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TLR5 AND ANTI-FLAGELLIN IGA ARE CRITICAL ELEMENTS TO QUENCHING BACTERIAL MOTILITY IN THE MAMMALIAN GUT

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The mammalian gut contains an incredibly diverse and abundant bacterial community that makes essential contributions to host metabolism and immune system priming. The close proximity of the intestinal epithelium with the gut microbiota poses serious challenges to the host, who in turn has developed a complex set of physical and immunological mechanisms to effectively manage this host-microbe relationship. A defect in any one of these mechanisms can result in opportunistic invasion of host tissues by otherwise commensal bacteria and this breakdown in homeostasis may contribute to numerous metabolic and gastrointestinal pathologies. Here, I investigate the role of two such mechanisms in maintaining homeostasis: Toll-like receptor 5 (TLR5), the innate immune receptor for bacterial flagellin, as well as IgA, which has previously been indicated to be aberrant in abundance and antigen specificity in the absence of TLR5. Using metatranscriptomics and biochemical assays, I found that TLR5^{-/-} mice harbor a bacterial community that exhibits increased flagellation. I also found that TLR5 is required to synthesize the amount of anti-flagellin IgA observed in healthy WT mice, and flagellin load appears to share an inverse relationship with levels of anti-flagellin IgA. I performed 16S rRNA analysis of the gut microbiota in mice with or without the capacity for producing IgA to better understand how IgA influences the stability and resilience of the bacterial community. Indeed, the lack of IgA resulted in reduced temporal stability and reduced community evenness, as well as increased susceptibility to antibiotic-induced perturbation. To specifically investigate the influence of TLR5 on IgA coating of the gut microbiota, I used

fluorescence activated cell sorting (FACS) combined with 16S rRNA analysis and found that the bacterial community of TLR5^{-/-} mice displays an aberrant profile of IgA coating, including reduced coating of Proteobacteria and increased coating of Firmicutes. With a combination of immunohistochemistry, fluorescent *in situ* hybridization, and fluorescent microscopy of intestinal tissues, I observed that TLR5^{-/-} mice have a defective mucosal barrier that results in bacterial invasion of villi in the small intestine and penetration deep into the colonic mucus layer. Together, these results highlight the critical role of TLR5 in maintaining gut homeostasis through directing the synthesis of anti-flagellin IgA and thus reducing levels of flagellation and motility in the bacterial community.

BIOGRAPHICAL SKETCH

Tyler Cullender was born and raised in Dallas, Texas, the son of Terry and Shirley Cullender. He graduated from the Texas Academy of Mathematics & Science and began his college career at Texas A&M University where he studied the endangered star cactus, *Astrophytum asterias*. Tyler then transferred to Rhodes College in Memphis, Tennessee where he obtained his Bachelors of Science degree in biology, with a minor in environmental science. While at Rhodes, Tyler was involved with the study of crayfish behavior and the invasive crayfish species *Orconectes rusticus*, as well as investigating the cell cycle of the yeast *Saccharomyces cerevisiae*. His interest in ecology and microbiology led Tyler to pursue a graduate program where he could meld the two fields.

In 2008, Tyler was accepted to the microbiology graduate program at Cornell University. The program allowed him to rotate through several microbial ecology labs before committing to a focus on the gut microbiome under the tutelage of Dr. Ruth Ley. Tyler honed his research to the interaction between the host immune system and genomics of the gut bacterial community, with an emphasis on secretory IgA and Toll-like receptor 5.

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THIS THESIS IS DEDICATED TO MY GRANDPARENTS,
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1. Background

The gastrointestinal tract of mammals is densely populated by a phylogenetically diverse suite of bacteria, commonly termed the gut microbiota. These bacteria outnumber our own cells by a factor of ten to one [1, 2] and harbor roughly 100 times as many genes as are found in the human genome [3]. The gut microbiota provides such benefits to the host as protection from infection by enteropathogens [4, 5], increased nutrient and energy extraction from the diet [6, 7], and priming and regulation of the immune system [8-10]. Conversely, the host provides its resident microbiota with a diversity of glycan nutrient sources and a protected anoxic environment [3]. The vast majority of the gut microbiota consists of strict anaerobes and this community is dominated by two phyla: Bacteroidetes and Firmicutes [11]. Minor populations of Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, Cyanobacteria, as well as methanogenic archaea are also common residents of the mammalian gut [11, 12]. As a whole, the gut microbiota can be considered as an additional “organ” that provides a multitude of essential functions to the host [3].

Toll-like receptor 5 (TLR5) is one of twelve known evolutionarily conserved TLRs in mammals [13]. TLRs are a critical part of the innate immune system that function as membrane-bound antigen-recognition receptors by recognizing conserved structures originating from bacteria, viruses, and fungi and collectively termed microbe-associated molecular patterns (MAMPs) [14]. TLR5 specifically recognizes bacterial flagellin, the protein monomer that comprises the bulk of the flagellar filament responsible for motility [15]. TLR5 is predominantly expressed by epithelial cells, monocytes, and dendritic cells [16-18], where upon binding to flagellin TLR5 induces the signaling adaptor MyD88 and activates a proinflammatory response

via the transcription factor, NF- κ B [15]. This signaling cascade induces the inflammatory cytokines TNF- α and IL-6 [15], which in turn stimulate various immunological responses such as acute phase response, recruitment of neutrophils, and increased phagocytosis by macrophages [19]. The recruitment of neutrophils to mucosal surfaces is thought to serve as a particularly important mechanism protecting against the spread of infiltrating bacteria, but may also drive the intestinal inflammation observed in models of immune dysregulation [20].

Deletion of TLR5 in mice has resulted in such diverse pathologies as intestinal inflammation [20], metabolic syndrome [21], and impaired CD4 T cell responses against flagellated pathogens [22]. The determinants that steer this outcome are unclear, but rederiving TLR5^{-/-} mice in order to change their gut microbiota to be more similar to mice housed at Jackson Laboratory (the world's largest supplier of research mice) resulted in attenuation of colitis and an increased prevalence of metabolic syndrome [21]. Placing TLR5^{-/-} mice on broad-spectrum antibiotic therapy alleviates many of the symptoms of metabolic syndrome [21]. Likewise, transfer of the gut microbiota of TLR5^{-/-} mice to germ-free WT recipient mice induces symptoms of metabolic syndrome, including increased weight gain [21]. TLR5^{-/-} mice display an inability to maintain a stable community of gut bacteria and elevated levels of Proteobacteria [23], suggesting that the commensal microbiota of these mice may also be less resistant to environmental perturbation. These findings suggest that the composition of the gut bacterial community is a major determinant in the physiological consequences of loss of TLR5.

Antibodies, also known as immunoglobulins, are large proteins produced by the B cells of all vertebrates and are developed to recognize unique structures, or antigens. There are five antibody isotypes produced in mammals, with IgA being the most abundant isotype by far [24,

25]. The bulk of IgA is secreted across mucosal surfaces in dimeric form [26], where it aids in barrier defense through a multitude of mechanisms. Over 95% of IgA that is secreted across mucosal surfaces is produced locally, either in Peyer's patches, isolated lymphoid follicles, or nonorganized lamina propria [25, 27]. As a result, anywhere between 24%-74% of bacteria in the gut appear to be coated with IgA [28, 29], which serves to limit bacterial adherence to the host epithelium and penetration of the mucosal layer. IgA is able to limit undue immune responses through mechanisms collectively termed "immune exclusion", which includes agglutination, entrapment in mucus, and peristaltic clearance of microbial antigens [30], thus preventing these antigens from interacting with proinflammatory receptors [31]. IgA is even capable of transcytosing bacterial antigens back into the lumen after they have invaded host tissues [32]. IgA is also thought to aid in temporal stability of the bacterial community through the establishment of biofilms in the gut [33, 34]. Together, these observations support the idea that IgA promotes gut homeostasis by defending against pathogens as well as promoting a stable gut microbiota without eliciting damaging proinflammatory responses [35].

There is accumulating evidence to suggest that TLR5 and IgA cooperate to maintain a homeostatic relationship between the gut microbiota and host. Germ-free mice express incredibly low levels of TLR5, which is upregulated upon colonization of the gut by commensal bacteria [36]. A prominent subset of dendritic cells (DCs), termed CD11c^{hi}CD11b^{hi}, reside in the lamina propria and express high levels of TLR5 [37]. Upon exposure to flagellin, these DCs promote the differentiation of B cells into flagellin-specific IgA-producing plasma cells [37]. Indeed, TLR5^{-/-} mice display defects in flagellin-specific adaptive immunity [38] and IgA-deficient mice display

a deleterious overactive innate immune response [39, 40], suggesting that a loss of either component negatively impacts the performance of the other.

It is not well understood how IgA functions once it is secreted into an external environment such as the intestinal lumen, despite the fact that IgA deficiency is the most common primary immunodeficiency in humans [41]. What is more, 5% of the human population carries an allele for a dominant nonfunctional TLR5 polymorphism [42], though the health effects of this trait are not well documented. New information on this subject is critical if effective therapeutic tools are to be developed. In this thesis, I investigate how anti-flagellin IgA and TLR5 cooperate to maintain barrier integrity at the interface between host intestinal tissues and the lumen. I hypothesize that TLR5 deficiency results in reduced production of anti-flagellin IgA, causing an increase in bacterial flagellation and inflammation of the gut. To address this hypothesis, I use a combination of biochemical assays, bacterial culturing, microscopy, and next-generation sequencing to assess how gut bacterial communities respond to host TLR5 deficiency and the consequences of this response on host health.

This dissertation is divided into three chapters that each focus on a particular facet of the interactions between the gut microbiota and its host, with an emphasis on the TLR5^{-/-} mouse model. In chapter one, I discuss my findings that TLR5 is required in order to produce sufficient anti-flagellin IgA to inhibit bacterial motility. I expand on my research of IgA in chapter two to better understand which bacteria IgA targets and the role of IgA in maintaining a gut microbiota that displays temporal evenness and stability. Finally, in chapter three I bolster my hypothesis that TLR5 deficiency results in decreased mucosal integrity and increased flagellation with the

use of *in situ* microscopy of the epithelium-lumen interface and show that a diverse phylogeny of commensal bacteria display reduced flagellation in the presence of anti-flagellin antibodies.

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CHAPTER 1

IGA QUENCHES FLAGELLA-BASED BACTERIAL MOTILITY IN THE GUT IN A TLR5-DEPENDENT MANNER

2.1 Summary

The mammalian gut uses a combination of physiology and immunity to inhibit its trillions of resident bacterial cells from eliciting harmful inflammatory responses. Immunoglobulin A (IgA) forms part of host barrier defense by binding bacteria to mucus, but questions remain about how IgA interacts with bacterial populations. Here, I report that IgA specific to bacterial flagellin directly suppresses motility across the microbiome and I confirm this finding *in vitro*. Host expression of Toll-like receptor 5 (TLR5) is required to synthesize sufficient anti-flagellin IgA, and flagellin load is inversely proportional to levels of anti-flagellin IgA in the mouse intestine. These results expand the role of IgA in barrier protection to include the quenching of gut microbial motility, and present a novel target for reducing the risk of metabolic inflammation.

2.2 Introduction

The spatial segregation of the microbiota away from epithelial surfaces is a critical element of gut homeostasis, as microbial penetration through the mucosal barrier causes inflammatory responses that can lead to colitis or metabolic inflammation [1, 2]. One important mechanism for maintaining barrier protection is immunoglobulin A (IgA), which both binds cells to mucus and induces their agglutination [3]. A healthy human adult secretes between 3-6 grams of IgA into the lumen daily, an amount that far exceeds the combined total of all other

antibodies' production in the body [4, 5]. Consequently up to three quarters of the 10-100 trillion microbes residing in the gut are coated with IgA [6, 7]. Recent work has shown that each host has an individual IgA repertoire [8] that is thought to mirror the individuality of their gut microbiome [9]. However, bacterial penetration into mucus and the resulting inflammation have been reported in animal models with deficiencies in the innate immune system [10], even when IgA levels in the gut are high [11]. Thus, IgA alone cannot inhibit the microbiota from penetrating the mucus, raising questions about how the innate immune system interacts with the adaptive immune system to maintain the spatial segregation of host and microbiota.

Directed motility in the gut may provide distinct advantages to host-adapted bacteria, such as increased penetration into the mucosa, but little is known about the fitness advantages of motility other than specific models of pathogenic infection. The ability to produce flagella is a trait widely shared by commensal gut bacteria representing a wide taxonomic diversity (e.g., many members of the phyla Firmicutes, Actinobacteria, and Proteobacteria are motile). Their capacity for motility is also evident from the motility genes recovered in healthy gut metagenomes [12, 13]. However, flagellin is a potent immune stimulator that could cause the host to mount an unwanted proinflammatory response against flagellated commensal bacteria. Furthermore, the assembly and use of flagella is metabolically expensive [14] and the intestinal environment nutrient-dense, such that flagellation in the gut is widely assumed unnecessary outside of select pathogens. In fact, flagellin levels are low in healthy human stool [15], suggesting low levels of motility overall. These observations suggest that a large fraction of the microbiota have the capacity for motility but do not express motility genes in the gut.

High levels of flagellin, along with barrier breakdown, have been associated with inflammation in Crohn's disease and colitis [16, 17]. Research is unable to link a specific

pathogen to these diseases and it has been suggested that a breakdown of host-microbiome homeostasis is responsible for their onset. It is unclear how a symbiotic microbiome develops into a bacterial community with pathogenic characteristics, but the increased rate of immunodeficiency among individuals with Crohn's disease, inflammatory bowel disease, and colitis suggest that loss of homeostasis is likely to stem from improper immune responses.

A few species of bacteria have been shown to downregulate the expression of specific antigens to evade antibody coating [3, 18]. Also, some reports have shown that IgA can alter bacterial gene expression [18-20], but whether IgA impacts the expression of motility genes is unknown. Unlike constitutively expressed antigens such as lipopolysaccharide, the expression of flagella is optional [21, 22], and motility is selected against in the healthy gut [23]. These results indicate that a failure of the host's adaptive immune system to restrain the inherent capacity for flagellation in the gut microbiome may be a risk factor for such diseases. We have shown that the microbiota of mice deficient in Toll-like receptor (TLR) 5 induce metabolic inflammation when transferred to wildtype (WT) germ-free recipients, and that this inflammatory microbiota is less temporally stable, and more enriched in Proteobacteria, compared to WT microbiota [24]. However, the host-microbial interactions underlying the shift in microbiota remain unclear.

Here, I investigate the importance of TLR5 in regulating gene expression of the gut microbiome and the role of IgA directed against bacterial flagella. I combine metagenomics and metatranscriptomics to analyze the functional capacity and active gene expression of the gut microbiome of TLR5^{-/-} mice compared to WT mice. I then use enzyme-linked immunosorbent assays (ELISAs) and a reporter cell assay to quantify the amount of flagellin and anti-flagellin antibodies present in the fecal and cecal contents of these mice. To determine the potential influence of antibodies on the fitness of flagellated bacteria, I employ a gnotobiotic mouse model

and compare the ability of a flagellated *E. coli* to thrive in the presence or absence of host antibodies. Next, I investigate the potential of anti-flagellin antibodies to stimulate downregulation of bacterial flagellin and inhibit bacterial motility using an engineered *E. coli* flagellin reporter strain as well as motility plate assays. Lastly, I extend my scope of investigation to find out if the phenotypic markers of TLR5^{-/-} mice are applicable to NLRC4^{-/-} mice, which lack the ability of intracellular flagellin detection, as well as normal weight and obese humans.

2.3 Results and Discussion

To assess how the absence of TLR5 alters the active function of the gut microbiome, I profiled the community-level gene expression of the entire microbiomes of five eight-week old TLR5^{-/-} mice and six WT mice of the same age using shotgun metatranscriptomic sequencing. I purified total RNA from cecal microbiomes and used custom probes to selectively pull down rRNA, thus enriching for mRNA, prior to cDNA synthesis and Illumina sequencing [25]. This approach resulted in between 289,970 to 2,670,042 reads per mouse (Table 1).

Table 1. Number of sequences generated for the metagenomic and meta-transcriptomic analyses. Sequences were generated the 454 Titanium and Illumina HiSeq2000 sequencing platforms. Values are linked to their corresponding samples on the MG-RAST server.

Metagenome		Metatranscriptome					
454 Titanium	Illumina HiSeq2000						
WT1	62,079	WT1	50,265,870	WT1	1,854,639		
WT2	81,981	WT2	36,401,312	WT2	867,799		
WT3	59,515	WT3	31,012,847	WT3	1,682,469		
WT4	74,533	WT4	53,119,526	WT4	391,021		
				WT5	633,254		
				WT6	1,059,826		
		TLR5-/-1	55,151	TLR5-/-1	29,535,962	TLR5-/-1	1,236,464
		TLR5-/-2	73,413	TLR5-/-2	33,884,214	TLR5-/-2	2,231,408
		TLR5-/-3	64,550	TLR5-/-3	14,850,852	TLR5-/-3	2,670,042
TLR5-/-4	27,581	TLR5-/-4	38,445,732	TLR5-/-4	289,970		
				TLR5-/-5	3,293,206		

Sequences were filtered for quality and uploaded to the metagenomics analysis server MG-RAST and potential functions were assigned using the Clusters of Orthologous Groups (COGs) annotation source. Taken as a whole, the metatranscriptomes of TLR5^{-/-} and WT mice were not significantly different. I then selected the COG categories that displayed at least a two-fold difference between the genotypes. Now, the metatranscriptomes segregated clearly by host genotype (Fig. 1), based on unsupervised hierarchical clustering of samples based on the enrichment or depletion of COGs in the metatranscriptomes. Notably, of the six COG categories significantly enriched in TLR5^{-/-} samples, three were associated with the manufacture and assembly of bacterial flagella.

Taxonomic assignment of the flagellum-associated genes over-expressed in TLR5^{-/-} transcriptomes compared to WT showed that these motility-related gene transcripts were expressed by phylogenetically diverse bacteria, including commensal bacteria such as butyrate-producing Firmicutes (*e.g.*, *Roseburia*, *Eubacterium*, *Clostridium*), Bacteroidetes (*Bacteroides pectinophilus*), and Proteobacteria (*e.g.*, *Desulfovibrio* spp.) (Figure 2). In total, 18 COGs in the TLR5^{-/-} metatranscriptome had significantly different abundances relative to WT. In addition to presenting an enrichment in flagellar motility in TLR5^{-/-}-mice, these COGs indicate a depletion in carbohydrate metabolism and antimicrobial peptide transport functions and are sufficient to reliably segregate samples by host genotype (Figure 1).

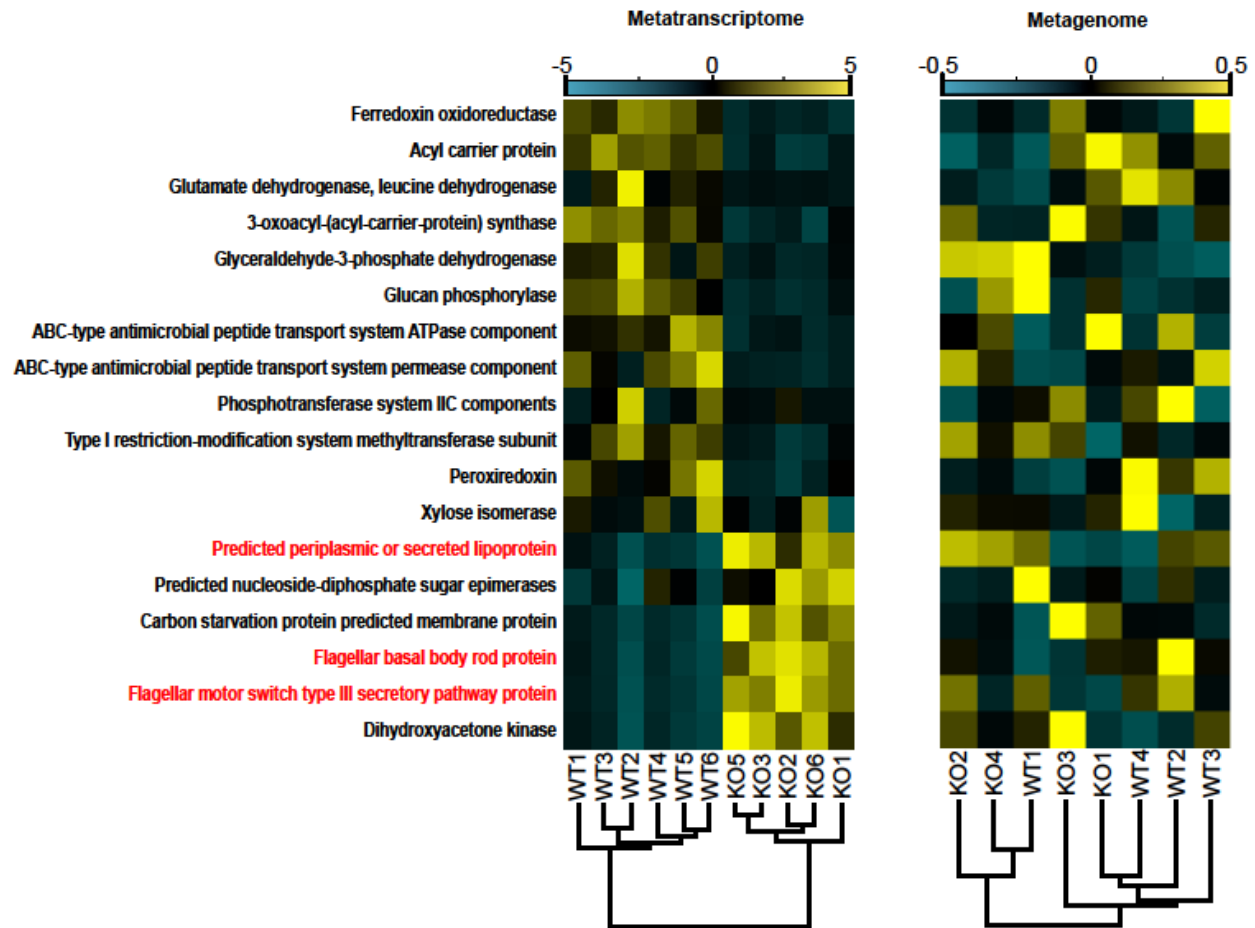


Figure 1. Flagella-related genes are upregulated in the TLR5^{-/-} metatranscriptome, but not in the metagenome. cDNAs assigned to the 18 most significant COG categories were normalized and hierarchically clustered. Dendrogram (bottom) shows clustering of samples based on the uncentered correlation similarity metric. Correlation coefficients are represented by color ranging from blue (-5x depletion relative to mean values across genotypes) to yellow (5x enrichment). Metagenomic reads from the same 18 COG categories were processed similarly. Functional categories are indicated in the legend; flagella-associated gene categories are highlighted in red.

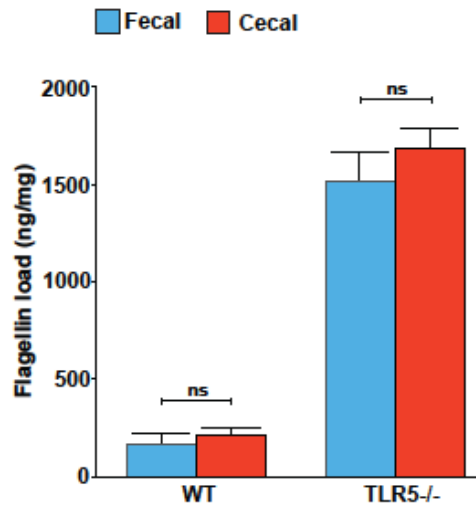


Figure 2. Metatranscriptomic reads annotated as flagellin are phylogenetically diverse. I used hierarchical classification and the Subsystems database in MG-RAST to annotate function. I isolated reads with an annotation of flagellin and assigned taxonomy using BLASTX with default arguments. Taxonomic assignments are shown to the species level, with abundance of reads corresponding to the key at bottom. Note that *Bacteroides pectinophilus* is a Firmicute based on 16S rRNA gene sequence analysis but misclassified in the phylum Bacteroidetes in the MG-RAST database.

