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THE ROLES OF MICROBIOTA AND INNATE IMMUNITY DURING ROTAVIRUS INFECTION AND HUMORAL IMMUNTIY

By

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Doctor of Philosophy

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THE ROLES OF MICROBIOTA AND INNATE IMMUNITY DURING ROTIVIRUS INFECTION AND HUMORAL IMMUNITY

By

Robin Uchiyama

B.S., University of Missouri-Columbia, 2009

Advisor: Andrew T. Gewirtz, Ph.D.

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ABSTRACT

THE ROLES OF MICROBIOTA AND INNATE IMMUNITY DURING

ROTAVIRUS INFECTION AND HUMORAL IMMUNITY

By

Robin Uchiyama

Rotavirus (RV) infection is the leading cause of viral gastroenteritis amongst children globally and is responsible for upwards of 500,000 deaths annually. While 2 vaccines are available, they do not adequately protect against disease amongst children in developing countries. With this in mind, we aimed to determine if environmental factors, specifically gut microbiota, contribute to infection and induction of humoral responses. Using an infection model, we found that microbiota-ablation, achieved through antibiotic treatment or germ-free (GF) conditions, experienced a delay in virus shedding. Postponed shedding correlated with less virus at the small intestine, demonstrating that microbiota speeds infection. Microbiota promoted RV entry as antibiotics had no effect on replication. Antibiotics also protected against RV diarrhea, demonstrating a negative role for microbiota during disease. Microbiota inhibited humoral immunity as antibiotics enhanced RV antibody production and small intestinal, IgA-producing antibody-secreting cells (ASCs) frequencies. To examine if microbiota exposure reversed the antibody-enhancing effects of antibiotics, dextran sodium sulfate (DSS) was administered. DSS increased inflammation and weakened RV antibody responses, indicating that microbiota-derived inflammation inhibits humoral immunity. Thus, the microbiota could be an environmental factor involved in vaccine inefficacy and serve as a target for therapeutics.

We also aimed to elucidate how innate immunity controls RV infection and humoral immunity, as innate immunity to RV is understudied. When mice lacking MyD88, an adaptor protein for most TLRs and inflammasome cytokine receptors, were infected, they shed more virus and experienced virus spread, demonstrating MyD88 controls infection and spread. Control of primary infection was independent of inflammasome cytokines IL-1 and -18, indicating that TLRs were responsible for limiting infection. MyD88-deficient neonates experienced greater incidence of and days with diarrhea, demonstrating that MyD88 protects against disease. Mice lacking MyD88 also experienced slowed systemic antibody responses and skewed IgG subisotype switching, with a bias towards Th2-associated IgG1 and away from Th1-associated IgG2c. MyD88's influence on antibody responses originated from bone marrow-derived MyD88, but not epithelial MyD88, and IL-1 and -18 signaling prompted proper subisotype switching. Insights into RV-specific innate immunity uncovered novel therapeutic targets, including MyD88, TLRs, and inflammasome cytokines, during RV infection and vaccination.

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CHAPTER 1: INTRODUCTION

RV is a major cause of acute gastroenteritis in infants and young children and accounts for approximately one-third of cases of severe diarrhea requiring hospitalization and up to 500,000 deaths per year (1). RV-associated economic burdens are high with RV-related disease responsible for around 500,000 physician visits, 50,000 hospitalizations, and over $1 billion in healthcare costs each year in the United States alone (1). Introduction of RV vaccines in the 2006 has largely reduced health and economic burdens, but such burdens in developing nations have not decreased to the same extent as in developed nations (2).

Since the discovery of RV, scientists have intensely studied RV virology and adaptive immunity as it pertains to vaccine development and largely ignored the influences of microbiota and innate immunity. With these gaps in knowledge in mind, we aimed to (1) determine the role of intestinal microbiota during RV infection, disease, and humoral immunity and (2) determine if RV infection, disease, and humoral immunity is mediated by MyD88, an adaptor protein responsible for most TLRs and inflammasome cytokine receptors. Understanding how microbiota and innate immunity influences RV infection and humoral immunity would provide opportunities for improving vaccine efficacy in developing nations and creating antiviral therapeutics for those who do not benefit from vaccination or are experiencing severe disease.

Here we show that microbiota depletion using antibiotics and GF conditions manipulates RV infectivity as well as subsequent humoral immune responses. Antibiotic administration before and throughout infection slowed the rate of initial infection, reduced rates of disease, and enhanced virus-specific humoral immunity months after primary infection. These results were replicated in GF mice, indicating that the effects of microbiota depletion were independent of

selection or loss of a specific bacterial species and instead were dependent on reduced microbiota. Furthermore, increased immune system exposure to microbiota provided by DSS treatment decreased virus-specific humoral immunity, illustrating that microbiota-derived inflammation interferes with immunity. This led us to conclude that the presence of microbiota negatively influenced RV immunity and microbiota manipulation during vaccination or natural infection may promote RV immunity as well as protection against disease.

Using mice deficient in MyD88, we found that control of RV infection, disease, and subsequent humoral immunity, including proper IgG subisotype switching, was dependent on MyD88 signaling. MyD88-deficient mice infected with RV saw increased virus shedding as well as increased incidence of and days with diarrhea, indicating that MyD88 signaling protects against infection and disease. The absence of inflammasome cytokines IL-1 and -18 had no effect on RV infection, demonstrating that MyD88-mediated TLR, but not MyD88-mediated inflammasome cytokines, influences infection. Furthermore, the absence of MyD88 slowed antibody generation after adult and neonatal inoculations, and the absence of MyD88 signaling in bone marrow-derived cells, but not epithelial cells, slowed and inhibited robust humoral responses. Moreover, the absence of MyD88 promoted IgG1 instead of IgG2c antibody production, indicating skewed subisotype switching and potentially a bias towards Th2 differentiation and responses. Mice deficient in inflammasome cytokines showed similar viral infection kinetics but had markedly lowered IgG2c responses than control mice, illustrating that inflammasome cytokines do not influence infection but influence IgG2c production.

1.1 Rotavirus Virology

1.1.1 Rotavirus structure and classification

RV is a genus of the *Reoviridae* family and as such, has a double-stranded (ds) RNA (dsRNA) genome composed of 11 segments. RV's genome encodes 8 structural proteins, also known as viral proteins (VPs), and 6 non-structural proteins (NSPs). The VPs perform a variety of functions from target cell attached to genome replication. In greater detail, VP1, an inner core protein, functions as an RNA-dependent RNA polymerase; VP2 acts as a scaffolding protein and also makes up the core layer; VP3 is a guanylyl transferase and methylase which facilitates RV transcription and replication; VP4 makes up the outer capsid with VP7 and attaches to target cells after proteolytic cleavage into VP5 and VP8; VP6 comprises the middle capsid; VP7 is an outer capsid protein that may be involved in binding an intracellular receptor. There are 7 groups of RV, A-G, based on VP6 antibody reactivities, and groups are further sub-classified based on VP7 and VP4 genes, known as G and P types, respectively (3).

There are 6 non-structural proteins, NSP1-6, which are only expressed in host cells and function to elude RV from the immune system and promote its lifecycle. A brief explanation of the functions of each NSP is given here, but a more detailed review is provided by Hu and others (4). NSP1 induces IRF3 degradation and prevents production of type I IFN as wells as interferon-stimulated genes (ISGs); NSP2 is a multifunctional enzyme which regulates RV replication and transcription and eluding RIG-1 detection; NSP3 has been proposed to antagonize host protein synthesis thus facilitating transcription of viral mRNA; NSP4 induces diarrhea and electrolyte loss through the release of chloride ions by intestinal epithelial cells (IECs) but is also involved in double-layered particle assembly and disruption of plasma membrane integrity; NSP5 and 6 have not been characterized to the same extent as other NSPs, but NSP5 interacts with NSP2 in viroplasms (4).

1.1.2 Rotavirus replication

RV replication has been widely studied but not completely understood; replication is similar to other dsRNA viruses and Desselberger discusses RV replication in greater detail in *Rotaviruses: Methods and Protocols* (3). RV, upon binding to receptors, enters the cell by either receptor-mediated endocytosis or direct penetration. Once in the cytoplasm, a viroplasm is formed where transcription of VPs and NSPs and replication takes place. Double-layered particles assemble followed by triple-layered particles, and the virion further matures with VP7 glycosylation. The virion releases upon cell lysis (3).

1.2 Rotavirus Pathogenesis

1.2.1 Rotavirus transmission, target cell, and kinetics of infection

RV transmission occurs via the fecal-oral route, and primarily infects mature IECs lining the small intestine (3). As a result of infection, IECs at the tip of intestinal villi rapidly undergo apoptosis and are shed, causing a blunted appearance; new cells replace infected, apoptotic cells (5, 6). In neonatal mice, virus shedding lasts approximately 10 days depending on the initial dose, and diarrhea occurs in 2 peaks of intensity within the first 5 days of infection (5). In contrast to neonatal mice, adult mice do not develop disease and infection resolves within 1 week (7).

1.2.2 Rotavirus-mediated induction of diarrhea

The mechanism as to how RV causes diarrhea is not fully understood, but NSP4 has been shown to play a prominent role in diarrhea by causing carbohydrate maldigestion, reducing IEC barrier function, activating the enteric nervous system, and mediating chloride ion loss, a key characteristic of secretory diarrhea (8). RV infection, but not replication, is required for diarrhea indicating that the amount of RV present does not necessarily

correlate with disease severity and that RV possesses a bacterial toxin-like ability to cause disease (9). RV infects mice of all ages, but unlike other diarrhea-inducing pathogens, RV diarrhea in mice is age dependent; after 2 weeks of age, diarrhea cannot be induced (7).

1.3 Rotavirus Treatment and Prevention

There are no antiviral medications currently available to treat RV disease, however, oral rehydration salts and supportive care is effective at treating and preventing dehydration caused by diarrhea and vomiting (10).

Two FDA-approved vaccines are available to infants and young children for the protection against RV-associated disease: RotaTeq and Rotarix. RotaTeq contains 5 attenuated reassortant RVs developed from human and bovine parent RV strains, and 3 doses are given orally in the series. Rotarix is formulated from an attenuated human RV strain, and 2 doses are administered orally (11).

1.4 Rotavirus Vaccine Efficacy in Developing Nations

1.4.1 Rates of vaccine efficacy in developing nations

In developing countries, the efficacy of RV vaccines is less than in developed countries. For example, Rotarix only provides 50% protection in Malawi, 77% protection in South Africa, and 85% protection in Latin America against RV diarrhea. This is in contrast with protection rates in developed nations where protection reaches upwards of 96% (12). In addition to protection rates, vaccine seroconversion rates are also blunted in developing nations; serum IgA seroconversion after Rotarix administration is markedly reduced in low-income countries than in high-income countries (13). This difference in vaccine efficacy between nations is not

attributed to a discordance between circulating RV strains and RV strains included in the vaccines and instead suggests that differences between individuals may contribute to vaccine in efficacy (14).

1.4.2 Proposed explanations for lack of vaccine efficacy

While the reasons for the lack of RV vaccine efficacy in developing countries are unknown, many explanations have been proposed and include the following: environmental enteropathy, pathogenic organism co-infection, malnutrition, and maternal antibody interference.

1.4.2.1 Environmental enteropathy

Environmental enteropathy, an increasingly common condition amongst individuals of low income that live in unsanitary environments, has been attributed to decreased RV vaccine efficacy. Afflicted individuals have small bowels characterized by blunted epithelial villi, greater inflammation, bacterial overgrowth, nutrient malabsorption, and microbiota composition alterations, and such characteristics interferes with not only proper digestive activities, but also gut immune functioning (14, 15). For example, microbiota composition alters immunity in the gut, and bacterial overgrowth inhibits oral vaccine responses (16, 17). The roles of nutrient malabsorption on oral vaccine efficacy are discussed further below, as is greater inflammation in the context of pathogenic co-infections.

1.4.2.2 Pathogenic organism co-infection

Co-infections with pathogenic organisms have also been hypothesized as a reason for lowered RV vaccine efficacy. Ongoing or recurrent infections with parasites, viruses, and other organisms may produce enough inflammation such that RV vaccine strains cannot

"infect" recipients, restricting antibody production and permitting disease. This phenomenon was observed in roundworm (*Ascaris*)-infected children receiving the oral inactive cholera vaccine; children who did not receive treatment for their roundworm infections had lowered vaccine seroconversion rates than treated children (18). Due to the relatively primitive health care systems and infrastructure in most developing natures, treating infections prior to vaccination may not be an option. Instead, increasing vaccine dosage, which would overcome pathogen-derived inflammation, may increase the likelihood of vaccine seroconversion. This strategy was employed in Chilean children that were hyporesponsive to an oral cholera vaccine; a higher dose cholera vaccine induced greater antibody titers than a lower dose (19).

1.4.2.3 Malnutrition

The absence of immune-promoting nutrients, like vitamin A and zinc, has also been hypothesized as a source of decreased RV vaccine protection in developing nations. Studies have demonstrated that vitamin A metabolites, like retinoic acid, promote T and B cell homing to the gut and a balance of gut regulatory to inflammatory T cells (20). Like vitamin A, zinc has also been shown to enhance immunity. Zinc supplementation increases T and B cell activity, enhances antibody responses, and also stimulates oxidative bursts and phagocytosis in macrophages during bacterial infection (21-23). One could imagine that vaccination in the absence of these nutrients could prevent macrophage functioning, inhibit immune cell homing and activity, encourage an inflammatory environment, and potentially, hinder RV vaccine seroconversion.

A vitamin newly recognized to regulate immunity and modulate infection is vitamin D. Like supplementation with vitamin A and zinc, vitamin D supplementation may

increase protection against infection and promote antiviral immunity. In fact, vitamin D supplementation among Mongolian school children reduced the risk for acute respiratory diseases (24). Moreover, low vitamin D levels have long been associated with the susceptibility to *Mycobacterium tuberculosis* infection, and vitamin D was once used to treat such infections before the development of antibiotics (25). It is unclear if vitamin D deficiencies are common in children from developing countries and if those deficiencies would affect RV vaccine seroconversion; however, in light of the study in Mongolian children, further studies addressing the prevalence of vitamin D deficiencies and the mechanisms behind vitamin D-mediated immune cell functioning are clearly warranted.

1.4.2.4 Maternal antibody interference

Maternal antibody, by blocking vaccine strain infection and seroconversion, has been hypothesized to reduce RV vaccine efficacy in developing countries. In a vaccine trial where RV vaccination was delayed from 6 to 10 weeks of age, recipients who began vaccination at 10 weeks, as opposed to 6, experienced increased seroconversion (26). While the study did not show that maternal IgG blocked vaccine strain infection, it was inferred that systemic IgG degradation contributed to greater seroconversion. Breast milk has long shown RV-neutralizing activity, and all mothers, no matter the degree of wealth, produce RV-neutralizing IgA. Mothers from developing nations, however, produce more breast milk antibody and antibody with greater neutralizing activity, indicating that mothers from developing nations have a higher propensity to block vaccine "infection" and consequent immunity (26).

1.5 The Immune Response to Rotavirus Infection

The immune response to RV infection involves both the innate and adaptive immune systems with components of the adaptive system necessary for clearance of virus and protection against reinfection. Here I outline the known involvement of the immune system from innate to cellular immunity.

1.5.1 The innate immune response

Not much is understood regarding innate immunity and RV infection. Studies related to pattern recognition receptor (PRR)-mediated RV sensing in humans and animals and PRR-mediated type I IFN production are explored below.

1.5.1.1 TLRs

Similar to other pathogens, RV expresses pathogen-associated molecular patterns (PAMPs) capable of binding PRRs and activating protective signaling. Up to 2 days post inoculation of IECs to RV, upregulation of TLR2, 3, 7, and 8 was observed (27). This pattern of TLR upregulation was also observed in peripheral blood mononuclear cells (PBMCs) of children infected with RV. Up to 3 days after illness onset, PBMCs upregulated TLR2, 3, 4, 7, and 8 with TLR3 and 8 remaining upregulated long after illness onset (28). Neither study addressed the consequence of TLR involvement, however, both demonstrated potential roles for a variety of TLRs during infection.

TLR3, a sensor for dsRNA, has been more thoroughly investigated during RV infection. *In vivo* exposure to RV-derived dsRNA induced IEC apoptosis, prevented timely cellular repair, and provoked IL-15 production thought to attract natural killer (NK) cells to the site of infection (29, 30). Such TLR3 expression and signaling could be considered somewhat beneficial to the host. Despite a protective role for TLR3, a more recent study demonstrated a

much different finding; Pott and colleagues found that TLR3 expression is limited in mice most susceptible to infection, and thus TLR3 is incapable of participating in a protective response (31). Clearly, more research is needed to define which TLRs control of RV infection and influence immunity during infancy and beyond.

1.5.1.2 PKR

Protein kinase R (PKR), an intracellular sensor of dsRNA, has also been shown to mediate the IEC response to RV infection. In the presence of a pharmacologic inhibitor to PKR, RV inoculation of IECs reduced IL-8 expression; such reduction in IL-8 indicated a novel role for PKR in the IEC-mediated recognition of RV infection. Surprisingly, reduced IL-8 production was not seen with pharmacologic inhibition of TLR3 or other intracellular TLRs indicating that PKR alone may be involved in RV genome-facilitated IL-8 induction (32).

1.5.1.3 RLRs

RLRs were recently found to be necessary for type I IFN production during RV infection. RIG-I, MDA5, and their common adaptor, MAVS, were required for IFN-β production by IECs upon infection with RV. Moreover, the absence of MAVS enhanced RV replication *in vivo*, demonstrating a role for RLR-facilitated in RV genome recognition (33, 34).

1.5.2 The adaptive immune response

Unlike the innate response to RV infection, the adaptive response to RV has been thoroughly studied. A summary of the T cell, B cell, and antibody responses to RV infection is discussed below.

1.5.2.1 CD4 T cells

In those most susceptible to RV infection, infants and young children, the RV-specific CD4 T cell response is blunted. Infants aged 16 days to 6 months lack proliferative, functional RV-specific CD4 T cells in circulation despite the presence of neutralizing antibody. During convalescence and as an individual ages, more proliferative, functional, RV-specific CD4 T cells are found in circulation (35, 36).

The necessity of RV-specific CD4 T cells during virus clearance is not clear. McNeal and others depleted CD4 T cells in mice and found that such depletion prevented RV clearance; transfer of VP6 and adjuvant-primed CD4 T cells to chronically-infected mice or mice depleted of CD4 T cells were able to clear infection (37, 38). Two separate studies, on the other hand, demonstrated opposing conclusions; depleting CD4 T cells had no effect on clearance of RV infection (39, 40).

CD4 T cells are critical for robust RV-specific mucosal and systemic antibody responses. CD4 T cell depletion in animal models reduced RV-specific intestinal and serum IgA production; such low levels of antibody produced in the absence of CD4 T cells were similar to levels observed in T cell-deficient mice (37, 39, 40).

1.5.2.2 CD8 T cells

Like other adaptive immune cells, the function of CD8 T cells during RV infection has been well characterized. In mice deficient of functioning CD8 T cells, virus was shed 2 days longer than immunocompetent mice, however, mice lacking functional CD8 T cells completely resolved primary RV infection. Protection against reinfection and intestinal IgA production was not dependent on CD8 T cells, as mice deficient in functioning CD8 T cells were resistant to reinfection and produced similar amounts of IgA (40, 41).

1.5.2.3 T regulatory and γδ T cells

The role of other T cells beyond CD4 and 8 T cells in the context of RV infection has been somewhat explored. Suckling mice inoculated with RV saw increased T regulatory cell number and percent in the spleen and mesenteric lymph nodes (MLNs); depletion of T regulatory cells increased RV-specific CD4 and CD8 T cell functionality, including proliferation and IFN-γ secretion and B cell numbers (42). γδ T cells do not play a role in RV infection as mice deficient in the γδ T cell receptor shed similar virus and induced similar amounts of fecal RV-specific IgA (40).

1.5.2.4 B cells

The RV-specific B cell response has been widely studied as mucosal IgA production and frequency of memory B cells in gut-associated lymphoid tissue (GALT) correlates to protection against RV diarrhea (43). RV infection in mice dramatically increases B cell size and frequency in Peyer's patches (PPs) and MLNs. This increased frequency of activated B cells was seen in the absence of activated T cells, and the primary antibody produced was IgM, not IgA, together indicating that initial RV clearance may be mediated by a T cell independent mechanism (44).

While a T cell independent mechanism has been implicated for early antibody production, B1 B cells are not responsible for IgA needed to completely clear RV and protect against reinfection; severe combined immune deficiency mice reconstituted with B1 B cells were unable to totally clear chronic infection (45).

1.5.2.5 Antibody response

Intestinal IgA production after natural infection or vaccination is a correlate to protection against RV disease, and because of intestinal IgA's imperative role in protection, it has been thoroughly investigated (46). One early study found that IgA was dispensable for protection against infection; mice deficient in IgA cleared infection at a similar rate as control mice and were protected with rechallenge 6 weeks after primary infection (47). Such results were not replicated in a more recent investigation by Blutt and colleagues where IgA was necessary for timely clearance and protection against reinfection (48).

1.5.3 IFN response to rotavirus infection

IFNs are typically made during viral infections to prevent the spread of infection, promote elimination of infected cells, and stimulate antiviral immunity. Similar to many viruses, RV elicits IFN production, including type I, II, and III IFNs.

1.5.3.1 Type I IFN

After RV infection, type I IFN is readily produced *in vitro* and *in vivo*; whether type I IFN is beneficial or detrimental during infection is not clearly understood. *In vitro* studies by Frias and others demonstrated that type I IFN signaling is readily observed with RV infection of human IEC lines, and aforesaid IFN signaling promotes IEC apoptosis while simultaneously increasing RV replication (49). *In vivo* studies have generally shown that type I IFN somewhat influences infection and disease. Mice deficient in STAT1 had similar rates of clearance as control mice, however, STAT1 deficient mice shed 100 times more virus. Despite differences in the amount of virus shed, type I IFN does not affect the duration or severity of diarrhea as mice deficient in type I IFN receptor or STAT1 had similar duration and severity of diarrhea to control mice (50-52).

1.5.3.2 Type II IFN

Type II IFN does not affect disease in neonatal mice as RV infection of type II IFN receptor and STAT1 deficient neonates had similar disease duration and diarrhea intensity to controls; in addition, type II IFN did not affect the length of RV antigen shedding in adult mice (50-53). Type II IFN receptor KO neonates, however, were susceptible to extra-intestinal virus spread to the liver, MLN, bile duct, and blood (51). Type II IFN seemed to be most important in the RV antibody response. Mice deficient in type II IFN produced less RV-specific IgG2a; IgA production, however, was similar to controls (53).

1.5.3.3 Type III IFN

Type III IFN, which was only discovered a little more than a decade ago, has shown to protect against virus infection. The functions of type III IFN are similar to that of type I, and influences susceptibility to RV infection. Pott and colleagues recently demonstrated that type III IFN is readily produced after infection and protects against RV susceptibility in both neonatal and adult mice, as mice deficient in the type III IFN receptor are more susceptible to infection (54).

1.6 The Importance of Microbiota on Immunity and Immune System Development

The microbiota in the last decade has gained notoriety as a factor involved in immunity at mucosal surfaces and in the development of the immune system in the gut. Since our aims included investigating how gut microbiota influences RV infection and immunity, a thorough discussion of how the microbiota influences immune system development and immunity is discussed below.

All epithelial surfaces of the human body are colonized by bacteria, but the gastrointestinal tract has the highest bacterial burden; an average human has 10^{14} bacteria inhabiting their gut, which is 10 fold greater than the total number of cells comprising the entire human body (55). Novel sequencing methods have revealed the diversity of the microbiota anywhere from 1,000 to 40,000 species depending on the method used (56). Such diversity is highly dependent on the location along the intestinal tract with species at the small intestine vastly different than species at the colon or rectum (55). The microbiota and its composition are largely acquired early in life, specifically during vaginal delivery and breastfeeding (57-59). Environmental factors also influence microbiota composition, and factors include antibiotic use, diet, and the presence of siblings during childhood (60-62). Uncontrolled innate factors, like genetics, gender, and age have also shown to influence microbiota composition (60, 63, 64).

The functions of gut microbiota are wide-ranging and thought to promote normal healthy bodily processes. It has been demonstrated to be involved in angiogenesis, food digestion, enteric nervous system functioning, epithelial and immune cell homeostasis, and resistance to pathogenic infections (65-70). Microbiota with a dysregulated composition or increased burden has shown to be involved in and contribute to a variety of diseases, including allergies, asthma, cancer, diabetes, inflammatory bowel disease, and obesity (62, 71-74).

1.6.1 Intestinal architecture and gut-associated lymphoid tissue

In the absence of microbiota, either through GF conditions or with antibiotic treatment, intestinal architecture is altered. Cecum size in GF mice is increased due to the accumulation of bile and mucus glycoproteins, which are normally digested by bacteria (75-77). Villi in the cecum and colon are shortened due to reduced cellularity and epithelial cell turnover as well (78,

79). Also, functioning of the intestines is disturbed in the absence of or a change in microbiota, as enteric nerve function and peristalsis are both impaired (67, 80, 81).

