INTERKINGDOM COMMUNICATION OF A BACTERIAL MUTUALIST AND ITS MAMMALIAN HOST

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This dissertation is dedicated to my parents for their love,

endless support and encouragement

throughout my life

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Abstract

Microbial molecules have evolved to promote transient and/or permanent associations with mammals. Although numerous examples of secretion systems employed by pathogens during infection have been described, mechanisms by which commensal bacteria export molecules during symbiosis remain unknown. The human gut mutualist *Bacteroides fragilis* produces a capsular polysaccharide (PSA) that directs host immune development. We reveal herein that outer membrane vesicles (OMVs) deliver PSA to dendritic cells (DCs), promoting development of regulatory T cells and inducing anti-inflammatory cytokines during *in vivo* protection of intestinal disease. OMV mediated regulatory responses required the Growth Arrest and DNA-Damage-Inducible protein (Gadd45α) in DCs. DCs treated with OMVs containing PSA protect mice from experimental colitis, whereas Gadd45α-/- DCs are unable to support T cell regulatory response and are defective in suppressing proinflammatory cytokine production and host pathology. Our findings demonstrate DC-induced protection from disease via interaction with a beneficial microbial molecule delivered by OMVs, uncovering a novel paradigm for interkingdom communication between the microbiota and mammals.

In another effort to test the immunomodulatory activity of PSA outside of the gut, we found systemic treatment with PSA protects animals from experimental sepsis, a model for systemic inflammatory disease. More interestingly, this protection is mediated by B cells but not T cells because Rag-/- mice reconstituted with B cells gained the protection by PSA while those reconstituted with T cells were not protected. We further showed that a subset of B cells, marginal B cells, which are known to produce natural antibodies against bacterial antigens, were sufficient in mediating this protection.

Preliminary data also suggested that secretion of IgM and/ or expression of type II Interleukin 1 receptor (IL-1R2) from marginal zone B cells might be critical for the suppression of the excessive inflammation during disease. This study will help to uncover the systemic effect of PSA, a microbial molecule from a gut commensal, and its potential as a novel therapy for human sepsis.

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Chapter 1

Introduction

1.1 Host-Microbial Homeostasis: Active Immunomodulation by Gut Microbiota

Immediately after birth, all mammals are initiated into a life-long colonization of foreign microorganisms on almost all environmentally exposed surfaces including skin, mouth, gut and vagina (Dethlefsen et al., 2007). Among these areas, the gastrointestinal tract is the primary site of host-bacterial interactions. It is evident that maintenance of the association between the gut microbiota and host tissue is established via constant and complex crosstalk between the commensal bacteria, the gut epithelium and the intestinal immune cells.

The gut-associated immune system has evolved to tolerate the commensals through various mechanisms, including the secretion of a thick mucus layer by goblet cells in the intestine (Deplancke and Gaskins, 2001), the production of antimicrobial peptides by Paneth cells and the production of IgA by B cells (Macpherson et al., 2000; Macpherson et al., 2001a; Macpherson et al., 2001b; Macpherson and Uhr, 2004). Such mechanisms restrict the microbiota from directly contacting host tissues and prevent the penetration of commensals across the epithelial barrier.

Nevertheless, the constant interaction between the commensals and the epithelium is inevitable because stable colonization of the microbiota requires close contact of bacteria with mucosal surfaces. Pattern Recognition Receptors (PRRs), such as Toll-like Receptors (TLRs) and Nucleotide-binding, oligomerization domain (NOD)-like receptors (NLRs), are expressed on intestinal epithelial cells to specifically monitor the microbial components in the intestinal lumen. These receptors can trigger downstream immune responses once bound with Microbes Associated Molecular Patterns (MAMPs) in the

lumen, which may lead to host inflammation and elimination of gut microbes. Therefore, stable colonization of gut commensals requires regulatory mechanisms to dampen the constant inflammatory responses that the microbiota may induce in healthy hosts. Indeed, such mechanisms are being extensively studied and are shown to vary among bacterial species. For example, some commensals have evolved to have surface molecules that are less immunogenic, which protect them from being recognized as foreign antigens by the host immune system. Moreover, since germ-free mice do not have a fully functional immune system (Round and Mazmanian, 2009a; Smith et al., 2007), it was strongly implicated that the gut commensals may actively shape the innate and/ or adaptive immune responses and promote the health of the host.

1.1.1 Downregulation of Innate Immunity by Commensal Bacteria

PRRs, such as TLRs, play essential roles in innate immunity in response to microbial ligands. They are cell surface or intracellular molecules that recognize microbe-specific molecules and trigger intracellular signaling cascades, which may lead to the activation of several transcription factors (e.g., NF-κB, AP-1, IRF-3, -7). These transcription factors can drive the transcription of genes involved in proinflammatory responses and hence induce inflammation (Takeda and Akira, 2005).

Recently, several studies have demonstrated that commensal organisms may target and inhibit NF-κB activation to suppress inflammation. By analyzing the composition of the intestinal microbiota of Crohn's disease patients, Sokol et al. identified *Faecalibacterium prausnitzii*, which is greatly reduced in Crohn's disease patients, as an anti-inflammatory commensal bacterium in the gut. They showed that the supernatant of *F. prausnitzii* inhibited NF-κB activation in a human intestinal epithelial

cell line and suppressed proinflammatory cytokine production both *in vitro* and in a mouse model of colitis (Sokol et al., 2008). However, the underlying molecular mechanism remains unknown.

The activation of NF-κB is regulated by its inhibitor, IκB. Phosphorylation, ubiquitination, and degradation of IκB allow NF-κB to translocate into the nucleus, bind to specific DNA motifs, and induce transcription of target genes (Karin and Ben-Neriah, 2000). An *in vitro* study by Neish et al. showed nonpathogenic *Salmonella typhimurium* could inhibit IκBα degradation and in turn prevent NF-κB from entering the nucleus in human epithelial cells so as to reduce NF-κB-mediated proinflammatory response (Neish et al., 2000). Furthermore, a prevalent commensal bacterium of the human intestinal microbiota, *Bacteroides thetaiotaomicron*, was shown to attenuate proinflammatory cytokine production from epithelial cells *in vitro* and prevent pathology in epithelial tissues *in vivo*. The anti-inflammatory activity of *B. thetaiotaomicron* is achieved by facilitating the nuclear export of NF-κB subunit RelA in a PPARγ-dependent manner, which largely decreases the transcription of NF-κB-mediated proinflammatory genes (Kelly et al., 2004).

Although TLR/NOD signaling typically results in proinflammatory responses toward microbes, there are studies suggesting that they might also play important roles in sensing molecules derived from commensals and eliciting responses counteracting the inflammation. For example, Lai et al. found that after skin injury, *Staphylococcus epidermidis*, a member of the skin microbiota, could suppress the release of proinflammatory cytokines in keratinocytes and inhibit tissue inflammation *in vivo*. LTA derived from *Staphylococcus epidermidis*, which is responsible for the anit-inflammatory

effect, was found to signal through TLR2 to induce the expression of TRAF1, a negative regulator of TLR3-mediated NF-κB activation in keratinocytes (Lai et al., 2009). However, this and other studies have also shown that LTA can induce proinflammatory responses in other cell types, such as macrophages, monocytes (Timmerman et al., 1993), and mast cells (Yoshioka et al., 2007). This brings up the question as to how TLRs differentiate signals from commensals and pathogens and initiate signaling cascades with seemingly opposite consequences. One explanation proposed by Lai et al. was that TLRs might function differently depending on their proximity to microbes. Therefore, since keratinocytes are constantly exposed to the skin microbiota and have a greater chance of interacting with commensal-derived molecules, their TLRs may act as anti-inflammatory mediators.

In addition to directly targeting TLR-mediated signaling to suppress inflammation in cells that are in close contact with the microbiota, commensals have also evolved ways to modulate innate immune responses of other cell types, such as neutrophils. Maslowski et al. found that short chain fatty acids (SCFAs) produced during fermentation of dietary fiber by intestinal commensals, could interact with G-protein-coupled receptor 43 (GPR43) expressing neutrophils to attenuate inflammation during experimental colitis, arthritis, and asthma (Maslowski et al., 2009). This is an interesting finding because it connects the diet with the composition of the microbiota and the host immune response. In addition, it may also explain the difference among individuals in their susceptibility to human inflammatory or autoimmune diseases in different geographical regions.

Taken together, the innate immune system of the host, while serving as the early defense mechanism against pathogenic agents, also provides a platform for commensal

organisms to exert their influence on during the establishment of stable colonization, therefore promoting host–microbial homeostasis.

1.1.2 Induction of T cell-dependent Regulatory Responses by Commensal Bacteria

Beside their effects on innate immune responses, commensals also play a role in shaping the adaptive immune responses to achieve host–microbial homeostasis.

Regulatory T cells (Tregs) are a major cell population involved in adaptive regulation of inflammatory responses. Tregs are marked by the expression of a master transcription factor, Foxp3 (Fontenot et al., 2005). Once differentiated and activated, Tregs downregulate inflammatory responses by secreting anti-inflammatory cytokines (e.g. IL-10), suppressing effector T cell responses, and inhibiting the activation of dendritic cells or macrophages (Vignali et al., 2008). Recently, several studies have demonstrated that some commensal bacteria or molecules derived from them may induce Treg differentiation and/ or release of anti-inflammatory cytokines. For example, O'Mahony et al. showed that mice fed with *Bifidobacterium infantis* were protected from pathogenic *Salmonella typhimurium*-stimulated-inflammation by the induction of a Treg population. Normally the inflammation induced by pathogenic bacteria could be excessive and result in tissue damage if host immune system fail to control it. Mechanistically, adoptive transfer of Tregs induced by *B. infantis* greatly suppressed NF-κB activation *in vivo* and depletion of these Tregs abolished this suppression, suggesting that these Tregs are both required and sufficient for the anti-inflammatory activity of *B. infantis* (O'Mahony et al., 2008). Similarly, an *in vitro* study showed that *B. infantis* inhibited the production of proinflammatory cytokine IL-17 and induced the release of

anti-inflammatory cytokine IL-10 in cultured murine splenocytes stimulated by TGF-β and IL-6 (Tanabe et al., 2008). Interestingly, a pathobiont *Helicobacter hepaticus*, can also induce a Treg population and adoptive transfer of these Tregs into immunocompromised hosts prevented colitis triggered by *H. hepaticus* infection in an IL-10-dependent manner (Kullberg, 2002). In addition, new evidence has further suggested that Tregs may also help to promote IgA production from B cells in order to maintain host-microbial homeostasis (Cong et al., 2009).

In our lab, an *in vivo* study revealed that polysaccharide A, a surface molecule of a prominent commensal bacterium of the human gut, *Bacteroides fragilis*, could protect animals from experimental colitis induced by either CD4+ $CD45Rb^{high}$ T cell transfer or trinitrobenzene sulfonic acid (TNBS). And such protection was mediated by the induction of IL-10 producing CD4+ T cells (Mazmanian et al., 2008a). How that immunomodulatory molecule, PSA, is delivered from the bacterium to the immune cells is part of this thesis.

The maturation and activation of DCs direct T cell differentiation into various lineages. Several studies have investigated the influence of commensal organisms on DC functioning. Christensen et al. showed that various *Lactobacillus* strains differentially modulated DC maturation and cytokine production. *L. reuteri* DSM12246 was incompetent in eliciting proinflammatory cytokines, such as IL-12, IL-6, and TNFα, from DCs. But it could induce anti-inflammatory cytokine IL-10 production and suppress those proinflammatory cytokines triggered by *L. casei* CHCC3139 (Christensen et al., 2002). Such observations indicate that regulation between closely related commensal organisms might be a mechanism to limit inflammation toward beneficial bacteria and maintain

homeostasis. Likewise, Baba et al. tested the effect of eight different commensal bacteria on the maturation of DCs. Although different species induced distinct cytokine profiles in DCs, most of them directed DCs to promote suppressive CD4+ T cell differentiation *in vitro* (Baba et al., 2008). But the cellular mechanisms underlying these observations remain unknown. Taken together, these studies suggest that DCs may be an important mediator for Treg induction during the interaction between commensals and host adaptive immune system.

One remaining question is whether the induction of Tregs by commensal bacteria can render the host immunocompromised. If not, how is the immunosuppressive activity of the commensal-induced Tregs regulated? One intriguing hypothesis is that commensal bacteria induce antigen-specific Tregs so that Tregs induced by one bacterial species only suppresses the inflammatory response toward this particular baterium. This hypothesis offers a new angle to examine the influence of the microbiota on host adaptive immune system. However, it is still unclear whether the Treg response exhibits antigen specificity, and if so, what the underlying mechanism is.

Commensal organisms actively interact with both the innate and the adaptive immune systems to achieve their long-term colonization within the host. Investigations of how commensal organisms and their hosts communicate will continue to enhance our understanding of how the host-microbial homeostasis is achieved. In addition, a better understanding of the underlying molecular mechanisms may greatly advance the development of new therapies for inflammatory or autoimmune diseases.

1.2 Host-Microbial Communication: Outer Membrane Vesicles (OMVs)

Microbes have evolved complex adaptations to inhabit and replicate in numerous ecological niches on Earth. Animals are no exception, and provide an ideal environment for both transient and permanent microbial colonization. Many pathogens cause acute infections as a means to propagate and disseminate, and accordingly have developed myriad mechanisms to avoid, subvert and/or prevent host immune responses (Merrell and Falkow, 2004). Conversely to pathogens, symbiotic bacteria have taken a different evolutionary route to promote bacterial replication. The gastrointestinal tract of mammals is colonized for life with a diverse commensal microbiota that provides essential benefits to the host such as nutrient and caloric extraction from food, development of the immune system and protection from immunologic and metabolic diseases (Dethlefsen et al., 2007; Flint, 2004; Round and Mazmanian, 2009b). Therefore unlike most pathogens that acutely infect animals for days or weeks, symbiotic bacteria have evolved to improve host health, providing themselves a protected niche where they flourish for decades. The dichotomy between beneficial and harmful microbes is illustrated by studies showing that gut colonization by the microbiota inhibits infection by enteric pathogens (Reid and Bruce, 2006; Servin, 2004; Tilman, 2004).

How do bacteria establish molecular communication with their hosts to promote microbial replication? In addition to the export of single proteins (such as toxins) by the general secretion pathway, gram-negative bacteria have evolved intricate mechanisms that can deliver a concert of microbial molecules to the host. Well-studied examples include type III secretion systems (T3SS), T4SS and T6SS, which assemble

macromolecular surface appendages that translocate bacterial effectors following direct contact with host cells (Galan, 2009). In addition, the cell membrane of most gram-negative bacteria and some gram-positive bacteria have the dynamic feature of releasing outer membrane vesicles (OMVs) (Lee et al., 2009). OMVs were first described in the 1970s in *Escherichia coli* (Hoekstra et al., 1976) and later were found in both pathogenic and nonpathogenic bacterial species (Beveridge, 1999), such as *Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge, 1995) and *Helicobacter pylori* (Fiocca et al., 1999).

During the past four decades, more and more studies on OMVs revealed their role in mediating bacterial-bacterial communication and more significantly, in delivering virulence factors to host cells during infections (Kuehn and Kesty, 2005). For example, heat-labile enterotoxin (LT), which disrupts electrolyte balance in the gut endothelium, is found to be associated with and even enriched in enterotoxigenic *E. coli* (ETEC) OMVs (Horstman and Kuehn, 2000). However, there are no reports so far showing whether or how bacterial OMVs could mediate beneficial effects during microbial-host interactions. Notably, a fundamental difference between surface secretion systems and OMVs is the distance that microbial molecules can travel. As commensal bacteria generally do not make intimate contact with host cells, OMVs appear to provide a suitable mechanism for members of the microbiota to deliver molecules to distant targets in the host.

1.3 Microbiota and Diseases: Inflammatory Bowel Diseases (IBDs) and Sepsis

1.3.1 Inflammatory Bowel Diseases (IBDs)

IBDs are chronic and relapsing inflammatory conditions of the gastrointestinal tract. The major types of IBDs are Crohn's disease and ulcerative colitis. Crohn's disease could affect any part of the digestive system while ulcerative colitis is usually restricted to colon. Clinical symptoms of both diseases are similar, including abdominal pain, diarrhea, weight loss and so on. Current treatments of IBDs focus on reducing the uncontrolled inflammatory responses (e.g. TNF inhibitors) or suppressing the immune responses in general (e.g. steroids).

Clinical studies of human IBD patients as well as studies using animal models of IBDs have revealed that the pathogenesis of the disease is driven by multiple factors, including host genetic background and various environmental triggers, especially the intestinal microbiota (Packey and Sartor, 2008).

Family and twin studies showed that offspring of two IBD parents has more than 30% risk of developing disease and monozygotic twins exhibited concordance rate of 20% to 50%. These observations strongly suggested that host genetics significantly contributes to disease susceptibility (Halme et al., 2006). Indeed, genetic studies, especially genome wide association studies, have identified several genes that are closely linked to IBD susceptibility in human (Van Limbergen et al., 2009). For instance, Nod2 was the first gene found to be associated with IBD and therefore also named IBD1 (Hampe et al., 2007; Hugot et al., 2001; Ogura et al., 2001). Nod2 is an intracellular PRR for recognizing muramyl dipeptide (MDP), the minimal motif of bacterial peptidoglycan.

Mutations that disrupt the function of Nod2 impair the detection of bacteria by host cells, and have been implicated in the pathogenesis of IBD (Kufer et al., 2006). Many other PRRs, such as TLR1, TLR2, TLR4 and TLR6, were also found to be associated with the disease (Franchimont et al., 2004; Pierik et al., 2006; Rioux et al., 2007). These studies strongly implicated that some gut bacteria might be one of the causes of the disease. However, so far there is no clear evidence showing the involvement of a certain gut bacteria in the initiation of IBD (Macfarlane et al., 2009).

Despite the potential link between the gut microbiota and the pathogenesis of IBD, antibiotic treatment has not been very successful in treating IBD. The failure of antibiotic treatment for IBD as well as the observation that the composition and diversity of the intestinal microbiota were stustaintially altered in IBD patients (Scanlan et al., 2006) has led to a hypothesis that the imbalance of the normal microbiota, also known as dysbiosis, may also contribute to the disease (Krinos et al., 2001; Ley et al., 2007; Mazmanian et al., 2008b). Although it is still not clear that whether dysbiosis of the microbiota is a result of IBD or the cause of inflammation during disease, some commensals were found greatly reduced in human IBD patients, leading to a notion that gut commensals could also be protective rather than pathogenic in IBD. Recent studies demonstrated that some members of the normal microbiota, e.g. *Bacteroides fragilis* and *Clostridium* species, have the capacity to induce regulatory T cell response (Atarashi et al., 2011). Therefore the reduction of *Firmicutes* and *Bacteroidetes* in the microbiotas of IBD patients comparing to healthy individuals (Frank et al., 2007) supported the idea that IBD may result from the loss of one or more protective microbes in the microbiota.

1.3.2 Sepsis or Septic Shock

When excessive inflammation occurs systemically and starts causing pathology in multiple organs, it is considered as sepsis or septic shock (Stearns-Kurosawa et al., 2010). Sepsis is the third-most common cause of death in the U.S. and kills more than 215,000 people per year. According to a report published by the Centers for Disease Control and Prevention (CDC) in 2011, sepsis-related hospitalization has increased more than two times from 2000 to 2008 (621,000 to 1,141,000).

When an infection occurs, the host mounts an appropriate inflammatory response to control the infection. The inflammation normally reduces once the source of infection is cleared. However, sepsis develops following an infection that is uncontainable and unclearable by the host. As a result, the uncontrolled inflammation causes endothelial damage or dysfunction, which could lead to multiple organ failure or even death. The mortality rate of sepsis ranges from 28% to 47% (Buras et al., 2005). Current treatments for sepsis include broad-spectrum antibiotics, anti-TNFα monoclonal antibody, etc., most of which are aimed to limit aberrant inflammation. However, none of them proved to be highly effective because of the heterogeneity of the causes or the stages of the disease in human patients.

The development of sepsis can be divided into two stages. Right after infection, a rapid inflammatory response is crucial for bacterial clearance. Therefore, during the first stage, limiting inflammation does not help to control the disease. But once the infection is cleared, inflammation should be reduced to a minimum level in order to prevent tissue damage. So anti-inflammatory therapy could be useful at the second stage. In all, choosing appropriate treatments for patients at different stages of the disease is critical in

the current medical practice as well as in the research and development of new therapies.

Although a lot of the sepsis cases are initiated by severe infections caused by pathogenic bacteria, translocation of microbiota from the gut into systemic circulation under some circumstances may also be an important factor of disease development (MacFie et al., 1999). Being one of the causes for sepsis, microbiota can also be protective against sepsis during surgeries. It has been shown that peri-operative modulation of the gut microbiota may beneficially influence surgical outcome (Kinross et al., 2009). Therefore, investigating the protective influence of gut commensals or the molecules derived from them on sepsis would improve our understanding of microbiota's role in the progression or the treatment of this systemic inflammatory disease.