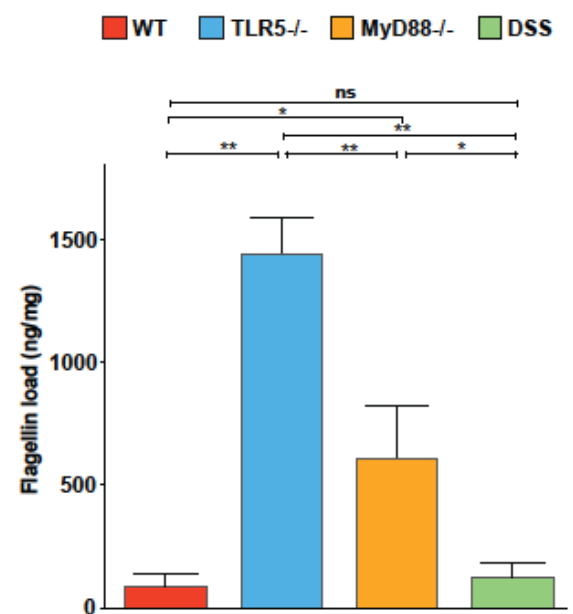
A difference in gene transcripts between genotypes could reflect differences in metagenomes (total gene content of the microbiome) rather than gene expression, so it is important to check the proportion of motility genes. Thus, I also characterized the gene content of the gut microbiomes of four eight-week old male TLR5^{-/-} mice and age/gender matched WT mice using shotgun metagenomics and the Illumina HiSeq2000 sequencing platform. This analysis did not reveal any significant differences between TLR5^{-/-} and WT microbiome functional gene content, including motility genes, suggesting that the shift in microbial diversity was too slight to impact the functional potential of the microbiota (Figure 1). I confirmed this finding and increased the sample size and average read length by repeating the analysis with new mice and sequencing using the 454 Titanium platform. The 454 dataset agreed with the functional assignments given to the Illumina reads. Thus, although the metagenomic analysis showed that the overall functional potential of the microbiota was similar between TLR5^{-/-} and WT hosts, the metatranscriptomic analysis revealed that motile bacteria belonging to a suite of different phyla upregulate their motility genes when anti-flagellin IgA production is low.

To verify that the greater transcription of flagella-associated genes in the TLR5^{-/-} host resulted in elevated levels of flagella, I estimated flagellin load using a TLR5-reporter cell assay [26]. This assay uses HEK293 cells that are engineered to secrete alkaline phosphatase in response to TLR5 stimulation, which can then be measured in the culture supernatant as a proxy for units of flagellin stimulation. I standardized flagellin levels by using the TLR5-stimulation potential of flagellin purified from *Salmonella typhimurium* strain 14028. I observed that flagellin levels were significantly elevated in the cecal contents and fecal pellets of TLR5^{-/-} mice (Figure 3A).

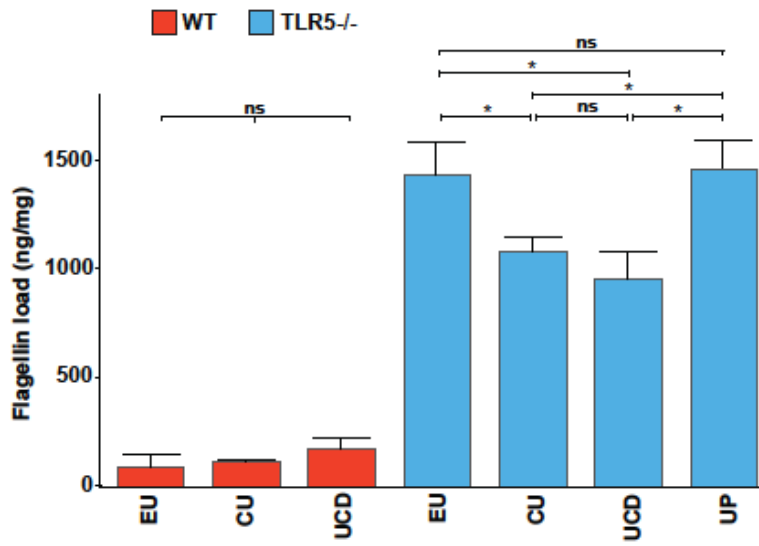
A.



B.



C.



D.

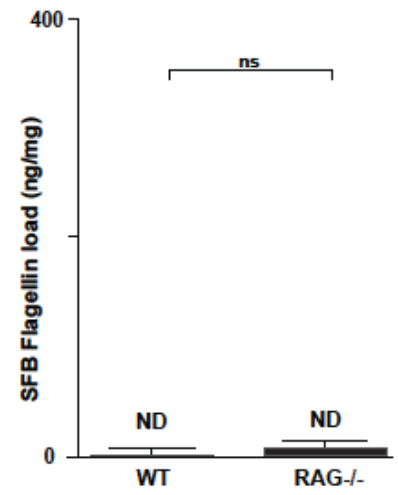


Figure 3. Flagellin load is consistently higher in TLR5^{-/-} mice relative to WT mice. I quantified flagellin load in the cecal contents of TLR5^{-/-}, MyD88^{-/-}, RAG1^{-/-} and WT mice with or without DSS treatment (n=5-8), aged 10-23 weeks. **A)** Quantification of flagellin load in TLR5^{-/-} and WT mice as well as cecal contents of the same mice. **B)** Quantification of flagellin load in MyD88^{-/-} and DSS-treated WT mice in addition to TLR5^{-/-} and non-treated WT mice for reference. **C)** Quantification of flagellin load in the feces of TLR5^{-/-} and WT mice from four different facilities. EU, Emory University; CU, Cornell University; UCD; University of California, Davis; UP, University of Pittsburgh. University of Pittsburgh was unable to provide a WT control sample. Columns represent mean concentrations of flagellin (\pm s.e.). **D)** Quantification of flagellin in WT and RAG1^{-/-} mice monocolonized with segmented filamentous bacteria (SFB). Values are below limit of detection. Mouse type is indicated on the x-axis in A) and C) and in the top legend in B) and D). *p < 0.01, **p < 0.001; two-tailed t-test; n.s., nonsignificant.

Since TLR5^{-/-} mice display elevated inflammatory markers, I needed to differentiate whether increased flagellin levels were a result of innate immune deficiency or simply inflammation. To do this, I used MyD88^{-/-} mice, which also have impaired TLR5 signaling (though partial *via* TRIF [27]) but display no elevation of inflammatory markers. I also used immunocompetent WT mice that were treated with dextran sulfate sodium (DSS) to chemically induce inflammation. Flagellin levels were significantly elevated in MyD88^{-/-} mice, but DSS-treated WT mice were similar to untreated WT (Figure 3B), supporting the role of TLR5 in this trait. To ensure that the increased flagellin load observed in TLR5^{-/-} mice was not simply due to aberrant microbiota in a single facility, I also measured flagellin load in TLR5^{-/-} from three additional facilities (Figure 3C). TLR5^{-/-} mice displayed increased flagellin load compared to WT, regardless of facility. Though still significantly elevated, flagellin levels in TLR5^{-/-} mice housed at Cornell University and University of California, Davis are reduced compared to the flagellin levels of TLR5^{-/-} mice housed at Emory University and University of Pittsburgh. This suggests that, though the trend is the same, there still may be variations in these facilities at the level of bacterial community or gene expression profiles.

Segmented filamentous bacteria (SFB) are common members of the gut microbiome of rodents, where they adhere tightly to the epithelium of the small intestine [28]. I became interested in SFB due to their potent effect on the host immune system [29] as well as the recent finding that the SFB genome possesses genes for flagella [30]. They have been shown to expand in abundance in mice lacking IgA [31] and in mice lacking MyD88 [32]. However, I did not identify gene transcripts from SFBs in our metatranscriptomes, despite the fact that they have been detected at low abundance in mice from this facility and are likely present [33]. SFB cannot currently be cultured *ex vivo* [34], so to determine if SFB flagellin is capable of stimulating

TLR5, I obtained cecal contents from gnotobiotic mice that were monocolonized with SFB by Daniel Peterson at the University of Nebraska–Lincoln. In addition to WT mice, I also used the cecal contents of RAG1^{-/-} mice that are incapable of producing IgA and may therefore maintain a higher bacterial load of SFB. I tested for the ability of cecal contents to simulate TLR5 *in vitro*, and could not detect any TLR5 stimulatory capacity for the microbiota of either mouse type (Figure 3D). These results suggest that either SFB do not express flagellin when in the ceca of mice or that SFB flagellin does not stimulate TLR5. Despite the abundance of SFB observed in rodents, there are only two reports of SFB recovered from the human intestine [28, 35]. An additional possibility is that the TLR5 protein used in this assay, which is of human origin, is unable to recognize SFB flagellin even though murine TLR5 is capable of doing so.

With the exception of a subset of mechanisms [36], B cells require CD4⁺ T cell activation before they can mature into antibody-producing plasma cells [37]. TLR5^{-/-} mice have an impaired T cell response to flagellin [38], suggesting that they may have insufficient plasma cells producing flagellin-specific antibodies. Furthermore, reduced anti-flagellin IgA levels have been noted in the serum of TLR5-deficient human hosts [39]. To determine if TLR5^{-/-} mice are impaired in their ability to produce the same proportion of flagellin-specific antibodies as is found in WT mice, I assessed the levels of total and flagellin-specific IgA and IgG in the feces of TLR5^{-/-} mice. I measured significantly lower levels of anti-flagellin IgA and IgG in the feces of TLR5^{-/-} compared to WT mice, despite higher total antibody concentrations (Figure 4). Anti-flagellin IgA levels were also low in MyD88^{-/-} mice, whereas levels in DSS-treated mice were normal. These data suggest that loss of TLR5 signaling leads to reduced anti-flagellin IgA production in the gut regardless of inflammation.

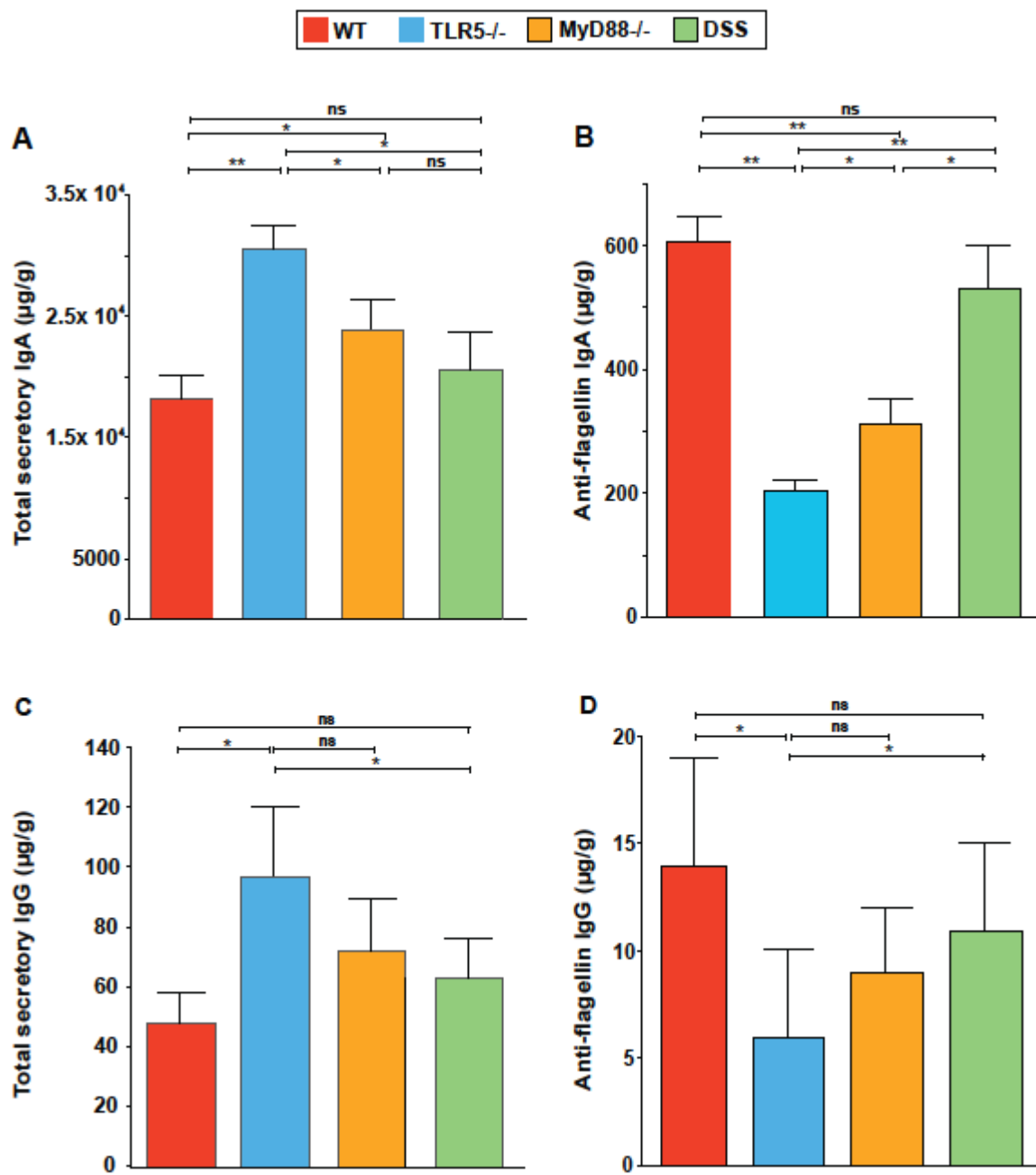
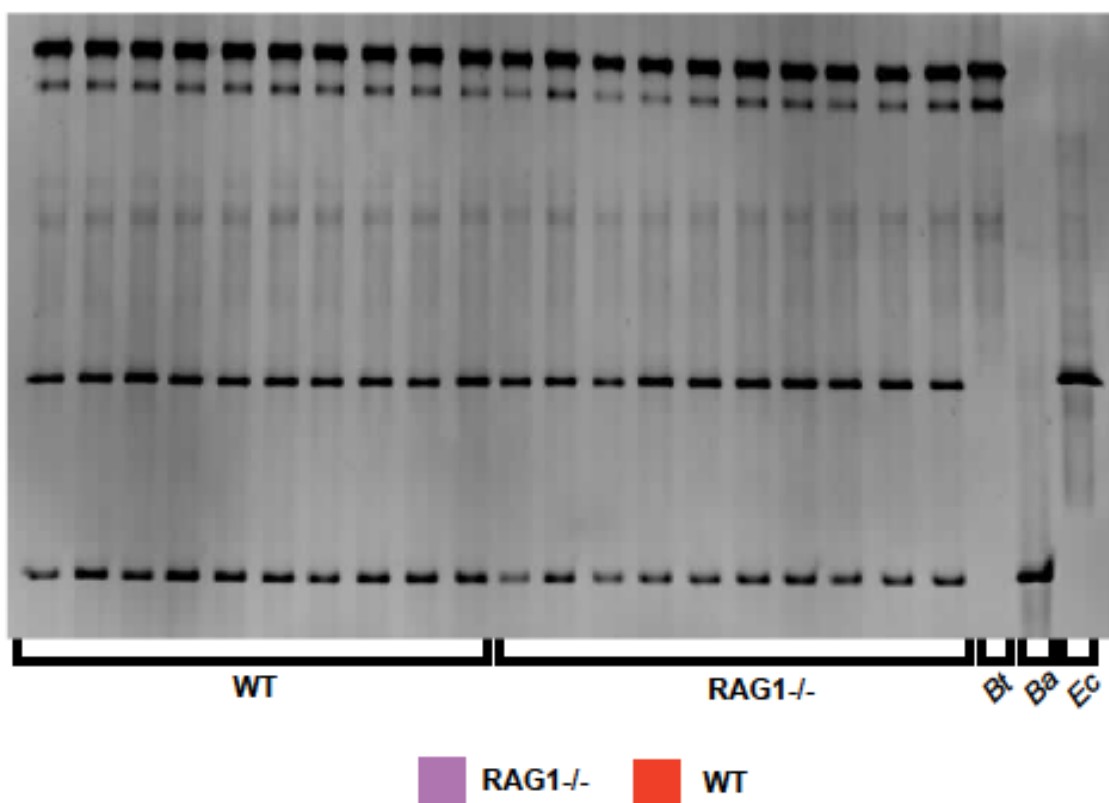


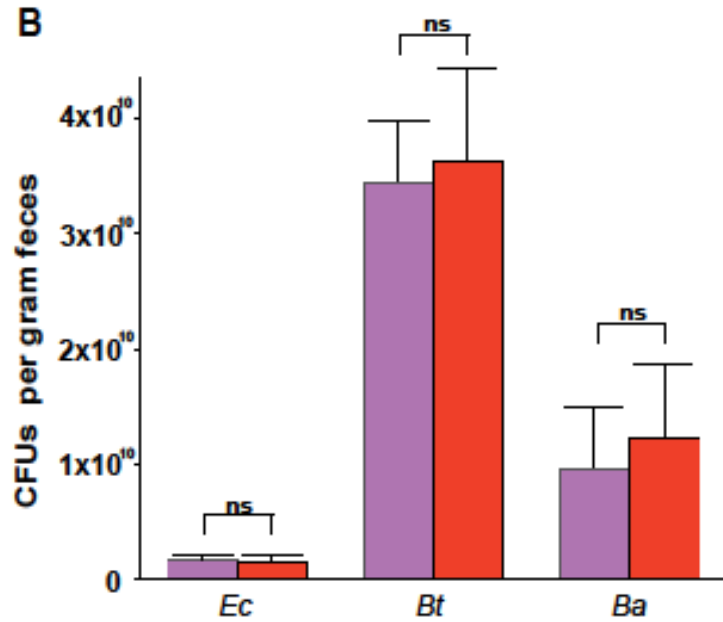
Figure 4. TLR5^{-/-} mice have reduced anti-flagellin immunoglobulins compared to WT mice, despite higher total immunoglobulin levels. I quantified anti-flagellin IgA and IgG as well as total IgA and IgG concentrations in the cecal contents of TLR5^{-/-}, MyD88^{-/-}, and WT mice with or without DSS treatment (n=8), aged 10-23 weeks. **A)** Total IgA concentration. **B)** Flagellin-specific IgA. **C)** Total IgG concentration. **D)** Flagellin-specific IgG. Mouse type is indicated in the top legend. *P < 0.01, **P< 0.001; two-tailed t-test; ns, nonsignificant.

There are several examples of IgA negatively impacting the fitness of commensal gut bacteria [31, 40]. To test if the competitive balance of the microbiota is altered in the absence of anti-flagellin IgA, I collaborated with Jens Walter and Laura Junker at University of Nebraska–Lincoln in using a simplified gnotobiotic system to reliably quantify the population of flagellated bacteria in RAG1^{-/-} mice. Jens Walter and Laura Junker colonized germ-free RAG1^{-/-} and WT mice with the motile *E. coli* MG1655 as well as the non-motile *Bifidobacterium adolescentis* FST-1 and non-motile *Bacteroides thetaiotaomicron* VPI-5482. The three bacterial species were quantified by Jens Walter and Laura Junker after one week using both plate culture methods and denaturing gradient gel electrophoresis (DGGE), (Figure 5A, B). Both methods showed that each of the three species colonized WT and RAG1^{-/-} mice equally well. I then measured flagellin in the RAG1^{-/-} mice and found them to be significantly higher than WT despite similar population levels of *E. coli* (Figure 5C). This suggests that the increased motility of *E. coli* did not significantly improve its competitiveness against the two non-motile species.

A



B



C

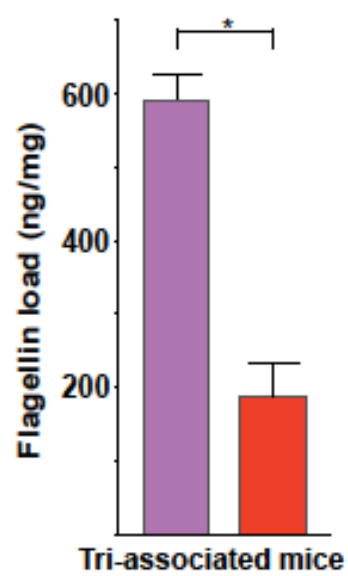


Figure 5. A lack of IgA is associated with increased flagellin load but does not significantly alter fitness of flagellated bacteria. Gnotobiotic WT and RAG1^{-/-} mice were colonized with *Bacteroides thetaiotaomicron* (Bt), *Bifidobacterium adolescentis* (Ba), and *E. coli* (Ec) and sacrificed after one week to assess bacterial communities (n=10). **A)** Denaturing gel gradient electrophoresis. Equal intensity bands across samples indicate equal population sizes, within a given species. **B)** CFU counts of bacteria cultured from the ceca. **C)** Flagellin load in ceca. Bars are means \pm s.e., * $p < 0.001$; two-tailed t-test; ns, non-significant.

To verify that antibodies impacted flagella production directly, I used a flagellin gene (*fliC*) reporter *E. coli* (courtesy of Anthony Hay, Cornell University). This *E. coli* is engineered to have a green fluorescent protein (GFP) gene that is regulated by a *fliC* promoter sequence and allows GFP signal to serve as a proxy for flagellin production. I measured the GFP signal of the *E. coli* in liquid medium to which I added anti-FliC antibody, anti-LPS antibody as a control, or glucose (a known inhibitor of flagellin expression [41, 42]). The addition of anti-LPS antibodies to the reporter culture had no effect on flagellin gene expression, whereas glucose and anti-FliC antibodies each reduced the expression of flagellin significantly within two hours of addition (Figure 6A). These data suggest that it is not simply antibody recognition of bacteria that causes a reduction in flagella, but rather the specific binding of the flagellar structure by anti-flagellin antibodies. Interestingly, the *E. coli* grew equally well with or without the addition of antibody, supporting the observation that motility did not offer *E. coli* a fitness advantage in the gnotobiotic mouse model.

Though this bioreporter provides important insight to the regulation of flagellin by *E. coli*, it does not address the question of whether anti-flagellin antibodies can actually affect motility. I confirmed the relevance of this bioreporter to the motility of *E. coli* with the use of 0.3% agar motility plates. I stab inoculated *E. coli* into the center of each culture plate either with or without the addition of anti-FliC or anti-LPS antibodies. Whereas anti-LPS antibodies had a partial impact on the size of the resulting motility rings, anti-FliC appeared to inhibit motility completely (Figure 6B). The discrepancy between anti-LPS reducing motility without inhibiting flagellin expression as observed with the bioreporter may be due to steric hindrance caused by large amounts of antibody binding to the cell surface of *E. coli*.