Generally, organs composing the GALT are smaller in size and fewer in number in microbiota-depressed conditions. MLNs are smaller, less cellular, and have fewer germinal centers in GF conditions. Upon microbiota recolonization, MLN size and cellularity recovers (82). PPs number and cellularity are decreased in GF conditions or with antibiotic treatment, and like MLNs, increase in size with recolonization (83, 84). GF conditions lower the number of isolated lymphoid follicles (ILFs), and peptidoglycan exposure sufficiently induces ILF formation (85).

1.6.2 Innate immune cells

1.6.2.1 Macrophages

Microbiota seems to have a variable influence in intestinal macrophage frequency and function. In the absence of bacteria, macrophage numbers in the intestine are either similar or reduced (86, 87). Qualitatively, macrophage activation status and antiviral functionality is reduced in GF conditions or with antibiotic treatment as the expression of activation markers is lessened (88, 89).

1.6.2.2 Dendritic cells

Dendritic cell (DC) numbers in the intestine are aided by the presence of the microbiota; in GF conditions, total DC numbers in the intestine are reduced (86, 87).

1.6.2.3 Group 3 innate lymphoid cells

Group 3 innate lymphoid cells (ILCs3) are a recently-described cell type which are distinguished by the production of IL-22 and their NK-like effector functions. ILCs3 have also been shown to be imperative in the development of GALT, specifically cryptopatches and ILFs, and in regulating immune responses to commensals and pathogens (90). The development and presence of ILCs3 are independent of microbiota, as GF mice have similar numbers of ILCs3 (91). The microbiota, however, does influence ILCs3 function; GF mice make less IL-22 (92).

1.6.2.4 Intestinal epithelial cells

The microbiota plays a key role in maintaining the IEC barrier and supporting IEC-mediated immune responses to pathogenic organisms. *Bacteroides thetaiotaomicron* colonization enhanced the expression of a transcript involved in the fortification of the IEC barrier (93). Such bacteria-mediated fortification of the IEC barrier utilized TLR2 signaling, which inhibited IEC apoptosis and enhanced IEC repair (94, 95).

PRR sensing of microbiota causes a cytokine, chemokine, and antimicrobial protein response essential for IgA class switch and other host defense mechanisms. The mucosal IgA and antimicrobial proteins produced, in turn, maintains the IEC barrier and protects cells against infection (96-98).

1.6.3 Adaptive immune cells

1.6.3.1 B cells

Generally, the presence of the gut microbiota promotes intestinal B cell number and function as well as antibody production. In GF conditions, mice have reduced numbers of small intestinal plasma cells and systemic germinal cell formation when compared to control mice (82, 99). GF mice also produce fewer antibodies than controls as the amount of small intestinal IgA

and systemic antibody of all isotypes are decreased excluding IgE (100-103). T dependent, but not T independent, antibody production is lessened in the absence of microbiota (104, 105).

1.6.3.2 Th17-T regulatory immunity bias

Intestinal microbiota clearly affects Th17-Treg immunity bias as GF mice have altered ratios of Th17 to Treg cells. In GF mice, the number of small intestinal Th17 cells is reduced, while Treg cells are greater (106). Moreover, specific bacteria are able to induce Th17 or Treg cells; colonization with segmented filamentous bacteria expanded Th17 cells, while *Bacteroides fragilis* expanded Treg cells (107, 108).

1.6.3.3 Th1-Th2 immunity bias

In addition to controlling the Th17-Treg immunity bias, the microbiota also regulates the bias of Th1-Th2 immunity. Specifically, intestinal microbiota promotes Th1 immunity and GF conditions or antibiotic treatment stimulates Th2 immunity. Mice treated with antibiotics for only 1 week experienced a shift from Th1 to Th2 immunity, with increased IL-4 and IgE expression and decreased type II IFN and IgG2a expression (109). Such Th2 bias can be reversed by colonization, specifically with *Enterococcus faecalis* (110).

1.7 Interactions Between the Microbiota and Viruses

Since the microbiota plays significant role populating the gut with immune cells and controlling immune cell function, it can be inferred that the microbiota also highly influences responses to pathogen infection. Some studies have investigated the role of the microbiota and virus infections, but very few have shown how the gut microbiota affects viral infections in the

gut. Below I discuss recent publications that give mechanisms behind microbiota-mediated antiviral immune responses at mucosal surfaces, specifically at the lungs and in the gut.

1.7.1 PR8 influenza A virus

Two independent studies have both demonstrated that microbiota influences immunity to PR8 influenza A (H1N1) infection in mice. With antibiotic administration and in GF conditions, mice experience greater susceptibility to H1N1 infection and delay in virus clearance. This was accompanied by reduced immunity to H1N1, including lowered CD4 and CD8 T cell numbers in the lungs and lowered virus-specific antibody production. Rectal exposure to TLR ligands reversed the effects of antibiotic treatments, and as a result, virus-specific immunity was recovered in the lungs (88, 111).

Both studies indicated phagocytic, antigen-presenting cells are responsible for reduced immunity and control of virus infection with antibiotic treatment and in GF conditions. Specifically, DCs from lymph nodes proximal to the lungs had lowered frequencies and diminished expression of proteins involved in DC functioning, including CD80, CD86, and MHC II. Such expression patterns were accompanied by a lessened ability to prime and expand T cells (111). Moreover, macrophages from lymph nodes proximal to the lungs were also found to have a reduced antiviral activity as a microarray of macrophages from antibiotic-treated mice showed decreased expression of IFN signaling proteins and IFN-stimulated gene proteins (88).

1.7.2 Poliovirus, reovirus, and mouse mammary tumor virus

The microbiota also influences virus infection at the gastrointestinal tract, however, responses are opposite to what occurs at the lungs. Kuss and colleagues found that antibiotic-treated mice infected with either poliovirus or reovirus are less susceptible to infection and have

increased survival than infected control mice. Poliovirus exhibited decreased infectivity with antibiotic treatment as virus obtained from treated mice replicated less *in vitro*. Exposure of poliovirus to bacterial products, including chitin, LPS, or peptidoglycan, recovered virus infectivity; direct virus-bacteria interactions were demonstrated, but recovery of infectivity was not shown to be dependent on such interactions (112).

Kane and others published a similar showing that antibiotic-treated mice are less susceptible to mouse mammary tumor virus (MMTV) infection than control mice. LPS bound to MMTV, and after virus association with LPS, LPS signaled through TLR4 on IECs. TLR4 signaling elicited IL-6 production, which initiated IL-10 expression. The presence of IL-10 created a tolerant, less inflamed environment, more suitable for infection (113).

While the above-mentioned articles defined novel mechanisms viruses utilize to infect along microbe-rich surfaces, a critical weakness to both studies is that they did not address the effect of microbiota on the virus-specific immune responses.

1.7.3 HIV

Since HIV transmission most often occurs at the vaginal and rectal mucosa, studies focusing on the role of the microbiota at theses surfaces have become of wide interest and are discussed below.

Lactic acid-producing bacteria primarily dominate the vaginal mucosa, and microbiota composition at this surface is less diverse than in the gut. Lowered diversity, however, is associated with greater female reproductive tract health (114). Unlike gut microbiota, very few studies have examined how vaginal microbiota influences immunity at the vagina. Exposure of the vagina to lactic acid-producing bacteria strains was found to decrease inflammation at the

lower vaginal epithelium (115, 116). Reducing inflammation would likely decrease the numbers and migration of the primary targets of HIV, including macrophages, DCs, and CD4 T cells, and thus, lower susceptibility to infection at the vaginal mucosa.

Some investigators have proposed that vaginal microbiota may regulate susceptibility to HIV infection by making antipathogen products that could directly inhibit virus viability. Indeed, studies have shown that lactic acid, hydrogen peroxide, and bacteriocins made by lactic acid-producing bacteria may prevent infection at the vaginal mucosa (117-119). Furthermore, vaginal microbiota has been shown to make lectins, a group of carbohydrate-binding proteins, which adheres to the surface of HIV, and as a result, lectins may prevent infection of macrophages and DCs by competitively binding to free HIV (120).

Microbiota along the gastrointestinal tract has been studied after HIV infection; unfortunately little is known about how microbiota influences initial HIV infectivity. After HIV infection, gut microbiota dramatically changes with higher numbers of pathogens present than beneficial microorganisms (121-123). Furthermore, more microbial translocation from the gut to the blood is observed in HIV-infected individuals; this translocation positively correlates and contributes to chronic inflammation and worsening of many infection-related conditions (124).

1.8 Probiotics in Rotavirus Disease, Infection, and Immunity

Probiotics have been employed in the clinic and animal models to prevent and treat RV infection and disease and enhance virus-specific immunity. Some studies have been rather successful in eliciting benefits from probiotics, others less so; the highlights of studies using probiotics in the context of RV infection is outlined below.

1.8.1 Preventing disease

Probiotics have had varying degrees of success when used to prevent RV disease. *Lactobacillus rhamnosus* administration in hospitalized children aged 1 month to 18 years lowered rates of symptomatic RV gastroenteritis (125). While *Lactobacillus rhamnosus* administration lowered rates of disease, administration of *Lactobacillus reuteri* in a similar hospital setting had no effect on the overall incidence of nosocomial diarrhea, including RV-associated diarrhea (126).

1.8.2 Treating disease

In addition to using probiotics to prevent RV disease, probiotics have been utilized to treat ongoing disease. In a study of hospitalized Brazilian infants aged 6 to 48 months, *Saccharomyces boulardii* administration early after diarrhea onset showed a reduction in RV diarrhea duration when compared to placebo (127). In a similar study, *Saccharomyces boulardii* administration not only decreased RV diarrhea duration but also decreased fever duration as well (128).

Species belonging to *Lactobacillus* genus have also shown varying effectiveness in treating RV-associated diarrhea. Administration of *Lactobacillus rhamnosus* shortened the duration of RV diarrhea in Polish children aged 2 months to 6 years; moreover, *Lactobacillus rhamnosus* administration also shortened the need for intravenous rehydration (129). *Lactobacillus reuteri* intervention in hospitalized Finnish children aged 6 to 36 months shortened the duration of diarrhea when compared with placebo treatment. In addition, the effects of *Lactobacillus reuteri* administration was dose-dependent; the more probiotic given, the greater reduction in diarrhea (130). Such beneficial effects of bacteria belonging to the *Lactobacillus*

genus was not seen in children from developing nations; *Lactobacillus rhamnosus* treatment in hospitalized Indian infants had no effect on diarrhea duration or severity (131).

1.8.3 Limiting infection

In addition to preventing and shortening RV diarrhea duration, probiotics have also been shown useful in inhibiting RV infectivity. *Bifidobacterium longum* subspecies *infantis* was able to inhibit RV infectivity in human IEC lines and mice (132). Furthermore, hospitalized children with RV diarrhea treated with a high dose of *Lactobacillus rhamanosu*s saw RV infectivity decline by 86% after 3 days when compared to RV obtained before treatment (133).

1.8.4 Enhancing virus-specific B cell and antibody responses

The ability of probiotics to enhance RV-specific antibody and B cell responses in infants and animals has also been studied but with varying results. *Bifidobacterium animalis* subspecies *lactis* was administered via formula to 6-week-old healthy, cesarean-delivered infants for 6 weeks. When RV-specific fecal IgA was assessed at 2 and 6 weeks after *Bifidobacterium animalis* feeding, IgA increased among the RV-vaccinated infants that were treated (134). In another study, infants with RV gastritis received different combinations of probiotics. The group receiving *Lactobacillus rhamnosus* saw enhanced levels of systemic virus-specific IgA and IgA-producing ASCs during disease recovery (135). This finding is supported by other studies and indicates that certain members of the *Lactobacillus* genus have the unique ability to increase RV-specific antibody production and B cell responses (136, 137). Despite these positive outcomes, many investigators have found that strains of *Lactobacillus* genus had no effect on humoral responses after RV infection either in animal models or the clinic (130, 138, 139).

CHAPTER 2:

Antibiotic treatment suppresses rotavirus infection and enhances specific humoral immunity

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Abstract

Background: Rotavirus causes 500,000 deaths and millions of physician visits/hospitalizations per year with worse outcomes and reduced vaccine efficacy in developing countries. We hypothesized that microbiota might modulate rotavirus infection and/or antibody response thus potentially playing a role in such regional differences. **Methods**: Microbiota was ablated via germ-free or antibiotic approaches. Enhanced exposure to microbiota was achieved via low dose dextran sodium sulfate (DSS) treatment. Rotavirus infection/replication was assessed by ELISA and qRT-PCR. Diarrhea was scored visually. Rotavirus humoral responses were measured by ELISA and ELISpot. **Results**: Microbiota elimination delayed infection and reduced infectivity by 42%. Antibiotics did not alter (+):(-) rotavirus strand ratios suggesting entry rather than replication was influenced. Antibiotics reduced diarrhea incidence and duration indicating reduced rotavirus antigen was biologically significant. Despite lowered antigen, antibiotics resulted in a more durable rotavirus mucosal/systemic humoral response. Increased rotavirus antibody response durability correlated with increased small intestinal rotavirus-specific, IgA-producing antibody-secreting cell concentration in antibiotic-treated mice. Conversely, DSS treatment impaired generation of RV-specific antibodies. **Conclusion**: Microbiota ablation resulted in reduced rotavirus infection/diarrhea and a more durable rotavirus antibody response suggesting antibiotic administration before rotavirus vaccination could raise low seroconversion rates that correlate with its inefficacy in developing regions.

Key words: Vaccine, microbiota, antibiotics, germ-free, mucosal immunity

Introduction

Rotavirus (RV), a double-stranded, non-enveloped RNA virus that preferentially infects intestinal epithelial cells, is the world's leading cause of acute gastroenteritis in young children (46). Prior to the recent introduction of RV vaccines, RV universally infected children under age 5. Most cases resolve within 8 days although some result in more severe complications eventuating in 2-4 million hospitalizations per year globally (140, 141). However, the RV disease burden is greatest in developing countries where RV causes 500,000 deaths annually (2, 142). Such disparity in RV disease burden is generally assumed to reflect general health/nutritional status, access to supportive care, and/or potential co-infections (143).

Widespread introduction of RV vaccines has greatly reduced RV disease burden in developing countries. For example, in Malawi, one of the world's least developed nations, administration of Rotarix has lowered RV-associated deaths by 43%. Yet, vaccine efficacy in developing countries is markedly lower than that observed in Europe and the Americas; 49% in Malawi vs. greater than 95% in Europe and Americas (140). Much of this difference appears attributable to vaccines eliciting lesser immune responses as Malawi only exhibited 57% anti-RV IgA seropositivity after Rotarix vaccination while RotaTeq induced 95% anti-RV IgA seropositivity in Americas and Europe (12, 144).

Various hypotheses may explain why RV vaccines are less immunogenic in some regions. Lack of proper nutrition, which influences immune responses, may be responsible for decreased vaccine efficacy. In addition, high titers of maternal-derived transplacental antibody and breast milk IgA has RV vaccine strain neutralization potential and may lower RV antigen exposure by preventing "infection" by the RV vaccine. To overcome the presence of neutralizing antibody, which blocks “infection” and subsequent protective immune responses,

higher RV doses have been proposed (13). Another possibility is that chronic infection, for example with helminthes, suppresses the immune response to the RV vaccine (2, 145). A more general form of the latter hypothesis is that the infection of, or immune response to, RV vaccines is influenced by the gut microbiota, which is thought to differ considerably between developed and developing countries. Indeed, the microbiota exerts broad and varied influence on immune system development and function (146). For example, in models of influenza and lymphocytic choriomeningitis infection, virus-specific adaptive responses, including both T and B cell responses, were lessened with antibiotic treatment owing to a decline in dendritic cell and macrophage function (88, 147). In the case of enteric viruses, namely poliovirus and reovirus, ablation of microbiota resulted in relative resistance to infection, which might lessen the generation of protective immune responses (112). Such ability of the microbiota to influence both viral infection, which is necessary for the immune response, and the immune response suggest that microbiota may mediate disparities in RV disease severity and/or vaccine efficacy.

Our goal was to investigate the possibility that microbiota might influence RV infection and/or immune responses. Neonatal mice served as a model of RV disease while the adult model of RV infection was considered analogous to RV vaccination, wherein protection against infection best correlates with levels of intestinal anti-RV IgA (46). Our results indicate that commensal microbiota promotes RV infection and influences RV-induced immune responses.

Materials and Methods

Animals

All experiments, except those using neonatal or germ-free mice, utilized 6-8 week-old, male C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, Maine). Experiments involving neonatal mice used 6-day-old offspring (male and female) of C57BL/6J mice. Experiments on germ-free mice used C57BL/6 mice derived via embryo transfer as previously described (148), and mice were maintained in sterile isolators at Georgia State University, which approved all procedures.

Virus and inoculations

Mouse RV, EC strain was provided by Mary Estes (Baylor College of Medicine). Adult mice received 10^5 SD_{50} RV, preceded by 1.33% (w/v) sodium bicarbonate (Sigma-Aldrich) by oral gavage. Neonates received 2 DD_{50} RV by oral gavage.

Antibiotics and antibiotic regimens

Adult mice were administered ampicillin (Sigma-Aldrich) at 1g/L and neomycin (Sigma-Aldrich) at 0.5g/L in drinking water *ad libitum* 1 week prior to inoculation and 1, 7, or 11 weeks after inoculation. Neonates received 200µg ampicillin and 100µg neomycin in 100µL water by oral gavage 1-day pre and post inoculation. Additionally, mothers received 1g/L ampicillin and 0.5g/L neomycin in their drinking water 1 week prior to giving birth through weaning.

Germ-free RV infection

Six- to 8-week -old male and female germ-free mice were inoculated and maintained in gnotobiotic isolators (Park Bioservices) during and up to 9 weeks post inoculation. Germ-free status was monitored by qPCR for 16s rDNA and fecal culture in BHI broth (BD).

Bacterial load quantification

Fecal bacterial DNA was isolated using QiAamp DNA Stool Kit (Qiagen), and 16s rDNA was amplified by qPCR (Bio-Rad).

Fecal rotavirus antigen detection

Supernatants of fecal homogenates (100mg/mL) were frozen or immediately analyzed by ELISA as described (149).

Duodenal RV genome qRT-PCR

Duodenal samples were harvested, washed in PBS, and homogenized in TRIzol (Ambion). Duodenal RNA was probed for RV genome as described (150).

Single stranded qRT-PCR for RV replication

RV replication ability in duodenal RNA samples was determined by the ratio of RV (+) to (-) RV strands as described (150).

Antibody ELISAs

Fecal and serum relative anti-RV antibody production and titer was analyzed as described (151). Total IgA levels were measures as described (152).

Small intestinal, RV-specific, IgA-producing antibody secreting cell ELISPOT

Whole small intestines and lamina propria and Peyer's patches cells were harvested as described (153). Cells were applied to filter plates (Millipore) coated with purified rhesus RV. RV-specific IgA was probed by anti-IgA secondary antibody (SouthernBiotech).

Dextran Sodium Sulfate (DSS) administration

DSS (MP Biomedicals) was diluted in drinking water at 1% (w/v) and administered *ad libitum* 4 days before and 3 days after RV inoculation.

Lipocalin-2 ELISA

Fecal supernatants were made as above, and Lipocalin-2 was assessed as described (154).

Statistics

Except where indicated otherwise, data are shown are from a single experiment (n=5 mice per condition) that was performed multiple times and yielded a similar pattern of results. Statistical significance was evaluated via Student's t-test. Asterisk (*) indicates $p<0.05$.

Results

Ablation of microbiota delays RV infectivity and ameliorates RV-induced disease

Infection of adult mice with rotavirus (RV), does not result in severe disease manifestations but serves as a well-defined infection model (46). Accordingly, oral inoculation of 6-8 week-old C57BL/6 mice with 10^5 50% shedding doses (SD_{50}) of murine RV strain EC resulted in RV antigen becoming detectable in feces 1-2 days post-inoculation (PI). Such RV shedding, which peaks 3-4 days PI and lasts 6-8 days PI, is proportional to infectivity, i.e. the level of viral genome in intestinal lysates (155). To investigate the role of microbiota, we administered RV to mice that had been treated with antibiotics, specifically a combination of ampicillin/neomycin, 1-week prior to inoculation and maintained throughout infection. PCR-based quantitation indicated such antibiotics reduced gut bacterial loads by 99% (Supplemental Figure 1a). Such reduction of microbiota levels consistently resulted in a 1-day delay in the appearance of fecal RV antigen and an approximate 40% decrease in total RV shedding, i.e. area under the curve (Figure 1a). Considering that antibiotic-treated mice still had a significant bacterial load in their gut and that antibiotics can alter relative proportions of bacteria potentially resulting in increases in some species, use of germ-free mice, was also employed. Germ-free C57BL/6 mice, maintained in sterile isolators throughout the experiment, and conventionally-housed control mice, were orally inoculated with filter-sterilized RV. Fecal culture and qPCR verified the absence of bacteria in these mice. Similar to antibiotic-treated mice, germ-free mice exhibited a 1-day delay in initial appearance of infection. Germ-free mice also exhibited delayed RV clearance, likely reflecting their immature intestinal adaptive immune system (146), which is important for RV clearance (Figure 1b) (151). Thus, delay of RV infection delay may be a uniform consequence of microbiota ablation. To verify that reduced levels of fecal RV antigen

reflected reduced infectivity, we quantitated the level of RV genomes in duodenal lysates by qRT-PCR. Antibiotic treatment resulted in a 10-fold reduction in RV genomes at 2 and 3 days PI (Figure 1c) thus confirming that antibiotics reduced RV infectivity. We next measured ratio of (+) to (-) RV strands, which reflects the extent of active RV replication (150). This parameter did not significantly differ between control and antibiotic treated mice (Figure 1d) suggesting microbiota ablation reduced viral entry rather than RV replication.

Analogous to the case for humans, RV infection in neonatal mice induces secretory diarrhea arising 2-3 days PI and lasting for 3-8 days. To determine if the reduced RV infectivity resulting from antibiotic treatment impacted upon RV disease, neonatal mice were administered antibiotics via oral gavage 1-day prior and 1-day post inoculation and then inoculated with RV on day 0. Mice were then monitored daily for diarrhea as indicated by the presence of runny, profuse, yellow-colored feces upon application of light pressure to the abdomen (Figure 2a). Antibiotic treatment resulted in lower daily rates of diarrhea on days 4 to 8 days post inoculation (Figure 2b) and a 34% reduction in total diarrhea incidence (Figure 2c). Within mice that developed diarrhea, antibiotic treated mice showed roughly 1 day less diarrhea when compared to untreated mice (Figure 2d). Thus, reducing RV infection via microbiota ablation resulted in reduced diarrheal disease.

Absence of microbiota results in a more durable RV-specific mucosal antibody response

RV infection initiates robust adaptive immunity that clears primary infection and provides protection against future infection (46). Such infection-induced immunity is the basis of currently used RV vaccines, which are live attenuated viruses. Such protective immunity best correlates with RV-specific fecal IgA, whose levels often parallel those of serum IgG and IgA,

which are typically measured in clinical studies (46, 151). The adult mouse model of RV infection can be considered a model of RV vaccination in that both are asymptomatic infections that do not result in diarrhea but provide protective immunity (46). Considering that antibiotics can reduce antibody responses to systemically administered antigens and that reduced infectivity likely reduced exposure to antigen, we hypothesized that antibiotic treatment might reduce RV-specific antibodies (88, 147). To investigate this possibility, we treated mice with antibiotics 1 week prior to and up to 11 weeks post-inoculation, collected feces and serum weekly, and assayed samples for RV-specific IgG and IgA. Antibiotic treatment did not affect antibody production at early times following RV inoculation but enhanced levels of RV-specific antibodies, particularly serum and fecal IgA at 9 weeks post-inoculation and beyond (Figure 3). Such enhancement was observed upon measuring RV immune reactivity at a single dilution of serum or fecal supernatant or quantitating titer following a range of dilutions. This increase was specific for RV in that, in accord with other studies, antibiotic treatment resulted in modest reduction in total IgA (Supplemental Figures 2a-b) (146).

An alternate approach to use of antibiotics to study the microbiota is the use of germ-free mice, although a caveat is that the gut-associated lymphoid tissue that mediates adaptive immunity is lacking in these mice (146). In accord with this knowledge and the delayed clearance of RV observed in these mice, germ-free mice exhibited a marked delay in production of fecal anti-RV IgA. Despite lack of GALT, this impairment of germ-free mice to produce fecal anti-RV IgA was overcome with time. Moreover, analogous to antibiotic-treated mice, germ-free mice exhibited a serum anti-RV antibody response that was initially similar to that of conventional mice but became greater several weeks PI (Figure 4). We next examined the effect of antibiotics on acquisition of RV-specific antibodies following pathogenic, i.e. diarrhea-causing, RV

infection in neonatal mice. Pathogenic viral infection is typically a strong inducer of adaptive immunity. Yet, despite reducing RV-induced diarrhea, antibiotic treatment enhanced serum anti-RV IgA, and higher titers, particularly at later times following infection (Figure 5). Thus, in contrast to our initial prediction, microbiota ablation resulted in enhanced RV specific systemic and mucosal antibody responses.

