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Mechanism by Which Commensal Bacteria Limit Inflammation

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LOYOLA UNIVERSITY CHICAGO

MECHANISM BY WHICH
COMMENSAL EXOPOLYSACCHARIDES
LIMIT INFLAMMATION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

MALLORY LYNN PAYNICH

CHICAGO, ILLINOIS

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LIST OF ABBREVIATIONS

AMP	antimicrobial peptide
ANOVA	analysis of variance
AP-1	activator protein 1
APC	antigen-presenting cell
APRIL	A Proliferation-Inducing Ligand
Arg-1	arginase-1
BHI	brain heart infusion
BrdU	bromodeoxyuridine
C	celcius
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony-forming units
Clod-L	clodronate liposomes
COX2	cyclooxygenase 2
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CTV	CellTrace violet
CXCL1	chemokine (C-X-C motif) ligand 1
d	Day
DC	dendritic cell

DMSO	dimethyl sulfoxide
DNBS	dinitrobenzene sulfonic acid
dpi	days post-infection
dpt	days-post treatment
DSS	dextran sodium sulfate
EAE	experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
EPS	exopolysaccharide
FACS	fluorescence-activated cell sorting
FIZZ-1	found in the inflammatory zone 1
Foxp3	forkhead box P3
FSC	forward scatter
GALT	gut-associated lymphoid tissue
GIT	gastrointestinal tract
Gr	granulocytes
GVHD	graft-versus-host disease
h	hour
HCl	hydrochloric acid
HSC	hematopoietic stem cell
i.p.	intraperitoneal
i.v	intravenous
IBD	Inflammatory Bowel Disease

IFN- γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IL-4R α	interleukin 4 receptor alpha
ILC	innate lymphoid cell
ILF	isolated lymphoid follicle
iNKT	invariant Natural Killer T
iNOS	inducible nitric oxide synthase
IPEX	Immunedysregulation, Polyendocrinopathy, Enteropathy, X-linked
IRF4	interferon regulatory factor 4
iTreg	inducible regulatory T cell
LAP	latancy-associated peptide
LB	Luria-Bertani
LPM	large peritoneal macrophage
LPS	lipopolysaccharide
LTBP	latent TGF- β -binding protein
LTi	lymphoid tissue inducer cells
Ly6C	lymphocyte antigen 6 complex, locus C
Ly6G	lymphocyte antigen 6 complex, locus G
Lym	lymphocytes
MAM	microbioal anti-inflammatory molecule
MHCII	major histocompatibility complex class II

mL	milliliter
MLN	mesenteric lymph node
MMP	matrix metalloproteinase
MS	Multiple Sclerosis
MyD88	Myeloid Differentiation Primary Response 88
mye	myeloid
N	Normal
NaOH	sodium hydroxide
ND	not determined
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NK	natural killer
NOD	Non-obese diabetic mice
NOD-like	nucleotide-binding oligomerization domain-like receptors
nor-NOHA	N(omega)-hydroxy-nor-l-arginine
nos2	nitric oxide synthase 2
NT	no treatment
nTreg	natural regulatory T cell
o/n	overnight
OVA	ovalbumin
PBS	phosphate-buffered saline
PBS-L	PBS liposomes

PD-1	programmed cell death protein 1
PD-L1/2	programmed death ligand 1/2
pg	picogram
PGE ₂	prostaglandin E ₂
PPAR-γ	peroxisome proliferator-activated receptor gamma
PRR	pattern recognition receptor
PSA	polysaccharide A
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
RSV	respiratory syncytial virus
rTGF-β	recombinant TGF-β
SCFA	short-chain fatty acids
SFB	segmented filamentous bacteria
SPF	specific-pathogen free
SPM	small peritoneal macrophage
<i>spp.</i>	species
SSC	side scatter
ST2	suppressor of tumorigenicity 2
STAT	signal transducer and activator of transcription
TAM	tumor-associated macrophage
TCR	T cell receptor
TGF-β	transforming growth factor β

Th	T helper
TLR	toll-like receptor
TNBS	2,4,6-trinitro benzene sulfonic acid
TNF- α	tumor necrosis factor alpha
Tr1	type 1 regulatory T cells
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon- β
UC	Ulcerative Colitis
μg	microgram
μL	microliter
μM	micromolar
WT	wild-type

ABSTRACT

Trillions of bacteria live within the gastrointestinal tract and are critical for maintaining intestinal homeostasis; however, the mechanisms utilized by specific bacterial molecules to contribute to homeostasis are not well understood. We utilize a mouse model in which a single oral dose of the probiotic, *Bacillus subtilis*, protects mice from acute colitis induced by the enteric pathogen *Citrobacter rodentium*. Our goal is to elucidate the mechanism by which *B. subtilis* prevents inflammation.

We identified exopolysaccharides (EPS) to be the active molecule of *B. subtilis*, and a single dose of EPS protects mice from disease. EPS binds F4/80⁺CD11b⁺ peritoneal macrophages, and adoptive transfer of macrophage-rich peritoneal cells from EPS-treated mice confers protection from disease to recipient mice. Following EPS treatment, macrophages increase expression of CD206, arginase-1, YM-1, FIZZ-1, and IL-4R α , markers indicative of anti-inflammatory M2 macrophages. EPS does not protect TLR4-deficient mice from *C. rodentium*-induced disease, and as expected, M2 macrophages do not develop in TLR4^{-/-} mice following EPS treatment.

CD4⁺ T cells drive much of the inflammation associated with *C. rodentium* infection, and we hypothesized that EPS-induced M2 macrophages inhibit CD4⁺ T cell responses *in vivo*. Accordingly, we measured levels of IFN- γ (Th1), IL-17 (Th17), and IL-13 (Th2) in splenic T cells following EPS treatment and found

decreased levels of these cytokines. *In vitro*, EPS-induced M2 macrophages inhibit activation and proliferation of both CD4⁺ and CD8⁺ T cells. The inhibition of CD4⁺ T cells is dependent on TGF- β , whereas inhibition of CD8⁺ T cells is dependent on both TGF- β and PD-L1. We suggest that administration of *B. subtilis* EPS can be utilized to broadly inhibit T cell activation and thus control T cell-mediated immune responses in numerous inflammatory diseases.

CHAPTER ONE

LITURATURE REVIEW

Introduction.

Humans are home to trillions of bacteria that inhabit virtually all surfaces and mucosal tissues of the body. The gastrointestinal tract (GIT) shares the majority of the bacterial load, providing a home to 100 trillion individual organisms at a density of 10^{11} to 10^{14} cells per gram of luminal contents (Ley et al. 2008). Millions of years of co-evolution have interwoven many aspects of the microbiota and host; humans provide a nutrient rich environment and safe-haven and in exchange, bacteria provide paramount benefits to the host. Beneficial bacteria aid in food digestion, synthesize vitamins, provide essential nutrients, promote angiogenesis and enteric nerve function, defend against opportunistic pathogens, and contribute to the development and maintenance of the immune system (Hooper, Wong, A. Thelin, et al. 2001).

Disruption of the normal microbiota which can occur through diet, antibiotic use and other environmental factors, contributes to a wide range of diseases (Hooper et al. 2012). Western civilization has brought an era of increased sanitation, widespread overuse of antibiotics, and alterations in diet. These changes have been accompanied by dramatic increases in allergy and asthma, IBD, obesity, and diabetes, all of which are associated with dysbiosis of

the microbiota, often in individuals with increased genetic susceptibility to these diseases.

Currently, we have limited knowledge of the mechanisms by which bacteria exert beneficial effects or how they can be used to treat disease. The co-evolution of the microbiota and the immune system has given rise to very complex and intricate mutualistic-symbiotic relationships, while many bacterial species may be true commensals. Of the thousands of different species that reside in the GIT, only a handful of organisms have been identified to have immunomodulatory functions. Additionally, several organisms not typically thought to be a part of the microbiota, such as those present in fermented foods such as yogurt, possess beneficial effects as probiotics.

Given the complexity of the established interactions, many other species undoubtedly exert a wide array of effects not only on the immune system, but other physiological processes, as well. Additionally, some species may promote similar pathways to those already identified to maintain homeostasis.

Outstanding questions: how does the host respond to bacterial colonization? Do bacteria promote normal development of the host? How do different bacteria promote immunity? Can beneficial bacteria be used as therapeutics? All of these are important questions in understanding how the microbiota contributes to the health of the individual, and importantly, how the microbiota can be used to treat disease.

Section 1: Role of the Microbiota in the Development of the Immune System

Early-life Microbial Exposure.

Our current understanding states that the fetal gastrointestinal tract is sterile. At birth, upon passage through the birth canal, the baby is exposed to microbes and seeding of the skin and gastrointestinal microbiota begins. This marks the first interaction between the immune system and bacteria, viruses and fungi, commencing the training of the immune system to tolerate these microbes. In infants born vaginally, *Lactobacillus* species, similar to the vaginal microbiome, comprise the majority of the gut microbiota. In infants born via cesarean section, the gut microbiota resembles the skin microbiome of the mother, including *Staphylococcus*, *Streptococcus* or *Propionibacteria* (Dominguez-Bello et al. 2010). Additionally, Pre-term birth is often associated with intrauterine infection and a breakdown of maternal-fetal tolerance, leading to an altered microbiome (Romero et al. 2014). By two years of age, the microbiota compositions between these groups are fairly similar; however, a delay in exposure to certain bacteria during this crucial developmental period for the immune system highly correlates with immune disorders later in life (Bäckhed et al. 2015; Weng & Walker 2013).

The tolerogenic environment of the neonate allows colonization of the microbes to occur, without eliciting a strong inflammatory response. Neonates have blunted inflammatory cytokine responses and a high percentage of regulatory T cells, which help maintain tolerance to the influx of microbes. This

immunosuppressed state, however, leaves the infant highly susceptible to infection and unable to mount protective responses following vaccination. Recently, impaired neonatal host defense to bacterial infections has been attributed to highly-suppressive erythroid cells (Elahi et al. 2013). These cells actively protect against excessive inflammation triggered by commensal microbes, allowing colonization to occur, which seemingly outweighs the risk of infection.

Following birth, nutrition, environmental factors, host genetic factors, and antibiotic use impact the developing microbiota. During this immunosuppressed state, breast milk provides nutrients, bacteria and immune protection to the infant (Koenig et al. 2011). Oligosaccharides present in breast milk influence the final composition of the infant microbiota to an adult-like state by promoting the expansion of *Bifidobacteria* and *Bacterioides* species (Marcobal et al. 2010). Additionally, maternal IgA in the breast milk shapes the neonatal microbiota by binding to nutrients or bacteria, allowing selection of certain bacteria, including *Bifidobacteria* and *Bacterioides* species, in the GIT (Peterson et al. 2007).

Bacteria that colonize the infant GIT play a vital role in fine-tuning of the developing immune system. The mode of delivery and host genetics contribute to the colonization of the microbiota in infants and development of immune responses. Recent evidence suggests that infants may also be exposed to microbial products that cross the placenta (Aagaard et al. 2014), suggesting that microbes may begin to shape the immune system prior to birth. At birth, T helper (Th)-2-dominant immune responses prevail, but colonization with

Bacterioides spp. contributes to Th1 skewing (Weng & Walker 2013). In formula-fed infants compared to breast-fed infants, and more dramatically in infants born via cesarean section compared to vaginal delivery, the microbiome is less diverse and Th1-skewing does not occur (Dominguez-Bello et al. 2010). Indeed, many of the diseases associated with early-life alterations in the microbiota are Th2-driven immune disorders.

Environmental factors also contribute to the maturation of the microbiota. In Western civilizations, relatively sterile living environments and good hygiene limit early childhood exposure to microbes. Additionally, the high-prevalence of antibiotic use in infants and in early childhood dramatically alters colonization. This dysbiosis is associated with increased risk of asthma, atopic dermatitis, allergy and autoimmune disorders (Sellitto et al. 2012). Children with early-life exposure to cats and dogs, presumably a less clean environment, have a decreased risk of developing asthma and allergy (Ownby et al. 2002). Further, children with siblings or living on or near a farm have lower rates of hay fever and atopic disorders compared to children raised in cities (Genuneit et al. 2013).

These observations and studies in humans demonstrate the importance of the microbiota early in life, but have not elucidated the mechanisms by which the microbiota shapes the immune system, and in turn, the immune system shapes the microbiota.

Influence of Microbiota on Development of Lymphoid Tissues.

Animal models have provided mechanistic insights for these correlations seen in humans. Germ-free animals have extensive immune system defects

compared to conventionally raised animals. In rabbits, development of gut associated lymphoid tissue (GALT) requires the microbiota (Perey & Good 1968; Stramignoni et al. 1969). Further, interactions between GALT and the intestinal microbiota stimulate development and expansion of the preimmune antibody repertoire (Lanning et al. 2000; Rhee et al. 2005). Similarly in mouse GALT, Peyer's patches, mesenteric lymph nodes (MLN), the cecal patch and isolated lymphoid follicles (ILF) all show developmental defects in size and function in germ-free mice compared to mice housed under normal conditions (specific-pathogen free (SPF) mice) (Falk et al. 1998; Bouskra et al. 2008). Although lymphoid tissue inducer (LTi) cells (reclassified as ROR γ t⁺ Innate Lymphoid Cells (ILC)-3, discussed later) induce development of lymph nodes and Peyer's patches prior to birth, selective colonization of germ-free mice restores these underdeveloped tissues. In some cases, such as ILFs, bacterial molecules appear to be sufficient. In contrast to GALT that begins development in a sterile environment, ILFs develop from LTi-like cryopatches and recruited B cells in the lamina propria (Pabst et al. 2006; Eberl & Littman 2004), and induction of this entire process requires the gut microbiota (Hamada et al. 2002; Pabst et al. 2006; Pabst et al. 2005). Specifically, peptidoglycan from Gram (-) bacteria induces intestinal ILF formation in germ-free mice in a NOD1-dependent manner (Bouskra et al. 2008).

Interactions between the Immune System and Microbiota During Development.

Humoral immune responses. Developing lymphoid tissues support important cellular responses to the microbiota. The generation of IgA-producing B cells that recognize the microbiota occurs in GALT. Normally, dendritic cells within the Peyer's patch phagocytose bacteria and promote IgA production by B cells. Secretion of IgA then limits bacterial penetration through the epithelium (Macpherson & Uhr 2004; Macpherson et al. 2001). Germ-free mice have decreased antibody responses; in particular, secretory IgA in the colon (Moreau et al. 1978). Colonization with several different bacterial species restores IgA production, as well as expression of the polymeric IgA receptor (Moreau et al. 1978; Hooper, Wong, Thelin, et al. 2001; Macpherson & Uhr 2004). In the absence of class switch recombination, somatic hypermutation and IgA production, a 100-fold expansion of anaerobic flora occurs in the small intestine, demonstrating a vital role for IgA in promoting homeostasis (Fagarasan et al. 2002).

T Helper cell responses: Th1 and Th2 balance. *Bacteriodes fragilis* is the best-characterized commensal in terms of immune regulatory and developmental effects. *Bacteriodes* species colonize early in development. As discussed above, these species rapidly proliferate in response to oligosaccharides present in breast milk thereby constituting a major portion of the bacteria present in the GIT. *B. fragilis* is a Gram (-) anaerobic bacteria that produces several capsular polysaccharides. One particular polysaccharide, polysaccharide A (PSA), is zwitteronic, possessing both positive and negative charges (Mazmanian et al. 2005). *In vitro*, antigen-presenting cells (APCs) internalize,

process and present PSA, which leads to proliferation of CD4⁺ T cells (Brubaker et al. 1999; Tzianabos & Kasper 2002; Cobb et al. 2004). Due to these immunomodulatory effects, the Kasper lab examined the effects of PSA and *B. fragilis* in germ-free mice. SPF mice have a greater proportion of CD4⁺ T cells compared to germ-free mice. Upon mono-colonization of germ-free mice with *B. fragilis*, CD4⁺ T cells expand and normal development of splenic lymphoid follicles occurs. These effects require PSA production by *B. fragilis*, and purified PSA is sufficient to recapitulate these effects. *In vitro*, PSA upregulates the production of the cytokine, IFN- γ , in T cells (Mazmanian et al. 2005).

Naïve CD4⁺ T cells differentiate into several T helper cell effector subsets based on their environment. Thus far, six subsets have been identified: Th1, Th2, Th17, Th9, Th22 and Treg cells, but new subsets of T helper cells are continuously being described (Nakayamada et al. 2012). Th1 cells differentiate in the presence IL-12 and IL-18 and produce IFN- γ and TNF- α to protect against intracellular pathogens. Th2 cells are induced by IL-4 and produce IL-4, 5, 6 and 13. They mediate responses to extracellular pathogens such as helminthes, and have been implicated in allergy and asthma. TGF- β and IL-6 promote Th17 cells. These cells are involved in immunity to mucosal pathogens and contribute to autoimmune disorders. Th9 and Th22 cells have recently been identified to contribute to tumor and skin/barrier immunity, respectively. Th9 cells differentiate in response to IL-4 and TGF- β , and produce IL-9, whereas Th22 cells differentiate in the presence of IL-6 and TNF- α and produce IL-22 (Nakayamada et al. 2012).

At the time when Kasper and colleagues determined the involvement of PSA in CD4⁺ T cell development, Th1 and Th2 cytokines were the only well-established subsets. Th1 cytokine production suppresses Th2 responses; conversely, Th2 cytokine expression inhibits Th1 responses. Proper balance of T helper cell effector responses plays a critical role in human health. As mentioned above, at birth, prior to colonization, humans have a Th2-skewed environment, which is also seen in germ-free mice. Colonization of germ-free mice with *B. fragilis* restores the Th1/Th2 balance to that of SPF mice in a PSA-dependent manner (Mazmanian et al. 2005). These studies demonstrate an essential role for PSA in shaping mammalian immune development by stimulating normal splenic CD4⁺ T cell numbers, establishing and maintaining Th1/Th2 balance, and thereby directing splenic organogenesis. Since other T helper subsets have been identified, new studies are underway to determine the role of the microbiota in the development and maintenance of each subset.

Invariant natural-killer T cells. Microbial exposure early in life also diminishes excessive responses of inflammatory cells. Invariant natural-killer (iNKT) cells accumulate in the lamina propria and the lung in germ-free mice. iNKT cells are implicated in the pathogenesis of ulcerative colitis (UC). In mouse models, iNKT cells mediate oxazolone-induced colitis and germ-free mice have exacerbated disease compared to SPF mice (Olszak et al. 2012; An et al. 2014). Similar results are seen in asthma models, suggesting that microbiota are required to control iNKT cell responses. Interestingly, colonization of neonatal, but not adult mice, with a conventional microbiota alleviates disease. iNKT cells

express CD1d, an MHC class I-like molecule that presents lipid antigens. The protective effects have been attributed to a single sphingolipid produced by the *B. fragilis*. The sphingolipid GSL-Bf717, purified from *B. fragilis*, reduces iNKT cell proliferation, and neonatal mice treated with this sphingolipid are protected from oxazolone-induced colitis later in life (An et al. 2014). These studies demonstrate that early-life exposure to microbes establishes tolerance and without these exposures, stimulation of iNKT cells, and perhaps other inflammatory cells, later in life can be detrimental to the host.

Section 2: Maintaining Homeostasis Between the Immune System and Microbiota

Epithelial cells. Intestinal epithelial cells provide a physical barrier to the luminal contents of the GIT. Although thick layers of mucus separate a vast majority of microbial species from direct contact with the epithelium, specialized epithelial cells including paneth cells and M cells can directly sense or take up bacteria. Further, dendritic cell protrusions through the epithelial layer sample the microbiota and produce cytokines to alter epithelial cell responses. Germ-free mice exhibit decreased epithelial cell turnover, altered microvilli formation, and decreased expression of cytokines and PRRs, suggesting bacteria contribute to epithelial cell responses (Artis 2008).

Interactions between intestinal epithelial cells and immune cells are critical in maintaining homeostasis with the microbiota. T cell cytokines contribute to epithelial barrier integrity by regulating epithelial permeability, proliferation, repair, and expression of tight junctions, mucins and anti-microbial

peptides (Artis 2008). Distribution of TLRs and other PRRs on epithelial cells remains controversial. Epithelial cells minimally express TLRs on the luminal surface, or, in some studies, the expression is below the limits of detection. In contrast, these cells seem to express TLRs on the basolateral side of the cells, allowing the innate immune system to rapidly detect bacteria that may breach the barrier (Abreu 2010). Specialized epithelial cells, however, directly sense bacteria via innate immune mechanisms. Paneth cells detect *Bacteroides thetaiotaomicron*, and potentially other bacteria, leading to a MyD88-dependent upregulation of the anti-microbial peptide RegIIIγ (Vaishnava et al. 2008; Sonnenburg et al. 2006). In the absence of this pathway, mice are highly susceptible to infection with *Listeria monocytogenes* (Brandl et al. 2007).

Epithelial cells also contribute to IgA production. Direct sensing of commensal bacteria by TLR upregulates A Proliferation-Inducing Ligand (APRIL) expression in epithelial cells, which activates dendritic cells (DCs) to promote class switch to IgA₂ (He et al. 2007). It is unclear if these bacteria were sensed by TLR in the lumen or if bacteria translocate through M cells and are then detected by epithelial cells.

Immunoglobulin A. IgA contributes to homeostasis at mucosal surfaces and is highly produced in the gut. As discussed above, the microbiota contribute to IgA production during development. IgA not only functions to eliminate pathogens and limit their ability to infiltrate the mucosa, but also directs the composition of the microbiota. IgA contributes to the selection of Firmicutes in a complex process: Firmicutes induce expansion of Treg cells, that

induce germinal centers and Firmicute-specific IgA, leading to their retention in the GIT, demonstrating a feedback loop between the microbiota and adaptive immune cells (Kawamoto et al. 2014). Other studies suggest that location within the GIT, rather than specific bacterial species, induced commensal-specific IgA. B1b cells that traffic from the peritoneal cavity to the small intestine and B2 cells give rise to T-independent IgA-producing plasma cells specific to commensal bacteria. Certain bacteria, including Segmented Filamentous Bacteria (SFB), evade T-independent responses and instead induce T-dependent B2 cell production of IgA (Bunker et al. 2015).

Innate lymphoid cells (ILCs). More recently, ILCs have been identified to play a critical role in maintaining intestinal homeostasis through direct and indirect interactions with the microbiota. ILCs are most likely the main source of 'T cell cytokines' previously established, and discussed above, to contribute to intestinal homeostasis. Development of the three ILC subsets appears to occur independently of commensal bacteria, although there are many conflicting reports (Monticelli et al. 2011; Vonarbourg et al. 2010; Bouskra et al. 2008; Tsuji et al. 2008). More consistently, it seems each subset of ILCs has impaired function in the absence of bacteria. Recently, genome-wide screens of intestinal ILCs in SPF mice compared to antibiotic-treated or germ-free mice revealed that the microbiota contribute to epigenetic regulation and gene expression in intestinal ILCs (Gury-BenAri et al. 2016).

ILCs and microbiota interactions occur through both direct and indirect mechanisms. Some ILCs have limited expression of TLRs that can respond to

viruses and bacteria to enhance anti-viral and bacterial responses. Additionally, metabolic by-products generated by commensals also stimulate ILC responses (Stockinger et al. 2011). ILCs can also shape the microbiota through indirect interactions by producing cytokines that influence epithelial cell permeability and production of antimicrobial peptides (Clark et al. 2005; Mullin & Snock 1990; Monticelli et al. 2011). Conversely, microbes stimulate cytokine production by epithelial cells and myeloid cells that can activate ILCs (Vonarbourg et al. 2010; Hughes et al. 2010).

T helper cell responses: Th17 induction. Several years after the discovery of the immunomodulatory functions of PSA, Th17 cells were identified as a new T helper cell subset (Mangan et al. 2006). These cells produce IL-17A, IL-17F and IL-22 and have been implicated in both inflammatory and anti-inflammatory processes. During bacterial and fungal infections, these cells provide vital mucosal immunity, but excessive activation of these cells contributes heavily to autoimmune disorders (Mangan et al. 2006). Th17 cells accumulate in high numbers in the GIT and are notably absent in germ-free mice, suggesting their development is induced by the microbiota (Ivanov et al. 2008; Ivanov et al. 2009). Indeed, re-colonization of germ-free mice restores numbers of Th17 cells within the lamina propria and colon. Interestingly, identical mouse strains purchased from different commercial vendors had strikingly different levels of Th17 cells within the small intestine. Upon analysis of the microbiota of mice from each vendor, mice with no Th17 cells also had undetectable levels of SFB compared to mice with Th17 cells from a different vendor (Ivanov et al.

2008). In germ-free mice or in conventional mice lacking Th17 cells, colonization with SFB potently induces Th17 cells and associated cytokine production in the lamina propria (Ivanov et al. 2009). SFB are Gram (+) spore-forming anaerobic bacteria highly prevalent in the gut microbiota. SFB colonize the terminal ileum of mice, humans, and a variety of other vertebrates including non-human primates, birds, frogs and fish (Klaasen et al. 1993), suggesting SFB and the immune system have evolved this symbiotic relationship for a vast period of time. In fact, SFB colonization appears to be species specific. Whereas most bacteria lie in the upper of two mucus layers, SFB penetrate through the thin upper and thick lower mucus layers and intimately attach to epithelial cells, leading to actin reorganization. In response to SFB binding, epithelial cells upregulate serum amyloid A and reactive oxygen species, which act on myeloid cells to upregulate Th17-inducing cytokines, thereby inducing Th17 cells (Atarashi et al. 2015). This tight adhesion with epithelial cells is species specific in that mouse SFB bind specifically to mouse epithelial cells and cannot be transferred to rats, and vice versa (Atarashi et al. 2015; Prakash et al. 2011). These studies suggest that SFB have undergone host-specific adaptation. In exchange for potent immune modulation, the host provides the auxotrophic SFB with amino acids and nutrients essential for its survival (Sczesnak et al. 2011).

Prior to the discovery that SFB induce Th17 cells, SFB were identified to stimulate secretory IgA production and recruitment of intraepithelial lymphocytes (Umesaki et al. 1999; Talham et al. 1999). Recently, Hirota et al. established a link between SFB induction of Th17 cells and IgA production. In

Peyer's patches, SFB-induced Th17 cells acquire a T follicular helper cell phenotype and induce IgA production by germinal center B cells (Hirota et al. 2013). These studies highlight nearly thirty years of research on independent diverse subjects coalescing into a remarkable mechanism by which a commensal bacterium has evolved a highly complex interaction with the host immune response.

The development of tolerance between the colonizing bacteria and the host response is pertinent for the health of the host. The picture of how the immune system and commensal bacteria work together to maintain this environment is constantly evolving as our understanding of new immune cells and bacteria change with newly developed technologies. What is clear is that defects in any of these arms of immunity and host defense leave the host susceptible to an array of immune disorders.

Section 3: Induction of Regulatory Responses by Commensal Bacteria

Aberrant immune responses to commensal bacteria are prevented through multiple different mechanisms. The mucus layers and IgA prevent direct association of the bacteria with epithelial and immune cells; T helper cells and ILCs produce cytokines to reinforce the barrier through antimicrobial peptide and mucin production; but what maintains these inflammatory cells from excessive responses? A failure to regulate these responses can lead to diseases including IBD, allergies, and metabolic syndromes.

T Helper Cell Responses: Regulatory T cells.

Regulatory T cells (Treg) maintain peripheral and mucosal tolerance.