Animal models of septic shock are widely used in studying the development and treatment of human sepsis. Lipopolysaccharide (LPS)-induced endotoxic shock model is a relatively simple model (Wichterman et al., 1980). LPS is the major immunogenic component of the cell wall of gram-negative bacteria and it can trigger rapid pro-inflammatory cytokine production, for example, IL-6 and TNF-α, of the host cells. When administered in high dose, it induces a hypodynamic cardiovascular state, which mimics clinical symptoms of human sepsis. However, this model has quite a few caveats. First of all, a single toxin (LPS) may not induce the same responses as bacterial pathogens do in human sepsis. Secondly, LPS induces an extremely rapid response while human sepsis is a prolonged process. Lastly, human is much more susceptible than mouse toward LPS-induced endotoxic shock. Therefore, findings from this particular mouse model may not directly apply to human sepsis. Another experimental sepsis model is cecal ligation and puncture model (Rittirsch et al., 2009). Basically, a longitudinal incision is made in the

middle of the mouse abdomen. Then cecum is pulled out of the body cavity and its tip (1/2 to 2/3 of the entire length of the cecum) is ligated before one or two punctures are made by needles to allow cecal content that includes tons of bacteria to get into the body cavity to induce systemic infection. Different levels of the disease can be induced by varying the length of the ligated cecum, the size of the needle and the number of punctures. This is considered a better model than the LPS model because it mimics an infection caused by a group of naturally existing bacteria in the mouse.

As discussed earlier, the gut microbiota has profound influences on host immune responses. Although the mechanism underlying the interaction between pathogenic bacteria and the host has been extensively studied, how commensals communicate their regulatory signals to the host, and how such interaction benefits the development of the host immune system are still largely unknown. Moreover, how this beneficial communication differs from that between pathogenic bacteria and the host also remains to be uncovered. Herein I used *Bacteroides fragilis* as a model commensal organism to address these questions. It has been shown that PSA, an immunomodulatory molecule of *B. fragilis*, contributes to the development of the host immune system and protects the host from experimental colitis. My research aimed to further understand the delivery mechanism of PSA to the host, and how this mechanism benefits the host.

In Chapter 2, I describe my work that led to the discovery of outer membrane vesicles (OMVs), a single membrane bound structure present in most gram-negative bacteria and some gram-positive bacteria, as a possible mechanism for *B. fraglilis* to deliver PSA to the host and the anti-inflammtory responses induced by PSA-containing

OMVs. It was the first time that OMVs has been shown to mediate beneficial effect between microbes and the host.

In Chapter 3, I present that, by whole genome microarray analysis on DCs that were treated with PSA-containing OMV, I identified Growth Arrest and DNA Damage Protein (Gadd45α) as a key component in DCs that mediates the immunomodulatory activity of PSA. This was the first intracellular signaling molecule that was found to be required for PSA's activity. And this study will help us unravel the entire signaling pathway that PSA triggers in the host.

Taken together, Chapters 2 and 3 uncover a novel communication paradigm between commensal bacteria and the host, and shed the first light on the molecular mechanism that underlies such beneficial communication.

In Chapter 4, I show the work from Dr. June Round and myself, investigating the systemic effect of PSA in two murine models of sepsis. We show that systemic treatment with PSA protects animals from experimental sepsis via Marginal Zone (MZ) B cells. The fact that the protective activity of PSA during experimental colitis (local) and experimental sepsis (systemic) is mediated by T cells and B cells, respectively, suggesting that PSA from gut commensal *B. fragilis*, may have evolved to employ independent cellular and molecular pathways to influence the immune system of the host. Additionaly, this study has also provided supporting evidence that PSA, as a potent anti-inflammatory microbial molecule, could be a potential therapy for human sepsis.

Finally, Chapter 5 concludes with a discussion of the implication of my study (Chapters 2 and 3) and some potential directions for further studies (Chapter 4).

Chapter 2

Outer Membrane Vesicles Deliver a Microbial Symbiosis Factor to Dendritic Cells and Protect Host against Intestinal Inflammation

2.1 Summary

Of the numerous microbial species that inhabit the gastrointestinal tract of mammals, *Bacteroidetes* are the most abundant gram-negative bacterial phylum (Ley et al., 2008). *Bacteroides fragilis* is a commensal that aids in host health by ameliorating inflammatory bowel disease (IBD) and multiple sclerosis (MS) in animal models ((Mazmanian et al., 2008b; Ochoa-Reparaz et al., 2010a; Ochoa-Reparaz et al., 2010b). Polysaccharide A (PSA) is an immunomodulatory molecule produced by *B. fragilis* that is required and sufficient for treatment of experimental disease ((Mazmanian et al., 2008b; Ochoa-Reparaz et al., 2010a). *B. fragilis* protects animals by inducing the development of interlekin-10 (IL-10)-producing CD4+Foxp3+ regulatory T cells (Tregs) that suppress inflammation (Ochoa-Reparaz et al., 2010a; Round and Mazmanian, 2010). By promoting Treg development, PSA suppresses immune responses that drive inflammation, representing a novel candidate therapy for IBD and MS. Moreover, the production of PSA suppresses intestinal immunity directed toward *B. fragilis* during normal homeostatic colonization of animals (Round et al., 2011). Thus PSA is the first identified bacterial molecule that promotes mutualism by providing benefits to both microbe and mammals during symbiosis. However, the mechanism by which *B. fragilis* delivers PSA to the immune system remains unknown.

Since the genome of *B. fragilis* does not encode for known secretion system genes (Cerdeno-Tarraga et al., 2005; Kuwahara et al., 2004) and PSA is a large capsular polysaccharide (Tzianabos et al., 1992), we reasoned that PSA might be delivered to the immune system by OMVs. Indeed, *Bacteroides fragilis* has been observed to produce OMVs during growth (Lutton et al., 1991). Related study also showed that *B. fragilis*

OMVs carry outer membrane associated epitopes, including some polysacchrides by immunoblot analysis (Patrick et al., 1996). But whether *B. fragilis* OMVs specifically package PSA, or are able to mediate communication between the bacteria and the host immune system, remains unknown. We show herein that PSA is selectively packaged in OMVs that are released by *B. fragilis*. *In vivo*, OMVs containing PSA prevent experimental colitis by suppressing tumor necrosis factor (TNFα) production and T helper 17 (Th17) cell development, unlike OMVs that do not contain PSA. OMVs are internalized into dendritic cells (DCs), and program DCs to induce the differentiation of IL-10-producing Treg cells in a PSA-dependent manner. *In vitro*, Tregs induced by OMVs are functionally suppressive and inhibit T cell proliferation.

2.2 Results

2.2.1 PSA is Packaged into Outer Membrane Vesicles

Outer membrane vesicles (OMVs) have been observed in many Gram-negative bacteria. For *Bacteroides fragilis*, OMVs are mostly produced by a subset of bacteria, known as the electron dense layer (EDL), which comprises <5% of the population in laboratory culture (Patrick et al., 1996). We imaged negatively stained EDL-enriched bacteria by transmission electron microscopy. OMVs were abundantly produced by *B. fragilis* (Figure 1A), and could be observed budding from the bacterial envelope (Figure 1A, higher magnification). Previous studies have shown that deletion of PSA abrogates the immunomodulatory capacity of *B. fragilis* (Mazmanian et al., 2005; Mazmanian et al., 2008b). Electron micrographs of a PSA mutant strain (*B. fragilis*ΔPSA) illustrated no defect in OMV production, and the size, shape and abundance of OMVs produced were indistinguishable from wild-type bacteria (Figure 1A and Figure S1A). To determine if PSA is associated with OMVs of *B. fragilis*, we purified vesicles and probed vesicle extracts with anti-sera specific for PSA. OMVs from wild-type bacteria displayed immunoreactivity to PSA, unlike OMVs from *B. fragilis*ΔPSA, confirming antibody specificity (Figure 1B). In addition to PSA, *B. fragilis* produces at least 7 other capsular polysaccharides that coat the surface of bacterial cells (Krinos et al., 2001; Liu et al., 2008). While PSB was also detected in vesicle preparations, PSG was absent, possibly suggesting regulation of polysaccharide packaging into OMVs (Figure 1B). Immunogold labeling of purified vesicles confirmed that PSA is physically associated with OMVs (Figure 1C). To verify that the absence of PSA did not alter the molecular composition of vesicles, we performed proteomic analysis by mass spectrometry that showed negligible

differences in the protein composition between vesicles from wild-type or PSA-mutant bacteria (Figure S1B). Together, these findings reveal that *B. fragilis* packages PSA into OMVs, potentially representing a mechanism to deliver immunomodulatory signals to its mammalian host.

2.2.2 OMVs Containing PSA Protect Animals from Experimental Colitis

Crohn's disease and ulcerative colitis (forms of IBD) are painful and medically incurable illnesses of the digestive system (Xavier and Podolsky, 2007). PSA has been shown to protect and treat colitis in animal models (Mazmanian et al., 2008b; Round and Mazmanian, 2010), including intestinal inflammation induced by 2,4,6- trinitrobenzene sulfonic acid (TNBS) (Neurath et al., 2000; Wirtz et al., 2007). To investigate if OMVs have therapeutic activity, we orally treated mice with vesicles containing PSA during experimental colitis. Control animals rapidly lost weight following rectal administration of TNBS (Figure 2A; PBS+TNBS), and did not recover compared to vehicle-treated mice that lost minimal weight (Figure 2A; PBS+EtOH). Remarkably, OMVs given orally to TNBS animals significantly protected from weight loss (Figure 2A; WT-OMV+TNBS). Importantly, when OMVs from *B. fragilis*ΔPSA were administered, weight loss was indistinguishable from TNBS-treated animals (Figure 2A; ΔPSA-OMV+TNBS), demonstrating that PSA is responsible for preventing wasting disease. Reduction in colon length is a hallmark of TNBS colitis (Diaz-Granados et al., 2000). Measurements of intact colons (resected from cecum to rectum) showed normal intestinal lengths in animals treated with PSA-containing vesicles, but not OMVs lacking PSA (Figure 2B). These results show that oral feeding of OMVs corrects weight loss and gross pathology of the colon in a preclinical model of IBD.

IBD in humans, and colitis in animals, features inflammation driven by innate immune cells and CD4+ T cells, resulting in severe intestinal pathology (epithelial hyperplasia, sub-mucosal thickening, infiltration of leukocytes) (Xavier and Podolsky, 2007). Tumor necrosis factor (TNF-α) causes damage to the epithelium and disrupts barrier function, and IL-17 promotes neutrophil migration into the gut mediating tissue damage. In contrast, IL-10 is protective in numerous animal models of colitis (Lindsay et al., 2002), and PSA requires IL-10 expression during protection from disease (Round and Mazmanian, 2010). TNFα and IL-17 levels were elevated in colonic tissues, and CD4+ T cells from the mesenteric lymph nodes (MLNs) and colons of TNBS-treated animals; WT-OMVs suppressed pro-inflammatory cytokines and enhanced IL-10 production compared to ΔPSA-OMVs (Figure S2A-C). Upon histological analysis of colonic tissues, we observed significant disease in TNBS-treated animals that was ameliorated by oral administration of PSA-containing vesicles (Figure 2C). TNBS colitis manifests in focal lesions throughout the colon that mimic the pathology observed in Crohn's disease. Finally, clinical symptoms were evaluated by a blinded pathologist to assess disease (Wirtz et al., 2007). While all TNBS and ΔPSA-OMV treated animals were severely affected, oral administration of WT-OMVs significantly ameliorated disease (Figure 2D). We conclude that PSA packaging into OMVs protects animals from the pathological and immunological manifestations of experimental colitis, and that OMVs from *B. fragilis* represent a novel candidate therapy for IBD.

2.2.3 OMV-associated PSA Induces Foxp3 and IL-10 from CD4+ T cells

The mucosal immune system constitutively surveys the intestinal environment and dendritic cells (DCs) sample intestinal contents to coordinate T cell reactions

(Rescigno et al., 2001). Purified PSA administered orally to animals is associated with CD11c+ DCs in the MLN (Mazmanian et al., 2005). We show that vesicles were taken up by bone marrow-derived DCs (BMDCs) regardless of PSA expression (Figure 3A and Figure S3A). Confocal microscopy revealed that OMVs (labeled with FITC) appeared as punctuate foci throughout the cytoplasm of cells (Figure S3A). DCs rapidly internalized vesicles in an actin-dependent manner, as treatment of cells with cytochalasin D significantly inhibited vesicle uptake (Figure S3B). Expression of DC activation markers (MHCII, CD86) were increased following internalization of OMVs regardless of PSA expression (Figure 3B and Figure S3C), indicating that differences in immune effects (shown below) are not a result of the inability of DCs to be activated by ΔPSA-OMVs. As IL-10 production is known to prevent colitis by suppressing unwanted inflammation (Maynard and Weaver, 2008), we examined if OMVs promote anti-inflammatory immune responses in cell culture. BMDCs were treated with OMVs, washed, and cultured with naïve CD4+ T cells. WT-OMVs induced the expression of IL-10 during *in vitro* DC-T cell co-culture, while minimal IL-10 was detected from DCs alone. Addition of anti-CD3 to the co-culture greatly increased IL-10 production, indicating the main source of IL-10 was from CD4+ T cells (Figure 3C). Vesicles purified from *B. fragilis*ΔPSA induced significantly less IL-10 than WT-OMVs, although an increase was observed over media controls. IL-10 production by DCs is known to promote CD4+IL-10+ T cell development both *in vivo* and *in vitro* (O'Garra et al., 2004). Accordingly, we observed no increase in IL-10 production when wild-type T cells were co-cultured with IL-10-/- DCs following treatment with OMVs (Figure 3D), showing that IL-10 expression by DCs is required to induce IL-10 from CD4+ T cells.

CD4+CD25+ T cells that express the transcription factor Forkhead box P3 (Foxp3) are an important regulatory T cells (Treg) subset shown to protect animals from inflammatory and autoimmune diseases (Izcue et al., 2009). Recent studies have shown that CD4+CD25+Foxp3+ Tregs can express IL-10, and IL-10 production from Tregs is required to prevent intestinal inflammation (Rubtsov et al., 2008). To determine the source of IL-10 induced by OMVs, we purified CD4+CD25+ and CD4+CD25- T cells following co-culture with DCs and measured the expression of Foxp3 and IL-10 by qRT-PCR. Remarkably, PSA containing OMVs significantly induced IL-10 expression in the CD4+CD25+ Treg population, but not from CD4+CD25- T cells (Figure 3E). Vesicles purified from *B. fragilis*ΔPSA were unable to enhance IL-10 production from either T cell population compared to media controls. Expression of Foxp3 was increased exclusively in CD4+CD25+ T cells by OMVs in a PSA-dependent manner (Figure 3F). Furthermore, flow cytometry revealed that WTOMVs stimulated increased IL-10 production from CD4+CD25+ T cells compared to ΔPSA-OMVs, whereas vesicles with or without PSA induced similar baseline levels of IL-10 from CD4+CD25- T cells (Figure 3G).

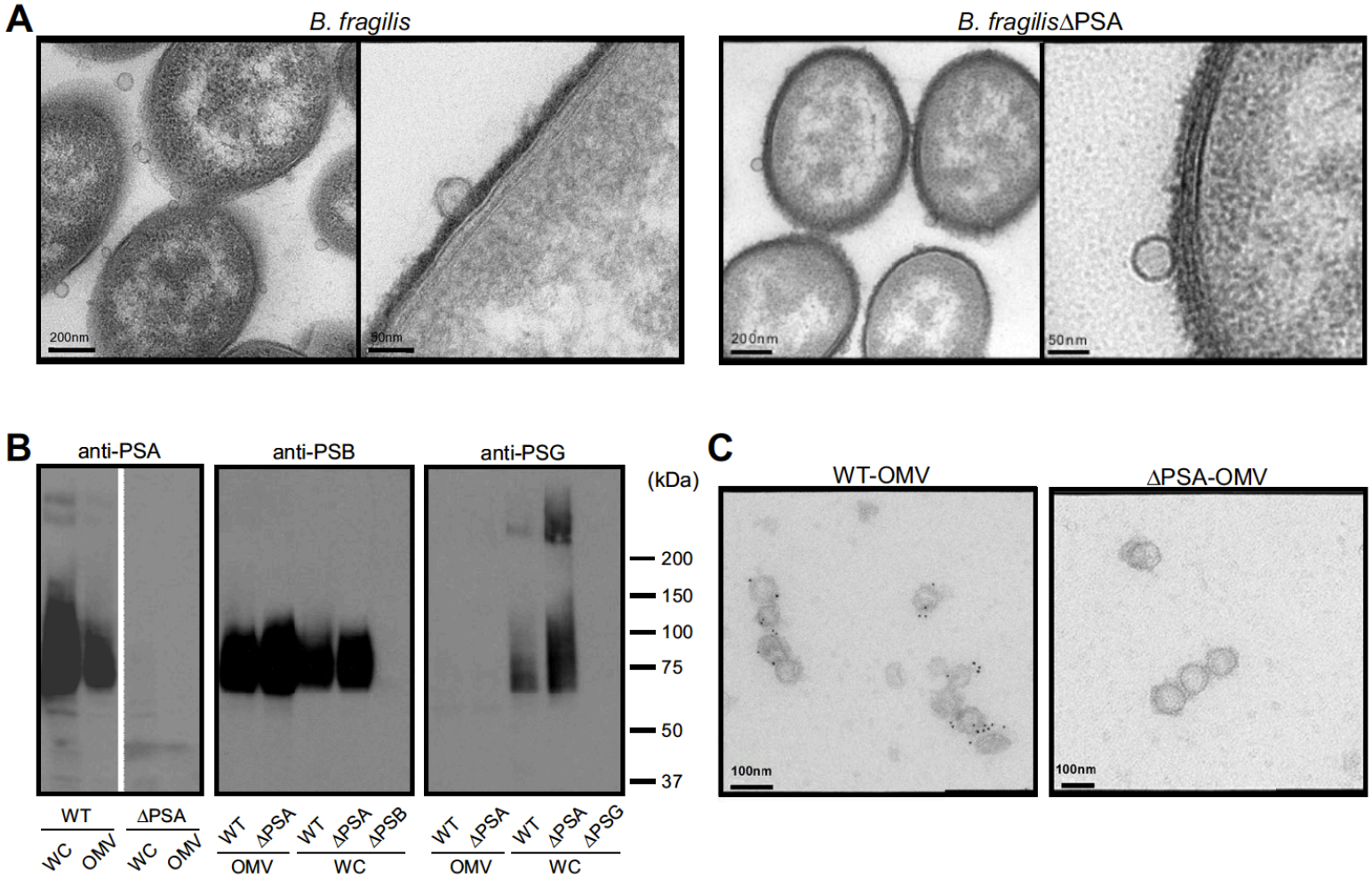
We sought to determine if PSA could enhance the anti-inflammatory capacity of Tregs by measuring their function in cell culture suppression assays. CD4+CD25- responder cells were labeled with the intracellular dye CFSE (Carboxyfluoresceinsuccinimidyl ester), the fluorescence of which decreases proportionally as cells proliferate (Figure 3H; No Tregs). Addition of CD4+CD25+ Tregs suppresses cell division (Figure 3H; Media). DCs were pulsed with OMVs, washed and cultured with naïve CD4+ T cells; subsequently, CD4+CD25+ Tregs were purified after

co-culture and added to CFSE labeled responder CD4+CD25- T cells; proliferation was measured by flow cytometry. Tregs recovered from conditions with WT-OMV-treated DCs displayed significantly enhanced suppressive capacity compared to CD4+CD25+ T cells treated with ΔPSAOMVs (Figure 3H and Figure S3D). Incubation of *in vitro* suppression cultures with a neutralizing anti-IL-10 receptor antibody partially abrogated Treg function induced by WT-OMVs, but had no effect on ΔPSA-OMV stimulated Tregs (Figure S3E). Collectively, we conclude that OMVs specifically induce functional Tregs in cell culture, and that the absence of PSA from OMVs abrogates the ability of *B. fragilis* vesicles to promote Treg activity by dendritic cells.

Our findings show that delivery of PSA by OMVs underlies the unique probiotic properties of *B. fragilis*, revealing a novel mechanism by which the commensal microbiota communicates with the immune system during host-bacterial mutualism.

