responses to colitogenic bacteria are largely T cell dependent. To address this issue, we examined the capacity of serum IgA from PENN-SPF *B6.TCRβ*^{-/-}*δ*^{-/-} mice to coat fecal bacteria from cohoused (PENN-SPF B6 plus PENN-SPF *B6.RAG1*^{-/-}) *B6.RAG1*^{-/-} mice. Whereas serum from PENN-SPF B6 mice stained substantial numbers of fecal bacteria as expected, serum from *B6.TCRβ*^{-/-}*δ*^{-/-} mice did not (Figure 5A). Moreover, very similar to germ-free B6 mice, we were unable to detect BM IgA⁺ plasma cells in PENN-SPF *B6.TCRβ*^{-/-}*δ*^{-/-} adults (Figure 5B). We conclude that systemic IgA responses are largely T cell dependent.

Serum IgA-Dependent Protection against Polymicrobial Sepsis

We hypothesized that elevated concentrations of serum IgA, as observed in PENN-SPF mice, would protect against mucosal barrier disruption and the resulting polymicrobial sepsis. Consistent with this idea, a recent report suggests that people with IgA deficiencies are more likely to succumb to sepsis (Ludvigsson et al., 2016). To test this hypothesis, we employed an experimental model of polymicrobial sepsis, namely cecal ligation and puncture (CLP), which results in mortality due to systemic inflammation caused by overwhelming bacteremia (Xiao et al., 2006). Accordingly, we performed CLP on PENN-SPF and JAX-SPF B6 cohoused briefly (1.5 weeks), 4 weeks, or 10 weeks with PENN-SPF B6 mice. The brief 1.5-week cohousing period was insufficient to induce elevations in serum IgA (data not shown), but allowed for the transfer of the PENN-SPF microbiota to the JAX-SPF mice. JAX-SPF mice are particularly susceptible to CLP resulting in >85% death, in contrast the PENN-SPF mice that were largely resistant to CLP-induced death with <20% succumbing over 14 days (Figure 6A). The cohousing the JAX-SPF for 4 or 10 weeks led to significant levels of protection from death, with the 10-week cohort recapitulating the phenotype of

PENN-SPF mice (Figure 6A). To test the relevance of IgA in this context, we next performed CLP on PENN-SPF *B6.IgA*^{-/-} mice and PENN-SPF B6 mice. Similar to JAX-SPF mice, PENN-SPF *B6.IgA*^{-/-} mice were susceptible to sepsis-induced mortality (Figure 6B). To determine whether serum IgA mediates protection against sepsis, we performed CLP on JAX-SPF mice that subsequently received sera from either PENN-SPF B6 or PENN-SPF *B6.IgA*^{-/-} mice. All but one animal receiving *B6.IgA*^{-/-} serum died within 2 days, whereas survival of JAX-SPF mice receiving B6 serum was extended significantly (Figure 6C). We conclude that serum IgA responses provide a constitutive defense mechanism against bacterial sepsis.

DISCUSSION

Our work demonstrates that a variety of commensal bacterial taxa elicit serum IgA responses, resulting in protective activity against polymicrobial sepsis and a marked remodeling of the BM plasma cell pool. In contrast to conventional models that predict a critical role for MZ and B1 B cells in generating IgM antibodies to blood-borne bacteria (Martin et al., 2001; Pillai et al., 2005), we demonstrate that pre-existing serum IgA antibodies play a unique role in the context of bacterial sepsis, especially when the microbes in question originate from the gut microbiota. Moreover, MZ and B1 B cells require stimulation via the B cell receptor or pattern recognition receptors to secrete large quantities of immunoglobulin (Fairfax et al., 2007; Oliver et al., 1997). By comparison, IgA⁺ plasma cells actively secrete antibody without receptor engagement, thus providing a constitutive humoral shield against systemic bacterial invasion. Notably, both pools of IgA-secreting cells consist mainly of long-lived cells (Bemark et al., 2016) (unpublished data), and therefore provide a mechanism for the continuous production of serum antibodies with antimicrobial activity. These ideas therefore build on recent evidence that serum IgG antibodies