These cells develop in the thymus (nTreg) under control of the master regulator, Foxp3, but can also be induced (iTreg) from naïve CD4⁺ T cells in the periphery. In the absence of Treg cells, the immune system runs rampant, leading to exacerbated autoimmune disorders; these mice develop a fatal lymphoproliferative disease, leading to multi-organ failure, and die within 3-5 weeks of age (Brunkow et al. 2001).

In humans, mutations in Foxp3 lead to IPEX autoimmune syndrome (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome), and most children die within the first two years of life (Wildin et al. 2001). Treg cells suppress immune responses through the effector molecules CTLA-4, Transforming Growth Factor- β (TGF- β) and IL-10. The latter anti-inflammatory cytokines act on a wide array of cells and potently inhibit cell activation and proliferation. In the GIT, Treg cells also promote class switch to IgA via TGF- β production, demonstrating another mechanism by which this essential molecule is induced (Cong et al. 2009).

Inducible regulatory T cells (iTreg). In the gut, iTreg cells play an essential role in the induction of tolerance to food and other oral antigens (Coombes et al. 2007; Mucida et al. 2005). The microbiota help generate the regulatory environment necessary to induce iTreg cells; Treg cell numbers and production of effector cytokines are reduced in germ-free mice. Gut-resident DCs promote iTreg development through TGF- β and vitamin A (Coombes et al. 2007) and many of the signals driving the regulatory DCs come from the microbiota. Clostridia, which comprise a large portion of the gut microbiota, play an

instrumental part in iTreg induction. The Clostridia clusters IV, XIVa and XVIII provide bacterial antigens and induce a TGF- β -rich environment, resulting in substantial increases in iTreg cells (Atarashi et al. 2011; Atarashi et al. 2013).

Induction of regulatory T cells by *Bacteroides fragilis*. In addition to expanding CD4⁺ T cell populations within the spleen of germ-free mice, *B. fragilis* also generates iTreg cells in a process requiring PSA. *B. fragilis* secretes PSA-filled outer membrane vesicles that are taken up by dendritic cells in a TLR2-dependent manner. DCs process and present PSA to T cells via MHC class II, thereby inducing IL-10–producing Treg cells (Shen et al. 2012). PSA can also directly induce and expand nTreg cells when administered to germ-free mice, or in germ-free mice monocolonized with *B. fragilis* (Round & Mazmanian 2010; Mazmanian et al. 2008; Round et al. 2011). *B. fragilis* PSA is a single bacterial molecule capable of both inducing development of immune responses and regulating immune responses. Although this molecule is the best characterized, it is likely not alone; more bacteria and specific bacterial molecules are likely to be identified to critically regulate immune responses.

Induction of regulatory T cells by microbial metabolites. A long understood benefit afforded by the microbiota is aiding in food digestion. As they break down food, the microbiota produces many metabolites, including short-chain fatty acids (SCFA). SCFA, and in particular butyrate, not only induce differentiation of iTreg cells, but also expand colonic nTreg cells (Smith et al. 2013; Furusawa et al. 2013), demonstrating that bacterial metabolites as well as physiological factors such as polysaccharides contribute to shaping immune

responses. In Westernized countries, plant-based diets have decreased in popularity, which may contribute to dysregulation of the microbiota and Treg cell development, providing a possible link to disease correlations mentioned previously. Although much of the focus on the capacity for the microbiota to regulate immunity has been focused on Treg cells, other regulatory cells undoubtedly play a part in sustaining this environment.

Section 4: Probiotics and Disease

Clearly, commensal microbes provide integral benefits to the development and regulation of the immune system and other bacteria undoubtedly share these beneficial properties. Can we harness the power of these bacteria to treat human disease? Probiotics are live microorganisms considered to benefit the health of the host. The mechanisms by which probiotics benefit the host, and which specific bacterial species among the hundreds found in different probiotics currently on the market are not well appreciated. As we grow in our understanding of how commensal bacteria influence the immune system, these bacteria can be rationally be used as probiotics to treat and prevent different ailments.

History of the Microbiota as a Therapeutic.

The use of the gastrointestinal microbiota as a therapeutic was first documented in ancient Egypt in 1700 BC in papyrus texts, although it most likely dates back further (Parkins, 2001). Excrement of different animals was used for a variety of internal and external ailments and thought to magically repel demons (Parkins, 2001). In the 4th century, Chinese physicians treated diarrhea with

yellow soup, a broth consisting of dried stool from a healthy individual. These anecdotes continue throughout history, from Antiquity to the Italians during the Renaissance. From these early examples of ‘fecal transplants,’ modern physicians and scientists developed invaluable treatments for many more diseases.

Early in the 1900s, Ilya Mechnikov developed the first probiotics in today’s understanding of the term. Fearing toxic byproducts of the GIT poisoned tissues and contributed to senility, he believed fermented dairy products possessed health benefits. He hypothesized that the microbiota was the main cause of the poisoning and that if consumed regularly, the bacteria in fermented milk would replace the damaged microflora by altering the intestinal phagocyte population (Metchnikoff & Metchnikoff 1908).

Decades later, the idea would take hold again, with the return of fecal transplants to treat enteric disease. The first documentation in a modern medical journal comes from Dr. Ben Eiseman who used fecal transplants to cure four patients suffering from pseudomembranous colitis induced by *Clostridium difficile* infection in 1958 (Eiseman et al. 1958).

Clearly the microbiota possessed a powerful therapeutic benefit that took modern scientific advances to begin to tease apart. For years, people speculated that the benefits exerted by these transplants were due to antibiotic production or metabolites produced by the bacteria. Today, hundreds of fecal transplants have been performed to treat *C. difficile* infection, and probiotics constitute a multi-billion dollar industry world-wide. The numbers of implications are growing, but

how the microbiota and specific probiotics contribute to health benefits is not understood. Finally, after significant technological advances, we now understand that the mechanisms of the benefits exerted by the microbiota are more complex and far-reaching into the immune system than we had ever imagined.

Prevention of Colitis by Probiotics.

As we have already discussed, germ-free mice are wrought with problems in the immune system. As expected, germ-free mice are highly susceptible to bacterial, viral and fungal infections (Carthew & Sparrow 1980; Taguchi et al. 2002; Kamada et al. 2012). These studies suggest that when dysbiosis of the microbiota occurs, a normal microbiota or probiotics can be added back to restore the normal homeostatic balance between the immune system and the microbiota.

Studies of probiotics in disease prevention, as with fecal transplants, have mainly focused on models of inflammatory bowel diseases (IBD), or pathogen-induced colitis. IBD are chronic inflammatory disorders of the small intestines and colon, in which patients suffer from rectal bleeding, severe diarrhea, abdominal pain, fever, and weight loss (Strober et al. 2007). In many cases of IBD, the mucosal barrier to the microbiota breaks down, and the immune system mounts improper responses to the microbiota. Host genetics and dietary influences can also contribute to IBD. All of these factors can lead to a loss of tolerance to the microbiota, resulting in excessive inflammation and disease. Mouse models of IBD attempt to take into account many of these different facets of disease. Chemically-induced colitis models utilize a variety of chemicals and

haptens to cause damage in the intestines and mimic the breakdown of the mucosa and homeostasis, similar to what occurs in IBD. Exacerbated T helper cell responses drive many of these models of chemically-induced colitis, including 2,4,6-trinitro benzene sulfonic acid (TNBS)-, dinitrobenzene sulfonic acid (DNBS)-, and oxazolone-induced colitis. DSS is believed to be directly toxic to colonic epithelial cells and induce gut leakiness, allowing the bacterial products and bacteria to infiltrate the lamina propria (Wirtz et al. 2007). Other models more directly exploit the T cell-mediated nature of colitis or host genetic contributions. IL-10^{-/-} mice spontaneously develop colitis at 8-10 weeks of age, since IL-10 is a main contributor to mucosal tolerance. Additionally, adoptive transfer of naïve T cells into *Rag1*^{-/-} mice induces a similar pathology to Crohn's disease in humans (Ostanin et al. 2009), as do SAMP1/YitFc (SAMP) mice which have chronic Crohn's Disease-like ileitis (Pagnini et al. 2010).

In models of pathogen-induced colitis, pathogens including *Helicobacter hepaticus*, *Salmonella enterica*, and *Citrobacter rodentium* induce damage, inflammation and disease through complex immune responses, although inflammatory T cell responses contribute to much of the inflammation. Many of these are human enteric pathogens or mouse models of human pathogens, and give clues as to how probiotics can be used to treat human infections.

Probiotics can limit disease in several different ways. First, in the case of pathogen infection, they can block colonization of the invading pathogen or compete for nutrients, not allowing the pathogen access to the specific niche required for infection. Additionally, probiotics can increase barrier integrity.

This can occur by promoting antimicrobial peptide (AMP) production (or producing AMPs themselves), increasing mucus production, or upregulating tight junctions between epithelial cells, decreasing permeability. Last but not least, probiotics can alter the host immune response, leading to protection from disease.

Colonization. Many pathogens need to occupy a certain niche within the gastrointestinal tract to upregulate production of toxins or virulence factors and induce disease. Many probiotics are thought to block adherence and colonization of pathogens, ultimately preventing disease. *Bifidobacterium breve* produces exopolysaccharide to promote its own colonization within the GIT, which in turn prevents colonization with the enteric pathogen *C. rodentium* (Fanning et al. 2012). Although *B. breve* limits pathogen colonization, it must first modulate the immune response to promote its own persistence. *B. breve* produces exopolysaccharide (EPS) that suppresses B cell responses to *B. breve*. In a strain of *B. breve* deficient in EPS production, high titers of antibody to *B. breve* are present in the feces, and colony-forming units (CFUs) of *B. breve* are drastically reduced, indicating that EPS production is crucial for *B. breve* persistence. Further, EPS-deficient strains do not limit *C. rodentium* colonization, demonstrating the importance of EPS production in disease prevention.

SFB also help to limit infection with *C. rodentium* by decreasing bacterial colonization in the colon. Induction of Th17 cells by SFB leads to upregulation of a signaling cascade that increases expression of IL-23, IL-22 and the AMP RegIII γ that are vital to controlling *C. rodentium* infection (Ivanov et al. 2009).

Commensals and probiotics can also inhibit pathogen colonization by competing for nutrients or producing inhibitory molecules including anti-microbial peptides and antibiotics. *B. thetaiotaomicron* competes with *C. rodentium* for carbohydrates (Kamada et al. 2012), directly inhibits toxin production by enterohaemorrhagic *E. coli* (De Sablet et al. 2009), and also upregulates RegIII γ , an anti-microbial peptide that binds Gram (-) bacteria (Vaishnava et al. 2008). *Bifidobacterium* species produce peptides that serve as antibiotics to pathogenic bacteria (Trejo et al. 2006; Gagnon et al. 2004), demonstrating that certain species exert a plethora of beneficial effects.

Antibiotic treatment increases susceptibility to a wide-range of infections and allows for proliferation of antibiotic-resistant pathogens such as vancomycin-resistant *Enterococcus* species and *C. difficile* (Stecher et al. 2005; Barthel et al. 2003; Buffie & Pamer 2013). Cocktails of probiotics decrease pathogen colonization and limit disease (Felley & Michetti 2003; Siggers et al. 2008). Even though these bacteria decrease pathogen colonization, many of the identified mechanisms involve innate immune modulation. More in depth analysis of the molecular mechanisms by which probiotics limit pathogen colonization may identify novel pathways of immune modulation.

Epithelial barrier integrity. Probiotics also promote epithelial barrier integrity to prevent colitis; however, similar to prevention of colonization, these effects are often due to alterations in innate immune responses. The probiotic cocktail VSL#3 stimulates TNF α production in epithelial cells to promote barrier integrity, which prevents ileitis in SAMP mice. Several studies have

demonstrated that probiotics such as *E. coli* Nissle, *Lactobacillus* species, and VSL#3 increase barrier function and anti-microbial peptide expression in cell-culture models *in vitro* (Schlee et al. 2008; Wehkamp et al. 2004; Schlee et al. 2007). *Lactobacillus* species and VSL#3 require MAP kinase, and NF- κ B and AP-1 pathways, demonstrating that these effects require innate immune sensing of bacteria or bacterial products (Schlee et al. 2008).

Modulation of immune responses. Probiotics clearly prevent enteric diseases by modulating innate immune response, leading to decreased pathogen colonization and increased barrier integrity. But how do probiotics limit IBD? Many beneficial microbes appear to enhance anti-inflammatory responses through modulation of adaptive immune responses (Table 1.1).

Table 1.1. Commensal Bacteria with Beneficial Immunomodulatory Effects on Inflammatory Diseases.

Bacteria	Elicited Immune Response	Bacterial Molecule Responsible for Protection	Inflammatory Disease	Ref
<i>Bacillus subtilis</i>	TLR4-dependent induction of M2 macrophages	Exopolysaccharide (EPS)	<i>C. rodentium</i> -induced colitis	Jones et al.
<i>Bacteriodes fragilis</i>	TLR2-dependent induction of iTreg by IL-10 ⁺ DCs	Polysaccharide A (PSA)	<i>H. hepaticus</i> -induced colitis, TNBS-induced colitis, EAE	Mazmanian et al.; Round et al.; Shen et al.; Ochoa-Rodriguez et al.
<i>Bifidobacterium spp.</i>	Induction of Tr1 and Treg cells; reduction of <i>B. breve</i> -specific antibody production	Possibly Exopolysaccharide	<i>C. rodentium</i> -induced colitis	Jeon, et al.; Fanning, et al.
<i>Bacteriodes fragilis</i>	Negative regulation of iNKT cells in neonatal mice	Sphingolipid GSL-Bf717	Oxazolone-induced colitis	An et al.
<i>Faecalibacterium prausnitzii</i>	Inhibition of NF-κB in intestinal epithelial cells	Anti-inflammatory protein MAM	DNBS- induced colitis	Sokol et al.; Quévrain et al.
<i>Clostridium butyricum</i>	TLR2-dependent induction of IL-10 ⁺ F4/80 ⁺ CD11b ⁺ CD11c ^{int} cells	n/a	DSS-induced colitis	Hayashi et al.
<i>Clostridia spp.</i>	Induction of IL-10 ⁺ Treg; upregulates IL-22 by ILC	n/a	DSS-induced colitis, food allergy	Sterka et al.; Atarashi et al.; Cao et al.
<i>Lactobacillus spp.</i>	Reduction of pro-inflammatory mucosal cytokines	n/a	<i>H. hepaticus</i> -induced IBD	Peña et al.
Segmented filamentous bacteria	Induction of Th17 cells; IgA production	n/a	<i>C. rodentium</i> -induced colitis, T1D	Ivanov et al.; Kriegel et al.
VSL#3	Induction of TNF-α production by epithelial cells; IL-10 ⁺ TGF-β ⁺ T cells	n/a	Chronic CD-like ileitis (SAMP mice), TNBS-induced colitis	Pagnini et al.; Di Giacinto et al.

Abbreviations: CD- Crohn's Disease; DCs- Dendritic cells; DSS- Dextran sulfate sodium; DNBS- Dinitrobenzene sulfonic acid; EAE- Experimental autoimmune encephalomyelitis; GIT- Gastrointestinal tract; iTreg- Inducible regulatory T cells; IBD- Inflammatory bowel disease; ILC- Innate Lymphoid Cell; MAM- Microbial anti-inflammatory molecule; n.d.- not determined; TNBS- 2,4,5-Trinitrobenzenesulfonic acid; T1D- Type 1 Diabetes

As discussed above, *B. fragilis* and Clostridia strains help maintain homeostasis between the immune system and microbiota through induction of Treg cells. Upon disruption of this homeostatic environment with enteric pathogens or in models of chemically induced colitis, administration of *B. fragilis*, or the active component PSA, alleviates disease induced by *H. hepaticus* and TNBS-induced colitis (Mazmanian et al. 2008; Shen et al. 2012). Further, Clostridia strains induce and expand Treg cells through butyrate production, and protect mice from DSS- and TNBS-induced colitis, as well as T cell transfer-induced colitis (Atarashi et al. 2011; Furusawa et al. 2013; Atarashi et al. 2013; Smith et al. 2013).

Other probiotics, including *B. breve* and other Bifidobacteria species, VSL#3 and *Lactobacillus rhamnosus* GG, also induce IL-10 or TGF- β producing Tr1 cells and iTreg cells that ameliorate disease in several inducible mouse models, both pathogen and chemical, of colitis (Jeon et al. 2012; O'Mahony et al. 2008; Peña et al. 2005; Di Giacinto et al. 2005). These studies demonstrate the importance of regulatory T cells in maintaining intestinal homeostasis and their versatility in limiting inflammation.

Few Treg cell-independent mechanisms have been established, but probiotics potentially modulate other regulatory cells. The probiotic *Clostridium butyricum* mediates protection from DSS-induced colitis in a Treg-independent manner, through TLR2-dependent, IL-10-producing F4/80⁺CD11b⁺CD11c^{int} macrophages (Hayashi et al. 2013). Interestingly, inhibition of iNKT cells by *B. fragilis* spingolipids at birth is essential for control of oxazalone-induced colitis

in adulthood. If mice are not exposed to *B. fragilis*, increased iNKT cells develop, leading to exacerbated disease (An et al. 2014). *Faecalibacterium prausnitzii* is one of the few bacteria for which an inhibitory bacterial molecule has been identified and appears to alter innate immune signaling in epithelial cells. The microbial anti-inflammatory molecule (MAM) is a peptide produced by *F. prausnitzii* that prevents DNBS colitis. MAM is thought to inhibit NF- κ B signaling in epithelial cells, decreasing inflammation; but the precise molecules mediating protection have not been identified (Quévrain et al. 2015; Sokol et al. 2008).

The overarching theme of the beneficial effects of probiotics seems to be control of inflammation and induction of tolerance. Indeed, many other probiotics, including *Lactobacillus* species, *B. lactis*, and *E. coli* Nissle, have been shown to limit experimental colitis by altering the cytokine balance, even though the precise mechanisms are undetermined (Peran et al. 2007; Grabig et al. 2006; C et al. 2005; Peña et al. 2005).

Probiotics and Disease Outside the Intestine.

Pathogens and chemical models of colitis alike, promote pro-inflammatory T helper cell responses. Probiotics promote Treg and Tr1 cells, or other regulatory cells, and control exacerbated T cell responses, and restore homeostasis to limit disease. Inflammatory T cells drive many different diseases outside of the GIT, but can probiotics be used to treat systemic diseases? Antibiotic-mediated disruption of the microbiota accelerates development of Type-1 diabetes in mice, and is also linked to increased pathology of numerous

systemic diseases including autoimmune diseases, e.g., lupus, arthritis, and chronic obstructive pulmonary disease. These studies and the mechanistic studies detailed above suggest that restoring a healthy microbiota with probiotics may alleviate disease outside the GIT.

Many probiotics will likely be useful for treating or preventing other inflammatory diseases, including diabetes, allergy and experimental autoimmune encephalomyelitis (EAE) (Ochoa-Repáraz et al. 2010; Hu et al. 2015; Stefka et al. 2014; Cao et al. 2014). In fact, SFB-induces Th17 cells in the gut and lung that regulate immunity to fungal infections (McAleer et al. 2016). Disruption of IL-17 receptor signaling, however, results in outgrowth of SFB and enhanced disease severity during autoimmune diseases, namely, EAE (Kumar et al. 2016). This study highlights the importance of a balance between the microbiota and the immune system in regulating dysbiosis. SFB-induced Th17 cells can also prevent the spontaneous development of type 1 diabetes in non-obese diabetic (NOD) mice (Kriegel et al. 2011). In cases of type 2 diabetes, increased systemic inflammation, often associated with obesity, drives insulin resistance and metabolic syndrome, promoting the development of diabetes. Insulin resistance in non-diabetic humans and patients with type 2 diabetes is associated with increased levels of serum branched-chain amino acids. The presence of *Prevotella copri* and *Bacteroides vulgatus*, species with enhanced biosynthetic potential for branched-chain amino acids, correlates with insulin resistance in humans. In mice, administration of *P. copri* increased insulin resistance and glucose intolerance, suggesting that detrimental members of the microbiota can

expand during dysbiosis (Pedersen et al. 2016). Restoring a healthy microbiota with probiotics or targeting pathogenic bacteria may reduce insulin resistance.

Oral administration of *B. fragilis* PSA prevents development of paralysis in EAE, a mouse model of multiple sclerosis (MS), by inducing IL-10-producing Treg cells (Ochoa-Repáraz et al. 2010). Further, *Clostridia* species alter innate lymphoid cells to secrete IL-22, which decreases epithelial permeability and prevents development of food allergy (Stefka et al. 2014). Additionally, *Clostridia* species-induced Treg cells prevent allergen-induced diarrhea (Atarashi et al. 2011; Atarashi et al. 2013). These studies are of particular importance given the rise of asthma and allergies in children raised in clean environments and not exposed to a wide range of bacteria to train their immune responses. As our understanding of the mechanisms by which specific commensal bacteria modulate the immune system increases, we will likely identify more diseases for which these probiotics will be beneficial.

Purified Bacterial Molecules as Therapeutics.

B. fragilis PSA and sphingolipids, and *F. prausnitzii* MAM protein (Mazmanian et al. 2005; An et al. 2014; Sokol et al. 2008), are the only bacterial molecules identified to have immunomodulatory effects. *B. fragilis* PSA and sphingolipids have been purified, administered to mice and shown to exert the given effects. MAM protein has been expressed in *E. coli* and shown to prevent colitis; *F. prausnitzii* superantigens containing MAM have anti-inflammatory effects, but an active molecule has not been purified. *B. breve* modulates IgA responses in an EPS-dependent manner, but this molecule has not been purified.

Most likely, the majority of bacterial molecule(s) and metabolic products responsible for protection have not yet been identified or purified, but perhaps some share characteristics of the established molecules described above. Undoubtedly, additional molecules from a large number of commensal bacteria were expected to regulate immune responses. Utilizing purified molecules as therapeutics will be a useful alternative to the entire bacteria, especially for immune-compromised individuals unable to tolerate or risk the entire organism. Fecal transplants and now probiotics seem to be efficacious in treating severe *C. difficile* infection, but many people suffering from recurrent *C. difficile* are elderly or immune-compromised. These individuals could benefit immensely from an anti-inflammatory molecule instead of an entire bacterium that could potentially cause detrimental effects not seen in healthy individuals.

Section 5: *Bacillus subtilis*: From Soil to Bench to Probiotic

B. subtilis is a Gram (+), spore-forming bacterium ubiquitous in the environment. *B. subtilis* has been extensively studied for many years as a model organism for Gram (+) physiology, sporulation and biofilm formation. It is highly amenable to genetic engineering, and also has the capacity to secrete large amounts of proteins, making it very attractable in biotechnology and industrial settings. Many purified enzymes on the market are produced in *B. subtilis*. It is also widely used in agricultural and aquaculture to prevent pathogen colonization, support bioremediation, and reduce abdominal fat in broiler chickens (Samanya & Yamauchi 2002). New fields of study revolve around the

capacity of *B. subtilis* to serve as a probiotic and a vaccine vector (Duc et al. 2003; Jones & Knight 2012).

Due to its high prevalence in soil, air and drinking water, *B. subtilis* is often found in insects, animals, and humans. Although vegetative cells would not survive transit through the GIT, *B. subtilis* in spore form is virtually indestructible, and would transit unimpeded. Once in the intestine, spores germinate, proliferate, and then resporulate within the GIT (Spinosa et al. 2000; Tam et al. 2006). Although not typically considered a true commensal or resident bacteria in the GIT, many studies report *Bacillus* species present in the majority of individuals surveyed, at levels from 10^3 to 10^8 CFU/gram of feces (Macfarlane et al. 1986; Tam et al. 2006). Approximately 30% of the 84 *Bacillus* isolates in humans were determined to be *B. subtilis* (Tam et al. 2006). Additionally, *B. subtilis* is often found in the elderly and breast-fed infants (Benno et al. 1986; Benno et al. 1984). Much importance has been placed on *Bifidobacteria*, *Bacteroides* and *Lactobacillus* species since they persist in high numbers and can reach 10^{11} colony-forming units (CFU)/gram of feces. Less prevalent species may play an important role in modulating immune responses, and clearly *B. subtilis* has adapted to living in the gut.

***Bacillus subtilis* as a Probiotic.**

B. subtilis is present in high levels in the soil, and as expected, *B. subtilis* and plants have evolved a symbiotic relationship. Upon infection with the plant pathogen *Pseudomonas syringae*, the leaves of the Arabidopsis plant turn yellow. If *B. subtilis* is added to the soil, the plants are perfectly healthy. The plant

secretes malic acid that acts as a distress signal to attract *B. subtilis* to the roots, where it forms a biofilm. During biofilm formation, *B. subtilis* secretes a surfactin with antibacterial activity against *P. syringae* as well as other bacteria (Bais et al. 2004). Innate immune responses are remarkably conserved in insects, mammals and plants. Bacterial infection triggers the plant flagellin PRR, leading to activation of the highly conserved MAP kinase signaling pathway and innate response genes. *B. subtilis* secretes a peptide that suppresses the innate immune response of Arabidopsis and over 1000 other plants, allowing itself to efficiently colonize the plant roots. Interestingly, suppression of plant PRR-stimulated genes is dependent on TasA, the molecule that tethers exopolysaccharide to the bacteria's surface, however *epsG* and *epsO* mutants deficient in EPS production still exerted suppressive activity (Lakshmanan et al. 2012). This immune suppression, however, could potentially leave the plant susceptible for a short period of time to pathogens before *B. subtilis*, and the plant itself, can mediate anti-bacterial effects.

B. subtilis limits pathogen infection in several other species including fish and chickens (La Ragione et al. 2001; La Ragione & Woodward 2003; Tactacan et al. 2013). Both of these are highly cultivated for commercial use, and as many regulatory agencies begin limiting antibiotic use in animal and fish farming, *B. subtilis* has been widely studied for probiotic potential as an alternative to antibiotic treatments. Administration of *B. subtilis* to chicks 24 h prior to infection with an avian *E. coli* infection suppresses disease and reduces pathogen colonization. Interestingly, the window of protection is about 5 days (La Ragione

et al. 2001). Pretreatment of chicks with *B. subtilis* also suppresses colonization and persistence of *Salmonella enteritidis* and *Clostridium perfringens*, demonstrating the versatility of this response (La Ragione & Woodward 2003; Tactacan et al. 2013). In shrimp, *B. subtilis* controls pathogenic *Vibrio* species through production of a secreted molecule that reduces shrimp mortality in shrimp feeding ponds by 90% (Vaseeharan & Ramasamy 2003). Clearly, *Bacillus subtilis* exerts a wide-range of effects that are beneficial to hosts across kingdoms and species.

B. subtilis has been available as a probiotic in many European countries for decades. The Japanese also regularly consume *B. subtilis* in the form of Natto. Natto is made by fermenting soybeans with *B. subtilis* and is believed to have probiotic properties, including vitamin synthesis. Several studies have demonstrated that *B. subtilis* from Natto stimulates the immune system and contributes to growth of other commensals (Hosoi et al. 2000; Tsukamoto et al. 2001; Hosoi et al. 1999).

Several clinical trials using multiple *Bacillus* species have shown some beneficial effects in several disease states. *B. coagulans* has been used successfully to prevent antibiotic-associated diarrhea in children (La Rosa et al. 2003). In other studies, *B. subtilis* reduced bacterial load in urine in the elderly with urinary tract infections, though the probiotic used, Enterogermina, is actually made of *B. clausii* and not *B. subtilis* (Coppi et al. 1985). In a clinical trial to determine if *B. subtilis* improved immunity in the elderly, patients taking *B. subtilis* had increased fecal and salivary IgA compared to placebo controls and

decreased respiratory infections, although no changes were seen in gastrointestinal disorders (Lefevre et al. 2015). *B. subtilis* did prevent antibiotic-associated diarrhea in a clinical trial in Russia. Patients receiving *B. subtilis* and antibiotics had a 7.8% occurrence of antibiotic-associated diarrhea compared to 32% in the placebo group. Patients receiving *B. subtilis* also reduced abdominal pain, nausea, bloating and vomiting (Horosheva et al. n.d.). Several other *Bacillus* species are effective in treating symptoms of IBD and diarrhea; however, no mechanistic insights have been identified outside of the demonstrated antibacterial properties of *Bacillus* products in other species.