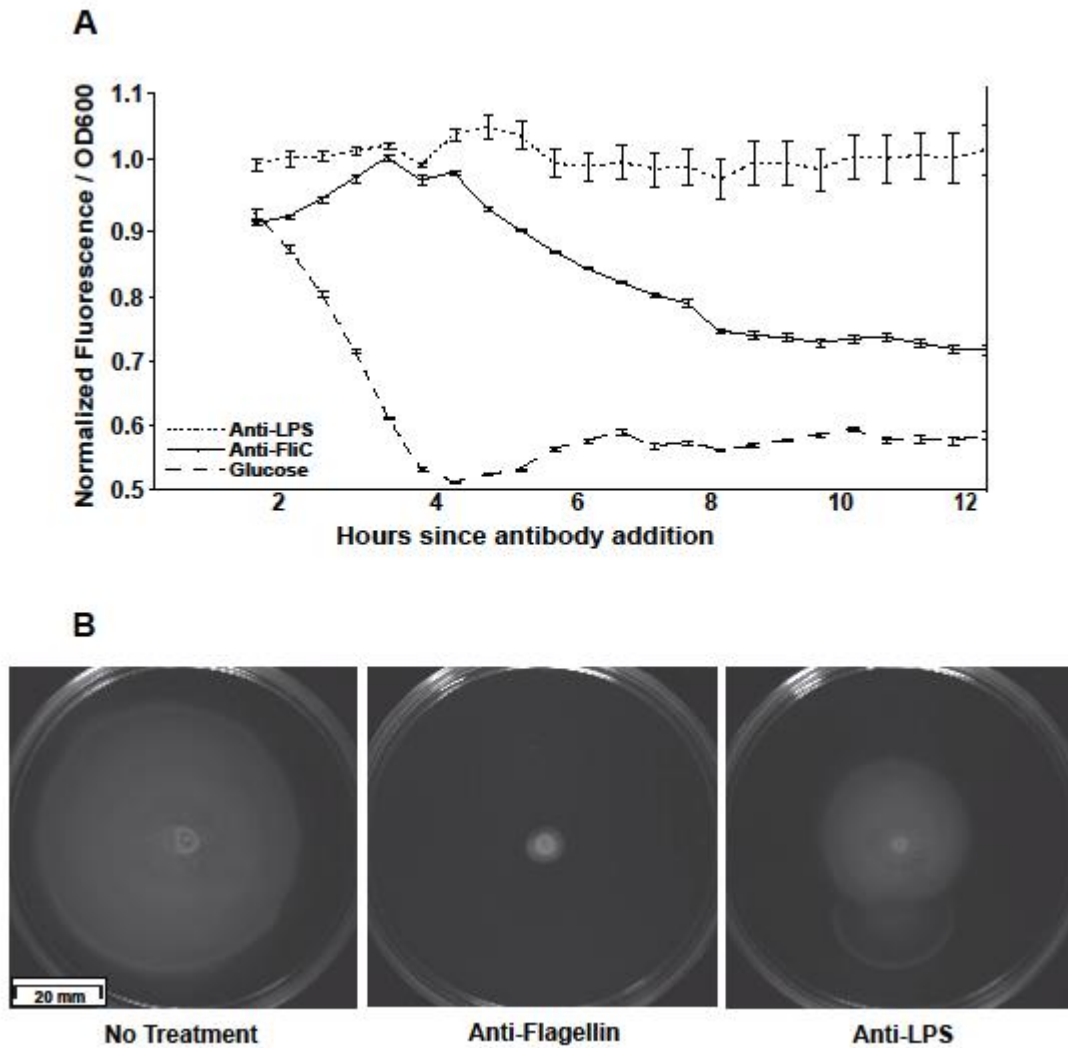


Figure 6. Anti-flagellin antibodies cause reduced expression of flagellin and reduced motility in *E. coli*. **A)** Normalized GFP signal from the engineered FliC-reporter *E. coli*, monitored for 12 hours after treatment with anti-LPS or anti-flagellin antibodies. Glucose is a negative control as it downregulates flagella expression. Means \pm s.e. for ratios of normalized GFP fluorescence:OD are plotted, $n=3/\text{group}$. **B)** I assessed inhibition of motility by plating *E. coli* onto motility plates containing 0.3% agar. Liquid culture with or without the addition of antibody was stabbed into the center of each plate. Plates were incubated at 37° for 14 hours.

The prevalence of metabolic syndrome and obesity among adults in the United States is reported to be 23.7% and 35.7%, respectively [48]. Obesity in humans is commonly associated with low-grade metabolic inflammation [49]. To better understand if my findings in mice with metabolic syndrome are transferrable to the human condition, I measured levels of anti-flagellin IgA and flagellin in fecal samples obtained from 43 healthy adults (62.5 ± 1.3 years old) with a range of body mass indices (BMI) from normal weight to obese. Consistent with my observations in mice, I found that anti-flagellin IgA levels were inversely proportional to flagellin in human feces ($R^2=0.39$, Figure 7A). Interestingly, fecal samples from normal-weight subjects ($18.5 \leq \text{BMI} < 25$) contained significantly higher levels of anti-flagellin IgA and lower flagellin compared to obese subjects ($\text{BMI} \geq 30$), while overweight subjects ($25 \leq \text{BMI} < 30$) had intermediate values (Figure 7B). Thus, I propose that excessive gut flagellin, which can result from an impaired adaptive immune response to flagellin, could be a risk factor for metabolic syndrome in humans. An alternative explanation might be that increased host adiposity stimulates increased flagellin production. However, reports of the ability of the gut microbiota to promote metabolic syndrome and insulin desensitization through stimulating host proinflammatory signaling [50, 51] suggest that an aberrant microbiota is more likely a cause than an effect of such phenotypes.

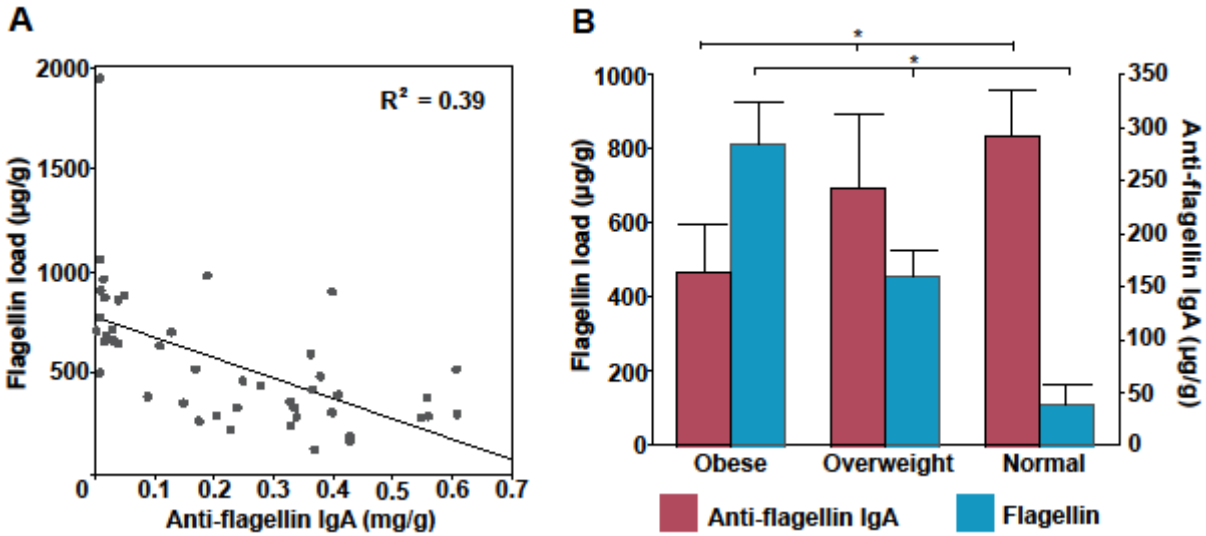


Figure 7. Flagellin load in feces is inversely correlated with anti-flagellin IgA in humans. A) Flagellin load (y-axis) correlated with anti-flagellin IgA concentration (x-axis) for 43 human stool samples. R^2 represents the coefficient of determination. **B)** Mean concentration \pm s.e. of flagellin load and anti-flagellin IgA concentration for human subjects segregated by their BMI of normal (18.5-24.9, N=17), overweight (25-29.9, N=11) or obese (>30 , N=15). Flagellin concentration is denoted on the left and anti-flagellin IgA concentration is denoted on the right. * $P < 0.05$; one-way analysis of variance (ANOVA).

2.4 Conclusion

I describe a dramatic shift in the actions of the gut microbiota due to a lack of the innate immune receptor TLR5. In TLR5^{-/-} mice the gut microbiota express increased transcripts relating to the structure and regulation of flagella, despite the lack of any discernible shift in the metagenomic potential of the bacterial community in relation to WT mice. I confirmed the increased flagellin load in the TLR5^{-/-} mouse gut using a cell reporter assay and suggest that this increase is in response to host immunodeficiency rather than inflammation. Not only are TLR5^{-/-} mice deficient in their innate immune response to flagellin, but they also secrete significantly fewer flagellin-specific antibodies into the gut.

These results suggest that antibody quenching of bacterial motility is a fundamental component of homeostasis in the mammalian gut, and that bacteria are active participants in this process. Several studies have shown that bacteria can evade IgA coating by halting production of the antigen to specific IgA [18-20]. IgA binds bacterial cells to mucus, inhibiting their penetration through the mucosal barrier and into host tissues and thereby staving off damaging inflammatory responses [1, 2, 52]. IgA also limits the association of bacteria with the epithelial cell surface [31] and plays a role in directing bacteria from the lumen to dendritic cells in the mucosal compartment [53]. My results suggest an additional, specific role of IgA: down-regulation of motility genes (*i.e.*, quenching motility), reducing the microbiota's ability to penetrate mucus and reducing the amount of highly immunoreactive protein (*i.e.*, flagellin) in the gut. This results in an active barrier of protection for the healthy host to maintain gut homeostasis and limit inflammation.

Flagella are critical in the pathogenesis of certain disease-causing bacteria [54, 55], but little is known about the role of flagella in commensal bacteria of mammalian hosts. Flagella are

metabolically expensive [14] and elicit powerful immune responses from the host [56], suggesting that commensal bacteria would only remain flagellated if this conferred a significant fitness advantage inside the mammalian gut. In fact, flagellated commensal *E. coli* are at such a disadvantage in the WT mouse gut that aflagellate mutants reach 90% prevalence within one week of colonization [23, 57]. Even so, it is possible that a flagellated commensal bacterium would have a selective advantage in an immunodeficient host. However, I did not observe that flagellated *E. coli* were more competitive in the RAG1^{-/-} mouse, which lacks antibodies. Since this *E. coli* still produced significantly more flagellin in the absence of antibodies, it is possible that the conferred fitness advantage was too subtle to detect with DGGE or plated colony counts.

Many of the healthy human gut's 100 trillion bacteria are capable of motility. Commensal members of the phyla Firmicutes and Proteobacteria possess motility genes that are readily detectable in healthy gut metagenomes [12, 13]. However, flagellin is only a small component of a healthy gut proteome [15]. The bacterial response to anti-flagellin antibodies (*i.e.*, reducing the expression of genes related to the structure, assembly, and regulation of flagella) appears to be evolutionarily conserved across phyla. I suggest that normal gut homeostasis requires a balance between motility allowing chemotaxis and immune pressure to quench production of the highly inflammatory flagellin protein. The inability to control commensal motility in the gut may be a factor predisposing a host to inflammation, and may represent a target for therapeutic intervention for the treatment and prevention of inflammation in the context of chronic intestinal inflammatory conditions and metabolic disease.

2.5 Materials and methods

Animal experiments: All mice used in this study were C57BL/6 strain. All mice used in metagenomics and metatranscriptomics analyses were maintained at Emory University. Mice

used in flagellin and antibody quantification were either (1) obtained from the Jackson Laboratories and maintained at Cornell University, (2) derived as previously described [24] and housed at Emory University, or (3) housed at the University of Nebraska–Lincoln. All gnotobiotic mice were developed and maintained at University of Nebraska–Lincoln. All animal experiments were approved by the local IACUCs. Additional TLR5^{-/-} ceca used to quantify flagellin load across facilities were obtained from University of Pittsburgh and University of California Davis (provided by David Hackam and Stephen McSorley, respectively). MyD88^{-/-} and DSS-treated mice were maintained at Emory University. For DSS treatment, C57BL/6 WT mice were administered 2.5% DSS (MBL Biomedicals) in drinking water for 7 days immediately preceding sample collection.

Metatranscriptomic analysis: For my samples, I used six age- and gender-matched eight week old male TLR5^{-/-} mice and WT mice, housed at Emory University. These mice were sacrificed and their cecal contents were immediately placed in RNAlater solution (Qiagen) and frozen. Samples were express shipped to Cornell University on dry ice. To prepare the samples for nucleic acid extraction, I thawed and briefly centrifuged the samples and pipetted off the supernatant containing the RNAlater solution. I divided cecal contents in half and extracted bulk RNA and bulk DNA in parallel using the Powersoil RNA Isolation kit and Powersoil DNA isolation kit, respectively, as described by the manufacturer (MoBio Laboratories Ltd, Carlsbad, CA).

I enriched the bulk RNA for nonribosomal RNA using a modification of a technique previously described [59]. This technique involves the construction of sample-specific probes to selectively deplete ribosomal RNA prior to cDNA synthesis. Briefly, I used a 2100 Bioanalyzer

(Agilent, Santa Clara, CA) and RNA 6000 Pico chip kit (Agilent, Santa Clara, CA) to detect the presence of ribosomal subunits based on size. Eukaryotic and Archaeal ribosomal subunits were not detected in the bulk RNA, thus no efforts were made to remove them. For each mouse, I generated sample-specific ribonucleotide probes targeting bacterial 16S and 23S rRNA genes by PCR amplifying these gene sequences from the bulk DNA using the universal primers 27F and 1492R for 16S and 189F and 2490R for 23S. I separately converted these 16S and 23S rRNA gene sequence amplicons to biotinylated antisense rRNA probes, which hybridized to complementary rRNA molecules in the total RNA sample. I confirmed subtraction efficiency by observing the absence of 16S and 23S rRNA peaks from the total RNA profiles using a 2100 Bioanalyzer and the RNA 6000 Pico chip kit. Next, I converted rRNA-subtracted samples to double-stranded cDNA, amplified via *in vitro* transcription, and converted back to double-stranded cDNA. This cDNA was used directly for pyrosequencing at the Core Laboratories Center at Cornell University using the Illumina HiSeq 2000 platform.

I used the LSU and SSU reference databases from SILVA (<http://www.arb-silva.de/>) to separate Illumina reads with >70% similarity to a database rRNA sequence. Using this approach, I identified 40.2% of the reads as ribosomal and removed them from downstream analysis. I uploaded the non-ribosomal reads to MG-RAST (33) with the default quality filtering. COG relative abundance data for protein-coding reads were summarized using MG-RAST (e-value < 10^{-5} ; ID > 50%; length > 20 aa). I uploaded functional assignments to Cluster 3.0 and centered and normalized counts before performing hierarchical clustering using the uncentered correlation similarity metric. This output was converted to a heatmap using Java Treeview 1.1.4r3 (34). To assign taxonomy to sequences annotated as flagellin, I used hierarchical classification and the Subsystems database in MG-RAST to annotate function using default cutoff parameters. I

isolated reads with an annotation of flagellin and assigned taxonomy using BLASTX (35) with default arguments. I visualized the BLAST results using MEGAN version 3.2.1 (36).

Metagenomic analysis: Four age/gender-matched 8-week old TLR5^{-/-} mice and WT mice were sacrificed and their ceca were immediately removed and snap frozen. I extracted bulk DNA as described above. I quantified the purified DNA using Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA). The products were assigned unique Multiplex Identifiers (MIDs) and sequenced at the Core Laboratories Center at Cornell University on the Roche 454 FLX platform. An additional 4 mice of each genotype were similarly processed and sequencing was performed at the DNA Sequencing Lab (Columbia University Medical center) using the Illumina HiSeq 2000 platform. I quality filtered sequence reads (trimmed ends at quality scores with code "B" or any ambiguous base) and uploaded to MG-RAST (33) with the default quality filtering and without identical read dereplication. Taxonomy assignments (LCA) and COG relative abundance data for protein-coding reads were summarized using MG-RAST (e-value < 10⁻⁵; ID > 50%; length > 20 aa).

Measurement of antibodies via ELISA: I measured total IgA, total IgG, flagellin-specific IgA, and flagellin-specific IgG in fecal and cecal samples by ELISA as previously described [60, 61]. Briefly, ELISA plates (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were coated overnight at 4°C with 1 µg/ml of purified flagellin from *Salmonella* Typhimurium strain 14028 (Enzo Life Sciences, Inc., Farmingdale, NY) or left uncoated for total antibody quantification. I prepared fecal and cecal samples by diluting to 0.25 mg/ml in PBS and homogenizing for 10 seconds using a Mini-Beadbeater-24 (BioSpec, Bartlesville, OK) without the addition of beads. The

detection antibodies that I used were goat anti-mouse IgA or anti-mouse IgG-HRP (Sigma, St. Louis, MO, USA) and I used TMB as a colorimetric reagent (Sigma, St. Louis, MO, USA). I stopped reactions with 0.18M H₂SO₄ before reading at 450 nm with a Synergy H1 plate reader (Biotek, Winooski, VT). Results presented here are representative for three total runs of the experiment, each of which included one technical replicate per sample.

Flagellin quantification: I quantified flagellin using HEK-Blue-hTLR5 cells according to the manufacturer (Invivogen, San Diego, CA). Briefly, I suspended between 10-100 mg of either cecal or fecal material in PBS at a concentration of 25 mg/ml and homogenized for 10 seconds using a Mini-Beadbeater-24 without the addition of beads (BioSpec, Bartlesville, OK). I then centrifuged the samples at 8,000g for 2 min, performed two 10-fold serial dilutions on the resulting supernatants, and applied 20 µl of each dilution to 180 µl of mammalian cells. After 20-24 h of incubation, I applied cell culture supernatant to HEK-Blue Detection medium (Invivogen, San Diego, CA) and measured alkaline phosphatase activity at 620 nm every 30 min for 3 h on a Synergy H1 multiple detection microplate reader (Biotek Instruments Inc., Winooski, VT). Presented results are representative for one repeat of the experiment and both experiments included one technical replicate per sample. I used purified *Salmonella typhimurium* strain 14028 flagellin (Enzo Life Sciences, Inc., Farmingdale, NY) to produce a standard curve. As a control for TLR5 specificity, I included one additional replicate per sample, per plate, to which I added anti-hTLR5 neutralizing antibody (5 µg/ml) (Invivogen, San Diego, CA). Alkaline phosphatase measurements in these wells represented nonspecific activity and were subtracted from the final quantification.

Monitoring flagellin expression of *E. coli* bioreporter: I grew *E. coli* strain PHL628 in minimal salts medium (MSM) containing 0.2% glucose (as an inhibitor of flagellin expression) and ampicillin (15 mg/L) at 37°C to an OD600 of 0.8 ± 0.2 . I then diluted the culture 1:100 in MSM containing either 0.2% glucose or 0.2% casamino acids. Casamino acids aid *E. coli* to grow in the absence of glucose without affecting flagellin production. I divided cultures into 100 μ l aliquots in 96 well clear flat-bottom plates (Costar, Corning, NY) and incubated at 37°C with periodic shaking on a Synergy H1, multiple detection microplate reader (Biotek Instruments Inc., Winooski, VT). OD600 and fluorescence of the *fliC* promoter fusion (Abs/Em: 488/509 nm) were measured every 30 minutes over the course of 15 hours. I added antibodies after 2 hours of growth at a concentration of (5 μ g/ml) [64]. The fluorescence values were normalized to growth. Results presented are representative for three repeats of the experiment, each of which included one technical replicate per sample.

Motility plates: I confirmed downregulation of flagella using Luria-Bertani (LB) broth motility plates containing 0.3% agar. I grew *E. coli* strain BW25113 in liquid LB at 37°C to an OD600 of 0.8 ± 0.2 . I then stab inoculated 5 μ l of liquid culture with or without the addition of antibody (0.1 mg/ml) into the center of each plate. Plates were incubated at 37°C for 14 hours and imaged immediately using a BioDoc-It Imaging System (UVP, Upland, CA). Results are representative for two repeats of the experiment.

Statistics: I performed statistical analysis and linear regression analysis in Microsoft Excel 2011, StatPlus 2009, R version 2.11.1, and IBM SPSS Statistics. Statistical tests were 1-way ANOVA, unpaired student '*t*'-test, or generalized mixed linear model, as indicated. Significance level of

p<0.05 unless otherwise indicated.

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CHAPTER 2

LOSS OF TLR5 ALTERS MICROBIOTA-ANTIBODY ASSOCIATIONS IN THE MOUSE GUT, WHICH NORMALLY AID IN MAINTAINING A STABLE AND RESILIENT GUT MICROBIOME

3.1 Summary

Temporal stability of the gut microbiota, as well as the microbiota's resilience in the face of environmental perturbation, are increasingly acknowledged as hallmarks of a healthy gut ecosystem. This homeostasis requires constant inputs from the host through a healthy lifestyle and immunological response - particularly, the effective production and secretion of IgA into the intestinal lumen. Defective IgA responses to commensal bacteria are implicated in multiple gastrointestinal disorders including inflammatory bowel disease and colitis. To better understand the role of IgA in maintaining a stable bacterial community, I performed 16S rRNA sequencing of gut bacteria in mice with and without IgA-producing capabilities over a period of two months. I found that IgA is critical in maintaining community evenness and resiliency to environmental perturbation. TLR5 is critical to the proper functioning of IgA, so I combined fluorescence activated cell sorting (FACS) with 16S rRNA sequencing to investigate which members of the gut bacterial community were coated with IgA in TLR5^{-/-} mice and how this differed from immunocompetent WT mice. I found that Proteobacteria, which are in greater abundance in the gut of TLR5^{-/-} mice as well as other immunodeficient models, were significantly less coated with IgA in TLR5^{-/-} mice compared to WT mice, while the opposite pattern was true for Firmicutes. These results increase our understanding of the role of IgA in maintaining gut homeostasis, suggesting that metabolic syndrome and the dysbiosis observed in TLR5^{-/-} mice result from defects in both the innate and adaptive immune systems.

3.2 Introduction

Previous studies have made the observation that gastrointestinal disorders are associated with temporally unstable bacterial communities [1, 2], while healthy individuals maintain a stable microbiome [3, 4]. Of course, stability in this system is not absolute, and even healthy individuals experience some degree of fluctuation in the composition of their microbiota due to environmental factors like diet and medication [5]. The important differences between a stable bacterial community and one that is unstable are that a stable community has a significant amount of functional redundancy [6] and an ability to effectively revert to the previous community dynamics after a perturbation [7]. These observations should expand our understanding of gut homeostasis to not only refer to the dynamic cross-talk between host tissues and the microbiota to effectively limit inflammation, but to also encompass host mechanisms that allow the microbiota to remain stable over time. However, little is known about the mechanisms that maintain temporal community stability or allow for effective recovery after an environmental perturbation.

Immunoglobulin A (IgA) is the most abundant antibody produced in mammals, and the majority of IgA is secreted across mucus membranes, such as the lining of the gastrointestinal tract [8, 9]. IgA protects the host through a multitude of mechanisms, including inhibiting host absorption of immunogenic particles like toxins and viruses [10-12] and interference with microbial adherence to the epithelium [13]. IgA is also argued to aid in the establishment and temporal stability of the commensal microbiota through such functions as assistance in biofilm development [14, 15]. The relationship between IgA and the commensal microbiota has received little attention, but the status of IgA deficiency as the most common primary immunodeficiency

in humans, coupled with the high incidence of infection and allergy in individuals with this deficiency [16], points to the value of investigating this host-microbe relationship.

IgA recognition of the commensal microbiota is a normal mechanism in mammalian physiology and between 24-74% of fecal bacteria are coated with IgA in healthy humans [17]. Attempts to correlate IgA coating frequency of the commensal microbiota with various states of health suggest that IgA coating is elevated in inflammatory bowel disease and reduced in irritable bowel syndrome [18], while lifestyle changes resulting in weight loss are also reported to reduce IgA coating frequency [19]. A decreased frequency of IgA coating of bacteria may indicate that the host immune response is underactive, which could correlate with increased susceptibility to pathogens. Conversely, a high frequency of IgA coating may indicate an overactive immune system that mounts defenses unnecessarily against commensal bacteria, food antigens, or even self-antigens. However, the frequency of bacteria coated with IgA in these disease states is still within the range reported for healthy individuals, so it is unlikely that such measurements are of clinical significance on their own. A healthy host-microbiota interaction is likely to include elements of immunological tolerance to antigens as well as directed and preferential IgA coating [20], which suggests that the types of bacteria or bacterial antigens coated with IgA are more indicative of disease state than is the total proportion of the microbiota coated with IgA.