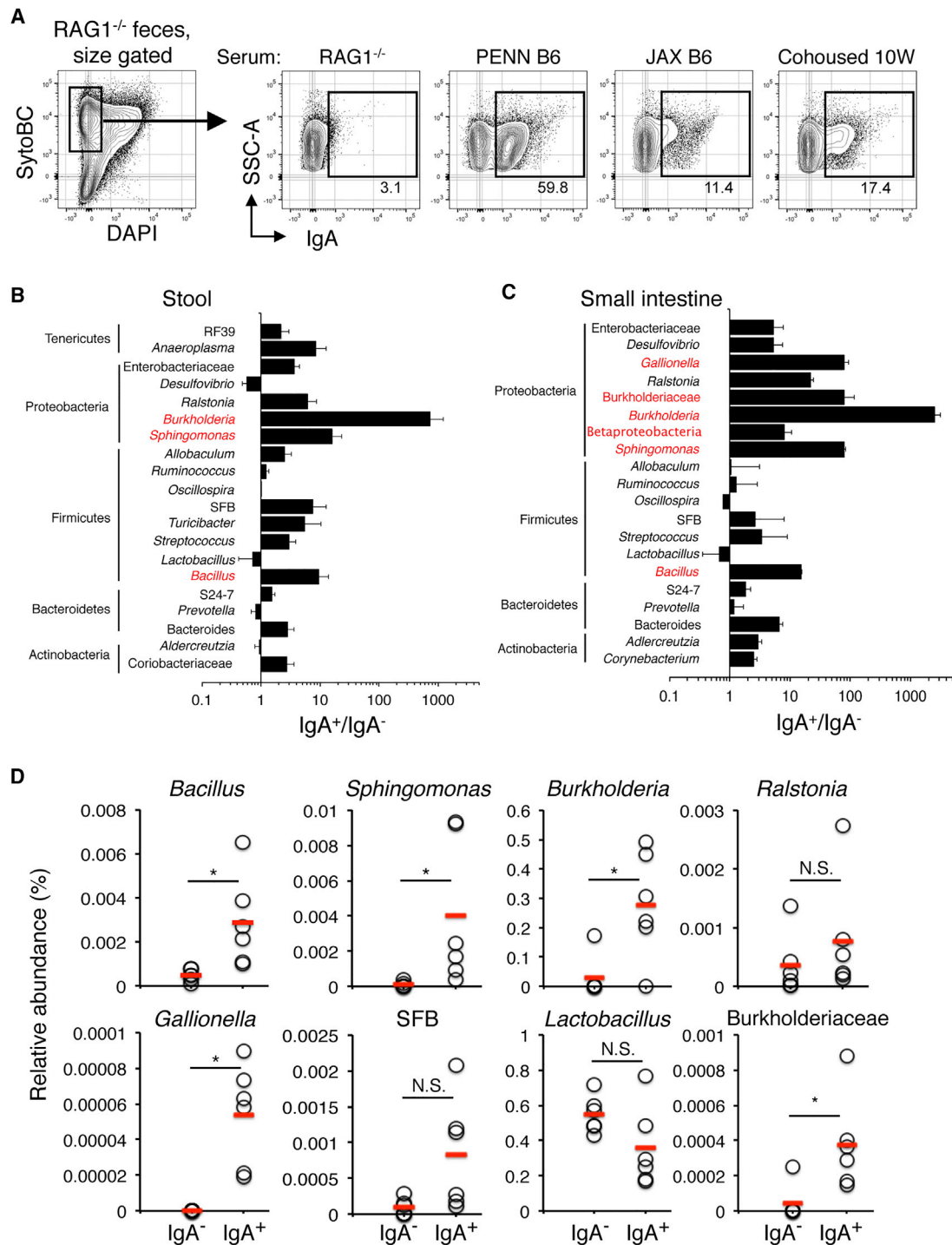


Figure 4. Serum IgA Binding to Select Commensal Bacteria Including Proteobacteria

(A) Fecal bacteria from B6.RAG1^{-/-} mice were stained with sera from the indicated mice, and IgA⁺ bacteria were identified with PE-anti-IgA antibodies by flow cytometry. Bacterial viability was determined by positive staining with SytoBC and minimal DAPI staining, as shown.

(B) Fecal bacteria from RAG1^{-/-} mice were stained with sera from PENN-B6 adults and both IgA⁺ and IgA⁻ fraction sorted twice. DNA from these samples was then subjected to 16S V4 sequencing. The ratio of the relative abundance for each taxon in each fraction (IgA⁺ and IgA⁻) was used to identify taxa enriched for IgA binding. Bacterial genera that were highly enriched (IgA⁺/IgA⁻ ≥ 10) are highlighted in red. Genera shown were selected because they possessed IgA coating index scores above or below 3, or because of potential relevance based on previous studies.

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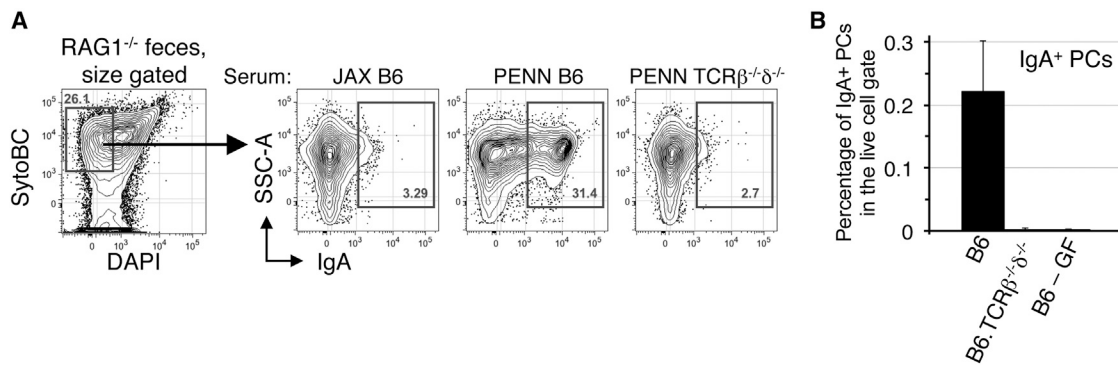


Figure 5. Systemic IgA Responses Are T Cell Dependent

(A) Fecal bacteria from *B6.RAG1^{-/-}* mice were stained with sera from the indicated mice as in Figure 4A, then IgA-bound bacteria were detected with anti-IgA antibody. Data are representative of two independent experiments with 3–4 mice per group.

(B) Percentages of IgA⁺ plasma cells within the live cell gate of the BM were determined by flow cytometry for PENN-SPF B6, *B6.TCRβ^{-/-}δ^{-/-}*, and germ-free B6 female adults. Data are means and SE for each group and are representative of two independent experiments with 4–5 mice per group.

specific for intestinal microbes form a barrier of protection against systemic invasion by commensal bacteria (Koch et al., 2016; Zeng et al., 2016). Therefore, we propose that serum IgA and IgG antibodies may play roles similar to the protective role proposed for natural IgM antibodies (Ochsenbein et al., 1999), with the IgA component providing a non-inflammatory mechanism to keep invading bacteria in check.

Our results also shed light on the role of systemic IgA responses for enteric pathogens. Systemic antibody responses may be of little advantage for certain enteric pathogens. However, in scenarios associated with breakdown of intestinal barrier function, certain enteric pathogens readily spread to distant tissues to cause or exacerbate disease. One classic example is poliovirus (Blondel et al., 2005). Even rotavirus, thought originally to remain localized to the intestine, was shown recently to spread to extra-intestinal tissues (Blutt and Conner, 2007). Thus, heightened serum IgA responses, induced perhaps through appropriate adjuvants, could increase availability of neutralizing antibodies throughout the body, and consequently limit the spread of infection for a variety of enteric pathogens.