Immune Modulation by *B. subtilis*.

B. subtilis produces a variety of molecules shown to directly target pathogenic bacteria and reduce pathogen colonization of the symbiont, but does *B. subtilis* alter immune responses?

Investigating if specific bacteria mediated the ability of the rabbit microbiota to promote GALT development, the Knight Lab determined that *B. subtilis* in combination with *B. fragilis* promoted B cell proliferation and development of the preimmune antibody repertoire (Rhee et al. 2004). Spores are also immunogenic in mice, but the dosing and administration schemes differ vastly between studies resulting in a variety of immune responses. Mice dosed three days in a row, every 3 weeks, develop systemic IgG and mucosal IgA responses and rapid increases in TNF- α and IFN- γ in the MLN. Antibodies have specificities for both spores and vegetative cells (Duc et al. 2004). *In vitro*, *B. subtilis* upregulates APRIL production in intestinal epithelial cells, which

promotes IgA class switch by germinal center B cells, providing mechanistic insight for changes seen *in vivo* (He et al. 2007).

Other cell types have also been shown to respond to *B. subtilis*. Murine macrophages phagocytose *B. subtilis* spores *in vitro*. Spores germinate within the phagosome and may initiate protein synthesis. However, vegetative cells fail to grow and divide and are destroyed within 5 h (Duc et al. 2004). This amount of time however, seems to be enough to trigger innate immune responses. Cells rapidly upregulate IL-6 mRNA upon treatment; minimal changes occur in TNF- α and IL-1 α .

Protection from *C. rodentium*-induced Disease by *B. subtilis*.

To further investigate the immune-stimulatory properties of *Bacillus subtilis*, the Knight lab focused on a mouse model of the enteric human pathogen, EPEC. *C. rodentium* is an attaching and effacing pathogen that induces acute colitis. Disease is characterized by colonic hyperplasia, mucosal infiltration, an increase in chemokines and pro-inflammatory cytokines, and diarrhea, similar to the pathology of EPEC infection in humans. During infection, CD4⁺ T cells infiltrate the intestinal mucosa and drive much of the pathology associated with disease. CD4⁺ T cells increase the proliferation of intestinal epithelial cells and deplete mucus-producing goblet cells in an IFN γ -dependent manner (Chan et al. 2013; Higgins et al. 1999). Additionally, infected mice display an increase in Th1 cytokines including IL-12, IFN γ and TNF α in the colon, as well as an increase in the Th17 cytokine, IL-17A, in the Peyer's patches (Li et al. 2014). These cytokines lead to increased production of other chemokines that recruit innate immune

cells to the site of infection, furthering the progression of disease. In most strains of immunocompetent mice, *C. rodentium* is not lethal and is cleared by both innate and adaptive immune responses 3 to 4 weeks post-inoculation; pathogen clearance requires MyD88-dependent signaling, CD4⁺ T cells and IgG (Bhinder et al. 2013; Kamada et al. 2015).

To test if *B. subtilis* protects mice from *C. rodentium*-induced disease, mice were orally administered 10^9 *B. subtilis* spores 24 h prior to infection with 5×10^8 CFU of *C. rodentium* by oral gavage. Jones & Knight then assessed levels of serum pro-inflammatory cytokines, colonic hyperplasia, and the development of diarrhea 10 days post-infection. *C. rodentium* infected mice had increased levels of the pro-inflammatory cytokine KC or CXCL1 in serum, mucosal infiltration and loss of goblet cells in the colon, and develop soft stool. In mice treated with a single oral dose of *B. subtilis* prior to infection, mice had no evidence of disease. Serum KC and colonic histology of mice treated with *B. subtilis* prior to *C. rodentium* infection are comparable to naïve mice. Further, these mice have normal stool, suggesting that *B. subtilis* prevents *C. rodentium*-induced inflammation (Jones & Knight 2012). In a separate study, D'Arienzo et al. found that *B. subtilis* spores protected suckling mice from *C. rodentium*-induced disease, but in adult mice, found no differences in disease progression (D'Arienzo et al. 2006). The doses of *B. subtilis* and *C. rodentium*, as well as the strains of mice, differed in these studies, which likely contributed to different experimental outcomes.

Jones et al. (2014) first tested if *B. subtilis* reduces pathogen colonization,

thereby limiting disease. In fact, D'Arienzo et al. did find reductions in *C. rodentium* colonization in both suckling and adult mice following repeated doses of *B. subtilis*. In our model using a single dose of *B. subtilis*, we see no alterations in pathogen colonization in the lumen or mucosa, demonstrating that the pathogen is still present at the same levels, but not causing disease. Jones et al. also tested if *B. subtilis* alters the localization of *C. rodentium* in the GIT using a luciferase-expressing strain of *C. rodentium*. Mice were monitored using an *in vivo* imaging system throughout the course of disease. Administration of *B. subtilis* did not change the localization or quantity of luminescence of *C. rodentium* (Jones et al. 2014). These experiments demonstrate that *B. subtilis* does not protect mice by altering the localization, adherence or density of the pathogen.

Jones et al. (2014) also tested if *B. subtilis* increases epithelial barrier integrity to limit disease. During *C. rodentium* infection, epithelial cell loss leads to gut 'leakiness'. We measure this by orally administering FITC-dextran and assessing the serum for fluorescence. If *B. subtilis* functions by preventing epithelial damage, then little to no FITC-dextran should be present in the serum. During infection, we find about a two-fold increase in serum FITC-dextran compared to PBS-treated mice, regardless of *B. subtilis* administration or not (Jones et al. 2014). These data suggest that *B. subtilis* does not protect by preventing epithelial barrier damage. We have not, however, explored the possibility of increased IgA in the mucosa due to *B. subtilis*, as other groups have seen, which may help to limit *C. rodentium*.

Dr. Jones then asked which bacterial molecules produced by *B. subtilis* mediate the protective effects, hypothesizing that *B. subtilis* formed a biofilm within the GIT, blocking *C. rodentium* from attaching to epithelial cells and causing damage. To test this hypothesis, Dr. Jones screened mutants deficient in biofilm production, *epsH* and *tasA*, for protective effects in the *C. rodentium* model. The *epsH* mutant did not protect mice from disease, however, the *tasA* mutant, that also cannot form a biofilm, did protect mice from disease (Jones & Knight 2012). These data suggest that the *epsH* gene product, a glycosyltransferase, is required for protection, but formation of a biofilm is not.

As a negative control, mice were treated with *hag* mutant spores, which do not produce flagella. Interestingly, the *hag* mutant also did not protect mice from disease, suggesting flagella are required for *B. subtilis*-mediated protection. There are two possibilities by which the flagella could provide protection: first, TLR5 recognizes flagella and *B. subtilis* flagella could potentially stimulate TLR5 to activate the innate immune response. The second possibility is that *B. subtilis* needs to localize to a particular niche within the GIT to mediate protection. To test these possibilities, Dr. Jones utilized a *motAB* *B. subtilis* mutant, which produces structurally intact flagella that cannot rotate. Therefore, if the bacteria need to stimulate TLR5, this mutant will be protective, but if protection requires localization of *B. subtilis* to a given niche, this mutant will not be protective. The *motAB* mutant did not protect mice from *C. rodentium*-induced inflammation, suggesting protection requires functional flagella (Jones & Knight 2012). To further support this conclusion, mice were administered purified *B. subtilis*

flagella, which did not protect mice from disease (Dr. Sara Jones, unpublished observations). These studies demonstrated that *B. subtilis* spores germinate within the GIT and vegetative cells express functional flagella to localize to a certain niche, where cells also express *epsH*, which together induce a protective response during *C. rodentium* infection.

Exopolysaccharide.

EPSs are secreted heterogeneous structures composed primarily of carbohydrates that often coat bacteria and are major components of the biofilm matrix. The role of EPS during pathogen infection is well appreciated. For example, pathogenic *Staphylococcus aureus* are coated with an EPS-containing capsule that prevents phagocytosis and allows adherence of the bacteria to host tissues and subsequent immune evasion. Much less is known about EPS from commensal bacteria. EPS might be important for probiotic or commensal organisms to establish and maintain an intestinal niche that could prevent pathogen colonization, as is the case with *B. breve* (Fanning et al. 2012). Alternatively, gut metabolism of EPS could contribute to short chain fatty acid synthesis, a process that regulates intestinal permeability and Treg cell induction (Smith et al. 2013).

The *epsH* gene is encoded within the 15 gene *eps* operon required for exopolysaccharide (EPS) production. *B. subtilis* produces EPS in the transition from planktonic cells to cell aggregates known as biofilms. Biofilms contain non-motile cells encapsulated within an extracellular matrix often composed of polysaccharides, proteins and DNA. EPS and the amyloid protein TsaA are the

main components of the *B. subtilis* biofilm and serve to stabilize the biofilm (Romero et al. 2010; Branda et al. 2006; Kearns et al. 2005). The *epsH* gene encodes one of several glycosyltransferases within the operon, which are most likely required for EPS biosynthesis (Guttenplan et al. 2010). In the absence of *epsH*, *B. subtilis* does not produce EPS (Guttenplan et al. 2010).

We tested if *B. subtilis* EPS is required for protection by purifying EPS from *B. subtilis*. We collaborated with the laboratory of Dr. Daniel Kearns at Indiana University, where they produced a *sinRtasA B. subtilis* mutant. The *sinR* gene encodes a DNA binding protein that binds to the promoter region of the *eps* operon, and represses transcription of genes responsible for production of EPS (Kearns et al. 2005). In the *sinRtasA* mutant, EPS is constantly being produced and is no longer tethered to the surface of cells, allowing us to purify EPS from culture supernatants. The Kearns Lab also produced a *sinRtasAepsH* mutant to serve as a negative control, since it would produce all the background polysaccharide, but would not produce EPS. Using these mutants we harvested *B. subtilis* supernatants, digested nucleic acids and protein, and further purified the remaining polysaccharide on a size exclusion column. Mice were injected intraperitoneally (i.p.) with EPS or the same volume of material purified from the *sinRtasAepsH* material then infected with *C. rodentium* 1 day later and disease was assessed 10 days post-infection (dpi). Mice treated with EPS showed no evidence of disease, whereas mice treated with the negative control material from the EPS-deficient strain had increased levels of serum KC, colonic hyperplasia and soft stool (Jones et al. 2014). These data indicate that EPS mediates

protection from inflammation by *B. subtilis*.

How does EPS limit *C. rodentium*-induced inflammation? Bacterial polysaccharides from both pathogens and commensal bacteria are ligands for many types of PRRs, including TLRs, NOD-like receptors (NLRs), and C-type lectins. Because many of these PRRs signal through MyD88, an adaptor protein that often leads to NF- κ B activation, we tested if EPS-mediated protection requires MyD88. MyD88 is essential for controlling *C. rodentium*-induced inflammation and MyD88-deficient mice are highly susceptible to disease (Gibson et al. 2008). Jones et al. (2014) titrated the dose of *C. rodentium* to a level where disease was comparable to that of wild-type (WT) mice, then tested if EPS alleviated disease symptoms. *MyD88*^{-/-} mice treated with EPS prior to pathogen infection develop disease comparable to mice not treated with EPS, suggesting EPS requires MyD88 to prevent disease (Dr. Sara Jones, unpublished observations).

We further explored the dependency on MyD88 for protection using cell-type-specific *MyD88*^{-/-} mice. Mice lacking MyD88 in myeloid cells (*LysM-cre*) and mice lacking MyD88 in epithelial cells (*villan-cre*) were treated with EPS one day prior to infection with *C. rodentium* and disease was assessed 10 dpi. Myeloid-specific *MyD88*^{-/-} mice treated with EPS had elevated serum KC, crypt hyperplasia and diarrhea; in comparison, EPS protected epithelial-specific *MyD88*^{-/-} mice from disease (Jones et al. 2014). These data suggest that EPS utilizes MyD88 signaling in a myeloid lineage cell to mediate protection. Many TLRs signal through MyD88. Other bacterial polysaccharides signal through

TLR4, therefore we tested if EPS requires TLR4 signaling for protection. *TLR4*^{-/-} mice treated with EPS develop *C. rodentium*-induced disease (Jones et al. 2014). These studies suggest that *B. subtilis* prevents *C. rodentium*-induced inflammation by producing EPS that signals through TLR4 on a myeloid cell to elicit a protective response.

Section 6: Macrophages

Metchnikoff identified macrophages over 100 years ago as ‘phagocytes’ that phagocytose bacteria and apoptotic cells (Metchnikoff & Metchnikoff 1908; Gordon 2008). He viewed these cells as essential components to maintaining homeostasis. Today, we know that macrophages play a crucial part in homeostasis by clearing apoptotic cells and promoting tissue development and repair. Macrophages are also critical for host defense to intracellular and extracellular pathogens; these cells produce antimicrobial factors that can directly target pathogens within endosomes in the cell (Lawrence & Natoli 2011). Additionally, macrophages produce cytokines and chemokines that initiate and regulate adaptive immune responses.

Macrophage Development.

Tissue-resident macrophages. Macrophages are present in most tissues throughout the body and have important functions in tissue development, as well as sensing tissue damage and orchestrating tissue repair. Each tissue has a specialized subset of macrophages, with distinct transcriptional master regulators, that contribute to tissue-specific maintenance, even in immune-privileged sites such as the eye, brain and testes (Okabe & Medzhitov 2016).

Tissue-resident macrophages highly express PRRs including TLRs and NLRs, but relative amounts of each PRR varies between tissues. Normal development and function of some tissues and organs is dependent on macrophages that reside in these organs, but macrophages in every tissue sense tissue damage and orchestrate tissue-repair responses. Microglia, resident CNS macrophages, function in synaptic formation and remodeling, which are vital to memory and learning (Parkhurst et al. 2013; Blank et al. 2014). Bone macrophages, or osteoclasts, perform bone resorption, which is imperative in bone remodeling and tissue repair. In the liver, Kupffer cells clear pathogens and toxic metabolites. Alveolar macrophages clear bi-products of neighboring cells such as surfactins, as well as dust, allergens and microorganisms. In all tissues, macrophages clear apoptotic debris, especially in the spleen where marginal zone macrophages suppress immune responses to apoptotic cells and red-pulp macrophages clear senescent red blood cells (McGaha et al. 2011). Intestinal macrophages aid in tolerance to food antigens and the microbiota and help to remove enteric pathogens (Schreiber et al. 2013).

Tissue-resident macrophages arise from the yolk sac, fetal liver, and hematopoietic stem cells (HSCs) in the bone marrow. Until recently, macrophage dogma dictated that the majority of tissue macrophages differentiated from infiltrating blood monocytes. We now understand that the majority of tissue-resident macrophages seed tissues before birth and are maintained for the life-span of the organism by self-renewal (Jenkins et al. 2011; Jenkins et al. 2013; Yona et al. 2013; Hashimoto et al. 2013). Judith Allen and

colleagues first established the proliferative capacity of tissue-resident macrophages during steady state and their increased proliferation in response to parasite infection in the pleural cavity (Jenkins et al. 2011). Fate-mapping and parabiosis studies have provided insight into the actual origins of tissue-resident macrophages versus macrophages that differentiate from monocytes (Yona et al. 2013; Hashimoto et al. 2013). In both of these studies, there was no evidence that monocytes contributed to liver, skin, splenic, or alveolar macrophages in steady-state conditions. The majority of peritoneal macrophages appear to arise prior to birth, but a small population of monocyte-derived macrophages resides within the peritoneum and is discussed in detail below (Ghosh et al. 2010).

Whether macrophages within different tissues have different rates of proliferation and survival has not been established. In cases of irradiation or extreme inflammation, bone marrow-derived macrophages may repopulate certain macrophage tissue populations.

Monocyte-derived macrophages. Monocytes differentiate from HSCs in the bone marrow and circulate in the blood. Upon infection or acute inflammation, monocytes infiltrate the site of damage and differentiate *in situ* into macrophages or dendritic cells. Within the tissue, monocyte-derived macrophages respond to environmental stimuli and contribute to inflammation or help to resolve inflammation and promote repair (Schreiber et al. 2013). Tumor-associated macrophages also differentiate from monocytes and are highly anti-inflammatory. As discussed, very few macrophages arise from an influx of monocytes to a given tissue under steady-state conditions. It is unclear, however,

whether monocyte-derived macrophages dissipate with the resolution of inflammation or if they persist and self-maintain in the tissue.

Certain tissues, including the intestine, rely on infiltrating monocytes as the main source of macrophages (Little et al. 2014). The main population of CX₃CR1^{hi} macrophages in the intestine differentiates from Ly6C^{hi} monocytes that expand within the gut lamina propria (Bogunovic et al. 2009; Jaensson et al. 2008). These cells have a half-life of roughly 3 weeks. Intestinal macrophages help maintain homeostasis and prevent inflammatory responses to commensal bacteria and food proteins (Murai et al. 2009). During colitis, macrophages further infiltrate and accumulate in the gut and drive pathogenesis. The skin and splenic marginal zone also contain resident macrophages that are monocyte-derived (Tamoutounour et al. 2013; A-Gonzalez et al. 2013). These tissues, along with the gut, are regularly exposed to microbes and microbial products, which may explain the need to be regularly replenished by monocytes.

Peritoneal Macrophages.

Macrophages comprise over half the cells within the peritoneal cavity of mice. Due to their large numbers and the easy accessibility of the peritoneum, these cells have been extensively studied in characterizing macrophage innate immune responses, and for their ability to phagocytose pathogens. Peritoneal macrophages are not a homogeneous population, but rather two distinct populations. Small peritoneal macrophages (SPMs) are bone marrow-derived F4/80^{low}, CD11b^{low}, and Ly6C⁺, and present in small numbers within the peritoneal cavity. During infection, monocytes infiltrate the peritoneal cavity and

differentiate into SPMs (Ghosn et al. 2010). As expected, SPMs highly express CCR2, the chemokine receptor critical for monocyte recruitment. These cells are highly phagocytic and produce large amounts of nitric oxide. Large peritoneal macrophages (LPM) are the main population of macrophages within the peritoneal cavity. As are most tissue-resident macrophages, these cells develop during embryonic development in the yolk sac and are maintained through self-renewal. During infection, these cells undergo rapid proliferation (Jenkins et al. 2013); however, they are less phagocytic and produce less nitric oxide compared to SPMs. Following peritoneal injection of TLR agonists or even PBS, LPMs rapidly disappear from the peritoneal cavity and the influx of monocytes leads to an increased percentage of SPMs (Ghosn et al. 2010). Okabe and Medzhitov determined that when macrophages disappear in the peritoneal cavity following intraperitoneal injection of LPS, LPMs accumulate within milky spots of the omentum, a fatty tissue within the peritoneum (Okabe & Medzhitov 2014). These milky spots are clusters of mostly lymphoid cells and some other leukocytes. Here, the omentum provides retinoic acid to macrophages, allowing them to migrate back to the peritoneal cavity. Retinoic acid regulates expression of the transcription factor GATA-6. GATA-6 controls expression of peritoneal macrophage-specific genes in LPMs and localization of LPMs within the peritoneal cavity under normal conditions and their return following peritoneal inflammation (Okabe & Medzhitov 2014). Among these genes are *Tgfb2* and other genes related to deposition and activation of the latent form of TGF- β . Since TGF- β and retinoic acid promote B cell homing to the gut and induce IgA

class switching (Baumgarth 2011), Okabe and Medznikov investigated the function of GATA-6 and LPM in gut IgA production. Interestingly, they determined that GATA-6 in macrophages controls IgA production by B1 cells that migrate to the lamina propria. Overall, these studies demonstrate that macrophage subsets within the peritoneal cavity have diverse roles in maintaining immunity inside and outside of the peritoneum.

Peritoneal cell trafficking. In contrast to other tissue-resident macrophages, peritoneal macrophages float within the peritoneal fluid that circulates toward lymphatics (Avraham-Chakim et al. 2013), allowing them to constantly survey abdominal organs. Trafficking into the peritoneal cavity has been much better characterized than cells leaving the peritoneal cavity to travel to other parts of the body. Peritoneal cells are thought to traffic from the peritoneal cavity to other organs through two routes. By one route, cells traffic through milky spots in the omentum and into the lymphatics. Early studies demonstrated that capillaries within these lymphoid aggregates are points of cellular traffic for multiple cell types, including monocytes (Hodel 1970; Beelen et al. 1980; Doherty et al. 1995). Another route seemingly exists, however, much less is understood. Recently, Wang and Kubes identified a non-vascular route by which macrophages from the peritoneal cavity directly enter the liver in a model of sterile inflammation. In this model, macrophages rapidly accumulate in the liver by upregulating CD44, an adhesion molecule that binds to exposed hyaluronan present on liver mesothelium (Wang & Kubes 2016). Although other organs were not examined in this study, it stands to reason that macrophages, and other

peritoneal cells, can directly enter other organs covered by the peritoneum, including the gut.

Macrophage Polarization.

Macrophages polarize into different activation states following microbial stimulation or environmental and cytokine stimuli. Activation phenotypes are thought to occur across a spectrum and be fairly plastic, allowing cells to rapidly adjust to different environments. Traditionally, two activation states, classical (M1) and alternative (M2) (Figure 1), encompassed most identified macrophages, but these subtypes are fairly heterogeneous. Different transcription factors have been identified to regulate classical versus alternative polarization.

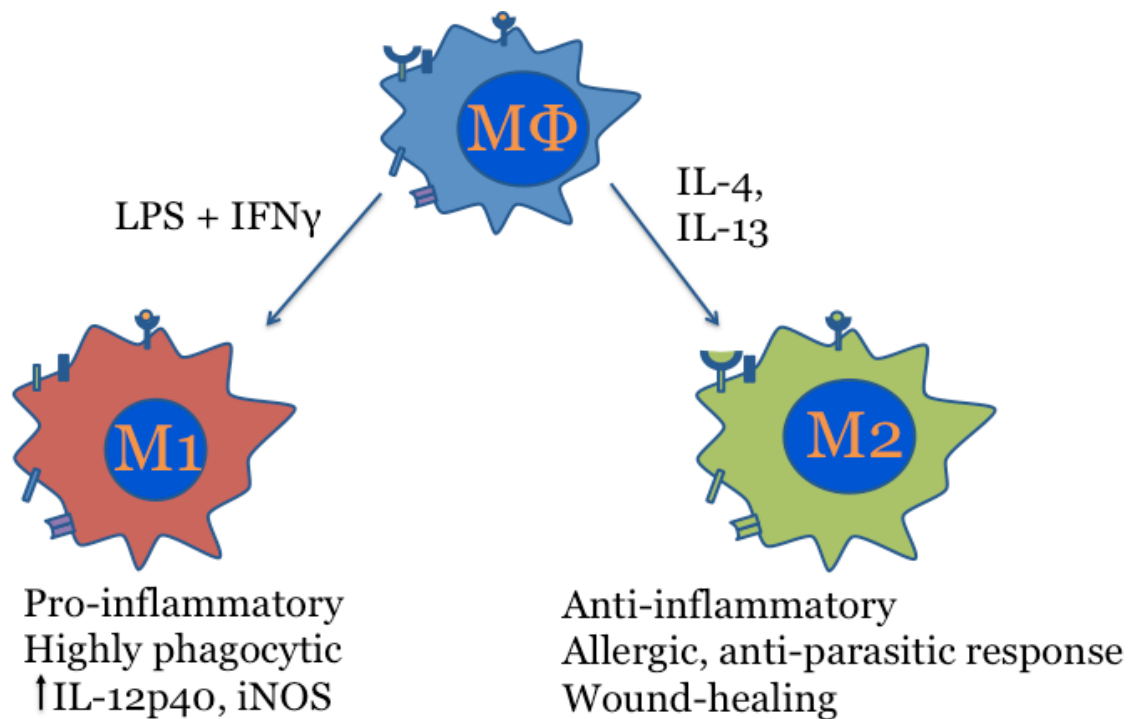


Figure 1.1. Macrophage Polarization. Tissue-resident macrophages (MΦ) adopt different activation states based on their environment. M1 macrophages are induced following LPS and/or IFN γ stimulation and produce pro-inflammatory cytokines and are highly phagocytic. M2 macrophages develop in the presence of IL-4, IL-13 and potentially other anti-inflammatory factors. They induce an anti-inflammatory environment.

M1 Macrophages.

M1, or classical, activation occurs following TLR ligation and/or IFN- γ stimulation, and cells express high levels of inducible nitric oxide synthase (iNOS). IFN- γ often comes from natural killer (NK) cells or other innate cells. M1 macrophages are pro-inflammatory, highly phagocytic, and mediate host defense to bacteria, viruses, and protozoa, and contribute to anti-tumor immunity. Upon phagocytosis of pathogens, the phagosome containing the pathogen fuses with the lysosome where nitric oxide, reactive oxygen species, and enzymes aid in destruction of intracellular pathogens. Depending on the pathogen, these cells produce large amounts of IL-12p40 and TNF- α that promote Th1 effector cell differentiation, or IL-23 that can promote Th17 differentiation (Italiani & Boraschi 2014). These effector T cell subsets produce cytokines that help to maintain the M1 phenotype. As the pathogen is eliminated, the reduction in M1-stimuli and increase in M2 stimuli are thought to give rise to M2 macrophages that promote wound healing and repair tissue damage that occurred during inflammation.

M2 Macrophages.

M2, or alternatively-activated, macrophages are anti-inflammatory and regulate wound healing and tissue repair. M2 activation primarily occurs in response to IL-4 and IL-13 (Dyken & Locksley 2013). These cytokines are produced by granulocytes such as basophils, eosinophils and mast cells, and lymphoid cells including Th2 cells, ILC2 and NKT cells (Jenkins & Allen 2010). Several other M2 macrophage-inducing stimuli have been described. Each

resulting ‘subtype’ exhibits anti-inflammatory properties, but often differ in marker expression and often produce varying effector molecules. Other M2-inducing factors include IL-10, TGF- β , immune complexes, and glucocorticoids (Cao et al. 2010). Additionally, TLR stimulation also can drive M2 polarization. Another population of macrophages, regulatory macrophages, produce large amounts of IL-10 in response to Fc receptor ligation. Within the tumor microenvironment, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) have been identified and are quite functionally similar to M2 macrophages (Mills 2012).

M2 macrophage markers include arginase-1, CD206, Fizz-1, YM-1, and effector cytokines; these molecules are described in detail below. Table 1.2 contains a summary of M2 macrophage markers that are upregulated in response to different stimuli, however, different studies utilize various combinations of these markers or as little as one marker to define these cells.