Antibiotic enhancement of the duration of the antibody response to RV suggests the possibility of incorporating antibiotic treatment into vaccine campaigns. However, given the negative potential consequence of prolonged antibiotic administration, we next sought to determine whether briefer exposures might also enhance RV antibody generation. Thus, we compared the effects of a 2-week course of antibiotics (1 week pre and post inoculation) to control conditions (i.e. no antibiotics) and antibiotics maintained throughout the experiment (1 week pre and 7 weeks post inoculation). Mice receiving only 2 weeks of antibiotics displayed a return of fecal bacterial load within 2 weeks after antibiotic cessation whereas mice maintained on antibiotic continued to exhibit bacterial suppression (Supplementary Figures 1b-c). While maximal enhancement of RV-specific antibody responses was observed with maintained antibiotic treatment, a 2-week course was sufficient to significantly enhance serum anti-RV IgG and IgA levels and titers (Figures 6a-d). A further reduced course of antibiotics, 2-days prior to and 3 days post RV inoculation resulted in a trend toward increased fecal and serum anti-RV IgA titers that was not statistically significant (Supplemental Figure 3). We hypothesized that antibiotic-mediated enhancement of the durability of the mucosal antibody response involved more sustained levels of RV-specific antibody secreting cells (ASCs). Thus, we next performed ELISPOT analysis to quantitate levels of RV-specific IgA producing ASCs from small intestinal lamina propria and Peyer's patches. In control mice, inoculation with RV resulted in RV-

specific ASC going from undetectable level to 4000 cells per million lamina propria and Peyer's patches cells, which then declined by about 40-fold by 7 weeks PI. Antibiotic treatment did not significantly affect generation of RV-specific IgA ASC at 2 weeks PI. However at 7 weeks PI, antibiotic-treatment markedly enhanced levels of such cells. The degree of enhancement (about 20-fold) was greatest in mice maintained on antibiotics but nonetheless (10-fold) robust in mice on the 2-week course of antibiotics (Figure 6e).

Microbiota's impact upon basal state of innate immune activation may regulate RV-specific adaptive immunity

We hypothesize that lowering bacterial load through antibiotic treatment is reducing the extent of basal state of innate immune activation thus allowing for RV-specific danger signals to more readily active GALT during infection; this should result in stronger activation of virus-specific cells and the persistence of ASC in the gut. The converse of this hypothesis is that inducing a greater extent of innate immune activation prior to virus administration might attenuate the antibody response. To test this possibility, mice were exposed to dextran sodium sulfate (DSS) via drinking water, which compromises the integrity of the gut epithelial barrier resulting in increased exposure of immune cells to microbiota and its products. While DSS treatment can result in robust life-threatening colitis, exposure to low levels of DSS such as 1.0% results in only modest histopathological changes in the gut but still induces readily detectable activation of pro-inflammatory gene expression, which can be monitored by measuring levels of fecal Lipocalin-2 (154). Accordingly, exposure to 1.0% DSS for 1 week did not result in apparent symptoms of colitis but nonetheless induced robust expression of fecal Lipocalin-2 (Figure 7a). Such DSS treatment did not alter the course of RV infectivity (Figure 7b).

Moreover, DSS-induced low-grade inflammation did not affect initial generation of serum RV-specific IgG (figures 7c-d). However, such DSS treatment resulted in significantly lower levels of RV-specific fecal and serum IgA from 3 to 9 weeks PI (Figures 7e-h). These results suggest that the state of basal innate immune activation at the time of RV inoculation may modulate the levels of RV-specific antibodies. Accordingly, manipulation of this parameter may be a way to modulate generation of these protective responses.

Discussion

The gut microbiota is increasingly appreciated to modulate numerous infectious and immunologic processes. In many circumstances, the gut microbiota serves to protect the host from infectious disease such that use of antibiotics results in increased susceptibility to a number of bacterial infections. Moreover, generation of immune responses to some viruses is impaired in mice subjected to microbiota ablation (88, 147). Thus, we originally hypothesized that elimination of microbiota might increase susceptibility to RV infection. In accord with observation that microbiota are essential for development of GALT (146), which generates the fecal IgA that promotes RV clearance (151), germ-free mice exhibited a delayed generation of fecal anti-RV IgA and concomitantly delayed RV clearance. Nonetheless, the overall consequence of microbiota ablation, particularly when achieved via the more clinically relevant approach of antibiotic treatment, seemed to benefit the host. Specifically, treatment with antibiotics resulted in a delay in RV infection and a reduction in total infectivity. Such reduction in infection was associated with, and likely resulted in, a substantial reduction in the incidence and duration of RV-induced diarrhea.

The mechanism by which ablation of microbiota retards RV infectivity is not clear but may be similar to the case for poliovirus, reovirus, and mouse mammary tumor virus, which were all recently reported to infect less efficiently in the absence of a microbiota (112, 113). In such cases, bacterial-derived LPS was observed to bind the virus and facilitate its entry (112). While our experiments have not, to date, demonstrated a direct role for bacterial ligands in promoting RV entry, antibiotics had a clear effect on viral loads with little effect on RV replication, which support that a similar paradigm might be operative. Another possibility is that absence of bacteria may be lessening the expression of RV receptors needed for viral entry.

Indeed, it is known that microbiota promotes expression of certain TLRs (156, 157), which have been postulated to facilitate enteric virus entry (112, 113).

The reduction in RV infectivity upon antibiotic treatment likely resulted in reduced exposure of the immune system to RV antigens, which we predicted would reduce the antibody response to the virus. In contrast, antibiotics enhanced the antibody response, particularly resulting in one that was more durable. Such higher titers at later time points would likely offer more lasting and broader protection against subsequent infection by heterologous RV strains. The mechanism mediating this effect is not entirely clear but might reflect that RV "danger signals" provide a greater stimulatory effect on the antigen presenting cell-lymphocyte interactions when such signals occur in the context of reduced basal/background signaling. In support of this possibility, microbiota ablation results in reduced fecal levels of the immune inflammatory markers such as Lipocalin-2 (data not shown) and Relm-β(158), while use of DSS to increase the state of immune activation prior to RV infection resulted in a less durable antibody response. In this context, we speculate that failure of RV vaccines to consistently elicit strong antibody responses in developing countries may reflect altered microbiota composition, possibly enriched with pathobionts that result in chronic immune activation upon RV vaccine administration. Another possibility is that microbiota ablation reduces regulatory T-cell functioning allowing for heightened inflammatory T cell responses (159). Deciphering such complex mechanisms remains an important research challenge.

Presuming the RV mouse model is translatable to humans, our study has several implications on antibiotic use in the context of RV infection and vaccination. First, given that many clinical diagnoses of infections are based on symptoms rather than laboratory-based detection of a specific pathogen, our results suggest that decisions to prescribe antibiotics need

not fear that this would increase susceptibility to RV. Moreover, our results suggest that administration of RV vaccines need not be postponed in the event that an individual is presently taking antibiotics. Rather, our study suggests this might be an ideal time to administer RV, and perhaps other orally administered vaccines. Furthermore, the low cost of antibiotics may justify their select use in managing RV disease. Perhaps an active outbreak of RV might be managed by administration of antibiotics quickly followed by RV vaccination. This action might reduce severity of arising infections and increase the vaccine efficacy. Alternatively, incorporating antibiotics into a broader RV vaccine campaign might increase seroconversion rates. Indeed, based upon our proposed mechanism by which antibiotics promote RV antibodies, we would envisage the effect might be greatest in areas with low seroconversion rates such as Malawi that may have higher levels of basal immune activation. Even a modest increase in vaccine efficacy might result in dramatic reduction in societal disease burden due to herd immunity. In the event that our observations in mice prove to be relevant in humans, careful consideration of the detrimental consequences of antibiotic use, including increased susceptibility to bacterial infections (160) and promotion of antibiotic resistant bacteria (161) is warranted before wide scale deployment of antimicrobial agents to manage the RV disease burden. In light of these concerns, development of approaches to selectively manipulate the microbiota may provide a better means to safely enhance immune responses to RV vaccines. Such selective manipulations might take the form of more specifically-acting antibiotics and/or administration of probiotics. Indeed, administration of a probiotic increased seroconversion rates in a cohort of RV-vaccinated Finnish infants (162). Thus, while mechanistic understanding and optimization require additional experimentation, the approach of manipulating the microbiota may be a useful strategy to combat RV.

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Conflict of Interests

The authors declare no conflicts of interest related to this work.

Figure 2-1

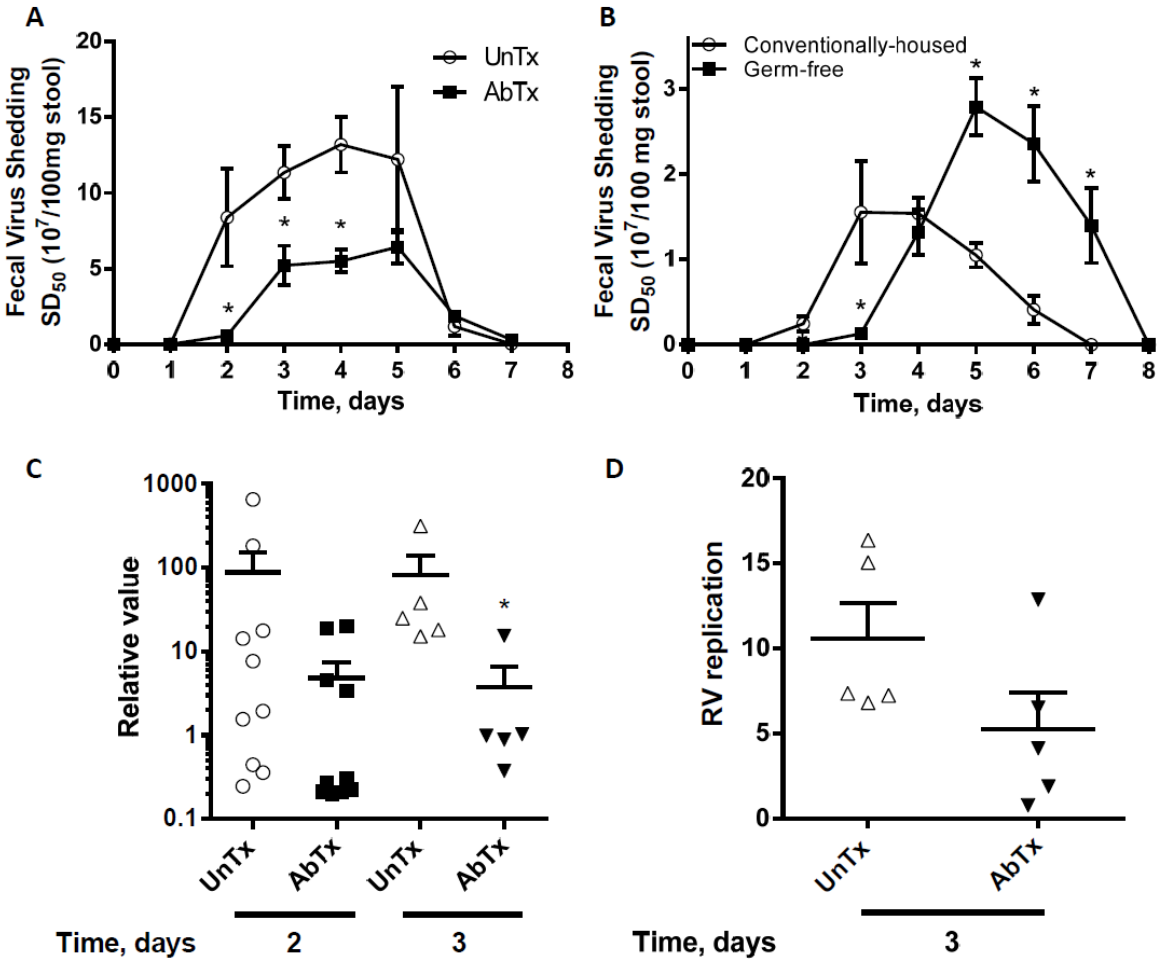
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Figure 2-2

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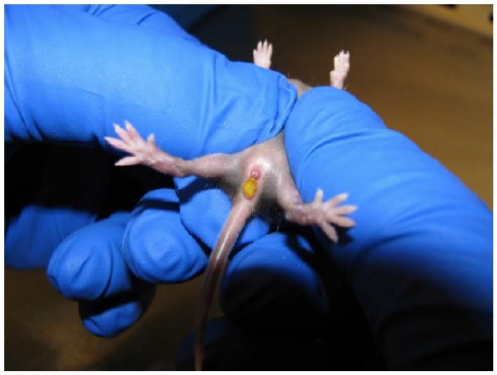

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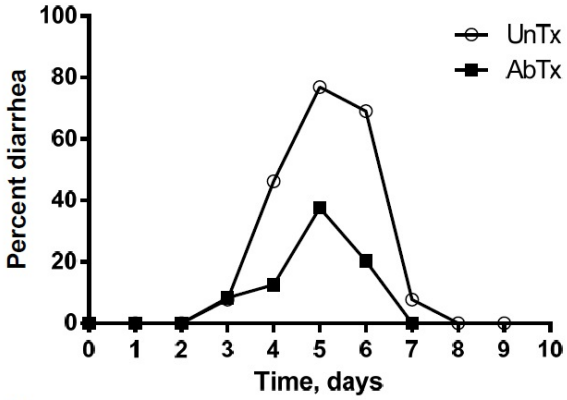
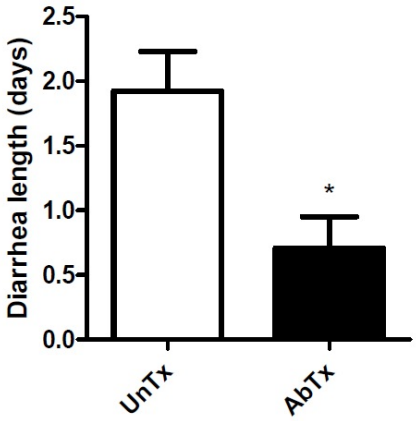


C

	Fraction Diarrhea	Percent Diarrhea
UnTx	12/13	92%
AbTx	7/12	58%

D



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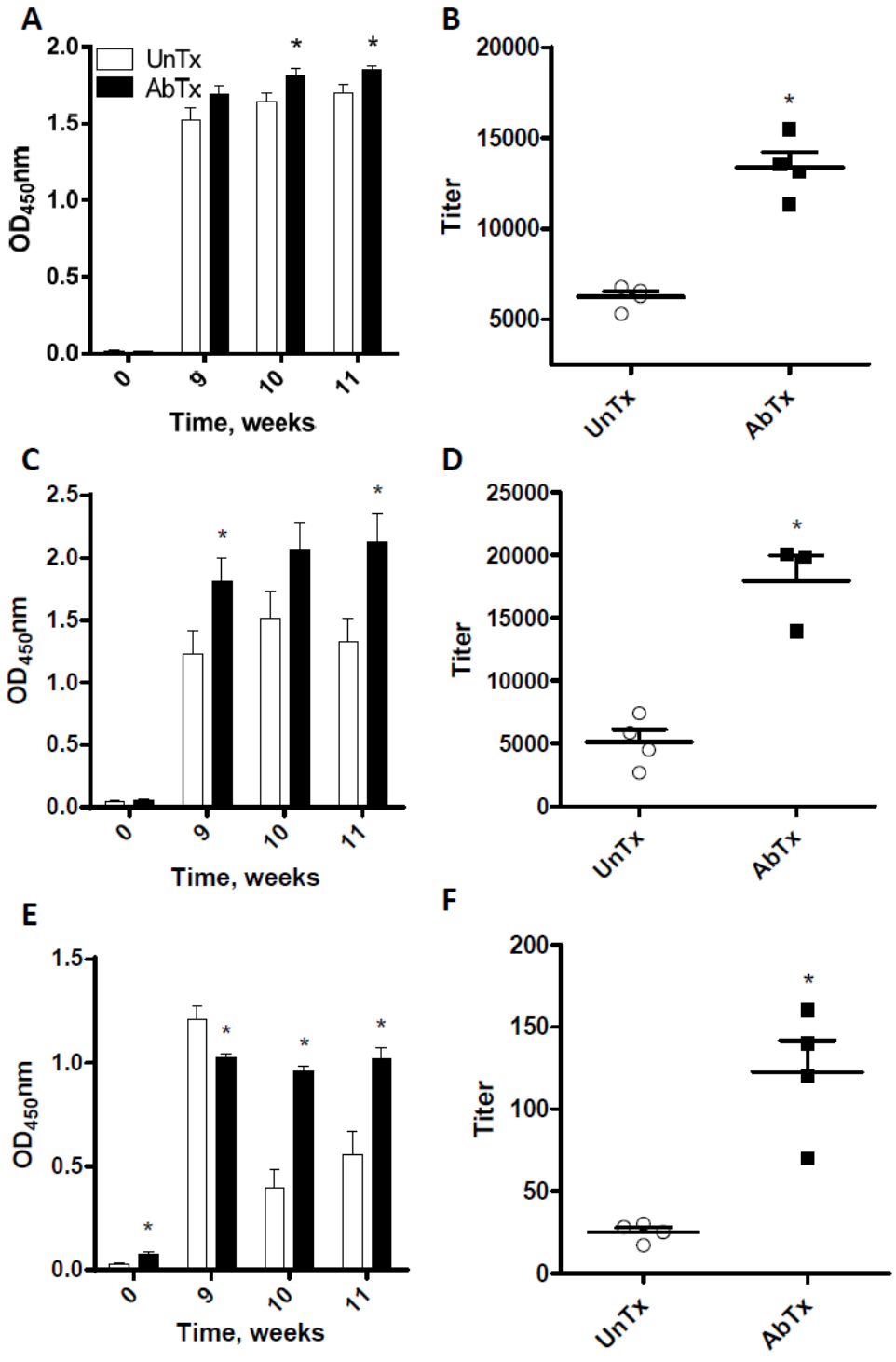
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Figure 2-4

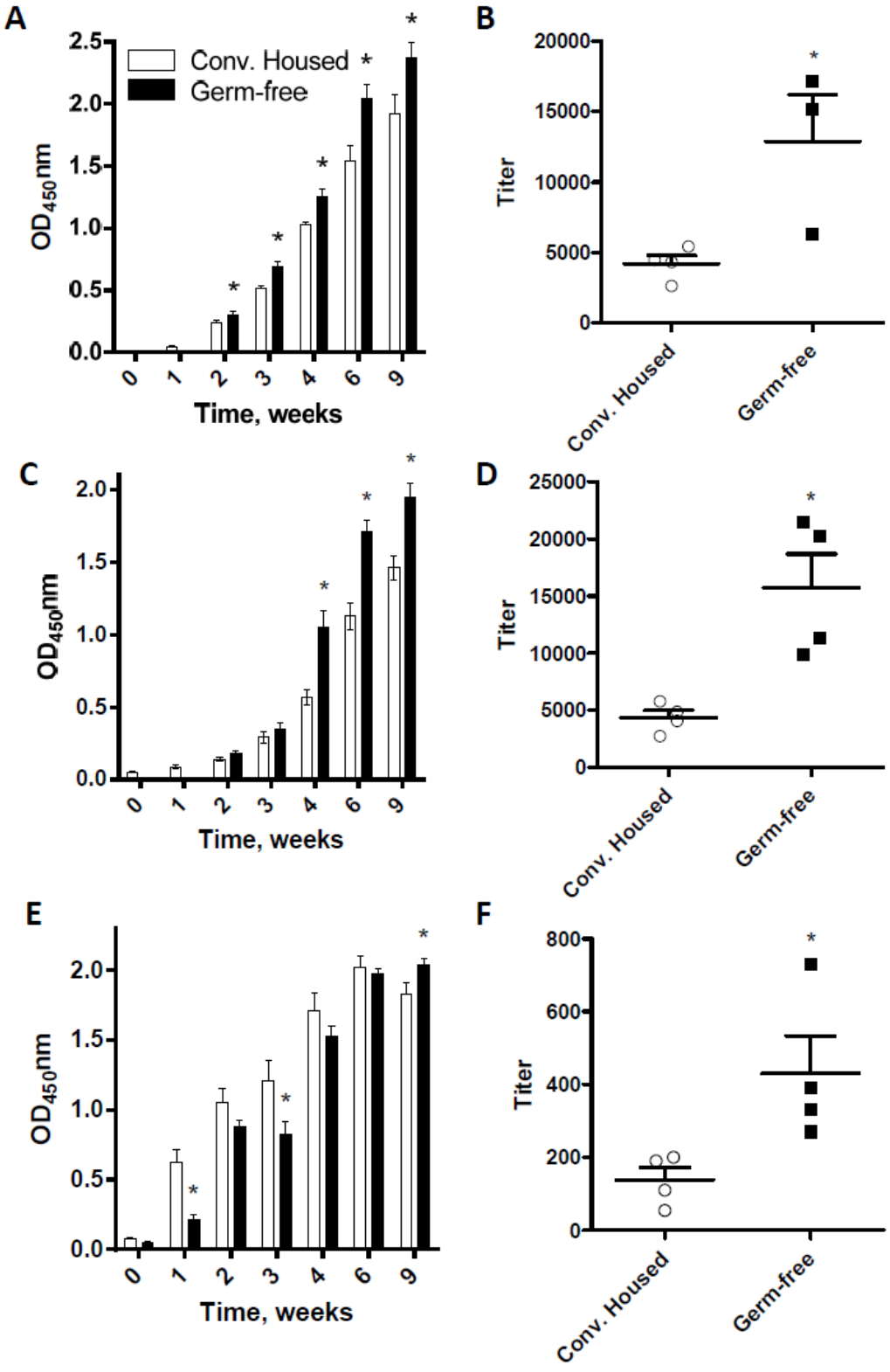
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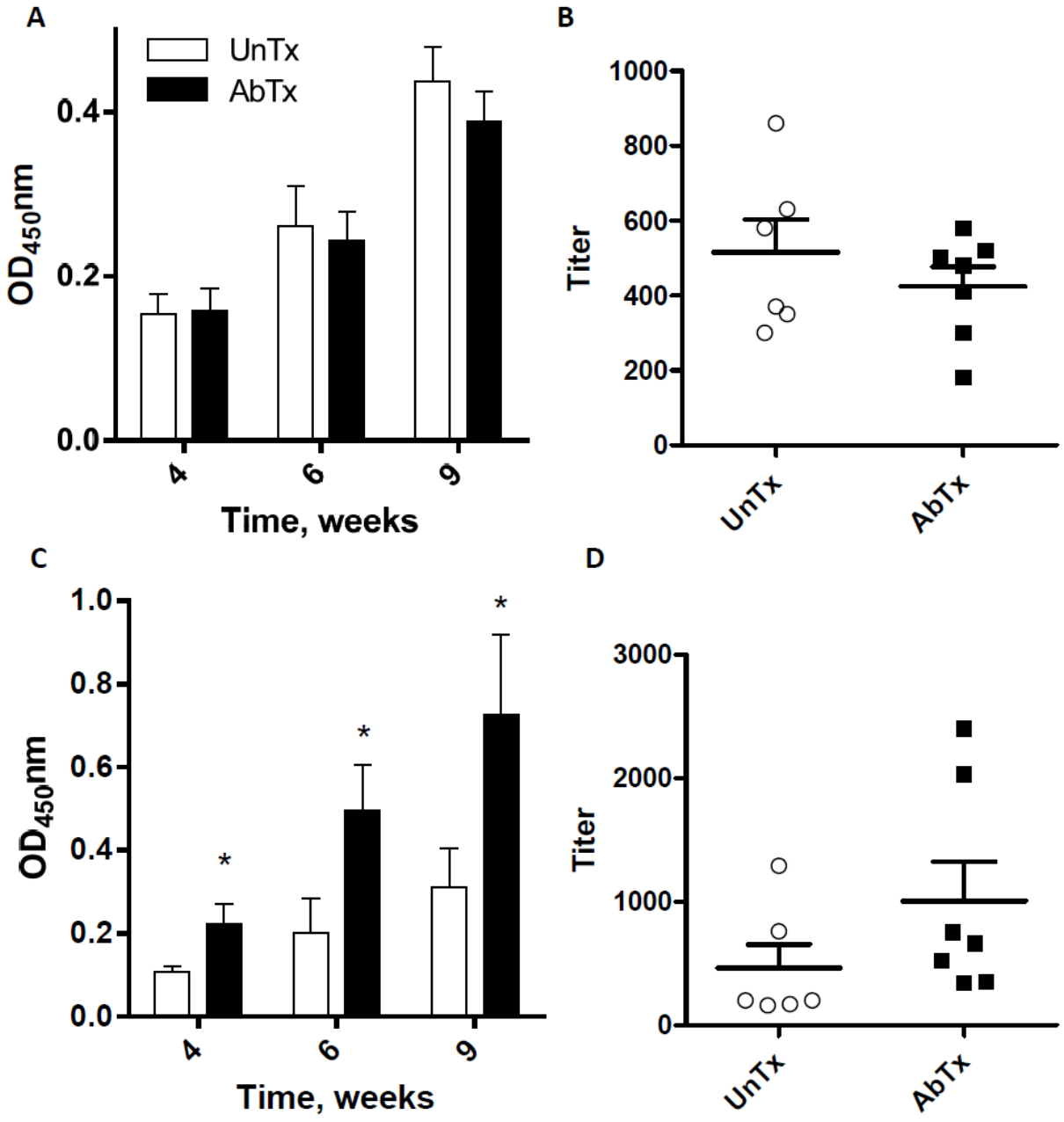
Figure 2-5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0.4
0.2
0.0
OD450nm
4
6
9
Time, weeks
B
1000
800
600
400
200
0
Titer
UnTx
AbTx
C
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
*
*
*
4
6
9
Time, weeks
D
3000
2000
1000
0
Titer
UnTx
AbTx