Past work indicates that recently generated mucosal plasma cells readily travel through the efferent lymph and into the blood before eventually returning to mucosal sites (Gowans and Knight, 1964). Consistent with this idea, more recent work indicates that mucosal B cells are induced to express gut homing receptors by specialized dendritic cells (Mora and von Andrian, 2008). Our data reveal another layer of complexity to these processes and suggest that certain consortia of commensal microbes push the boundaries between the mucosal and systemic immune systems, increasing the production of mucosal IgA-secreting plasma cells and the infiltration of many of these cells into the BM. Notably, colitogenic bacteria characterized by a propensity to invade the inner GI mucus layer are preferentially coated with IgA (Palm et al., 2014). Such microbes include members of the *Helicobacter*

genus such as *H. muridarum*, shown here to induce the generation of IgA⁺ BM plasma cells.

A recent study highlights the strong propensity of *Helicobacter* species to induce mucosal T cell differentiation (Chai et al., 2017). Additionally, Atarashi et al. (2015) showed that SFB, *Citrobacter rodentium*, and *Escherichia coli* are especially adept at attaching to intestinal epithelial cells, which in turn enhances the local generation of T_H17 cells. Given that mucosal T_H17 cells can support the induction of large numbers of IgA⁺ GCs (Hirota et al., 2013), it follows that select members of the GI microbial community possess an intrinsic propensity to invade the inner mucus layer and drive the generation of T_H17-supported IgA responses, which in turn lead to increases in the differentiation of IgA⁺ plasma cells and serum IgA titers. This idea is consistent with our data demonstrating that bacterial microbes are readily and uniquely detected in the siLP of PENN-SPF mice. Additional work is ongoing to identify these microbes and characterize their capacity to generate local and systemic IgA responses. It is tempting to speculate that these microbes will include members of the *Burkholderia*, *Sphingomonas*, and *Bacillus* genera, identified by our serum IgA-seq experiments.

Several studies show that certain intestinal bacterial taxa influence T cell-mediated processes beyond local mucosal tissues (Klaasen et al., 1993; Wu et al., 2010). In this regard, serum IgA can increase with age or exposure of germ-free mice to commensal bacteria. However, these results have been attributed to increased numbers of IgA⁺ siLP plasma cells (Kamata et al., 2000). By contrast, our results with conventional SPF mice show that changes in the commensal microbiota lead to increases in serum IgA without altering frequencies of siLP IgA-secreting cells, suggesting that the bulk of the increased serum IgA is due to increased production of IgA⁺ BM plasma cells.

Moving forward, how B-lineage cells regulate intestinal homeostasis as microbial communities evolve throughout life while also providing protection for dangerous enteric pathogens

(C) Small intestine fecal samples from *B6.RAG1^{-/-}* mice were stained, sorted, and sequenced as in (B).

For (B) and (C), data are means and SE for each group.

(D) Relative abundance of the indicated taxa for the serum IgA⁺ and serum IgA⁻ fraction derived from the data in (C). Red lines indicate means for each group. N.S., not significant; *p < 0.05 (Wilcoxon rank-sum test).

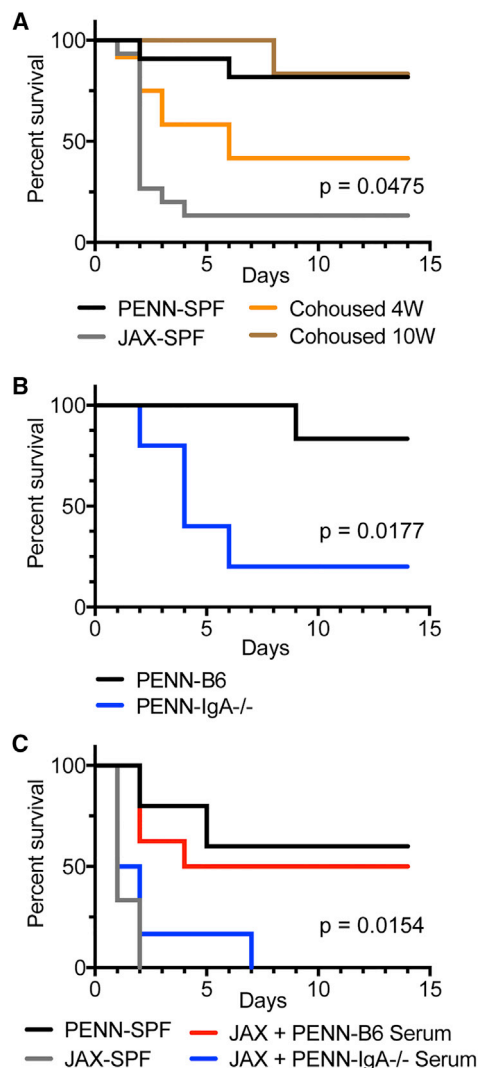


Figure 6. Serum IgA-Mediated Protection from Induced Sepsis

(A) JAX-SPF and PENN-SPF were cohoused for 1.5 (labeled JAX-SPF), 4, or 10 weeks to allow for mice to achieve similar intestinal microflora and in the case of the 4- and 10-week cohoused groups to develop increased serum IgA. Mice were then subjected to CLP surgery with a 1-cm ligation followed by 2 punctures with a 21G needle. Survival was monitored daily for 14 days. JAX-SPF, n = 15; PENN-SPF, n = 11; Cohoused 4W, n = 12; Cohoused 10W, n = 6. Reported p value is between JAX-SPF and Cohoused 4W.

(B) PENN-SPF B6 and PENN-SPF *B6.IgA^{-/-}* mice were subjected to CLP as in (A). B6, n = 6; *B6.IgA^{-/-}*, n = 5.