Table 1.2. M2 Macrophage Markers and Functions

Molecule	Full Name	Function
CD206/ MRC1	Macrophage mannose receptor, C-type mannose receptor-1	C-type lectin, phagocytosis of pathogens
IL-4R α	---	Dimerizes with the common γ -chain or IL-13R α 1 to form IL-4 and IL-13 receptor
Arginase-1	---	Competes with iNOS to catalyze L-arginine hydrolysis to urea and ornithine, production of prolines and polyamines to promote tissue repair/fibrosis
Relm α / FIZZ-1	Resistin-like molecule alpha or found in inflammatory zone protein	Cysteine-rich small proteins, not well characterized
Ym-1/ Chi3l3	chitinase-3-like protein 3	Lectin, chitinase-like molecule, not well characterized

Abbreviations: Chi3l3: chitinase-like 3 ; FIZZ-1:found in inflammatory zone 1 ; IL-4R α : interleukin-4 receptor α ; IL-13R α 1: interleukin-13 receptor α 1; iNOS: inducible nitric oxide sythase; MRC1: ; Relm α : resistin-like molecule α

Arginase-1. Arginase-1 (Arg-1) catalyzes the conversion of L-arginine into L-ornithine and urea. IL-4 and IL-13 upregulate Arg-1 expression in macrophages via STAT6, STAT3 and PPAR- γ , supporting the fact that various stimuli drive Arg-1⁺ M2 macrophages. Arg-1 competes for substrates with iNOS, which is induced by M1 stimuli. These factors, therefore, have become defining characteristics in the M2 versus M1 macrophages. Additionally, Arg-1 contributes to M2 macrophage-anti-inflammatory functions. Arg-1 production by macrophages inhibits CD4⁺ T cell proliferation and IFN- γ production, since T cells require arginine to proliferate (Pesce et al. 2009; Gobert et al. 2004).

Mannose receptor. The mannose receptor, or CD206, is a C-type lectin expressed by macrophages in many tissues and upregulated by IL-4/IL-13. CD206 binds serum glycoproteins and contributes to their clearance (Lee et al. 2002). CD206 also contributes to endocytosis and possibly antigen presentation (Gazi & Martinez-Pomares 2009). Expression of this receptor contributes to immunity to fungal and parasitic infections, most likely by serving as a PRR for mannosylated ligands.

Ym-1. Ym-1, or Chi3l3, is a chi-lectin that is induced in an IL-4/IL-13 and STAT6-dependent manner in macrophages. Chi-lectins, including Ym-1, are homologous to chitinases that bind and degrade chitin; chi-lectins bind chitin, but lack enzymatic activity. Chi-lectins bind β -1,4-N-acetylglucosamine that can be found on parasites, fungi and dust mites that elicit Type 2 immune responses. Although Ym-1 upregulation on M2 macrophages occurs during allergic responses and helminth infections and could potentially recognize chitin motifs,

Ym-1 accumulates intracellularly. It is unclear if it can contribute to M2-mediated anti-inflammatory effects.

FIZZ-1. FIZZ-1, found in inflammatory zone (FIZZ-1), or resistin-like molecule α (Relm α), is upregulated during helminth infection or allergen-induced inflammation in epithelial cells, eosinophils and macrophages. As with Ym-1, FIZZ-1 is consistently described as an M2 marker, but the function of this molecule is not well understood. Some evidence suggests that FIZZ-1 is a negative regulator of Type 2 inflammation and that recombinant FIZZ-1 can directly inhibit Th2 cell cytokine production (Nair et al. 2009), while contrasting studies suggest FIZZ-1 promotes allergy-induced lung inflammation (Doherty et al. 2012). Both of these studies use recombinant FIZZ-1. The actual function of FIZZ-1 production by macrophages has not been identified.

A recent publication discussed the difficulties across the macrophage field due to various methods of generating and polarizing macrophages and their transient nature (Murray et al. 2014). Instead of classifying macrophages into M1 versus M2 macrophages, this large group of macrophage biologists suggest that future studies directly reference the stimulus in the name, i.e. M(IFN- γ) or M(IL-4). This will undoubtedly be difficult to establish as a new basis of naming macrophages. As our understanding grows of transcriptional programs controlling macrophage polarization in different environments and disease states, perhaps new nomenclature methods will be identified.

M2 Macrophages in Health and Disease.

M2 macrophages have primarily been characterized during helminth

infections, and allergy, but recent studies have linked them to an array of normal processes including systemic metabolism, cold adaptation and tissue homeostasis. Dysregulation of these normal functions is linked to atherosclerosis, osteoporosis, obesity and type 2 diabetes, fibrosis and cancer (Martinez et al. 2008). Interestingly, many of these diseases are also linked to disruptions of the microbiota.

Metabolism. Immune cells can regulate metabolic homeostasis. In a lean state, Th2 cells, Treg cells, ILCs, and eosinophils promote M2 macrophages that all help maintain an anti-inflammatory environment within brown adipose tissue, and maintain insulin sensitivity (Carey et al. 2013; Molofsky et al. 2013). These cells that maintain adipose tissue homeostasis are dysregulated in obesity when fat tissue becomes highly inflamed. M1 macrophages are highly prevalent, as well as Th1 cells, ILC1s and CD8⁺ T cells (O'Sullivan et al. 2016). These cells secrete proinflammatory cytokines that contribute to insulin resistance and Type 2 diabetes.

There are three main types of adipose tissue; white adipocytes store energy as lipids and contribute to metabolic disease in obesity, whereas brown adipocytes, or 'healthy fat,' dissipate chemical energy for heat production in response to cold and counteract obesity and metabolic disease. White fat can undergo a 'beiging' process, increasing energy expenditure, and become beige adipocytes. Brown and beige adipocytes function to prevent weight gain in mice and humans (Feldmann et al. 2009; Carey et al. 2013). During cold exposure, M2 macrophages produce norepinephrine and epinephrine that promote beiging

of white adipocytes and decreases adiposity (Wu et al. 2011; Qiu et al. 2014).

Allergy and asthma. Allergic disease is caused by the immune system inappropriately reacting to innocuous substances. Allergic reactions occur upon exposure to an allergen in pre-sensitized individuals. Antigen-presenting cells (APC), such as dendritic cells (DC), macrophages or B cells, initiate the process of allergic sensitization by capturing and internalizing allergen. APC present antigen, especially to T cells which secrete cytokines, interleukin-4 (IL-4), IL-5, and IL-13 that help drive the inflammatory response. IL-4 is critical for class switch to IgE, which mediates allergic symptoms (Galli & Tsai 2012).

Numerous studies have identified roles for M2 macrophages in allergic responses driven by IL-4 and IL-13. However, their function in allergy and asthma remains controversial, with some studies suggesting that M2 macrophages promote allergic inflammation and others indicating a suppressive role for these cells. In several types of allergic inflammation, mice deficient in M2 polarization (myeloid-specific IL-4R α ^{-/-}), showed few differences in allergic antibody and cytokine production, mucus secretion, and eosinophil infiltration that are detrimental during allergic responses (Heller et al. 2007). Other studies have shown that M2 macrophages can contribute to eosinophil recruitment (Falcone et al. 2001; Huffnagle et al. 1998). In cases of asthma, by facilitating the uptake and removal of fungal conidia, M2 macrophages have been shown to inhibit asthma symptoms associated with chronic fungal infections (Moreira et al. 2010). In any event, the upregulation of IL-4 and IL-13 during allergy and asthma contributes to accumulation of these cells.

Helminth infection. Helminth infection in the GIT, pleural cavity and peritoneal cavity elicit strong Type 2 immune responses that are required to expel the worms. Infection leads to increased IL-4 and IL-13 production, proliferation of macrophages, and upregulation of M2 phenotypic markers (Jenkins et al. 2011). IL-4 and IL-13 also promote IgE class switch, increased mucus production and eosinophilia (Jenkins & Allen 2010). In the gut, *Trichiuris muris* infection results in monocyte recruitment that ultimately contributes to macrophage accumulation and obtain an M2 macrophage phenotype (Little et al. 2014). Although robust Type 2 immune responses can be detrimental in the case of allergy, these responses are necessary to clear helminth infection and limit detrimental responses. Deficiency of IL-4 receptor α (IL-4R α)-signaling specifically in myeloid cells results in early mortality to *Schistosoma mansoni*, due in part to exacerbated disease pathology from Th1 cells (Herbert et al. 2004), and Th2 inflammation and fibrosis, which are limited by Arg-1 (Pesce et al. 2009). Infection with other helminthes can impair eosinophil recruitment and tissue repair, but worms are cleared normally, suggesting that M2 macrophages play different roles in various helminth infection models (Martinez et al. 2008).

Mechanisms of Inhibition by M2 Macrophages.

In addition to Arg-1, M2 macrophages produce several other inhibitory molecules including IL-10, PD-L2, and TGF- β . These molecules can potentially inhibit a variety of cell types, but their inhibitory properties have been primarily studied with T cells.

Interleukin-10. IL-10 potently inhibits T cell cytokine production and proliferation, rendering the T cells unresponsive, or anergic. IL-10 also downregulates MHC class II and co-stimulatory molecules on APCs (A. Taylor et al. 2006), and induces immunoregulatory phenotypes, i.e. M2 macrophages, iTregs (Murai et al. 2009; Italiani & Boraschi 2014). Administration IL-10 to mice alleviates a plethora of diseases, including colitis, graft-versus-host disease (GVHD), and allergic responses to name a few, however clinical trials in humans have not been as successful (Min et al. 2007; Murai et al. 2009). Induction of IL-10 within a given cell-type or tissue versus administration of recombinant IL-10 may have better therapeutic effects.

Programmed death-ligand 1/2. Immune checkpoints are critical for maintaining self-tolerance, and much of this is mediated by membrane receptor-ligand interactions. Programmed cell death protein 1, PD-1, is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors expressed on T cells and B cells. PD-1 is upregulated in activated T cells and binds to PD-L1 and PD-L2 (Latchman et al. 2001; Liang et al. 2003). PD-1 interacts with its ligands to prevent T cell activation and proliferation by promoting apoptosis (Carter et al. 2002). Interestingly, PD-1/PD-L1 interactions also maintain an anti-inflammatory environment by reducing apoptosis in Treg cells (Carter et al. 2002). PD-L1 and PD-L2 are part of the B7 family of co-stimulatory molecules and are expressed on macrophages and dendritic cells. IL-4 upregulates expression of PD-L2 on macrophages in a STAT6-dependent manner (Liang et al. 2003). Inhibition of PD-L2 during infection with the

helminth *Nippostrongylus brasiliensis* enhances Th2 responses in the lung, demonstrating that PD-L2 is required to limit an exacerbated Th2 response (Huber et al. 2010).

Transforming growth factor- β . TGF- β is a pleiotropic cytokine that acts on virtually all cell-types. TGF- β potently inhibits T cells by blocking IL-2 production, a required growth factor for T cells, via smad3 signaling (A. Taylor et al. 2006). This induces anergy, which is characterized by T cells that fail to activate upon antigen-stimulation. TGF- β contributes to peripheral tolerance by promoting Treg cell generation and increasing their suppressive function. In combination with IL-6, TGF- β promotes differentiation to Th17 cells (Mangan et al. 2006), whereas TGF- β promotes survival in memory T cells. In B cells, TGF- β suppresses allergen-specific IgE and induces class-switch to allergen-specific IgA (Mucida et al. 2005). Granulocytes, including mast cells and eosinophils, and NK cells increase chemotaxis and often survival in the presence of TGF- β , which may account for some detrimental effects of TGF- β in allergy models (Gorelik & Flavell 2002). In NK cells, TGF- β also decreases cytokine production and their cytolytic activity (Bellone et al. 1995). Dendritic cells and macrophages decrease antigen presentation following TGF- β stimulation, but these cells can obtain regulatory phenotypes and inhibit T cells and/or induce Treg cells (Gandhi et al. 2007; Jaensson et al. 2008). Several studies demonstrate that TGF- β drives M2 macrophage polarization and TAM development or mediate the anti-inflammatory effects of M2 macrophages (Derynck et al. 2001; Calon et al. 2014). M2 macrophages induced during infection with the nematode *Litomosoides*

sigmodontis suppress CD4⁺ T cell proliferation through production of TGF- β (M. D. Taylor et al. 2006). Following phagocytosis of apoptotic cells, macrophages upregulate TGF- β to suppress proinflammatory cytokine production that may occur in response to the apoptotic bodies (Fadok et al. 1998). Additionally, macrophages promote wound healing and repair through TGF- β production.

TGF- β has three subtypes, TGF- β 1, TGF- β 2 and TGF- β 3, all with similar functions, that are synthesized as precursor molecules containing a propeptide region and the TGF- β homodimer (Gorelik & Flavell 2002). TGF- β non-covalently associates with a latency-associated peptide (LAP). This complex binds to Latent TGF- β -binding Protein (LTBP), forming a large complex that gets deposited on the extracellular matrix. TGF- β can also be expressed on the cell surface when associated with LAP; this form mediates TGF- β suppression of T cells by Treg cells (Nakamura et al. 2001). TGF- β remains inactive when bound by LAP and LTBP; these molecules must be cleaved to release active TGF- β , but the mechanisms by which activation occurs are poorly defined. Components of the extracellular matrix including proteases release active TGF- β , as do pH changes, ROS and thrombospondin in serum (Nunes et al. 1995; Hyytiäinen et al. 2004; Taylor 2009). Cell-surface bound TGF- β in complex with LAP mediates T cell suppression and Treg induction by dendritic cells, but how TGF- β is activated on cell surfaces is unknown (Gandhi et al. 2007). Integrins on epithelial cells also cleave LAP from TGF- β and could potentially contribute on cell surfaces (Taylor 2009).

T cell responses in disease. Maintaining a balance between induction and inhibition of T cell responses is vital to the health of the host. Immunity to several pathogens requires Th1 and Th17 responses, but excessive responses can contribute to pathology, as is the case in colitis (Giacinto et al. 2005; Higgins et al. 1999; Yang et al. 2014). Similarly, exacerbated Th2 responses, or inappropriate responses to antigens, lead to allergy and asthma, but Th2 inflammation is required to clear helminth infections (Paula et al. 2013). M2 macrophages possess the capabilities to dampen excessive immune responses through several different mechanisms, and can also promote tolerance through induction of Treg cells.

Unchecked, T cells are main drivers of autoimmune disorders, multiple sclerosis (MS) and colitis. EAE, an animal model of MS, is driven by pathogenic Th1 and Th17 responses that target the central nervous system (Langrish et al. 2005). The therapeutic agent, fasudil, alleviates the symptoms of EAE by promoting M2 macrophages that inhibit pathogenic T cell responses and increasing Treg cells (Liu et al. 2013; Liu et al. 2015). However, many of the factors produced by M2 macrophages to inhibit inflammatory responses have been implicated in tumorigenesis, demonstrating the importance of the inducible and reversible nature of M2 macrophages. Within the tumor microenvironment, M2 macrophages or TAM that develop within the tumor suppress cytotoxic T cells and NK cells that could help to eliminate the tumor (Noy & Pollard 2014). Further, M2 macrophage functions that normally contribute to wound healing and tissue repair promote tumor growth and metastasis through increased

stroma remodeling and angiogenesis (Martinez et al. 2008). Treg cells are also increased in the tumor microenvironment. M2 macrophages may directly induce these cells, or enhance their suppressive functions within the tumor.

The microbiota alters and induces development of several subsets of T cells, and perhaps others that induce inflammation and alter inflammation. Understanding the balance of T cell responses during infection and disease will help us determine how specific microbes can augment or limit certain responses.

Concluding Remarks.

The microbiota is vital to the development of lymphoid tissues and immune responses, the induction of tolerance, and disease prevention. The molecular mechanisms by which intestinal bacteria guide these beneficial responses are just beginning to be discovered. Continued research into the interactions between specific bacteria and the immune system will undoubtedly uncover novel interactions that contribute to homeostasis in the gut, and other microbiota/host interfaces such as the skin, eyes, lung, vagina and bladder.

CHAPTER TWO

MATERIALS AND EXPERIMENTAL METHODS

Mice.

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Loyola University Medical Center (Maywood, IL). Specific pathogen-free (SPF) C57BL/6, TLR4^{-/-}, TLR2^{-/-}, CD4^{-/-}, and OT-II transgenic mice founders were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. Mice lacking MyD88 in myeloid cells were generated by crossing *Lyz2*-Cre transgenic mice to MyD88-floxed mice as described (Gais et al. 2012). Sterile standard chow and tap water were given to mice *ad libitum*.

Reagents.

All base media and supplements were from Life Technologies (Grand Island, NY). All antibodies were from Biolegend (San Diego, CA) unless otherwise indicated. The fluorescent antibodies used for flow cytometry include anti-CD16/32 (FC block), anti-F4/80 (BM8), anti-CD25 (PC61), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD44 (IM7), anti-IL-4R α (I015F8), anti-CD206 (Co68C2), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-PD-L1 (10F.9G2), anti-CD11c (N418), anti-siglecF (1RNM44N, eBioscience, San Diego, CA), anti-Ly6G (1A8), anti-Ly6C (HK1.4), anti-MHCII (M5/114.15.2), anti-PD-L2 (TY25), anti-IgM (RMM-1), anti-IL-4 (11B11), anti-IL-13 (eBio13A, eBioscience,

San Diego, CA), anti-IFN- γ (R4-6A2), anti-LAP (TW7-16B4), anti-Foxp3 (FJK-16s, eBioscience, San Diego, CA), anti-CD68 (ICFC), anti-CD80 (human, 2D10), anti-CD163 (GHI/61), anti-PD-L1 (human, 29E.2A3) and sheep anti-mouse/human arginase-1 (R&D systems, Minneapolis, MN). Enzyme-Linked Immunosorbent Assay (ELISA) reagents for quantitation of IL-10, IFN- γ , TNF- α , and KC/CXCL1 were from R&D Systems; IL-17A, IL-2, IL-13 and TGF- β matched-pair reagents were from Biolegend. Clodronate and PBS liposomes were from VU medisch centrum (Amsterdam, The Netherlands). The anti-CD3 ϵ (145-2C11) for *in vivo* experiments was from Leinco Technologies, Inc, (St. Louis, MO). The anti-CD3 ϵ used for *in vitro* experiments was LEAF-purified anti-CD3 ϵ (145-2C11, Biolegend). Cytochalasin D, NS-398, and SB-431542 were from Sigma (St. Louis, MO). All primers were from IDT (Coralville, IA). Apoptosis reagents were from BioLegend (San Diego, CA). The MFB-F11 cell-line and RAW264.7 cell-line were generous gifts from the Iwashima and Weithoff laboratories, respectively. QUANTI-blue reagent was from Invitrogen (Carlsbad, CA). Cytokines for *in vitro* assays were from Peprotech (Rocky Hill, NJ).

The following neutralizing antibodies were used for *in vitro* assays: TGF- β inhibition, 5 μ g/mL anti-TGF- β (1D11, R&D Systems, Minneapolis, MN) or equivalent concentration of LEAF-purified mouse IgG1 isotype control; PD-L2 inhibition, 3 μ g/mL anti-PD-L2 (TY25) or isotype control LEAF-purified rat IgG2a; PD-L1 inhibition, 5 μ g/mL anti-PD-L1 (10F.9G2) or isotype control LEAF-purified rat IgG2b. Experiments with blocking antibody included the addition of 1 μ g/mL LEAF-purified anti-CD16/32 (Fc γ RII/III) to all wells. For small molecule

inhibitors, the following concentrations were used: Nor-NOHA (12 μ M), exogenous L-arginine (2 mM), NS-398 (1 μ M).

Purification of Exopolysaccharide.

Exopolysaccharide was isolated from *B. subtilis* DS991 (Δ *sinRtasA* mutant), a strain that produces and secretes large amounts of EPS (Guttenplan et al. 2010). The negative control, designated Δ EPS, DS5187 (*sinRtasAepsH* mutant), does not produce EPS and does not protect from *C. rodentium*-induced disease (Jones et al. 2014). EPS was isolated from stationary phase supernatants of bacteria grown in Luria–Bertani (LB), BHI (brain-heart infusion) or MSgg medium isolated by 50% EtOH precipitation at -20° C. The precipitate was pelleted (15,000xg, 4° C, 20 min), and resuspended in 0.1 M Tris (pH 8.0) and samples were treated with DNase (67 mg/ml) and RNase (330 mg/ml) at 37° C for 2 h followed by proteinase K (40 mg/ml) digestion at 55° C for 3 h. EPS was further purified by gel filtration on an S1000 column. Dialyzed EPS was quantified by dry weight and phenol sulfuric acid assay.

Generation of EPS-specific Antibodies.

A New Zealand White rabbit was bled for preimmune serum then immunized by intramuscular and subcutaneous injection of 100 μ g EPS from BHI medium in TiterMax Gold adjuvant. Three weeks post primary immunization, the rabbit was boosted with 100 μ g EPS in adjuvant. Eight days later, serum was collected. We assessed antibody recognition of EPS and EPS purity by immunoelectrophoresis and western blot. For immunoelectrophoresis, EPS from BHI was electrophoresed in a 0.9% agarose gel, then anti-EPS and

preimmune serums were loaded into troughs and incubated o/n in a humidity chamber. The gel was dehydrated and stained with Coomassie brilliant blue to visualize antigen/antibody arcs of precipitation. We found one arc of precipitation between anti-EPS serum and EPS and no precipitation occurring between the EPS and preimmune serum, or elsewhere on the gel (Figure 2.1A). Also, we see little migration of EPS through the gel. These data demonstrate that anti-EPS serum recognizes EPS and neither serum recognizes other material in the sample at the level of detection. For western blot analysis, EPS and Δ EPS from BHI were electrophoresed on a 10% SDS-PAGE gel for 3 h at 80 Volts and transferred to nitrocellulose overnight at 100 milliamps. The blot was incubated with rabbit anti-EPS serum for 2 h, then the secondary reagent, donkey anti-rabbit IgG (H&L)-HRP (The Jackson Laboratory, Bar Harbor, ME). Similar to our immunoelectrophoresis results, we find that EPS does not migrate in polyacrylamide either, but by western we do see faint bands of contamination in EPS (Figure 2.1B). Importantly, the EPS smeared band is not present in the Δ EPS sample. Further, the EPS band is comparable to published results from the Kern's laboratory of EPS purified from the *B. subtilis* 991 strain assessed on an agarose gel with StainsAll (Guttenplan et al. 2010). For certain experiments, anti-EPS IgG was purified from anti-EPS serum using protein G beads and biotinylated using NHS-EZ-Link.

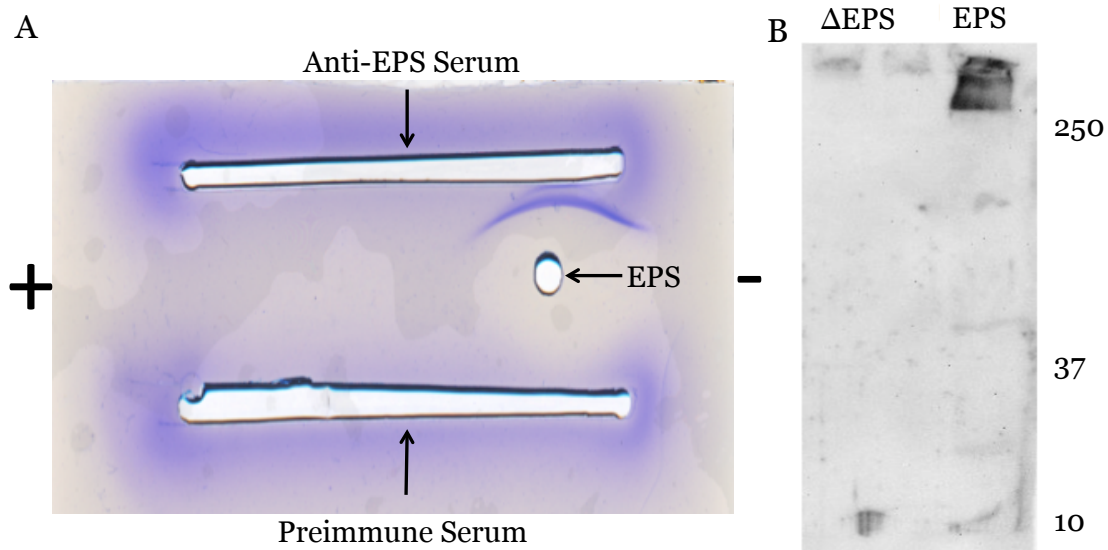


Figure 2.1. Recognition of EPS by anti-EPS rabbit serum.

A. Immunoelectrophoresis of EPS. EPS (BHI) was electrophoresed on 0.9% agarose. Troughs were cut for anti-EPS and pre-immune serum. Troughs were loaded with either serum and incubated in a humidity chamber overnight. The gel was dehydrated and stained with Coomassie blue. **B.** Western blot of EPS and Δ EPS (BHI). EPS and Δ EPS were loaded into a 10% SDS-PAGE gel and electrophoresed for 3 h at 80 Volts. The gel was transferred to nitrocellulose o/n at 100 milliamps, and blotted the next day with rabbit anti-EPS serum, detected using goat-anti-rabbit Ig – HRP.

***B. subtilis* Spore Preparation.**

B. subtilis wild-type 3610, DS76 (*espH* mutant) were germinated via exhaustion as described previously (Jones & Knight 2012). On the day of administration, *B. subtilis* spores were washed with ice-cold water, resuspended in 100 mL PBS, and administered to mice via oral gavage. Cells were isolated 4-6 days post-gavage for analysis.

EPS Binding and Internalization.

Peritoneal cells were obtained from mice (4–6 weeks of age) by peritoneal lavage with 5 ml DMEM (10% FBS). It is imperative that these experiments were

performed with cells on ice at all times or at 4°C in the centrifuge for washes.

After lysing RBCs, cells were incubated with EPS (10 µg) on ice for 1h, washed, and then incubated with anti-F4/80 (clone BM8), anti-CD11b (clone M1/70), or rabbit anti-EPS serum, followed by donkey anti-rabbit Ig as secondary antibody. In some experiments, purified IgG anti-EPS that was biotinylated was used. For internalization experiments, cells were incubated with Cytochalasin D (5 µM) for 20 minutes on ice. EPS was added to cells, incubated on ice for 30 minutes, then experimental tubes were shifted to 37°C for 15 minutes and returned to 4°C to stain for EPS, as described. Fluorescence intensity was assessed by flow cytometry.

Adoptive Transfer Studies.

For adoptive transfer, peritoneal cells were isolated by lavage (with RPMI/50%FCS) from mice 3 days post-treatment with EPS (i.p.). Cells in the granulocyte and lymphocyte gates were FACS-sorted based on forward scatter (FSC) and side scatter (SSC) and injected i.p. into mice. For macrophage depletion studies, mice were injected i.p. with 200 µL clodronate-loaded or PBS-loaded liposomes (Stock 5 mg/mL). Four to 6 h later, mice were treated with EPS, and then 3 days later, peritoneal cells were isolated by PBS lavage. By flow cytometry, less than 1% of the transferred cells were macrophages.

C. rodentium ATCC 51459 was cultured 16 hr in LB medium and washed once in PBS. An infectious dose (5×10^8 CFUs) was resuspended in 100 µL sterile PBS and administered to mice by oral gavage. Disease was assessed 11 days post-infection (dpi). Serum cytokine levels were assessed by ELISA and distal colons

were collected and processed for histological analysis as described (Jones & Knight 2012). To assess diarrhea, feces were examined and scored 1–4 (Jones & Knight 2012): 1, no diarrhea (hard, dry pellets); 2, slightly soft stool (mild diarrhea); 3, very soft stool (moderate diarrhea); and 4, unformed stool (severe diarrhea).

Flow Cytometry.

For flow cytometry, cells were treated with anti-CD16/32 Fc Block and then stained with surface antibodies. To assess intracellular cytokines, cells were fixed using perm/fix reagents from BD Biosciences according to manufacturer's protocol. For Foxp3 staining, cells were fixed using Foxp3/ Transcription Factor Staining Buffer Set (Ebioscience, San Diego, CA). Cells were fixed for 16 h, then permeabilized as per manufacturer's protocol. Anti-Foxp3 antibody was added to cells for 1 h at room temperature. Cells were analyzed on FACSCanto II or LSRFortessa flow cytometers; cell sorting was performed on a FACS Aria cell sorter (BD Biosciences). Analyses were performed using FlowJo software (Tree Star, Ashland, OR) by first gating on single cells, and then analyzing each population as described.