Figure 2-6

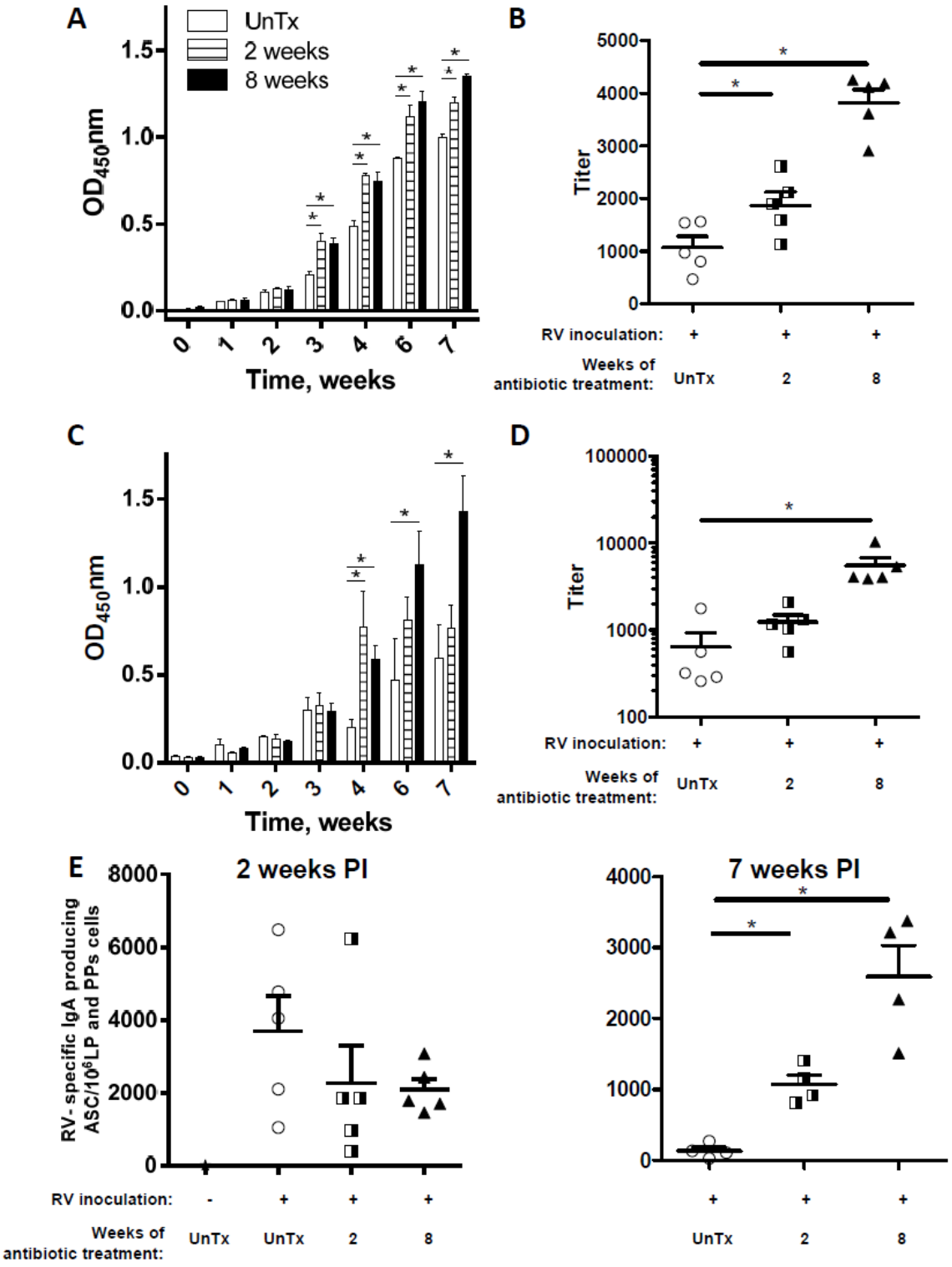
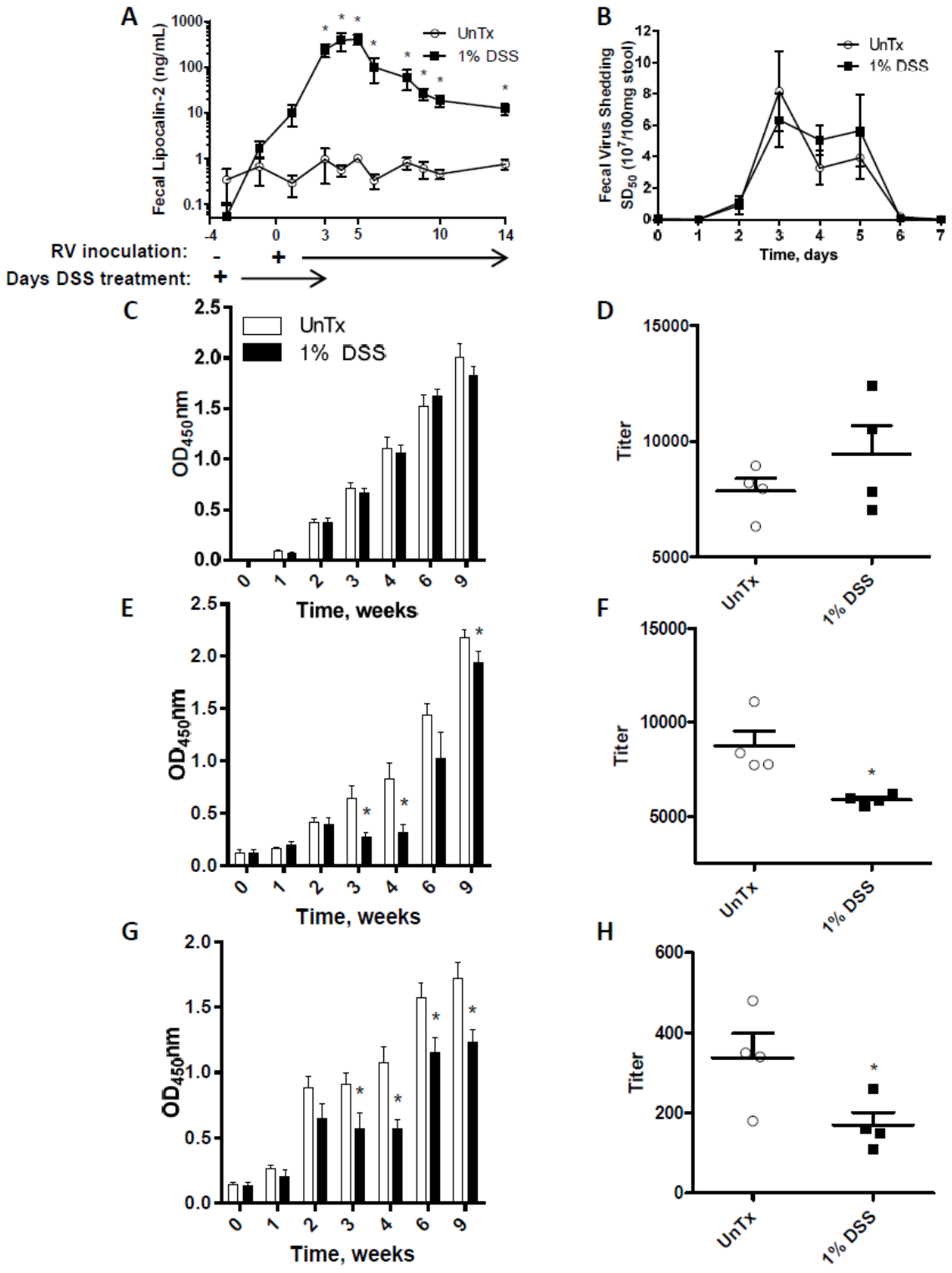

A
UnTx
2 weeks
8 weeks
OD450nm
Time, weeks
B
Titer
RV inoculation:
Weeks of antibiotic treatment:
UnTx
2
8
C
OD450nm
Time, weeks
D
Titer
RV inoculation:
Weeks of antibiotic treatment:
E
2 weeks PI
RV- specific IgA producing ASC/10^6 LP and PPs cells
RV inoculation:
Weeks of antibiotic treatment:
7 weeks PI

Figure 2-7


A
Fecal Lipocalin-2 (ng/mL)
1000
100
10
1
0.1
UnTx
1% DSS
-4
0
3
5
10
14
RV inoculation:
-
+
Days DSS treatment:
+
B
Fecal Virus Shedding
SD50 (10^7/100mg stool)
12
10
8
6
4
2
0
UnTx
1% DSS
0
1
2
3
4
5
6
7
Time, days
C
2.5
2.0
1.5
1.0
0.5
0.0
OD450nm
UnTx
1% DSS
0
1
2
3
4
6
9
Time, weeks
D
15000
10000
5000
Titer
UnTx
1% DSS
E
2.5
2.0
1.5
1.0
0.5
0.0
OD450nm
0
1
2
3
4
6
9
Time, weeks
F
15000
10000
5000
Titer
UnTx
1% DSS
G
2.0
1.5
1.0
0.5
0.0
OD450nm
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1
2
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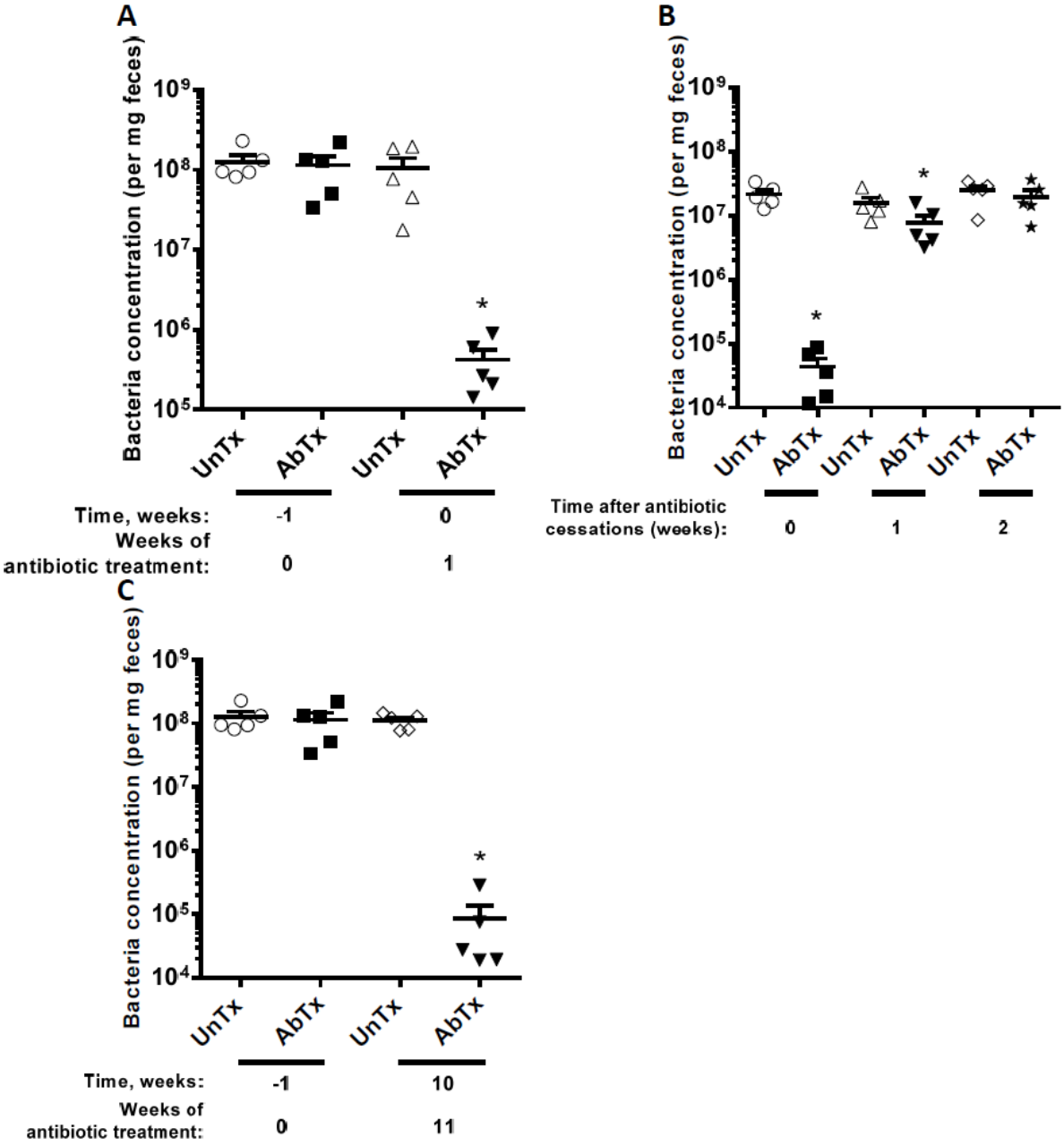
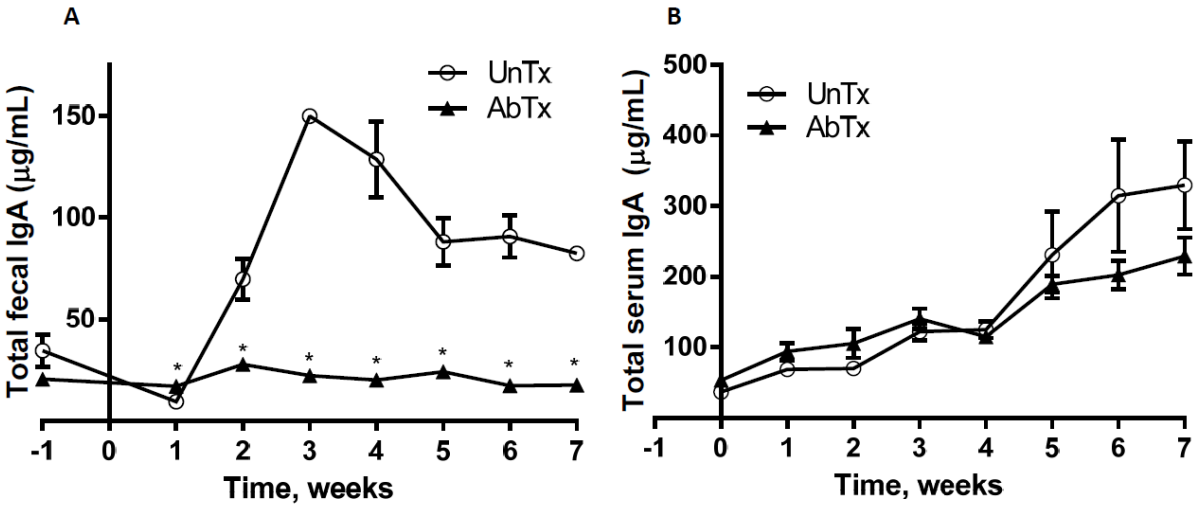
Time, weeks
H
600
400
200
0
Titer
UnTx
1% DSS

Supplemental Figure 2-1



Supplemental Figure 2-2



Supplemental Figure 2-3

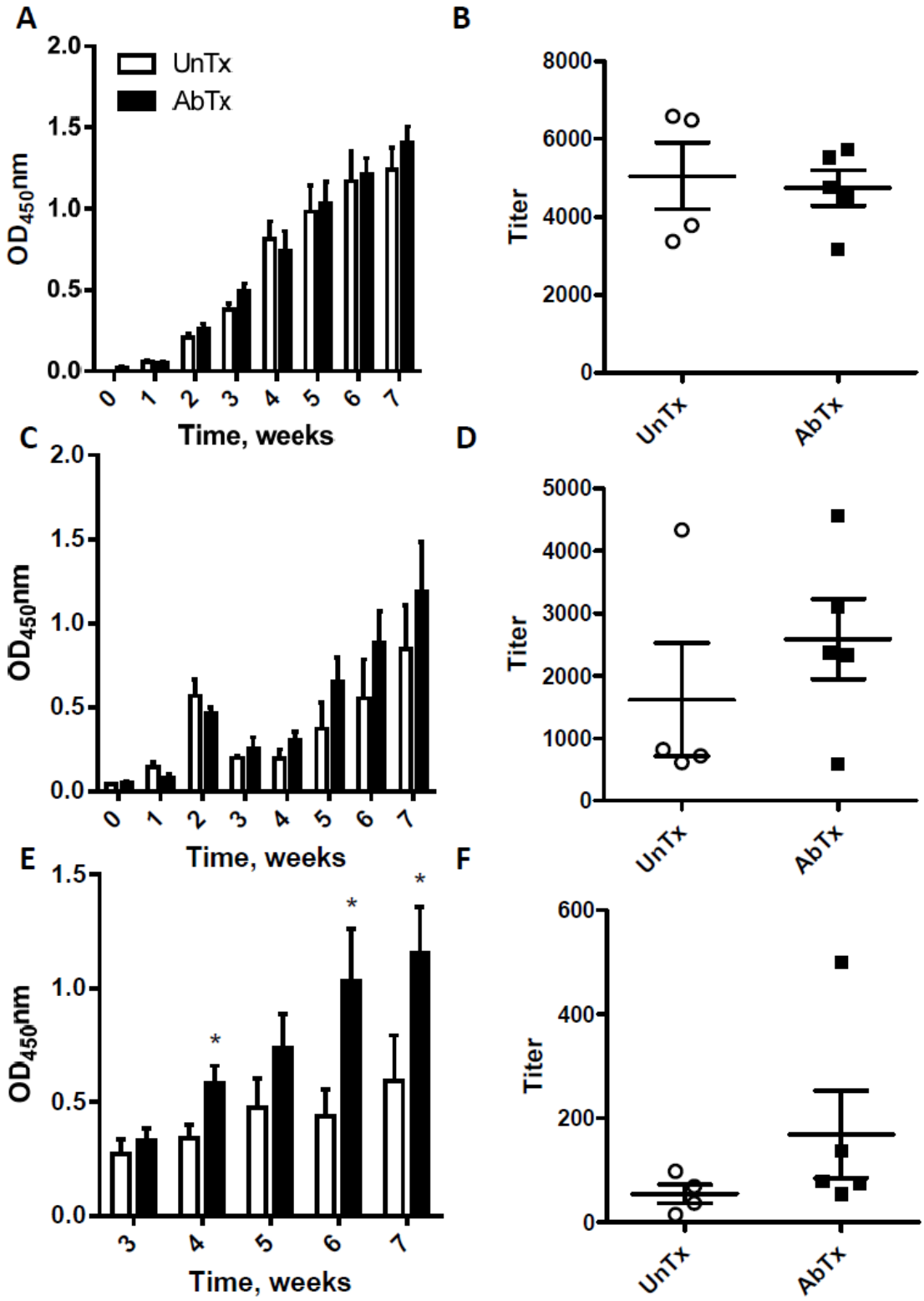


Figure Legend

Figure 2-1. Ablation of microbiota retards RV infectivity. Six- to 8-week -old C57BL/6 male mice were treated with ampicillin and neomycin 1 week prior to RV inoculation and orally inoculated with 10^5 SD_{50} mouse RV, EC strain. **A**, Feces were collected daily and assayed for RV antigens by ELISA. **B**, Six- to 8-week-old male and female germ-free were infected with filter-sterilized RV, feces were collected daily, and feces were assayed for RV antigen by ELISA. **C**, Total RNA from the duodenum was prepared and cell lysate of antibiotic-treated mice was probed for NSP3 mRNA, representative of RV genome, by qRT-PCR. **D**, Duodenal cell lysate was prepared and each sample was analyzed for (+):(-) RV strand ratios by ss-qRT-PCR. Ratio value positively correlates with RV replication.

Figure 2-2. Antibiotic treatment reduces RV-induced diarrhea in neonatal mice. C57BL/6 pregnant dams were treated with ampicillin and neomycin in drinking water *ad libitum* 1 week prior to giving birth and kept on antibiotics until offspring weaning. Offspring were treated with 100μg neomycin and 200μg ampicillin in 100μL water by oral gavage 1 day prior and 1 day post inoculation. Six-day-old mice were inoculated with 2 DD_{50} mouse RV and were visually observed for diarrhea daily. **A**, Typical RV diarrhea in neonates. **B**, Daily rates of observable diarrhea days 0 to 9 days PI. **C**, Incidence of diarrhea represented as both a fraction and percentage of total mice inoculated. **D**, Of the mice that showed evidence of diarrhea, the numbers of days each mouse had diarrhea was averaged.

Figure 2-3. Antibiotic treatment enhances durability of antibody response to RV. C57BL/6 mice were treated with antibiotics as described and remained on antibiotics until 11 weeks PI. **A**, Serum RV IgG, (**C**) serum RV IgA, and (**E**) fecal RV IgA production, as measured by RV immune reactivity at a single dilution of serum or fecal supernatant, 0, 9, 10, and 11 weeks post

inoculation, reflective of late systemic and mucosal RV antibody responses. **B**, Serum RV IgG, (**D**) serum RV IgA, and (**F**) fecal RV IgA titer, as measured by the sample dilution at which OD_{450}nm equaled 0.2 over blank, at 11 weeks PI.

Figure 2-4. Germ-free mice exhibit enhanced serum antibody response to RV. Germ-free C57BL/6 male and female mice were inoculated with filter sterilized RV, feces and serum were collected weekly up to week 9 PI, and samples were analyzed for the presence of RV antibody by ELISA. Mice were monitored by fecal culture for germ-free status weekly until experiment endpoint. **A**, Serum RV IgG, (**C**) serum RV IgA, and (**E**) fecal RV IgA production, as measured by RV immune reactivity at a single dilution of serum or fecal supernatant, 0 to 9 weeks PI, reflective of late systemic and mucosal RV antibody responses. **B**, Serum RV IgG, (**D**) serum RV IgA, and (**F**) fecal RV IgA titer, as measured by the sample dilution at which OD_{450}nm equaled 0.2 over blank, at 9 weeks PI.

Figure 2-5. Antibiotic-treated neonatal mice exhibit enhanced serum IgA following RV inoculation. Neonates were treated with antibiotics as described, inoculated, feces and serum collected at various weeks PI, and samples probed for RV antibody. **A**, Serum RV IgG, serum RV IgA (**C**) production, as measured by RV immune reactivity at a single dilution of serum, 4, 6, and 9 weeks PI. **B**, Serum RV IgG titer, as measured by the sample dilution at which OD_{450}nm equaled 0.2 over blank, at 9 weeks PI. **D**, Serum RV IgA titer, as measured by the serum dilution at which OD_{450}nm equals 0.2 over blank, at 9 weeks PI.

Figure 2-6. Antibiotic treatment results in greater maintenance of RV-specific antibody producing cells in intestine. C57BL/6 mice were treated with antibiotics as described, however, one group remained on antibiotics for only 1 week after inoculation (for 2 weeks total)

while another group of mice stayed on antibiotics throughout the duration of the experiment (for 8 weeks total). **A**, Serum RV IgG and (**C**) serum RV IgA generation weekly until 7 weeks post inoculation, reflective of late systemic and mucosal RV antibody responses. **B**, Serum RV IgG titer and (**D**) serum RV IgA titer as measured by the sample dilution at which OD_{450}nm equaled 0.2 over blank, at 7 weeks PI. **E**, Small intestinal lamina propria and Peyer's patches cells were isolated and applied to coated plates at 2 weeks and 7 weeks PI. The concentration of RV specific, IgA-producing ASCs were calculated in each group.

Figure 2-7. Increasing basal immune activation impairs RV-induced antibody generation. C57BL/6 mice were treated with DSS as described and inoculated with RV. **A**, Feces were collected daily and RV antigen was assayed. **B**, Fecal Lipocalin-2 expression was also analyzed with ELISA. Serum and fecal supernatant was obtained and (**C**) serum RV IgG, (**E**) serum RV IgA, and (**G**) fecal RV IgA production, as measured by RV immune reactivity at a single dilution of serum or fecal supernatant, was probed up to 9 weeks PI. **D**, Serum RV IgG, (**F**) serum RV IgA, and (**H**) fecal RV IgA titer, as measured by the sample dilution at which OD_{450}nm equaled 0.2 over blank, at 9 weeks PI.

Supplemental figure 2-1. Antibiotic treatment reduces fecal bacterial load by 99% and cessation of antibiotics results in rapid restoration of bacterial loads in feces. **A**, Feces were collected before and 1 week after antibiotic administration, and bacteria load was assessed for 16s rDNA by qPCR. C57BL/6 mice were treated with antibiotics for 2 weeks and then removed from antibiotics; feces were collected the day antibiotics were removed and 1 and 2 weeks after antibiotic cessation, and (**B**) bacterial load was assessed by 16s rDNA qPCR. Mice were treated with antibiotics 1 week prior to inoculation and kept on antibiotics up to 11 weeks PI. **C**, Fecal bacterial load at 10 weeks PI (after 11 weeks of antibiotic administration).

Supplemental Figure 2-2. Antibiotic treatment lowers total fecal IgA. C57BL/6 mice were treated with antibiotics 1 week prior and up to 7 week PI. **A**, Fecal and (**B**) serum samples were probed for total IgA weekly by ELISA.

Supplemental Figure 2-3. A 5-day antibiotic treatment modestly enhances RV antibody generation. C57BL/6 mice were treated with antibiotics 2 days before and 3 days after RV inoculation and serum and fecal supernatant was obtained weekly. **A**, Serum RV IgG, (**C**) serum RV IgA, and (**E**) fecal RV IgA production as measured by optical density (OD) at one sample dilution up to 7 weeks PI. **B**, Serum RV IgG, (**D**) serum RV IgA, and (**E**) fecal RV IgA titers at 7 weeks PI were determined by the dilution at which the sample produced an OD value of 0.2 over blank.