2.3 Figures and Legends

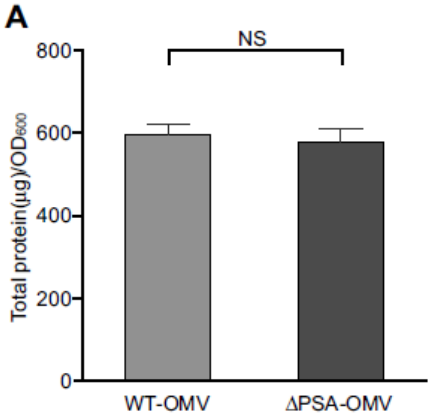
2.3.1 Figure 1. Outer Membrane Vesicles (OMVs) from *Bacteroides fragilis* Contain PSA



(**A**) Transmission electron microscopy of electron dense layer (EDL) enriched *B. fragilis* (WT-OMV) and *B. fragilis*ΔPSA (ΔPSA-OMV) reveals vesicles budding from the bacterial surface. (**B**) Immunoblot analysis of whole cell (WC) and OMV extracts of bacteria show that PSA and PSB are associated with vesicles, while PSG is only found associated with the bacterial cell. Deletion mutants for PSA (ΔPSA), PSB (ΔPSB) and PSG (ΔPSG) confirm specificity of each anti-sera. (**C**) Immunogold labeling of purified OMVs, stained with anti-PSA and anti-IgG-colloidal gold conjugate (5 nm), analyzed by electron microscopy shows specific staining for PSA only in OMVs from wild-type bacteria but not *B. fragilis*ΔPSA. See also Figure S1.

2.3.2 Figure S1, related to Figure 1. Wild-type *B. fragilis* and *B. fragilis*ΔPSA Deletional Mutant Produce Similar Amounts of OMVs and A Similar Proteomic Profile

A

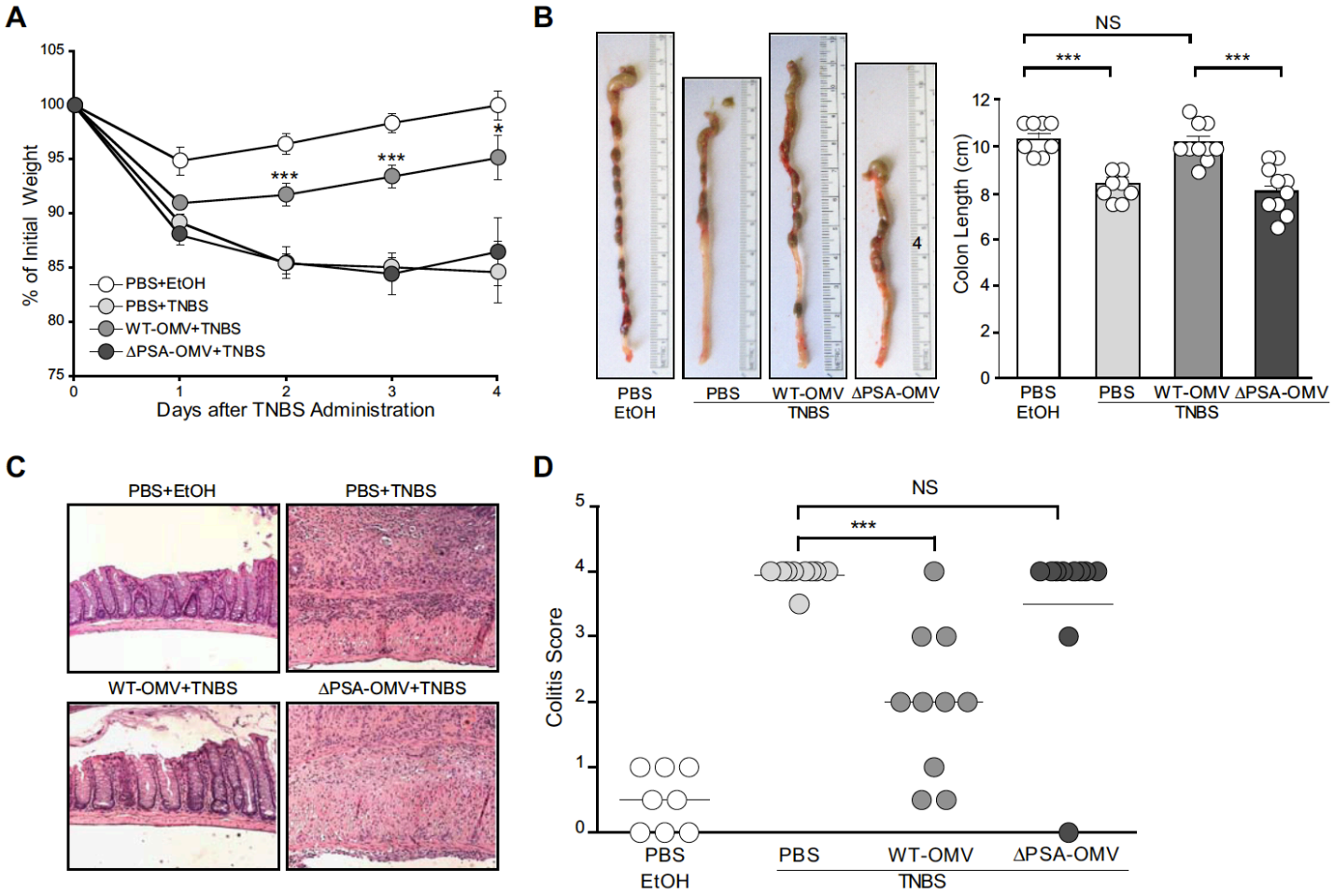


B

No.	Accession No.	Protein Name	WT-OMV	ΔPSA-OMV
BF3567	gi|60683022	hypothetical protein	864±58	452±65
BF2157	gi|60681636	putative lipoprotein	647±45	630±37
BF0595	gi|60680161	hypothetical protein	341±25	296±18
BF2161	gi|60681640	hypothetical protein	213±24	85±5
BF2706	gi|60682179	putative lipoprotein	178±18	128±12
BF1956	gi|60681445	putative outer membrane protein	161±41	129±9
BF0594	gi|60680160	hypothetical protein	142±12	202±8
BF1957	gi|60681446	hypothetical protein	134±10	157±17
BF3067	gi|60682536	putative lipoprotein	124±31	62±6
BF0589	gi|60680155	hypothetical protein	124±17	119±12
BF3432	gi|60682894	hypothetical protein	117±9	96±11
BF2023	gi|60681124	putative ATP/GTP-binding protein	117±13	74±1
BF1619	gi|60681115	hypothetical protein	117±9	147±14
BF3144	gi|60682613	putative lipoprotein	107±22	110±14

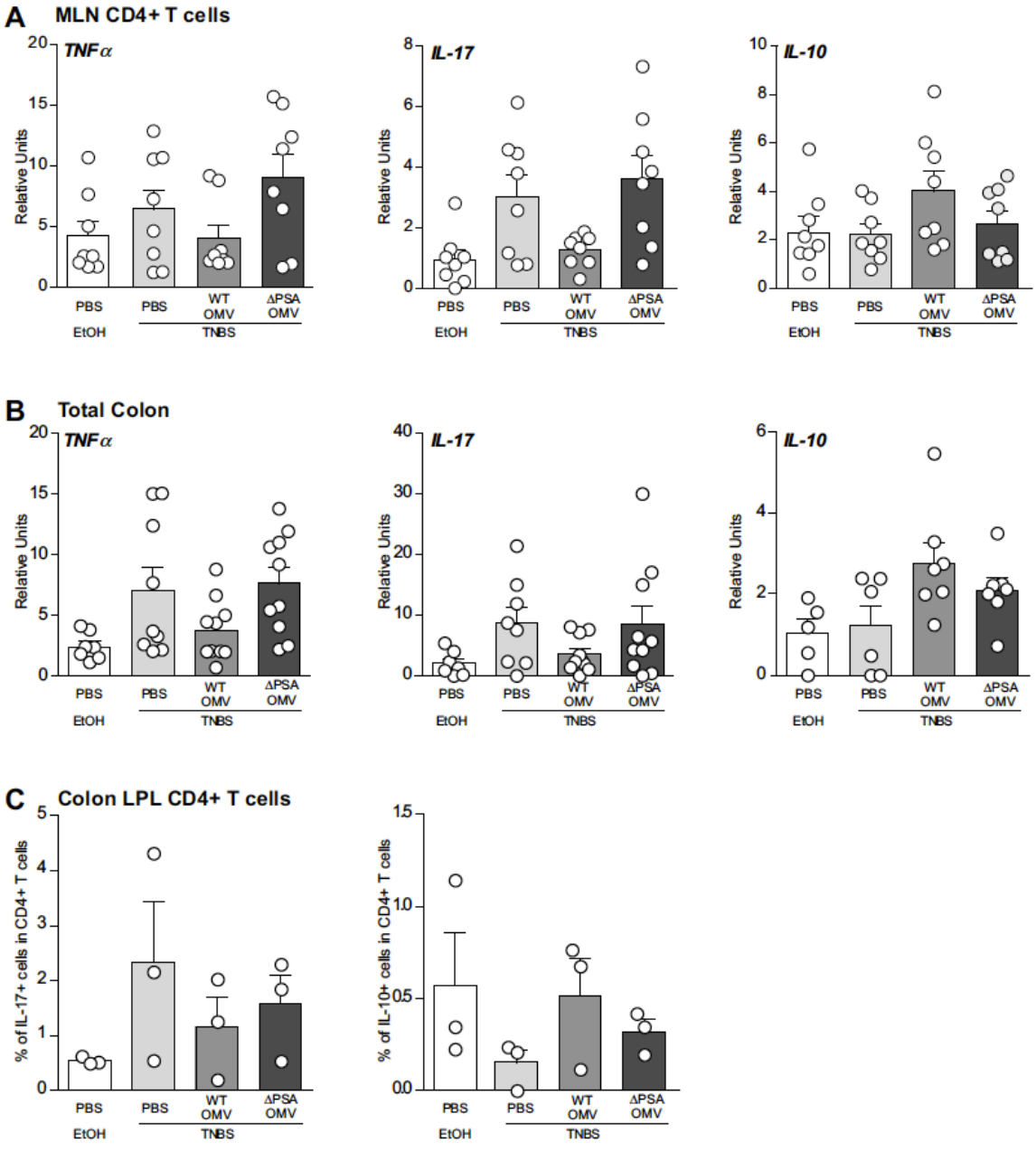
(**A**) Wild-type *B. fragilis* and the PSA deletion mutant (*B. fragilis*ΔPSA) produce similar amounts of OMVs during *in vitro* culture. Total protein recovered from each OMV preparation was normalized by OD600 of the culture at the time of harvest. Error bars indicate SEM. Result is shown from >10 combined experiments performed independently. *p* value determined by Student's t-test. NS: not significant. (**B**) OMVs from wild-type or PSA deletional mutant *B. fragilis* show no significant difference in protein composition. Proteome mass spectrometry shows 100% overlap of the identified proteins (>1 unique peptide identified for each protein) between WT-OMVs and ΔPSA-OMVs. Among all of the identified proteins, we semi-quantitatively compared the amount of those relatively abundant proteins according to the number of unique peptides identified. The majority of them show no difference between WT-OMV and ΔPSA-OMV. Results are shown from 3 combined experiments performed independently.

2.3.3 Figure 2. OMVs Protect Animals from Experimental Colitis and Intestinal Inflammation in a PSA-dependent Manner



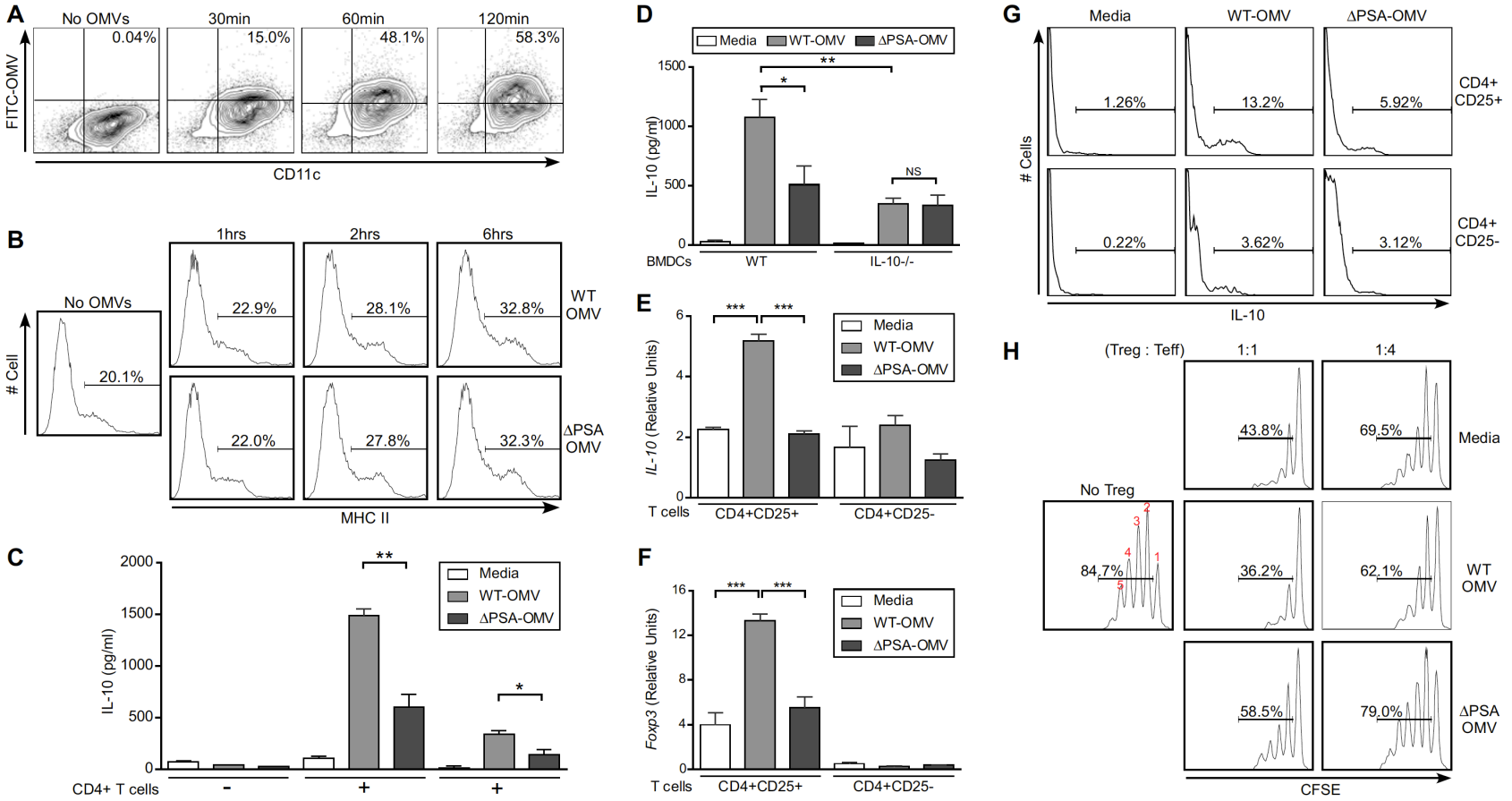
(**A**) Weight loss in animals following the induction of TNBS colitis (day 0) measured as reduction from initial weight until day of sacrifice (day 4). All groups contained at least 4 animals, with error bars indicating standard error (SEM). Results are representative of 3 independent trials. * $p<0.05$; *** $p<0.001$. (**B**) Images of colons immediately following resection and quantification of length indicated in the bar graph) from vehicle treated (EtOH) and TNBS groups (n=4 animals/group). Error bars indicate SEM. Results are shown from 3 combined experiments where each was performed independently. *** $p<0.001$. NS: not significant. (**C**) Images from hematoxylin and eosin (H & E) stained colon sections representative of each treatment group. (**D**) Colitis scores from animals assigned by a blinded pathologist (G.W.L) according to a standard scoring system (see Experimental Procedures). Each symbol represents an individual animal. Results are shown from 3 combined experiments, each performed independently. *** $p<0.001$. NS: not significant. See also Figure S2.

2.3.4 Figure S2, related to Figure 2. PSA-containing OMVs Suppress Pro-inflammatory Cytokines and Induce Anti-inflammatory Cytokine Levels in Colon Tissue during Experimental Colitis



(A) Cytokine transcript analysis by qRT-PCR of RNA recovered from purified CD4+ T cells from mesenteric lymph nodes. Each symbol represents a single animal. Error bars indicate SEM from 4 animals/ group. Results are shown from 3 combined experiments, each performed independently. **(B)** Cytokine transcript analysis by qRT-PCR from RNA recovered from whole colons of each treatment group. Each symbol represents a single animal. Error bars indicate SEM. Results are shown from 3 combined experiments performed independently. **(C)** Cytokine analysis by ICCS on CD4+ T cells from colon LPL preparations of each treatment group. Each symbol represents a single animal. Error bars indicate SEM. Results are representative of 2 independent trials.

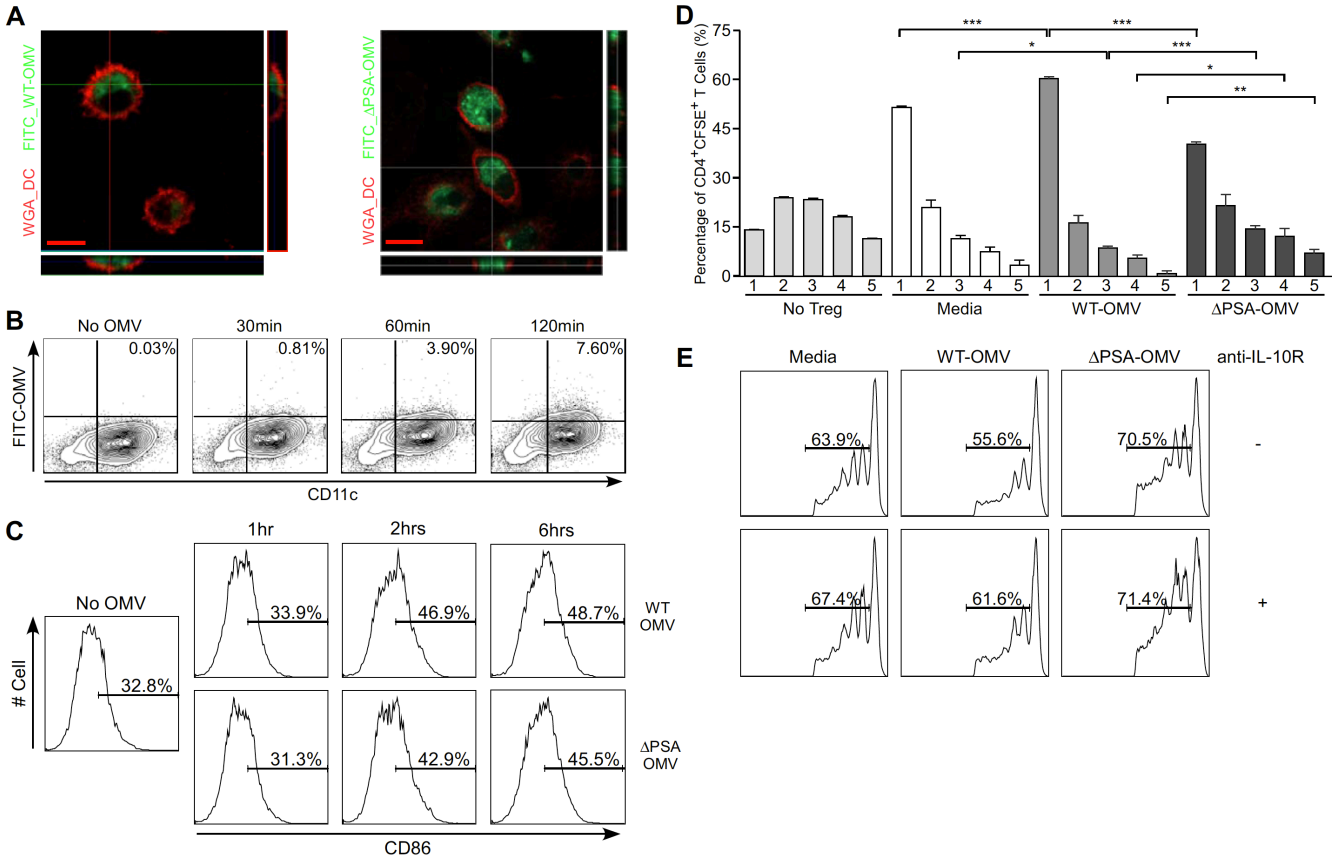
2.3.5 Figure 3. Treatment of DCs with PSA-containing OMVs Induces IL-10 Production and Foxp3 Expression from T Cells



(**A**) Flow cytometry (FC) analysis of OMV internalization by DCs. OMVs were labeled with FITC (Fluorescein isothiocyanate) and incubated with cultured DCs for various times. Cells were stained with anti-CD11c. Percentages show CD11c+OMV+ cell populations. (**B**) FC plots of DCs incubated with WT-OMVs and ΔPSA-OMVs for various times and stained with anti-CD11c and anti- MHCII (Major histocompatibility complex II). Percentages show MHCII+ populations among CD11c+ cells. (**C**) ELISA analysis for IL-10 production from culture supernatants of DCs or DC-T cell co-cultures, where DCs were pulsed with OMVs for 18 hours, washed and incubated with or without splenic CD4+ T cells. Supernatants were collected at day 4 of culture. Media samples indicate DCs that were not pulsed with OMVs, but otherwise treated identically. Anti-CD3 was added to some samples to demonstrate T cell-specific responses. Error bars indicate SEM from quadruplicate samples. Results are representative of over 5 independent trials. * $p<0.05$; ** $p<0.01$. (**D**) ELISA analysis of DC-T cell co-cultures similar to (C), with IL-10-/- DCs. Error bars indicate SEM from quadruplicate samples. Results are representative of 3 independent trials. * $p<0.05$. ** $p<0.01$. NS: not significant. (**E, F**) Transcript levels of IL-10 (E) and Foxp3 (F) of RNA recovered from purified CD4+ T cell subsets following *in vitro* culture with DCs. Co-cultures were set up as in (C); on day 4, CD4+CD25+ and CD4+CD25- T cells were purified by magnetic bead separation (>95% purity) and RNA extracted for qRT-PCR. Relative values were normalized to β-actin. Error bars indicate SEM. Results are shown from 3 combined experiments each performed independently. * $p<0.05$; *** $p<0.001$. NS: not significant. (**G**) FC histograms of IL-10 expression by CD4+ T cell subsets following 4 days of co-culture with DCs treated with OMVs. Splenic CD4+ T cell were purified from IL-10-GFP mice, stained with anti-CD4 and anti-CD25 following co-culture, and IL-10 expression measured by GFP expression.