***In vitro* and *in vivo* Treatment with EPS.**

For *in vitro* treatment of peritoneal cells, total peritoneal cells were isolated by lavage with 5 mL PBS and plated in 24-well plates for 2 h to allow macrophages to adhere. Non-adherent cells were aspirated and washed away. The remaining cells were 80-90% F4/80⁺CD11b⁺ macrophages. Peritoneal cells or RAW264.7 cells were treated for 16 h with 5 µg/mL EPS purified from DS991

B. subtilis grown in MSgg medium. For intracellular cytokine analysis, cells were cultured for an additional 2 h with Brefeldin A, and analyzed by flow cytometry. For *in vivo* treatment, mice were injected i.p. with EPS (200 µg) and 3 days later, peritoneal cells obtained by lavage as described (Jones et al. 2014). The level of TGF-β production in serum (1:100) was determined by ELISA after activating TGF-β with 5 µL 1 N HCL/100 µL for 15 min at RT, followed by neutralization with 1 N NaOH.

T cell Proliferation Assay.

Splenocytes were labeled with 5 µM CellTrace Violet (Life Technologies) according to manufacturer's directions and cultured either alone (3×10^5 cells) or with total peritoneal cells (10^4) or purified macrophages (5×10^3) in 96-well flat-bottom tissue culture plates coated with 2 µg/mL anti-CD3. Plates were coated with anti-CD3 using borate buffer pH 8.5 for at least 1 h at 37°C or room temperature o/n. Three days later, non-adherent cells were collected, stained and analyzed by flow cytometry. For Treg cell induction experiments, this same assay was performed with the addition of IL-2 (50 µg/mL). For transwell experiments, 1×10^6 splenocytes were cultured in anti-CD3-coated 24-well plates with or without transwell inserts (Corning) containing 5×10^5 peritoneal cells. For antigen-specific T cell proliferation assay, peritoneal cells were pulsed o/n with 1 mg/mL ovalbumin (OVA). CD4⁺ were purified by positive selection from OT-II mouse spleen using magnetic beads (Streptavidin Particles Plus-DM, BD Biosciences, San Jose, CA) using biotinylated anti-CD4. Purified cells were

labeled with CTV and incubated with OVA-pulsed peritoneal cells in anti-CD3 (2 µg/mL)-coated wells with soluble anti-CD28 (5 µg/mL) for 6 d.

Cell Purification using Magnetic Beads.

F4/80⁺ and CD4⁺ cells were isolated, as indicated, by positive selection using magnetic beads, Streptavidin Particles Plus-DM (BD Biosciences, San Jose, CA) as per manufacturers protocol. Total cells were incubated with anti-CD16/32 (Fc Block) for 15 minutes on ice, then incubated with biotinylated antibodies. Cells were then washed twice and magnetic beads were added based on cell numbers (15 µL per 5x10⁶ cells) and incubated on ice for 30 minutes. Without washing, samples are placed on the BD IMag Cell Separation Magnet for 8 minutes. Negatively-selected cells are collected in the supernatants, and positively-selected cells are resuspended in PBS and placed on the magnet two more times.

Real-time Quantitative PCR.

RNA was isolated from flow cytometry-sorted F4/80⁺CD11b⁺ macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was prepared. PCR was performed on a C1000 thermal cycler with CFX96 real-time detection system (Bio-Rad, Hercules, CA) using the following primers (forward and reverse): *Arg1*, 5'-AGACCACAGTCTGGCAGTTG-3' and 5'-CCACCCAAATGACACATAGG-3'; *Nos2*, 5'-CAGCTGGGCTGTACAAACCTT-3' and 5'-CATTGGAAGTGAAGCGTTTCG-3'; *Ym-1*, 5' – CATGAGCAAGACTTGCGTGAC-3' and 5' – GGTCCAAACTTCCATCCTCCA-3'; *FIZZ-1*, 5' – TCCCAGTGAATACTGATGAGA-3' and 5' –

CCACTCTGGATCTCCCAAGA-3'; *IL-12p40*, 5' - GAAGTTCAACATCAAGAGCAGTAG-3' and 5' - AGGGAGAAGTAGGAATGGGG-3'; *Actb* (β -actin), 5'-GGCTGTATTCCCCTCCATCG-3' and 5'-CCAGTTGGTAACAATGCCATGT-3'. Expression of each target gene was normalized to β -actin expression, and data are presented relative to F4/80⁺CD11b⁺ cells isolated from untreated mice.

TGF- β Bioassay.

MFB-F11 cells (2×10^4) were seeded into 96-well plates in RPMI with 10% FCS. The next day, cells were washed extensively with PBS and co-cultured with peritoneal cells (1×10^5) in serum-free RPMI for 24 h. Recombinant human TGF- β (Biolegend, San Diego, CA) was used as a positive control. Supernatants (30 μ L) were mixed with QUANTI-blue reagent (170 μ L) and incubated at 37°C for 24 h until A630 was measured. Background absorbance (PBS-treated cell supernatants) was subtracted from all samples. Data are represented as fold-increase over peritoneal cells from untreated mice.

Apoptosis Analysis.

For apoptosis staining, cells were collected from the T cell proliferation assay after 1 d of culture. Following extracellular cell-surface staining, cells were resuspended in Annexin V Binding Buffer (BioLegend, San Diego, CA) and 3 μ L of Annexin V and 3 μ L of 7-AAD were added to 1×10^6 cells for 15 minutes at room temperature. Cells were then analyzed by flow cytometry without washing.

Statistical Analysis.

Statistical significance was determined by unpaired two-tailed Student's *t*

test unless otherwise indicated using Prism software (GraphPad Software; La Jolla, CA). A p value <0.05 was considered statistically significant with $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *. Not significant denoted as ns ($p > 0.05$).

CHAPTER THREE

EXPERIMENTAL RESULTS

Section 1: Identification of Cell(s) Required for EPS-mediated Protection from Disease

Binding of EPS to Peritoneal Cells.

EPS administered via intraperitoneal (i.p) injection prevents disease associated with *C. rodentium*. Additionally, adoptive transfer of total peritoneal cells from EPS-treated mice into recipient mice prevents disease (Jones et al. 2014). We therefore hypothesized that EPS modulates a cell within the peritoneal cavity to exert a protective effect. The peritoneal cavity is comprised of a large number of innate-like B1 cells, macrophages, and a small percentage of T cells, DCs, and eosinophils. To determine which cell-type is involved in EPS-mediated protection, we tested which cells bind EPS by incubating peritoneal cells from a wild-type (WT) untreated mouse with EPS. We then identified cells bound to EPS using antiserum from rabbits immunized with EPS, detected with fluorescent goat anti-rabbit Ig antibody and cell-specific antibodies. As a negative control, cells were incubated with material derived from the mutant *B. subtilis* (Δ EPS) that does not produce EPS. These strains are described in greater detail in Chapters One and Two. This mutant strain of *B. subtilis* produces 5 to 10-fold less total polysaccharide compared to the hyper-producing strain used to

isolate EPS, but the background polysaccharide levels are the same. We added a volume of Δ EPS equivalent to the volume of EPS added, relative to the starting cultures of each strain. E.g., we purify both EPS and Δ EPS from 500 mL starting cultures; if we need 10 μ L of EPS for a final concentration of 5 μ g/mL, we add 10 μ L of Δ EPS, even though the actual polysaccharide concentration of this material is less. By flow cytometry, we found an increase in EPS binding on F4/80⁺CD11b⁺ macrophages (Figure 3.1A), with little to no binding to other cell-types within the peritoneal cavity (Figure 3.1). As negative controls, we do not see any increase in fluorescence intensity by Δ EPS or by preimmune rabbit serum. These data show that EPS binds F4/80⁺ cells and suggest that macrophages may be involved in EPS-mediated protection from *C. rodentium*-induced disease.

Macrophages rapidly internalize antigens that bind to the cell surface, leading us to test if macrophages internalize EPS. To test this, cells were incubated with EPS as described above at 4°C, and then experimental tubes were shifted to 37°C for 15 minutes; cells were then returned to 4°C. Purified, biotinylated anti-EPS IgG was added to cells and the presence of EPS on the cell surface was assessed by flow cytometry. If macrophages internalize EPS, we would expect to see a decrease in surface EPS following incubation at 37°C.

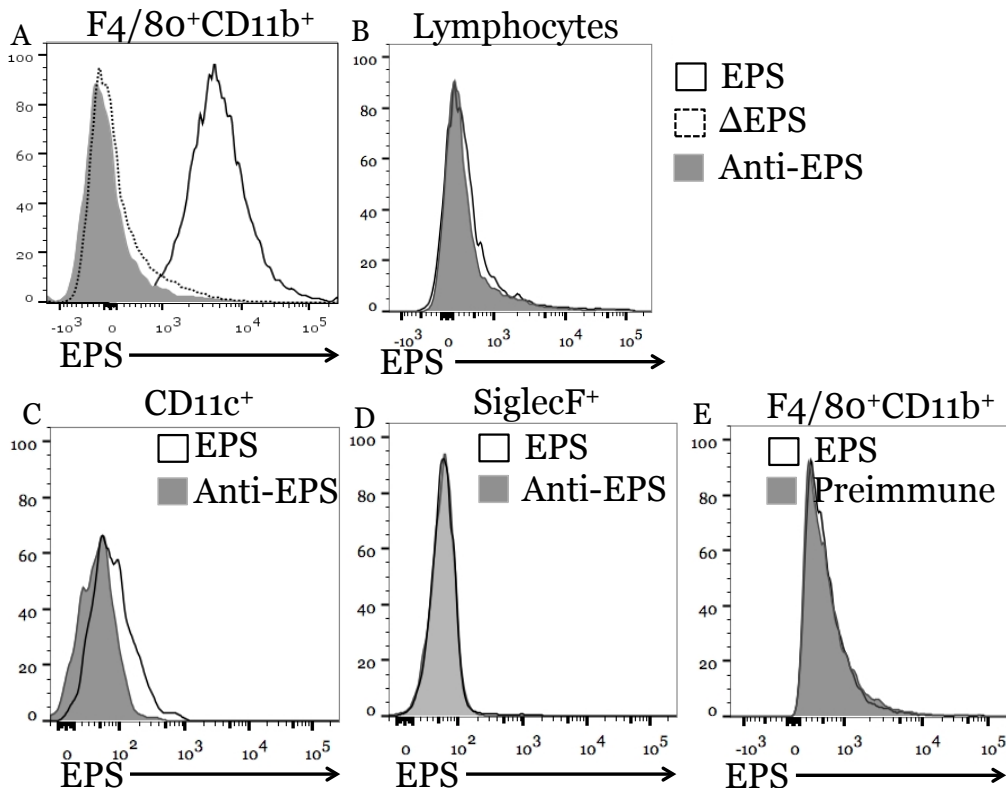


Figure 3.1. EPS binding to peritoneal cells. EPS (10 μg) or an equal volume of ΔEPS purified from BHI media was added to total peritoneal cells from untreated WT mice for 1 h. EPS binding was detected using anti-EPS rabbit serum and goat FITC-anti-rabbit-Ig antibody. Fluorescence Intensity was measured by flow cytometry gated on specific subsets of cells. A. F4/80⁺CD11b⁺ macrophages. B. Lymphocyte gate. C. CD11c⁺ dendritic cells. D. SiglecF⁺ eosinophils. E. EPS, total peritoneal cells, and preimmune serum gated on F4/80⁺CD11b⁺ macrophages. Representative flow cytometric profiles from 5 independent experiments.

As expected, we found a decrease in surface EPS in samples incubated at 37°C (green line) compared to samples that remained at 4°C (red line) for the duration of the experiment (Figure 3.2), suggesting that EPS was internalized by the macrophages. To confirm this idea, we inhibited phagocytosis by adding Cytochalasin D (5 μM), a small molecule inhibitor of actin polymerization, to cells before incubation with EPS. We expected that Cytochalasin D would block

internalization of EPS at 37°C, and we would see similar levels of surface EPS comparable to samples that remained at 4°C. As shown in Figure 3.2, Cytochalasin D blocks much of the internalization of EPS at 37°C (yellow line), although some EPS may still be internalized as surface levels of EPS are not as high as cells that remain at 4°C. Biotinylated anti-EPS IgG with streptavidin-PE (present in each tube) alone serves as the negative control (blue line). These data suggest that peritoneal macrophages bind and rapidly internalize EPS.

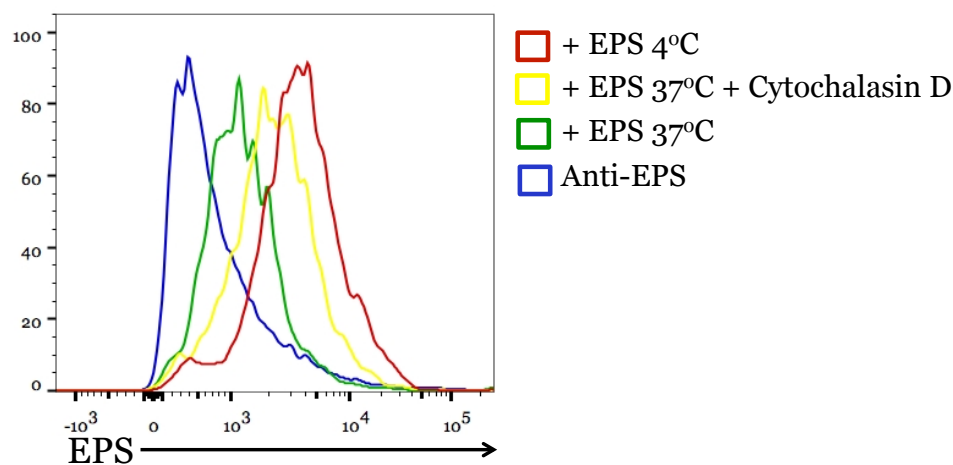


Figure 3.2. Internalization of EPS by peritoneal macrophages. EPS (10 μ g) was added to total peritoneal cells and incubated at 4°C for 30 minutes. Indicated samples were then shifted to 37°C for 15 minutes. Binding was detected using purified, biotinylated anti-EPS IgG and streptavidin-PE. Fluorescence Intensity was measured by flow cytometry of granulocyte-gated cells. Representative flow cytometric profiles from 4 independent experiments.

Pattern recognition receptors, including Toll-like Receptors, NOD-like receptors, and C-type lectins, etc., recognize bacterial and viral products, leading to activation or inhibition of innate immune responses. Our lab has previously shown that EPS does not protect TLR4-deficient mice from disease (Jones et al. 2014), suggesting that EPS utilizes TLR4 to induce a protective response. Since

TLRs are highly expressed on peritoneal macrophages, we hypothesized that EPS binds to TLR4 on peritoneal macrophages. To test this hypothesis, we incubated peritoneal macrophages from TLR4^{-/-} mice with EPS and anti-EPS IgG, and assayed for an increase in fluorescence intensity by flow cytometry. If EPS binds to TLR4 on macrophages, we do not expect to see an increase in fluorescence in TLR4^{-/-} macrophages compared to WT macrophages. Surprisingly, EPS binds TLR4^{-/-} F4/80⁺CD11b⁺ cells (Figure 3.3A) to the same extent as we see in WT F4/80⁺CD11b⁺ cells (Figure 3.1A). We also assessed EPS binding to TLR2^{-/-} macrophages and found that EPS binds macrophages in the absence of TLR2 (Figure 3.3B). These data suggest that EPS does not directly bind to TLR4 or TLR2 and perhaps utilizes a co-receptor of the TLR4 signaling complex, such as carbohydrate-binding lectins, to bind to macrophages.

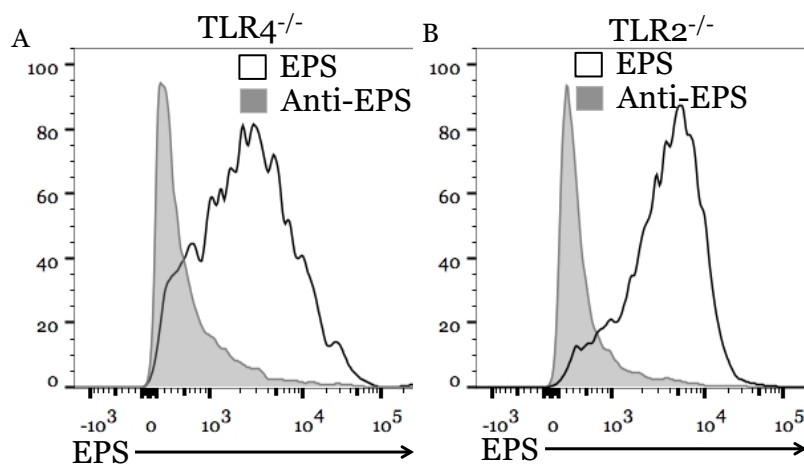


Figure 3.3. EPS Binding to peritoneal cells from TLR2- and TLR4-deficient mice. EPS was added to total peritoneal cells from untreated mice, and binding was detected using rabbit anti-EPS anti-serum and FITC-goat anti-rabbit-Ig antibody. Fluorescence Intensity was measured by flow cytometry. **A.** F4/80⁺CD11b⁺ macrophages from TLR4^{-/-} mice. **B.** F4/80⁺CD11b⁺ macrophages from TLR2^{-/-} mice. Representative of 4 independent experiments.

Requirement of Macrophages for EPS-mediated Protection from *C. rodentium*-induced Disease.

Since EPS binds to peritoneal macrophages and total peritoneal cells from EPS-treated mice prevent disease caused by *C. rodentium*, we next asked if macrophages purified from the peritoneal cavity of EPS-treated mice prevent disease. Macrophages are readily activated by antibodies or magnetic beads; therefore, we decided to purify macrophages by FACS-sorting the granulocyte gate and the lymphocyte gate based on forward scatter (FSC) and side-scatter (SSC) in order to minimally manipulate the cells. Cells within the granulocyte gate are 80-90% macrophages (Figure 3.4), therefore we expect that the granulocyte population will prevent disease, but the lymphocyte population will not (<5% macrophages, Figure 3.4). Sorted cells (3×10^4) were adoptively

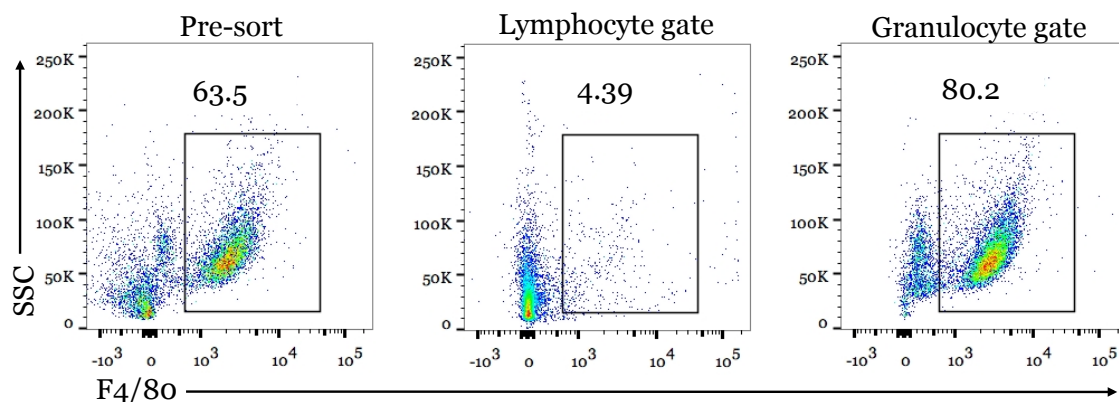


Figure 3.4. F4/80 expression on sorted peritoneal cells. Peritoneal cells were sorted using FACS based on FSC and SSC from the lymphocyte and granulocyte gates from untreated or EPS-treated mice. F4/80 expression on total peritoneal cells prior to sort (left), or cells obtained from the sort in the lymphocyte gate (center), or granulocyte gate (right). Representative of 3 sorts in each of 2 independent experiments.

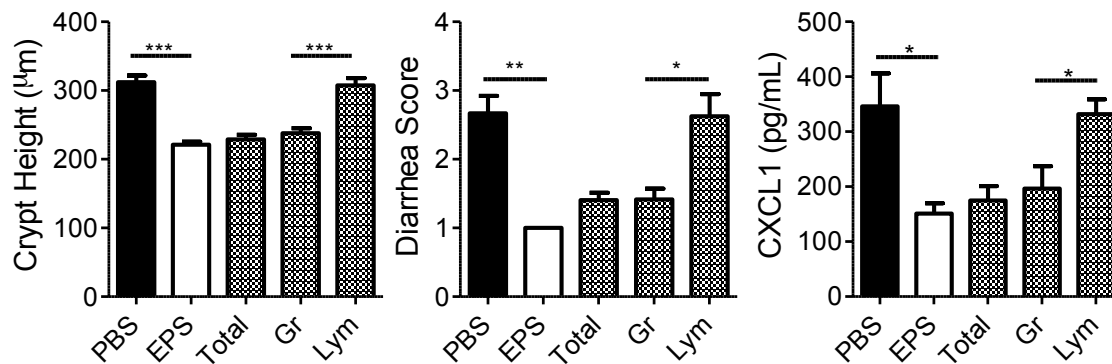


Figure 3.5. Assessment of *C. rodentium*-induced disease following adoptive transfer of peritoneal cells from EPS-treated mice. Crypt height (left), diarrhea score (center), and serum CXCL1 (right) on day 11 post *C. rodentium* infection. Peritoneal cells (3×10^4) from EPS-treated mice were transferred days -1, +1, and +3 relative to *C. rodentium* infection. (■) PBS-injected mice, no cell transfer (- control); (□) EPS-injected mice, no cell transfer (+ control); (▣) cells transferred from EPS-injected mice, total unsorted (total), granulocytes (Gr), lymphocytes (Lym). Data are from 2 independent experiments; N = 5-7 mice total per treatment. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

transferred to recipient mice -1, +1, and +3 days post-infection (dpi) with *C. rodentium*, and disease was assessed 11 dpi. The cells in the granulocyte gate protected recipient mice from disease, whereas lymphocytes did not, as evidenced by increased colonic crypt heights, loose stool, and increased levels of serum pro-inflammatory chemokine CXCL1 (Figure 3.5). These data suggest that cells in the granulocyte gate, which are predominately macrophages, mediate protection by EPS.

We further assessed the requirement of macrophages in EPS-mediated protection using clodronate liposomes. Macrophages phagocytose the liposomes,

releasing clodronate inside the cells, which induces apoptosis (Rooijen & Sanders 1994). Intraperitoneal injection of the liposomes results in rapid depletion of peritoneal macrophages. The liposomes gradually disseminate and deplete macrophages in the spleen, liver, and bone marrow (van Rooijen & Hendrikx 2010).

Mice were injected with clodronate liposomes to deplete macrophages, or PBS liposomes as a control, 4-6 hr prior to EPS treatment. Three days later, we adoptively transferred peritoneal cells (6×10^4) to naïve recipients -1, +1, and +3 dpi with *C. rodentium*; disease was assessed 11 dpi. If macrophages are required for EPS-mediated protection, we would expect that in the absence of macrophages, peritoneal cells would no longer protect mice from disease. As expected, all mice that received macrophage-depleted peritoneal cells from EPS-injected mice had evidence of disease, whereas only 1 of 7 that received peritoneal cells from EPS-injected mice treated with PBS liposomes developed disease (Figure 3.6). These data indicate that macrophages are required for EPS-mediated protection from *C. rodentium*-induced inflammation.

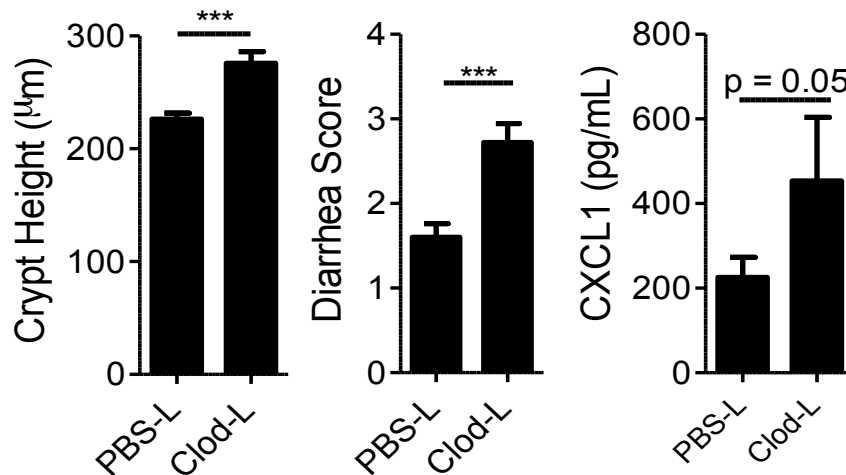


Figure 3.6. Adoptive transfer of macrophage-depleted peritoneal cells into *C. rodentium*-infected Mice. Mice were treated with clodronate liposomes (Clod-L) or PBS liposomes (PBS-L) 4-6 hr before injection with EPS (100 µg); peritoneal cells (6×10^4) were transferred to naïve recipients -1, +1 and +3 days relative to *C. rodentium* infection. Crypt height (left), diarrhea score (center), and serum CXCL1 (right) were measured 11 dpi. Data are from 2 independent experiments with N= 7-9 mice total per treatment. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

How does EPS alter macrophages to induce protection? Macrophages are classified into two main subsets. M1, classically-activated, macrophages differentiate in response to TLR stimulation and IFN- γ , and are pro-inflammatory and highly phagocytic. They produce effector molecules including IL-12p40 and iNOS. In contrast, M2, alternatively-activated, macrophages are anti-inflammatory, and involved in immunity to parasites and wound-healing responses. They differentiate in response to glucocorticoids, TLR stimulation, the anti-inflammatory cytokines IL-10 or TGF- β , or more traditionally, IL-4 and IL-13. We hypothesize that EPS induces M2 macrophages, which prevent disease

by providing an anti-inflammatory environment that diminishes a hyper-inflammatory immune response induced by, for example, *C. rodentium*.