Several findings indicate that the absence of TLR5 causes profound alterations in host IgA, such as significantly increased IgA production [21], decreased anti-flagellin IgA (Chapter 1), and an impaired T cell response to flagellin [22]. Furthermore, TLR5^{-/-} mice exhibit a compensatory upregulation of TLR4 [23], which indirectly spurs a further increase in IgA production [24]. These observations suggest that TLR5 is critical not only to the effective

production of anti-flagellin IgA but also other components of the IgA repertoire and their relationship with the commensal microbiota. If the development of gut inflammation observed in TLR5^{-/-} is due not only to their inability to manage bacterial flagellin production but also improper interactions between IgA and the gut commensal microbiota, this mouse model might exhibit an immune defect applicable to a whole host of gastrointestinal disorders.

In this study, I investigate the implications of IgA deficiency on the composition of the gut bacterial community as well as the community's temporal stability and response to perturbation. A two month time course of 16S rRNA-based sequencing and analysis was performed on the gut microbiota of RAG1^{-/-} mice, which cannot produce T cells or B cells, and thus do not produce antibodies. In addition to quantifying the temporal stability of the gut microbiota of these mice, the antibiotic clindamycin was administered for a portion of the time course in order to investigate how well the bacterial community recovers from environmental perturbation. Finally, I used fluorescence activated cell sorting (FACS) combined with 16S rRNA pyrosequencing to compare the IgA-bound gut bacterial population to the population not bound to IgA, and how these two fractions of the microbiota differ between WT and TLR5^{-/-} mice.

3.3 Results and Discussion

To gain a better understanding of the influence of the host's adaptive immune system on the composition, stability, and resilience of the gut microbiota, I performed a 16S rRNA-based analysis of the fecal pellets of RAG1^{-/-} mice and age-matched WT mice over the course of two months. These mice were maintained by Daniel Peterson at the University of Nebraska–Lincoln.

A UniFrac principal coordinates analysis (PCoA) plot, which measures the phylogenetic similarity between communities based on sequence divergence, reveals that the two mouse genotypes harbor fecal microbiotas that do not clearly segregate (Figure 1). Curiously, the microbiotas of RAG1^{-/-} and WT mice begin to diverge after the twelfth time point and this divergence is also apparent in the cecal contents that were collected at the end of the study. The divergence does not appear to be genotype specific, but, rather, the bacterial communities of both genotypes shift in similar directions. On the date of the twelfth time point there was heavy building construction close to the housing facility (introducing noise and vibration) as well as the weekly cage change and a thorough floor washing of the facility. This collection of disturbances may have been sufficient to profoundly alter the composition of the gut bacterial community. Since these factors cannot be defined or quantified, these confounding time points after the eleventh fecal collection were excluded from downstream analysis.

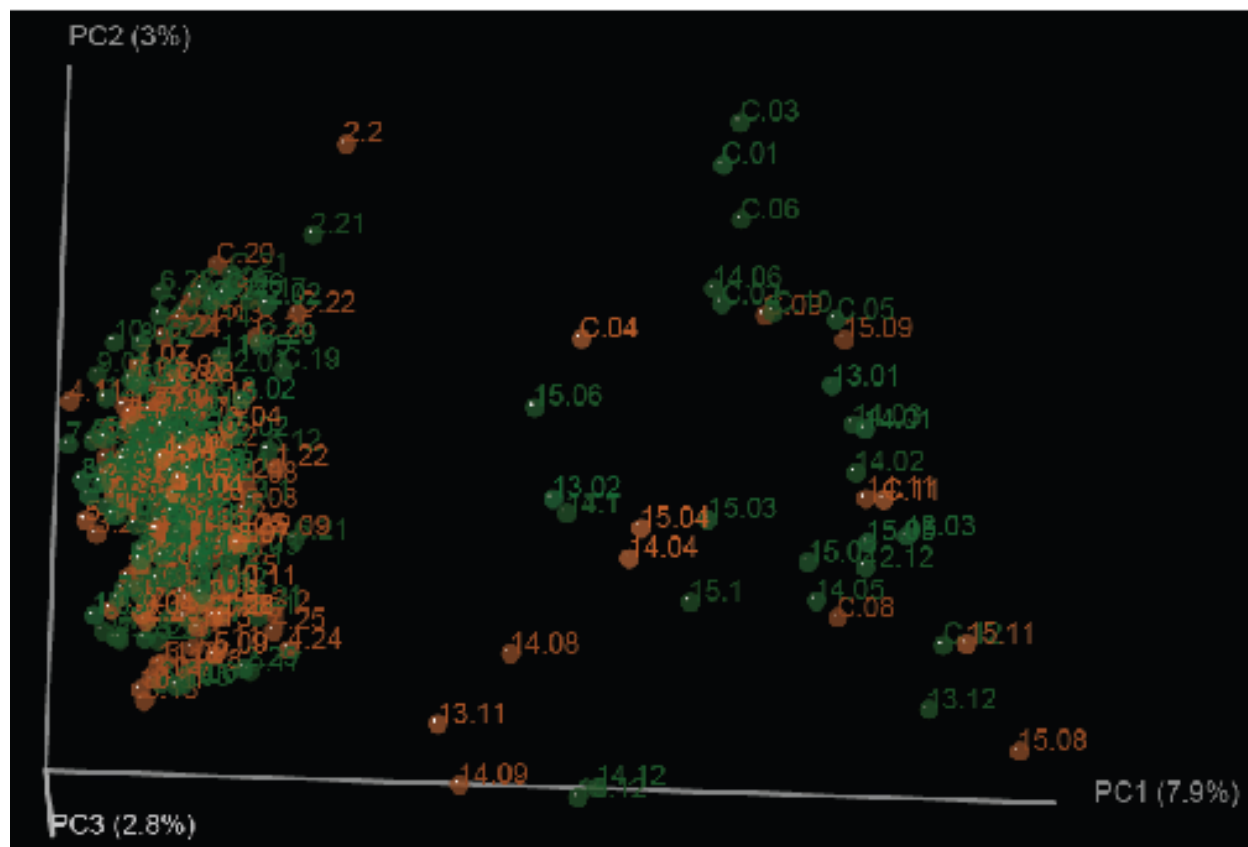


Figure 1. Principal coordinates analysis of 16S rRNA sequences does not clearly segregate the gut bacterial communities of RAG1^{-/-} mice and WT mice. The first three principal coordinates (PC1, PC2, PC3) from the principal coordinates analysis constructed from the unweighted UniFrac are plotted for each sample. Each dot represents the bacterial community at a particular time point and is labeled as 'time point.mouse number' for RAG1^{-/-} (orange) or WT (green). The variance explained by the PCs is indicated in the parentheses on the axes.

To investigate how the gut microbiota respond to a defined perturbation in the absence of the host's adaptive immune system, clindamycin was administered subcutaneously for four consecutive days, beginning on time point seven. I chose clindamycin because administration of this antibiotic is often associated with post-therapeutic *Clostridium difficile* infection and colitis in clinical settings [25], suggesting that clindamycin is a good candidate for studying perturbation and resiliency of the microbiota. It is unlikely that clindamycin was responsible for the dramatic shift in the microbial communities that was observed after time point twelve because two weeks separate the first antibiotic administration and the dramatic community shift, which would be an extremely delayed onset for clindamycin [26, 27]. The antibiotic did not appear to alter the bacterial community dramatically based on the similar UniFrac distances and phylum compositions before and after administration of clindamycin (Figure 1, Figure 2), but administration did correspond with an increase in the frequency of Proteobacteria. Further, the increase in Proteobacteria was significantly greater in RAG1^{-/-} mice than in WT mice ($p = 0.013$, paired 2-tailed t-test). Interestingly, many γ -Proteobacteria including the genera *Pseudomonas* and *Enterobacter* show resistance to clindamycin [28, 29], which may explain why this phylum was able to expand its presence in the community following the antibiotic challenge. None of the other phyla were significantly different between the mouse genotypes before or after the antibiotic challenge. An expansion of Proteobacteria in the gut microbiota has been repeatedly observed in numerous intestinal disorders [30], even when a pathogen cannot be identified or the pathogen does not belong to the Proteobacteria phylum [31, 32]. These findings suggest that Proteobacteria are particularly adept at expanding during or following gut perturbations and the inability of a host to manage populations of commensal Proteobacteria could be indicative of a disease state.

Though I did not directly measure the functional stability of the gut microbiota of RAG1^{-/-} mice and WT mice over time, I did calculate the Gini coefficient of these communities. The Gini coefficient is a metric of community evenness, with lower numbers representing increasing evenness. Previous studies of bacterial communities have used this metric and found that increased community evenness correlates well with functional stability [33, 34], suggesting that the gut microbiota of a healthy host should have a lower Gini coefficient compared to the microbiota of diseased or immunocompromised hosts. Indeed, the Gini coefficient of the microbiota of RAG1^{-/-} mice is significantly higher than the microbiota of WT mice ($p = 0.008$, paired 1-tailed t-test), especially after the administration of clindamycin ($p = 0.004$, paired 1-tailed t-test), (Figure 3). Thus, I observed that higher evenness and stability of the gut microbiota was associated with healthy WT mice, while reduced evenness and stability of the gut microbiota was associated with RAG1 immunodeficiency, which predisposes the host to disease symptoms such as chronic skin inflammation and granuloma development [35]. Therefore, these data suggest that the adaptive immune system is critical to maintaining a gut microbiota that is both functionally stable and more resistant to perturbation—two characteristics of healthy communities. In this sense, a properly functioning immune system is critical not only to protection from pathogenic insult, as conventional understanding holds, but also to maintaining community evenness of the gut microbiota as a means of minimizing damage from nonpathogenic environmental changes.

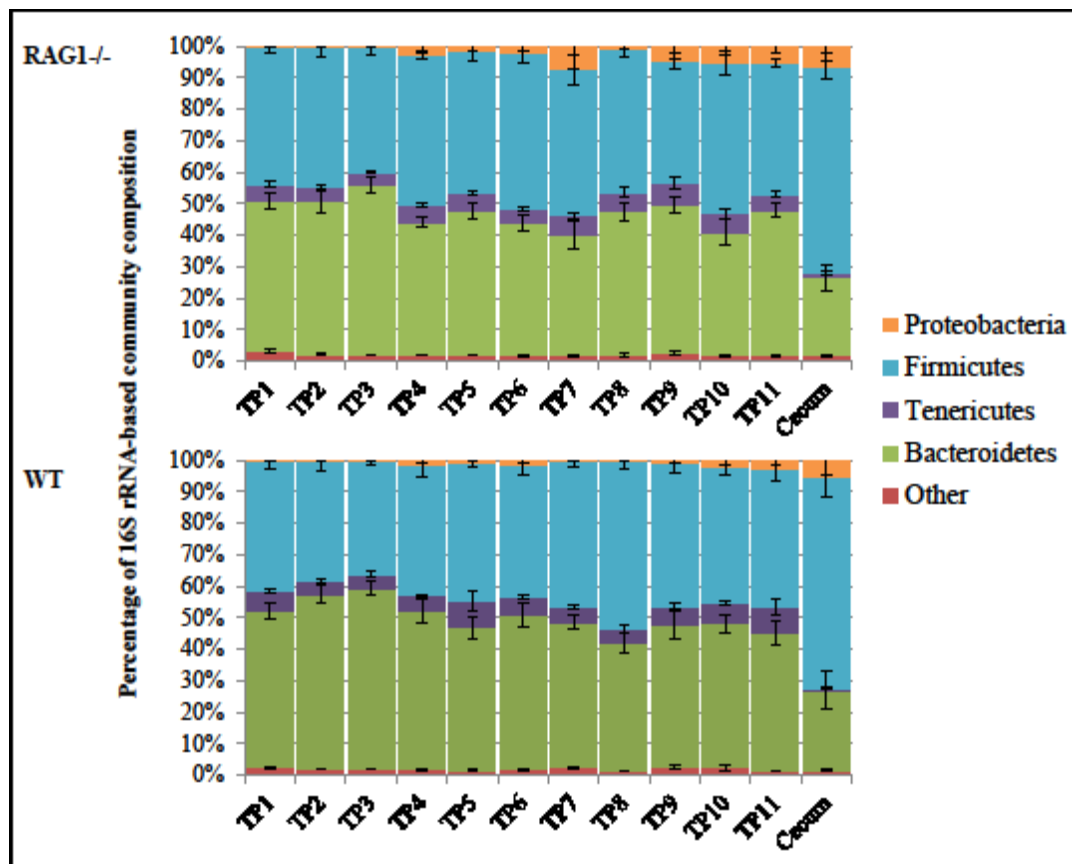


Figure 2. 16S rRNA-based composition of the gut microbiota at the phylum level reveals an increased frequency of Proteobacteria in RAG1^{-/-} mice compared to WT mice. The percentages of the community comprised of each of the listed phyla at each time point (abbreviated as TP) are represented by the variously colored bars (mean \pm se), with RAG1^{-/-} mice above and WT mice below.

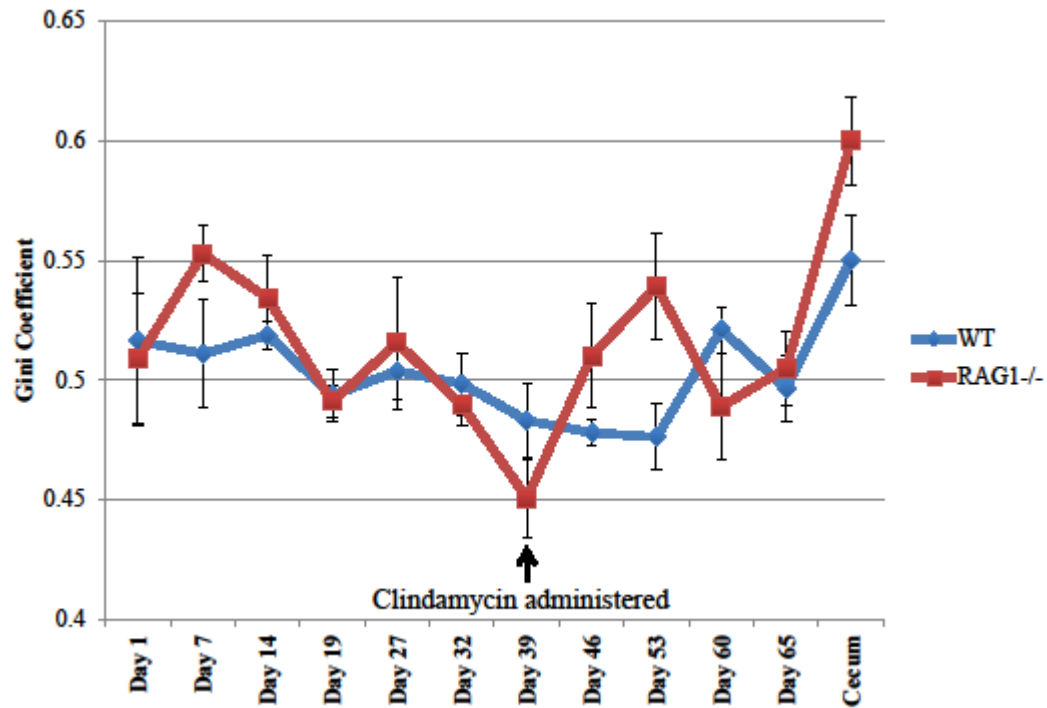


Figure 3. The community evenness of the gut microbiota of RAG1-/- mice is significantly lower than in WT mice over time. The Gini coefficient is represented on the y-axis, with lower values representing increased evenness. The Gini coefficient is given at each time point for RAG1-/- mice (red squares) and WT mice (blue diamonds) as means \pm se.

I previously showed that TLR5^{-/-} mice have an aberrant IgA response that is characterized by increased IgA production and reduced anti-flagellin IgA (Chapter 1). Since IgA is implicated in gut homeostasis and the maintenance of a commensal microbiota [14, 15] and TLR5^{-/-} mice display a temporally unstable gut microbiota [36], I wanted to investigate the possibility that the IgA repertoire of TLR5^{-/-} mice interacts with the gut microbiota in a different manner than in WT mice. To do this, I first assessed the proportion of the bacterial biomass coated by IgA in TLR5^{-/-} and WT mice by sorting cecal bacteria into IgA-coated (IgA⁺) and uncoated (IgA⁻) fractions using fluorescence-activated cell sorting (FACS), (Figure 4) [17]. The proportion of IgA⁺ bacteria was similar for TLR5^{-/-} and WT (21.7%±5.9 vs. 18.8%±4.2, n=4/group, n.s.). This suggests that, despite the discrepancies in IgA production between TLR5^{-/-} and WT mice, the gut microbiota is equally bound with IgA in both mouse types. However, this data does not provide information on the average number of IgA molecules bound to each IgA⁺ bacterial cell, the epitopes recognized by IgA and their binding affinity, or the taxonomy of the IgA⁺ and IgA⁻ bacteria.

As a gross comparison of the specificity of IgA from the TLR5^{-/-} and WT hosts, I incubated IgA derived from the intestines of these mice with a bacterial biomass previously unexposed to IgA (derived from RAG1^{-/-} mice). Results indicate that IgA from TLR5^{-/-} and WT mice is equivalently able to bind the bacteria derived from RAG1^{-/-} mice (55.7%±7.8% and 50.1%±7.8% of cells, respectively, n=4/group, n.s.). This relatively crude assay suggests that IgA produced by TLR5^{-/-} is sufficiently able to recognize and bind a portion of the microbiota similar to what is observed in WT mice.

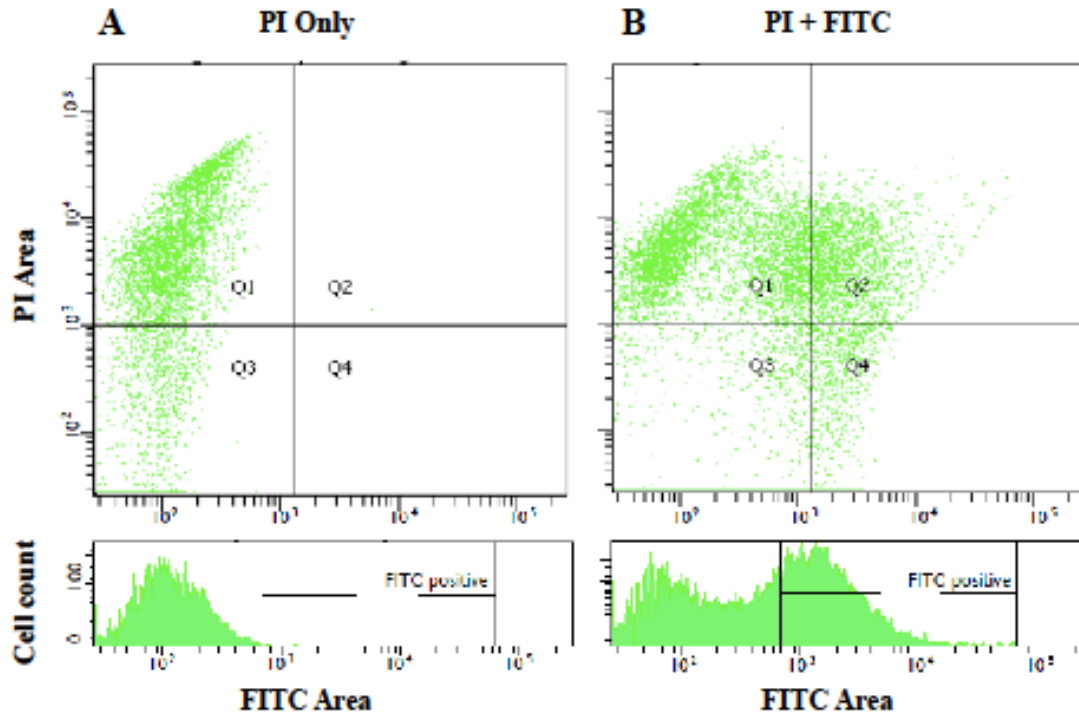
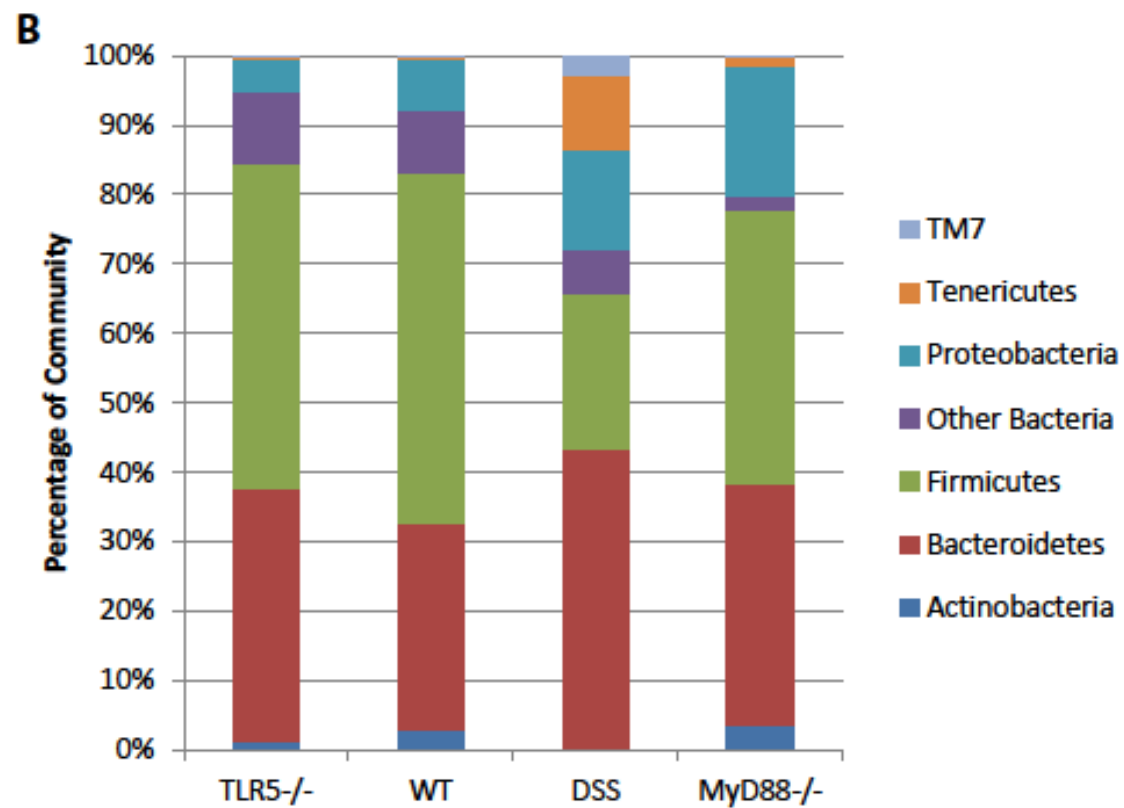
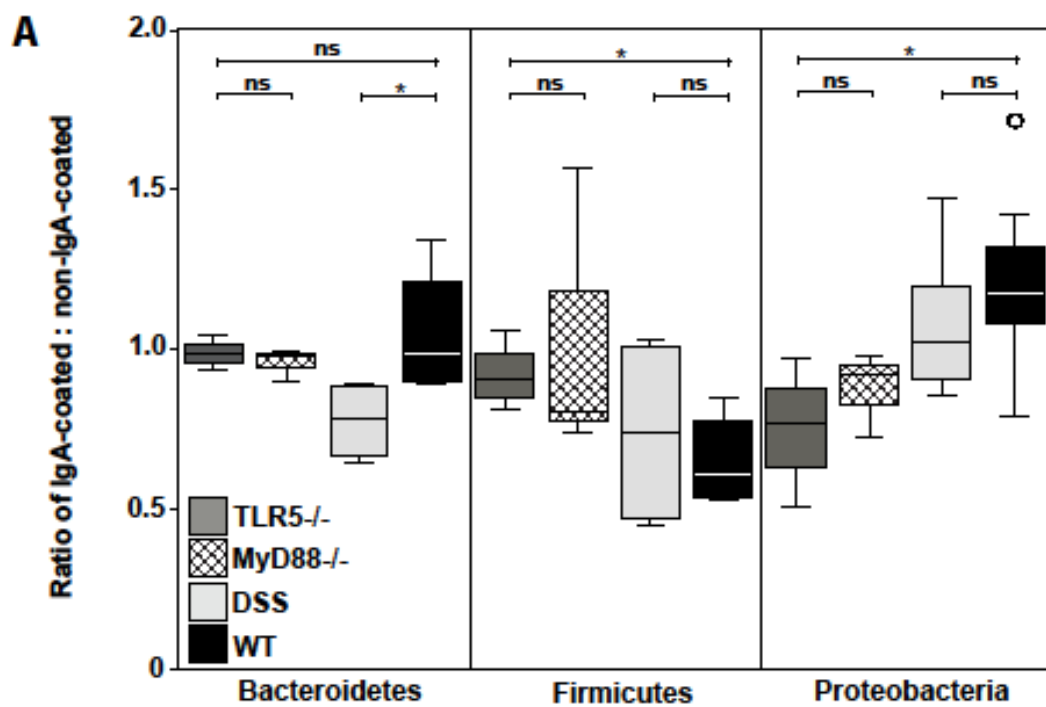


Figure 4. Proof of concept for fluorescence activated cell sorting of IgA-coated bacteria. I recovered bacteria from the cecal contents of a WT mouse via selective centrifugation and incubated with a FITC-labeled anti-mouse-IgA antibody. (A) Propidium iodide (PI) was used as an indiscriminate bacterial stain and (B) cells were separated based on the exhibition of FITC/PI fluorescence. Top: dot plots represent 10,000 recorded events. Q1: PI positive (IgA- cells); Q2: PI/FITC positive (IgA+ cells); Q3: PI/FITC negative (debris); Q4: FITC positive (IgA+ debris). Bottom: histograms represent count-binned FITC fluorescence.