(C) CLP was performed on PENN-SPF and JAX-SPF control mice as in (A) while additional JAX-SPF mice received sera from B6 or *B6.IgA^{-/-}* PENN-SPF mice: 400 μ L on day zero, and 300 μ L daily for the next 4 days. Reported p value is between B6 and *B6.IgA^{-/-}* serum transfer groups. Sample sizes were as follows: Penn-SPF controls, n = 5; JAX-SPF controls, n = 6; JAX-SPF + B6 serum, n = 8; JAX-SPF + *B6.IgA^{-/-}* serum, n = 6. p values were determined using a log-rank test. Representative of two independent experiments.

remains unclear. Defining the cell-cell interactions and molecular mechanisms through which effective and lasting IgA responses to enteric microbes are generated is essential for establishing the fundamental mechanisms underlying intestinal homeostasis, and for improving approaches to treat and avoid inflammatory

bowel disease and intestinal barrier disruption. We suggest that defining the mechanisms whereby intestinal homeostasis is maintained will require elucidation of the roles played by long-lived IgA-secreting plasma cells in this process, including a better understanding of how and why certain commensal microbes promote the induction of systemic IgA responses.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <https://doi.org/10.1016/j.chom.2018.01.005>.

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AUTHOR CONTRIBUTIONS

J.R.W. and D.A. designed experiments and wrote the manuscript; B.T.G. performed bioinformatics analyses; J.R.W., T.H., D.D.J., and C.A.G. performed experiments; D.G.A. performed all imaging experiments; S.D.C. cultured and provided anaerobic bacteria; and A.M.M. and D.P.B. analyzed 16S data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat Anti-Mouse Ig, Human ads-UNLB	Southern Biotech	Cat# 1010-01; RRID: AB_2687542
Anti-Mouse IgA (α -chain specific) – Peroxidase (goat)	Sigma-Aldrich	Cat# A4789; RRID: AB_258201
Lectin from <i>Arachis hypogaea</i> (PNA) FITC conjugated	Sigma-Aldrich	Cat# L7381
Biotin anti-mouse IgA	BioLegend	Cat# 407003; RRID: AB_315078
Brilliant Violet 785 anti-mouse CD19	BioLegend	Cat# 115543; RRID: AB_11218994
APC/Cy7 anti-mouse IgD	BioLegend	Cat# 405715; RRID: AB_10660304
Brilliant Violet 605 anti-mouse CD90.2 (Thy-1.2)	BioLegend	Cat# 140317; RRID: AB_11203724
PE/Cy7 anti-mouse TER-119	BioLegend	Cat# 116221; RRID: AB_2137789
Brilliant Violet 421 Streptavidin	BioLegend	Cat# 405226
PE Rat Anti-Mouse CD138 Clone 281-2	BD Biosciences	Cat# 553714; RRID: AB_395000
CD19 Monoclonal Antibody (6D5), APC-Cyanine5.5	Thermo Fisher	Cat# RM7719; RRID: AB_10373567
IgM Monoclonal Antibody (II/41), PerCP-eFluor 710	Thermo Fisher	Cat# 46-5790-82; RRID: AB_1834435
F4/80 Monoclonal Antibody (BM8), PE-Cyanine7	Thermo Fisher	Cat# 25-4801-82; RRID: AB_469653
CD4 Monoclonal Antibody (GK1.5), PE-Cyanine7	Thermo Fisher	Cat# 25-0041-82; RRID: AB_469576
CD8a Monoclonal Antibody (53-6.7), PE-Cyanine7	Thermo Fisher	Cat# 25-0081-82; RRID: AB_469584
CD38 Monoclonal Antibody (HIT2), Alexa Fluor 700	Thermo Fisher	Cat# 56-0389-41; RRID: AB_10852837
Ly-6G (Gr-1) Monoclonal Antibody (RB6-8C5), PE-Cyanine7	Thermo Fisher	Cat# 25-5931-82; RRID: AB_469663
APC Anti-Human/Mouse CD45R (B220) (RA3-6B2)	Tonbo Biosciences	Cat# 20-0452; RRID: AB_2621574
Bacterial and Virus Strains		
<i>Helicobacter muridarum</i>	Lee et al., 1992; obtained from American Type Culture Collection	ATCC 49282
Chemicals, Peptides, and Recombinant Proteins		
Liberase TL Research Grade	Sigma-Aldrich	Cat# 5401020001
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich	Cat# D5025
ExtrAvidin –Alkaline Phosphatase	Sigma-Aldrich	Cat# E2636
BCIP/NBT Liquid Substrate System	Sigma-Aldrich	Cat# B1911
SYTO BC Green Fluorescent Nucleic Acid Stain	Thermo Fisher	Cat# S34855
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermo Fisher	Cat# D1306
ProLong Diamond Antifade Mountant with DAPI	Thermo Fisher	Cat# P36962
AccuPrime Pfx SuperMix	Thermo Fisher	Cat# 12344040
SequalPrep Normalization Plate Kit, 96-well	Thermo Fisher	Cat# A1051001
BD OptEIA TMB Substrate Reagent Set	BD Biosciences	Cat# 555214
Zombie Aqua Fixable Viability Kit	BioLegend	Cat# 423101
PhiX Control Kit v3	Illumina	Cat# FC-110-3001
Critical Commercial Assays		
MasterPure Yeast DNA Purification Kit	Epicentre	Cat# MPY80200
DNeasy PowerSoil Kit	Qiagen	Cat# 12888
PureLink Genomic DNA Mini Kit	Thermo Fisher	Cat# K182000
MiSeq Reagent Kit v2 (500 cycle)	Illumina	Cat# MS-102-2003
Experimental Models: Organisms/Strains		
C57BL/6J	Jackson Laboratory	Stock# 000664
B6;129P- <i>Tcrb</i> ^{tm1Mom} <i>Tcrd</i> ^{tm1Mom} /J	Jackson Laboratory	Stock# 002121