Following M2 macrophage polarization, macrophages upregulate expression of CD206, IL-4R α , arginase-1, FIZZ-1, and Ym-1 (Table 1.2). To determine if EPS induces an M2 macrophage phenotype, we took a step back from the *C. rodentium* model and asked how EPS modules the immune system in naïve, uninfected mice. We examined peritoneal cells from mice 3 days after i.p. injection with EPS, and we expected to find an increase in M2 macrophages at this time point since total peritoneal cells 3 days post-EPS treatment protect recipients from disease. We found increased M2 macrophage marker expression on F4/80⁺CD11b⁺ cells compared to treatment with the negative control, Δ EPS (Figure 3.7A). Additionally, we FACS-purified F4/80⁺CD11b⁺ macrophages from EPS-injected mice and found that transcripts of *Ym-1* and *FIZZ-1* were

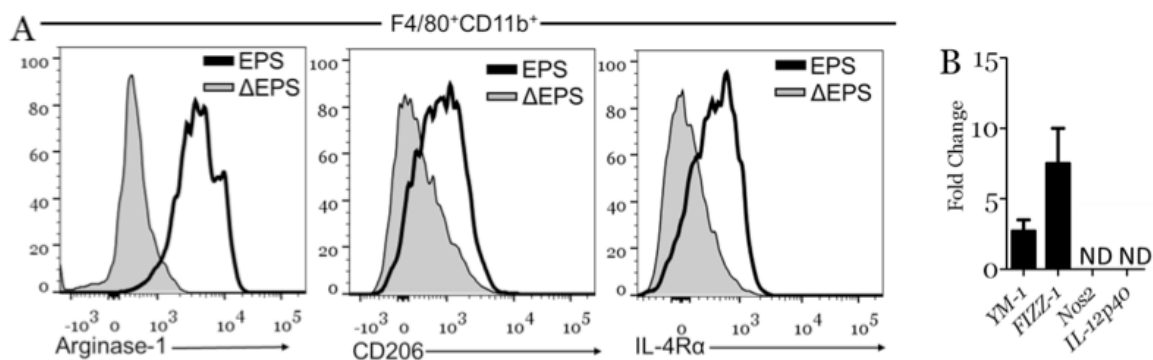
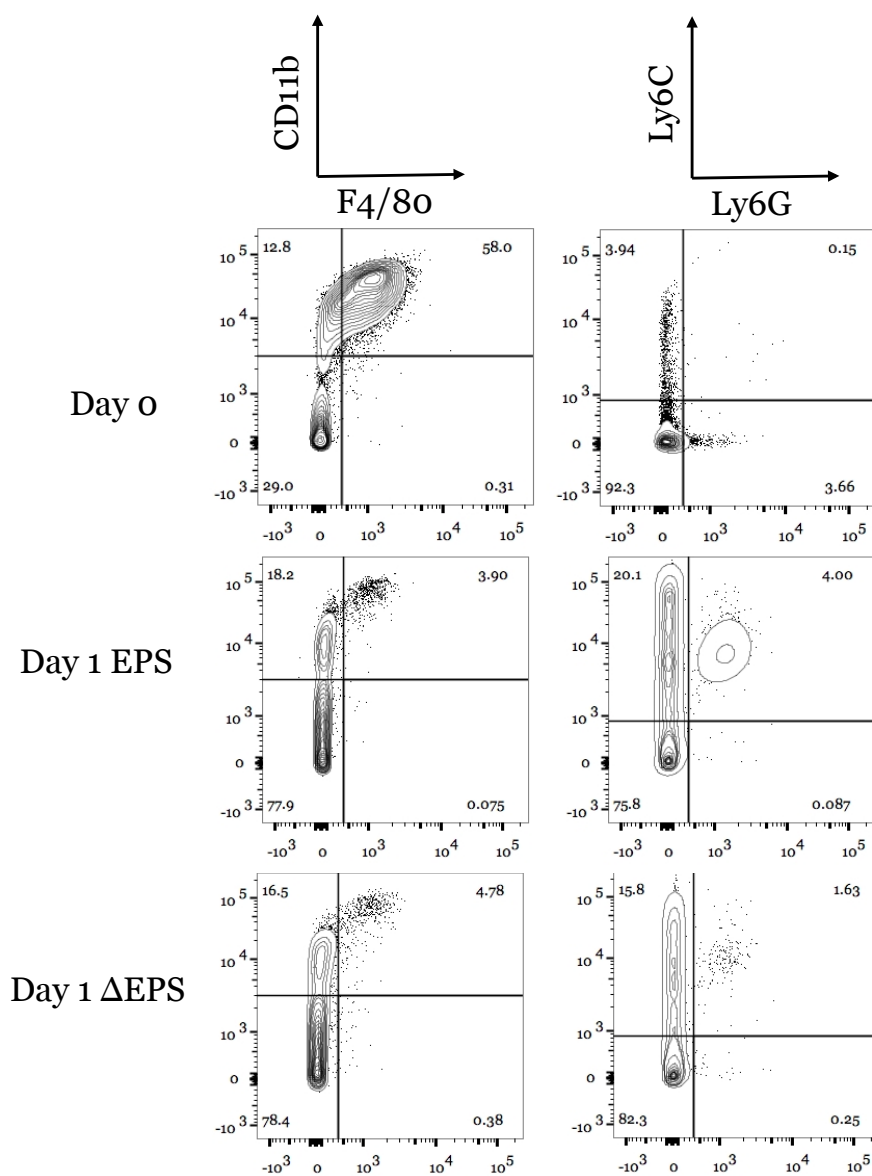


Figure 3.7. EPS-induced changes in phenotype of peritoneal macrophages *in vivo*. WT mice were injected with EPS and 3 days later peritoneal cells were examined by flow cytometry and qRT-PCR. **A.** Representative flow cytometric profiles of M2 macrophage marker expression (Arg-1, CD206, and IL-4R α) on F4/80⁺CD11b⁺ cells from WT mice. **B.** Fold change by qRT-PCR of *Ym-1*, *FIZZ-1*, *IL-12p40* and *Nos2* expression in F4/80⁺CD11b⁺ cells in WT mice injected with EPS (relative to mice injected with Δ EPS) (average of 4 independent experiments, N = 7 mice total per group). ND = not detectable.

upregulated 5-fold and 9-fold, respectively, compared to macrophages from Δ EPS-treated mice. In contrast, no upregulation of the M1 macrophage markers *IL-12p40* or *Nos2* (iNOS) occurs (Figure 3.7B). We conclude that administration of EPS induces cells with an M2 macrophage phenotype in peritoneal macrophages.

The peritoneal cavity is a dynamic environment with cells continuously entering, surveying, and trafficking to other parts of the body. We sought to characterize the distribution of cells in the peritoneal cavity in response to EPS to better understand when M2 macrophage induction occurs. We performed a time-course study and analyzed the cells present in the peritoneal cavity 1, 2, and 3 days following EPS treatment. Previous reports have shown that regardless of what material is administered, an i.p. injection results in an influx of monocytes and neutrophils and an efflux of B1 cells (Doherty et al. 1995; Ghosn et al. 2010; F et al. 2012). Similar to these findings, we find a rapid increase in Ly6C⁺ monocytes 1 day after treatment with EPS and Δ EPS (Figure 3.8). By day 2, the percentage of macrophages increases in both treatment groups, which may be attributed to the differentiation of infiltrating monocytes into macrophages. Further, the majority of F4/80⁺ cells are CD11b⁺F4/80^{lo} and have yet to reach full differentiation into CD11b⁺F4/80^{hi} macrophages that bear the M2 phenotype in EPS-treated mice (Figure 3.8). By day three, the peritoneal cell composition in Δ EPS-treated mice looks similar to an untreated mouse, whereas EPS-treated mice display an increase in M2 macrophages, as described in Figure 3.7. These

data suggest that it takes 3 days to fully restore macrophages to the peritoneal cavity and induce an M2 phenotype following an i.p. injection. Although the small percentage of macrophages at two days bear the M2 phenotype, the influx of other cell types that occurs due to the injection drowns out the number of macrophages within the peritoneal cavity.



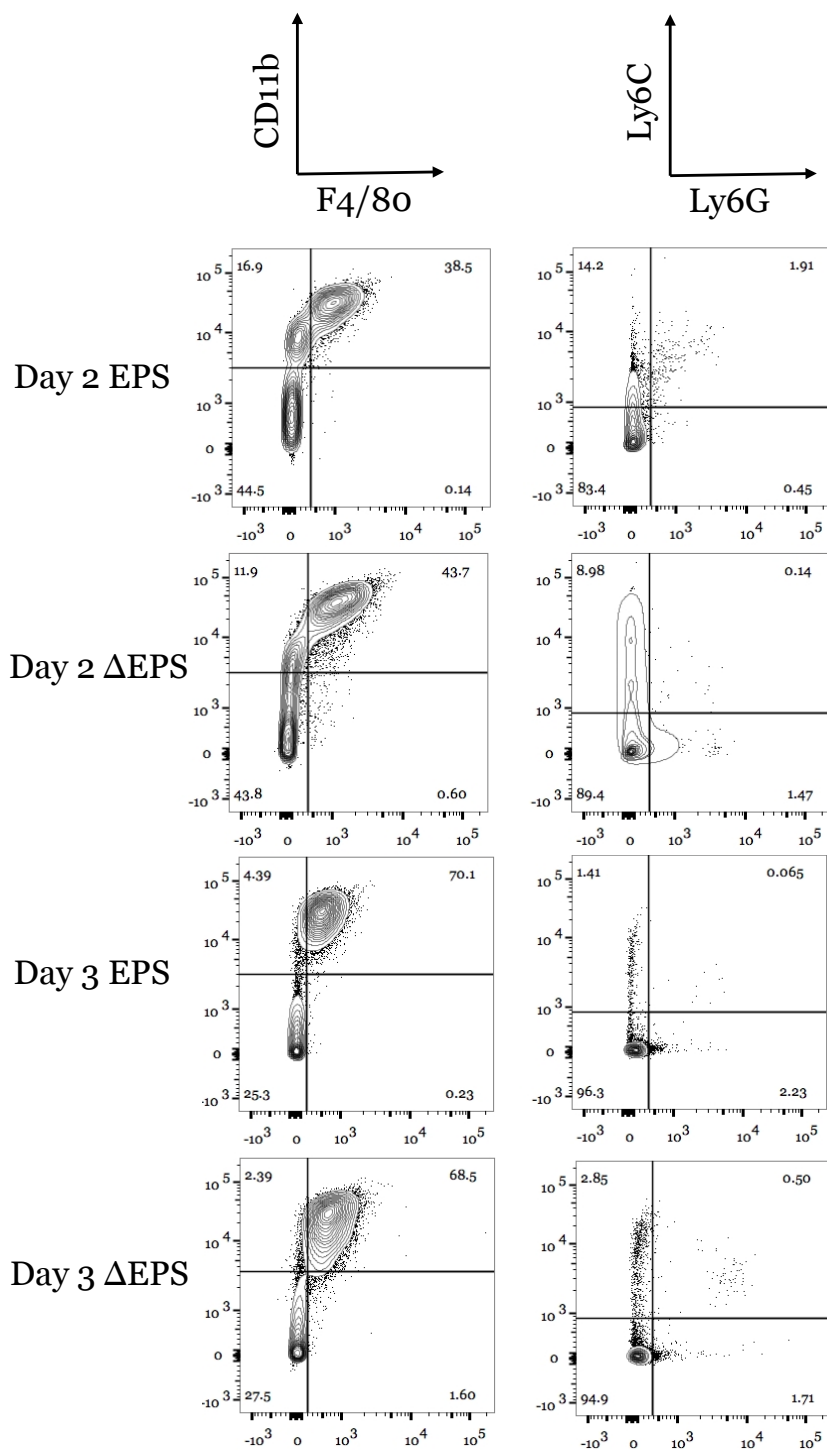


Figure 3.8. Kinetics of macrophage accumulation following i.p. injection with EPS. Macrophages (F4/80⁺CD11b⁺), monocytes (Ly6C⁺) and neutrophils (Ly6G⁺Ly6C^{lo}) present in the peritoneal cavity were assessed in untreated (day 0) or days 1-3 post-injection with EPS or an equal volume of ΔEPS. Representative of 3 independent experiments, 2 mice per group per experiment.

Does EPS act directly on macrophages? Although most reports suggest that M2 macrophages develop in response to IL-4 and IL-13 produced by a variety of cell types, a diverse set of activators including cytokines, immune complexes, and glucocorticoids have been shown to induce an M2 phenotype. Since EPS binds to macrophages, we expect EPS to directly induce M2 macrophage polarization. We tested if EPS acts directly on macrophages to induce the M2 macrophage phenotype *in vitro* by culturing peritoneal F4/80⁺ cells overnight with EPS (1 $\mu\text{g}/\text{mL}$) or an equal volume of ΔEPS , with the expectation that we would see upregulation of the markers listed in Table II in EPS-treated cells. By flow cytometry, we found upregulation of Arg-1, CD206 and IL-4R α in EPS-treated cells compared to untreated cells (Figure 3.9A).

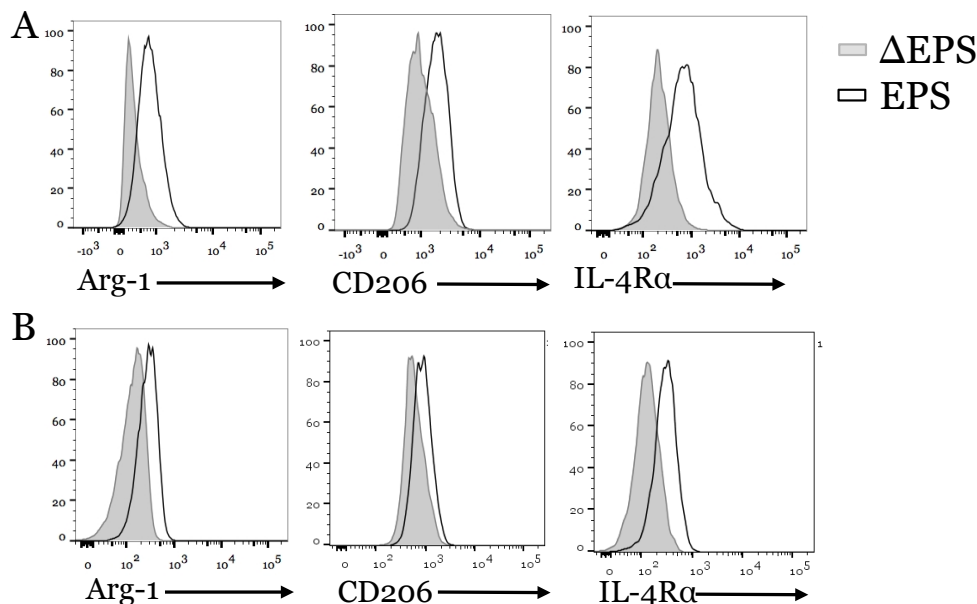


Figure 3.9. EPS-induced changes in phenotype of macrophages *in vitro*. Peritoneal F4/80⁺ macrophages (A) or RAW264.7 cells (B) were incubated 16 h with EPS or ΔEPS *in vitro* and analyzed by flow cytometry for M2 marker expression (Arg-1, CD206 and IL-4R α). Data are representative of 5 independent experiments.

These data indicate that EPS induces M2 macrophages *in vitro* by acting directly on macrophages.

To confirm that EPS acts directly on macrophages, we cultured RAW264.7 cells, a mouse macrophage cell line, with EPS (1 $\mu\text{g}/\text{mL}$) or ΔEPS for 16 h *in vitro* and assessed upregulation of M2 macrophage markers. By flow cytometry, we found that the expression of these markers was upregulated in EPS-treated cells compared to cells treated with ΔEPS (Figure 3.9B). These data further demonstrate that EPS acts directly on macrophages to induce M2 macrophages.

We further characterized the phenotype of M2 macrophages by assessing the expression of co-stimulatory or co-inhibitory molecules CD80, CD86, PD-L2

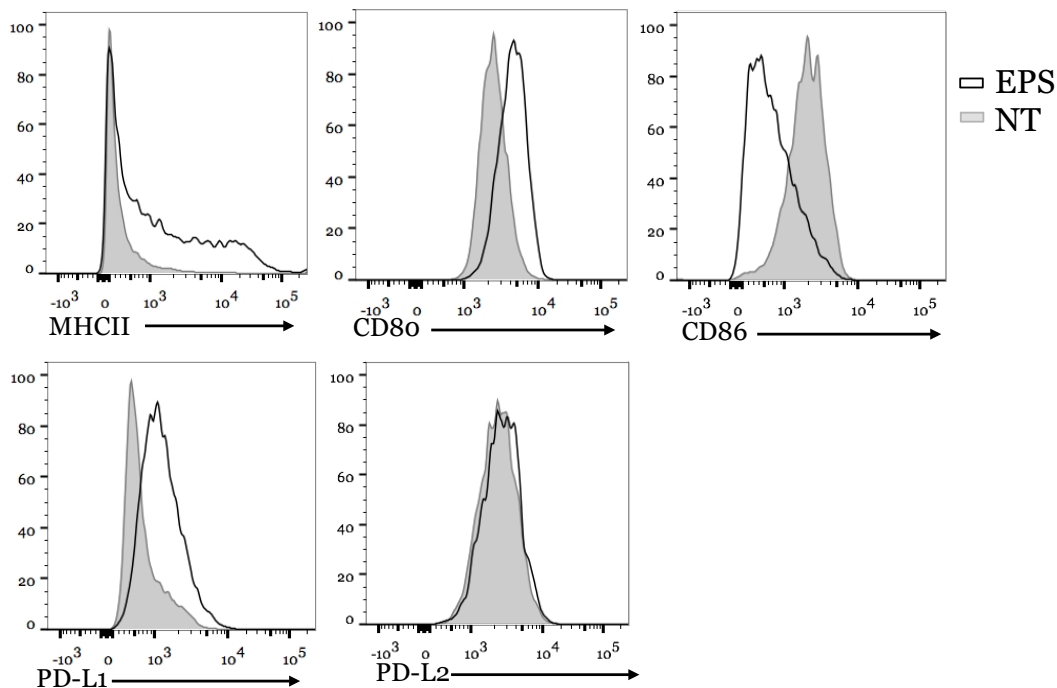


Figure 3.10. EPS-induced changes in macrophage receptor expression. Peritoneal cells were isolated from untreated, or EPS-treated mice 3 days post-treatment (dpt). Expression of MHC class II, CD80, CD86, PD-L1 and PD-L2 on F4/80⁺ macrophages was assessed by flow cytometry. Data are representative of 5 independent experiments.

and PD-L1, as well as MHC class II. These molecules could influence the anti-inflammatory response of M2 macrophages and interactions with target cells. We examined expression of these markers on peritoneal macrophages from EPS-treated mice 3 days post-treatment (dpt), as well as peritoneal macrophages and RAW264.7 cells treated *in vitro* with EPS. By flow cytometry, we find upregulation of CD80 and PD-L1 on macrophages from mice treated *in vivo* with EPS (Figure 3.10). In contrast, we find downregulation of CD86 in peritoneal macrophages from EPS-treated mice. MHC class II expression is slightly upregulated, but does not occur on all macrophages following EPS treatment. We do not find changes in PD-L2 expression.

Similar to peritoneal macrophages from EPS-treated mice, peritoneal cells and RAW264.7 cells treated with EPS *in vitro* upregulate CD80 and PD-L1

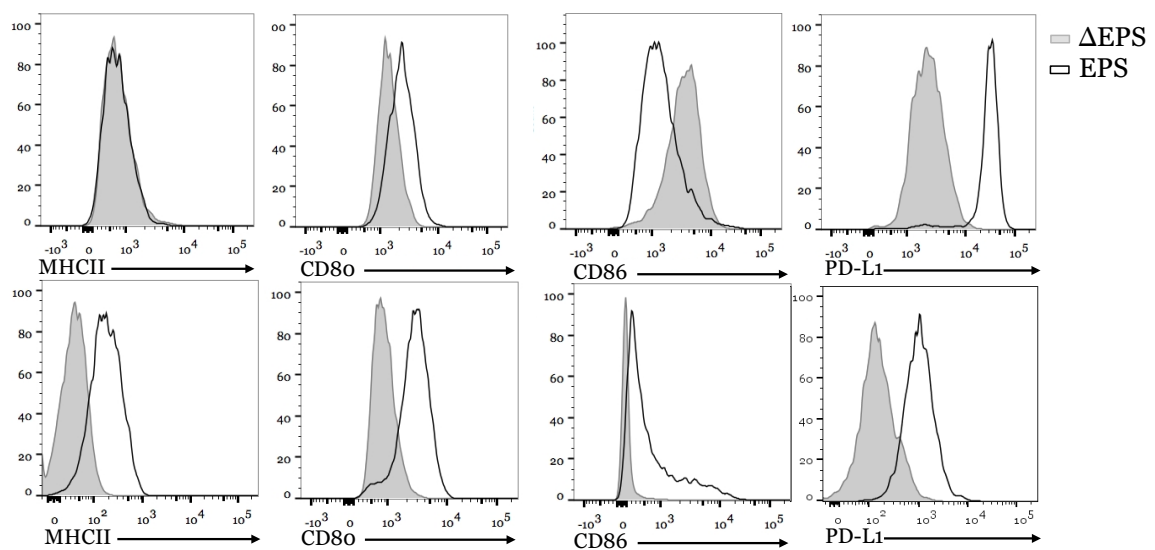


Figure 3.11. EPS-induced changes in macrophage co-stimulatory molecules *in vitro*. Peritoneal macrophages (top) or RAW264.7 cells (bottom) were incubated 16 h with EPS 5 $\mu\text{g}/\text{mL}$ or an equal volume of ΔEPS *in vitro* and analyzed by flow cytometry. Expression of MHC class II, CD80, CD86, PD-L1 and PD-L2 on F4/80⁺ macrophages was assessed by flow cytometry. Data are representative of duplicate wells in 4 independent experiments.

(Figure 3.11). EPS decreases expression of CD86 and does not alter MHCII in peritoneal macrophages treated *in vitro*, however, CD86 expression slightly increases, as does MHC class II in RAW264.7 cells treated with EPS. These data suggest that peritoneal macrophages respond similarly to macrophages from mice treated with EPS, but RAW264.7 cells are slightly altered in their response to EPS.

EPS-induced M2 Macrophage Localization *in vivo*.

Thus far, we have examined the generation of M2 macrophages *in vitro* in response to EPS and *in vivo* in the peritoneal cavity, but does EPS induce development of M2 macrophages in other organs and locations of the mouse? Further, can M2 macrophages induced in the peritoneal cavity travel to other locations, i.e., the colon, to protect from *C. rodentium*-induced disease?

To address these questions, we first examined the spleen and MLN of EPS-treated mice for the presence of M2 macrophages. The number of macrophages

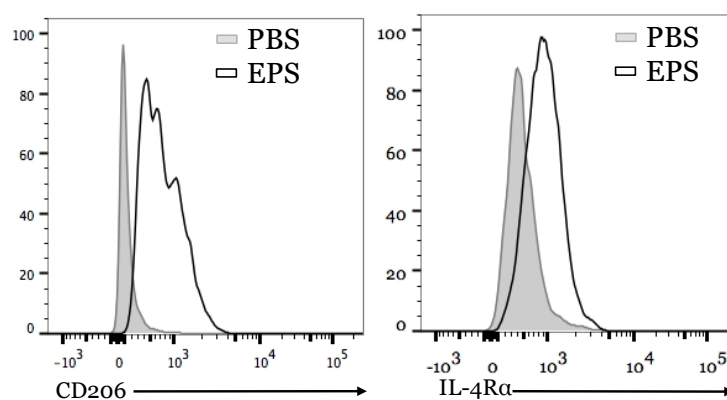


Figure 3.12. M2 macrophage development by intravenous injection with EPS. Mice were injected with 100 μ g of EPS in 100 μ L of PBS, or PBS alone, by tail vein injection. The phenotype of peritoneal macrophages (CD206 and IL-4R α) was assessed by flow cytometry 2 dpt. Representative data of 2 independent experiments with 2 mice per group in each experiment.

is small in both the spleen and the MLN. Although we sometimes find a slight increase in M2 macrophage phenotypic markers on macrophages in these organs following EPS treatment, the increases are not reproducible (data not shown). To verify that the injection site did not restrict the development of M2 macrophages, we injected mice with EPS by intravenous (i.v.) injection and examined the peritoneal cavity 2 dpt for M2 macrophages and found that M2 macrophages still develop when using an alternate route of injection (Figure 3.12).

To determine if EPS-induced M2 macrophages traffic to other organs from the peritoneal cavity, we isolated peritoneal cells from EPS-treated mice 3 dpt and adoptively transferred them to recipient mice by i.p. injection. Prior to transfer, the cells were labeled with the fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), that covalently binds free amines on the surface and within the cell. This allows us to track the transferred cells in the recipient mice.

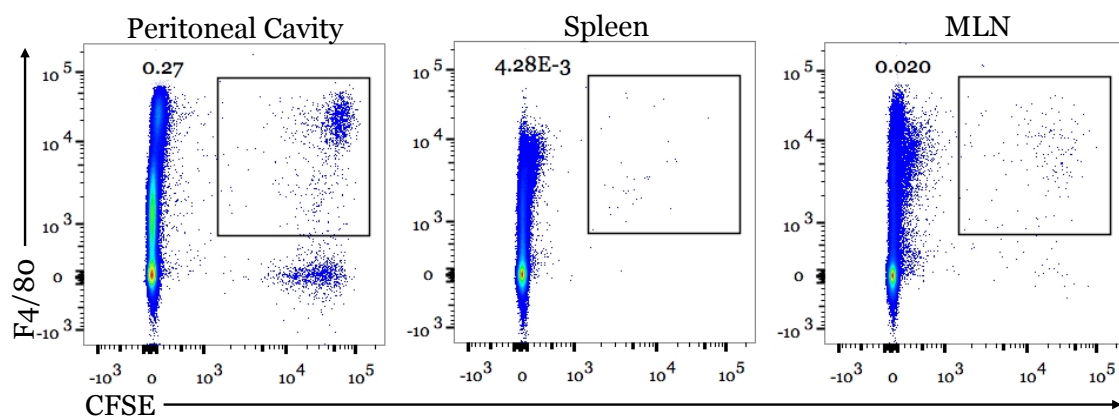


Figure 3.13. Trafficking of peritoneal macrophages from EPS-treated mice. Peritoneal cells were isolated from EPS-treated mice 3 dpt, labeled with CFSE and 10⁶ transferred (i.p.) into naïve mice. After 24 hours, peritoneal cells, spleen and MLN were isolated from recipient mice and analyzed for transferred CFSE⁺F4/80⁺ macrophages by flow cytometry. Representative flow cytometric profiles from 3 independent experiments.

At 24 h post-transfer, we analyzed the presence of CFSE⁺ cells in various organs by flow cytometry. As is shown in Figure 3.13, we find a small percentage of CFSE⁺F4/80⁺ cells that remain in the peritoneal cavity following injection, with a small percentage trafficking to the MLN at 24 h post-treatment. Very few transferred macrophages are found in the spleen. The majority of CFSE⁺F4/80⁻ cells in the peritoneal cavity and MLN are IgM⁺ B cells (data not shown). Since the MLN drains from the colon, we conclude that the macrophages from i.p.-treated EPS mice can migrate from the peritoneum and traffic to the MLN. Future experiments will determine if infection or injury enhance recruitment to various organs.

Mechanism by which EPS Induces M2 Macrophages.