Percentages show IL-10+ populations amongCD4+CD25+ and CD4+CD25- subsets. Results are representative of 2 independent trials. (**H**) *In vitro* suppression of naïve responder cells by purified CD4+CD25+ T cells following co-culture with DCs treated with media (control), WT-OMVs and ΔPSA-OMVs. CD4+CD25- responder cells (effector cells; Teff) were pulsed with CFSE, incubated with Tregs and stimulated with anti-CD3 and APC (CD4+ T cell depleted splenocytes) for 3 days. Cell proliferation was measured by FC as a function of CFSE dilution. Treg:Teff ratios are indicated, and percentages show total proliferating cells. No Treg: CD4+CD25- cells only. Numbers above peaks represent number of cell divisions. Results are representative of 3 independent trials. See also Figure S3.

2.3.6 Figure S3, related to Figure 3. OMVs are Internalized via Actin Polymerization and Localized in the Cytoplasm of DCs, and Upregulate Co-stimulatory Molecules; Neutralization of IL-10R Signaling Partially Abrogates PSA Activity *in vitro*



(**A**) Fluorescent micrographs of OMV (WT or ΔPSA) internalization by DCs. OMV were labeled with Fluorescein isothiocyanate (FITC, green) and incubated with cultured DCs for 2hrs. Cells were fixed and cell membrane was stained with Wheat Germ Agglutinin (WGA)-tetramethylrhodamine (red). Scale bar: 7.5μm. (**B**) Actin polymerization is required for OMV uptake by DCs. Flow cytometry analysis of OMV internalization by DCs pre-treated with Cytochalasin D. OMVs were labeled with FITC and incubated with cultured DCs for various times (as indicated). Cells were stained with anti-CD11c. Percentages show CD11c+OMV+ populations (compare to Figure 3A). (**C**) WT-OMVs and ΔPSA-OMVs up-regulate the co-stimulatory molecule CD86 (B7.2) for DC activation. FC plots of DCs incubated with WT OMVs and ΔPSA-OMVs for various times (as indicated) and stained with anti-CD11c and anti-CD86. Percentages show CD86+ populations among CD11c+ cells. (**D**) Quantification of percentage of CD4+ T cells in each proliferating peak (as is labeled in Figure 3H). Error bars indicate SEM. Results are representative of 3 independent trials. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (**E**) Neutralization of IL-10R signaling partially abrogates PSA activity. *In vitro* suppression assay was set up as in Figure 3H except that CFSE labeled responder cells (Teff) were incubated with 20μg/ml of anti-IL-10R (+) or isotype control (-) for 1 hour before addition of Tregs purified from DC-T culture under various conditions as indicated. Percentages show total proliferating cells. (Treg:Teff=1:4) Results are representative of 2 independent trials.

Chapter 3

Growth Arrest and DNA-Damage-Inducible Protein (Gadd45α) of Dendritic Cells Mediates the Protective Activity of PSA-containing OMVs

3.1 Summary

Microbial ligands are detected by pattern recognition receptors (Medzhitov, 2007), and PSA is sensed by toll-like receptor 2 (TLR2) (Wang et al., 2006). Unlike other microbial molecules, PSA promotes an anti-inflammatory response upon TLR2 signaling (Round et al., 2011). Innate immune cells (such as DCs) have been widely recognized for their expression of TLRs and their central role in alerting the innate and adaptive immune systems to microbial infection. Our lab recently reported that TLR2 on CD4+ T cells responds to purified PSA to promote Treg function (Round et al., 2011). However, a role for TLR2 expression by innate immune cells during OMV recognition remains unknown. Here, we reveal that TLR2 expression by DCs, and not T cells, is required for recognition of PSA within OMVs. Gene expression profiling further led to identification of the Growth Arrest and DNA-Damage-Inducible protein (Gadd45α) as being required for PSA-mediated TLR2 signaling in DCs. The transfer of OMV-treated DCs to mice induced for colitis protects from disease, demonstrating a critical function for DCs in promoting PSA activity. Gadd45α deletion in DCs abrogates IL-10 induction by PSA, and Gadd45α-/- DCs are unable to provide protection from experimental colitis.

3.2 Results

3.2.1 Toll-like receptor 2 on DCs is Required to Sense OMV-associated PSA

To test the requirement for DC-specific TLR2 in PSA recognition, we treated WT and TLR2-/- DCs with OMVs and subsequently co-cultured DCs with CD4+ T cells. Compared to wild-type DCs, the absence of TLR2 (DCs from TLR2-/- animals) almost completely inhibited IL-10 production in response to vesicles (Figure 4A). Both TLR2-sufficient and deficient DCs responded equally to super-antigen (SEA) stimulation, demonstrating a specific defect in PSA sensing, not a general lack of T cell activation by TLR2-/- DCs. In addition, when TLR2-/- T cells were cultured with OMV-pulsed WT DCs, production of IL-10 was unaffected (Figure S4A). When OMVs were added continuously to DC-T cell co-cultures (thus both cell types were exposed to OMVs), TLR2 deletion on DCs led to decreased IL-10 induction (Figure S4B). Furthermore, direct treatment of purified CD4+ T cells with PSA-containing OMVs resulted in no IL-10 up-regulation, unlike stimulation with purified PSA, which activated T cells in a TLR2-dependent manner as previously shown (Figure S4C) (Round et al., 2011). Therefore, while purified CD4+ T cells can respond directly to PSA, immune responses to OMV delivery of PSA require DC, and not T cell, expression of TLR2. Using this information, we sought to identify cellular factors involved in PSA-specific, TLR2-specific signaling by DCs.

3.2.2 TLR2 Signaling on DCs Induces OMV-specific Gene Expression

OMVs from Gram-negative bacteria have been shown to contain numerous TLR ligands, including lipopolysaccharide (LPS), lipoproteins, peptidoglycan, and bacterial DNA (Beveridge, 1999). These molecular patterns are recognized by DCs to drive

proinflammatory reactions. PSA represents a novel class of microbial molecule that induces immune tolerance. To differentiate between the contributions of PSA and other TLR ligands contained within vesicles, we performed global gene expression profiling, comparing mRNA levels induced by WT-OMVs and ΔPSA-OMVs in a TLR2- specific fashion. Using microarray analysis, 4,712 target probes representing mouse transcripts showed expression changes following treatment of DCs with OMVs (Figure 4B). The vast majority of these genes were classified as immune-related by the GO Biological Process enrichment and the KEGG Pathway enrichment algorithms (Figure 4C). Approximately two-thirds of the genes were either up- or down-regulated similarly by both WT-OMVs and ΔPSA-OMVs, representing PSA-independent transcript changes within DCs (Figure 4D and Figure 4E; blue shaded regions). Of the remaining one-third (roughly 1,200), which corresponds to PSA-dependent expression changes, the vast majority (98%) of genes were regulated in a TLR2-dependent fashion (Figure 4E). We currently do not know of other signaling molecules that sense PSA, and further studies are needed to validate the few transcripts that appear to be regulated independently of TLR2. However, a list of the top 12 genes that displayed greatest PSA-specific, TLR2-specific changes again yielded several molecules known to have immune functions (Figure 4F). Of these, transcriptional analysis by qRT-PCR verified the microarray results for 6 genes, which showed a pattern of being up-regulated by WT-OMVs and dependent on TLR2 expression (Figure 4G). Thus, by virtue of delivering PSA to DCs, OMVs signal through TLR2 to induce a highly specific gene expression profile.

3.2.3 DCs Require Gadd45α for Induction of IL-10

Downstream of TLR2, factors involved in the intracellular signaling pathway(s)

that respond to PSA remain completely unknown. Based on its reported role in the immune system, we assessed the function of Gadd45α (Growth Arrest and DNA-Damage- Inducible protein), whose expression by DCs has been shown to promote T cell responses (Jirmanova et al., 2007). Gadd45α was induced by OMVs in a PSA specific, TLR2- specific manner (see Figure 4G). Co-culture of Gadd45α-/- DCs with WT CD4+ T cells resulted in reduced IL-10 production compared to WT DCs treated with WT-OMVs (Figure 5A). The level of IL-10 expression upon co-culture of Gadd45α-/- DCs and CD4+ T cells was identical following treatment with either WTOMVs or ΔPSA-OMVs. IL-10 production from BMDCs alone treated with OMV was not detectable by ELISA (see Figure 3C); thus, using qRT-PCR analysis, we found that IL- 10 expression by BMDCs was increased by WT-OMVs, whereas Gadd45α-/- DCs displayed reduced IL-10 expression when treated with WT-OMVs or ΔPSA-OMVs (Figure 5B). Gene expression profiling had identified several other genes that were coregulated with Gadd45α (see Figure 4F and 4G). Of these, BpiI2 and Chac1 are downstream of Gadd45α signaling as their expression is lost in Gadd45α-/- DCs following treatment with OMVs (Figure S5A). Gadd45α thus appears to be a central signaling component of the TLR2 response to OMVs by DCs.

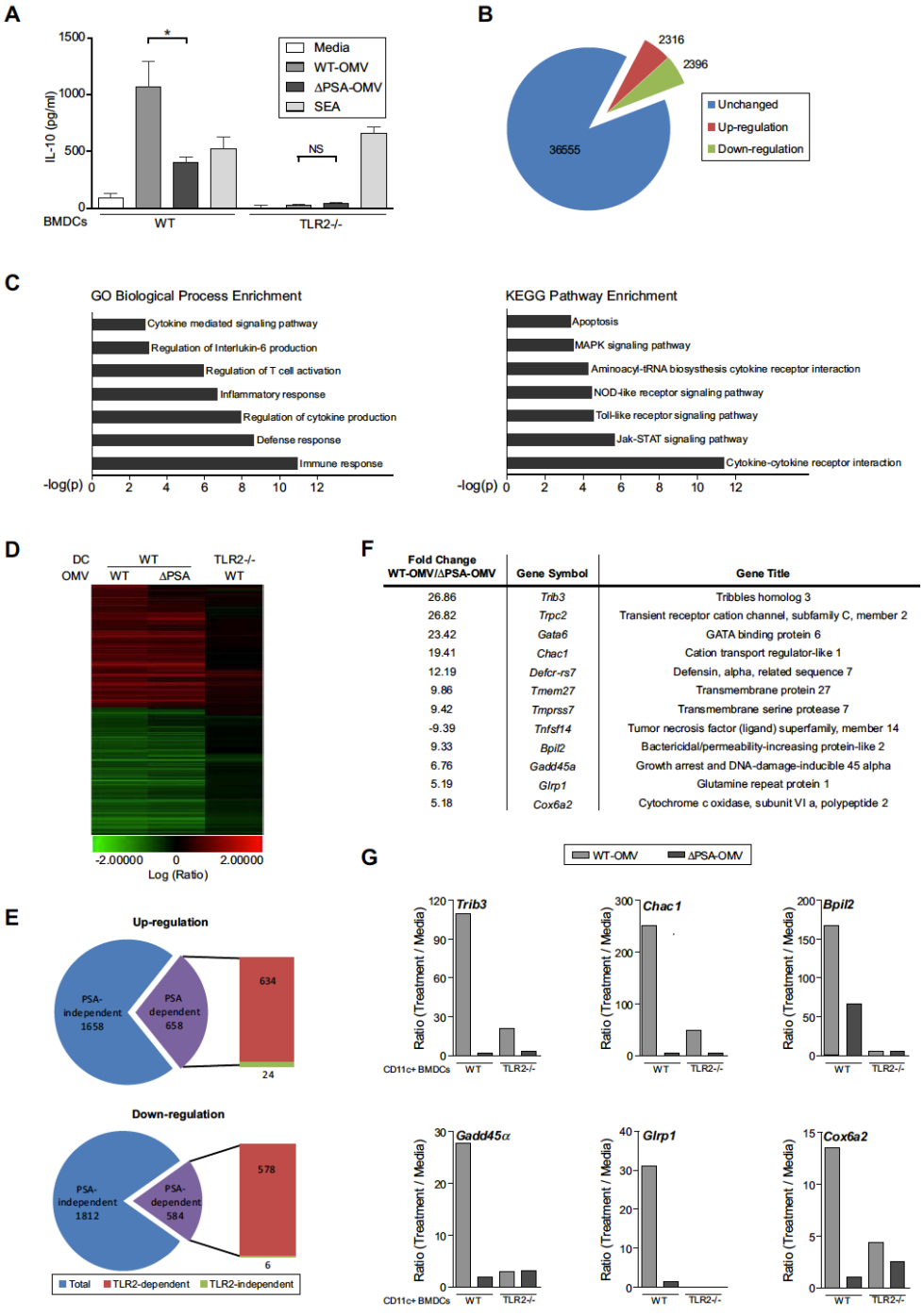
3.2.4 DCs Mediate Protection from Colitis Through a Requirement for Gadd45α

Colonization of animals with *B. fragilis* or treatment with PSA ameliorates experimental colitis (Mazmanian et al., 2008b; Round and Mazmanian, 2010). However, the role of DCs during PSA-mediated protection from disease remains unknown. To demonstrate whether DCs require Gadd45α for *in vivo* PSA activity, we incubated

BMDCs with vesicles and transferred cells into mice that were induced for TNBS colitis. WT-OMV treated DCs protected animals from disease compared to control (PBS) treated cells (Figure 5C). However, treatment of Gadd45α-/- DCs with OMVs prior to transfer into wild-type mice resulted in disease scores similar to untreated cells (last column of Figure 5C), demonstrating that Gadd45α is required by DCs for PSA-mediated protection. Representative histopathology is shown in Figure S5B. Accordingly, reduction in colon length is rescued by OMV treatment of WT, but not Gadd45α-deficient, DCs (Figure 5D). Finally, we analyzed pro-inflammatory molecules within intestinal tissues of TNBS colitis-induced animals following DC transfer. The cytokines IFNγ, IL-6 and TNFα were reduced in the colon following transfer of WT DCs incubated with OMVs (Figure 5E). Transfer of OMV-treated Gadd45α-/- DCs into animals resulted in cytokine and chemokine levels as high as untreated DCs (last column of Figure 5E). Collectively, these findings show that OMVs promote tolerogenic DC function during protection from colitis and further identify Gadd45α is a factor required for PSA activity, revealing a novel signaling pathway by which the human mutualist *B. fragilis* mediates immunologic health.

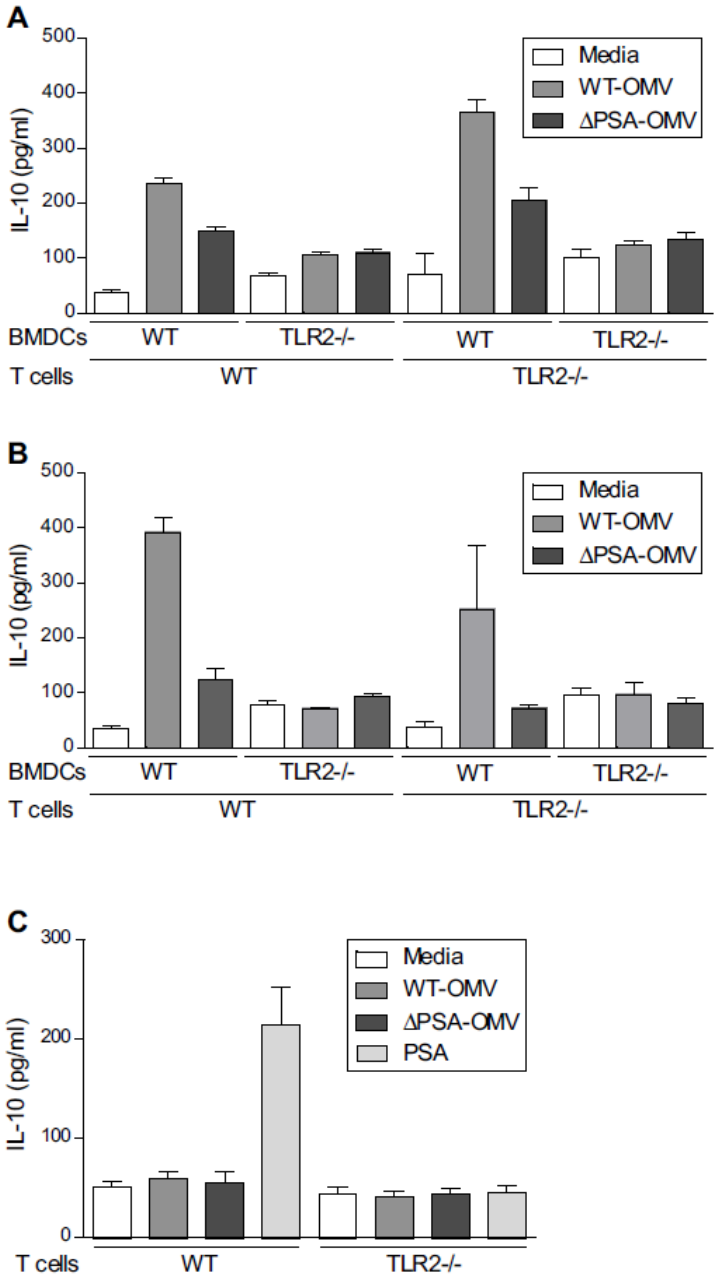
3.3 Figures and Legends

3.3.1 Figure 4. TLR2 on DCs is Required to Sense OMV-associated PSA and Induces Genes Specific for OMVs.



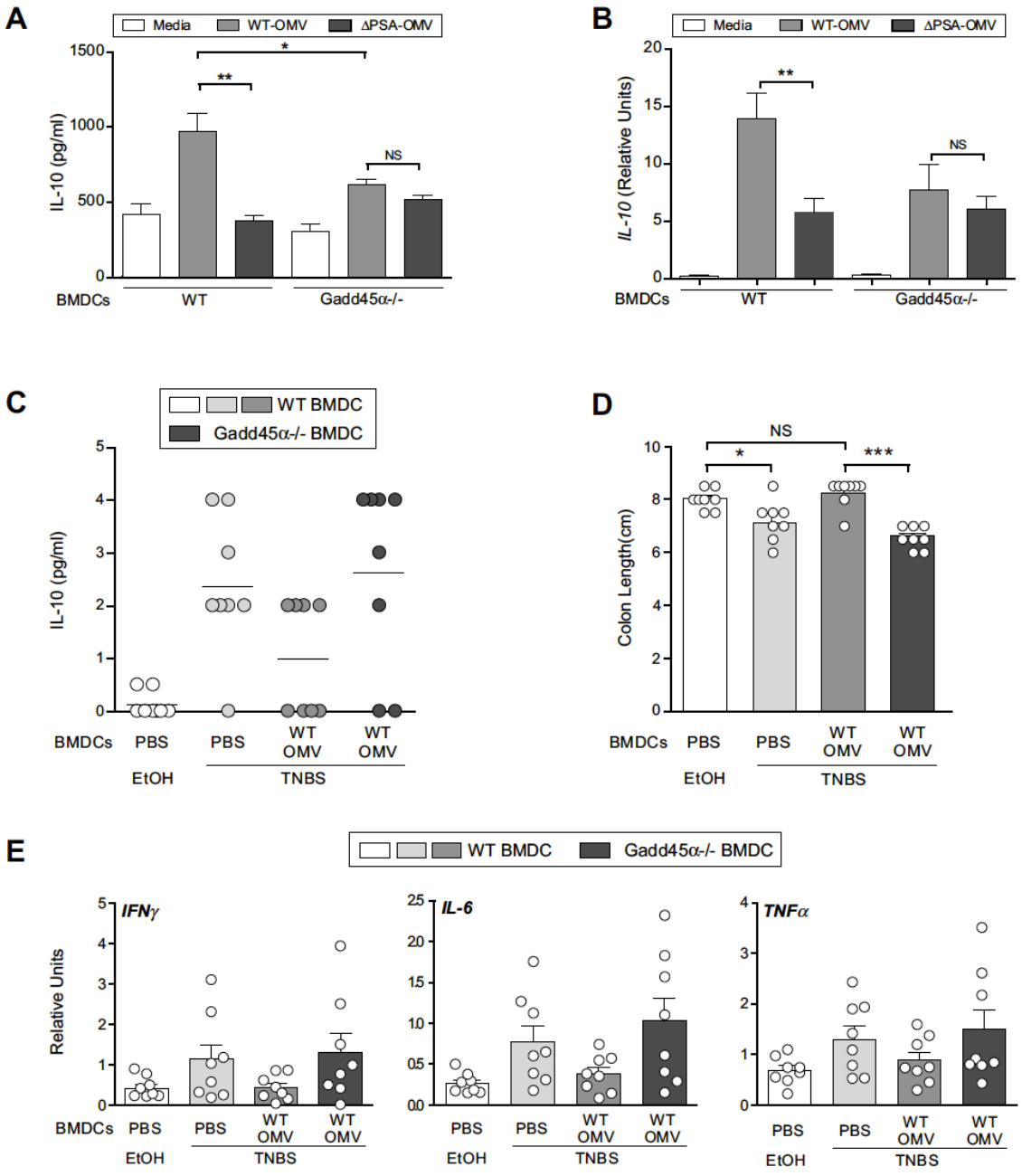
(**A**) ELISA analysis of DC-T cell co-cultures similar to Figure 3(C) from WT and TLR2-/- DCs. SEA: staphylococcal enterotoxin A. Error bars indicate SEM from quadruplicate samples. Results are representative of 3 independent trials. * $p<0.05$. NS: not significant. (**B**) Pie chart showing the number of up-regulated genes (red) and down-regulated genes (green) upon stimulation with PSA-containing OMVs during *in vitro* cultured with CD11c+ BMDCs, using a Whole Mouse Genome Microarray analysis. Unaffected genes (blue). Only genes with a p value of <0.01 and fold change of >2 were used for subsequent analysis. (**C**) Gene ontology analysis of changes in gene expression levels in BMDCs for various biological processes or pathways. (**D**) Heat-map analysis of gene expression in BMDCs from either wild-type or TLR2-/- animals upon OMV stimulation reveals that approximately 30% of the genes are either up-regulated or down-regulated in a PSA dependent manner and that the majority of the genes are TLR2 dependent. (**E**) Pie charts show that the majority of the genes in BMDCs that respond to PSA are dependent on TLR2. (**F**) List of select genes of highest fold change of expression level upon OMV stimulation in a PSA- and TLR2- dependent manner. (**G**) qRT-PCR analysis of the expression level of genes listed in (F) identified and confirmed 6 genes that are significantly up-regulated by OMVs in a PSA- and TLR2-dependent manner from purified CVD11c+ BMDCs. Results are representative of 3 independent experiments. See also Figure S4.