To gain a more detailed understanding of how loss of TLR5 signaling impacted the specific suite of bacteria coated in IgA, I profiled the bacterial diversity of cell-sorted IgA⁺ and IgA⁻ cells isolated from the cecal contents of TLR5^{-/-} and WT mice using pyrosequencing of 16S rRNA genes. Since TLR5^{-/-} mice have an immune deficiency and also display increased inflammatory markers [23, 37], I also included MyD88^{-/-} mice and DSS-treated WT mice in this assay to better discriminate between the effects of immune deficiency (MyD88^{-/-}) and inflammation (DSS-treated). Patterns of bacterial coating were similar for TLR5^{-/-} and MyD88^{-/-} mice, and DSS-treated mice were similar to WT mice (with the exception of Bacteroidetes, which is a nonflagellated phylum), (Figure 5A). These findings indicate that differences in IgA-coated populations were attributable to impaired TLR5 signaling rather than inflammation. A previous study found that, in healthy mice and humans, *Enterobacteriaceae* (Proteobacteria) is preferentially frequently coated with IgA in the gut while *Lactobacillus* and *Enterococcus* (both Firmicutes) were poorly coated [38]. This pattern agrees with my findings of IgA coating in WT mice with or without DSS treatment, while TLR5^{-/-} mice exhibit the opposite pattern. Further, the similarities in phylum composition between TLR5^{-/-} and WT mice (Figure 5B) indicate that the differences in IgA-coated populations are due to an altered IgA repertoire and not simply to differences in the bacterial community. Notably, TLR5^{-/-} mice showed an enrichment of IgA-coated Firmicutes and a depletion of IgA-coated Proteobacteria, even though both of these phyla contain many flagellated species. It is possible that the reduced IgA coating of Proteobacteria in TLR5^{-/-} mice is a factor that allowed this phylum to expand its population in these mice. If Proteobacteria are less bound by IgA and their flagellar motility is not inhibited by the immune system, they may be able to extract more nutrients and occupy a wider range of sites in the gut. This may be why I see Proteobacteria penetrating the villi of the small intestine and

deep into the mucosal layer in the colon of TLR5^{-/-} mice (Chapter 3). It is also possible that Proteobacteria are actively downregulating epitopes targeted by the IgA of TLR5^{-/-} mice in a similar fashion to what has been observed for *Bacteroidetes thetaiotaomicron* and *S. Typhimurium* [39, 40]. Despite the differences observed between the IgA⁺ and IgA⁻ fractions of the gut microbiota of these mice, the community diversity of these two fractions still cluster most tightly by individual mouse in a PCoA of the UniFrac distance matrix (Figure 5C). If the antigen specificities within the IgA repertoire were highly similar for a given mouse genotype, then I would expect the IgA⁺ fraction of the community to cluster together for that genotype.

If IgA were coating gut bacteria indiscriminately, as is argued by the “natural” IgA hypothesis that IgA in the lumen is spontaneously generated without affinity towards particular antigens [41], then one would expect the increased gut IgA of TLR5^{-/-} mice [21] to correspond to an increased proportion of all microbiota coated in IgA. However, I did not observe this, and more recent work refutes the claim of “natural” IgA, finding that most IgA in the gut is actually antigen-specific [42]. These findings indicate that the role of IgA in gut homeostasis is more nuanced than its mere presence or abundance in the intestine. In fact, the average number of IgA molecules secreted into the human gut each day is calculated to be 10⁷-fold higher than the total number of commensal intestinal bacteria [43] and less than 5% of these IgA molecules are actually bound to bacteria [44]. My observation that anti-flagellin antibodies are sufficient to cause bacterial downregulation of flagellin (Chapter 1), coupled with other recent findings of bacteria decreasing antigen expression in direct response to antibodies specific for that antigen [39, 40], suggests that IgA may have profound implications for gene regulation of the gut microbiota at a broad level.



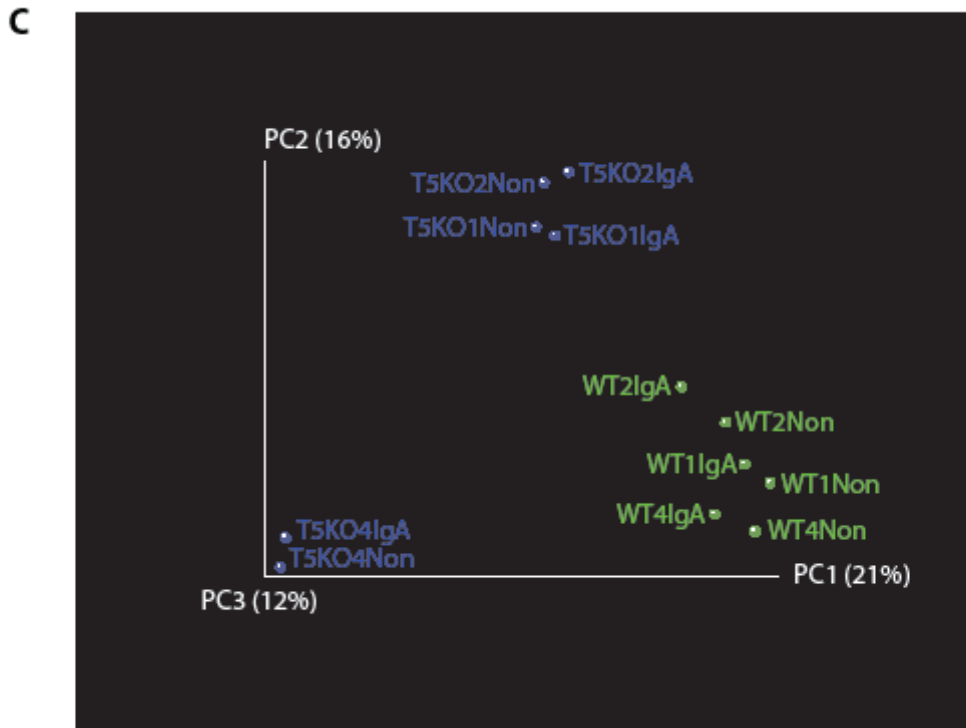


Figure 5. Effect of TLR5 signaling on IgA coating of bacterial populations. (A) Box plots represent the ratio of IgA-coated to non-IgA-coated cecal bacteria for the specified dominant bacterial phyla. A value of 1 indicates equal frequencies of IgA-coated and non-IgA coated bacteria in a particular phylum. N = 4 mice/group; *P < 0.05, two-tailed t-test; n.s., nonsignificant. (B) Bar graphs of the average composition of cecal bacteria for each genotype, with sequences pooled from IgA-coated and non-IgA-coated bacteria. Phylum key is on the right. (C) PCoA of the weighted UniFrac distance matrix using 16S rRNA sequences from IgA-coated to non-IgA-coated cecal bacteria of TLR5^{-/-} (blue) and WT (green) mice. The first three principal coordinates (PC1, PC2, PC3) are plotted. Each dot represents either the IgA⁺ or IgA⁻ fraction of the bacterial community for a particular mouse. The variance explained by the PCs is indicated in the parentheses on the axes.

If IgA binds widely to shared antigens, the coating frequency of bacterial genera is expected to be proportional to their abundance in a stepwise fashion [45]. This was the case for a majority of taxa, although a few outliers with high abundances were either more or less coated than expected, as calculated by Cook's distance (Figure 6). Several operational taxonomic units (OTUs) classified as bacteria of the Bacteroidetes phylum were consistently highly coated with IgA in WT and TLR5^{-/-} mice, while OTUs classified as bacteria of the Firmicutes phylum tended to be less coated with IgA in WT mice only. Despite containing many flagellated species and exhibiting expansion in the gut microbiota of TLR5^{-/-} mice [36, 46], no particular γ -Proteobacteria displayed significantly aberrant IgA coating profiles in either mouse genotype. Interestingly, an OTU classified as Alcaligenaceae, which is within the β -Proteobacteria phylum and contains many flagellated members, was highly coated in WT mice but not TLR5^{-/-} mice. Conversely, an OTU classified within the δ -Proteobacteria phylum as *Desulfovibrio* and an OTU classified within the ϵ -Proteobacteria as Helicobacteraceae, both of which are flagellated, were significantly highly coated and less coated in the TLR5^{-/-} mice, respectively. These results suggest that, while the taxonomic profiles of IgA-bound bacteria do not largely differ between TLR5^{-/-} and WT mice, there are still significant discrepancies in the frequency of IgA coating of specific OTUs.

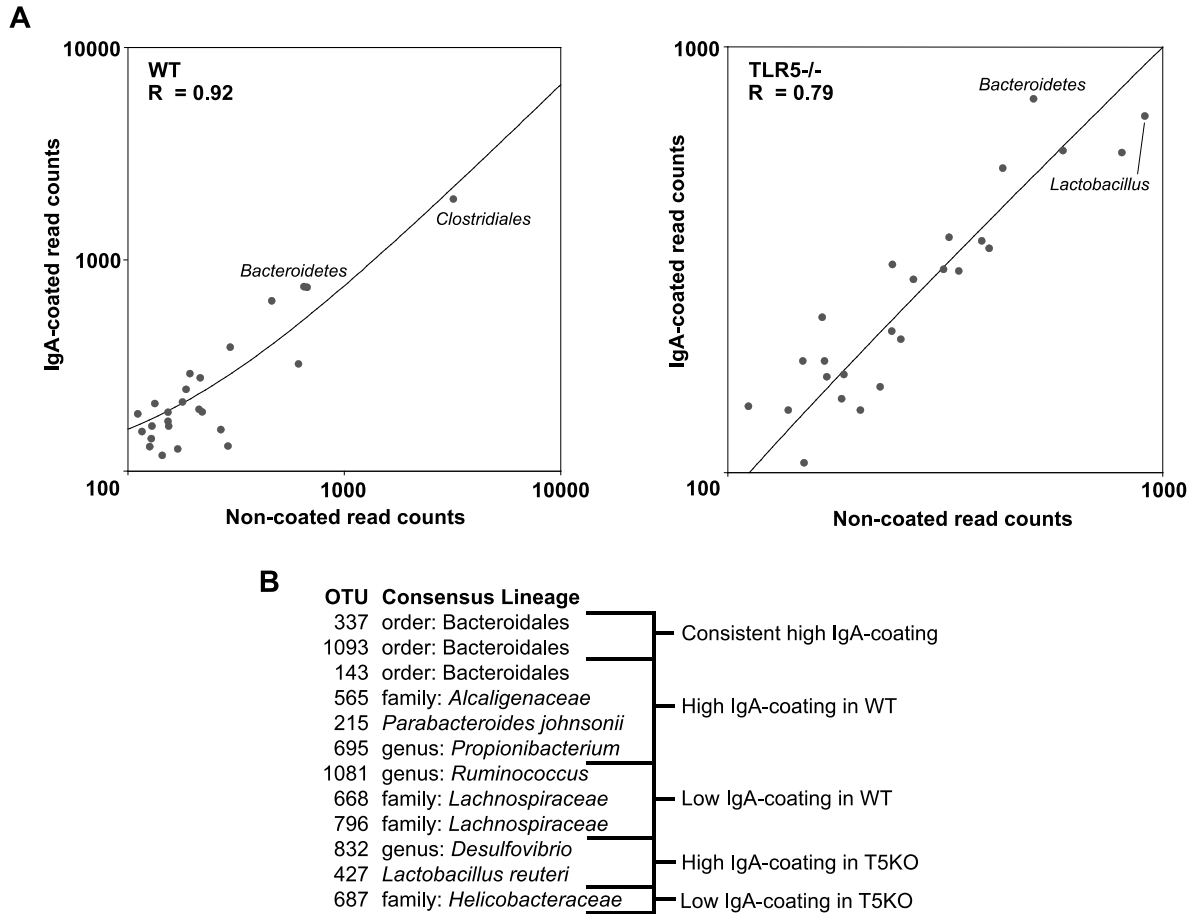


Figure 6. Effect of TLR5 signaling on IgA coating of bacterial populations. (A) The normalized abundance of 16S rRNA gene sequences from IgA⁺ bacterial cells are plotted against abundances for IgA⁻ cells for the 25 most abundant genera. The 2 most abundant genera in each mouse phenotype are labeled. (B) Taxonomic classification of genus-level OTUs (97%ID) with significantly aberrant IgA-coating frequencies (based on Cook's distance, See Methods).

2.4 Conclusion

These findings indicate that hosts need to develop an effective IgA repertoire with a broad range of antigen specificities in order to maintain temporal stability and resilience of their commensal gut microbiota. Consistent with previous surveys of the microbiota of TLR5 mice, I found that the guts of TLR5^{-/-} mice are slightly enriched in Proteobacteria and are more temporally unstable [36, 37]. I also found that the microbiota of RAG1^{-/-} mice were more temporally unstable and exhibited reduced community evenness compared to the microbiota of WT mice. Thus, the temporal instability of the TLR5^{-/-} mice may be linked to their inability to manage Proteobacteria populations [36], and this may be due to their reduced ability to coat these bacteria with IgA. IgA titers and bacterial biomass were previously reported to be higher in TLR5^{-/-} mice [21, 37]. My results generally support the view that IgA induction functions as a stepwise response tailored to bacterial load because most bacteria were equally represented in the IgA-coated and non-IgA-coated fractions [45], but they also show that specific bacterial species - particularly, abundant ones - can evade IgA coating in the gut.

I found that the IgA from TLR5^{-/-} and WT mice coated the same proportion of a naïve microbiota previously unexposed to IgA. The IgA produced by these two hosts has been trained on gut microbial communities that differ in the following ways: (1) greater overall abundance in TLR5^{-/-} mice and (2) slight enrichment of Proteobacteria in TLR5^{-/-} mice. I found that the Proteobacteria generally evade IgA coating, despite increased abundance in the TLR5^{-/-} mice. This suggests that the repertoire of IgA specificities is different between TLR5^{-/-} and WT mice. Alternatively, it is possible that Proteobacteria are more actively downregulating expression of surface antigens that are recognized by the IgA repertoire of the TLR5^{-/-} mouse. In summary, these results support the view that antibody coating of bacteria is a fundamental component of

homeostasis in the mammalian gut due to the increased temporal stability and resilience that result from microbe-antibody interactions.

2.5 Materials and Methods

Animal experiments: All mice used in this study were C57BL/6 strain. All mice used in the timeline study were maintained at University of Nebraska—Lincoln and all mice used in FACS analyses were maintained at Emory University. All animal experiments were approved by the local IACUCs. For the timeline study involving RAG1^{-/-} and WT mice, fecal pellets were collected approximately every five days for two months and mice were subcutaneously injected with 8 mg of clindamycin phosphate (Hospira, Inc., Lake Forest, IL) suspended in 0.2 ml of sterile saline (Bimeda – MTC Animal Health, Inc., Cambridge, Ontario). This work was performed by Clindamycin was administered for four consecutive days beginning on the day of the seventh fecal collection. For DSS treatment, C57BL/6 WT mice were administered 2.5% DSS (MBL Biomedicals) in drinking water for 7 days immediately preceding sample collection.

Sorting of IgA-coated bacteria: Four age/gender matched 8-week old TLR5^{-/-}, MyD88^{-/-}, WT DSS-treated, and WT mice were sacrificed and their ceca were immediately removed and snap frozen in liquid N₂. I prepared cecal contents for FACS of IgA-coated bacteria as described using methods in (25). Briefly, I suspended cecal contents of mice in 4.5 ml PBS, homogenized on a vortex mixer on high for 2 minutes, and centrifuged at low speed (40g, 20 minutes) to separate larger cecal particles from bacteria. I removed the resulting supernatant and centrifuged at 8,000g for 10 minutes and discarded the supernatant in order to remove non-bound IgA. I then resuspended each bacterial pellet in 2 ml of PBS containing 1% weight/volume of BSA with or

without FITC-labeled goat F(ab')₂ anti-mouse IgA (Sigma, St. Louis, MO) and incubated for 1 hour. I washed suspensions and resuspended in 2 ml of PBS with or without propidium iodide (PI, 100 mg/l) as an indiscriminate marker for all bacteria. Samples were stored on ice in the dark and analyzed within 2 hours.

I performed flow cytometry with a BD-Biosciences FACS Aria high speed flow cytometer/cell sorter utilizing a quartz cuvette for interrogation. Fluorophore excitation was performed by an Argon laser operating at 15 mW and 488 nm. Filter settings were 525 BP for FITC, 550 LP and 630 BP for measurement of PI. Standard ELITE software comprising the Immuno-4 program was used to determine the percentage of stained events. The discriminator was set on PI fluorescence as a specific probe for bacteria. The discriminator value was determined by a filtered, bacteria-free (0.22 µm Millipore, Molsheim, France) solution of PI / PBS (4 mg/l) and set at a level with minimal background noise. I analyzed a portion of each sample incubated with PBS (background fluorescence) and a portion incubated with FITC-labeled goat F(ab')₂ anti-mouse IgA. Both measurements were performed with a minimum of 10,000 events, at a flow rate of 1000 - 1500 events/sec. The percentages of stained bacteria were determined with Immuno-4 software (Coulter). Sorting experiments were performed with gates on FSC > 1000 and on FSC < 1000 in combination with gates on side scatter or PI. Furthermore, I performed a sorting experiment with a FSC – FITC fluorescence gate to isolate a strongly IgA-coated bacterial population. The cell sorter collected a minimum of 500,000 cells of each category (IgA-bound and non-bound).

16S rRNA gene sequencing and analysis: I extracted bulk DNA from the fecal pellets of mice used in the time course study with the PowerSoil DNA isolation kit as described by the manufacturer (MoBio Laboratories Ltd, Carlsbad, CA) and using a Mini-Beadbeater-24 (BioSpec, Bartlesville, OK) set on high for 2 minutes. I extracted bulk DNA of the FACS-sorted cells (see above) using a similar process except with a more appropriate kit: the PowerWater DNA isolation kit as described by the manufacturer (MoBio Laboratories Ltd, Carlsbad, CA). I amplified bacterial 16S rRNA genes from each sample using the 27F and 338R primers for the V1-V2 hypervariable region of the 16S rRNA gene (26). Primers included unique error-correcting 12-base barcodes used to tag PCR products from different samples (27). PCR reactions consisted of 2.5U Easy-A high-fidelity enzyme and 1X buffer (Stratagene, La Jolla, CA), 200 nM of each primer, and 2-3 ng DNA template; reaction conditions consisted of an initial denaturing step for 2 minutes at 95°C, followed by 32 cycles of 40 seconds at 95°C, 30 seconds at 57°C and 60 seconds at 72°C. I performed triplicate PCR reactions for each sample, combined and then purified with Ampure magnetic purification beads (Agencourt, Danvers, MA). I quantified the clean PCR products using a Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA). I had the pooled products sequenced at the Core Laboratories Center at Cornell University using the Roche 454 FLX platform.

For quality filtering I discarded sequences <200 bp or >1,000 bp, and ones containing >0 primer mismatches, uncorrectable barcodes, >0 ambiguous bases, or homopolymer runs in excess of 6 bases, using the open source software package Quantitative Insights Into Microbial Ecology (QIIME) (28). I checked sequences for chimeras (UCHIME) and assigned sequences to operational taxonomic units (OTUs) using Otupipe (29) with a 97% threshold of pairwise

identity, and then classified them taxonomically using the Greengenes reference database (30). I rarified samples to 7,000 reads per sample, calculated the ratio of IgA+ : IgA- for each OTU, and calculated Cook's distance in R to find OTUs with IgA coating ratios that diverged significantly from the mean (31).

Statistics: I performed statistical analyses and linear regression analysis in Microsoft Excel 2011, StatPlus 2009, and R version 2.11.1. Statistical tests were paired student 't'-test, unpaired student 't'-test, Cook's distance, and Gini coefficient, as indicated. Significance level of $p < 0.05$ unless otherwise indicated. All multiple comparison testing was accounted for with Bonferroni correction.

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CHAPTER 3

TLR5^{-/-} MICE DISPLAY REDUCED INTEGRITY OF THE GUT MUCOSAL BARRIER AND AN INABILITY TO QUENCH BACTERIAL MOTILITY

4.1 Summary

The maintenance of separation between host tissues and the bacterially dense intestinal lumen is critical to host health. Immunoglobulin A (IgA), Toll-like receptor 5 (TLR5), and a physical barrier of mucus are all important in preventing bacterial pathogens from invading host tissues, but it is unclear how these mechanisms interact with the comparatively abundant and persistent commensal microbiota. Here, I use a combination of fluorescent *in situ* hybridization and immunohistochemistry to show that the intestinal microbiota of TLR5^{-/-} mice invades the inner mucus layer of the colon and that flagellated bacteria are in direct association with the epithelium, despite increased secretion of IgA. Flagellin purified from an assortment of commensal Firmicutes revealed that many members of this phylum produce flagellins with TLR5 stimulatory capacity equal to that of γ -Proteobacteria. An antibody developed against a representative flagellated commensal Firmicutes, *Roseburia hominis*, displayed cross reactivity by binding to multiple flagellin proteins derived from both Firmicutes and γ -Proteobacteria. These results suggest that TLR5 is critical to the proper functioning of the mucosal barrier and development of an anti-flagellin IgA repertoire. In the absence of TLR5, it appears that the commensal bacteria increase their motility and penetrate host tissues without impedance from anti-flagellin IgA.

4.2 Introduction

The mammalian gut is densely populated by bacteria that are essential to host metabolism, the vast majority of which reside in the colon. When the gut epithelium is properly functioning, hosts are able to maintain a symbiosis with their resident microbiota without overstimulation of the innate and adaptive immune systems [1]. The mechanisms by which host tissues are healthily maintained in such close proximity to bacteria are not completely understood, though it has been recognized that mucus, IgA, and TLRs all play critical roles [2-4]. Through these interactions, commensal bacteria are maintained on the luminal side of the host-derived mucus layer.