Previously, we showed that TLR4 is required for EPS protection from *C. rodentium*-induced disease and that transfer of peritoneal cells from TLR4^{-/-} mice into WT mice did not prevent disease (Jones et al. 2014). These findings suggest that TLR4 signaling is required for the generation of EPS-induced M2 macrophages. To test this, we injected TLR4^{-/-} mice and TLR2^{-/-} mice with EPS, with the expectation that M2 macrophages would not develop in TLR4^{-/-} mice but would develop normally in TLR2^{-/-} mice. After injecting TLR4^{-/-} mice with EPS, we found no upregulated expression of the M2 markers IL-4R α , CD206, Arg-1, CD80, CD86 or PD-L1 by flow cytometry (Figure 3.14), whereas TLR2^{-/-} mice show upregulated expression of M2 macrophage markers, similar to WT mice (Figure 3.14, 3.9). Similarly, little to no upregulation of *FIZZ-1* and *Ym-1*

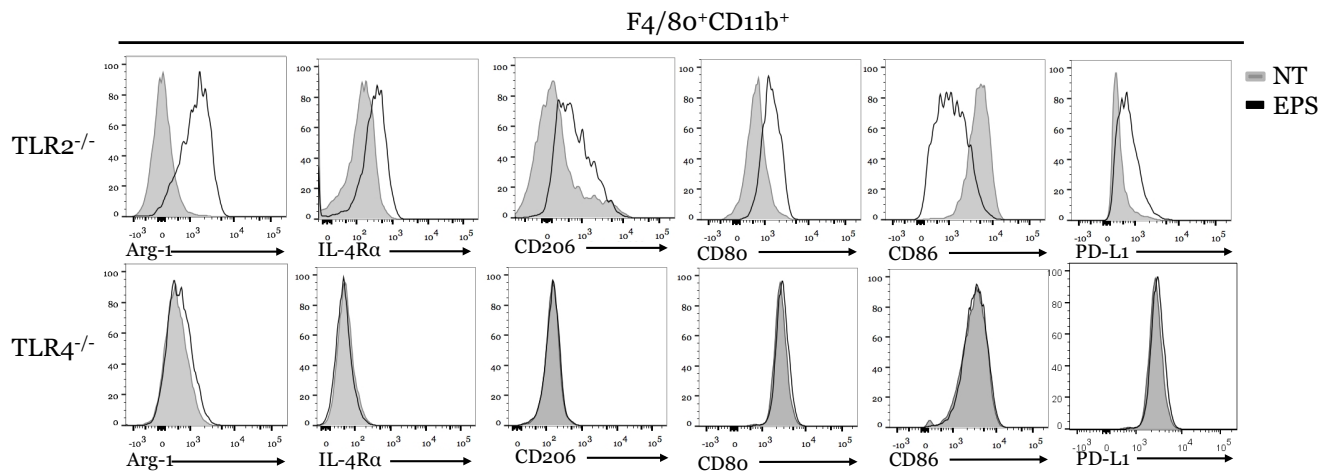


Figure 3.14. Induction of M2 macrophages in TLR-deficient mice.

TLR2^{-/-} and TLR4^{-/-} mice were injected with EPS and 3 days later peritoneal cells were examined by flow cytometry. Representative flow cytometric profiles of M2 macrophage marker expression (Arg-1, CD206, IL-4Rα, CD80, CD86 and PD-L1) on F4/80⁺CD11b⁺ cells from TLR2^{-/-} mice (top) or TLR4^{-/-} mice (bottom). Representative flow cytometric profiles of 6 independent experiments, at least two mice per group in each experiment.

transcripts occurs in macrophages from EPS- injected TLR4^{-/-} mice compared to WT mice, as well as no detectable levels of M1 macrophage markers (Figure 3.15).

We also tested if upregulation of M2 markers by EPS treatment of macrophages *in vitro* requires TLR4 signaling. Macrophages from WT, TLR2^{-/-} or TLR4^{-/-} mice were culture for 16 h with EPS and IL-4Rα and PD-L1 expression was assessed by flow cytometry. We expected to find upregulation of these markers in WT and TLR2^{-/-} macrophages, but not in TLR4^{-/-} macrophages. As expected, EPS upregulated expression of IL-4Rα and PD-L1 on WT and TLR2^{-/-} cells, but not on TLR4^{-/-} cells (Figure 3.16). These data suggest that the induction of M2 macrophages by EPS, both *in vitro* and *in vivo* requires TLR4 signaling.

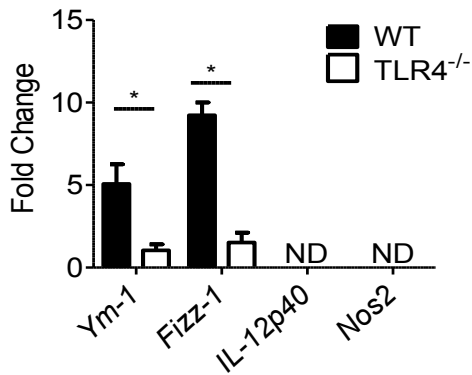


Figure 3.15. qRT-PCR analysis for M2 macrophage markers on TLR4^{-/-} macrophages after i.p. injection of EPS. Fold change by qRT-PCR of *Ym-1*, *FIZZ-1*, *IL-12p40* and *Nos2* expression in F4/80⁺CD11b⁺ cells in WT (■) and TLR4^{-/-} (□) mice injected with EPS (compared to mice injected with ΔEPS) (average of 4 independent experiments, N = 7 mice total per group). ND = not detectable.

TLR4 signals through two downstream adapter molecules: TRIF and MyD88.

Our lab previously showed that mice lacking MyD88 signaling specifically in myeloid cells are not protected from disease by EPS (Jones et al. 2014), which supports our conclusions that macrophages, a myeloid lineage cell, mediates protection, and that EPS signals through TLR4. Given these results, we asked if EPS induces M2 macrophages in myeloid-specific MyD88^{-/-} mice. Since these mice are not protected from EPS, we would not expect to see induction of protective M2 macrophages. As expected, no M2 macrophages were found in the peritoneal cavity of EPS-injected myeloid-specific MyD88^{-/-} mice (Figure 3.17). Taken together, these data suggest that both TLR4 and MyD88 in myeloid cells are required for the polarization of M2 macrophages by EPS.

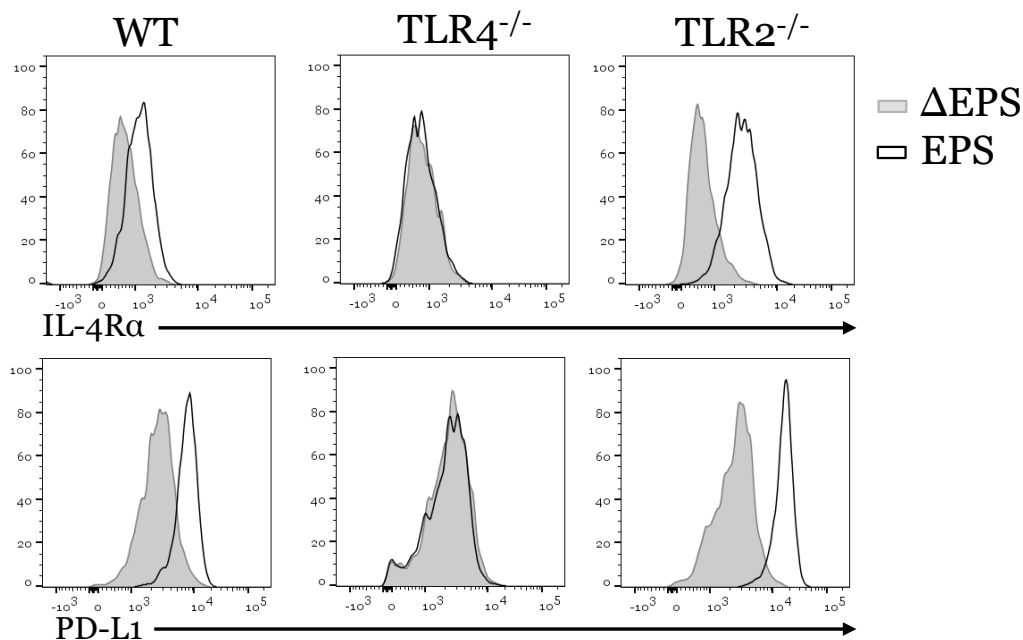


Figure 3.16. *in vitro* induction of M2 macrophages by EPS in TLR-deficient mice. Peritoneal macrophages from WT, TLR4^{-/-} or TLR2^{-/-} mice were incubated 16 h with EPS (5 μ g) *in vitro* and analyzed by flow cytometry. Expression of IL-4R α and PD-L1 were assessed by flow cytometry. Representative flow cytometric profiles of duplicate wells in 3 independent experiments.

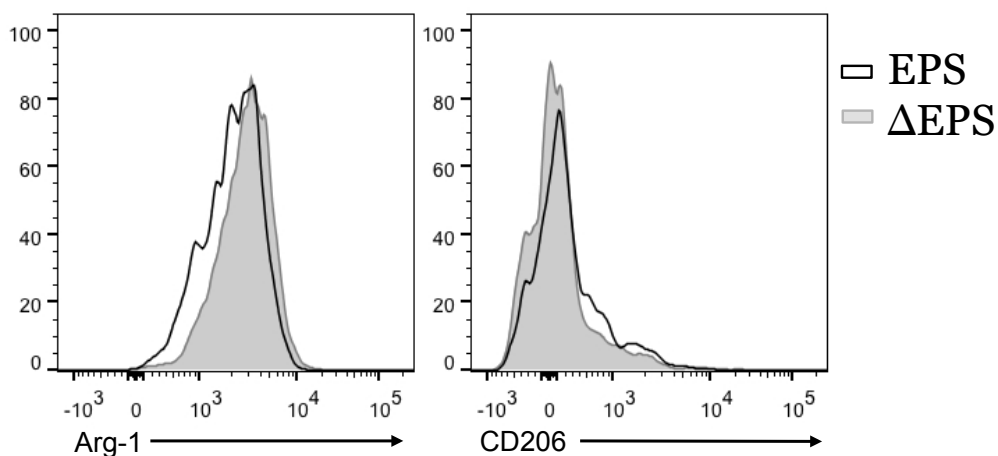


Figure 3.17. Flow cytometric analysis of M2 macrophage markers on peritoneal macrophages from EPS-treated myeloid MyD88^{-/-} mice. Representative profiles of 4 independent experiments; N = 8 mice total per treatment.

Polarization of M2 macrophages often requires IL-4 and IL-13, IL-10, or TGF- β . We find upregulation of IL-4R α on EPS-induced M2 macrophages, as well as increased levels of genes expressed downstream of IL-4 and IL-13 signaling, including CD206, Ym-1, and arginase-1, leading us to hypothesize that EPS induces M2 macrophages by upregulating IL-4 and IL-13 production in macrophages. To test if macrophages express IL-4 and IL-13 in response to EPS, we cultured total peritoneal cells with EPS overnight, and then added brefeldin A (5 μ g/mL) for 3 h to allow for retention of the cytokines intracellularly, and analyzed by flow cytometry. If EPS induces M2 macrophages using IL-4 and IL-13, we expect to see increased expression of these cytokines in macrophages in response to EPS. Figure 3.18 shows that F4/80⁺ peritoneal cells upregulate both

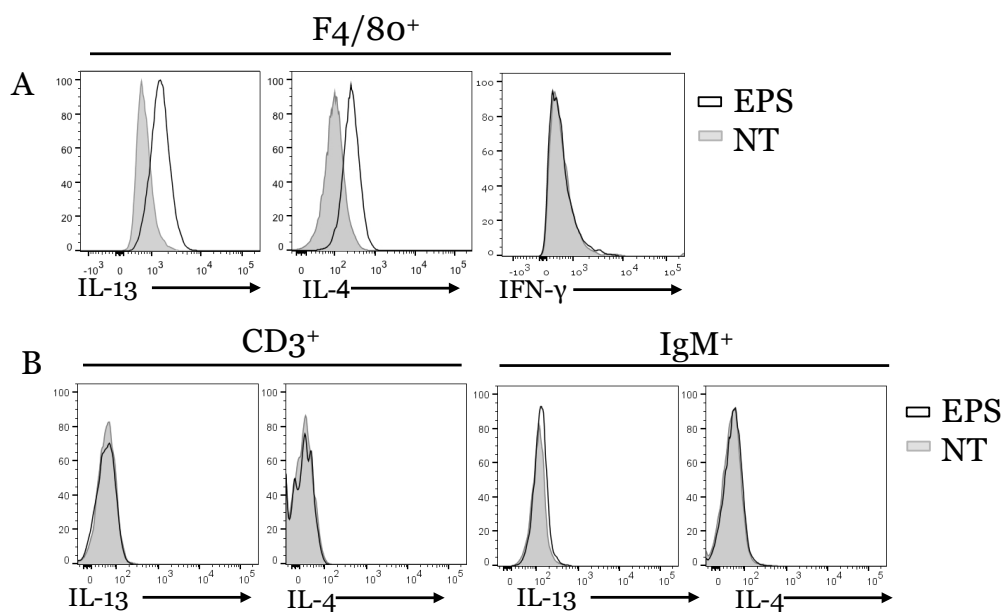


Figure 3.18. EPS-induced changes in cytokine phenotype of peritoneal cells *in vitro*. Peritoneal cells were incubated 16 hr with EPS *in vitro* and analyzed by flow cytometry. IL-13, IL-4, or IFN- γ expression in F4/80⁺ cells (A) IL-13 and IL-4 expression in CD3⁺ or IgM⁺ cells (B). Data are representative of duplicate wells in 4 independent experiments.

IL-4 and IL-13, whereas no increase in cytokine expression is seen in CD3⁺ T cells or IgM⁺ B cells, (Figure 3.18). Further, we find no changes in the M1 macrophage cytokine, IFN- γ . Consistent with this finding, EPS induces M2 macrophages in CD4^{-/-} mice (Figure 3.19), suggesting that macrophages do not require CD4⁺ T cells for M2 polarization in response to EPS. Taken together, these *in vitro* and *in vivo* data support the hypothesis that EPS directly drives M2 macrophage polarization by upregulating IL-4 and IL-13, although we cannot rule out the contribution of other type-2 immunity promoting cells, including ILC2s (innate lymphoid cells), DCs, and eosinophils, in helping to maintain these cells *in vivo*.

Recent studies have demonstrated that macrophages self-sustain within tissues by proliferating (Jenkins et al. 2011; Yona et al. 2013). Additionally, tissue-resident macrophages proliferate in response to M2 macrophage-polarizing conditions (Jenkins et al. 2013). We hypothesized that since EPS upregulates M2 macrophage-inducing cytokines, EPS will induce macrophage

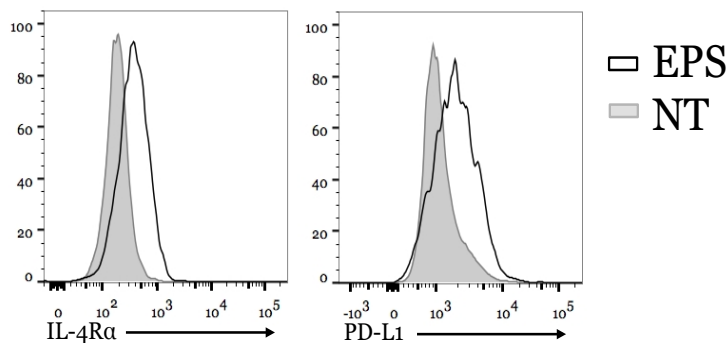


Figure 3.19. M2 macrophage induction in CD4^{-/-} mice. CD4^{-/-} mice were injected with 100 μ g of EPS and 3 dpt peritoneal F4/80⁺ macrophages were assessed by flow cytometry for M2 macrophage markers. Data are representative flow cytometric profiles of 8 independent experiments, 1-3 mice per group per experiment.

proliferation within the peritoneal cavity. To test this hypothesis, we isolated peritoneal cells from mice 3 dpt with EPS and labeled them with CellTrace Violet (CTV), a highly stable membrane-permeable fluorescent dye. As cells proliferate, CTV dilutes, which can be measured by flow cytometry. Once labeled with CTV, cells were transferred into naïve mice, and 48 h later, peritoneal cells were isolated and analyzed for proliferation by flow cytometry. If EPS stimulates macrophage proliferation, we expect to find increased dilution of CTV in peritoneal cells from mice treated with EPS compared to cells from untreated mice. As expected, in F4/80⁺ cells from donor mice treated with EPS, we find increased decreased levels of CTV, and hence increased proliferation compared to F4/80⁺ cells from untreated donor mice (Figure 3.20). CD3⁺ T cells and CD19⁺ B cells from either group of mice did not proliferate. In CD11c⁺ DCs, we find two peaks in CTV staining from EPS-treated cells, compared to only one peak from untreated cells (data not shown). Because only a few DCs are present within the peritoneal cavity and the population is fairly heterogeneous, it is difficult to draw a conclusion about these cells. *In vitro*, we do not find increases in macrophage proliferation following EPS treatment (data not shown). Macrophages tightly adhere to plastic in tissue-culture dishes; the vastly different environment *in vitro* compared to *in vivo* conditions, including infiltrating monocytes, may explain the differences in proliferation. Further studies are needed to optimize *in vitro* conditions to determine if macrophages proliferate *in vitro* and if DCs proliferate in response to EPS.

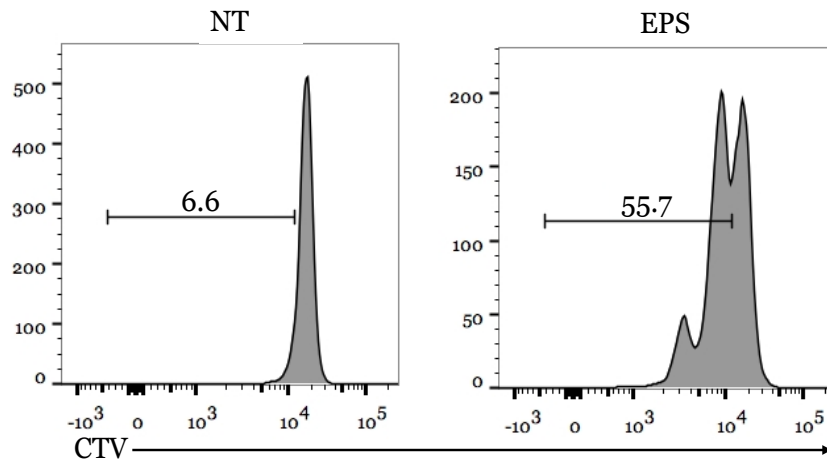


Figure 3.20. Proliferation of peritoneal macrophages following EPS treatment. Peritoneal cells were isolated from untreated or EPS-treated mice 3 dpt, labeled with CTV, and transferred (i.p.) into untreated recipient mice. Two days post-transfer, CTV dilution was assessed in CTV⁺F4/80⁺ macrophages by flow cytometry. Data are representative flow cytometric profiles of 2 independent experiments with 2 mice per group.

Conclusions from Section 1.

EPS induces M2 macrophages. These macrophages are required for protection from *C. rodentium*-induced disease and transfer of these cells to recipient mice prevents disease. EPS acts directly on macrophages to induce an M2 macrophage phenotype and also upregulates IL-4 and IL-13. Induction of M2 macrophages requires TLR4 and MyD88 signaling in myeloid cells.

Section 2. Mechanism by which EPS-induced M2 Macrophages Exert Anti-inflammatory Effect

Inhibition of T cell Responses *in vitro*.

EPS induces M2 macrophages, and adoptive transfer of these macrophages prevents disease associated with *C. rodentium*. Further, depletion

of macrophages alleviates these protective effects. How do EPS-induced M2 macrophages prevent inflammation induced by *C. rodentium*? M2 macrophages exert anti-inflammatory effects through a variety of different mechanisms, one of which is through inhibition of T cells. Because much of the damage caused by *C. rodentium* is due to a hyper-reactive CD4⁺ T cell response, as discussed in Chapter 1, we hypothesized that EPS prevents *C. rodentium*-induced inflammation by inducing M2 macrophages, which inhibit T cell responses.

To test this possibility, we developed an *in vitro* T cell proliferation assay. T cells proliferate *in vitro* in response to anti-CD3 and co-stimulation. T cells can be labeled with CTV for the purpose of tracking proliferation. Following activation with anti-CD3, T cells upregulate activation markers including CD25, the IL-2R α chain, and CD44, a glycoprotein involved in cell-to-cell adhesion, cytokine production increases, especially IL-2 and IFN- γ , and then the T cells proliferate rapidly. As the cells proliferate, the fluorescent dye associated with each cell is decreased, and this decrease can be measured by flow cytometry. We hypothesized that co-culture of T cells with peritoneal cells from EPS-treated mice would result in decreased T cell proliferation. To test this hypothesis, we labeled total splenocytes with CTV and added the cells to anti-CD3 ϵ -coated wells. The anti-CD3 ϵ stimulates the TCR, and B cells present in the splenocytes provide co-stimulation. We then added peritoneal cells from EPS- or Δ EPS-treated mice and tested for a decrease in T cell proliferation, activation and cytokine production. If EPS-induced M2 macrophages are inhibitory, we expected to see a

decrease in CD4⁺ T cell activation and proliferation in co-cultures with peritoneal cells from EPS-treated mice. By flow cytometry, we found a decrease in activated CD4⁺CD44⁺CD25⁺ T cells (Figure 3.21A, C middle) in co-cultures with peritoneal cells from EPS-treated mice compared to peritoneal cells from ΔEPS-treated mice. Further, CD4⁺ T cells do not proliferate when cultured with peritoneal cells from EPS-treated mice compared to cultures with peritoneal cells from ΔEPS-treated mice or splenocytes alone (Figure 3.21B, C left). Additionally, total IFN-γ levels are decreased in the supernatant of the cultures containing peritoneal cells from EPS-treated mice compared to peritoneal cells from ΔEPS-treated mice or splenocytes alone (no peritoneal cells) (Figure 3.21C right). These data indicate that peritoneal cells from EPS-treated mice inhibit CD4⁺ T cell responses.

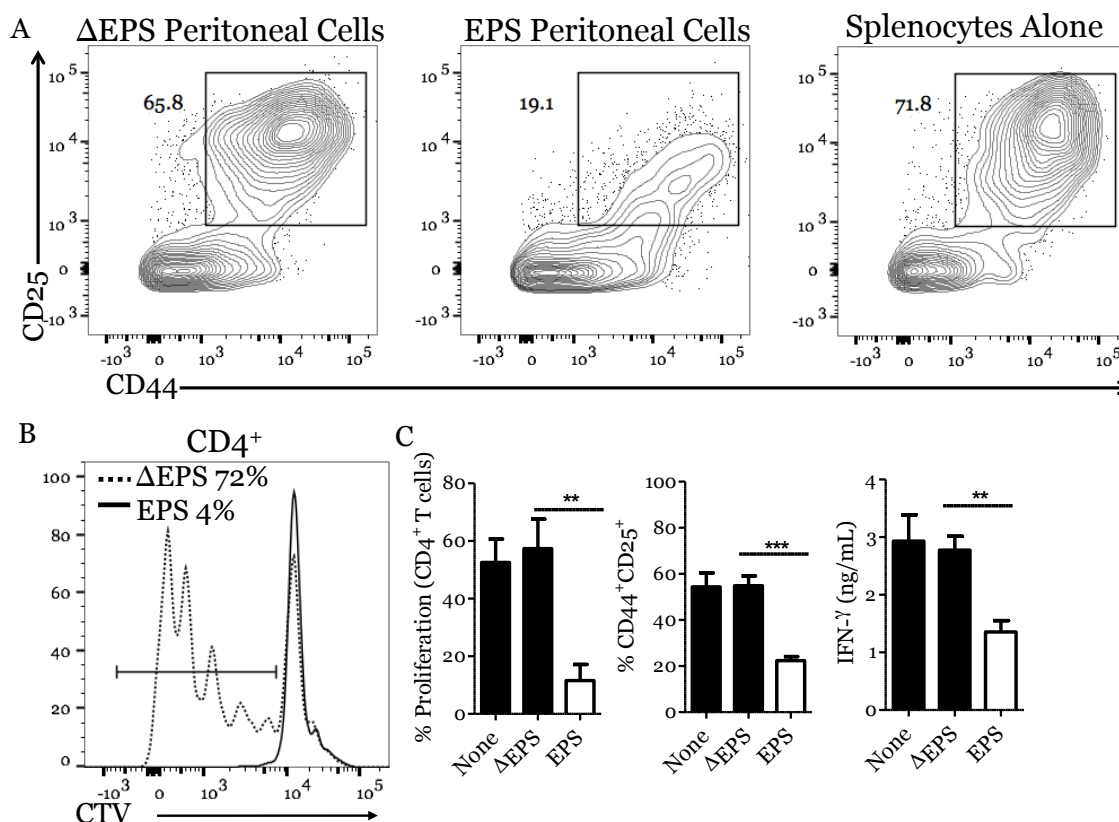


Figure 3.21. Inhibition of anti-CD3-stimulated CD4⁺ T cell proliferation by peritoneal cells from EPS-treated mice. CTV-labeled spleen cells were stimulated with anti-CD3, cultured with total peritoneal cells from EPS or Δ EPS-treated mice, and analyzed by flow cytometry. **A.** CD4⁺CD44⁺CD25⁺ Activated T cells. **B.** Proliferation of CD4⁺ T cells; Horizontal bar = % proliferating cells. **C.** (Left) % proliferating CD4⁺ T cells; (center) % activated CD4⁺ T cells (CD44⁺CD25⁺); (right) concentration of IFN- γ in culture supernatants as determined by ELISA. Average of 10 independent experiments; N = 15 mice total per group. Statistical significance determined by Student's *t* test. $p < 0.05$ denoted as *.

Since peritoneal cells from EPS-treated mice were co-cultured with total splenocytes (containing both CD4⁺ and CD8⁺ T cells), we also tested if CD8⁺ T cell responses were suppressed. We found that, like CD4⁺ T cells, CD8⁺ T cell activation was decreased in co-cultures with peritoneal cells from EPS-treated mice compared to peritoneal cells from Δ EPS mice (Figure 3.22A), although the

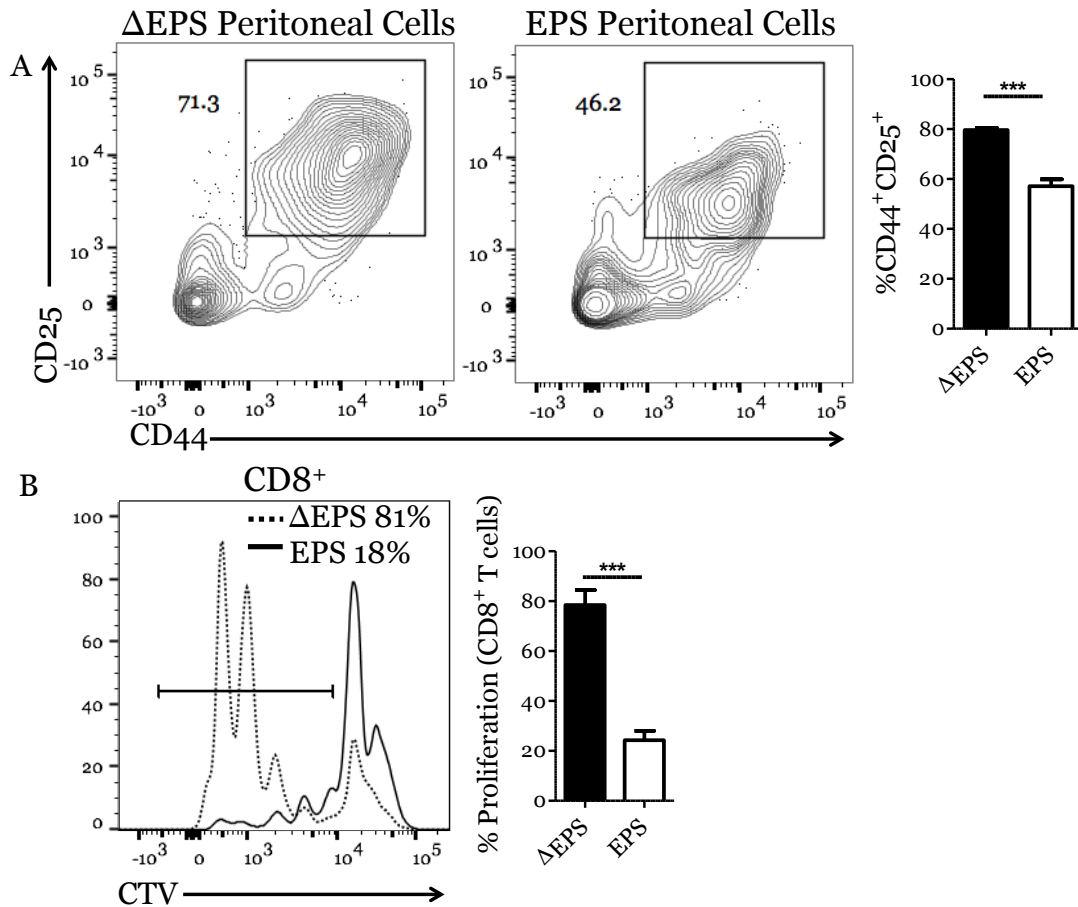


Figure 3.22. Inhibition of anti-CD3-stimulated CD8⁺ T cell proliferation by peritoneal cells from EPS-treated mice. CTV-labeled spleen cells were stimulated with anti-CD3 and cultured with total peritoneal cells from EPS or Δ EPS-treated mice. **A.** Activated CD8⁺ T cells (CD44⁺CD25⁺) in co-cultures with peritoneal cells from Δ EPS-treated mice (left) or EPS-treated mice (center); % Activated CD8⁺ T cells (CD44⁺CD25⁺) in 3 independent experiments; N = 7 mice total per group. **B.** Proliferation of CD8⁺ T cells; Horizontal bar = % proliferating cells; (right) % proliferating CD8⁺ T cells in 3 independent experiments; N = 7 mice total per group. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

reduction was not as much as for the CD4⁺ T cells. CD8⁺ T cell proliferation is also greatly reduced in co-cultures with EPS-treated mice compared to peritoneal cells from Δ EPS mice (Figure 3.22B). Since IFN- γ was measured in culture supernatants, the reduction in IFN- γ production in Figure 3.20C is likely due to

decreased expression by both CD4⁺ and CD8⁺ T cells. Taken together, these data demonstrate that peritoneal cells from EPS-treated mice inhibit both CD4⁺ and CD8⁺ T cell responses.