CHAPTER 3:

MyD88-mediated TLR signaling protects against acute rotavirus infection while inflammasome cytokines direct antibody response

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Running title: Role of MyD88 in protection against rotavirus

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Abstract

Rotavirus (RV), the world's leading cause of severe viral gastroenteritis amongst children, infects small intestinal epithelial cells and is responsible for over 500,000 deaths and millions of physician visits/hospitalizations per year. While successful resolution of infection and protection against future infection is mediated by adaptive immunity, relatively little is known how innate immunity contains acute infection and drives the adaptive immune response to RV. To investigate innate immunity to RV, we examined the consequence of absence of MyD88 in a mouse model of RV infection. Absence of MyD88, but not the combined blockade of IL-1β and IL-18, signaling resulted in greater infectivity as reflected by levels of RV in feces, intestinal lysates, and viremia. Such increased RV levels correlated with an increase in the incidence and duration of diarrhea in neonatal mice. Loss of MyD88 also markedly impaired humoral immunity to RV. Specifically, in both adults and neonates, MyD88-KO mice were impaired in generating RV-specific IgA and exhibited a profound reduction in the ratio of RV-specific IgG2c/IgG1 suggesting an important role in driving Th1 responses associated with RV infection. Study of MyD88 bone marrow chimeric mice indicated that MyD88-dependent control of acute RV infection was mediated by both hemopoietic and non-hemopoietic cells while generation of RV-specific humoral immunity was predominantly driven by MyD88 signaling in hemopoietic cells, which reflected loss of IL-1β and IL-18 expression by these cells. Thus, TLR signaling and inflammasome cytokines, drive innate and adaptive immunity to RV.

Introduction

Rotavirus (RV) is a non-enveloped, double-stranded RNA virus that preferentially infects small intestinal mature enterocytes. RV is the leading cause of severe viral gastroenteritis amongst children worldwide and causes moderate intestinal distress in adults. Before the introduction of RV vaccines in the 2000's, RV universally infected children under the age of 5 and caused 2-4 million hospitalizations and over 0.5 million deaths per year globally (46). While the introduction of RV vaccines has markedly reduced the disease burden caused by RV, it, nonetheless, remains a major public health problem especially in developing countries both because infections result in more disease manifestations, including death, and because current vaccines that have proven highly effective in North America and Europe do not elicit robust protective immune responses in many developing countries (2). While the reasons for these disparities remain unclear, the now well-appreciated role of innate immunity in both controlling initial infection and driving adaptive immunity suggest the possibility that innate immune responses may be involved. Better understanding of basic mechanisms that mediate innate immunity to RV might make it possible to investigate this possibility.

RV has been studied using a well-defined mouse model that recapitulates many aspects of RV disease and immunity in humans. Similar to humans, neonatal mice develop diarrhea with RV infection, however, they do not develop any features of severe disease, like fever, vomiting, or dehydration. Because mice only develop diarrhea with infection, this is the only method to expunge the body of virus. One key facet of innate immunity to RV revealed by use of this model is that, absence of type I and/or type II IFN has a significant effect on RV-induced clearance, but not pathogenicity, based upon mouse gene deletion; specifically, the deletion of STAT1, a signaling component for both type I and II IFN, renders mice unable to control virus

shedding as does the deletion of MAVS, a protein downstream from RIG-I which is partially responsible for type I IFN induction in response to dsRNA exposure (163, 164). The roles of IFNs during rotavirus infection are further discussed by Holloway and Coulson (165). TLR3-mediated production of type III IFN helps limits RV infectivity in adult, but lack of expression of TLR3 in the neonate gut indicates it is not involved in protecting the most susceptible hosts (166, 167). Moreover, adult mice remain readily infectable to RV indicating that TLR3-mediated signaling is not sufficient to protect against this pathogen suggesting the existence of additional means of innate immunity to RV. TLR 7 signaling, endosomal acidification, and IFNAR was recently found to activate dendritic and B cells after exposure to RV, but the authors did not address the role of TLR 7-mediated control of innate immunity or antibody generation following infection (168).

One of the most central molecules in innate immunity in general is the signaling adaptor MyD88. MyD88 mediates signaling for all TLRs, except TLR3, as well as receptors for inflammasome cytokines IL-1 and -18. Indeed, MyD88 signaling plays a role in immune responses to a wide variety of pathogens including viruses, bacteria, protozoa, and fungi both in terms of limiting initial infection and promoting pathogen-specific adaptive responses (169). One example of MyD88-mediated antiviral immunity is the case of influenza, in which MyD88-mediated inflammasome cytokine signaling protects against influenza mortality and induces specific antibody production, while MyD88-mediated TLR7 signaling induces type I IFN, which contributes to resistance against infection (170). Hence, the goal of this study was to investigate the extent to which MyD88 signaling contributes to the initial control of RV infection and induction of RV-specific antibody responses. The role of MyD88 signaling during RV infection, however, is understudied although it has been presented that upon RV infection, MyD88

signaling, independent of BCR, was able to induce activation of B cells and antibody generation (171). Adult mice, deficient in MyD88 and related signaling molecules, were employed in this study as a model of infection, and neonatal mice served as a model of RV-induced disease. Our results indicate that MyD88-mediated TLR signaling contributes to control of primary RV infection while inflammasome cytokine signaling mediates a robust and properly polarized RV-specific antibody response.

Materials and Methods

Animals

All experiments, excluding those using neonatal mice, utilized 6- to 8-week-old male MyD88 KO mice (gift of Jian-Dong Li, Georgia State University), IL-18 KO mice (Jackson Laboratories), IL-1R KO (Jackson Laboratories), NLRP3 KO mice (Jackson Laboratories), or C57BL/6 offspring from C57BL/6 originally purchased from Jackson Laboratories (Bar Harbor, Maine). Experiments involving neonatal mice used 6-day-old neonates bred in-house.

Virus and inoculations

Mouse RV (EC strain) was supplied by Mary Estes (Baylor College of Medicine). Adult mice received 1.33% (w/v) sodium bicarbonate (Sigma-Aldrich) by oral gavage, followed by 10^5 SD_{50} murine RV also by oral gavage. Neonates received 1 DD_{50} murine RV.

Fecal and serum RV antigen detection

Supernatants, made from fecal homogenates (100mg feces/mL PBS), or serum was frozen or immediately analyzed by ELISA as described (172).

RV genome qRT-PCR

Small intestinal and colonic samples were harvested, washed in PBS, homogenized in TRIzol (Ambion), and probed for RV genome as described (172). Blood was harvested, RNA from peripheral blood cells were isolated using QIAamp RNA Blood Mini Kit (Qiagen), and RV genome was amplified.

Single-stranded qRT-PCR for RV replication

RV replication in small intestinal and colonic samples were determined by the (+):(-) RV strand ratio magnitude as previously described (150).

Antibody ELISAs

Relative serum RV-specific antibody production (measured by optical density at a specified serum dilution) and titer (measured by the serum dilution at which optical density was 0.2 over blank) was analyzed as described (172).

Bone marrow chimeras

Recipient mice were irradiated with the equivalent of either 800-850 or 600Rads (C57BL/6 or MyD88 KO mice, respectively) using an X-ray irradiator (Rad Source) and were administered bone marrow cells from either C57BL/6, MyD88 KO, or IL-1/18 DKO mice as described (173). Bones used to generate cells from IL-1/18 DKO were provided by Gabriel Nunez (University of Michigan) who generated such mice by crossing IL-1β and IL-18 mice that had been backcrossed to C57BL/6 mice for more than 6 generations.

IL-1β and IL-18 detection

IL-1β was measured in *ex-vivo* small intestinal culture supernatants as described (154). IL-18 was captured using an unlabeled anti-mouse IL-18 antibody (MBL) and detected utilizing a biotinylated anti-mouse IL-18 antibody (MBL).

IL-1β neutralization

IL-1β was neutralized with intraperitoneal administration of human IL-1Ra, Anakinra (Kineret, Swedish Orphan Biovitrum), at a concentration of 100μg/g body weight once per day the day of

inoculation and up to 3 days post inoculation. This dosing regimen was equal to or higher than several studies found effectively neutralized IL-1β activity (148, 174).

Statistics

Statistical significance was evaluated via Student's t-test. Asterisk (*) indicates $p<0.05$. Grubbs test was applied to identify and remove outliers in situations where patterns were observed but no statistical significance was achieved. The chi square test was also applied with the plus sign (+) indicating $p<0.05$. Additionally, statistical significance was evaluated by the 2-way ANOVA test. Number sign (#) indicates $p<0.05$.

Results

MyD88 signaling contributes to protection against RV infection, dissemination, and diarrhea

Infection of adult mice with rotavirus (RV) serves as a well-defined infection model without causing severe manifestations of RV disease (46). An oral inoculation of a 6-8 week-old C57BL/6 mouse with 10^5 50% shedding doses (SD_{50}) of murine RV strain EC resulted in RV antigen becoming detectable in feces 1-2 days post-inoculation (PI), with a peak at 2-5 days PI, and antigen shedding lasting 6-8 days PI. Such RV antigen shedding is proportional to infectivity, i.e. the level of viral genome in intestinal lysates. To elucidate the role of innate immunity in RV infection, we inoculated adult and neonatal mice lacking MyD88, an adaptor protein that mediates all signaling by inflammasome cytokine receptors (IL-1β and IL-18) and a major portion of the signaling by all TLRs, except for TLR3. In the absence of MyD88, mice displayed greater antigen shedding from days 2-7 PI (Figure 1a). To confirm the increased fecal RV antigen shedding was in fact reflecting increased infection of RV, small intestinal lysates were assayed for levels of RV genomes by qRT-PCR at day 3 PI, which approximately corresponds to the peak of infection (Figure 1b). In the absence of MyD88, levels of RV virus genome increased by over 5-fold relative to WT mice thus verifying that absence of MyD88 resulted increased RV loads in IECs. Increased RV loads could reflect that absence of MyD88 enhanced RV replication ability and/or enhanced RV entry, with the latter possibility potentially reflecting reduced extracellular antiviral mediators expression. To help distinguish between such possibilities, we assayed intestinal lysate for RV replication levels by measuring the relative ratios of (+):(-) RV strands by ss qRT-PCR (Figure 1c). MyD88 KO mice exhibited a 10-fold

enhancement of in the (+):(-) RV strand ratio suggesting increased RV loads likely reflect increased ability of RV to replicate in IECs of MyD88 deficient mice.

While is RV infection normally predominates in the small intestine, it can disseminate more widely in immune-compromised mice. Thus, we next examined if loss of MyD88 resulted in increased spread of RV beyond the small intestine. Loss of MyD88 resulted in a marked increase in the amount of viral genome in the colon (Figure 2a) indicating a role for MyD88 signaling RV in preventing RV spread within the intestinal tract. The (+):(-) RV strand ratio indicated that the virus present in colon lysates, in both WT and MyD88 KO mice, was indeed actively replicating virus. Yet, the increased RV loads in MyD88 KO colon were associated with only a modest increase such ratios (Figure 2b) suggesting that enhanced colonic RV loads were, at least in part, driven by greater amounts of RV arriving from the small intestine. RV genomes were undetectable in the livers, spleens, and lungs of both WT and MyD88 KO mice (data not shown). However, in the absence of MyD88, serum RV antigen levels were increased (Figure 2c), and peripheral blood cells exhibited increased levels of RV genomes at 3 days PI (Figure 2d) supporting a role for MyD88 signaling in limiting viral spread to the blood. Thus, MyD88 signaling plays a role in restricting RV to the small intestine, likely, at least in part, by limiting its replication within this organ.

We next investigated the extent to which the impairment of MyD88 KO mice in controlling RV infection might reflect loss of TLR or inflammasome cytokine signaling. Loss of the best-studied inflammasome, NLRP3, which has been implicated in a broad array of innate immune recognition pathways, had no effect on the course of RV shedding suggesting lack of involvement in RV recognition (Supplemental Figure 1a). Next, we determine if RV induced an increase in IL-1β and IL-18 expression. While, in accordance with other studies, mature IL-1β

and IL-18 were detectable in intestinal supernatants (duodenum and jujenum), their levels were not significantly increased following RV inoculation (Supplemental Figures 1b-c). Moreover, neither groups of mice deficient in IL-18 or IL-1 receptor exhibited significant impairment in their ability to clear RV (data not shown). To address the possibility that perhaps both IL-1 and -18 might be required for efficient handling of RV, IL-18 KO mice were administered an IL-1R antagonist prior to and following inoculation with RV. Such combined blockade of IL-1 and -18 signaling did not alter the time course or extent of RV infectivity relative to that observed in WT mice (Supplemental Figure 1d) indicating the increased RV infectivity observed in the absence of MyD88 does not reflect loss of inflammasome cytokine signaling.

To further understand how MyD88 signaling was contributing to control of RV infection, we examined RV infectivity in MyD88 KO bone marrow irradiated chimeric mice. While this method has proven useful in understanding what types of cells are responsible for specific phenotypes, conclusions drawn from experiments using MyD88 bone marrow chimeric mice bring caveats. Specifically, MyD88 deficient mice are extremely susceptible to irradiation, developing a variety of health problems, making it difficult to compare irradiated WT and MyD88 mice. Hence, we focused on comparing irradiated mice of the same host genotype that received WT or MyD88 KO bone marrow. Reconstituting WT irradiated mice with MyD88 KO bone marrow, under conditions that give greater than 95% chimerism, did not impair clearance of RV relative to mice receiving WT bone marrow (Supplemental Figure 2a). Yet, administering WT bone marrow to irradiated MyD88 resulted in a moderate reduction in RV infectivity (Supplemental Figure 2b). Together, these results indicate MyD88 in both hemopoietic and radio-resistant cells, likely epithelial cells, contribute to MyD88-mediated clearance of RV infection.

Analogous to the case for human infants, RV infection in neonatal mice results in diarrhea that begins 2-3 days after virus exposure and lasts for approximately 1 week depending on initial dose. To determine if increased production of RV antigens that resulted from absence of MyD88 translates into greater disease in neonatal mice, we inoculated 6-day-old neonatal MyD88 KO mice with 1 DD_{50} RV and monitored mice for diarrhea, as indicated by the presence of profuse, yellow, runny feces upon application of light pressure to the abdomen (172). This relatively low dose of RV resulted in incidence of diarrhea in 53% of WT and 89% of MyD88 KO neonatal mice (Figure 3a). Moreover, absence of MyD88 resulted in a marked increase of the daily diarrhea rates from days 3 to 7 PI (Figure 3b), which corresponded to more than 3-fold increase in the average duration of diarrhea (Figure 3c). Thus, the increased RV replication within IECs, and concomitant increase in RV antigens in MyD88 KO mice indeed translated into greater disease.

MyD88 signaling drives RV humoral responses and controls proper sub-isotype switching

RV infection elicits a robust adaptive immune response that plays a critical role in clearing primary infection and confers protection against future infection. Such protective immunity correlates with and is largely mediated by generation of RV-specific antibodies (46). RV vaccines, which are live attenuated viruses, employ such protection and can be considered analogous to the adult model of RV infection in that both elicit antibody production by asymptomatic infection. It is now well appreciated that innate immune signaling is critical for initiation and regulation of adaptive immunity although the relative roles for MyD88 signaling is pathogen-dependent (175, 176). Hence, to investigate the role of MyD88 in the induction of RV-specific humoral responses, we infected MyD88 deficient mice and measured anti-RV IgG and IgA responses. Although MyD88 KO mice were likely exposed to considerably more RV

antigen, due to increased infectivity, absence of MyD88 resulted in a modest reduction of anti-RV IgG and IgA particularly within a few weeks following infection (Figures 4a-b). This modest deficit was overcome shortly thereafter and resulted in titers that did not significantly differ between WT and MyD88 KO mice at 9 weeks PI (data not shown). Total serum IgA concentrations revealed that MyD88 deficient mice produce less serum IgA than WT mice (Supplemental Figure 3) indicating that the weakened early RV-specific IgA response may be reflective a deficiency in total IgA. The absence of MyD88, however, resulted in a dramatic shift in IgG subtypes with MyD88 KO mice exhibiting a marked loss of IgG2c and increase in IgG1 (Figures 4c-d). Next, we examined antibody response in WT and MyD88 KO mice that had been infected as neonates. Somewhat similar to adult MyD88 KO mice, neonates exhibited a modest reduction in the anti-RV IgG and IgA response (Figures 5a and c), particularly at later time points when viewed as dilution titers (Figures 5b and d). Moreover, they exhibited a dramatic loss of RV-specific IgG2c and a corresponding marked increase in IgG1 (Figures 5e-f). These results indicate MyD88 signaling plays a key role in driving the Th1-mediated immune response normally expected with RV infection.

Bone marrow-derived MyD88 signaling enhances RV humoral responses and proper antibody class switching

To determine in which cells (radio-resistant/epithelial versus bone marrow-derived) MyD88 signaling contributes to RV-specific antibody responses, we utilized the above-described MyD88 KO bone marrow chimeric mice. In contrast to the case for controlling RV infectivity, proper anti-RV humoral response was largely a consequence of absence of MyD88 in bone marrow-derived cells. Specifically, reconstitution of WT mice with MyD88 KO bone marrow resulted in a marked attenuation of anti-RV IgG and IgA (Figures 6a-d) while, conversely,

administration of WT bone marrow to irradiated MyD88 KO enhanced anti-RV IgG (Figure 6e). Moreover, in such chimeric mice, regardless of whether the irradiated host was WT or MyD88 KO, the presence of MyD88 in bone marrow derived cells was necessary and sufficient for a robust anti-RV IgG2 response, whose absence correlated with an enhanced level of anti-RV IgG1 (Figures 7a-d). Thus, the Th1-polarized humoral immune response to RV was mediated by MyD88 in bone marrow-derived cells. To discern whether such role of MyD88 reflected loss of TLR or inflammasome cytokine signaling, we next investigated if reconstitution of irradiated WT mice with bone marrow from IL-1β/IL-18 DKO mice would result in a similar phenotype. Indeed, absence of IL-1βand IL-18 in bone marrow cells resulted in a modest reduction in anti-RV IgG and IgA (Figures 8a-b) and a dramatic shift in IgG subtypes, especially the complete loss of anti-RV IgG2c (Figure 8c). Such loss of anti-RV IgG2c was not observed in mice lacking only IL-1R or IL-18 (Supplemental Figures 4a-d) and was not be phenocopied in mice lacking the NLRP3 inflammasome (Supplemental Figures 5a-d). Thus, the combination of bone marrow-derived IL-1β and -18 supports proper anti-RV antibody class switching that optimizes the immune response to RV.

Discussion

Rotaviruses remain a world leading cause of severe diarrhea, resulting in substantial morbidity and death. Even though the vast majority of RV infections resolve without treatment and result in lasting protection against reinfection, adult/asymptomatic RV infections are an example of an effective immune response. Indeed, mimicking this response with live attenuated orally-administered RV has proven to be an effective vaccine strategy. Yet, the host determinants that mediate initial control of RV, including restricting it to the gut and drive the well-characterized antibody responses that mediate lasting protection have not been well defined. Herein, we investigated the potential involvement of MyD88 signaling pathways, which mediate signals by inflammasome cytokine receptors and TLRs, might mediate innate and/or adaptive immunity to RV. We observed that, indeed, MyD88 signaling plays a key role in both initial containment of RV and directing generation of RV-specific antibodies.

In adult mice, the absence of MyD88 enhanced RV infectivity and increased virus spread to the colon and blood. In neonate mice, loss of MyD88 prolonged and exacerbated RV-induced diarrhea. As diarrhea causes much of the mortality associated with RV infection and extra-intestinal spread of the virus correlates with more severe infections, we conclude that MyD88 signaling is a key part of the innate immune response that normally limits RV infection and disease. Such MyD88-mediated protection did not reflect a role for inflammasome cytokines as blockade of IL-1β and/or IL-18 signaling did not significantly impede containment and clearance of RV. Rather, it suggests a role for TLR signaling in initial control of RV infection. The mechanism of how MyD88-mediated TLR signaling controls RV infection is unclear but may resemble other viruses where specific or groups of TLRs limit virus infection and spread. For example, mice deficient in endosomal TLR7 and 9 show higher DNA virus murine

cytomegalovirus (MCMV) titers at the peak of infection and are more susceptible to virus-induced death (177). As such, it can be hypothesized that MyD88 mediated TLR signaling, most likely endosomal TLRs, may recognize RV and provide signaling necessary to control RV infection and spread. Indeed, Deal and colleagues depleted pDCs, a dendritic cell sub-type which secretes type I IFN production in a TLR 7 and/or 9 dependent manner, and found that it limited fecal RV shedding (178). The authors, however, did not identify which TLR or MyD88 as responsible for pDC-mediated control of virus infection. The contribution of nearby innate cells to the control of infection was evaluated but not presented here; DCs, neutrophils, NK cells, or macrophages were not found to be involved in limiting infection by various depletion methods. Based on our results with MyD88 bone marrow radiation chimeras and depletions, we speculate that activation of such MyD88 signaling by endosomal TLRs might occur in infected epithelial and nearby immune cells, like pDCs.

The other major consequence of absence MyD88 observed in our study was alteration in the humoral immunity elicited by RV. Considering that the increased infection of RV observed in MyD88 KO mice, which would presumably result in RV antigens reaching more immune cells, one might have expected a greater antibody response in MyD88 mice. Rather, we observed a modest reduction in total levels of RV-specific antibodies and a dramatic shift in IgG subtypes, including the near complete loss of IgG2c. This virus-specific skew in IgG profiles may be influenced by basal non-specific IgG profiles in the MyD88 KO mouse as Gavin and colleagues reported that mice lacking both MyD88 and Trif made less IgG2c and more IgG1 than control mice (179). Moreover, the absence of MyD88 may be influencing the differentiation of B cells into plasma cells as was observed by Guay and colleagues polyoma virus infection (176, 180). In contrast, the induction of anti-bacterial adaptive responses, like to *Toxoplasmosis gondii* and

Citrobacter rodentium, seems to be unchanged by the absence of MyD88 (175, 181). While the alteration in the response observed in MyD88 KO mice did not render them susceptible to reinfection with the homologous RV strain (data not shown), it might reduce protective efficacy against infection by heterologous RV strains.

In contrast to the case for MyD88 signaling in initial RV containment, MyD88-mediated control of RV-specific antibodies was mediated by MyD88 in bone marrow-derived cells and reflected a role for inflammasome cytokines rather than TLR signaling in that it was phenocopied by combined, but not individual, absence of IL-1 and IL-18. That neither IL-18 nor IL-1β expression was significantly induced by RV but yet seemed to direct the antibody response to RV is reminiscent of studies by Pierini and colleagues wherein IL-18-dependent IFN-γ production drove immunity to *Franciscella novicida* (182).

That, as observed herein and by others (182, 183), inflammasome cytokine signaling was needed for IgG2c generation, raises the question as to which inflammasome might be triggering inflammasome cytokine IL-1 and -18 maturation. Some inflammasomes have already been implicated in the recognition of various pathogens and consequent IL-1 and -18 maturation. For example, free cytosolic DNA from DNA viruses vaccinia virus and MCMV and from bacteria *Francisella tularensis* and *Listeria monocytogenes* activate the AIM2 inflammasome, and in the case of MCMV, AIM2 activation is necessary for IFN-γ production via IL-18 (184). Like MCMV activation of AIM2, it seems possible that an inflammasome may recognize RV infection and is involved in maturation of inflammasome cytokines and subsequent RV-specific sub-type switching. Indeed, Kanneganti and colleagues found that dsRNA analog poly(I:C) and dsRNA isolated from RV triggered NLRP3-mediated caspase-1 activation with poly(I:C) also capable of eliciting IL-1β and IL-18 secretion (185). We, however, did not elicit measurable IL-

1β or IL-18 with RV infection and found NLRP3 dispensable for IgG2c induction. In addition to IL-1 and -18 signaling, MyD88 has recently been shown to control class switch recombination via TACI signaling in B cells (186). It can be hypothesized that MyD88-mediated TACI signaling in B cells is the reason why less RV-specific antibody and IgG sub-type switching occurs in mice lacking MyD88 as shown by us and others (171). Further experimentation in TACI deficient models needs to be done to define the role of MyD88-mediated TACI signaling in IgG class switch.

Since MyD88-mediated TLR signaling limited RV infectivity and spread of infection, TLRs could serve as a potential target to control RV infection in those that are chronically infected or to prevent infection in those of danger of bioterrorism or viral epidemics. Since TLR7, 8, and 9 all recognize nucleic acids and induce potent antiviral cytokines, these TLRs could be a target for RV disease treatment and prevention. In fact, some pharmaceutical companies have already done this with other viruses. For example, the TLR7 agonist imiquimod is approved for the treatment of external genital and perianal warts from human papillomavirus infection (187). Other TLRs not canonically thought to be involved in antiviral immunity could also be targeted to treat and prevent RV infection. For instance, administration of the TLR5 agonist, Flagellin, prior to RV inoculation inhibits viral infectivity in mice (188). Thorough understanding of the role of MyD88 during RV infection not only expands our limited knowledge of RV-specific innate immunity but could also serve as doorways to potential therapeutics.