Mucus not only provides a physical barrier to protect the host, but also serves as a colonization site [3] and nutrient substrate [5] for much of the gut bacterial community. The mucus layer in the small intestine is relatively thin, so as not to impede nutrient absorption, and must be bolstered by antibacterial peptides and fast peristalsis in order to prevent bacterial penetration [6, 7]. Conversely, colonic mucus is approximately 4-5 times thicker and maintains a dense inner layer that tightly adheres to the epithelium and is impervious to bacteria [3, 6]. Mucus in the small intestine and colon is comprised largely of the mucin protein MUC2, which is produced by goblet cells that reside at the base of epithelial crypts [8]. Colonic MUC2 forms a dense polymer that expands and becomes more permeable over time, which results in a stratified mucus layer that provides a habitat for bacteria in the less dense layer while still protecting the epithelium [9]. Mice deficient in MUC2 display inflammation and cancer development that stems from constant bacterial contact with the epithelium and invasion of epithelial crypts [3].

Secretory IgA serves as an additional mechanism in maintaining barrier protection between host tissues and the gut microbiota. The reported actions of IgA in the intestinal lumen

are myriad and include entrapment of bacteria in the mucus layer and hindering bacterial association with the epithelial cell surface [10]. Hence, secretory IgA is generally associated with protecting the host from bacterially induced harm in a process termed ‘immune exclusion’. For example, mice with genetic IgA deficiency undergo an excessive expansion of anaerobic bacteria that results in an exaggerated immune response and hyperplasia of isolated lymphoid follicles [2, 11, 12]. However, evidence suggests that secretory IgA may play a much broader role in maintaining health by aiding commensal bacteria in colonization and increasing resiliency of the gut community to perturbation [13, 14]. Some gut bacteria reduce their expression of IgA binding proteins when growing in IgA-deficient hosts, suggesting that there may be selection pressure in the healthy gut for commensal bacteria that are able to bind IgA [15]. Further, secretory IgA was found to facilitate biofilm formation when microbial communities from healthy individuals were cultured on epithelial cells under conditions in which non-adherent bacteria were continuously washed away [13].

The innate immune system comprises the third major mechanism by which hosts maintain a barrier of protection between themselves and their gut microbiota. It relies on the recognition of microbial structures that are evolutionarily conserved and not represented in the host. In the case of TLR5, this recognized structure is the highly conserved D1 domain of the flagellin protein, which is required for flagellar protofilament assembly and thus, bacterial motility [16, 17]. However, several bacterial taxa, including the often pathogenic α - and ϵ -Proteobacteria, contain alternative residues in the D1 domain that allow them to evade TLR5 recognition [18]. Conversely, many species of the *Lactobacillus* genus that are regularly employed in food and probiotic applications produce flagellin proteins that are fully capable of stimulating TLR5 [19]. This suggests that TLR5 is unable to discriminate between flagellin

derived from pathogens and flagellin derived from commensal or symbiotic bacteria. The majority of flagellated commensal gut bacteria have not been assessed for their ability to stimulate TLR5, so it is unclear how this potential shortcoming of TLR5 affects gut health. Stimulation of TLR5 is an important factor in the development of flagellin-specific adaptive immunity [20], so an inability to discern between pathogenic and commensal bacteria may also have important implications in the development of an anti-flagellin IgA repertoire.

I have previously shown that TLR5^{-/-} mice have an overly flagellated microbial community and that anti-flagellin IgA, which I argue to be responsible for reducing bacterial expression of flagella, is deficient in TLR5^{-/-} mice (Chapter 1). However, it is unclear how these traits manifest at the mucosal interface. It is also unknown if flagellin proteins from taxonomically distinct commensal bacteria stimulate TLR5 to the same magnitude as more thoroughly studied pathogens. Here I use fluorescent *in situ* hybridization (FISH) and immunohistochemistry techniques to investigate the spatial organization of bacteria and host tissues, as well as flagellin and IgA, *in situ*, in an effort to better understand how the lack of TLR5 alters the integrity of the mucosal barrier. I also work to expand our understanding of the role of anti-flagellin IgA in gut homeostasis beyond its effects on γ -Proteobacteria to better understand how these antibodies affect Firmicutes, the most abundant flagellated phylum in the gut. I explore the cross-reactivity of commercial anti-flagellin antibodies as well as the potential of antibodies derived from healthy mice to bind various bacterial flagellins. Lastly, I use time-lapse microscopy to better understand the motility of the gut microbiota of TLR5^{-/-} mice and how the presence of anti-flagellin antibodies affects motility within this mixed community as compared to pure cultures.

4.3 Results & Discussion

To better understand how the increased bacterial load, increased flagellin load, and altered SIgA specificity of TLR5^{-/-} mice manifest in the gut, I first performed FISH microscopy in which I probed for 16S rRNA of the domain Bacteria. The images reveal that the colon of WT mice has a distinct layer of mucus that is populated by few to no bacteria and is generally absent or markedly reduced in the TLR5^{-/-} mouse colon (Figure 1). Thus, bacteria in the colon of TLR5^{-/-} mice appear to heavily infiltrate right up to the epithelial edge of the mucus layer. Previous studies suggest that the colonic inner mucus layer in healthy mice is approximately 50 μ m thick [3, 21], which agrees with my findings. This layer of mucus has been repeatedly observed in the colons of healthy mice and humans and absent or depleted in individuals with inflamed colons [3, 22, 23]. However, few studies have directly addressed whether reduced mucosal integrity is a cause or an effect of gut inflammation. Mice treated with dextran sodium sulfate (DSS) develop an impaired mucosal barrier within 3-5 days but display gut inflammation only after bacteria have penetrated the mucus [24], suggesting that reduced mucosal integrity is a cause of inflammation. Also, mice housed in germ-free conditions do not have elevated inflammatory markers despite a comparatively thin mucus lining of the colon [25]. The thin mucus layer is restored to typical thickness upon exposure to bacterial peptidoglycan or lipopolysaccharide [25], indicating that a commensal gut microbiota can actually help to maintain the mucus lining in healthy hosts. Both of these findings suggest that the increased inflammatory markers observed in TLR5^{-/-} mice [26, 27] are initially caused by the highly flagellated microbiota rather than an aberrant mucus layer.

A subset of TLR5^{-/-} mice has been found to spontaneously develop colitis [27], though we have not observed colitis in TLR5^{-/-} mice housed at Cornell University. Recent FISH studies

of TLR5^{-/-} colonic tissues have revealed that colitic mice have an intact, though relatively thin, inner mucus layer, but that this layer was penetrated by bacteria [22]. Conversely, the colons of non-colitic TLR5^{-/-} mice in the study displayed a relatively intact mucus layer without bacterial penetration. Insufficient information is provided about the mice used in the study to provide an explanation for the differences in mucosal integrity between our TLR5^{-/-} mice and those used by Johansson *et al.* [22]. However, the composition of the gut microbiota of mice is heavily influenced by their housing facility [28], which may explain why our TLR5^{-/-} display a defective mucosal barrier but fail to develop colitis.

The small intestines of TLR5^{-/-} mice revealed assemblages of bacteria invading the villi – these groups were completely absent in WT mice. To further investigate the type of bacteria present in these assemblages, I used probes specific for γ -Proteobacteria as well as the phyla Firmicutes and Bacteroidetes (Figure 2). The differential probes revealed that γ -Proteobacteria were responsible for the vast majority of observed invading bacteria. In several occurrences, the invading γ -Proteobacteria were found to co-occur with Firmicutes. The penetration of bacteria into the small intestines of TLR5^{-/-} mice further suggests that these mice have an insufficient mucosal barrier. The co-occurrence of γ -Proteobacteria and Firmicutes in the breach of the small intestine agrees with the inability of previous studies to identify a specific pathogen responsible for the TLR5^{-/-} phenotype [26, 29] and bolsters the idea that metabolic syndrome and inflammation correlates with a broad shift in the activity of the gut microbiome – namely, an increase in flagellar motility in at least two prominent phyla.

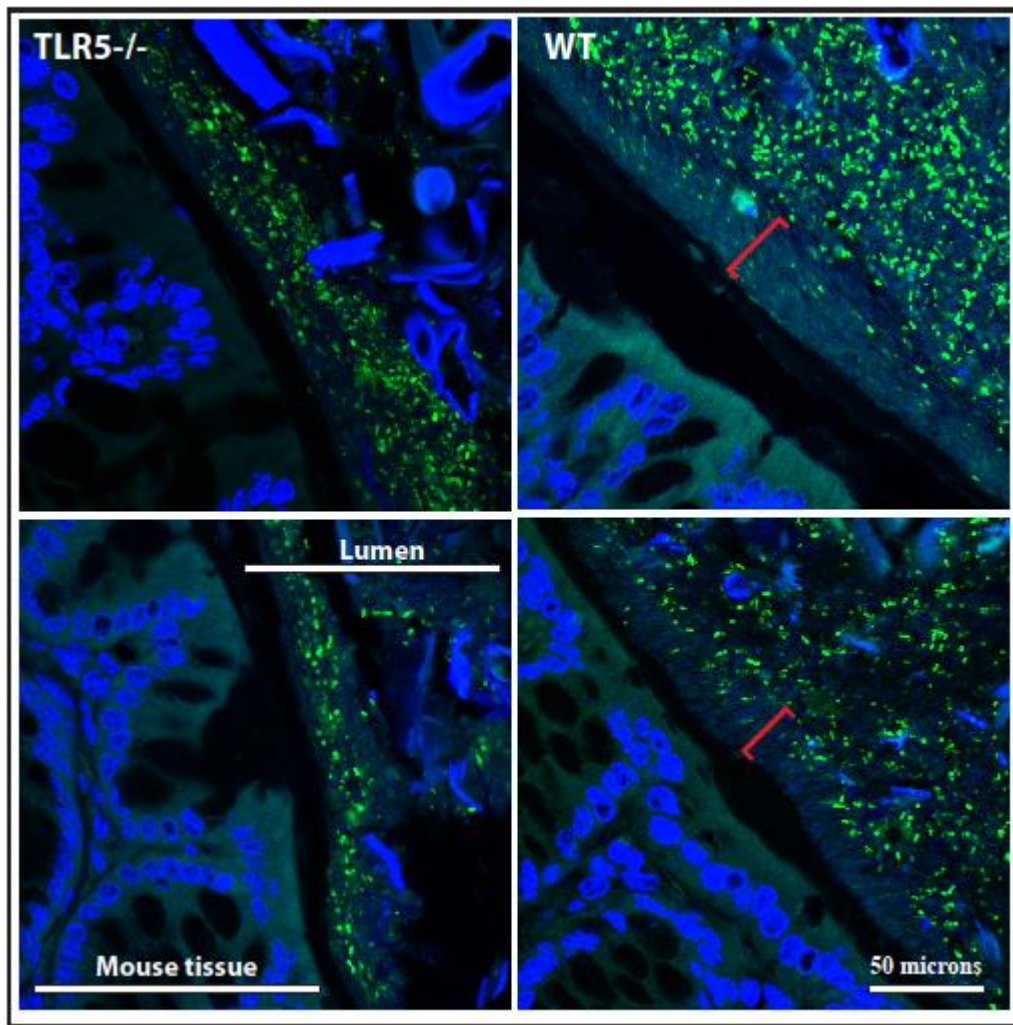


Figure 1. The large intestine of TLR5^{-/-} mice lacks mucosal integrity and displays increased bacterial penetrance. Each panel displays a fluorescent micrograph of a separate mouse large intestine section, with TLR5^{-/-} mice (LEFT) and WT (RIGHT) shown. The images are positioned with host tissues on the left and the lumen on the right. BLUE: Nuclei of cells comprising host villi are stained with Hoechst 33342. GREEN: bacterial cells imaged by *in-situ* fluorescent hybridization using fluorescently labeled oligonucleotides probes specific to the Domain Bacteria (EUB338). Food particles in the lumen display autofluorescence and may fluoresce blue due to DNA content. Red brackets annotate the bacterially sparse inner mucus layer of WT mice that is absent in TLR5^{-/-} mice. The top and bottom panels show two examples in each genotype and the scale bar applies to all images.

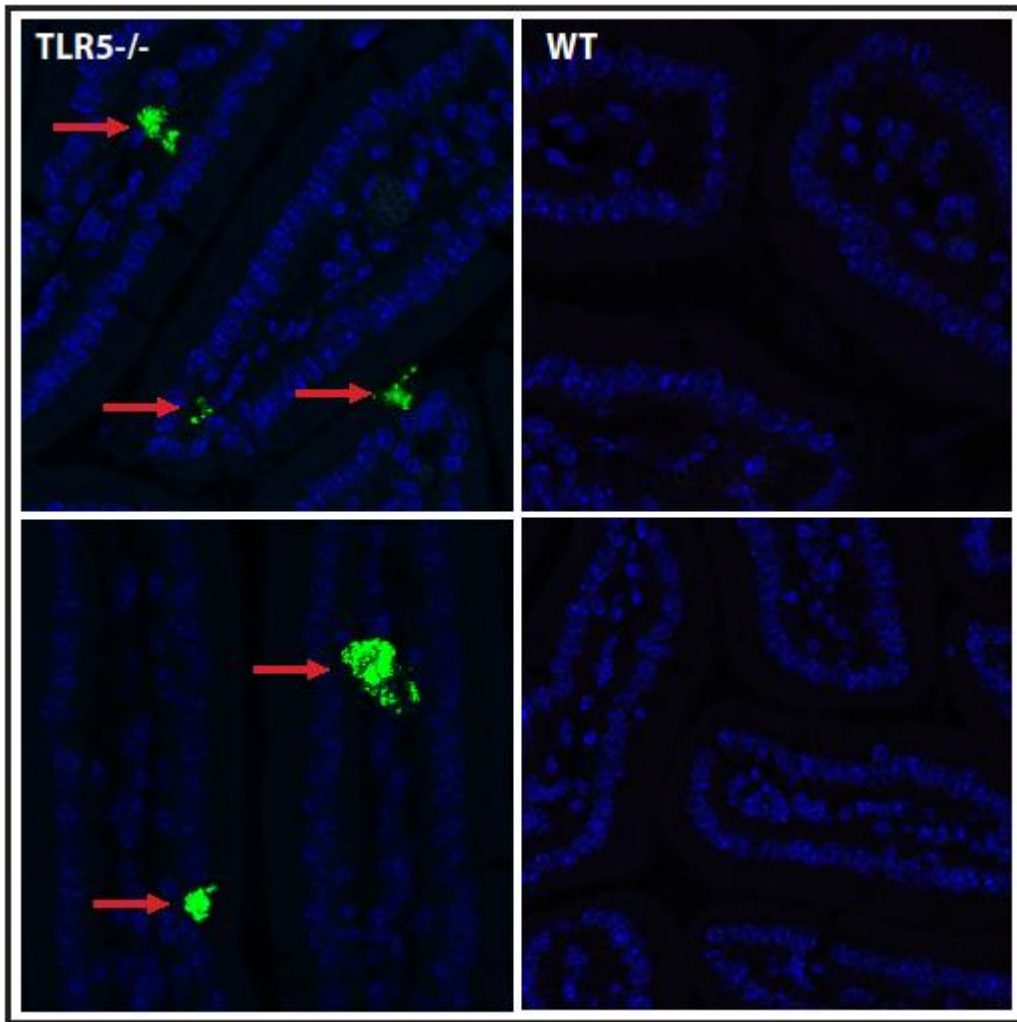


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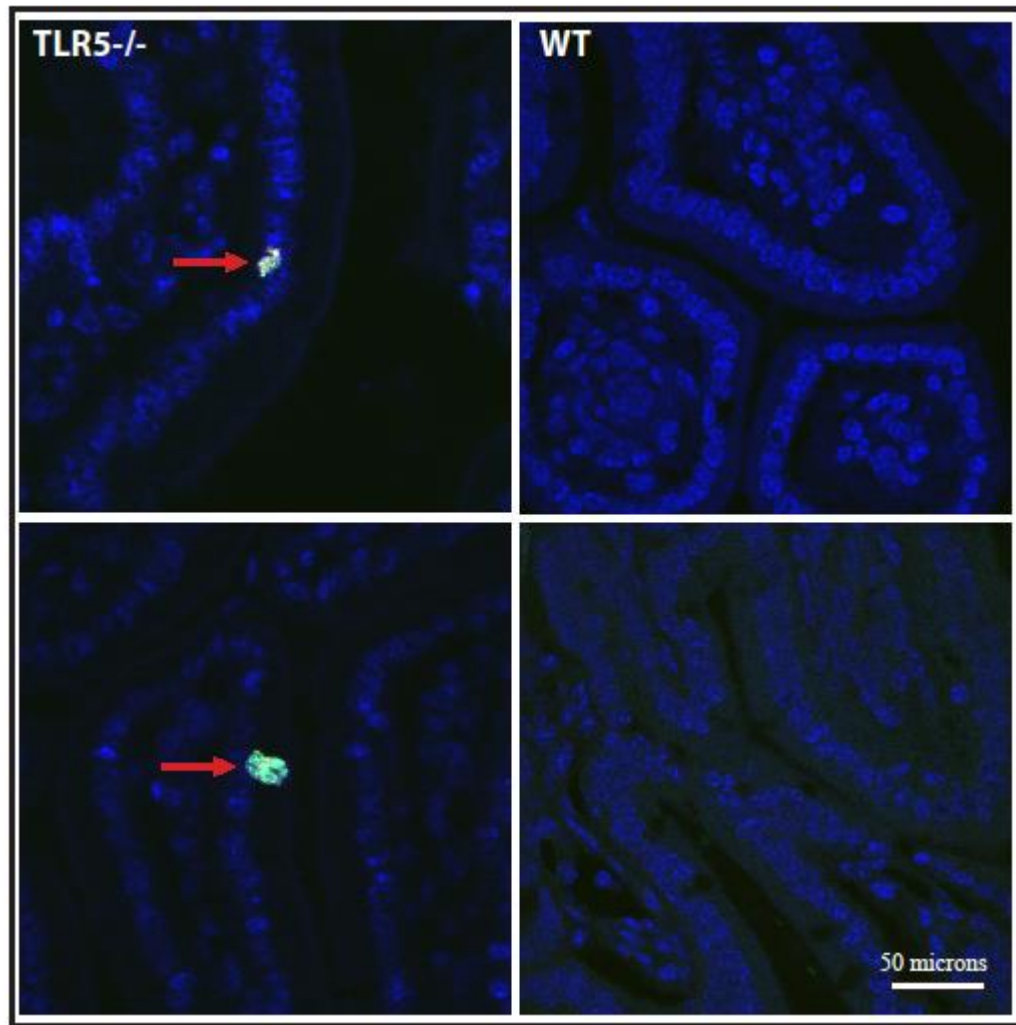


Figure 2. γ -Proteobacteria and Firmicutes display increased penetrance of the small intestine of TLR5^{-/-} mice. Each panel displays a fluorescent micrograph of a separate mouse small intestine section with TLR5^{-/-} (LEFT) and WT (RIGHT) shown. BLUE: Nuclei of cells comprising host villi are stained with Hoechst 33342. GREEN: bacterial cells imaged by *in situ* fluorescent hybridization using fluorescently labeled oligonucleotides probes specific to the phylum γ -Proteobacteria (GAM42a). ORANGE: bacterial cells imaged by *in situ* fluorescent hybridization using fluorescently labeled oligonucleotides probes specific to the phylum Firmicutes (LGC354a-c). The co-occurrence of γ -Proteobacteria and Firmicutes appears as white. Bacterial clusters are labeled with red arrows. The top and bottom panels show four examples in each genotype and the scale bar applies to all images.

Measurements taken from the cecal contents and fecal pellets of TLR5^{-/-} mice indicate that total IgA is significantly elevated compared to WT levels (Chapter 1). To better understand the location and bearing of secretory IgA, I performed immunohistochemistry with antibodies targeting mouse IgA in the large intestine. In agreement with previous observations, TLR5^{-/-} mice appear to have more total IgA present in the lumen (Figure 3). In both mouse types IgA appears dispersed and lacks noticeable patterning at the mucosal interface, except for the appearance of subepithelial host cells that present strong IgA signal and are likely IgA-producing plasma cells. If TLR5^{-/-} mice have impaired mucosal barrier function, as suggested, it is likely that their immune system encounters significantly more bacterial products as well as other luminal contents. The development of immune tolerance is not well understood, but it's possible that insufficient tolerance against luminal antigens is responsible for increased production of secretory IgA in TLR5^{-/-} mice. It's also possible that the TLR5^{-/-} immune system attempts to compensate for insufficient anti-flagellin IgA production by increasing the production of IgA with alternate specificities. The association of bacteria with the epithelium of the small intestine has been shown to increase host IgA production [7], suggesting that increased IgA levels in TLR5^{-/-} mice may be a consequence of epithelium-associated bacteria. Increased exposure of the epithelium to bacterial products coupled with a defective adaptive immune response against flagellin [20] may explain why the abundance of IgA observed in the colon of TLR5^{-/-} mice is insufficient to protect the mucosal barrier from being penetrated by a highly flagellated microbiota.

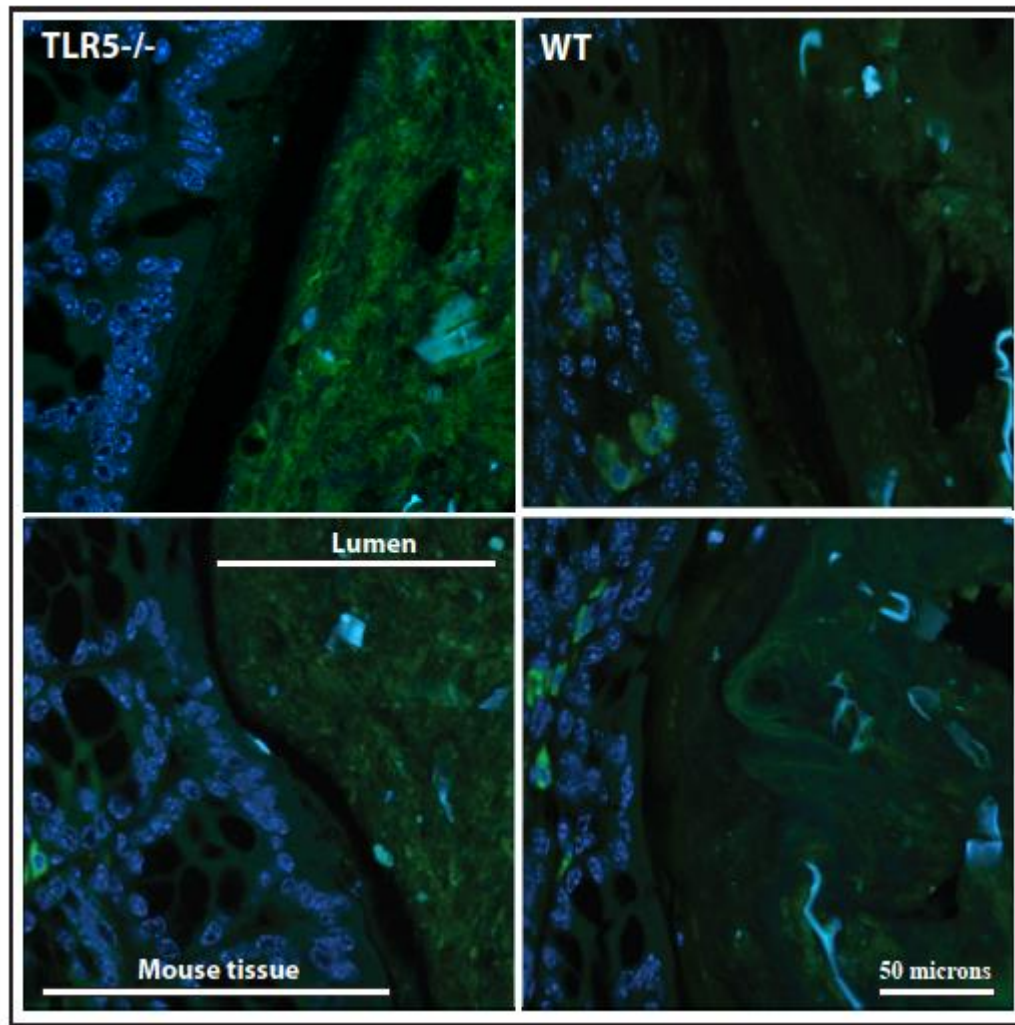


Figure 3. Elevated levels of secretory IgA are apparent in the large intestine of TLR5^{-/-} mice. Each panel displays a fluorescent micrograph of a separate mouse large intestine section with TLR5^{-/-} (LEFT) and WT (RIGHT) shown. The images are positioned with host tissues on the left and the lumen on the right. BLUE: Nuclei of host villi cells stained with Hoechst 33342. GREEN: IgA tagged with a fluorescent anti-IgA antibody (Alexa fluor 488). Food particles in the lumen display autofluorescence and may fluoresce blue due to DNA content. Green cells in the subepithelium are likely to be IgA-producing plasma cells. The scale bar applies to all images.