3.3.2 Figure S4, related to Figure 4. Purified PSA Elicits IL-10 Production from T cells in a TLR2-dependent Manner, While PSA-containing OMVs Do Not Induce IL-10 Directly from T Cell



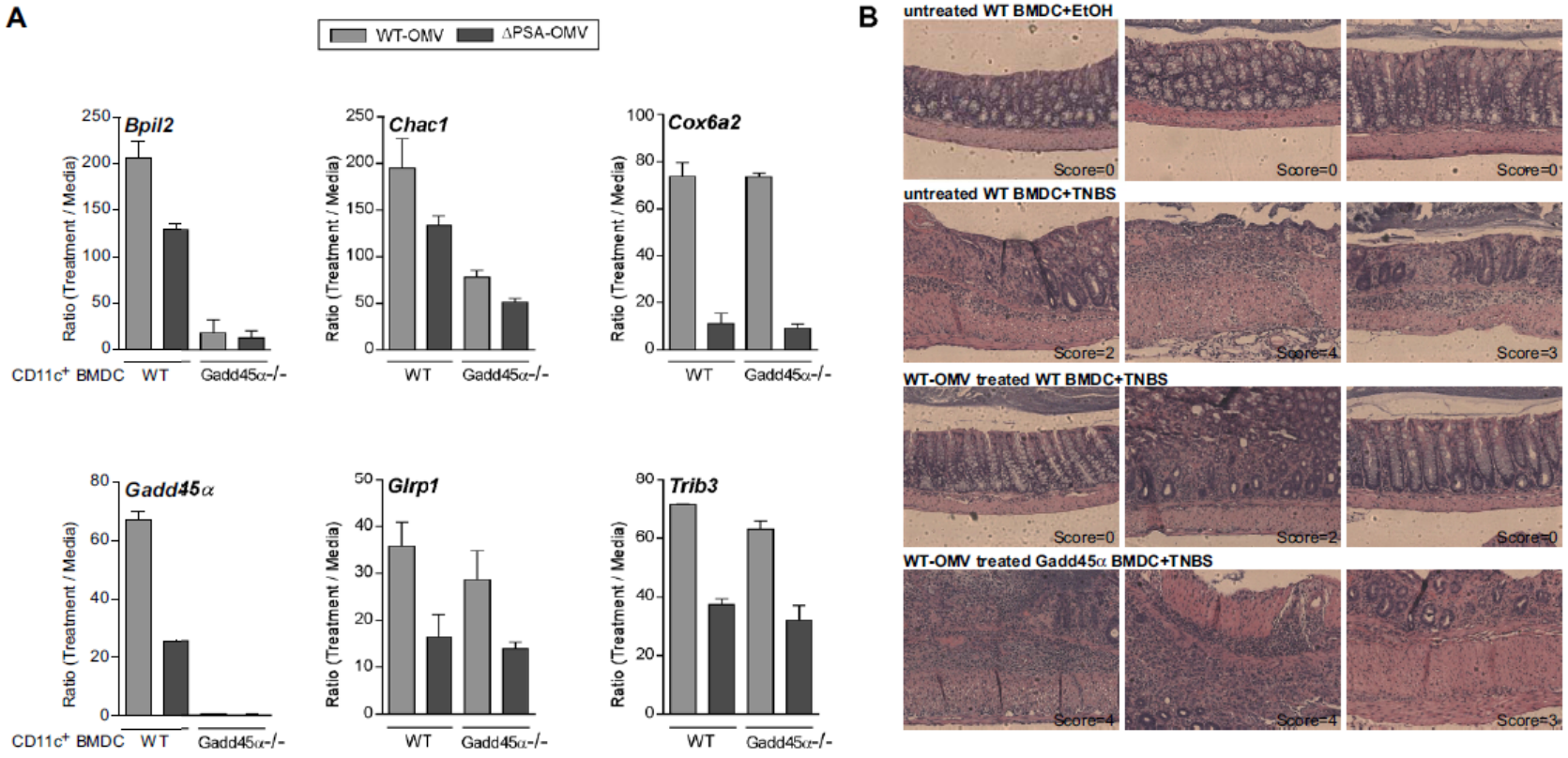
(A) ELISA analysis for IL-10 production from culture supernatants of DC-T cell co-cultures, where DCs were pulsed with OMVs for 18 hours, washed and incubated with primary CD4+ T cells. Supernatants were collected at day 4 of culture. Media samples indicate DCs that were not pulsed with OMVs, but otherwise treated identically. Error bars indicate SEM from quadruplicate samples. Results are representative of 2 independent trials. **(B)** ELISA analysis for IL-10 production from culture supernatants of DC-T cell co-cultures exposed to OMV or media control. Supernatants were collected at day 4 of culture. Error bars indicate SEM from quadruplicate samples. Results are representative of 2 independent trials. **(C)** ELISA analysis for IL-10 of culture supernatants from T cell cultures exposed to OMVs or purified PSA. Supernatants were collected at day 4 of culture. Media samples indicate T cells that were not stimulated, but otherwise treated identically. Anti-CD3 was coated on the culture plate to activate T cells. Error bars indicate SEM from quadruplicate samples. Results are representative of 2 independent trials.

3.3.3. Figure 5 Gadd45α on DCs is Required for *in vitro* IL-10 Production and Protection from Colitis by OMVs.



(**A**) ELISA analysis of DC-T co-cultures similar to Figure 3(C), but also including Gadd45α-/- DCs. Error bars indicate SEM from quadruplicate samples. Results are representative of 3 independent trials. *p<0.05. ** p<0.01. NS: not significant. (**B**) qRT-PCR analysis of IL-10 transcript levels from either WT or Gadd45α-/- BMDCs after 16-18hrs incubation with OMVs *in vitro*. Error bars indicate SEM from triplicate samples. Results are representative of 3 independent trials. ** p<0.01. NS: not significant. (**C**) Colitis scores from DC recipient animals induced for TNBS colitis. Each symbol represents an individual animal. Results are shown from 2 combined experiments, each performed independently. (**D**) Colon length from mice, at day 2 following TNBS treatment. Each symbol represents an individual animal. Error bars indicate SEM from 8 animals. Results are shown from 2 combined experiments each performed independently. * p<0.05. *** p<0.001. NS: not significant. (**E**) Cytokine analysis by qRT-PCR of RNA recovered from whole colon. Each symbol represents an individual animal. Error bars indicate SEM from 8 animals. Results are shown from 2 combined experiments, each performed independently. See also Figure S5.

3.3.4 Figure S5, related to Figure 5. Gadd45α is Required in BMDCs to Mediate PSA Activity during Protection from Intestinal Inflammation



(A) Transcript analysis by qRT-PCR in FACS sorted wild-type and Gadd45a-/- CD11c+ BMDCs of selected genes that were induced by PSA-containing OMVs in a TLR2 dependent manner from microarray studies. Error bars indicate SEM. Results are representative of 2 independent trials. **(B)** Images from hematoxylin and eosin (H & E) stained colon sections representative of each treatment group. Colitis scores from animals were assigned by a blinded pathologist (G.W.L) according to a standard scoring system (see Experimental Procedures).

Chapter 4

Systemic Treatment with PSA Protects Animals from Experimental Sepsis via Marginal Zone B cells

4.1 Summary

As shown in the previous chapters, *B. fragilis* and its symbiosis molecule PSA exerts robust immunomodulatory effects in the gut during both steady state colonization and intestinal inflammation (Mazmanian et al., 2008b; Round et al., 2011), We have also shown that PSA and PSA-containing OMVs (chapter 2-3) are very effective in protecting against intestinal inflammation and are representating potential therapies for IBDs (Mazmanian et al., 2008b; Round and Mazmanian, 2010). Nevertheless, we are always interested in testing whether *B.fragilis* or the microbiota have systemic influence on the host immune system, and if so, whether PSA can also be used to treat systemic inflammatory diseases that are beyond the gut, for instance, sepsis.

In this chapter, I will discuss our recent findings that uncover the systemic immunomodulatory effect of PSA. We found that systemically administrated PSA could protect the animals from LPS-induced endotoxic shock. To our surprise, we found that T cells, which had been found critical for the effect of PSA in protecting against experimental colitis (chapter 2), were not required for the protective activity of PSA against experimental sepsis. Instead, B cells, in particular, Marginal Zone B cells (MZ B cells), and the secretion of IgM, seemed to play an important role in mediating this protection. Furthermore, transcriptome analysis of MZ B cells isolated from both diseased animals and protected animals identified a list of genes that might be important players that mediated the protective effect of PSA against endotoxic shock.

Studies are still under way to reveal the molecular mechanism underlying this new protective effect of PSA. The findings will not only potentiate our understanding of

the immunomodulatory effect of PSA, but also provide new insight into the interaction between commensal bacteria and the immune system of their mammalian host.

4.2 Results

4.2.1 Systemic Treatment of PSA Protects Animals from Experimental Sepsis

As previsouly shown (Chapter 2-3), oral administration of PSA or PSA containing OMVs protects animals from experimental colitis by promoting regulatory responses in the host (Mazmanian et al., 2008b). Therefore we first asked whether pre-treatment with PSA could lead to protection against experimental sepsis. Here we used LPS-induced endotoxic shock model (Chapter 1). LPS induces rapid IL-6 and TNFα responses and mice could die from endotoxic shock within a few days (Buras et al., 2005). Interestingly, while oral treatment of PSA did not reduce the level of serum IL-6 or TNF-α, systemic administration (retro-orbital) of PSA before LPS induction led to a significant decrease of these proinflammatory cytokines (Figure 6A and 6B). Along with the reduction of serum IL-6 and TNF-α, systemic PSA treatment also resulted in a much higher survival rate (Figure 6C). Therefore, PSA, when administered systemically, is able to protect animals from experimental sepsis and greatly lower proinflammatory cytokines in the serum. More interestingly, we found mice treated with PSA simultaneously with LPS injection were still protected from endotoxic shock, as serum TNF-α and IL-6 were largely reduced (Figure S6A) and survival rate was much higher (Figure S6B). This result suggests the potential of PSA as a therapy for sepsis. PSA given one hour after LPS injection, however, appeared to be less effective (Figure S6A and S6B).

4.2.2 IL-10 or TLR2 is not Required for the Protective Activity of PSA against Experimental Sepsis

Previous studies have shown that both TLR2 and IL-10 are required for the protective activity of PSA against experimental colitis (Mazmanian et al., 2008b; Round

and Mazmanian, 2010). So we sought to test whether the same protective mechanism of PSA was shared against experimental sepsis. To our surprise, neither IL-10 nor TLR2 were required for the protection against experimental sepsis by PSA. Serum TNF-α level was still significantly lowered by PSA treatment in both IL-10-/- mice and TLR2-/- mice (Figure 7A and 7C). And upon LPS induction, both strains of mice treated with PSA survived endotoxic shock, much better than vehicle treated control mice (Figure 7B and 7D). This result suggested that the protective mechanisms of PSA against experimental colitis and sepsis may be largely divergent.

4.2.3 Marginal B Cells Mediate the Protection against Experimental Sepsis by PSA

We next asked whether lymphocytes were required for the protective effect of PSA against experimental sepsis (Mazmanian et al., 2008b; Round and Mazmanian, 2010). We found that in Rag-/- mice that had no T or B cells, PSA was no longer sufficient to reduce serum TNF-α level (Figure 8A) induced by LPS. Furthermore, the mortality of Rag-/- mice after LPS administration was not rescued by PSA (Figure 8B). Next, we asked whether T and/or B cells were sufficient for the protective activity of PSA against experimental sepsis. To do so, we transferred either T or B cells into Rag-/- recipient mice, and tested the effect of PSA to protect these mice from LPS-induced endotoxic shock. Interestingly, total T cells (CD3+ splenocytes) or CD4+ T cells (CD4+ splenocytes) reconstituted Rag-/- mice were as not responsive to the protective effect of PSA, similar to the vehicle treated animals (Figure 8C and 8D). In contrast, PSA could protect Rag-/- mice from experimental sepsis when they were reconstituted with CD4+ T cell depleted splenocytes (Figure 8C and 8D). Taken together, these data suggest that a

non-T cell population in the spleen could mediate the protective effect of PSA. Notably, in our experimental colitis model, PSA acts on CD4+ T cells to promote regulatory responses and to suppress the excessive inflammation during disease (Round and Mazmanian, 2010). Therefore, the notion that PSA may also influence a cell type other than T cells is very intriguing.

Since B cells are another major cell type in the spleen and represent more than half of the splenocytes, we sought to test whether B cells were sufficient to mediate the protective activity of PSA. To test this idea, we reconstituted Rag-/- mice with splenic B cells and asked whether these mice could be protected from endotoxic shock by PSA. Indeed, we found that PSA significantly reduced serum TNF-a levels in these mice upon LPS injection (Figure 8E). These mice were also less susceptible to endotoxic shock, evident by the increased survival rate compared to that of the vehicle treated ones (Figure 8F).

B cells can be categorized into several subsets based on their developmental routes and immune functions. By using this cell transfer method, we further tested which specific subset of B cells could mediate the protection against endotoxic shock. Marginal zone B cells (MZ B cells) attracted our attention because it was a subset of B cells known to participate in the first line of defense against systemic antigens (Pillai et al., 2005). Notably, all of the tested Rag-/- mice that were reconstituted with MZ B cells were protected against LPS-induced endotoxic shock by PSA (Figure 8G). It is also believed that MZ B cells can produce natural antibodies that are specific against bacterial cell wall components, such as polysaccharides (Pillai et al., 2005). Moreover, soluble IgM has been shown to be protective in experimental sepsis (Reid et al., 1997). Therefore, we

utilized knockout mice (sIgM-/-) in which B cells were capable of expressing but not secreting IgM (Boes et al., 1998) to test whether IgM secretion was required for MZ B cell to mediate the protective effect of PSA. Elimination of IgM secretion from B cells completely abolished the protective effect of PSA to reduce serum TNF-α or IL-6 after LPS injection (Figure 8H). All these data suggest that a specific B cell population, MZ B cells, and the secretion of natural antibody IgM are required for PSA to protect mice against experimental sepsis.

Moreover, reconstitution of Rag-/- recipient mice with MZ B cells harvested from mice that were treated with PSA systemically could protect these mice from experimental sepsis (Figure 8I and 8J). The fact that MZ B cells from PSA pre-treated animals could transfer protection to untreated recipients indicated that PSA directly or indirectly influenced the gene expression profile of MZ B cells and such molecular changes in the MZ B cells could be maintained and used to inhibit systemic inflammation.

4.2.4 Preliminary Data Exploring the Possible Molecular Mechanisms underlying the Protective Effect of PSA

In order to study the molecular mechanism underlying the protective effect of PSA mediated by MZ B cells, we examined the whole genome gene expression profiles of MZ B cells upon PSA treatment. We first treated animals with either PBS or PSA twice before induction of endotoxic shock by LPS. One hour after LPS injection, we sacrificed the mice, isolated MZ B cells from the spleens by fluorescent activated cell sorting (FACS), and purified mRNA from these cells. mRNAs of sorted MZ B cells from different treatment groups were then sequenced by RNAseq. (1: non-diseased group- PBS (i.v.)+PBS (i.p.); 2: diseased group- PBS (i.v.)+LPS (i.p.); 3: protected

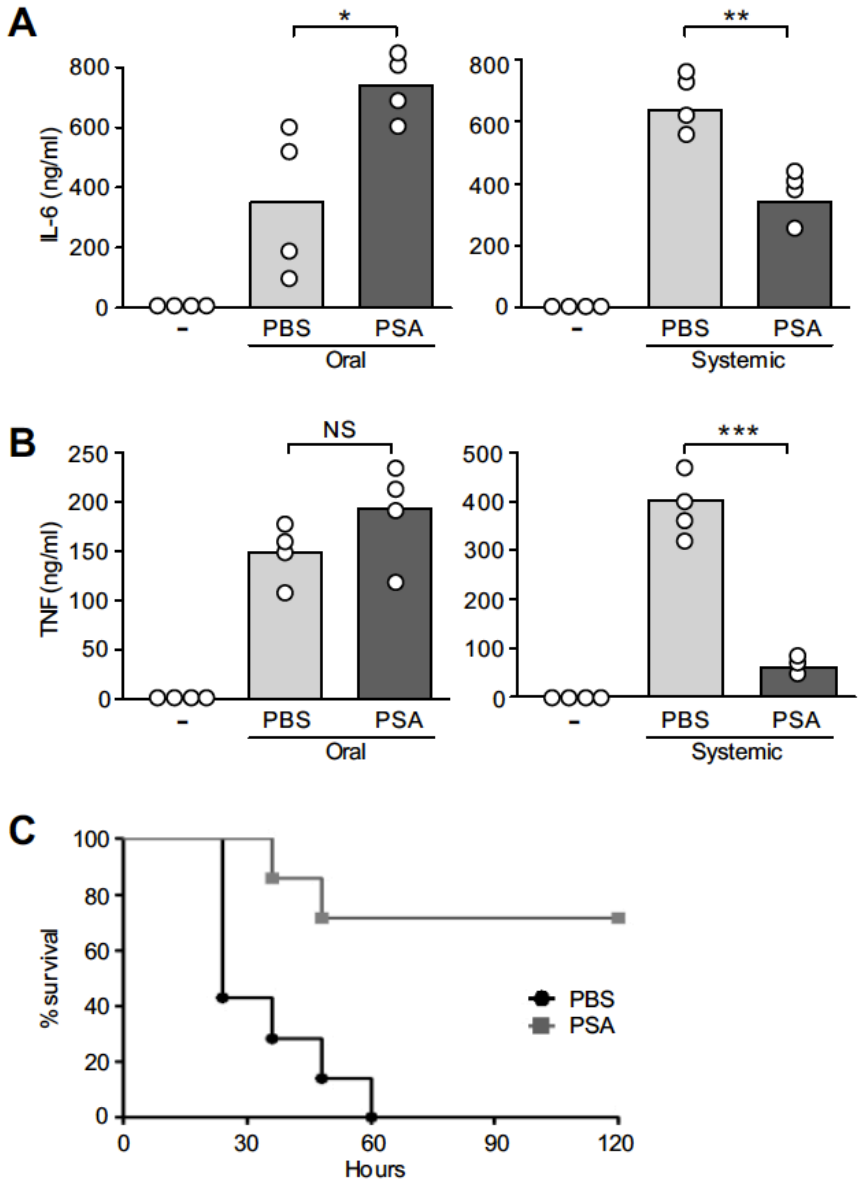
group- PSA (i.v.)+LPS (i.p.)) RNAseq analysis identified 1,740 genes across the whole mouse genome that showed significant difference in expression level between any of the two treatments. These genes were then categorized into 8 distinct expression patterns among all three treatment groups by K-means clustering analysis (Figure S7). Among the 8 clusters, cluster 1 represents the genes that were specifically up-regulated by PSA in protected animals but not in non-diseased or diseased animals (a full list of genes in this cluster is shown in Table S1). Induction of selected genes from this cluster by PSA treatment was confirmed by RT-qPCR (Figure 9A) performed on a new set of samples prepared independently.