(Continued on next page)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
B6.129S7-Rag1 ^{tm1Mom} /J	Jackson Laboratory	Stock# 002216
B6.Blimp1 ^{+/GFP} (Prdm1 ^{tm1Nutt})	Dr. Stephen Nutt (Kallies et al., 2004)	MGI:3510704
Oligonucleotides		
EUB338- AF594 /5Alex594N/GCTGCCTCCCGTAGGAGT	IDT (Amann et al., 1990)	NA
Software and Algorithms		
Prism v6.07	GraphPad	https://www.graphpad.com/
FlowJo v8.8.7	Treestar	https://www.flowjo.com/
Velocity v6.3	Perkin Elmer	http://cellularimaging.perkinelmer.com/downloads/detail.php?id=14
QIIME v1.8.0	Caporaso et al., 2010	NA
CD-HIT	Li and Godzik, 2006	NA
ImmunoSpot	CTL	http://www.immunospot.com/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David Allman (dallman@penmedicine.upenn.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Vertebrate Animals**

B6.Blimp1^{+/GFP} (Kallies et al., 2004), B6.RAG1^{-/-}, B6.TCRβ^{-/-}δ^{-/-}, B6.IgA^{-/-} and C57BL/6J (B6) mice were bred and maintained at the University of Pennsylvania. B6 mice termed 'JAX-SPF' were from Jackson Laboratories and housed in our SPF facility; these cages were kept in isolation and changed separately from other mice. Mice were all female to facilitate cohousing and aged 8-20 weeks at the time of euthanasia. All experiments were performed in accordance with the Office of Regulatory Affairs Institutional Animal Care and Use Committee.

Bacterial Strains

Helicobacter muridarum (ATCC 49282) was cultured on blood agar plates (BD BBL Prepared Plated Media R01202). Isolates were incubated for 48-72 hours at 37° C under microaerophilic conditions (Pack-Micro, Mitsubishi Gas Chemical America Inc R681005). Pure cultures were harvested and suspended in PBS before centrifugation and measurement of bacterial density by weight. Mice were given an oral gavage of 5mg of *H. muridarum* re-suspended in PBS.

METHOD DETAILS**Bacterial Flow Cytometry and Sorting**

For analyses and purification of serum IgA-bound bacteria samples were prepared and stained according to Palm et al (Palm et al., 2014). Bacteria were harvested by physical disruption of stool samples followed by light centrifugation to pellet debris. The resulting bacteria were stained with PE-anti-mouse IgA antibodies at a concentration of 1-12.5. Samples were then washed twice then stained with SytoBC and DAPI to identify viable bacteria (Ben-Amor et al., 2005). For serum IgA, samples were stained first with 50μl of a 1:2 serum dilution for 30 minutes on ice, then washed twice before adding PE-anti-IgA antibodies. Bacteria were run on LSRII or FACSARIA cytometers equipped with green (532nm) lasers. All purified bacteria were sorted twice as SytoBC⁺ DAPI^{low} IgA^{+/+} cells.

Tissue Preparation

Spleen and Peyer's patch cells were isolated by mechanical disruption of the tissue between frosted glass slides and filtration through 66μm nitex mesh. BM cells were isolated by flushing bones using a 23G needle and syringe with FACS buffer (PBS, 0.5% BSA, 1mM EDTA). siLP preparations were made using a modified protocol from Hall et al. (Hall et al., 2011). Small intestines were harvested and Peyer's patches, fat, and intestinal contents were removed. The cleaned small intestine was cut into ~2cm pieces before incubating at 37°C on a shaker for 20 minutes in RPMI 1640 with 20mM HEPES, 5mM EDTA, 1mM DTT, Penicillin/ Streptomycin, and 5% FBS. The small intestine pieces are then filtered over a large pore strainer and washed 2 times in RPMI with Pen/Strep, 2mM EDTA, and 20 mM HEPES. The pieces were minced and put into RPMI with Pen/Strep, 0.1mg/mL Liberase TL (Roche), 0.05% DNase I (Sigma D5025), and 20 mM HEPES for 30 minutes at 37°C while shaking. The Liberase reaction is then quenched with cold

RPMI with 10% FBS, 0.05% DNase I, Pen/Strep, 20mM HEPES and the small intestine is filtered through 70µm Corning cell strainers (Corning 352350). Before running siLP cells through the cell sorter or flow cytometer and before plating on ELISpot plates they are filtered again with 66µm nitex.

Flow Cytometry

Mouse cells were stained for flow cytometric analysis as previously described (Wilmore et al., 2017). Briefly, cell suspensions were stained with the Zombie Aqua Fixable Viability kit (BioLegend) according to the manufacturer's instructions. Then, cells were stained with a cocktail of optimal concentrations of antibodies in 100µl on ice for 30min., washed twice, and when needed stained with fluorescently labeled streptavidin at a predetermined concentration. The following antibodies were used for staining and analysis: PE-anti-CD138 (BD; 281-2), APC-Cy5.5-anti-CD19 (Thermo Fisher; RM7719), PerCP-eFluor710-anti-IgM (eBio; II/41), PE-Cy7-anti-CD4 (eBio; GK1.5), -CD8α (eBio; 53-6.7), -Gr1 (eBio; RB6-8C5), -F4/80 (eBio; BM8), -TER-119 (BioLegend), AF700-anti-CD38 (eBio; HIT2), APC-anti-B220 (Tonbo; RA3-6B2), FITC-PNA (Sigma), APC-Cy7-anti-IgD (BioLegend; 11-26c.2a), Biotin-anti-IgA (BioLegend; RMA-1), BrilliantViolet(BV)605-anti-Thy1.2 (BioLegend; 53-2.1), and BV785-anti-CD19 (BioLegend; 6D5). To detect biotin-conjugated antibodies we used streptavidin-BV421 (BioLegend).