Though I previously measured an increased flagellin load in the cecal contents and fecal pellets of TLR5^{-/-} mice, those quantitative assays did not provide information on the location of flagellin in relation to host tissues. To investigate the presence of bacterial flagellin *in situ*, I performed immunohistochemistry with antibodies targeting flagellin in large intestine tissues. In agreement with prior findings, the presence of flagellin in the colon of TLR5^{-/-} mice was strikingly elevated in comparison to WT (Figure 4). Similar to what was observed in the FISH images of bacteria in the colon, flagellin also appears in high abundance in the inner mucus layer of TLR5^{-/-} mice and virtually absent in the inner mucus layer of WT mice. Interestingly, the fluorescence signifying the presence of flagellin revealed what appear to be distinct bacterial cells which breached the mucus layer and associated directly with the epithelium in TLR5^{-/-} mouse tissues. This suggests that, not only does the gut microbiome of TLR5^{-/-} mice produce more flagellin, but that the bacteria breaching the mucosal barrier are indeed flagellated. While I did observe a lesser flagellin signal in the lumen of WT tissue preparations, I did not observe the presence of distinct bacterial shapes or the presence of flagellin signal in contact with the epithelium. This could suggest that the flagellin levels in WT mice are due to low-level expression from a diverse suite of bacteria, rather than the presence of highly flagellated bacteria that are simply in lower abundance relative to the TLR5^{-/-} microbiome.

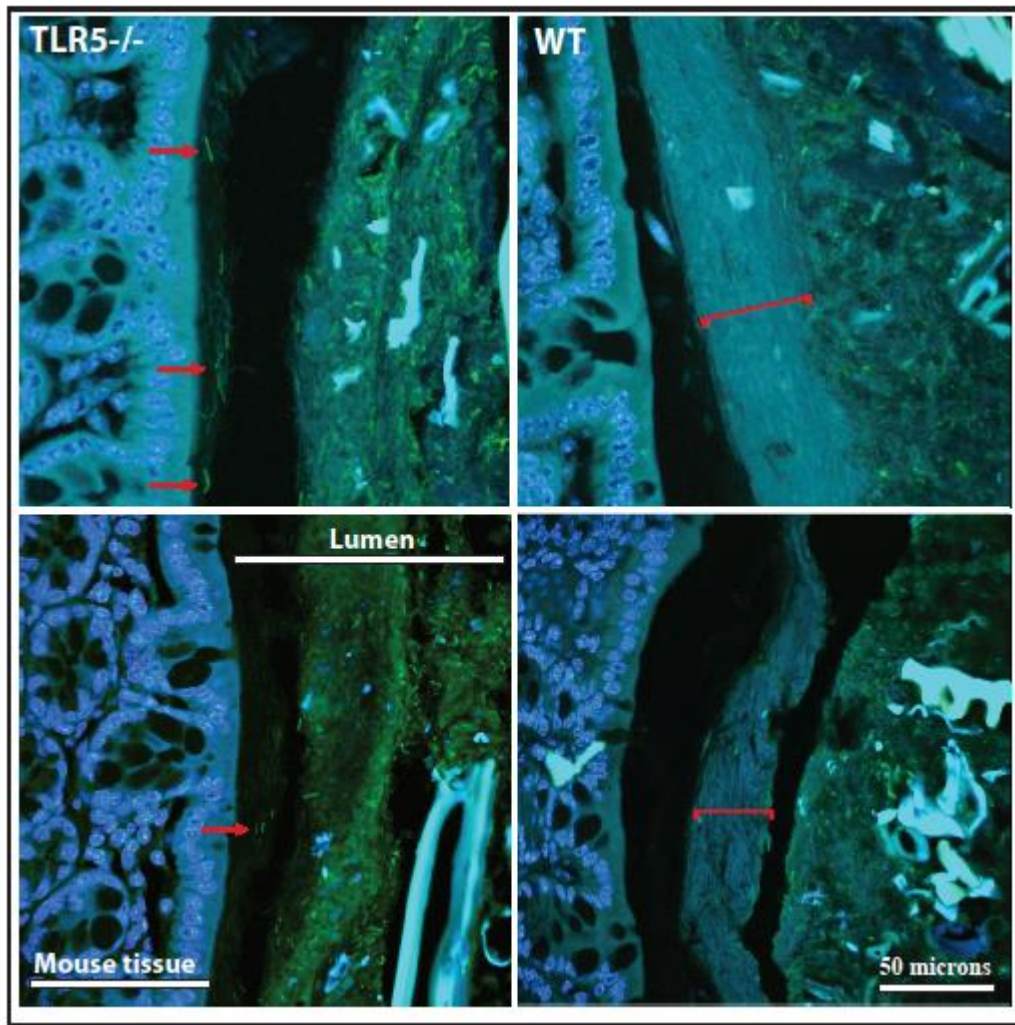


Figure 4. Bacteria display increased flagellin and penetrance in the large intestine of TLR5^{-/-} mice. Each panel displays a fluorescent micrograph of a separate large intestine section with TLR5^{-/-} (LEFT) and WT (RIGHT) shown. The images are positioned with host tissues on the left and the lumen on the right. BLUE: Nuclei of host villi cells stained with Hoechst 33342. GREEN: flagellin tagged with a fluorescent anti-flagellin antibody (Alexa fluor 488). Food particles in the lumen display autofluorescence and may fluoresce blue due to DNA content. Red arrows indicate bacteria in association with the epithelium and red brackets annotate the bacterially sparse inner mucus layer of WT mice.

Multiple studies indicate that intestinal pathogens cannot effectively penetrate the mucus layer and invade the epithelium without flagellar motility [30, 31]. Though the same data are not available for gut commensal bacteria, my observations suggest that a taxonomically widespread increase in flagellation in the gut microbiota of TLR5^{-/-} mice is responsible for the bacterial colonization of the colonic inner mucus layer and penetration into the villi of the small intestine.

My findings indicate that anti-flagellin antibodies quench bacterial motility and that these antibodies are significantly reduced in the intestines of TLR5^{-/-} mice. Unfortunately, commercially available anti-flagellin antibodies and purified flagellin are greatly limited to the γ -Proteobacteria species *E. coli* and *S. enterica*. This raises questions about the applicability of these reagents and the data they help to generate concerning the whole gut microbiome. Though γ -Proteobacteria are likely to be important to the development of metabolic syndrome and inflammation in TLR5^{-/-} mice, my metatranscriptomics data suggest that most of the flagellin present in the gut is produced by bacteria of the Firmicutes phylum. To extend the relevance of my findings to Firmicutes, I used a combination of genomic analyses, motility assays, and antibody cross-reactivity assays.

There are seven amino acid residues of the flagellin protein, spread across the amino acid positions 88-114 in most γ -Proteobacteria flagellins, that are critical for recognition by TLR5 [18]. To see if these residues are present in commensal Firmicutes, I aligned annotated flagellin peptide sequences with several well-characterized Proteobacteria (Table 1). The presence of critical amino acid residues appears to segregate by bacterial class: γ -Proteobacteria and Clostridia consistently have all of the critical residues, while α -Proteobacteria, ϵ -Proteobacteria, and Bacilli tend to have only a portion of the critical residues. These data suggest that Clostridia, which is the most abundant class of flagellated bacteria found in WT and TLR5^{-/-} mice

according to my 16S rRNA sequence analysis, produce flagellin that is capable of eliciting a TLR5 response. Interestingly, γ -Proteobacteria and Clostridia share more amino acid sequence homology in this region of the flagellin protein that either one shares with more closely-related members of their own phylum.

Table 1. Alignment of flagellin peptide sequences showing the amino acid residues critical for TLR5 recognition. Amino acid sequences from a variety of flagellin proteins were obtained from NCBI and aligned. Amino acid positions known to be critical for eliciting a TLR5 response have an asterisk at the top of the alignment and flagellin proteins with the correct residues at these positions are underlined red. The species are grouped by class. Note that sequences derived from Firmicute species (classes Bacilli and Clostridia) have most, if not all, of the necessary residues for TLR5 stimulation .

			***	*	*	*		*																						
α -Proteo	{	BARTONELLA BACILLIFORMIS	L	D	D	I	Q	K	S	M	V	S	A	R	E	--	K	S	D	--	D	D	I	M	K	I	Q	D	S	
		CAMPYLOBACTER JEJUNI	L	D	T	I	K	T	K	A	T	Q	A	A	Q	D	G	Q	S	L	--	K	T	R	T	M	L	Q	A	D
ϵ -Proteo	{	HELICOBACTER PYLORI	L	D	T	V	K	V	K	A	T	Q	A	A	Q	D	G	Q	T	--	E	S	R	K	A	I	Q	S	D	
		HELICOBACTER FELIS	L	D	T	I	K	V	K	A	T	Q	A	A	Q	D	G	Q	N	T	--	Q	S	R	K	A	L	Q	A	D
	{	WOLLINELLA SUCCINOGENES	L	D	T	I	K	T	K	A	T	Q	S	A	A	Q	D	G	Q	T	--	T	T	R	K	A	L	Q	A	D
		PROVIDENCIA STUARTII	L	O	R	I	R	E	L	T	V	O	A	A	N	G	S	N	S	D	--	S	D	I	T	S	I	R	D	E
γ -Proteo	{	ESCHERICHIA COLI	L	O	R	I	R	E	L	S	V	O	A	T	N	G	T	N	S	D	--	S	D	L	S	S	I	Q	D	E
		SALMONELLA TYPHIMURIUM	L	O	R	V	R	E	L	A	V	O	S	A	N	S	T	N	S	Q	--	S	D	L	D	S	I	Q	A	E
	{	LISTERIA MONOCYTOGENES	L	O	R	M	R	Q	L	A	V	O	S	S	N	G	S	F	S	D	--	E	D	R	K	Q	Y	T	A	E
		BACILLUS SUBTILIS	L	O	R	V	R	E	L	V	V	O	A	G	N	T	G	T	Q	D	K	A	T	D	L	Q	S	I	Q	D
Bacilli	{	LACTOBACILLUS MALI	I	E	R	M	R	D	L	S	V	O	S	A	N	S	T	L	S	D	--	S	D	R	Q	D	I	Q	K	E
		LACTOBACILLUS RUMINIS	L	E	R	M	R	D	L	A	V	O	G	S	N	G	T	L	S	--	T	D	R	G	S	I	N	K	E	
	{	CLOSTRIDIUM NOVYI	L	O	R	M	R	E	L	S	V	O	A	A	N	D	T	N	K	T	--	E	D	R	A	M	I	Q	K	E
		CLOSTRIDIUM PASTEURIANUM	L	O	R	M	R	E	L	A	V	O	S	A	T	D	T	N	T	D	--	D	D	R	A	K	I	Q	A	E
		ROSEBURIA HOMINIS	L	O	R	M	N	E	L	A	T	O	A	A	N	D	T	N	T	T	--	S	D	R	T	A	V	Q	Q	E
		ROSEBURIA INTESTINALIS	L	O	R	M	R	E	L	S	V	O	A	A	N	G	T	E	T	D	--	D	D	R	E	A	V	Q	N	E
		ROSEBURIA INULINOVORANS	L	O	R	M	N	E	L	A	V	O	S	A	N	G	T	N	A	Q	--	S	D	R	D	A	I	Q	A	E

To test if the presence of these critical amino acids was, in fact, sufficient for TLR5 recognition, I purified flagellin from a variety of cultured bacteria and tested their ability to stimulate TLR5 using a HEK cell reporter assay (see Table 2 for strain information). As expected based on previous studies and the presence of amino acid residues in the flagellin protein known to be important in TLR5 stimulation [18, 32], purified flagellin from all three tested species of γ -Proteobacteria elicited a TLR5 response (Figure 5, Table 3). I also tested six Clostridia species and three Bacilli species and found that flagellin from all of the Clostridia, but none of the Bacilli, stimulated TLR5. I included pure LPS from *E. coli* as well as a mock flagellin extraction of the aflagellate Bacillus, *Staphylococcus epidermidis*, to confirm that neither of these substances are capable of eliciting a TLR5 response in the HEK cell reporter assay. These data suggest that, while not all flagellated members of the gut microbiome cause TLR5 stimulation, many commensal bacteria belonging to a diversity of phylogenies produce flagellin with TLR5 stimulatory capacity.

Table 2. Strain information for bacteria used in this study.

Genus	Species	Strain	Type strain	Motile	Source
<i>Clostridium</i>	<i>ramosum</i>	DSM 1402	Y	Y	DSMZ
<i>Clostridium</i>	<i>scindens</i>	ATCC 35704	Y	Y	DSMZ
<i>Clostridium</i>	<i>bartlettii</i>	DSM 16795	Y	Y	DSMZ
<i>Roseburia</i>	<i>intestinalis</i>	L1-82	Y	Y	DSMZ
<i>Clostridium</i>	<i>bolteae</i>	ATCC BAA-613	Y	Y	DSMZ
<i>Providencia</i>	<i>stuartii</i>	ATCC 25827	N	Y	DSMZ
<i>Roseburia</i>	<i>inulinivorans</i>	A2-194	Y	Y	DSMZ
<i>Lactobacillus</i>	<i>brevis</i>	DSM 20054	Y	Y	DSMZ
<i>Lactobacillus</i>	<i>sakei</i>	NRRL B-1917	N	Y	ATCC
<i>Citrobacter</i>	<i>amalonaticus</i>	NRRL B-41228	Y	Y	ATCC
<i>Staphylococcus</i>	<i>epidermidis</i>	ATCC 14990	Y	N	ATCC

Table 3. Ability of flagellin from different bacteria species to stimulate TLR5 in an *in vitro* reporter cell assay. All species are commensal except for *Citrobacter amalonaticus* and *Salmonella enterica* serovar Typhimurium.

Species	Phylum	Class	Reactivity
<i>Citrobacter amalonaticus</i>	Proteobacteria	γ -Proteobacteria	+
<i>Salmonella</i> Typhimurium	Proteobacteria	γ -Proteobacteria	+
<i>Providencia stuartii</i>	Proteobacteria	γ -Proteobacteria	+
<i>Roseburia inulinovorans</i>	Firmicutes	Clostridia	+
<i>Roseburia intestinalis</i>	Firmicutes	Clostridia	+
<i>Clostridium scindens</i>	Firmicutes	Clostridia	+
<i>Clostridium ramosum</i>	Firmicutes	Clostridia	+
<i>Clostridium bolteae</i>	Firmicutes	Clostridia	+
<i>Clostridium bartlettii</i>	Firmicutes	Clostridia	+
<i>Staphylococcus epidermidis</i>	Firmicutes	Bacilli	-
<i>Lactobacillus brevis</i>	Firmicutes	Bacilli	-
<i>Lactobacillus sakei</i>	Firmicutes	Bacilli	-

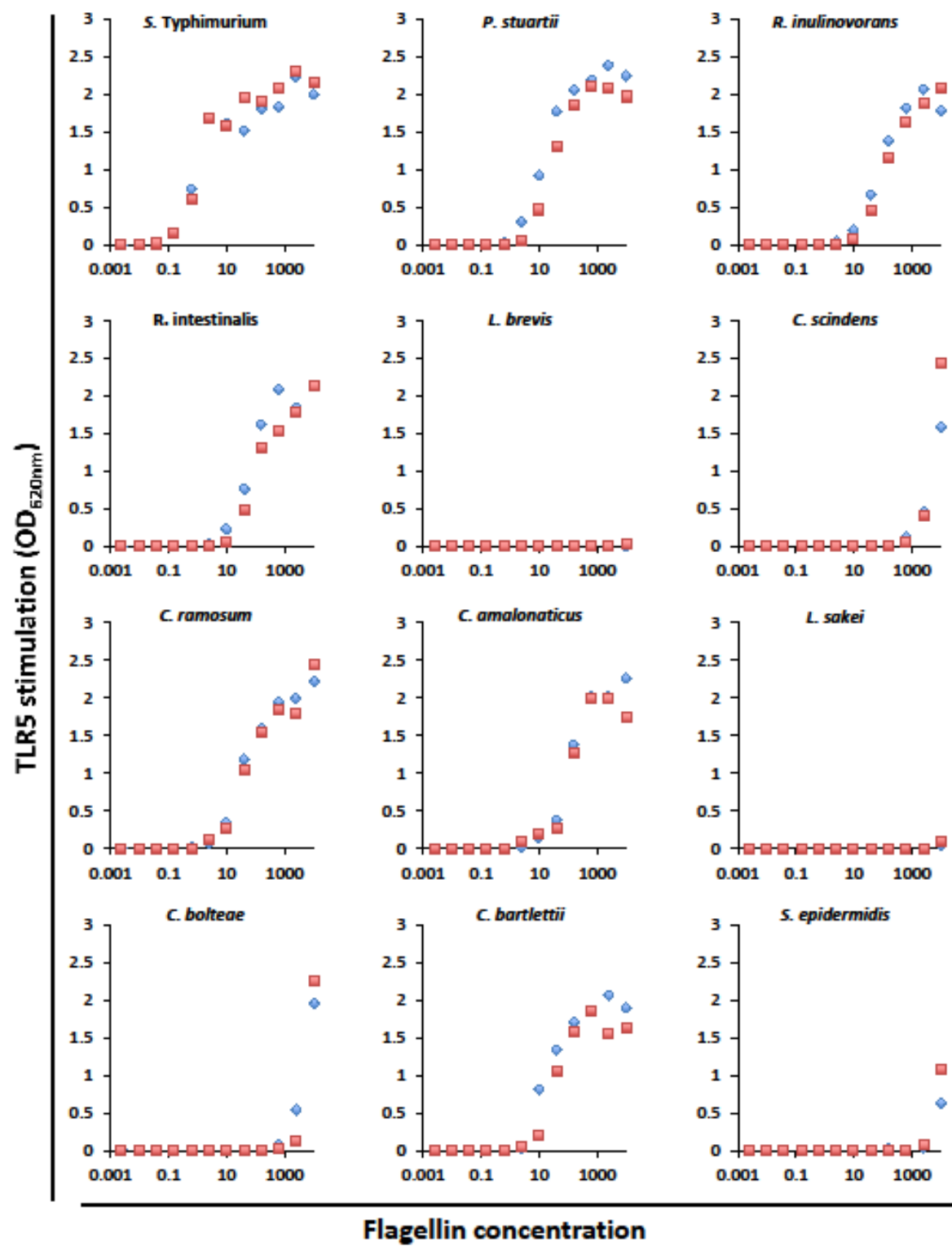


Figure 5. Flagella harvested from several species of gut commensal Firmicutes and Proteobacteria show a strong stimulatory potential for TLR5. We purified flagella from three Proteobacteria and nine Firmicutes and assayed for their ability to stimulate TLR5. Purified flagellin from *S. Typhimurium* serves as a positive control and *S. epidermidis* serves as a negative control because it does not produce flagella. Blue diamonds represent boiled flagella preparations and red squares represent unboiled preparations. The x-axis represents ng/mL of crude flagella extract, displayed on a log₁₀ scale. The y-axis represents alkaline phosphatase activity at 620 nm.

Next, I wanted to test the ability of anti-flagellin antibodies to cross-react with flagellin of bacteria other than the intended target species. To do this, I collaborated with Benoit Chassaing and Andrew Gewirtz of Georgia State University to perform Western blots. Benoit Chassaing used two different anti-flagellin antibodies either targeting *S. enterica* serovar Typhimurium (anti-FlaC) or *Roseburia hominis* (anti-Fla2) to probe flagellin extracts from a variety of species via Western blotting. The anti-*R. hominis* antibody, which was a component of polyclonal antiserum, recognized five of the six Clostridia, two of the three γ -Proteobacteria—including *S. Typhimurium*—and zero of the three Bacilli (Figure 6A). Interestingly, all flagellin types that were recognized by the anti-Fla2 antibody also elicited a TLR5 response. One Firmicute (*C. bolteae*) and one γ -Proteobacteria (*C. amalonaticus*) were not bound by anti-Fla2 antibody even though they elicited a TLR5 response, suggesting that there is not a perfect correlation between the cross reactivity of anti-flagellin antibodies and the immunogenicity of flagellin. A few of the bands on the Western are of differing size, but this may be explained by the differences in amino acid length of flagellin proteins from various species. For example, the flagellin protein of *P. stuartii* has an amino acid length of 362 while *R. intestinalis* and *R. inulinovorans* flagellins have lengths of 421 and 426 amino acids, respectively (Table 5) according to the online protein databases Uniprot and Patric [33, 34]. The anti-*S. Typhimurium* antibody, which was monoclonal, recognized *S. Typhimurium* flagellin, but none of the other flagellin extracts used (Figure 6B).