Figure 3-1

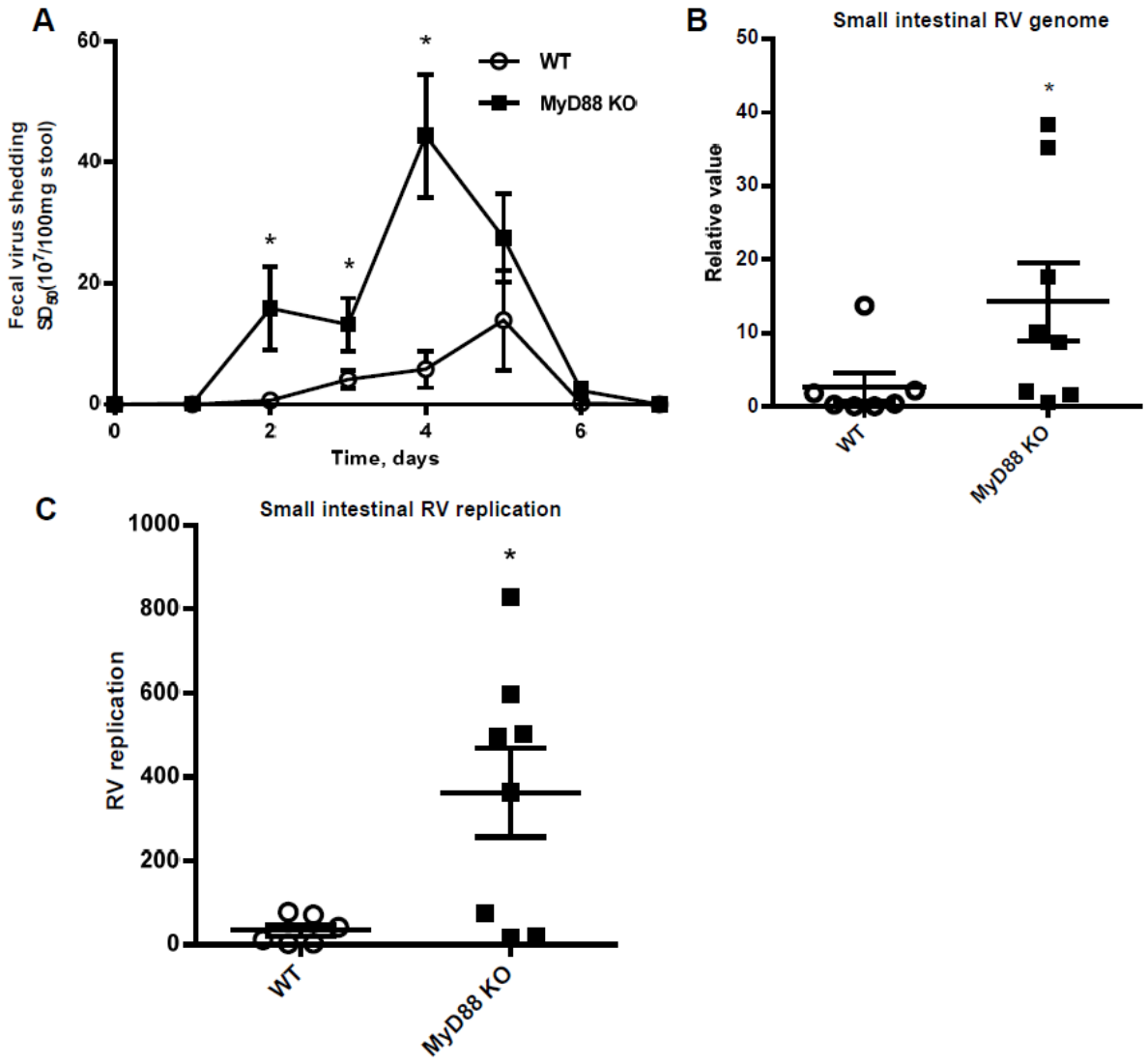

A
Fecal virus shedding
SD50(10^7/100mg stool)
60
40
20
0
0
2
4
6
Time, days
WT
MyD88 KO
*
*
*
B
Small intestinal RV genome
Relative value
50
40
30
20
10
0
WT
MyD88 KO
*
C
Small intestinal RV replication
RV replication
1000
800
600
400
200
0
WT
MyD88 KO
*

Figure 3-2

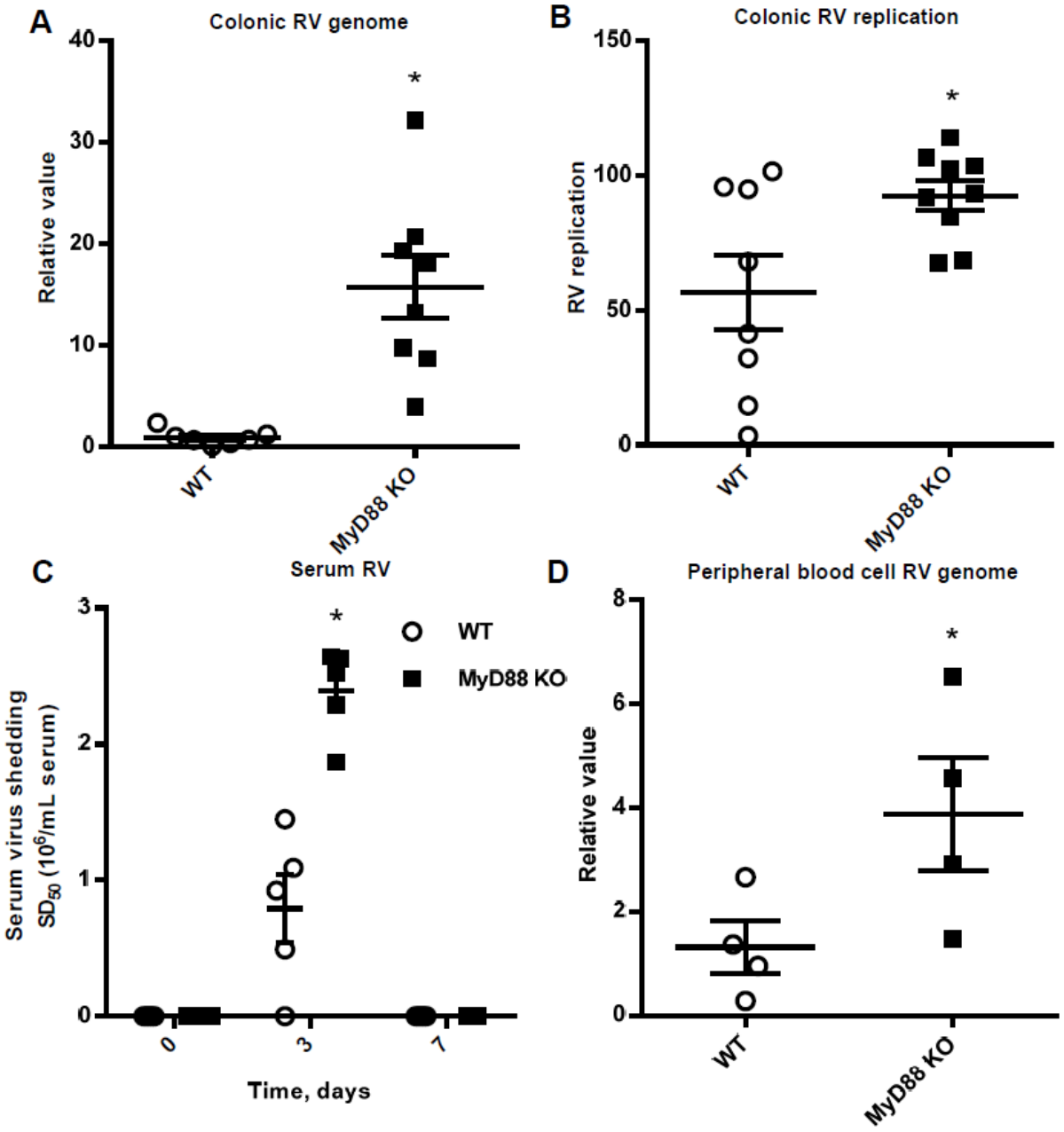

A
Colonic RV genome
Relative value
40
30
20
10
0
*
WT
MyD88 KO
B
Colonic RV replication
RV replication
150
100
50
0
*
WT
MyD88 KO
C
Serum RV
Serum virus shedding
SD50 (10^6/mL serum)
3
2
1
0
*
WT
MyD88 KO
0
3
7
Time, days
D
Peripheral blood cell RV genome
Relative value
8
6
4
2
0
*
WT
MyD88 KO

Figure 3-3

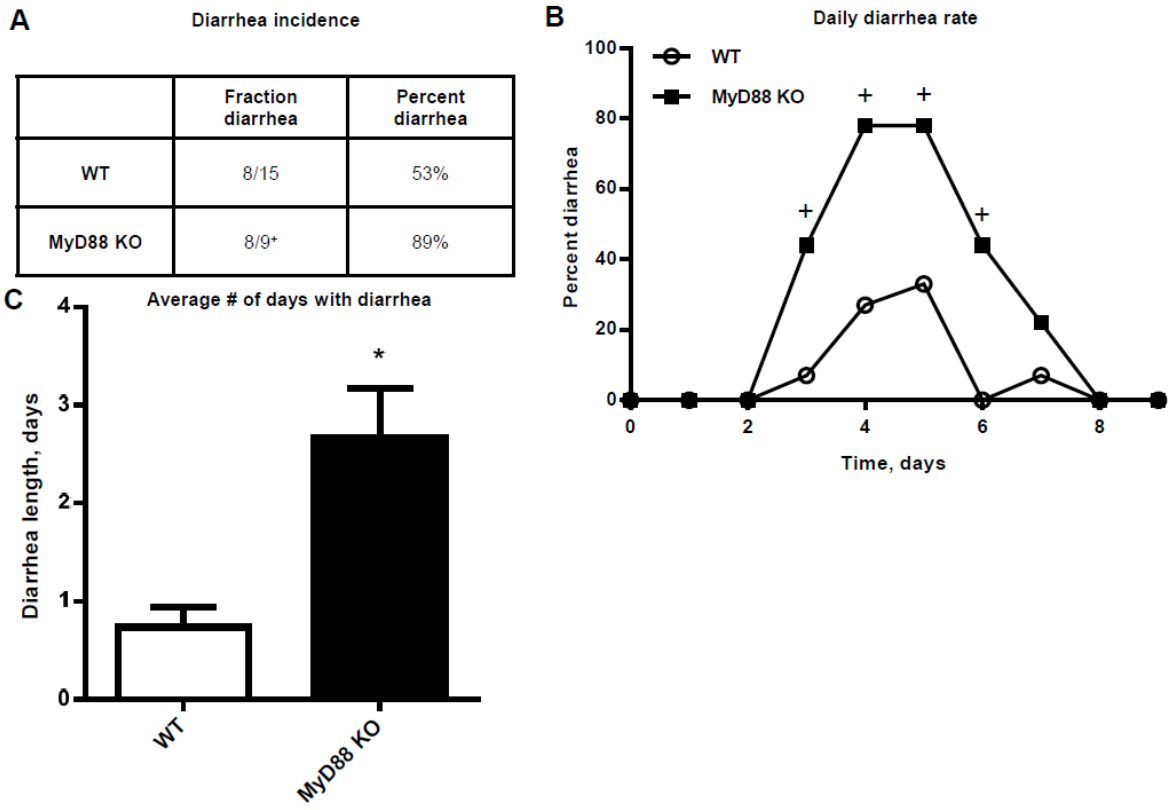

A
Diarrhea incidence
Fraction diarrhea
Percent diarrhea
WT
8/15
53%
MyD88 KO
8/9+
89%
B
Daily diarrhea rate
WT
MyD88 KO
Percent diarrhea
Time, days
C
Average # of days with diarrhea
Diarrhea length, days
WT
MyD88 KO

Figure 3-4

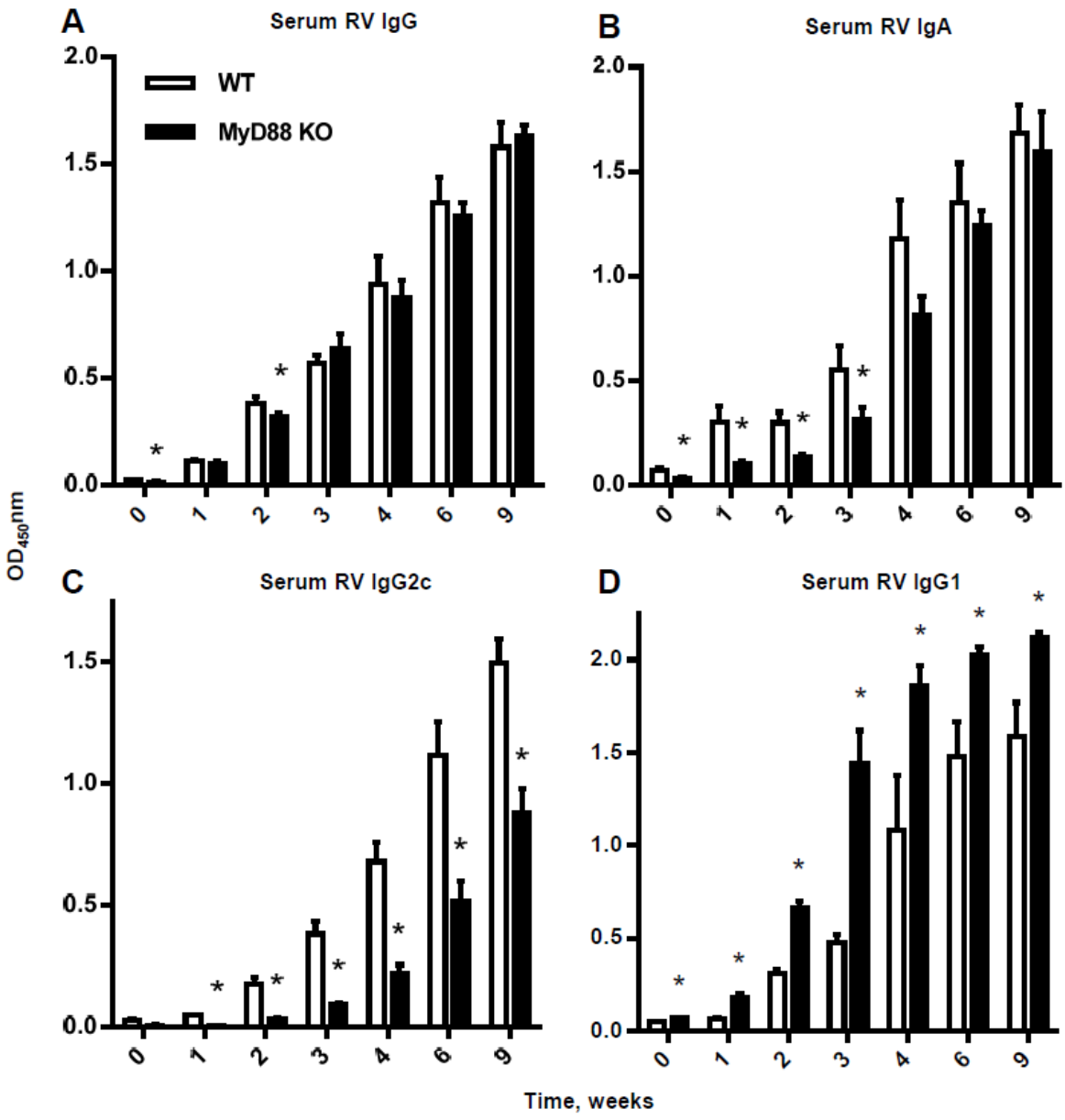

A
Serum RV IgG
WT
MyD88 KO
B
Serum RV IgA
C
Serum RV IgG2c
D
Serum RV IgG1
OD450nm
Time, weeks
0
1
2
3
4
6
9
0.0
0.5
1.0
1.5
2.0
*

Figure 3-5

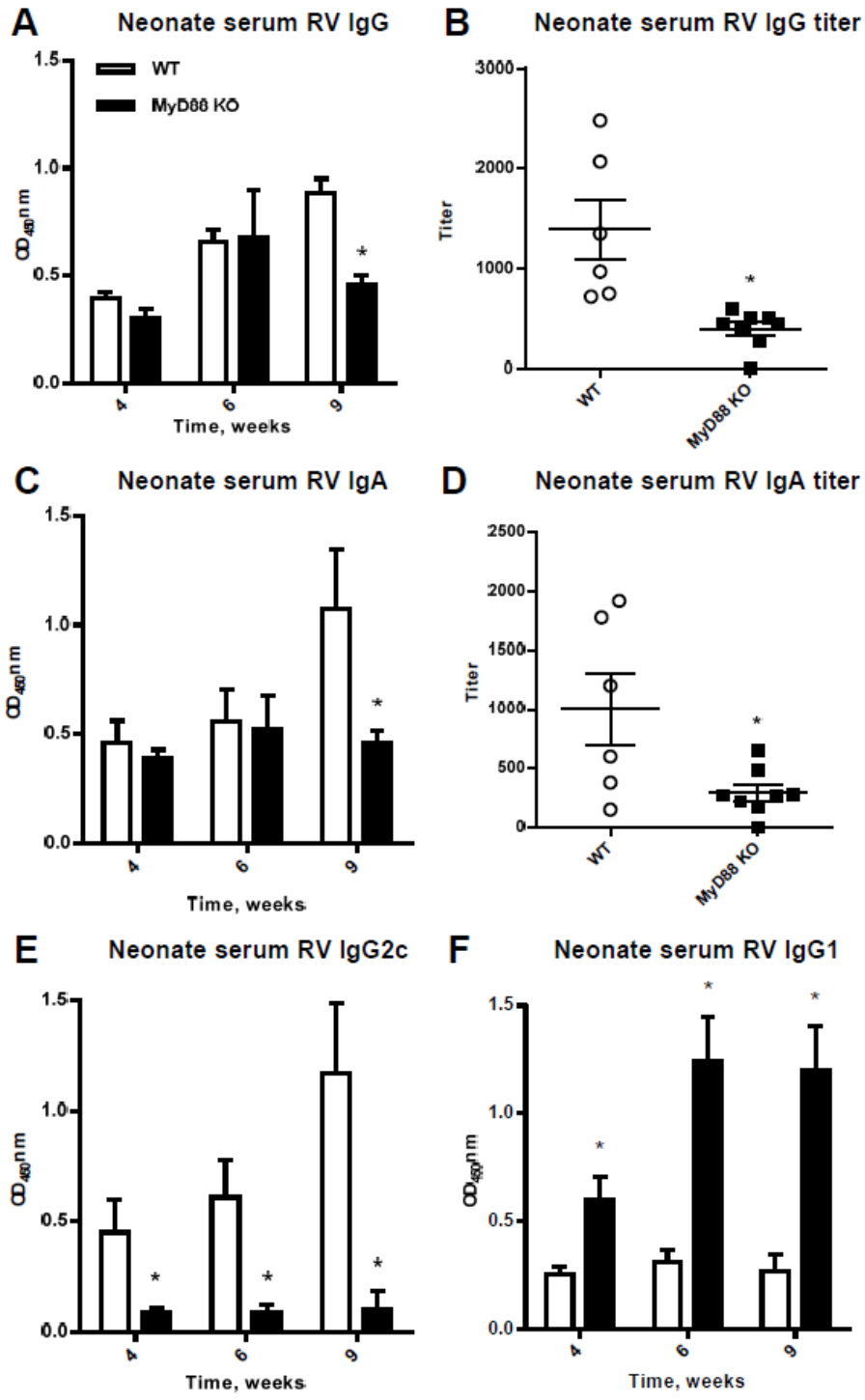

A
Neonate serum RV IgG
WT
MyD88 KO
OD 450 nm
Time, weeks
B
Neonate serum RV IgG titer
Titer
WT
MyD88 KO
C
Neonate serum RV IgA
D
Neonate serum RV IgA titer
E
Neonate serum RV IgG2c
F
Neonate serum RV IgG1

Figure 3-6

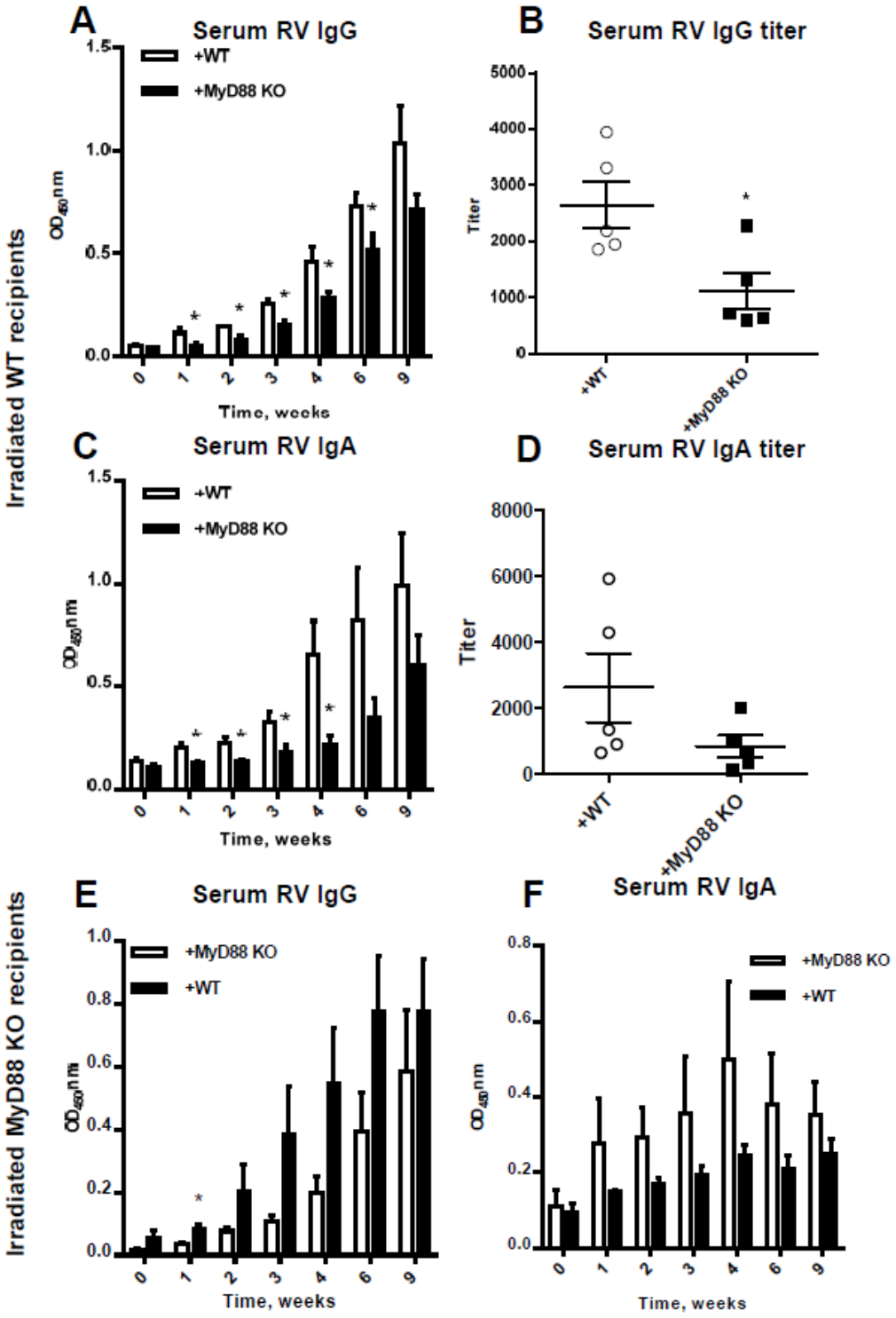

A
Serum RV IgG
+WT
+MyD88 KO
Time, weeks
B
Serum RV IgG titer
Titer
+WT
+MyD88 KO
C
Serum RV IgA
+WT
+MyD88 KO
Time, weeks
D
Serum RV IgA titer
Titer
+WT
+MyD88 KO
Irradiated WT recipients
E
Serum RV IgG
+MyD88 KO
+WT
Time, weeks
F
Serum RV IgA
+MyD88 KO
+WT
Time, weeks
Irradiated MyD88 KO recipients