ELISA/ELISpot Assays

ELISA and ELISpot assays were performed according to standard protocols. Briefly, plates (Thermo Fisher 442404) were coated with anti-mouse Ig(H+L) (Southern Biotech 1010-01) in a sodium carbonate/bicarbonate solution pH 9.6. *Helicobacter* ELISpot plates were coated with 200ng/well of heat-killed *H. muridarum*. Serum was serially diluted and detected using an HRP conjugated anti-mouse IgA antibody (Sigma A4789) and BD OptEIA TMB substrate (BD 555214). ELISpot plates (EMD Millipore MSIPS4W10) were coated the same as ELISA plates, but detection was performed with anti- IgM, IgA, IgG, κ, and λ antibodies conjugated to biotin (Southern Biotech). Biotinylated antibodies were revealed with streptavidin-alkaline phosphatase (Sigma E2636) and developed with BCIP/NBT (Sigma B1911). ELISpot plates were imaged and spots were counted using a CTL Immunospot Analyzer (Cellular Technologies Limited).

16S Sequencing

All sequencing analysis of the 16S V4 region was performed according to the SOP detailed by Kozich et al. (Kozich et al., 2013). Specifically, DNA from fecal bacteria was isolated using the DNeasy PowerSoil Kit (Qiagen). To isolate DNA from sorted bacteria we first used the MasterPure Yeast DNA Purification kit combined with a 10-minute high-speed vortex in 0.5mm glass bead tubes (Mo Bio). Then, the DNA extraction was completed with the PureLink Genomic DNA Mini Kit (Thermo Fisher). PCR was performed with barcoded 16S V4 oligonucleotides using AccuPrime Pfx SuperMix (Thermo Fisher), PCR products were then normalized using the SequalPrep Normalization Kit (Thermo Fisher), then pooled and sequenced on a MiSeq (Illumina) using a MiSeq Reagent Kit v2 500 cycle (Illumina) with 5% PhiX (Illumina).

Fluorescence In Situ Hybridization

4 mm-long pieces of the small intestine were cryoprotected in OCT compound (Fisher Health Care). 7 µm-thick transversal tissue slices were sectioned with a cryostat. FISH was performed following the protocol by Lowry et al. (Lowrey et al., 2015). Briefly, slides were fixed in 3.7% formaldehyde at RT and then incubated overnight at 4°C in 70% ETOH. Hybridization was performed overnight at 37°C in 10% formamide and 1X SSC (Saline Sodium Citrate) using 2 µg/mL of the probe EUB338 labeled with Alexa Fluor 594 (IDT). The EUB338 probe targets the 16S rRNA of ~90% of all bacteria found in the gut (Amann et al., 1990). Afterwards slides were washed thoroughly and mounted in ProLong Diamond Antifade with Dapi (Molecular Probes). All solutions were prepared with DEPC treated water. Images were obtained in a Zeiss LSM710 confocal microscope and analyzed using Volocity 6.3 (Perkin Elmer) software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

Data presented were analyzed with Prism v6 software (GraphPad) for statistical analysis. Data shown are presented as mean ± SEM and * denotes a significant p value of < 0.05. The p values were determined by a two-tailed unpaired student's t-test with Welch's correction or one-way ANOVA with Tukey's posttest where appropriate. Differences in survival were analyzed using a log-rank test.

16S Sequencing Analysis

Sequencing data was analyzed using the QIIME 1.8.0 software package (Caporaso et al., 2010). A total of 2,973,731 sequences were analyzed. Operational taxonomic unit (OTU) selection was performed using CD-HIT (97% identity threshold) (Li and Godzik, 2006). The RDP Classifier was used to assign taxonomy within QIIME at a 0.8 confidence threshold (Wang et al., 2007). Chimeric sequences were removed with ChimeraSlayer and rarefaction analysis was performed on a subsampled set of 15000 genes. PCoA plots were generated using unweighted UniFrac distances based on OTU tables generated in QIIME.

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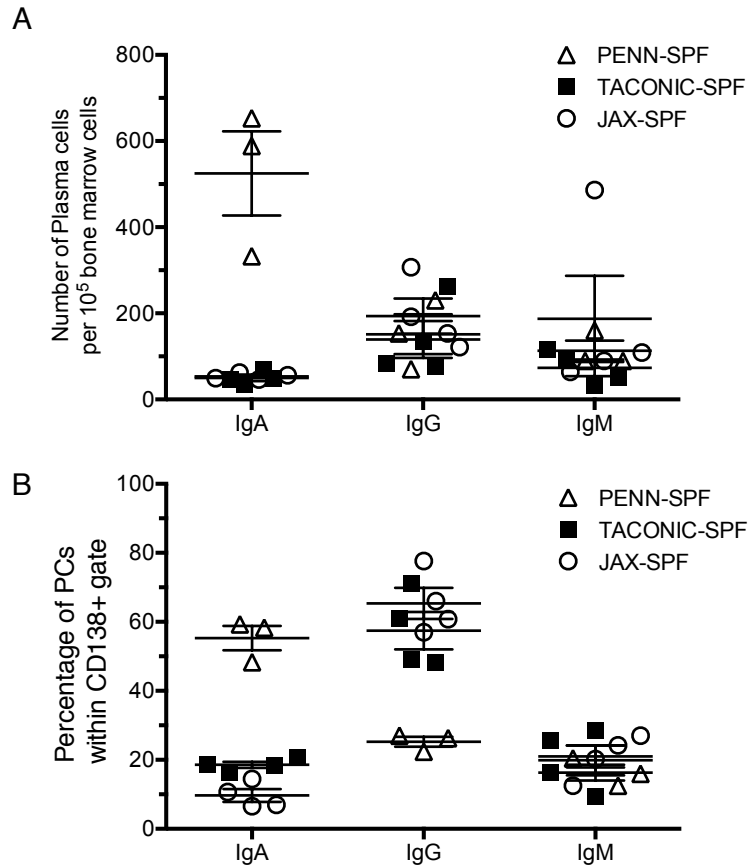
Supplemental Information