We also measured IgM level in the serum samples collected from recipient Rag-/- mice that were reconstituted with either PBS or PSA treated MZ B cells. Interestingly, we observed high levels of IgM from the mice reconstituted with PSA treated MZ B cells but not those reconstituted with PBS treated MZ B cells (Figure 9B). This result is consistent with the idea that IgM secretion is essential for MZ B cells to mediate the protective effect of PSA against sepsis.

Finally, we used CLP model, which is believed to better mimic human sepsis than LPS-induced endotoxic shock, to further examine the protective effect of PSA. We found that PSA could also protect mice against CLP (Figure 9C) as mice treated with PSA had a higher survival rate than the vehicle controls. Further experiments will be done to test if MZ B cells are also involved in this protection. We hope to use CLP model of sepsis to verify our findings from the LPS model that PSA protects experimental sepsis via MZ B cells and has the potential to become a novel therapy for human sepsis.

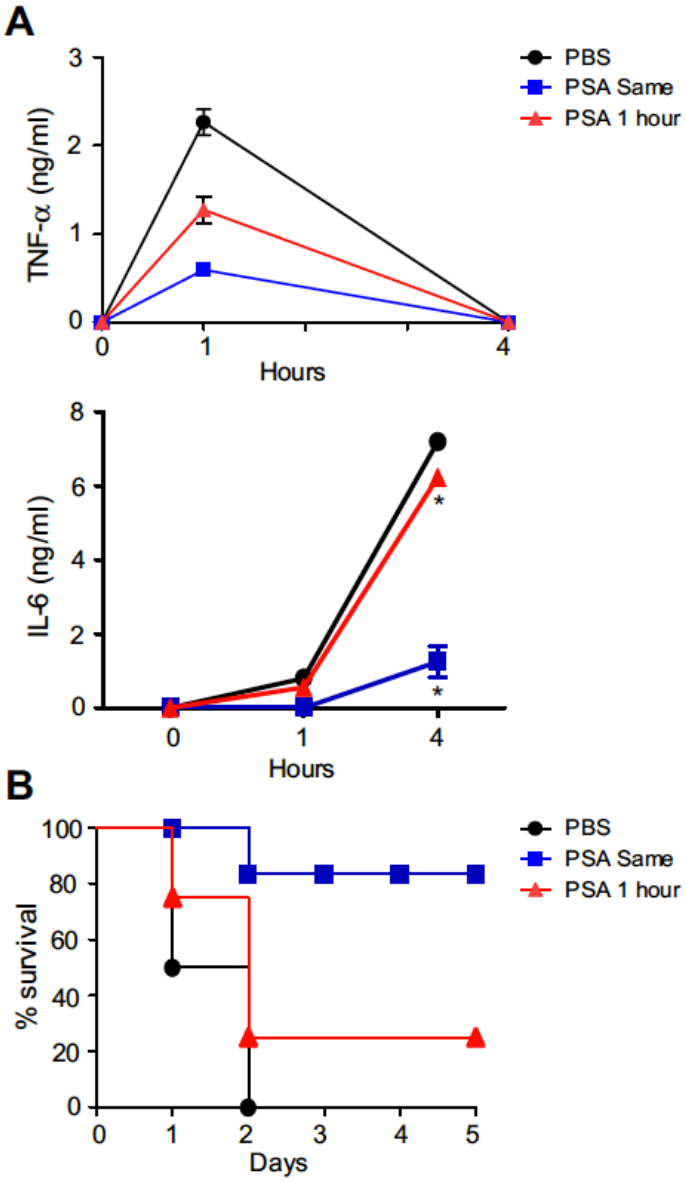
4.3 Figures and Legends

4.3.1 Figure 6. PSA can Protect Animal from Endotoxic Shock and Lowers Inflammatory Cytokines



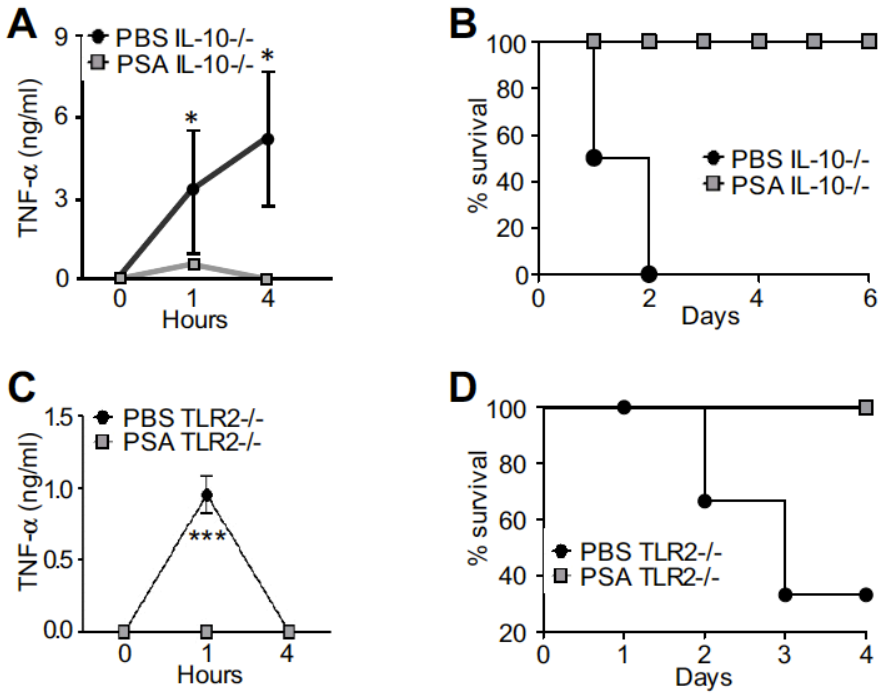
(A) Serum IL-6 level (4 hours after LPS induced endotoxic shock) measured by ELISA from animals that were either orally or intravenously pre-treated with PBS or PSA. * $p<0.05$; ** $p<0.01$. **(B)** Serum TNF-α level (1 hours after LPS induced endotoxic shock) measured by ELISA from animals that were either orally or intravenously pre-treated with PBS or PSA. *** $p<0.001$; NS: not significant. **(C)** Survival curves from animals that were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. Both groups contained at least 4 animals.

4.3.2 Figure S6, related to Figure 6. PSA can Still Lower Inflammation and Protect from Death Even When Given after LPS



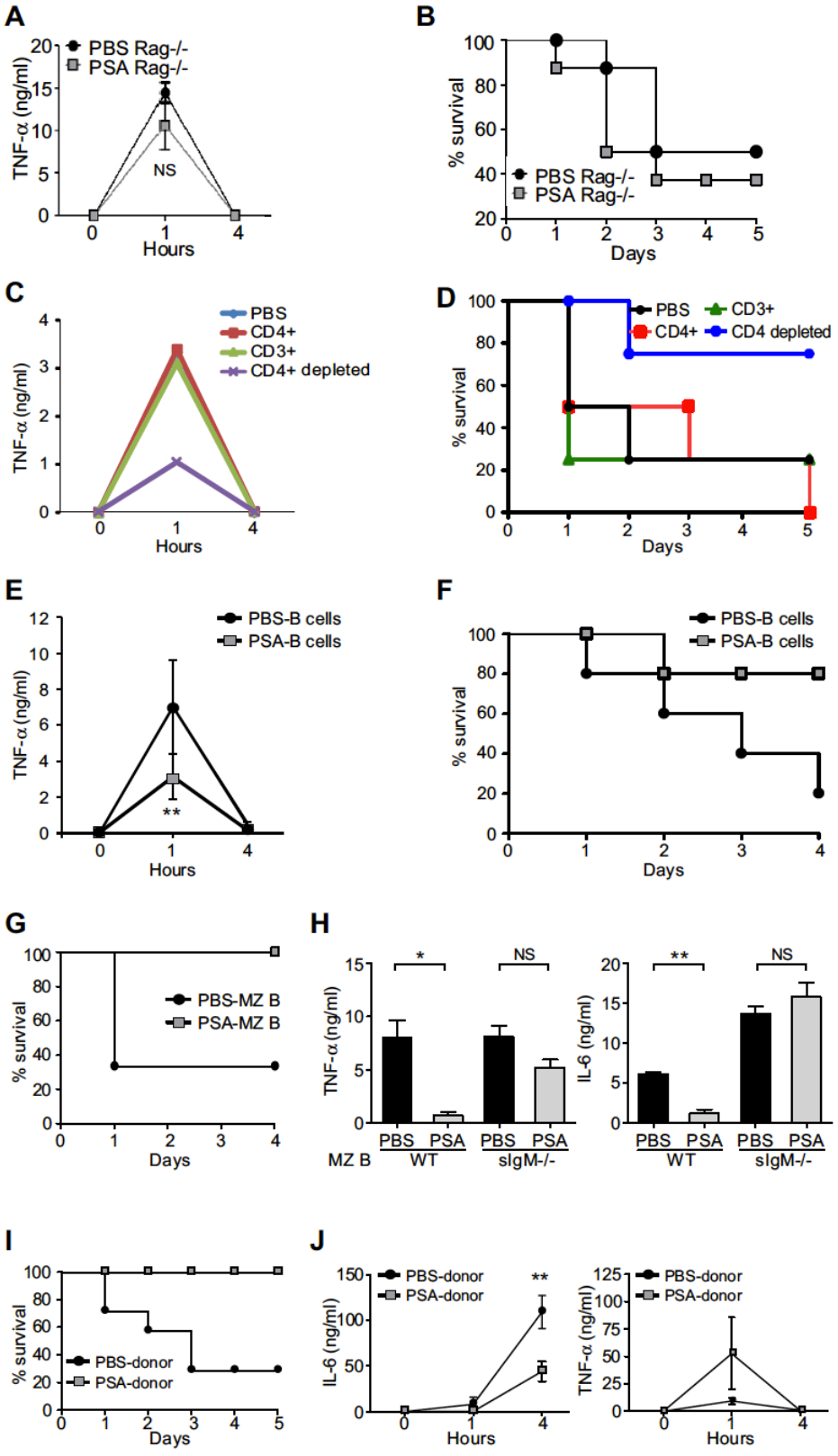
(A) Serum TNF-α and IL-6 levels measured by ELISA from animals that were intravenously treated with PBS or PSA either at the same time or 1 hour after LPS injection. * $p<0.05$. **(B)** Survival curves from animals that were intravenously treated with PBS or PSA at the same time or 1 hour after LPS injection. All groups contained at least 4 animals.

4.3.3 Figure 7. Neither IL-10 Nor TLR2 is Required for Protection by PSA



(A) Serum TNF-α level measured by ELISA from IL-10-/- animals that were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. * $p<0.05$. **(B)** Survival curves from IL-10-/- animals that were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. Both groups contained at least 4 animals. **(C)** Serum TNF-α level measured by ELISA from TLR2-/- animals that were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. *** $p<0.001$. **(D)** Survival curves from TLR2-/- animals that were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. Both groups contained at least 4 animals. **(E)** Serum TNF-α level measured by ELISA from TLR2-/- animals that were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. NS: not significant. **(F)** Survival curves from Rag-/- animals that were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. Both groups contained at least 4 animals.

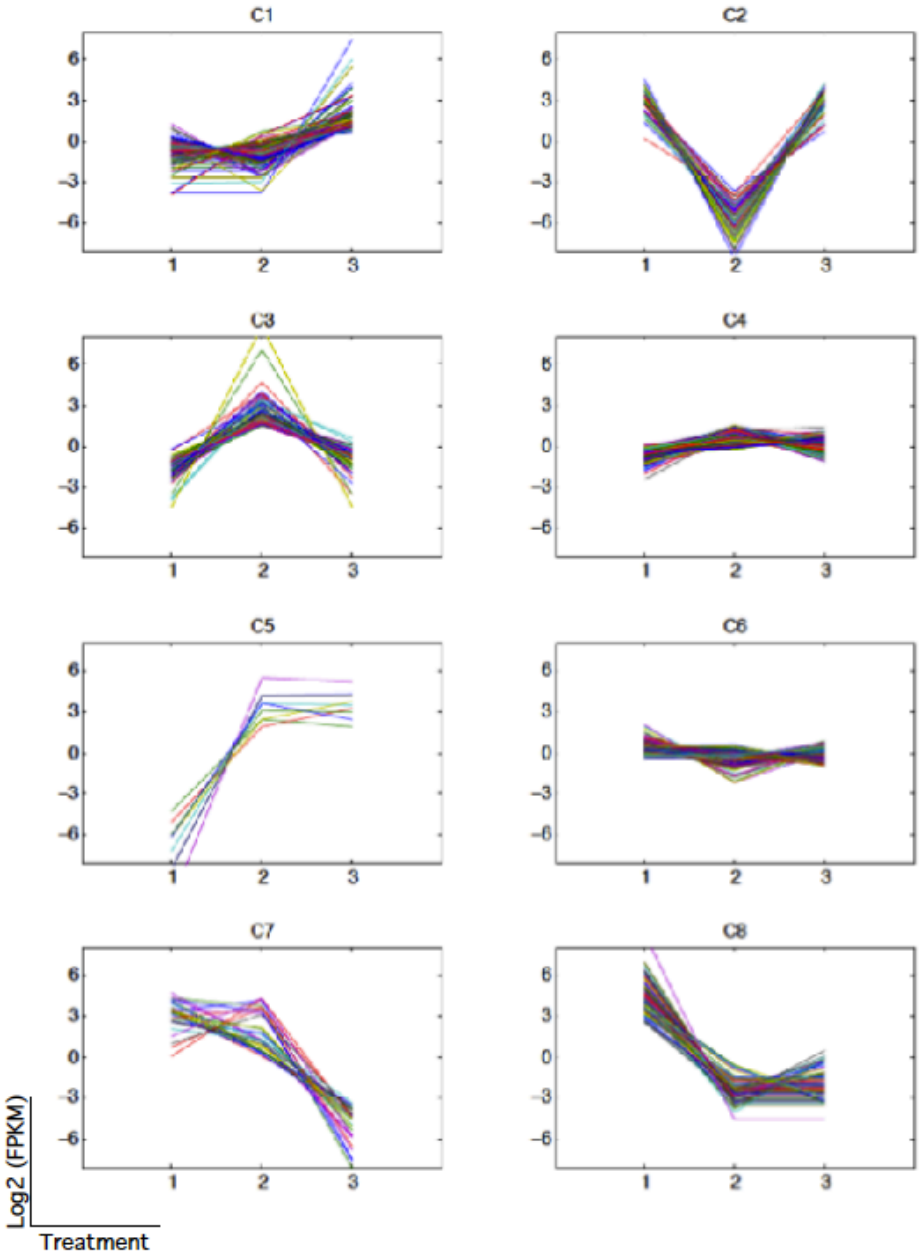
4.3.4 Figure 8. MZ B Cells can Mediate Protection by PSA



(A) Serum TNFα level measured by ELISA from Rag-/- animals that were reconstituted with PBS or indicated cells (CD4+ splenocytes, CD3+ splenocytes or CD4 depleted splenocytes) and then were intravenously treated with PSA, followed by LPS induced endotoxic shock. **(B)** Survival curves from Rag-/- animals that were reconstituted with PBS or indicated cells (CD4+ splenocytes, CD3+ splenocytes or CD4 depleted splenocytes) and then were intravenously treated with PSA, followed by LPS induced endotoxic shock. All groups contained at least 4 animals. **(C)** Serum TNF-α level measured by ELISA from Rag-/- animals that were reconstituted with B cells and then were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. **(D)** Survival curves from Rag-/- animals that were reconstituted with B cells and then were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. Both groups contained at least 4 animals. **(E)** Survival curves from Rag-/- animals that were reconstituted with marginal zone B cells and then were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. Both groups contained at least 4 animals. **(F)** Serum TNF-α and IL-6 level measured by ELISA from Rag-/- animals that were reconstituted with B cells from either WT or sIgM-/- spleens and then were intravenously treated with either PBS or PSA, followed by LPS induced endotoxic shock. Error bars indicate SEM from 4 animals. Results are representative of 2 independent trials. * $p<0.05$; ** $p<0.01$; NS: not significant. **(G)** Survival curves from Rag-/- animals that were reconstituted with marginal zone B cells sorted from wild-type donor animals, followed by LPS induced endotoxic shock. Both groups contained 6 animals. **(H)** Serum TNF-α and IL-6 level measured by ELISA from Rag-/- animals that were reconstituted with marginal zone B cells sorted from wild-type donor animals,

followed by LPS induced endotoxic shock. Error bars indicate SEM from 6 animals. ** $p<0.01$.

4.3.5 Figure S7, related to Figure 8. K-Means Clustering for Differentially Expressed Genes in Sorted Marginal Zone B Cells from Animals under Different Treatments



Differentially expressed genes (1,740) were subjected to K-means clustering analysis that inferred to 8 differentially expressed patterns. (1: PBS treated animals that were not induced disease; 2: PBS treated animals that were induced disease; 3: PSA treated animals that were induced disease.) The genes in C1 are listed in Supplemental Table 1.