Figure 3-7

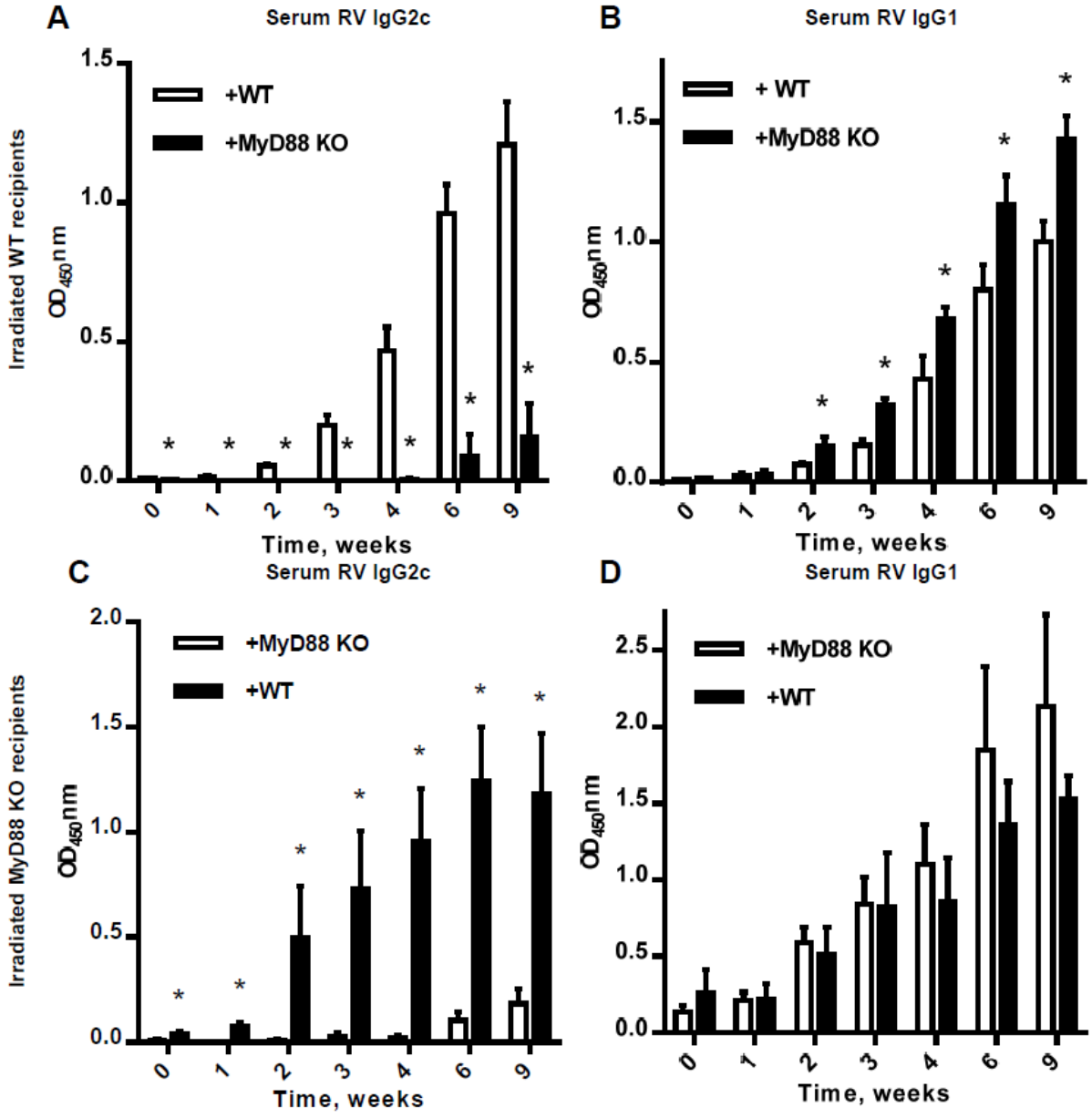
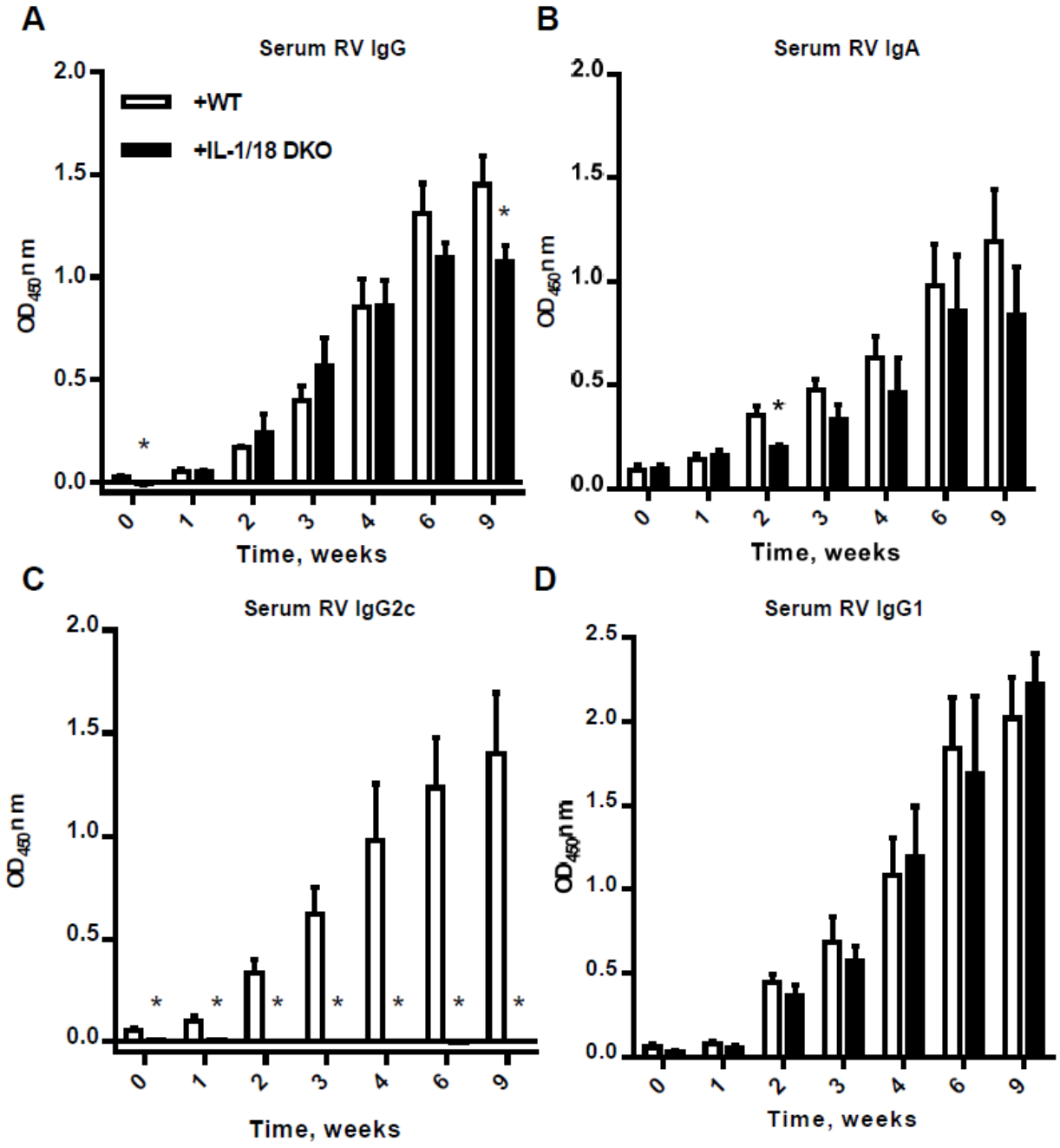
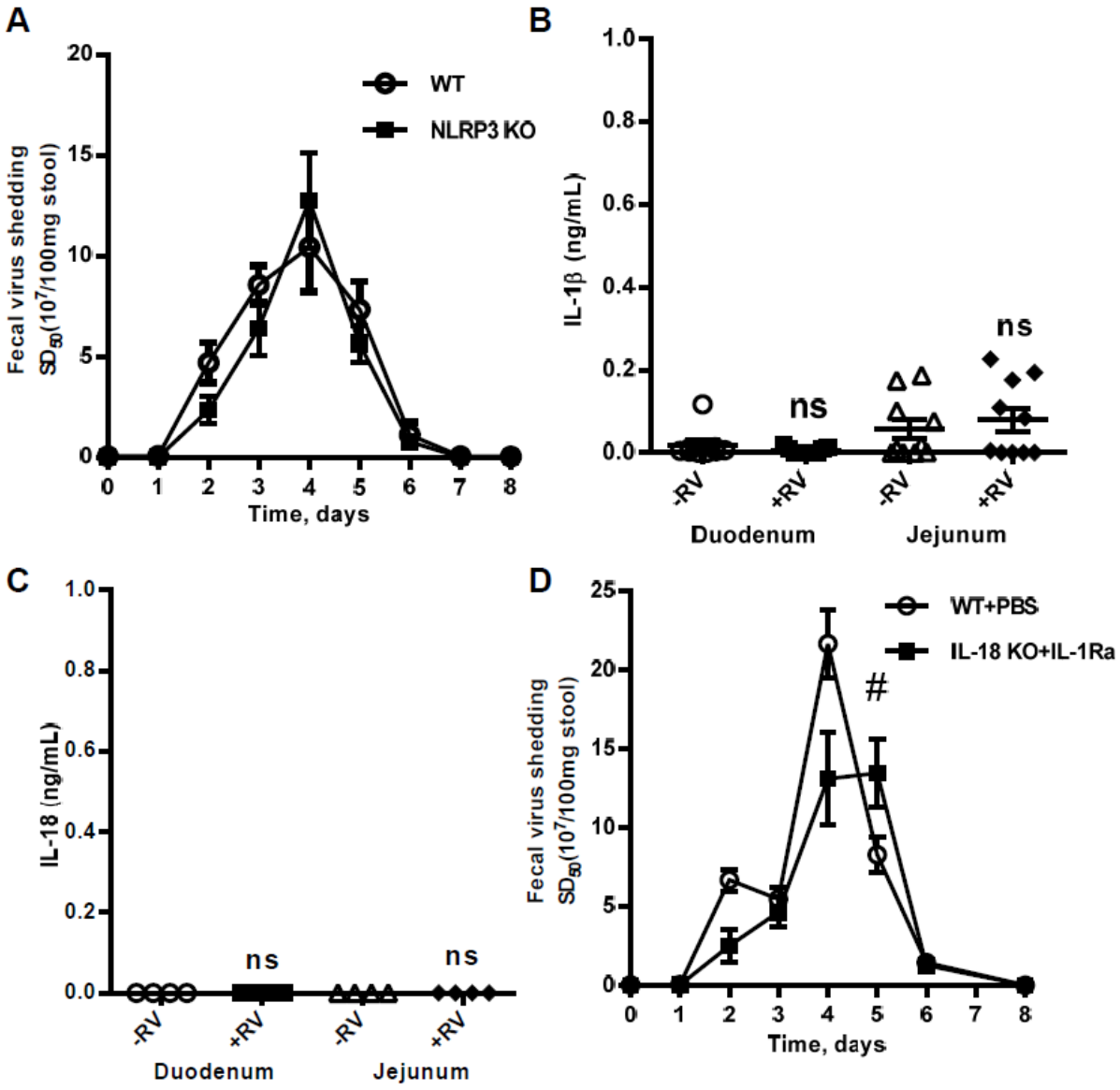

A
Serum RV IgG2c
Irradiated WT recipients
+WT
+MyD88 KO
OD450nm
Time, weeks
B
Serum RV IgG1
+ WT
+MyD88 KO
OD450nm
Time, weeks
C
Serum RV IgG2c
Irradiated MyD88 KO recipients
+MyD88 KO
+WT
OD450nm
Time, weeks
D
Serum RV IgG1
+MyD88 KO
+WT
OD450nm
Time, weeks

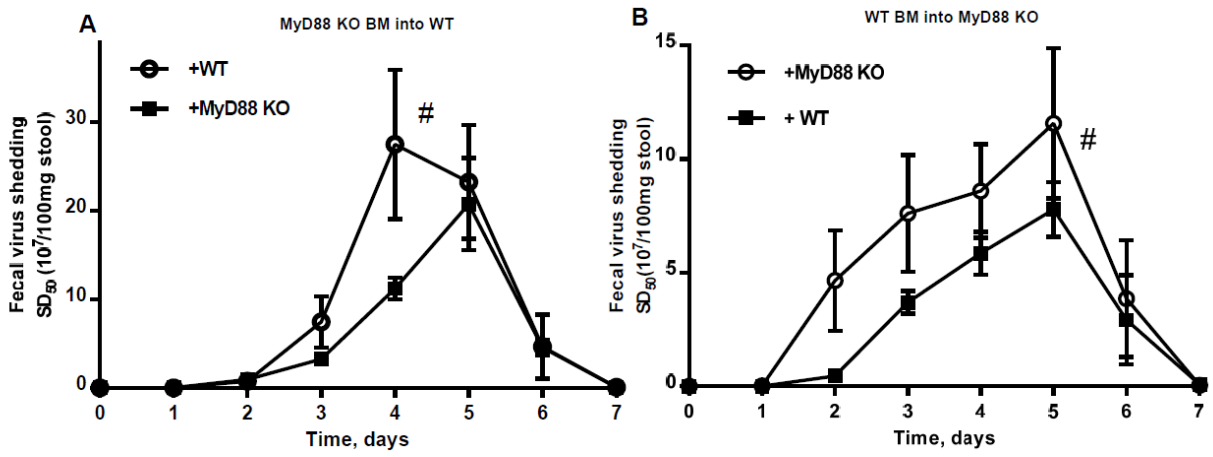
Figure 3-8


A
Serum RV IgG
+WT
+IL-1/18 DKO
OD450nm
Time, weeks
B
Serum RV IgA
OD450nm
Time, weeks
C
Serum RV IgG2c
OD450nm
Time, weeks
D
Serum RV IgG1
OD450nm
Time, weeks

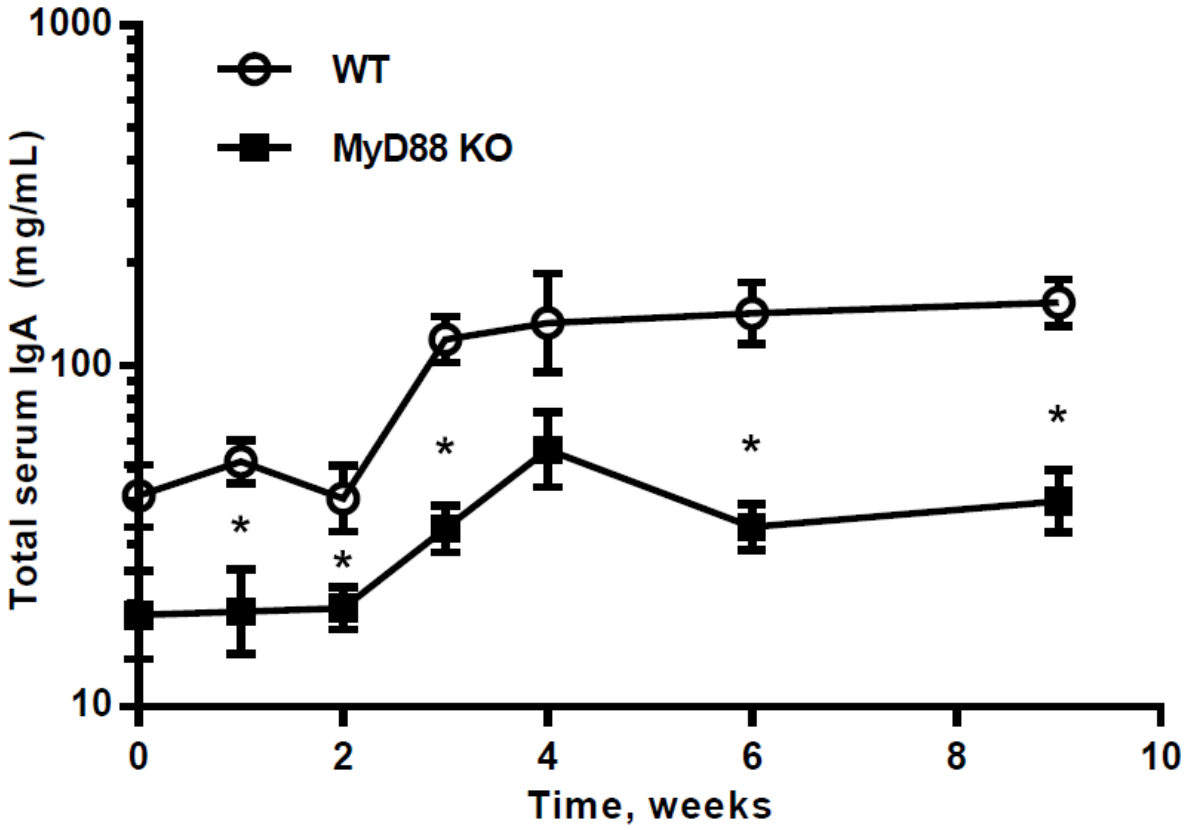
Supplemental figure 3-1



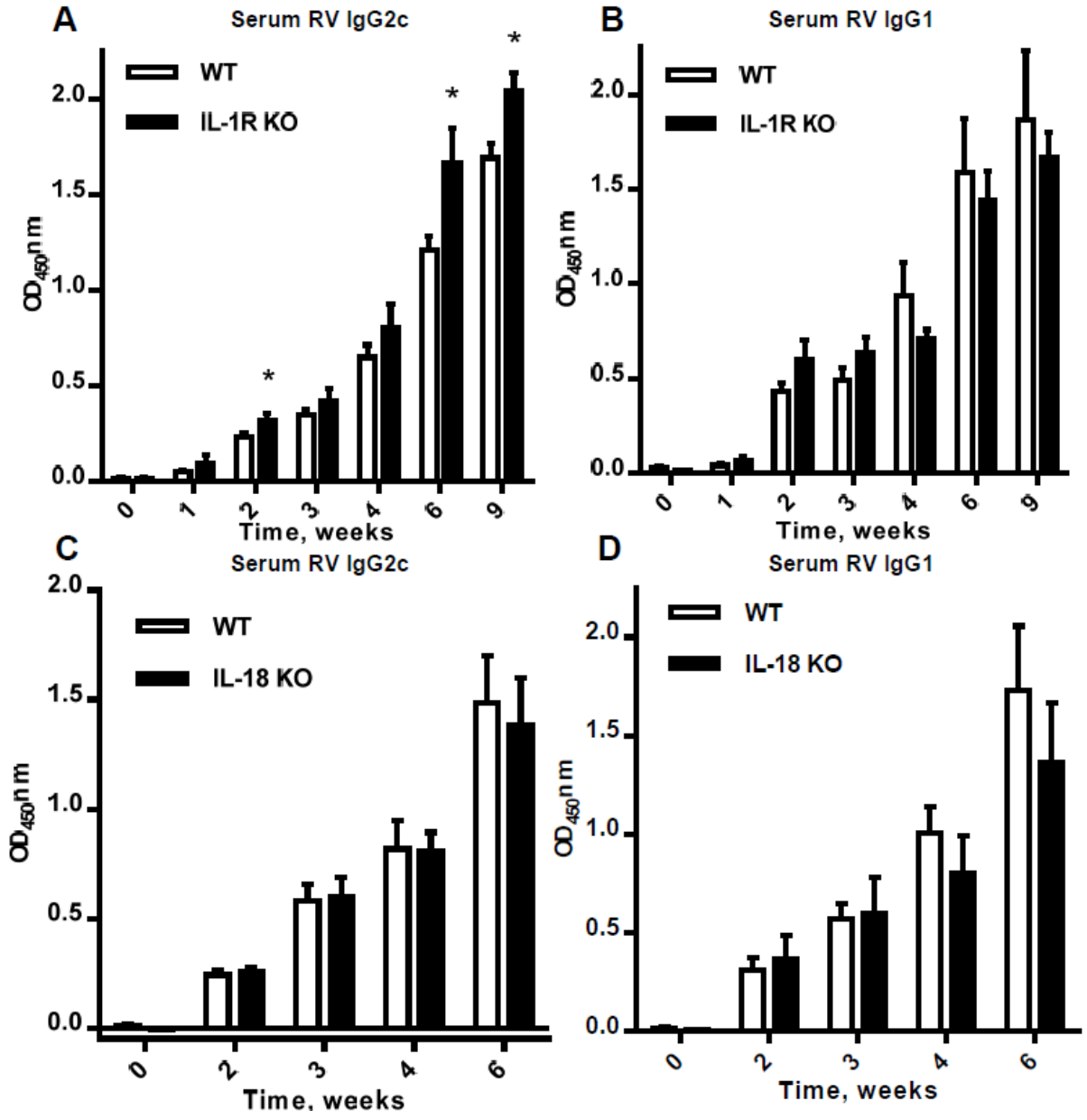
Supplemental figure 3-2



Supplemental figure 3-3



Supplemental figure 3-4



Supplemental figure 3-5

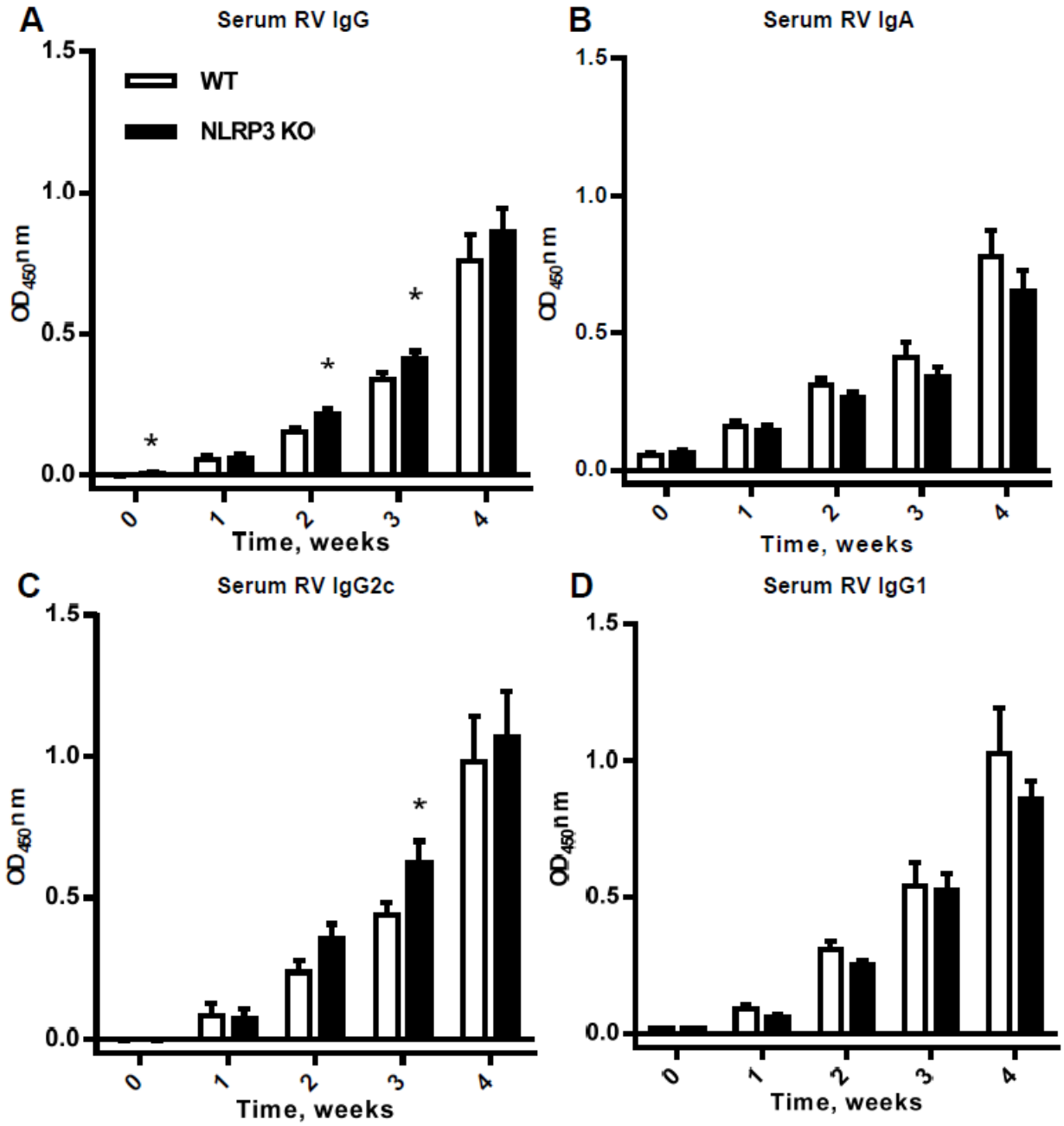


Figure Legends

Figure 3-1. MyD88 signaling contributes to the control of RV infection in adult mice. A, Six- to 8-week-old male mice were inoculated with 10^5 SD_{50} of murine RV, and feces were collected daily and probed for RV antigen by ELISA. **B,** On day 3 PI, duodenal lysates were probed for RV genome by the presence of NSP3 mRNA. **C,** Day 3 PI duodenal lysates were analyzed for RV replication (relative (+):(-) RV strand ratios) by ss qRT-PCR. Results in **(A)** are the mean +/- SEM of an individual experiment (n=5) and representative of 5 independent experiments. Results in **(B)** and **(C)** were generated from the combination of 2 independent experiments. Each point denotes an individual mouse, horizontal lines represent mean, error bars represent SEM, n=6-8.

Figure 3-2. MyD88 limits RV spread to the colon and blood. A, Day 3 PI colonic lysates were probed for RV genome by the presence of NSP3 mRNA. **B,** Day 3 PI colonic lysates were analyzed for RV replication (relative (+):(-) RV strand ratios) by ss qRT-PCR. **C,** Serum was harvested on day 0, 3, and 7 PI and analyzed for the presence of RV by ELISA. **D,** Mice were bled 3 days PI, RNA harvested as described and probed for RV genome by NSP3 mRNA qRT-PCR. Results in **(A)** and **(B)** are the combination of 2 independent experiments. **(C)** is the combination of 2 independent experiments where 1 group of mice was bled on day 0 and 7, and another was bled on day 3 PI. **D,** is representative of 1 experiment. Each point in **(A)-(D)** denotes an individual mouse, horizontal lines represent mean, error bars represent SEM, n=4-8.