Commensal Microbes Induce Serum IgA

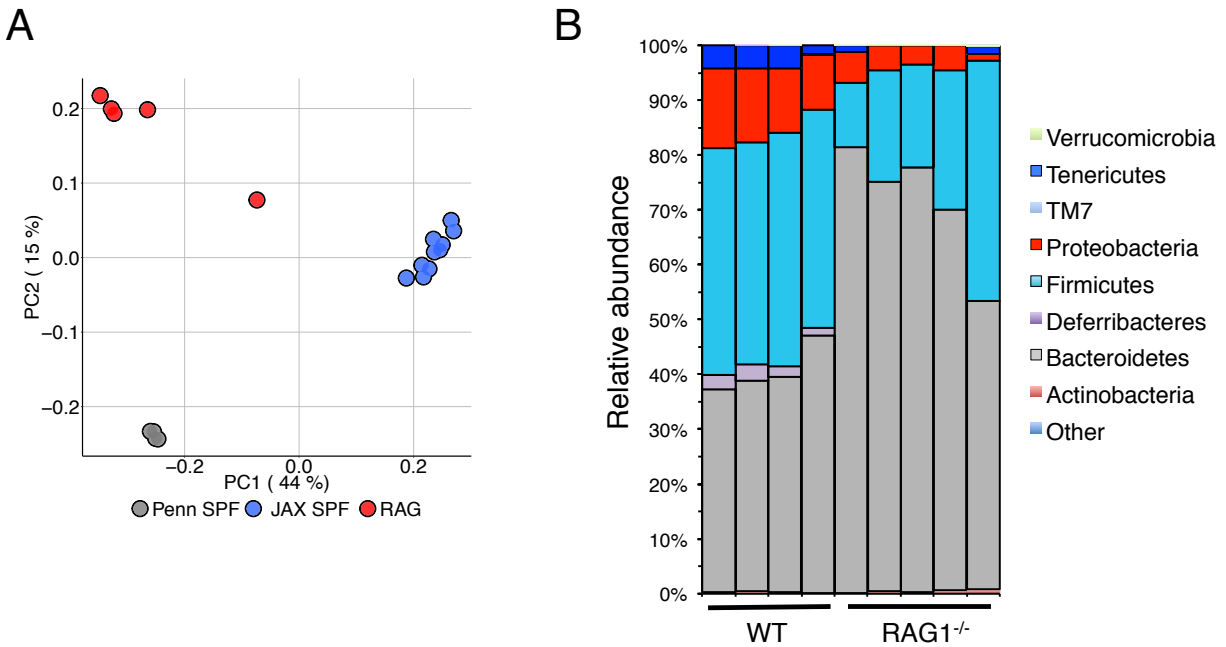
Responses that Protect against Polymicrobial Sepsis

Joel R. Wilmore, Brian T. Gaudette, Daniela Gomez Atria, Tina Hashemi, Derek D. Jones, Christopher A. Gardner, Stephen D. Cole, Ana M. Misic, Daniel P. Beiting, and David Allman

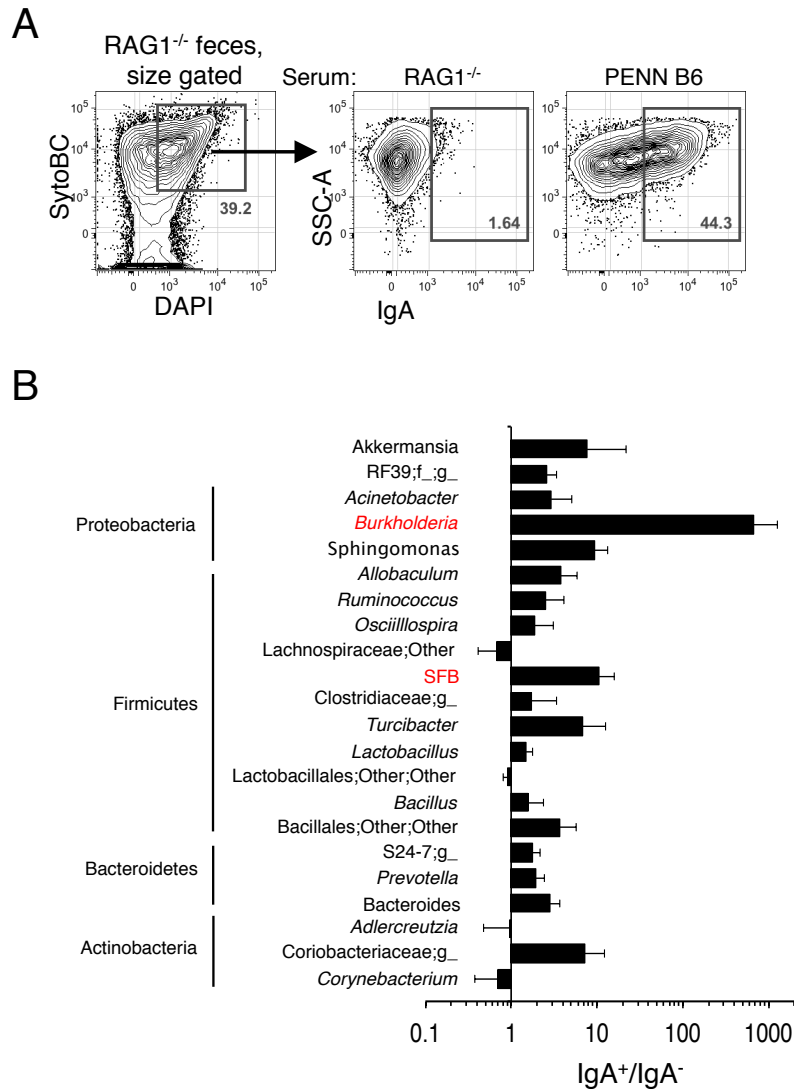
SUPPLEMENTAL INFORMATION



Supplemental Figure 1. Frequencies of IgA⁺ bone marrow plasma cells in B6 mice from Taconic Farms, related to Figure 1. 16-week old B6 mice were purchased from Jackson Labs, Taconic farms, or bred in house. **(A)** Total number of IgA, IgG, and IgM secreting PCs per 10⁵ BM cells determined by ELISpot. **(B)** Frequencies of the indicated plasma cell subset among all BM plasma cells were determined by flow cytometry focusing on Dump⁻ IgD⁻ Thy1.2⁻ CD138^{high} cells as illustrated in Figure 1. Horizontal lines indicate means and error bars SEMs for each group.