4.3.6 Table S1, Related to Figure 8. List of Genes that Were Induced by PSA (C1 in Figure S7)

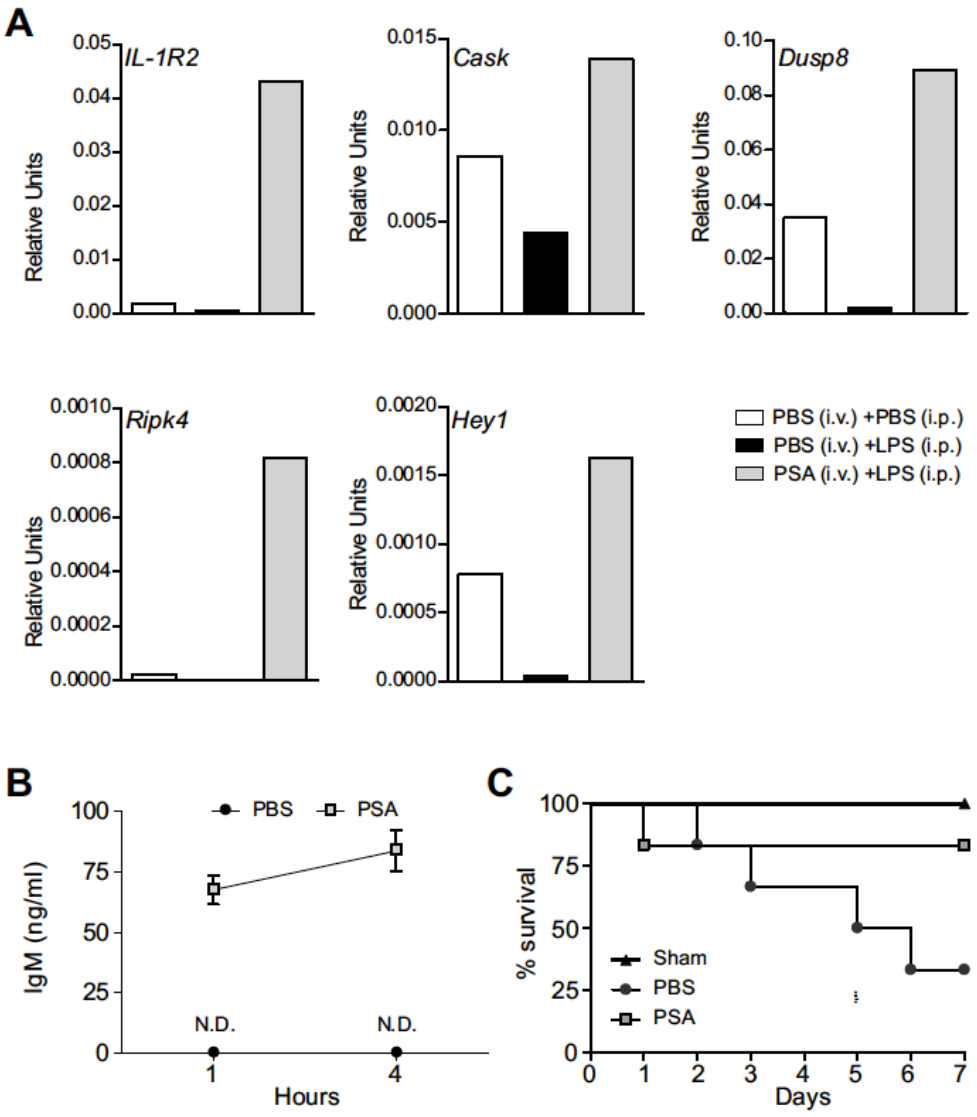
Gene Name	PBS+PBS (log2(FPKM))	PBS+LPS (log2(FPKM))	PSA+LPS (log2(FPKM))
1190002H23Rik	0.913515605	-0.686271644	1.997272536
1700017B05Rik	3.292141227	2.113724076	4.250523513
1700064E03Rik	1.714513697	1.274963395	3.675125515
1810032O08Rik	4.476938955	4.375183038	5.745060561
3230401D17Rik	4.151558495	3.754375803	5.26761074
4930415F15Rik	-0.054257472	-1.726214468	0.56672896
9830144P21Rik	-0.484081709	-0.677289616	0.911124797
Abca5	-2.031739638	-0.96096827	0.026413598
Adamtsl2	-6.64385619	-6.047740283	-4.411279339
Adm	1.678639854	1.228145852	2.891526898
Ahr	2.38297706	3.049812428	4.25500639
AI182371	-6.64385619	-6.640789457	-0.725562937
Ankrd33b	0.450168697	1.667396082	3.152860206
Ankrd57	-0.125591739	0.278050944	1.571223383
Apol8	-6.64385619	-6.640789457	-0.833400687
Arl5b	0.891045827	1.271168525	2.932184847
Atf3	4.883845609	3.321283487	5.776500742
B3gnt7	1.29190998	0.695216557	2.30413308
B4galt5	-6.64385619	-6.640789457	4.384441359
Bach1	1.762102144	2.089411476	3.09993487
Bag3	-0.348575811	-1.114608648	0.879658512
Bhlha15	-0.16854266	-1.941048702	0.544990085
Bhlhe40	4.979965872	3.849628052	7.196480596
Btg3	2.330437931	2.603402268	4.38373441
C230038L03Rik	-2.493678358	-5.12316511	-0.266088297
Cask	-6.64385619	-6.640789457	-0.925130821
Ccdc86	2.487133865	2.217245094	3.597651183
Ccdc99	1.188597153	1.307120635	2.710165076
Ccl3	2.484877983	1.868155534	3.67619451
Ccr7	5.267442162	3.947865867	6.223001544
Cdk5r1	2.515563604	2.707986658	3.843229938
Coq10b	1.523526829	1.678502652	3.168691496
Cxcr7	3.611526517	2.90548953	4.471486251

Ddit3	4.93929241	3.284743185	5.914001901
Dnajb4	2.882196968	2.437612018	4.299803759
Dnajb9	3.695281195	3.44411616	4.960447786
Dusp14	-2.741165134	-6.640789457	0.330545935
Dusp4	1.609613144	0.521829122	3.43789526
Dusp8	-3.646112164	-3.409925501	0.874208499
Dusp8	-4.568922667	-4.515495812	-1.709121094
Epha2	-1.833109771	-1.474167744	1.907647392
Errfi1	0.310025951	0.294184703	1.627582417
Ets2	3.103113206	2.497348587	4.597994418
Fam83d	-0.791317942	-1.033421681	0.873842701
Fosl2	-1.132052944	-0.544983891	3.006328836
Gadd45b	3.33309451	4.662231872	5.646704121
Gdf15	-6.64385619	-6.640789457	-3.977517057
Gla	1.963255854	1.603660772	4.770780845
Glt1d1	-6.078471535	-6.640789457	-4.707694706
Gm10501	-0.522459437	-0.390646347	2.594455915
Gm11870	-1.79741302	-3.979487589	0.349415297
Gm11974	5.541319683	5.867157939	7.008543302
Gm129	2.992730369	1.626837807	5.706065411
Gm14928	4.172087423	4.150770848	6.034513647
Gm15356	-4.497429054	-2.399957321	-1.433655986
Gm15470	2.737939806	2.986826325	4.536090421
Gm1564	-5.994315224	-6.640789457	-4.782539755
Gm15801	3.971497571	3.583310664	5.516887516
Gm16716	2.361372546	2.963375104	5.930946023
Gm17024	0.595150343	1.244897961	3.747618715
Gm17254	-1.691923428	-1.437219956	0.111852835
Gm17659	-0.106269687	-0.98722156	1.179229417
Gm614	2.956499061	2.762065359	4.169557368
Gm6568	2.984545604	2.736593977	4.633638197
Gm7148	2.345541149	3.2158677	4.786516831
Gm7278	3.035755858	3.128716358	4.906380253
Gm7334	2.400466886	2.763075386	4.34295396
Gm8783	3.971497571	3.583310664	5.516887516
Gm9115	3.512087794	3.665259752	5.540465445
Gpr132	3.746957642	3.735222199	4.96649833
Gpr3	-6.64385619	-6.640789457	-0.212439176
Grasp	0.855096704	0.160010918	3.389206267
Haus2	1.692404879	1.839976365	2.984315973

Hey1	-3.563843279	-5.335670429	-1.353887685
Hic1	-1.636579943	-3.441750123	-0.793585338
Hif3a	-1.792187092	-0.950435649	0.280892072
Hpn	-6.64385619	-6.640789457	1.476843609
Hspa1a	2.337893736	2.503846713	5.697884278
Hspa1b	2.709504598	3.004485465	6.082292003
Ifrd1	4.781092134	5.558831939	7.515306397
Ighv1-20	2.457906803	3.106420194	6.256076627
Il1r2	-2.17107601	-3.27605274	2.206828017
Impact	2.1314512	2.2445328	3.787309508
Ing3	4.290225327	4.516162068	5.633483498
Insig1	3.014192802	2.794548342	4.532842988
Irf4	4.571512996	4.787999395	6.086773739
Irs2	3.151025878	2.454471446	5.761164557
Kdm6b	3.329166661	3.92697432	5.514751519
Klf10	0.949004447	1.154161374	3.548366008
Klf11	1.611956087	1.194164719	3.329546239
Klf9	0.2131295	0.196974854	2.155853321
Litaf	3.318601736	3.258754598	4.418849108
Lrrc66	-6.140035285	-6.640789457	-4.927733151
Maff	0.890905792	0.279392252	4.220157725
Map1lc3a	2.977039912	1.377767121	3.806882671
Map3k6	-5.009025976	-6.640789457	-4.108104994
Mir155	-6.64385619	-6.640789457	1.534151953
Mreg	1.931293496	2.432922787	3.765801031
Mt1	2.784602393	0.506372281	3.424444749
Mt2	-3.635096822	-0.483484304	0.566986718
Nfil3	0.682726098	1.149842802	3.061186291
Ninj1	3.854434575	2.281117953	4.52585782
Nr4a1	5.821273974	4.653506298	7.245139269
Nr4a2	2.791511959	2.199403181	4.609263957
Nr4a3	-0.371885273	1.102559025	3.177143511
Nrarp	-0.633888767	-0.71012337	1.512711797
Odc1	5.11477512	4.973201224	6.711329443
Osgin1	2.247733116	1.068561825	3.920537714
Osgin2	2.458145736	2.354599592	3.989486906
Pdgfa	-6.64385619	-6.640789457	-5.041097059
Per1	4.276422221	2.898074698	5.961682434
Phlda1	0.94665601	0.06713726	1.955941076
Pim3	3.855142563	2.7123994	4.861433995

Plau	-0.072901515	-1.085776856	2.515240223
Plk2	2.958783296	2.050847479	4.04770379
Pmaip1	2.728493064	2.110667748	3.870387401
Prr7	2.730792097	2.088899856	3.716925534
Ptger4	3.070233027	2.771147776	4.268356152
Ptp4a1	3.394555299	3.061375325	4.706282387
Rem2	-6.64385619	-2.537344917	0.295879072
Ripk4	-6.64385619	-6.640789457	-1.834551203
Rnf125	-6.64385619	-2.360043802	0.54248062
Rrad	2.425647283	1.760212443	3.603615756
Sertad4	-4.720448355	-6.640789457	-2.102404502
Siah2	3.773236812	3.368939395	4.761999179
Slc16a1	0.81614092	0.946153053	2.280132884
Slc20a1	3.727386831	4.32276489	5.559928396
Slc38a2	3.654836488	3.924184755	5.047259518
Smad7	3.311238617	2.717693947	4.32901642
Snai1	-1.215234722	-0.537822093	1.700036462
Snora28	6.278999482	6.526145826	8.357527636
Snora75	4.929284451	5.608164562	7.668068884
Snord69	7.80469895	9.307017829	10.4348892
Suv39h2	0.158892797	0.226248955	1.549899683
Tex15	-6.64385619	-4.433875895	-2.37107081
Tfrc	3.159059163	3.489160486	4.602935506
Tgif2	2.745166325	3.651196234	5.168316528
Tmem88	-0.88576359	-1.004073373	1.005481915
Tnfrsf12a	1.172596322	-0.572349404	2.211469159
Trp53inp2	2.711378165	1.135706204	4.097537827
Utp14b	-0.32417865	-0.277022765	1.756762452
Vegfa	-6.64385619	-6.640789457	2.403340627
Vps18	2.889732465	1.878028037	4.042508598
Zbtb46	-1.621390721	-0.602326624	0.442434622
Zc3h12a	4.343208816	4.769499894	5.956545381
Zfand2a	2.46667932	2.248944415	3.63127555
Zfp295	1.684001802	1.691157848	3.454859003

4.3.7 Figure 9. Preliminary Data Implicating Future Directions



(A) qRT-PCR analysis of the expression level of selected genes listed in Supplementary Figure 9 identified and confirmed 5 genes that are significantly up-regulated by PSA from sorted marginal zone B cells. **(B)** Serum IgM level measured by ELISA from Rag-/- animals that were reconstituted with marginal zone B cells sorted from wild-type donor animals that were treated with either PBS or PSA. Error bars indicate SEM from 6 animals. **(C)** Survival curves from wild-type animals that were intravenously treated with either PBS or PSA, followed by Cecal Ligation and Puncture (CLP). Sham group has 2 animals and PBS or PSA group has 7 animals. Results are representative of 2 independent trials.

Chapter 5

Discussion and Perspective

5.1 Modulation of Intestinal Immune Responses by PSA-containing OMVs

5.1.1 Molecular Communication between the Microbiota and the Immune System

Bacteria shape diverse environments on land, in the oceans, and within plants and animals; often, these processes are mediated by secretion of bacterial molecules. Regarding interactions with mammals, decades of research has uncovered molecules produced by pathogens that promote bacterial invasion into tissues and cells, nutrient acquisition and subversion of the immune system. We know of numerous mechanisms by which virulence factors are secreted or delivered to mammalian targets. Only recently has it become appreciated that symbiotic bacteria also establish molecular communication with mammals. The archetypal symbiosis factor of the human microbiota is Polysaccharide A (PSA) from *Bacteroides fragilis*, which ameliorates inflammatory disease in animals by inducing immunologic tolerance (Deatherage et al., 2009; Lee and Mazmanian, 2010). However, it has remained unknown how PSA is delivered to the immune system. The studies described herein illustrate that outer membrane vesicles (OMVs) released from the bacterial surface are sufficient to mediate inter-kingdom interactions between *B. fragilis* and the immune system by delivering PSA to DCs. Oral treatment of mice with OMVs prevents experimental colitis, whereas vesicles from *B. fragilis*ΔPSA are unable to protect animals from wasting disease, histopathology and pro-inflammatory cytokine production. The requirement of OMVs for this process is presently difficult to address, as attempts to delete OMVs from bacteria have thus far been unsuccessful, as they may be necessary for viability (Deatherage et al., 2009; Kuehn

and Kesty, 2005). However, our work reveals the first paradigm by which a beneficial bacterium of the human microbiota selectively delivers a microbial molecule to its host. Future studies will aim to understand how other components of *B. fragilis* OMVs interact with the mammalian immune system.

5.1.2 OMVs Mediate Immunue Tolerance through Activation of Dendritic Cells

In response to sampling both self and foreign antigens, DCs interact with a variety of immune cells to coordinate diverse biological responses. The presentation of microbial molecules from pathogens to CD4+ T cells is a critical component of a productive immune response during infection. Conversely, antigens of the microbiota must be either ignored or actively tolerated during long-term colonization to prevent deleterious reactions that may adversely affect both microbes and mammals. Indeed, it is believed that a critical feature of IBD is uncontrolled immune reactivity to the microbiota. Our laboratory has previously shown that PSA induces immunologic tolerance through IL-10-producing Foxp3+ regulatory T cells (Tregs), a process that protects animals from colitis and promotes colonization by *B. fragilis* (Round et al., 2011; Round et al., 2010). Here we show that OMVs internalized by DCs induce tolerogenic DCs that produce IL-10, which in turn drive the development of IL-10-producing Tregs. In contrast, OMVs from *B. fragilis*ΔPSA do not promote Tregs. Thus, unlike immunity that results from recognition of microbial molecules from pathogens, PSA programs DCs to adopt an anti-inflammatory profile leading to T cell-mediated tolerance and protection from experimental colitis.

Recently, it was shown that intestinal colonization of animals with *B. fragilis*

prevents and treats experimental autoimmune encephalomyelitis (EAE), an animal model for human multiple sclerosis (MS) (Ochoa-Reparaz et al., 2010a; Ochoa-Reparaz et al., 2009). Both EAE and MS involve inflammation in the central nervous system (CNS). How does PSA, a symbiosis factor produced in the gut, mediate protection from immunity in the brain and spinal cord of animals? Mice protected from disease display an increase in the proportions of CD11c+CD103+ DCs, a subset that is known to promote Treg differentiation (Ochoa-Reparaz et al., 2010a; Ochoa-Reparaz et al., 2009). Interestingly, increased CD103+ DCs and Foxp3+ Treg proportions are only observed in the cervical lymph nodes that drain the CNS, but not in other systemic compartments. It is currently unknown if the gut is the site of tolerogenic DC induction followed by cell migration to the CNS, or if PSA somehow activates DCs outside the intestine. Further, it will be interesting to know the subset of DCs that respond to PSA/OMVs. As tolerogenic DCs promote Treg development, and Tregs have been shown to suppress inflammation in many tissues of the body, it is conceivable that PSA may represent a novel therapy for immunologic diseases beyond IBD and MS.

5.1.3 TLR2 Signaling by DCs is Required for OMV Sensing

Immune cells recognize microbial ligands through pattern recognition receptors to initiate immunologic responses. Previous studies have identified toll-like receptor 2 (TLR2) as being required for PSA-mediated induction of interferon (IFNγ) (Wang et al., 2006), as well as IL-10 production from Treg cells (Round and Mazmanian, 2010). Furthermore, TLR2-deficient animals are not protected from TNBS colitis by PSA treatment (Round and Mazmanian, 2010). Our studies now reveal that TLR2 expression by DCs is necessary for the induction of anti-inflammatory responses by OMVs. This

phenotype is largely dependent on PSA, although OMVs contain other molecules (perhaps lipoproteins) that also increase IL-10 expression through TLR2 (see Figure 4A). In contrast, we have previously shown that purified PSA is able to directly induce IL-10 from CD4+ T cells in the absence of DCs. The DC-dependent pathway described here is specific for OMVs, as treatment of T cells alone with OMVs (containing PSA) is unable to promote IL-10 production from CD4+ T cells, unlike purified PSA. Furthermore, TLR2 deletion on DCs abrogates IL-10 production during *in vitro* culture with OMVs.

The biological context by which DCs and T cells contact PSA during colonization by *B. fragilis* remains unresolved, and further studies involving *in vivo* metabolic labeling of PSA are required to distinguish when, where and how each cell type 'sees' PSA. However, we speculate that DCs internalize PSA associated with OMVs, which traffic through the endocytic pathway to contact T cells at the immunologic synapse. This notion is supported by evidence that PSA is presented to CD4+ T cells by major histocompatibility complex (MHC II) following internalization and processing in the endosome (Cobb et al., 2004). Perhaps deletion of TLR2 on T cells abrogates PSA recognition at the immunologic synapse following initial association of DCs with OMVs. Thus, both cell types may require TLR2, and addition of purified PSA directly to T cells bypasses the requirement for DC expression of TLR2 (Round et al., 2011). Other models are conceivable as well, and the details of this novel mechanism by which the immune system responds to anti-inflammatory microbial ligands are currently being addressed. What is clear from our results is that PSA, in the context of OMVs, directly activates DCs and not T cells, in a process that requires TLR2. The consequence of this interaction leads to the induction of tolerogenic DCs that enhance Treg function

and promote protection from inflammatory disease.

5.1.4 Identification of Gadd45a in the PSA Signaling Pathway

Following internalization into DCs and engagement of TLR2, OMVs initiate a gene expression program that results in IL-10 production by DCs. Although it is known that the outcome of PSA sensing by the immune system is Treg induction, nothing is known about the intracellular signaling pathway(s) activated by PSA within DCs. We analyzed the gene expression profile of DCs treated with OMVs to uncover factors that are expressed in a PSA-dependent, TLR2-dependent manner. Of the handful of candidates, we focused on Gadd45α (growth arrest and DNA-damage-inducible protein) due to its known role in immune responses. In addition to a function in stress responses and cell cycle control, Gadd45α has been shown to affect T cell activation by inhibiting the alternative p38 pathway in CD4+ T cells (Salvador et al., 2005). More recently, Gadd45α expression by DCs was implicated in Th1 polarization in response to *Toxoplasma gondii* antigen stimulation (Jirmanova et al., 2007). Levels of the Th1 skewing cytokine IL-12 are reduced in Gadd45α-/- DCs, resulting in a non-T cell intrinsic reduction in IFNγ+ T cells. We show that Gadd45α is up-regulated upon OMV stimulation in a TLR2-specific fashion, and that absence of Gadd45α in OMV-treated DCs leads to a defect in IL-10 production. Furthermore, this results in a lack of IL-10 expression from CD4+ T cells co-cultured with Gadd45α-/- DCs. Transfer of DCs treated with OMVs into wild-type animals ameliorates TNBS colitis, demonstrating the protective effects of PSA directly on DCs. Transfer of OMV-treated DCs deficient in Gadd45α results in no protection from the histopathologic damage and cytokine production associated with colitis, revealing that PSA signaling in DCs requires

Gadd45α. Intriguingly, Gadd45α deficient animals die at late ages from lupus-like autoimmunity (Salvador et al., 2002), a pathology that has been linked to defective Treg function (Long and Buckner, 2011). Collectively, these results suggest a model whereby PSA from OMVs activates TLR2 to induce expression of Gadd45α, which in turn modulates intracellular signaling cascades to promote tolerogenic DC function and possibly Treg development during protection from autoimmune and/or inflammatory disease. We have not yet directly shown that Gadd45a is required for Treg development induced by PSA-containing OMVs, which is currently being addressed in both DC-T coculture system and animal models of colitis. It is also worth noting that both PSA and Gadd45α could induce or mediate Th1 development (Jirmanova et al., 2007; Mazmanian et al., 2005) while here we show that these two molecules are involved in Treg development as well. This brings up a new question of how DCs, after sensing PSA, could direct T cells to commit to two phenotypically and functionally distinct lineages while both of the pathways require Gadd45α. Since Th1 development was observed in the spleen and Treg development was found in the intestine, one possibility is that the local environments in the spleen and the intestine differ, which influence the functioning of DCs and/ or T cells. As a result, T cells commit to either Th1 or Treg after integrating both microbial signals and environmental signals. Another possibility would be the difference in other unknown signaling molecules in PSA-Gadd45α pathway. As was described in Chapter 3, the microarray analysis has also yielded two other molecules that might be downstream of Gadd45α. Further study on the function of those molecules would help us to better understand the role of Gadd45α in DCs in mediating different T cell responses.

5.2 Modulation of Systemic Immune Responses by PSA

5.2.1 B Cells and Sepsis Protection by PSA

Although it is quite surprising that PSA can act through MZ B cells to achieve protection against experimental sepsis, the involvement of B cells in protecting against experimental sepsis is not entirely new. A recent study showed that B cell deficient mice exhibited decreased inflammatory cytokine and chemokine production and reduced survival after sepsis induction. Furthermore, B cells were activated during sepsis, and that the activation of B cells was critical for the early inflammatory responses essential to control the infection (Kelly-Scumpia et al., 2011). Another study identified a GM-CSF producing B cell population in reducing inflammation and limiting bacterial load during experimental sepsis. This population of B cell was named innate response activator (IRA) B cells because of GM-CSF's known role in activating innate leukocytes. The authors also showed that mice depleted of IRA B cells were extremely susceptible to Cecal Ligation and Puncture (CLP) and both inflammatory responses and bacterial burden were out of control during disease (Rauch et al., 2012). All these findings support the role of B cells in sepsis protection. Nevertheless, the identification of a microbial factor PSA as a trigger of protective B cell activation will advance our understanding of the function of B cells in sepsis and the development of B cell-mediated new therapies for human sepsis.