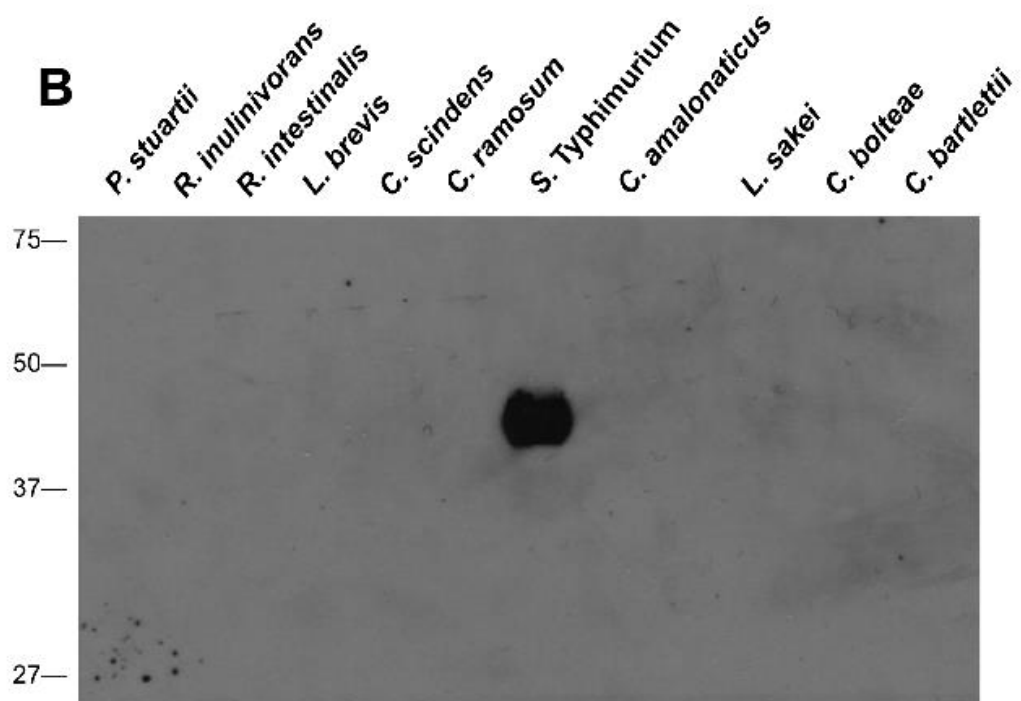
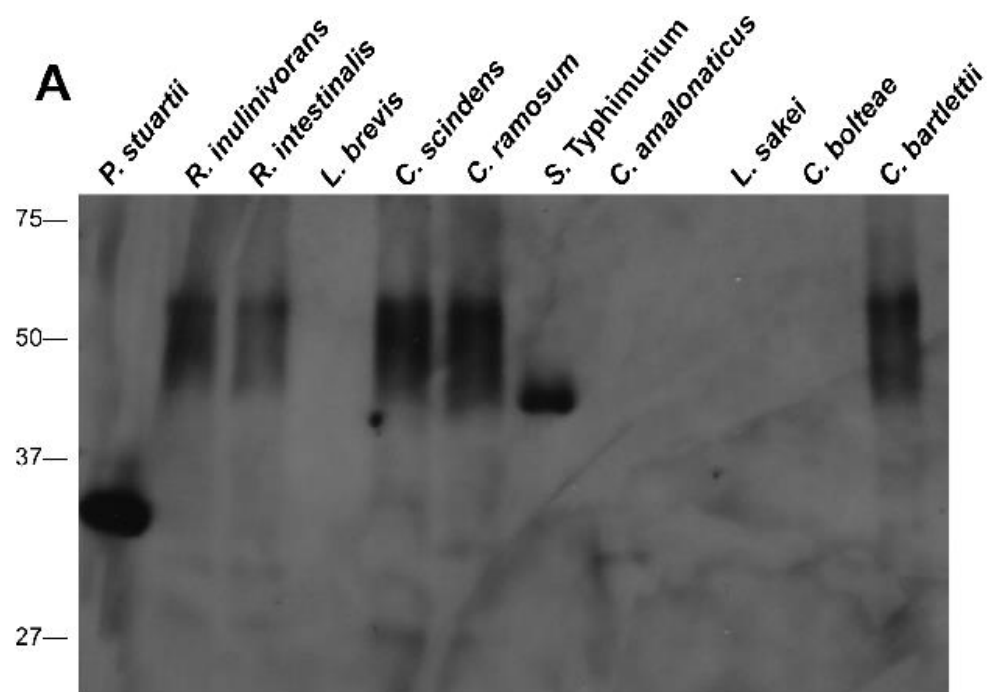


Figure 6. Immunogenic epitopes are shared between flagella from members of Proteobacteria and Firmicute phyla. Western blot analyses of crude flagellin preparations from Proteobacteria (1,7,8) and Firmicutes (2-6,9-11) probed with (A) primary polyclonal serum antibodies raised against a recombinant flagellin peptide from Firmicute *Roseburia hominis*, or (B) primary monoclonal antibody raised against *S. Typhimurium* flagellin. Protein size listed in kilodaltons to the left of each image.

Table 4. Molecule mass of predicted flagellin filaments from the bacteria that were used in Western blots. Bacteria are listed with their corresponding flagellin protein as annotated by either the Uniprot or Patric online databases. Amino acid length (AA) as well as approximate size (kD) are given. Note that not all bacteria used in Western blots had annotated flagellin proteins.

Species	Uniprot		Patric	
	AA	kD	AA	kD
<i>P. stuartii</i>	362	39.82	362	39.82
<i>R. inulinovornas</i>	392	43.12	426	46.86
<i>R. intestinalis</i> <i>Fla1</i>	445	48.95		
<i>R. intestinalis</i> <i>Fla2</i>	421	46.31		
<i>S. Typhimurium</i>	495	54.45	495	54.45
<i>C. amalonaticus</i>	553	60.83		
<i>C. bolteae</i>			501	55.11

If a healthy mouse with an intact immune system uses anti-flagellin IgA to quench the motility of its resident microbiota, then one would expect that antibodies derived from this mouse would bind to flagellin proteins from a variety of commensal bacteria. To test this, we performed Western blots in which the same panel of purified flagellins was probed with a cell-free fecal supernatant from a healthy WT mouse (Figure 7). The results are not definitive, likely because neither the flagellin purifications nor the fecal supernatant are completely pure products, but there are striking similarities between the Western blot probed with anti-Fla2 antibody and the blot probed with fecal supernatant. For example, both exhibit bands at approximately 60 kD in the lanes corresponding to *R. inulinovorans*, *R. intestinalis*, *C. ramosum*, and *C. bartlettii*. Confirming this result will likely require the development of protocols to further purify the flagellin and purify the fecal antibodies. However, the similarities suggest that healthy mice produce secretory antibodies that recognize and bind flagellin from a diversity of Firmicutes in much the same way that the antibody produced against *R. hominis* flagellin (Fla2) is cross-reactive in nature.

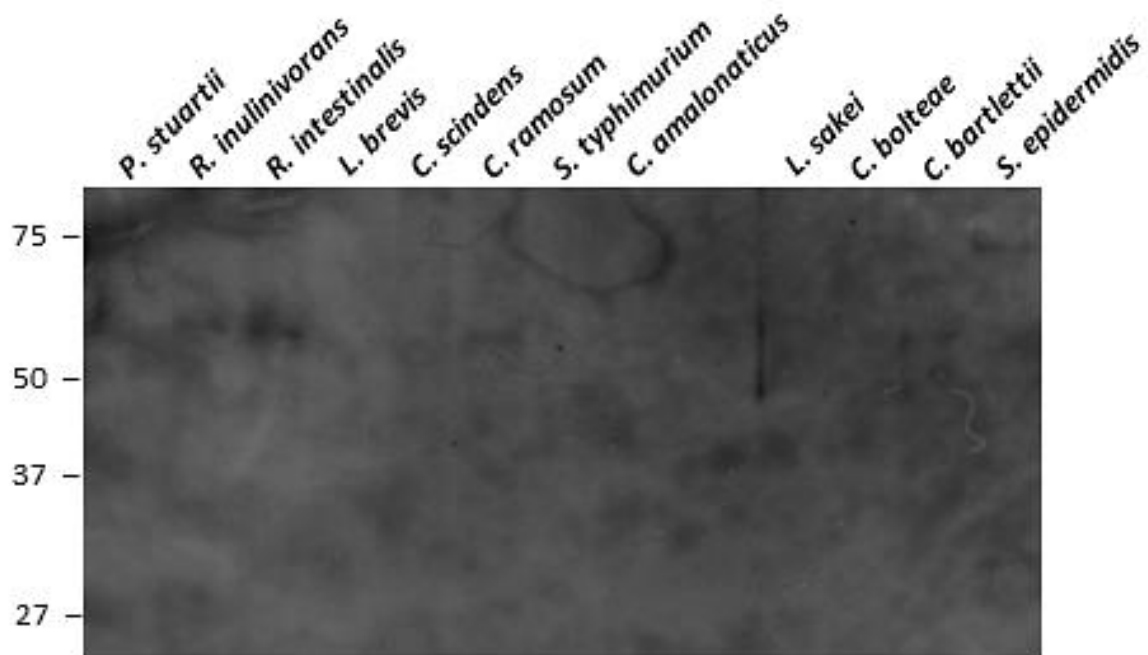


Figure 7. IgA from WT mice recognizes flagellin from multiple commensal bacteria.

Western blot analyses of crude flagellin preparations from Proteobacteria (1,7,8) and Firmicutes (2-6,9-12) probed with antibody-containing fecal supernatant from a WT mouse. Protein size listed in kilodaltons to the left of each image.

To better understand if anti-flagellin antibodies inhibit motility in flagellated Firmicutes in the same way that occurs in γ -Proteobacteria, I performed time-lapse microscopy of cultured bacteria after the addition of anti-flagellin antibodies. I used two flagellated Firmicutes common to the gut microbiota—*Roseburia intestinalis* and *Clostridium ramosum*—and observed that both displayed inhibited motility within 30 minutes after introducing anti-FliC antibody or anti-Fla2 antibody. This is in contrast to the control cultures that were mixed with water or anti-mouse antibody, which did not show decreased motility after 30 minutes. Videos are available for download and viewing on Cornell University Library eCommons website (<http://ecommons.library.cornell.edu/handle/1813/31547>), which is a digital repository. These data indicate that, similar to *E. coli* and *S. Typhimurium*, flagellated Firmicutes also experience inhibited motility when exposed to anti-flagellin antibodies.

As further confirmation that the microbiome of TLR5^{-/-} mice exhibits an increased frequency of motile bacteria compared to the WT microbiome, I performed similar time lapse microscopy using freshly dissected large intestines from each mouse type. Not only do bacteria harvested from the large intestine of TLR5^{-/-} mice appear to display increased motility compared to bacteria from WT mice, but bacteria from both mouse types displayed decreased motility within one hour after the addition of anti-FliC or anti-Fla2 antibody compared to a no-antibody control. Videos are available for download and viewing on Cornell University Library eCommons website (<http://ecommons.library.cornell.edu/handle/1813/31548>). These results agree with the finding that more flagella-related transcripts exist in the transcriptome of the bacterial community in TLR5^{-/-} mice. Further, the videos support the idea that anti-flagellin antibodies secreted into the gut can act to inhibit motility in a wide range of bacterial taxa by cross reacting with multiple flagellin types.

4.4 Conclusion

Together, these results expand our understanding of the phenotype presented by TLR5^{-/-} mice by revealing a disrupted mucosal barrier in the intestine and suggesting that commensal Firmicutes can function in much the same way as γ -Proteobacteria in their TLR5 stimulatory capacity and response to anti-flagellin antibodies. Simply due to a lack of TLR5, these mice fail to maintain functional mucus layers and adequate IgA repertoires. Previous studies indicate that gut IgA can protect the epithelium from commensal bacteria through steric hindrance, binding adhesion epitopes, anchoring bacteria to mucus via IgA secretory component, and agglutination [35, 36]. My results expand this view by suggesting that anti-flagellin IgA is also important in limiting flagellation of commensal bacteria, thus protecting against penetration into host tissues. Since the outer mucus layer is an important site of nutrients and colonization by commensal bacteria, the disrupted stratification of mucus observed in TLR5^{-/-} mice may serve to exacerbate the host damage caused by commensal bacteria as they attempt to occupy alternative niches. The similarities in symptoms presented by individuals with inflammatory bowel disease [37], primary antibody disorders [38], and TLR5^{-/-} mice suggest that the loss of gut homeostasis may have fairly universal causes at its core.

4.5 Materials and methods

Tissue preparation for microscopy: All procedures performed were reviewed and approved by the Cornell University IACUC in advance. Mice were euthanized individually and dissected by Mary E. Bell using CO₂ inhalation and large and small intestine were and immediately placed into Carnoy solution (60% absolute ethanol, 30% chloroform, 10% glacial acetic acid) without

luminal washing. All procedures were completed by approximately 12 minutes post-mortem. For motility assays, the large intestines were processed similarly, but instead of placing in Carnoy solution, they were immediately placed into an anaerobic chamber in order to best maintain the active motility of strict anaerobic bacteria. Carnoy-fixed tissues were allowed to fix for 24 hours before being transferred to 70% ethanol and submitted for processing at the Animal Health Diagnostic Center Histology Laboratory, Cornell University. There, the tissues were embedded in paraffin, sectioned at a width of 5 microns, and mounted on glass slides. To deparaffinize and rehydrate the tissues, I passaged the slides 3 times in 100% xylene, then 80% ethanol, then 50% ethanol, each for 20 min. I encircled the tissues on each slide with a Super PAP Pen (Daido Sangyo Co., Ltd. Tokyo, Japan) to increase liquid retention. I used this procedure for both FISH and immunohistochemistry assays.

Fluorescent in situ hybridization: I selected rRNA probes using probeBase [39]. I chose EUB338 (5'- GCT GCC TCC CGT AGG AGT -3') [40] as a universal 16S rRNA probe and GAM42a (5'- GCC TTC CCA CAT CGT TT -3') as a γ -Proteobacteria 23S rRNA probe [41]. There is not a single comprehensive probe for the phylum Firmicutes, so to more thoroughly detect the Firmicutes I used three 16S rRNA probes in an equimolar ratio: LGC354a (5' - TGG AAG ATT CCC TAC TGC - 3'), LGC354b (5' - CGG ATT CCC TAC TGC - 3'), and LGC354c (5' - CCG AAG ATT CCC TAC TGC - 3') [42]. As a specificity control, I chose NONEUB (5'- ACT CCT ACG GGA GGC AGC -3') which has been shown to not recognize bacterial ribosomal sequences [43]. The universal bacteria and γ -Proteobacteria probes were conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, California) and the Firmicutes and specificity probes were conjugated to Alexa Fluor 555. I mixed the specificity probe in

equimolar ratios with each of the target probes. To make the bacterial cells more amenable to FISH probing, I partially digested them by incubating the slides at 37°C for 1 hour with 150 µl proteinase buffer (0.05M EDTA, 0.1M Tris-HCl, 1.5% lysozyme from chicken egg white (Sigma-Aldrich, St. Louis, Missouri)). I then rinsed slides in sterile water and added 135 µl of hybridization buffer (30% formamide, 0.0002% SDS, 0.02M Tris-HCl, 0.9M NaCl) that I premixed with 15 µl of 50 ng/µl Alexa Fluor conjugated probe (Invitrogen, Carlsbad, California). Probes were allowed to hybridize overnight at 46°C in a humidified chamber without light. I constructed humidified chambers by wrapping 50 ml conical vials in aluminum foil and placing a strip of wetted filter paper inside, such that the vials each held a single slide and were laid sideways within a precision incubator. I then rinsed slides in 48°C washing buffer (0.02M Tris-HCl, 0.112M NaCl, 0.005M EDTA, 0.0001% SDS) and incubated slides in fresh washing buffer for 30 minutes at 48°C. After that, I rinsed slides in ice cold water and allowed them to dry without exposing to light. To stain host tissue, I incubated slides with 150 µl of 2 µg/ml Hoechst 33342 (Invitrogen, Carlsbad, California) for 5 minutes before rinsing with sterile water and allowing to dry once more. I mounted finished slides with the Prolong Antifade Kit (Invitrogen, Carlsbad, California) according to manufacturer's instructions.

Immunohistochemistry: I washed slides twice for 5 minutes in Tris-buffered saline (TBS) (50mM Tris-Cl, 150mM NaCl, pH 7.5) plus 0.025% Triton X-100 with gentle agitation. I then blocked the tissues for 2 hours at room temperature with 150 µl of 10% normal goat serum (Sigma-Aldrich, St. Louis, Missouri) with 1% BSA in TBS, after which I drained off the blocking solution and applied either rabbit anti-flagellin IgG (Abcam, Cambridge, Massachusetts) or FITC-conjugated goat anti-mouse-IgA (Sigma-Aldrich, St. Louis, Missouri)

diluted 1:100 in TBS with 1% BSA and incubated the slides overnight at 4°C. The following morning I rinsed the slides twice for 5 minutes in TBS plus 0.025% Triton X-100. I incubated the anti-flagellin slides for 1 hour at room temperature with 150 µl of TBS with 1% BSA and 0.1% CHROMEOSity 488-conjugated goat anti-rabbit IgG (Neuromics Antibodies, Edina, Minnesota). I then rinsed the slides 3 times for 5 minutes each in TBS, allowed them to dry, and incubated slides with 150 µl of 2 µg/ml Hoechst 33342 (Invitrogen, Carlsbad, California) for 5 minutes before rinsing with sterile water and allowing to dry once more. Finally, I mounted the slides using the Prolong Antifade Kit (Invitrogen, Carlsbad, California) according to manufacturer's instructions.

Fluorescent microscopy: All fluorescent microscopy was performed on a Zeiss LSM 710 confocal microscope equipped with Zen system software via the Microscopy and Imaging Facility at Cornell University. Unless stated otherwise, I used a 63x oil immersion objective and 405 and 488 nm emitting lasers. I adjusted laser power to 2% and held master gain between 500-750, with a digital offset of 0. To maintain high resolution, I used unidirectional scanning and a maximum pixel size of 1024×1024 .

Bacterial cultures: I cultured *Lactobacillus brevis* and *Lactobacillus sakei* in MRS liquid medium and all Proteobacteria in LB liquid medium in an aerobic environment with shaking at 37°C overnight. I cultured all other Firmicutes in M2GSC liquid medium in 100 ml anaerobic serum vials which were incubated at 37°C for 24 hours or longer depending on observed growth rate.

Flagellin purification: I transferred 80 mL of each culture into two 50 mL conical vials, divided evenly, and sheared flagellin from the bacteria with two minutes of vortex agitation. I then centrifuged the cultures at 2,754x (g) for 30 minutes at 4° C in order to pellet bacterial cells and debris. I transferred the resulting supernatants into 30 mL ultracentrifuge tubes and centrifuged the samples at 40,000 rev/minute for 1.5 hours at 4° C. I resuspended the resulting pellets in 1 ml of PBS and centrifuged the samples once more at 5,000 rev/minute for 30 minutes at 4° C in order to pellet any remaining large cellular debris. In order to reduce the confounding factor of endotoxin present in the flagellar purification of Gram-negative bacteria, I then detoxified the samples with polymyxin B using Detoxi-Gel AffinityPak Pre-packed Columns (Thermo Scientific, Rockford, Illinois).

Motility assays using time lapse microscopy: I cultured *Roseburia intestinalis* and *Clostridia ramosum* under strict anaerobic conditions before applying 10 µl of culture with either 1 µl of anti-flagellin antibody or water to a glass slide. I processed mouse colonic contents by opening the colon at its approximate midpoint with a razor, removing approximately 50 mg of colonic contents via scraping, and diluting the contents with 2 ml of PBS buffer, all under anerobic conditions. I prepared the diluted colonic contents in the same manner as the bacterial cultures. I sealed the cultures or colonic contents with a glass cover slip and acrylic sealant and allowed the sealant to dry fully before removing from the anaerobic chamber. I immediately captured a short time lapse video with bright field microscopy in order to achieve a sense for baseline motility. I captured a second time lapse video after 30 minutes (bacterial cultures) or 60 minutes (colonic contents) in order to observe potential changes in the frequency or degree of bacterial motility. All time lapse microscopy was performed on an upright Olympus BX-50 microscope with a 60x

magnification objective and the Metamorph imaging system. I made the videos with a spacing of 0.3 seconds over the course of 35 frames.

Statistics: I performed statistical analysis in Microsoft Excel 2011. Statistical test was unpaired student 't'-test, as indicated. Significance level of $p < 0.05$ unless otherwise indicated. All multiple comparison testing was accounted for with Bonferroni correction.

4.6 References

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5. Conclusion

My research presented here has been devoted to understanding how TLR5 directs the production of anti-flagellin IgA in order to quench bacterial motility in the gut and thus protect against gut inflammation. In the absence of TLR5, the host produces insufficient levels of anti-flagellin IgA and the microbiota responds by increasing flagellation. Using metatranscriptomics and metagenomics of the gut bacterial community, I found that this increase in flagellation is due to a shift in protein expression rather than a shift in phylogenetic composition and that a diverse suite of commensal bacteria are responsible for the increased flagellin observed in TLR5^{-/-} mice. The defective anti-flagellin adaptive immune response in TLR5^{-/-} mice is more strongly tied to their innate immune defect rather than their increased gut inflammation. I came to this conclusion because MyD88^{-/-} mice, which have impaired TLR5 signaling, display flagellin and anti-flagellin concentrations similar to TLR5^{-/-} mice whereas WT mice treated with DSS, which induces gut inflammation, display parameters more similar to non-inflamed WT mice.

I also observed this inverse relationship between flagellin and anti-flagellin antibodies in human fecal samples. Interestingly, flagellin load was positively correlated with BMI in these individuals, suggesting that obesity and a deficiency in anti-flagellin IgA may somehow be linked. As TLR5^{-/-} mice also display obesity, these findings promote the intriguing idea that obesity coupled with defective control of the flagellated gut microbiota could be a universal phenomenon among mice and humans. Future work may elaborate on these results by incorporating a larger human sample size and seeking out individuals with TLR5 deficiencies. Initial studies suggest that human TLR5 deficiency protects from obesity and Crohn's disease but predisposes to type II diabetes [1, 2], but the mechanisms behind these correlations remain unknown.

I found that the addition of anti-flagellin antibodies to *in vitro* bacterial cultures induced downregulation of flagellin and a severe decrease in motility, suggesting that the inverse relationship between anti-flagellin IgA levels and flagellin load in the gut is causal rather than just correlative. Despite the high metabolic expenditure required for flagellation, I was not able to observe a fitness advantage due to the presence of flagella, as flagellated bacteria were not better suited to colonizing the gut than aflagellate bacteria of the same strain. This may have been due to the relatively simple communities that I used to investigate this issue and future work involving more complex communities may be required to understand the benefits of flagellation for nonpathogenic gut bacteria.

The results in chapter two highlight the importance of IgA in maintaining temporal evenness and stability of the gut microbiota and suggest that a particular suite of IgA specificities is required in order to coat the proper bacteria. In the absence of antibodies, the gut microbiota was not phylogenetically distinct, but rather displayed more volatility and an increased susceptibility to change when confronted with environmental perturbation. Likewise, the bacterial community of TLR5^{-/-} mice was not distinct from the WT microbiota despite the observation that these host types display significantly different profiles of the bacterial types coated with IgA. Specifically, TLR5^{-/-} mice display increased IgA coating of Firmicutes and decreased IgA coating of Proteobacteria compared to WT mice. These findings have important implications for human health because a hallmark of gut homeostasis is the ability to maintain a stable community of symbiotic bacteria [3, 4] and IgA appears to be an important mechanism of achieving this stability. Despite IgA deficiency being the most common primary immunodeficiency, clinical measurements are not currently a part of routine screenings and are almost always performed on blood serum rather than at mucosal sites [5]. This, coupled with the

fact that circulatory IgA and secretory IgA are produced through fairly distinct mechanisms [5], suggests that many mucosal antibody deficiencies are likely to go undiagnosed.

In chapter three I provide evidence that TLR5^{-/-} mice indeed have a defective mucosal barrier that results in flagellated bacteria penetrating further into the mucus. This results in epithelia-adherent bacteria in the colon and invasion of villi tissues in the small intestine. I found that flagellin proteins from a variety of commensal bacteria are capable of stimulating TLR5 which, coupled with my observation that flagellin transcripts in the TLR5^{-/-} mouse belong to a diverse suite of bacteria, suggests that the pathogenesis of TLR5 deficiency is due to a broad shift in genetic expression of the gut microbiota. This corroborates an inability to detect a specific pathogen responsible for the phenotype of TLR5^{-/-} mice.

It has yet to be proven if TLR5-deficient individuals can be protected against inflammation by the therapeutic introduction of anti-flagellin antibodies. Another potential therapeutic strategy is the development of a vaccine that can bypass the requirement of TLR5 and induce the production of high amounts of anti-flagellin antibodies via alternative routes. One potential avenue for the creation of such a vaccine is the combination of short flagellin peptides with a proper adjuvant. Flagellin peptides processed to correspond to a highly conserved, immunogenic region of the protein have shown to elicit the maturation of flagellin-specific T cells when full-length flagellin failed to do so in TLR5^{-/-} mice [6], suggesting that these peptides do not require TLR5 to initiate an adaptive immune response. Such a vaccine might be successfully introduced into the gut via encapsulation and ingestion or a probiotic bacterium could even be engineered to secrete the peptide directly into the luminal environment [7]. If the positive correlation between flagellin load and BMI is a common trait in humans, as suggested by my findings, then the development of such a vaccine could have profound implications for

treating the increased inflammation exhibited by the growing percentage of the population living with obesity.

5.1 References

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