Figure 3-3. MyD88 signaling protects neonatal mice from RV disease. A-C, Six-day-old mice were inoculated with 1 DD_{50} murine RV and observed daily for diarrhea symptoms, including the presence of yellow, runny, and profuse feces with application of light pressure to

the abdomen. **A**, Total RV diarrhea incidence amongst MyD88 KO and control mice were calculated. **B,** The daily diarrhea rate, or the percentage of mice per day that displayed symptoms of diarrhea, was calculated. **C,** The number of days each neonate experienced symptoms of diarrhea was averaged for MyD88 KO and control groups. Results in **(A)-(C)** represent the combination of 2 independent experiments where n=9-15. In **(C)**, bars denote the mean, and error bars represent SEM.

Figure 3-4. MyD88 signaling in adult mice enhances anti-RV antibody generation and proper antibody sub-type switch. A-D, Adult MyD88 KO and control mice were inoculated with RV and bled until 9 weeks PI. **A**, Serum anti-RV IgG production, as represented by optical density value at a specified serum dilution, was analyzed by ELISA. The same serum was also probed for RV-specific (**B**) IgA, **(C)** IgG2c, and **(D)** IgG1 in the same manner. Results are from an individual experiment (n=5) and representative of 3 separate experiments that gave a similar pattern of results. Bars denotes mean, error bars represent SEM, n=5.

Figure 3-5. MyD88 signaling in neonatal mice enhances anti-RV antibody generation and proper antibody sub-type switch. A, 6-day-old mice were inoculated with 1 DD_{50} murine RV and bled at 4, 6, and 9 weeks PI. Serum was probed for anti-RV IgG production, as represented by optical density value at a specified serum dilution, and **(B)** IgG titer, as measured by the sample dilution at which optical density value equals 0.2 over blank. The same serum was also probed for RV-specific **(C)** IgA production, **(D)** IgA titer, **(E)** IgG2c, and **(F)** IgG1. Results are representative of 2 independent experiments where bars or horizontal lines represent mean, error bars denote SEM, n=6-8.

Figure 3-6. MyD88 signaling in radio-sensitive/bone marrow-derived cells but not radio-resistant cells contributes to the production of RV-specific IgG and IgA. A-D, Bone marrow chimeric mice were made where WT mice reconstituted with MyD88 KO bone marrow. After rest and confirmation of chimerism, experimental and control mice were inoculated with 10^5 SD_{50} murine RV and bled until 9 weeks PI. **A,** Serum was probed for anti-RV IgG production, and **(B)** IgG titer. The same serum was also probed for RV-specific **(C)** IgA production and **(D)** IgA titer. **(E)** Another group of bone marrow chimeric mice were made where MyD88 KO mice were reconstituted with WT bone marrow. Mice were treated as in **(A)-(C)**, and serum was probed for anti-RV IgG and **(F)** IgA production. Results in **(A)-(F)** are from an individual experiment (n=4-5) with bars or horizontal lines represent mean and error bars denote SEM.

Figure 3-7. MyD88 in radio-sensitive/bone marrow-derived cells but not radio-resistant cells assist in proper antibody sub-type switch. A, WT mice reconstituted with MyD88 KO bone marrow were made as described in methods, and serum was probed for RV-specific IgG2c and **(B)** IgG1 production. **C-D,** MyD88 KO mice reconstituted with WT bone marrow were also made as described, and serum was probed for RV-specific IgG2c and **(D)** IgG1. Figures (**A**)-(**B**) are representative of 1 independent experiment while **(C)-(D)** are representative of another independent experiment. Results are from an individual experiment (n=4-5) with bars or horizontal lines represent mean and error bars denote SEM.

Figure 3-8. Anti-RV IgG and IgA is independent of bone marrow-derived IL-1 and -18, however proper antibody sub-type switch is dependent on both IL-1 and -18. A-D, Bone marrow chimeric mice were made where WT mice were reconstituted with IL-1 and -18 DKO bone marrow. Mice were treated as described and serum RV-specific (**A**) IgG and **(B)** IgA production was probed by ELISA. The same serum was also probed for the production of anti-

RV **(C)** IgG2c and **(D)** IgG1. Results are from an individual experiment (n=4-5) with bars or horizontal lines represent mean and error bars denote SEM.

Supplemental figure 3-1. IL-1 or -18 alone is not important for control of primary RV infection. A, Adult mice were inoculated with RV as described, and small intestines were harvested and cultured. After 24 hours of *ex-vivo* culture, supernatants were probed for the presence of IL-1 and **(B)** -18 by ELISA. **C,** IL-18 KO mice were treated with an IL-1 receptor antagonist and inoculated with RV. Feces were collected and probed for RV antigen by ELISA. IL-1 and -18 expression ELISAs, **(A)-(B)**, respectively, were performed on the same culture supernatants and are representative of 1 experiment. Each point represents an individual *ex-vivo* culture supernatant, horizontal lines denote mean, error bars represent SEM, n=5. Results in **(C)** are representative of another independent experiment where each point denotes mean, error bars represent SEM, n=5.

Supplementa1 figure 3-2. MyD88 signaling in either radio-sensitive/bone marrow-derived cells or radio-resistant cells contributes to control of RV infection. A, WT mice reconstituted with MyD88 KO bone marrow were inoculated with RV, and feces were probed for RV antigen by ELISA. **B,** MyD88 KO mice reconstituted with WT bone marrow were inoculated with RV, and feces were analyzed as described in **(A)**. Data in **(A)** and **(B)** are from individual experiments with points denoting means, error bars representing SEM, n=4-5.

Supplementa1 figure 3-3. MyD88 induces non-specific (total) IgA production in the serum. MyD88 KO and control mice were inoculated with RV and bled until 9 weeks PI. Serum was probed for non-specific (total) IgA production by ELISA. Results are from and individual experiment where each point denotes mean, error bars represent SEM, n=5.

Supplemental figure 3-4. IgG sub-type switch is not dependent on either IL-1 or -18 signaling. A, IL-1R KO mice were inoculated with RV and bled until 9 weeks PI. Serum was probed for RV-specific IgG2c or **(B)** IgG1. **(C)** IL-18 KO mice were inoculated with RV and bled until 9 weeks PI. Serum was probed for RV-specific IgG2c or **(D)** IgG1. Data in **(A)** and **(B)** are representative results of 2 independent experiments, and data in **(C)** and **(D)** are from an independent experiment. **(A)-(D)** each bar denotes mean, error bars represent SEM, n=5.

Supplemental figure 3-5. IgG sub-type switch is not dependent on the NLRP3 inflammasome. A, NLRP3 KO mice were inoculated with RV and feces were probed for RV antigen by ELISA. Serum was probed for RV-specific **(B)** IgG, **(C)** IgA, **(D)** IgG2c, and **(E)** IgG1. Data in **(A)** through **(E)** are results of 1 independent experiment, with each bar denoting mean, error bars representing SEM, n=5.

CHAPTER 4: DISCUSSION

In the introduction, I aimed to determine the roles of intestinal microbiota and MyD88, an adaptor for many innate immune signaling pathways, during RV infection, disease, and immunity. The second chapter achieved the first aim as microbiota alteration through antibiotic treatment reduced RV infection and disease while increasing antiviral humoral immunity. Moreover, the third chapter accomplished the second aim with MyD88-mediated signaling found to protect against infection and disease, as well as promote antiviral humoral immunity.

In pursuing the first aim, we showed that antibiotic-treated mice shed virus later and shed less virus than controls indicating microbiota assists in RV infection. GF mice mirrored antibiotic-treated mice, who also experienced a delay of infection. Such delayed infection in GF mice demonstrated that a net decrease in bacterial loads, but not a loss or gain of a bacterial species, is responsible for the delay in infection. Antibiotic treatment had no influence on virus replication, illustrating that reduced virus entry but not an inability to replicate caused the reduction in virus shedding. When RV disease was assessed with antibiotic treatment, treatment shortened the length of diarrhea and reduced diarrhea incidence, indicating that microbiota facilitates disease.

Furthermore, antibiotic treatment enhanced virus-specific humoral immunity by boosting systemic and mucosal antibody production, demonstrating the negative impact of microbiota on this aspect of immunity. Increases in mucosal ASC frequency accompanied increases in antibody production, signifying that microbiota inhibits ASC generation needed for antibody production. Interestingly, improved humoral immunity with antibiotic treatment was not associated with lowered antigen levels during infection, negating the widely held thought that

more antigen leads to more antibody. Moreover, improvements in humoral immunity were not limited to the full length (8-weeks) antibiotic treatment; a short, 2-week treatment was able to significantly boost humoral immunity. Increasing inflammation in the gut by exposing the immune system to microbiota reduced antibody production. Such reduced antibody production revealed that microbiota-derived products and subsequent inflammation prevents the induction of humoral immunity by most likely masking virus-specific signals needed for robust antiviral humoral immunity.

Addressing this first aim led to the conclusions that (1) the microbiota promotes RV entry and infection and (2) microbiota inhibits RV-specific humoral immunity. While these findings are entirely novel, a few investigators have demonstrated that the microbiota promotes viral infection. Specifically, the presence of microbiota increased the ability of poliovirus and reovirus to infect and also increased virus-induced mortality. When the mechanism for increased infection was probed, poliovirus was found to associate directly with microbiota-derived bacterial products to enhance entry (112). MMTV infection followed a similar pattern; the presence of microbiota increased MMTV infection. Instead of binding to bacterial products to enhance entry, MMTV utilized the anti-inflammatory environment provided by microbiota to enhance entry. Our observation that RV uses the microbiota for infection of target cells, therefore, was not entirely novel, but gives a good framework for understanding how environmental factors influence natural RV infection and vaccine strain "infection."

Some investigators have explored how the microbiota influences antiviral immunity, however, most conclusions reached during their studies differ strongly from our conclusions. Two separate investigations revealed that the microbiota promotes H1N1influenza immunity in lymphoid tissues proximal to the site of infection. Specifically, antiviral adaptive immunity,

including adaptive cell frequencies and antibody production, was enhanced in the presence of microbiota. Such effects on adaptive immunity were attributed to more functional innate immune cells, including both DCs and macrophages. Furthermore, the effects of the microbiota on antiviral immunity were not limited to H1N1; microbiota also boosted LCMV-specific adaptive immunity after systemic infection (88, 111). Thus, our work demonstrating that microbiota inhibits mucosal and systemic antiviral immunity is both novel and contrary to modern beliefs regarding the microbiota's role in antiviral immunity and serves as a launching pad for further investigation.

When comparing the work of others to my own, it is easy to find areas for further investigation, especially when examining potential mechanisms for microbiota-mediated entry and inhibition of humoral immunity. First, I could have addressed the reversibility of the antibiotic-mediated delay of infection. More specifically, determining if the addition of microbiota to antibiotic-treated mice or sterile cultures increases infection would have further validated my conclusion that microbiota speeds infection. Second, my work did not identify the mechanism behind how the microbiota enhances viral entry. I could have evaluated if there is an interaction between RV and the microbiota using biochemical methods, and this would have provided a clearer view as to how microbiota mediates entry. Another potential explanation for increased viral entry is that microbiota may increase expression of RV receptors, and this also promotes promote entry. Comparing virus receptor expression between antibiotic-treated and untreated mice would have addressed alternate explanations for increased entry.

I could have also improved upon the explanation behind how antibiotic treatment increased virus-specific humoral immunity. I hypothesized that antibiotic treatment allowed virus-specific danger signals to dominate over microbiota-derived signals, which could increase

frequency and functionality of humoral immunity boosting DCs and helper T cells. Testing this hypothesis using flow cytometry or methods of cellular biology would have allowed us to improve our explanation of how antibiotic treatment increases immunity. Another potential explanation as to how antibiotic treatment enhanced humoral immunity was that treatment removed microbiota-derived antigens essential for the maintenance of microbiota-specific ASCs, and with the loss of microbiota-specific ASCs, virus-specific ASCs dominated, producing antibody at much higher levels. Addressing this explanation would have strengthened the conclusion that microbiota enhanced virus specific humoral immunity.

Based upon the positive effects of antibiotics on RV infection, disease, and humoral immunity, there are many clinical implications of this work. First, giving antibiotics to individuals susceptible to infection may not only delay RV infection, but also lower the amount of virus shed. As a consequence, antibiotic treatment could both ameliorate ongoing infections and also prevent virus spread by reducing the chance of exposing others. Second, giving antibiotics to those in risk of infection and those experiencing severe RV diarrhea may reduce the incidence of diarrhea and shorten the length of diarrhea, respectively. Like reducing the amount of virus shed, lessening incidence and length of diarrhea could also prevent spread of the virus. A third implication of this work is that antibiotics may boost immunity after either natural or vaccine strain infection. Since the current RV vaccines are incapable of providing wide-spread protection in many developing nations, giving antibiotics with vaccines may generate antibody responses potent enough to provide defense against disease. Logistically, the ease of introducing antibiotics during vaccination or natural infection would be relatively easy as there are many FDA-approved antibiotics available. Moreover, antibiotics have few inflexible storage

guidelines, have long shelf lives, are inexpensive, and have simple administration routes, making them ideal for health care settings in developing nations.

Beyond unwanted side effects like allergic reactions and yeast infections, an inescapable caveat to using antibiotics is the possibility of the development of antibiotic-resistant bacteria. Antibiotic-resistant bacteria are becoming more common and include clinically-relevant pathogens such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and bacteria belonging to the *Enterococci* genus. Therefore, antibiotics should only be considered in populations where there is high susceptibility for disease and chronic RV infection or who do not mount humoral responses to current vaccines.

Another caveat to using antibiotics to boost RV-specific humoral immunity is that the length of time needed to boost immunity (at minimum, 14 days) is much longer than conventional therapies (5-10 days). On top of increasing the risk of unwanted side effects and antibiotic resistance, the length of treatment may be logistically infeasible because clinics in developing nations may not have the resources to provide antibiotics for that length of time or because recipients may not adhere to such an extensive dosing schedule.

Future studies not only need to weigh the risks and benefits of prescribing antibiotics to at-risk populations, but also need to determine if the positive effects of antibiotics reach beyond RV infection and immunity. Because antibiotics protect against poliovirus, reovirus, MMTV, and, now, RV infections, it may be worthwhile to investigate if antibiotics also protect against other burgeoning diarrheal viruses, including norovirus, astrovirus, and gastrointestinal adenoviruses. On top of the infection and disease reducing benefits of antibiotics, the immune-enhancing benefits to antibiotic therapies should be evaluated in the context of clinically-relevant diarrheal

viruses and oral vaccines as well. Since there is no evidence demonstrating that antibiotics have similar effects on bacterial infection and immunity as they do on viruses, using antibiotics and to prevent/treat bacterial disease and promote humoral immunity may be ineffective. In fact, antibiotic use increases susceptibility to some bacterial infections, including *Clostridium difficile* and *Citrobacter rodentium*. While susceptibility to bacterial infection is increased with the use of antibiotics, some investigations have revealed that GF conditions, which is analogous to antibiotic treatment, enhances antibacterial immunity. Two studies demonstrated that bacterial antigen immunization under GF conditions increases bacterial antigen-specific adaptive immunity (189, 190).

Because of potential negative side effects of antibiotics, it may be more useful to study other, less harmful methods to prevent RV infection and improve humoral responses. Instead of altering the microbiota through antibiotics, changing microbiota composition through probiotic or prebiotic supplementation could prevent infection and improve immunity. Indeed, many have employed probiotics to prevent RV infection and disease, reduce disease length and severity, and boost humoral immunity with varied results (see Introduction, section 1.8). Our lab was recently recruited to help investigate how a probiotic strain of *Lactobacillus reuteri* influences the immunogenicity of RV vaccines in a cohort of Atlanta-area infants. Fifteen infants received 10^8 CFU of *Lactobacillus reuteri* or placebo daily, 10 days before their first immunization and up to 10 days after their last immunization; fecal and serum samples were acquired prior to the first immunization and after their last immunization for RV antibody titers. Unfortunately, the group receiving the probiotic did not experience any significant increases in antibody titer after vaccination, indicating that this particular probiotic was ineffective. Despite this finding, it remains possible that *Lactobacillus reuteri* administration increases vaccine-specific humoral

immunity; increasing the number of study subjects and evaluating antibody production at later time points could reveal probiotic efficacy.

Based on the observation that increased inflammation inhibits antibody responses, eliminating specific sources of inflammation may be another strategy to boost humoral immunity. More specifically, treating pathogen infections or inflammatory disorders that cause excessive inflammation prior to vaccination may be a more safe and logical method to eliminating inflammation than the ablation of microbiota through multiple non-specific antibiotics. Another method to preventing infection and boosting humoral immunity without causing negative outcomes may be to reduce malnutrition through micronutrient supplementation. Supplementation with immune-boosting vitamins A and D and zinc could provide protection against infection and boost immunity without causing unwanted side effects or antibiotic resistance.

Our work on antibiotics links microbiota with RV infection and immunity for the first time; prior studies simply had not shown the immense influence of the microbiota on infection and immunity to a clinically-relevant gastrointestinal virus. Furthermore, this work provides evidence that the microbiota could inhibit vaccine protection in developing nations and opens doors for further studies investigating how environmental factors like gut microbiota influence immunity. While I do not expect clinical trials investigating how antibiotics influence human RV infection, disease, and immunity, I do expect more work connecting the microbiota to infectious diseases and more work manipulating microbiota to treat infectious disease.

The second aim of our work was to investigate the role of MyD88 in RV infection, disease, and humoral immunity, and the aim was achieved as MyD88-mediated signaling pathways were

found to be involved in all 3 processes. MyD88 was specifically investigated because it is a vital component in many innate immune signaling pathways, including all TLRs (excluding TLR3) and inflammasome cytokines IL-1 and -18 receptors. Moreover, no studies to date have examined how MyD88 signaling pathways control RV infection and immunity, and because MyD88 signaling pathways are increasingly targeted by vaccines and antiviral drugs, elucidating the influence of MyD88 on infection and immunity opens opportunities for the development of novel preventatives and therapies.

When we infected mice in the absence of MyD88, we found that mice experienced a decreased ability to control the amount of virus shed, suggesting that MyD88 regulates virus infectivity. Such increased virus shed were accompanied by greater viral replication, indicating that MyD88 probably limits the amount of virus shed by regulating RV replication. MyD88 deficiency also promoted spread of the virus to sites beyond the small intestine to the colon and blood, indicating that MyD88 is imperative in localizing virus to the small intestine. Furthermore, the absence of MyD88 increased diarrhea incidence and length of diarrhea, demonstrating that MyD88 protects against RV disease. The MyD88-mediated signaling pathway responsible for controlling infection was determined to be TLRs and not inflammasome cytokine receptors as mice lacking the ability to signal through IL-1 or -18 shed no more virus as controls.

When the humoral response was probed, MyD88 was found to regulate both the intensity and quality of the systemic antibody response. In the absence of MyD88, mice infected as adults or neonates saw reduced virus-specific IgG and IgA, suggesting that MyD88 promotes robust antibody generation. In addition, MyD88 deficiency inhibited proper IgG subisotype switching as reflected by an increase of IgG1 and decrease of IgG2c production. Since IgG1 is associated

with IL-4 expression and IgG2c is associated with IFN-γ expression, the IgG1 seen in the absence of MyD88 is most likely due to expansion of Th2 cells instead of Th1 cells, and this suggests that MyD88 normally biases T helper responses towards Th1. When we investigated in which cells MyD88 signaling contributes to the RV-specific antibody production, MyD88 signaling in bone marrow-derived, but not epithelial cells, was found to mediate antiviral antibody production, including the amount of IgG, IgA, and IgG subisotypes produced. An investigation into which MyD88-mediated signaling pathways regulate antibody production revealed that IL-1 and -18, through an NLRP3-independent mechanism, promotes IgG subisotype switching to IgG2c, as the absence of IL-1 and -18 in bone marrow-derived cells decimated the IgG2c production.

While many studies have demonstrated the role of MyD88-mediated signaling pathways on virus infection, disease, and immunity, none have examined how MyD88 influences responses specifically to RV. Despite this absence in knowledge, current literature is consistent with our findings and generally shows that MyD88 is protective against viral infection and disease and promotes immunity; this is seen in a variety of clinically-relevant viruses, including influenza, as well as models of human viruses, including MCMV (180, 191, 192). What is not consistent with literature is the mechanism for MyD88-facilitated IgG2c induction; IgG2c induction is independent of the NLRP3 inflammasome but dependent on IL-1 and -18 maturation. This mechanism for inflammasome-independent IL-1 and -18 maturation has previously been observed after only bacterial infection. For example, *Francisella novicida* infection induced IL-18 independently of Caspase-1, a terminal component of all inflammasomes, and *Mycobacterium tuberculosis*-infected BDMCs induced IL-1 maturation independently of either Caspase-1 or -11 (182, 193). Both *Francisella* and *Mycobacterium*-mediated inflammasome cytokine production,

however, required the presence of the protein apoptosis-associated speck-like protein containing a CARD (ASC). Thus, it is possible that RV infection may induce IL-1 and -18 maturation in the absence of various inflammasome components, like NLRP3 or Caspase-1/11.

When looking back, it is easy to find alternate explanations for the conclusions made. We originally concluded that MyD88-deficient mice had an inability to induce robust antibody production and switch to correct IgG subisotypes. While a deficiency in MyD88 may lead to less RV-specific antibody, the defect in RV antibody production may actually reflect the genotype's inability to produce any antibody. Gavin and colleagues demonstrated that MyD88 KO mice, with no antigen exposure, produce less antibody of any isotype (179). We also concluded that IL-1 and -18 signaling, which was independent of the NLRP3 inflammasome, is needed to elicit IgG subisotype switch to IgG2c. However, we did not evaluate whether other components of the inflammasome, like ASC and Caspase-1/11, or other inflammasomes, like the AIM2 or NLRP1 inflammasomes, could be responsible for inflammasome cytokine maturation and subsequent IgG subisotype switch to IgG2c. Since both inflammasome-dependent and independent mechanisms mature IL-1 and -18, it is possible that the IL-1 and -18 signaling responsible for switching is mediated by an inflammasome other than NLRP3 or by a component of the inflammasome, like ASC or Caspase-1/11 (182, 193).

In future studies, it would be interesting to determine if MyD88 mediates RV-specific mucosal humoral immunity. Since RV-specific mucosal antibody and ASCs are correlates to protection, it would have made the findings regarding MyD88 and humoral immunity more clinically significant and noteworthy. However, based upon studies demonstrating that systemic antibody correlates with disease protection and the observation that systemic antibody production improves with MyD88 signaling, one could predict that MyD88 signaling also

improves mucosal humoral immunity (194). Determining the importance of IgG2c for protection against virus infection, dissemination, and disease with a heterologous RV strain, which previously-infected mice would have no immunity to, would have added further significance to my work. Since IgG2c is associated with antiviral Th1 responses, I believe that IgG2c would likely increase protection against a heterologous RV infection, dissemination, and disease.

An additional future direction would be to determine which MyD88-mediated TLR or set of TLRs mediate protection against RV infection and disease. Since TLR7 and 2 are known to detect components of viruses, one could hypothesize that TLR7 or 2 individually or together could be involved in the control of infection and disease. Indeed, Pane and colleagues recently demonstrated that RV activates B cells and APCs via TLR7, however, they did not demonstrate if TLR7 contributes to the control of infection or disease (168). TLR8 also recognizes microbial nucleic acid and could influence resistance to infection and disease, unfortunately, TLR8 is only functional in humans, not mice. The absence of functional TLR8 in mice complicates studying its effects on RV infection and disease *in vivo*. Beyond determining which TLR recognizes RV and controls infection and disease, it would be interesting to see which product downstream of TLR signaling is responsible for protection against disease and infection. While TLR7 and 2 lead to robust type I IFN secretion, type I IFN has only been shown to influence the amount of RV shed, but has no effect on disease or dissemination (50-52). Other IFNs, which are also induced in response to TLR signaling, provide similar protection against RV. For instance, type II IFN protects against RV extra-intestinal spread in the adult mouse model of infection, and type III IFN controls infection in both neonatal and adult mice (51, 54). With this in mind, it is

possible that combinations of IFNs are induced downstream of TLR7, TLR2, or another MyD88-mediated TLR, and together, the combination of IFNs contribute to protection.

Targeting MyD88, TLRs, or inflammasome cytokine signaling could treat serious RV disease and improve antibody production in response to RV vaccines. Because MyD88-mediated TLR signaling protects against infection, antiviral therapies which include MyD88 and TLR agonists could reduce infection and disease, as well as decrease the risk for viral dissemination. Since MyD88 and IL-1 and -18 induced robust antibody responses and proper IgG subisotype switch, incorporating MyD88 and inflammasome cytokine agonists in RV vaccines may enhance overall antibody responses and skew the subisotype response to antiviral, Th1-associated subisotypes, like IgG2c, and thus could increase rates of disease protection.

Our work, which elucidated how MyD88 signaling influences RV infection and immunity, provides a starting point for further investigations into host innate responses to RV. More specifically, researchers now have a foundation for studying how components of MyD88 signaling, including TLRs and inflammasome cytokines, influence infection and immunity. Moreover, since innate immunity has broad control over infection and immunity, a better understanding of how the innate immune system responds to RV will lead to better vaccine strategies for those that do not respond to current vaccines and to therapeutics for those experiencing severe or chronic RV disease.

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