5.2.2 Potential Directions for Future Studies

As discussed in Chapter 4, we found that systemic treatment with PSA could protect animals from LPS-induced endotoxic shock via MZ B cells. Future studies are required to figure out how MZ B cells mediate the protective activity of PSA during experimental sepsis.

Firstly, we have shown that IgM secretion from MZ B cells is required for protection by PSA because sIgM-/- MZ B cells failed to support the protective activity of PSA when transferred into Rag-/- mice. The immediate hypothesis following this piece of data is that PSA can promote IgM secretion from MZ B cells. More specifically, MZ B cells may make and secrete LPS-specific IgM upon LPS stimulation; and in PSA treated animals, elevated levels of LPS-specific IgM are produced to neutralize LPS. As a result, inflammation casued by LPS is reduced. In order to test this hypothesis, we need to either detect LPS-specific IgM or measure the residual free LPS level in the serum of protected animals. If we observe increased level of LPS-specific IgM or decreased amount of free LPS in PSA treated animals, the next question would be how PSA promotes IgM secretion from MZ B cells. Further studies on the direct or indirect influences of PSA on IgM secretion pathway would be of great interest.

Notably, with the induction of LPS-specific IgM by PSA being one plausible protective mechanism, the possibility that the secreted IgM could be specific to other molecules is not entirely ruled out. Since MZ B cells are known to have a BCR repertoire specific for bacterial antigens including polysaccharide, it is possible that PSA may also induce PSA-specific IgM secretion. And if PSA-specific IgM does exist, whether they are involved in the protection or how they contribute to the protection require further investigation.

In addition to the hypotheses that were derived from our experimental evidences, we also decided to take an unbiased approach to dissect the mechanism underlying the protective activity of PSA via MZ B cells.

A transcriptome analysis by deep sequencing was performed on the mRNA of MZ B cells isolated from PSA or vehicle treated animals that were induced endotoxic shock. We've identified and confirmed several genes that are specifically upregulated by PSA during protection. Among those genes, IL-1R2 attracted our attention because it was known as a decoy receptor of IL-1 that could block IL-1 signaling (Dinarello, 2009). Since IL-1β is also a major proinflammatory cytokine during sepsis, a possible hypothesis arose as PSA induced IL-1R2, a negative regulator of IL-1β, to inhibit IL-1β signaling so that inflammation was controlled once the infection was cleared. In order to test this hypothesis, we will measure the levels of IL-1R2 in the serum of PSA treated animals to see whether it is induced by PSA or not, and functionally neutralize IL-1R2 *in vivo* to see whether the protective activity of PSA is abolished or not. Besides, there are many more genes to be explored from this RNAseq analysis.

Additionally, we still do not know whether or not PSA acts directly on MZ B cells during protection against sepsis. It is possible that MZ B cells may sense PSA in a TLR2 independent manner (e.g. by employing an unknown PSA receptor). Alternatively, there might be other cell types involved, which may sense PSA and convey its protective effect to MZ B cells. Future studies are necessary to distinguish between these possibilities.

Lastly, we have not yet detected PSA or PSA-containing OMVs in the blood of *B. fragilis*-colonized animals. If PSA never reaches the systemic compartments of an animal under physiological conditions, what is the biological relevance of our finding that systemic treatment of PSA could protect animals from sepsis? One intriguing hypothesis would be that PSA has evolved a mechanism to rapidly control the inflammation under pathological conditions, e.g., during massive systemic infection. When the gut epithelial

barrier is disrupted, gut commensals including *B. fragilis* may leak out of the intestine. When the host mounts systemic proinflammatory responses to clear the massive bacteria translocated from the gut, PSA or PSA-containing OMVs derived from *B. fragilis* (now existing systemically) may directly or indirectly influence the activity of MZ B cells and produce a rapid anti-inflammatory response to control the inflammation, thus protecting the host tissue from being damaged. The advantage of this B cell mediated systemic response is its rapidness, as opposed to PSA-induced DC/ T cell-mediated regulatory response in the gut, which may take days. As mentioned in Chapter 4, PSA also shows protective activity in another murine model of sepsis, the CLP model. We can test the above hypothesis by colonizing SPF mice (which normally do not have *B. fragilis*) with *B. fragilis* and looking for protection following sepsis induction by CLP. This way, *B. fragilis* is released together with other bacteria into the systemic compartments. It provides a more "natural" setting to investigate whether PSA can protect animals from excessive systemic inflammation under circumstances that PSA is released into the circulation. We speculate that these experiments will further support the idea that gut commensals have evolved various mechanisms to promote the health of the host, thus to achieve their long-term colonization

5.3 Perspective

Eons of co-evolution have formed an inextricable connection between mammals and our microbiota. With over 100-fold more unique genes in the microbiome than the human genome, symbiotic bacteria posses the vast potential to produce molecules, destined for their host, which mediate various biological processes. We reveal that *B. fragilis* actively delivers PSA via outer membrane vesicles, uncovering a novel mechanism by which the immense molecular coding potential of the microbiome can be utilized during mutualism. Sustained, life-long molecular communication between the microbiota and mammals bridges the boundaries between kingdoms of life, and supports the notion that animals may be viewed as holobionts: a single unit of evolutionary selection comprised of a host and its associated microbes (Rosenberg et al., 2009).

Appendix

Experimental Procedures

Bacterial Strains and Culture Conditions. *Bacteroides fragilis* strain NCTC9343 was obtained from the American Type Culture Collection, its isogenic PSA deletion mutant (*B. fragilis*ΔPSA) has been described (Coyne et al., 2001). Bacteria were grown either in brain heart infusion (BHI) broth (BD Biosciences) supplemented with 0.5μg/ml Hemin (Sigma) and 0.5μg/ml Vitamin K (Sigma), or a customized minimum medium which contained 8g Glucose, 1% FBS, 0.5μg Hemin, and 0.5μg/ml Vitamin K in 1L of RPMI (Invitrogen).

Mice. C57BL/6 and BALB/c mice were purchased from Taconic Farms (Germantown, NY). TLR2 knockout and IL-10 knockout mice were purchased from Jackson laboratories. Gadd45α knockout mice were maintained in an NCI pathogen free animal facility. IL-10-eGFP mice (Vert-X mice) have been described previously. Foxp3-eGFP mice were obtained from Talal A. Chatila (University of California Los Angeles) (Lin et al., 2007). All procedures were performed in accordance with the guidelines and the approved protocol from the Institutional Animal Care and Use Committee at California Institute of Technology.

Isolation of EDL-enriched Bacteria. Percoll (GE Healthcare) discontinuous density gradient centrifugation was used for electron dense layer (EDL) isolation of both wildtype *B. fragilis* and *B. fragilis*ΔPSA, following minor modification from previous protocols (Patrick and Reid, 1983). Briefly, using a 20%, 40%, 60%, 80% Percoll gradient (diluted with PBS), *B. fragilis* cells were carefully added on top of the 20% Percoll layer. Subsequently, the gradient was centrifuged at 800 x g for 20 minutes at room temperature (RT). EDL-enriched bacteria can were recovered from the 40%-60%

interface of the gradient after centrifugation.

OMV Purification and Labeling. *B. fragilis* OMV isolation was adapted from a previously described protocol for the preparation of OMVs from *Escherichia coli* (Horstman and Kuehn, 2000). Briefly, EDL-enriched *B. fragilis* was grown in customized minimal media. OMVs were recovered from the bacteria-free supernatant of the culture by centrifugation at 40,000 x g for 2 hours at 4°C, washed twice with PBS and filtered through 0.45μm spin columns (Millipore). Total protein concentration of the purified OMVs was determined by Bradford assay (BioRad). FITC-labeled OMVs were prepared as previously described (Kesty et al., 2004). Briefly, OMVs were incubated in staining buffer containing 1mg/ml FITC (Thermo Scientific), 100mM NaCl, 50mM Na2CO3, pH 9.2) for 1 hour at RT. Labeled OMVs were collected by centrifugation and washed with PBS+200mM NaCl before use.

Electron Microscopy of Bacterial Ultrathin Sections. Ultrathin sections of EDL enriched *B. fragilis* were prepared as previously described (Patrick et al., 1996). Briefly, samples were fixed in 2.5% (v/v) glutaraldehyde (Sigma) in cacodylate buffer overnight at 4°C, followed by further fixation in osmium tetroxide (1%, w/v) for 3 hours at RT in the dark. Ruthenium Red (1mg/ml, Sigma) was included in both of the fixation processes. Then fixed samples were embedded in epoxy resin after dehydration in a graded series of alcohols. Ultrathin sections (100~200nm) were cut and negatively stained with 2% uranyl acetate and lead citrate on formvar/carbon coated copper grids (EMS) before visualization by transmission electron microscopy (TEM, FEI Tecnai T12).

Immunogold Labeling of Purified OMVs. Purified OMVs were applied to

formvar/carbon coated gold grids (EMS) and air-dried. Immunogold labeling was performed at RT by floating these grids with "OMVs"-side down on a series of small drops of antibody and wash solutions. Samples were blocked in 10% FBS for 10 minutes after 5 minute incubation in 0.12% glycine. After blocking, samples were further incubated with anti-PSA diluted in 10% FBS for 20 minutes, followed by 5 washes with PBS. Secondary antibody-IgG conjugated with 5nm gold (kind gift from Dr. Paul Webster, House Ear Institute, Los Angeles, CA) was applied to the samples for 20 minutes, followed again by 5 washes at 4 minutes each with PBS. After labeling, samples were fixed in 1% glutaraldehyde for 5minutes and washed extensively by transferring grids to drops of PBS and H2O. Contrast staining was performed by placing the grids on drops of 3-5% uranyl acetate in 2% methylcellulose for 10minutes on ice. Grids were removed from the staining solution and air-dried. Samples covered by a thin film of methylcellulose were removed from loop and used for visualization by TEM.

Chemical (TNBS)-induced Experimental Colitis. BALB/c mice were orally treated with PBS, WT-OMV (5μg) or ΔPSA-OMV (5μg) every other day for one week before TNBS administration. Mice were anesthetized with isofluorene and rectal administration of 2% TNBS (in 50% EtOH, Sigma) was applied through a 3.5F catheter (Instech Solomon; SIL-C35) as previously described (Wirtz et al., 2007). For BMDC transfer-TNBS experiments, WT or ΔPSA-OMV treated WT (C57BL/6) or Gadd45α-/- BMDCs were i.p. injected into C57Bl/6 recipient mice 2 hours before TNBS (125mg/kg) induction.

Tissue Pathology Analysis. Mouse colons were fixed in neutral buffered 10% formalin (ScyTek Laboratories), embedded in paraffin, sectioned and stained by Pacific Pathology

(San Diego, CA). Colitis scores for each colon section were evaluated in a blinded fashion by a veterinary pathologist (Dr. Gregory W Lawson) using a standard scoring system (Wirtz et al., 2007): 0: No signs of inflammation; 1: Low level of inflammation; 2: Low level of leukocytic infiltration; 3: High level of leukocytic infiltration, high vascular density thickening of colon wall; 4: loss of goblet cells, high vascular density, thickening of colon wall. Histology images were taken using light microscopy (Zeiss) at 20x magnification.

Quantitative Real-time PCR (qRT-PCR). RNA was collected either from mouse tissues using Trizol (Invitrogen) or from purified cells using RNeasy Mini Kit (Qiagen). iSCRIPTcDNA synthesis kit (BioRad) was used for conversion of cDNA and IQ SYBR Green supermix (BioRad) was used for qRT-PCR.

Colon Lamina Propria Lymphocytes (LPLs) isolation and Intracellular Cytokine Staining (ICCS). Full-length colon was dissected and luminal contents were carefully flushed out with ice-cold PBS. Colon epithelium was dissociated and the remaining tissue was subjected to enzymatic digestion with collagenase D (0.5mg/ml), dispase (3 units/ml) and DNase I (0.5mg/ml). Finally, a 40%-80% Percoll gradient was used to isolate LPLs. ICCS of IL-17 and TNFα was performed on LPLs that were re-stimulated *in vitro* with PMA/Ionomycin in the presence of golgi-plug for 4.5 hours, according to manufacturer's protocol (BD Biosciences).

***In vitro* BMDC-T Cell Co-culture.** Bone marrow was collected from different strains of mice and differentiated *in vitro* in the presence of 20ng/ml GM-CSF (MiltenyiBiotec) for bone marrow-derived dendritic cells (BMDCs) as described previously (Mazmanian et

al., 2005). Cell purity as assessed by CD11c staining was >90%. CD4+ splenic T cells were isolated by magnetic microbead purification (Miltenyi Biotec). Cell purity as assessed by CD4 staining was>95%. OMV-pulsed BMDCs (10μg/ml OMVs, 1x105 cells/ml, 12-24 hours) were washed with HBSS to remove unbound OMVs prior to incubation with CD4+ T cells (1x106 cells/ml). Co-cultures were performed in round bottom 96 well plates with addition of anti-CD3 (0.01μg/ml), TGFβ (2ng/ml), and with or without IL-2 (5ng/ml). Supernatants were collected for ELISA (eBiosciences) or cells were harvested for qRT-PCR analysis or flow cytometry (FC).

Flow Cytometry and Staining. BMDCs, for the OMV uptake assay or activation assay, were collected and blocked in 5% mouse serum for 30 minutes on ice. After blocking, cells were stained with anti-CD11c-APC, anti-MHCII-FITC or anti-CD86-PE (eBioscience) for 30 minutes on ice and washed twice with FACS buffer (HBSS without Ca2+/Mg2+, 1% FBS, 2mM EDTA, 10mM HEPES) at 4°C before FC analysis. Similarly, cells from *in vitro* BMDC-T cell co-culture were blocked, and stained with anti-CD4-APC and anti-CD25-PE similarly except that the cells were re-stimulated using PMA/Ionomycin for 4 hours before collecting. All FC was performed with BD FACSCalibur and results were analyzed using the FlowJo software (BD Biosciences).

***In vitro* Suppression Assay.** CD4+CD25+Treg cells purified from BMDC-T cell cocultures using magnetic microbeads (Miltenyi Biotec). CD4-depleted mouse splenocytes treated with Mitomycin C (Sigma) were used as APCs (1x105 cells/ml). CD4+CD25-responder T cells directly purified from mouse spleen were pulsed with CFSE for 10minutes at 37°C, followed by first wash with PBS and a second wash with culture media, and used immediately (5x105 cells/ml), reffered to as effector T cells

(Teff). This assay was conducted in round bottom 96 well plates with an addition of 5μg/ml of anti-CD3 (eBiosciences) in 200μl. Teff:Treg ratio was titrated and cells were collected after 2-3 days of culture for FACS analysis. Anti-IL-10R (20μg/ml, R&D Systems) was added as indicated in the results.

Microarray Hybridization and Data Analysis. RNA was prepared from WT or ΔPSAOMV treated CD11c+ BMDCs. RNA samples (1μg total RNA) were labeled with fluorescent dyes using the Quick Amp Labeling Kit (Agilent). Microarray (AgilentWhole Mouse Genome chip) hybridizations (65°C for 16 hours) and washes were performed with Agilent reagents following standard protocols. Microarrays were analyzed using an Agilent DNA Microarray Scanner G2565CA, and data were acquired using Agilent's Feature Extraction Software version 10.1.1.1. Significant genes were selected based on $p< 0.01$ and fold change >2.0. For enrichment analysis of biological process ontology, probe lists were analyzed in DAVID (The Database for Annotation, Visualization and Integrated Discovery annotation tools (http://david.abcc.ncifcrf.gov/)) and selected based on $p< 0.01$.

LPS Induced Endotoxic Shock Model. Balb/c or C57Bl/6 mice were retro-orbitally treated with either PSA (100μg/mouse/treatement) or PBS every other day for two times before intra-peritoneal injection of LPS (500μg/mice, Sigma). Blood was collected (through eye bleed) at both 1 hour and 4 hours after LPS injection to assess serum TNFα and IL-6 level. All animals were carefully monitored afterwards to obtain the survival curve. In cell transfer experiments, Rag-/- mice were reconstituted with different types of cells 12 hours before PSA or PBS treatment; Or Rag-/- mice were reconstituted with cells isolated from either PSA or PBS treated donor mice 12 hours before LPS injection.

Serum collection. Blood samples were centrifuged at 15,000g for 30 minutes at 4 degree. Supernatant (serum) was transferred into new microcentrifuge tubes and stored at -20 degree or used directly for cytokine ELISA.

Marginal Zone B Cell Sorting. Splenic B cells were isolated by magnetic microbead purification (Miltenyi Biotec). Cells then underwent surface staining of anti-IgM, anti-CD21 and anti-CD23. Cell sorting was performed on BD FACSAria to isolate marginal zone B cells that were IgM^{high} $CD21^{high}$ $CD23^{low}$.

mRNA Purification and cDNA Library Building. Mice were retro-orbitally treated with either PBS or PSA every other day for two times before intra-peritoneal injection of LPS. One hour after LPS injection, mice were sacrificed. Total RNA was extracted from 3-5 million sorted marginal zone B cells from the spleens of groups of mice undergone different treatments using RNeasy Mini Kit (Qiagen), and then subjected to two rounds of selection using OligodT coupled magnetic beads (Dynabeads) according to the manufacturer's protocol. About 100 ng polyadenylated mRNA per sample was obtained after double selection. cDNA library building was performed as previously described. Briefly, RNA was fragmented to an average length of 200 bp by Mg2+-catalyzed hydrolysis and then converted into cDNA by random priming. cDNA was then subjected to end repairing, adaptor ligation (using Illumina ChIP-seq sample preparation kit #IP-102-1001), size selection and one round of PCR amplification.

High-throughput Sequencing. Each cDNA library was sequenced with the Illumina Genome Analyzer IIX following the manufacturer's protocols (http://www.illumina.com).

RNAseq Data Analysis. Sequence reads from each cDNA library (38 bp, single-read) were trimmed to 32 bp long and mapped onto the mouse genome build NCBI37/mm9 using Bowtie (bowtie-0.12.1, http://bowtie-bio.sourceforge.net/index.shtml) with setting '-v 2 -k 11 -m 10 -t–best–strata'. The mappable data were then processed by the ERANGE v. 3.3 RNAseq analysis program (Mortazavi et al., 2008). Assuming total transcriptional activity is comparable between different cell types, the obtained data (data units in RPKM, reads per kilobase exon model per million mapped reads) were log2 transformed and linearly normalized among individual samples. At the same time, in order to find genes that were changed in expression between any two treatments to a statistically significant degree, ERANGE processed data were analyzed by the Bioconductor DEGseq program (Wang et al., 2010) (http://www.bioconductor.org/packages/2.6/bioc/html/DEGseq.html) (data units in FRPM, reads per million mapped reads, method = ''MARS,'' $p < 0.001$). This analysis yielded 1.740 DEGseq positive genes that had more than a 2-fold change in RNAseq reads (after normalization) between any two treatments, and these were defined as differentially expressed genes.

K-Means Clustering. To profile and categorize the behavior of clusters of similarly regulated genes by PSA treatment, we first normalized individual mRNA data for the 1,740 selected genes by the corresponding geometric mean of all three treatments, and then performed K-means clustering analysis on the results after log2 transformation (Figure S7). K was set at 8 and squared Euclidean distance was used (MATLAB 7.10.0).

Cecal Ligation and Puncture (CLP) Model. C57Bl/6 mice were retro-orbitally treated with either PSA or PBS every other day for two times before the surgery. Surgery was

performed according to a standard CLP protocol (Toscano et al., 2011). Briefly, mice were treated with eye ointment and buprenorphine before their abdomen were shaved. After scrubbing the shaved abdomen, a 1cm-incision was first made in the center of the abdomen. Cecum was then located and pulled out of the body cavity for ligation and puncture. In order to induce a medium-low level disease, we ligated the cecum at around 1 cm from the tip of the cecum and used 23G needle to made only one puncture at the tip of the cecum. Lastly, the incision was sutured up and mice were under close monitoring and regular injection of buprenorthine at each checkpoint. A scoring system was in place to assess the wellness of the animals in order to obtain the survival curve.

Statistical Analyses. Student's *t* test and one-way ANOVA were applied for pair-wise comparisons and comparisons among >2 groups, respectively. Significant differences among groups detected by ANOVA were analyzed using Newman-Keuls test as the post-hoc test to identify groups exhibiting statistically significant differences.

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