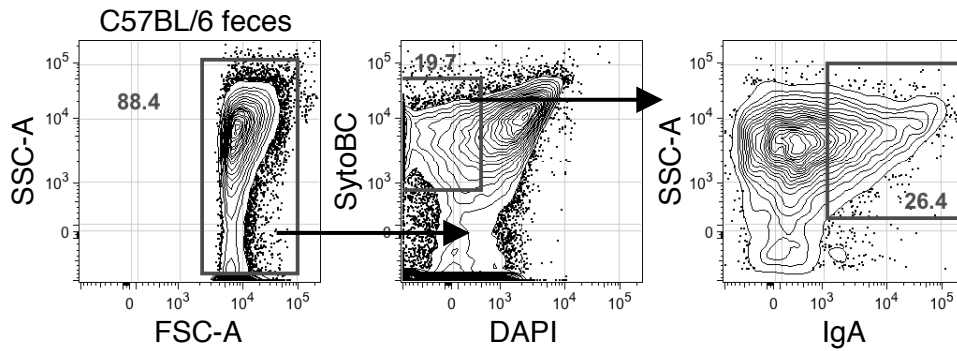
Supplemental Figure 2. Fecal microbiota in B6 and B6.RAG^{-/-} mice, related to Figure 4. Taxonomy for fecal bacteria in stool samples from individuals in the indicated group was determined by 16S V4 rDNA gene sequencing. **(A)** Principal coordinate analysis of the resulting data. **(B)** Relative abundance of the indicated phyla is shown.



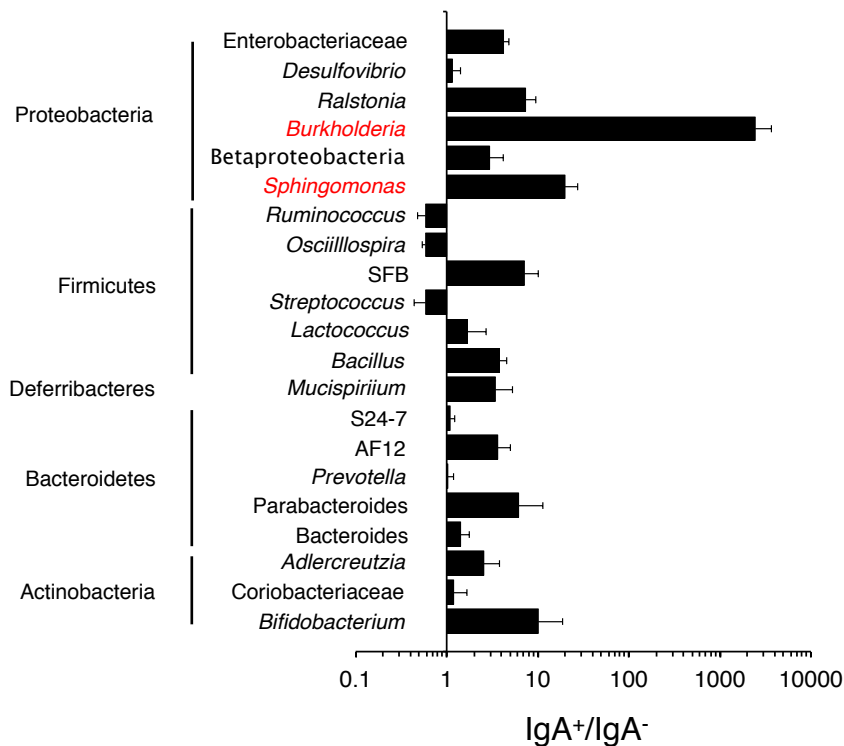
Supplemental Figure 3. Select taxa among serum IgA-bound bacteria, related to Figure 4.

(A) Fecal bacteria from B6.RAG1^{-/-} mice were stained with sera from the indicated mice, and IgA⁺ bacteria identified with PE-anti-IgA antibodies. (B) Fecal bacteria from RAG1^{-/-} mice were stained with sera from PENN-B6 adults and both IgA⁺ and IgA⁻ fraction within the DAPI⁺ SytoBC⁺ gate as shown in (A) sorted twice. DNA from these samples was then used for 16S V4 sequencing. The ratio of the relative abundance for each taxa in each fraction (IgA⁺, IgA⁻) was used to identify taxa enriched for IgA binding. Bacterial genera that were highly enriched (IgA⁺/IgA⁻ ≥ 10) are highlighted in red. Genera shown were selected because they possessed ICI scores above or below 3, or because of potential relevance based on previous studies.

A



B



Supplemental Figure 4. Select taxa bound by IgA in B6 mice, related to Figure 4. (A) Representative flow cytometric data of fecal bacteria stained with DAPI, Syto BC, and PE-anti-IgA from a Penn-SPF B6 adult. **(B)** Twice sorted bacteria from (A) were sequenced for the 16S V4 region to identify bacterial taxa in each population. As in Figure 5, highly coated bacteria (IgA⁺/IgA⁻ ≥ 10) are in red and the remaining groups were among the most enriched across all taxa.