Although unlikely that EPS would be present in the peritoneal cavity 3 dpt, we verified that EPS doesn't directly inhibit T cell proliferation by culturing with purified CD4⁺ T cells with EPS or ΔEPS for 16 h, then stimulated the cells with

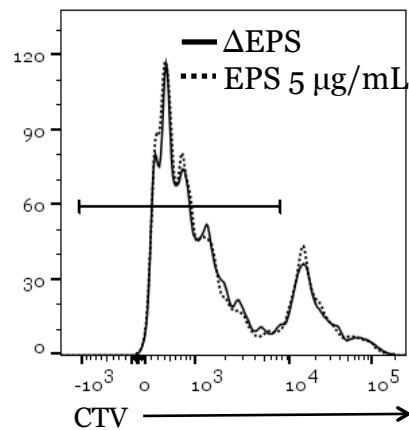


Figure 3.23. T cell proliferation following *in vitro* treatment with EPS. CTV-labeled CD4⁺ T cells were co-cultured with EPS (20 μg/mL) or an equal volume of ΔEPS for 16 h, then added to anti-CD3-coated plates with soluble anti-CD28. Three days later, proliferation was assessed by flow cytometry. Representative profile of duplicate wells in 3 independent experiments.

anti-CD3 and anti-CD28. Three days later, we assessed proliferation by flow cytometry and found no differences in CD4⁺ T cell proliferation in cells treated with EPS compared to ΔEPS-treated cells (Figure 3.23).

Anti-CD3 strongly stimulates T cells activation and proliferation. We asked if peritoneal cells from EPS-treated mice inhibit antigen-specific T cell proliferation, which is a more physiologically-relevant analysis. To do this, we utilized OT-II transgenic mice in which CD4⁺ T cells express an ovalbumin-

specific $\alpha\beta$ TCR. Peritoneal cells were isolated from PBS- or EPS-treated WT mice 3 dpt and pulsed overnight with ovalbumin (OVA). $CD4^+$ T cells were purified from OT-II mice and co-cultured with OVA-pulsed peritoneal cells for 6 days. We found that peritoneal cells from EPS-treated mice inhibit $CD4^+$ T cells proliferation compared to proliferation of T cells in culture with peritoneal cells from PBS-treated mice (Figure 3.24). Peritoneal cells from EPS-treated mice also inhibit OVA-specific T cell proliferation using bone marrow-derived dendritic cells as the antigen-presenting cell (APC) (data not shown). This experiment

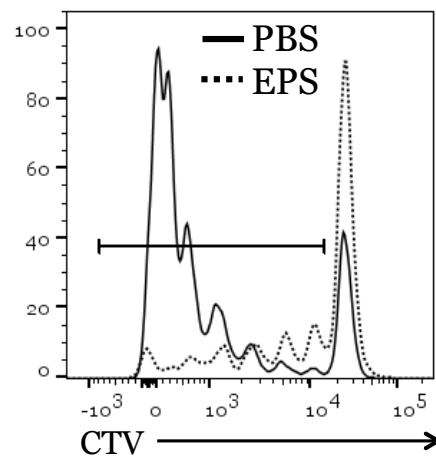


Figure 3.24. Inhibition of antigen-specific T cell proliferation by EPS-induced M2 macrophages. Anti-CD3-stimulated, CTV-labeled $CD4^+$ T cells from OT-II transgenic mice were co-cultured with OVA-pulsed peritoneal cells from PBS-treated or untreated mice. Three days later, proliferation was assessed by flow cytometry. Representative data from 3 independent experiments.

demonstrates that reduced proliferation was not due to decreased antigen presentation by peritoneal cells from EPS-treated mice, although we have not ruled out this possibility. These data suggest that peritoneal cells from EPS-treated mice inhibit antigen-specific T cell proliferation.

Since we hypothesize that EPS-induced M2 macrophages within the peritoneal cavity exert this anti-inflammatory effect, we next tested if purified macrophages from EPS-treated mice inhibit T cell proliferation. We FACS-sorted F4/80⁺CD11b⁺ cells from the peritoneal cavity of EPS-treated or ΔEPS-treated mice and co-cultured them in the T cell proliferation assay. We expected that if macrophages mediate the inhibitory activity within the peritoneal cavity in this assay, that macrophages would inhibit T cell responses and F4/80⁻ cells would not. As expected, macrophages from EPS-treated mice inhibited CD4⁺ T cell proliferation (18% proliferation), compared to F4/80⁺CD11b⁺ cells from ΔEPS-treated mice, which did not inhibit proliferation (75% proliferation). Further, FACS-sorted T cells (CD3⁺) and B cells (IgM⁺) from EPS-treated mice did not inhibit T cell proliferation (Figure 3.25).

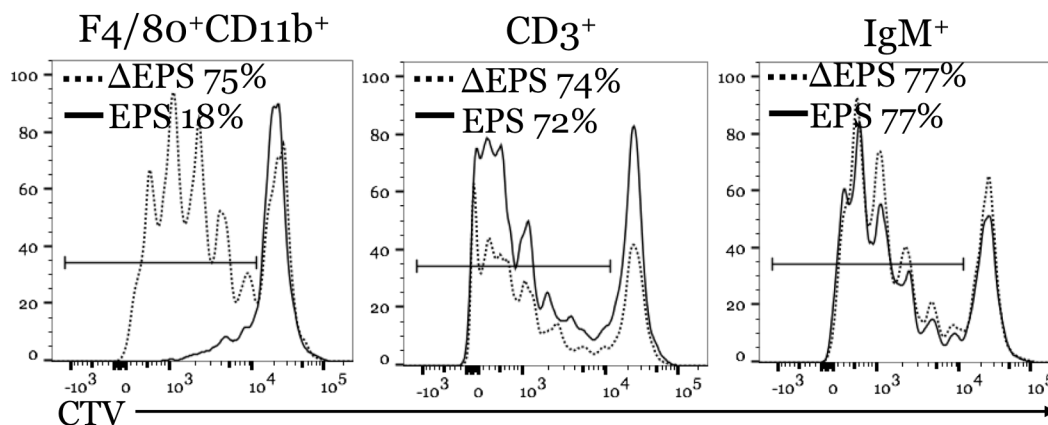


Figure 3.25. Proliferation of CD4⁺ T cells in co-cultures with FACS-purified peritoneal cell populations. CTV-labeled spleen cells were stimulated with anti-CD3 in co-cultures with FACS-purified cells from EPS or ΔEPS-treated mice. Proliferation of CD4⁺ T cells with F4/80⁺CD11b⁺ cells (left); CD3⁺ T cells (center) or IgM⁺ B cells (right). Horizontal bar = % proliferating cells. Representative flow cytometric profiles from 3 independent experiments; N = 7 mice total per group.

To further test if M2 macrophages are required for T cell inhibition, we co-cultured proliferating T cells with peritoneal cells from EPS-treated mice that were depleted of macrophages by clodronate liposomes. As a control, we co-cultured T cells with peritoneal cells from EPS-treated mice that were treated with PBS liposomes, with the expectation that peritoneal cells from these mice would inhibit proliferation. Further, we expected the cells from mice treated with clodronate liposomes and EPS would no longer inhibit proliferation. As expected, peritoneal cells from PBS liposome-EPS-treated mice inhibited CD4⁺ T cell proliferation, whereas peritoneal cells from EPS-treated mice depleted of macrophages did not inhibit proliferation (Figure 3.26).

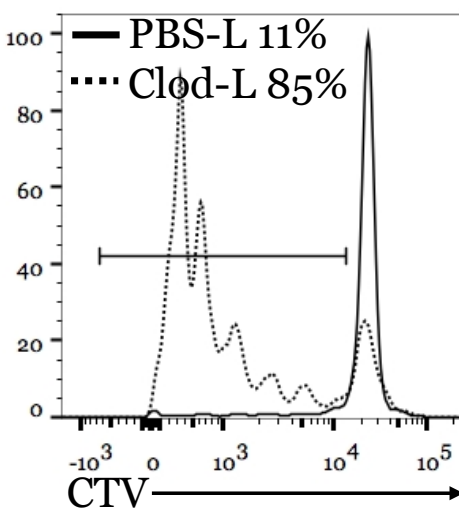


Figure 3.26. Proliferation of CD4⁺ T cells in co-cultures with peritoneal cells from macrophage-depleted mice. Mice were treated with EPS 4-6 h post-treatment with PBS liposomes (PBS-L) or clodronate liposomes (Clod-L). Peritoneal cells were co-cultured with CTV-labeled spleen cells and stimulated with anti-CD3. Proliferation of CD4⁺ T cells, horizontal bar = % proliferating cells. Representative flow cytometric profiles from 4 independent experiments; N = 6 mice total per group.

Similarly, peritoneal cells from EPS-treated $TLR4^{-/-}$ or myeloid $MyD88^{-/-}$ mice, which do not generate M2 macrophages, also did not inhibit T cell proliferation, whereas peritoneal cells from $TLR2^{-/-}$, which do develop M2 macrophages, inhibit proliferation (Figure 3.27). We conclude that EPS induces anti-inflammatory M2 macrophages that inhibit T cell responses *in vitro*.

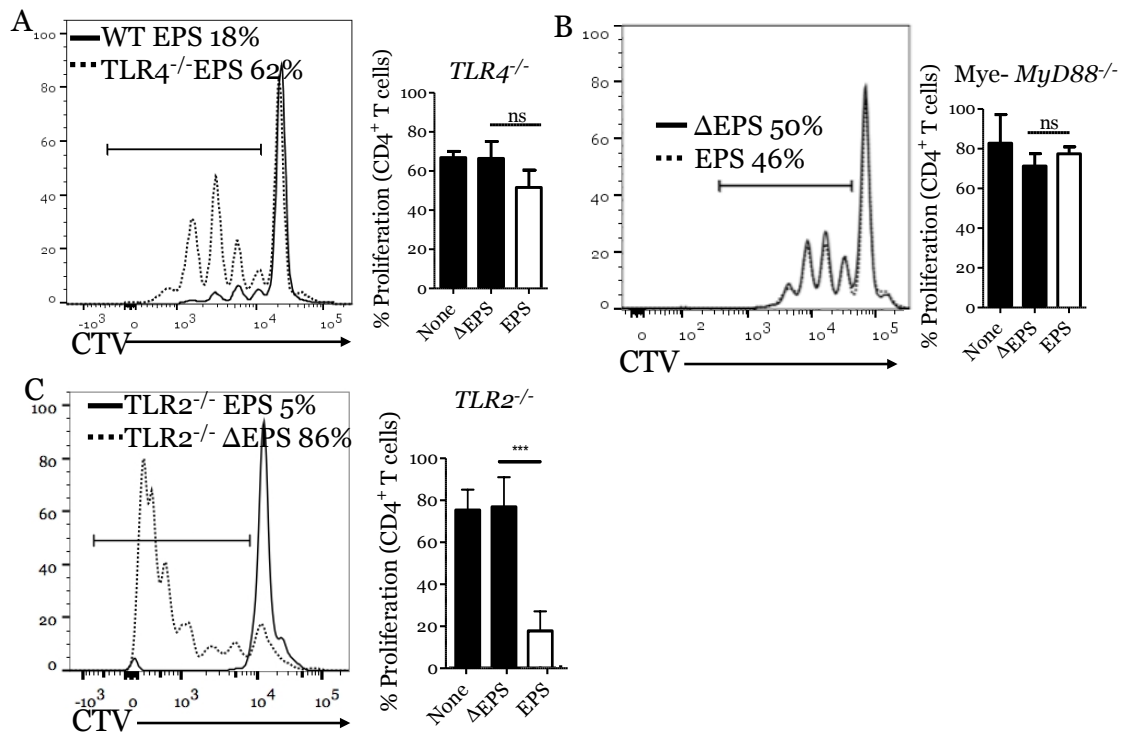


Figure 3.27. Proliferation of CD4⁺ T cells in co-cultures with peritoneal cells from EPS-treated TLR-deficient mice. CTV-labeled spleen cells were stimulated with anti-CD3 and cultured with total peritoneal cells from EPS- or ΔEPS-treated mice. Proliferation of CD4⁺ T cells cultured with peritoneal cells from $TLR4^{-/-}$ (A), myeloid $MyD88^{-/-}$ (B), or $TLR2^{-/-}$ (C) mice. Horizontal bar = % proliferating cells. None = splenocytes alone. 4-6 independent experiments with N = 7-10 mice total per group. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

Inhibition of T cell Responses *in vivo*.

The data above indicate that EPS-induced M2 macrophages have the

capacity to inhibit T cells *in vitro*, but does this occur *in vivo*? Naïve CD4⁺ T cells polarize into different T cell subsets based on their environment. Upon antigen-specific interactions, co-stimulation and the surrounding cytokine milieu, T cells activate, proliferate, and produce effector cytokines, leading to inflammation. We tested if EPS inhibited T cell responses *in vivo* by measuring levels of Th1, Th17, and Th2 inflammatory cytokines, IFN- γ , IL-17A and IL-13, respectively, in cultures of splenocytes from EPS- or Δ EPS-treated mice stimulated with anti-CD3 ϵ . The secretion of each of the cytokines was decreased in EPS-treated mice compared to Δ EPS-treated mice (Figure 3.28), indicating that EPS induces a systemic anti-inflammatory response that broadly suppresses Th1, Th17, and Th2 cytokine production.

To determine if EPS alters T cell responses during inflammation *in vivo*, we administered EPS to mice and 3 days later, after induction of M2

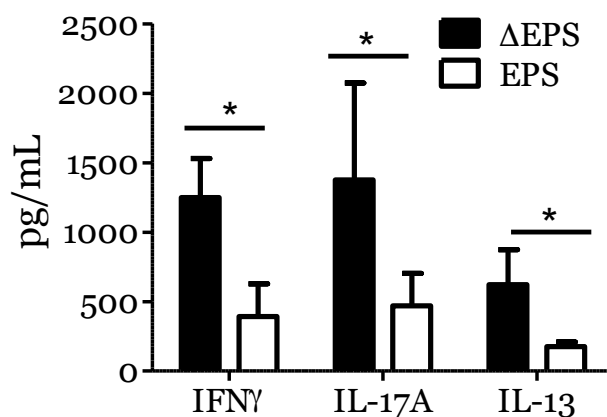


Figure 3.28. Inhibition of T cell responses in EPS-treated mice.

Quantification by ELISA of IFN γ , IL-17A, and IL-13 in culture supernatants of anti-CD3-stimulated splenocytes from EPS- or Δ EPS-treated mice. Average of 4 independent experiments with N = 8 mice total per group. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

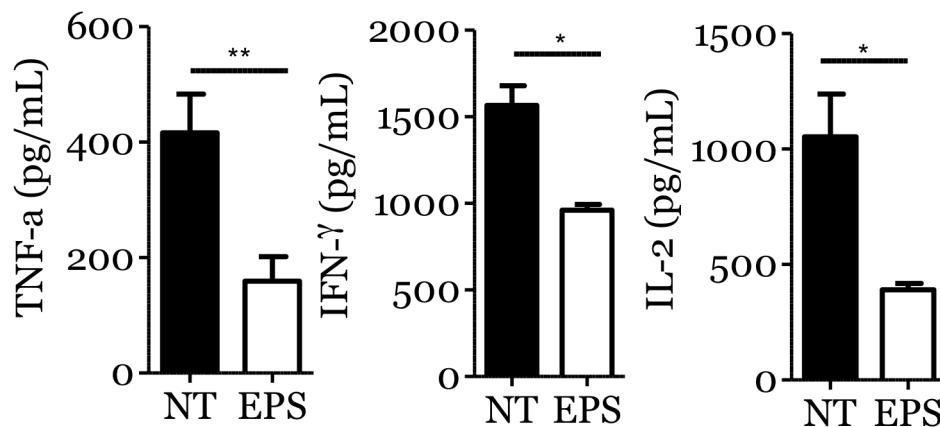


Fig. 3.29. Inhibition of T cell cytokine responses in serum from EPS-treated mice. Quantification by ELISA of serum TNF- α , IFN- γ and IL-2 in EPS-treated or untreated (NT) mice 2 h post-i.p. injection of anti-CD3. Average of 3 independent experiments with N = 9 mice total per group. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

macrophages, injected them i.p. with anti-CD3 ϵ , which induces potent T cell activation and release of TNF- α and IL-2 (Ferran et al. 1991). We expected decreased production of these cytokines in the blood of EPS-treated mice compared to Δ EPS-treated mice. Indeed, we found a two-fold decrease in production of serum TNF- α , IL-2 and IFN- γ in EPS-injected mice compared to mice injected with Δ EPS (Figure 3.29). Together, these data indicate that EPS inhibits T cell responses *in vivo*.

Mechanism by which EPS-induced M2 Macrophages Inhibit T cells.

Inhibitory molecules produced by EPS-induced M2

macrophages. M2 macrophages inhibit T cell responses by multiple factors, including Arg-1, PGE₂, IL-10, PD-L1/2 and TGF- β (Taylor et al. 2006; Pesce et al. 2009; Huber et al. 2010). In section 1, we established that EPS-induced M2 macrophages express Arg-1 and PD-L1, but these cells may also express other

inhibitory cytokines, such as IL-10, known to inhibit T cell proliferation. We used ELISA to test if EPS-induced M2 macrophages upregulate IL-10 production, and found no increase in IL-10 levels in culture supernatants compared to supernatants from untreated macrophages (Figure 3.30A).

TGF- β is secreted as a latent pro-peptide, and cannot interact with its receptor unless cleaved. TGF- β associates with latency-associated peptide (LAP) in the cytoplasm, but when associated with LAP, can be expressed on the cell surface, although the process is not well understood. Cells and tissues rapidly inactivate TGF- β following secretion making it difficult to detect; inactive TGF- β can be activated with acid, allowing detection. To determine if EPS upregulates

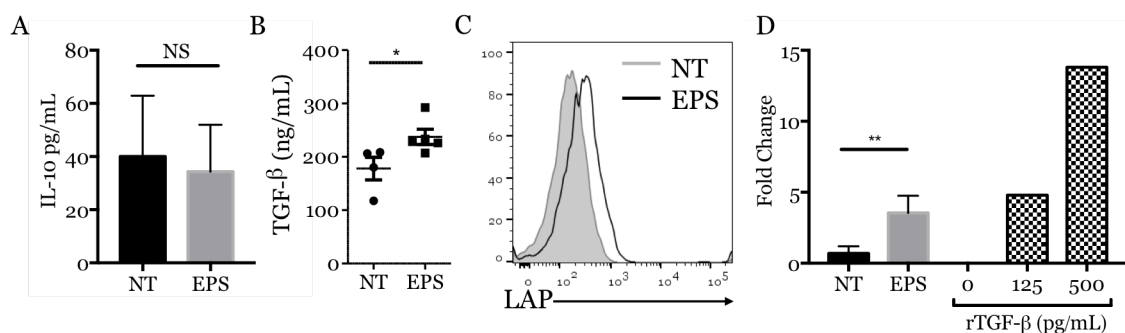


Figure 3.30. Identification of inhibitory molecules produced by EPS-induced M2 macrophages. A. Quantitation of IL-10 in cell supernatants of macrophages from untreated or EPS-treated mice. Average of 6 mice in 2 independent experiments. B. Quantification by ELISA of total TGF- β in serum after EPS-treatment or no treatment (NT). N = 4-5 mice total per group. C. LAP expression on F4/80⁺ macrophages 3 dpt with EPS compared to F4/80⁺ cells from untreated mice. D. Fold change in TGF- β reporter activity in co-cultures of MFB-F11 cells with peritoneal cells from EPS-treated relative to TGF- β activity in untreated mice. rTGF- β = recombinant human TGF- β . Average of 6 mice from 2 independent experiments. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

expression of TGF- β , we activated total TGF- β in serum by acid-treating with 1 N HCl, and then neutralizing with 1 N NaOH. By ELISA, EPS upregulates total TGF- β in serum compared to untreated mice (Figure 3.30B). To test if EPS upregulates TGF- β in macrophages from EPS-treated mice, we assessed LAP expression on F4/80⁺ cells from EPS-treated mice and untreated mice. Since LAP, and not TGF- β , has a transmembrane domain, if EPS upregulates cell-surface TGF- β , we would expect to see LAP upregulated. We find a very slight upregulation of LAP expression on the surface of macrophages following EPS treatment. (Figure 3.30C).

We further tested for increased TGF- β expression by co-culturing peritoneal cells from EPS-treated mice with MFB-F11 cells. This *Tgf-b1*^{-/-} fibroblast cell line stably expresses a reporter plasmid consisting of TGF- β responsive Smad-binding elements coupled to a secreted alkaline phosphatase reporter gene. Smad proteins are part of a signaling cascade activated upon TGF- β receptor signaling. Since this cell line originates from a *Tgf-b1*^{-/-} mouse, the assay has limited background activity. We expected that if EPS-induced M2 macrophages express TGF- β that we would see an increase in alkaline phosphatase activity compared to cells from untreated mice. Relative to cells from untreated mice, we find a 4-fold increase in Smad activity, and therefore TGF- β activity, in cells from EPS-treated mice (Figure 3.30D). The 4-fold increase in TGF- β was equivalent to 125 pg/mL of recombinant human TGF- β added to MFB-F11 cells. These data suggest that EPS-induced M2 macrophages

produce TGF- β , which is a potent inhibitor of T cell proliferation.

Since EPS-induced M2 macrophages express multiple inhibitory factors, including Arg-1, PD-L1 and TGF- β , we next tested if EPS-induced M2 macrophages inhibit via a soluble factor or by cell-to-cell contact. We used transwells to separate peritoneal cells from T cells. We expected that if inhibition requires cell contact, the peritoneal cells from EPS-treated mice would no longer inhibit T cell proliferation if placed in a transwell, separated from the splenocytes. As is seen in Figure 3.31, EPS-induced M2 macrophages no longer inhibited CD4⁺ or CD8⁺ T cell proliferation when separated from the T cells, indicating that cell-to-cell contact is required for inhibition (Figure 3.31); this finding does not rule out the possibility that soluble factors also contribute to the inhibition.

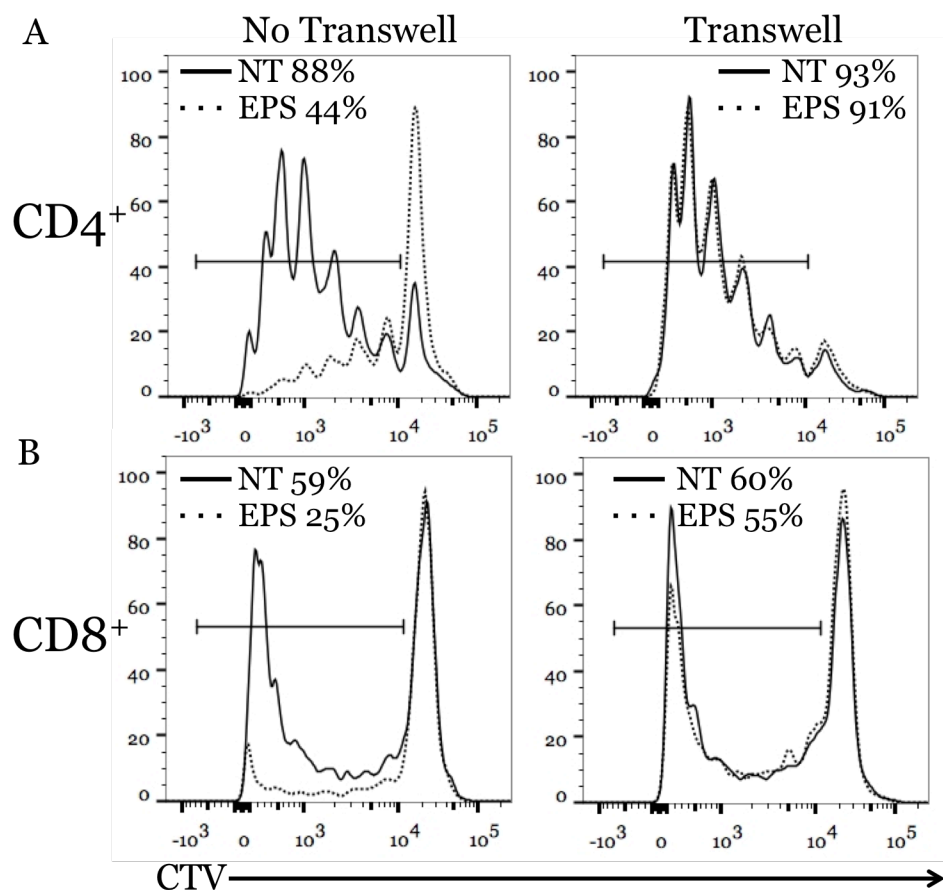


Figure 3.31. Proliferation of T cells in contact with, or separated from peritoneal cells. Proliferation of $CD4^+$ (A) or $CD8^+$ (B) T cells co-cultured with peritoneal cells from untreated (NT)- or EPS-treated mice in direct contact (no transwell, left) or with peritoneal cells in a transwell insert (right). Horizontal bar = % proliferation. Representative of three independent experiments.

Restoration of $CD4^+$ T cell proliferation by inhibitors of M2

macrophage function. To identify the molecule(s) produced by EPS-induced M2 macrophages that prevents T cell activation, we added inhibitors to the T cell inhibition assay, focusing on PD-L1 and TGF- β . To test if TGF- β is required for inhibition of T cells by EPS-induced M2 macrophages, we added neutralizing TGF- β antibody to co-cultures of peritoneal cells and proliferating T cells. If

TGF- β mediates the anti-inflammatory effect of EPS-induced M2 macrophages, we expected to find an increase in T cell proliferation in these co-cultures compared to co-cultures with isotype control antibody. Whereas only 14% of CD4⁺ T cells proliferated in cultures with peritoneal cells from EPS-treated mice, proliferation was greatly increased by the addition of anti-TGF- β (69% CD4⁺ T cells) (Figure 3.2A, B), indicating that EPS-induced M2 macrophages produce TGF- β , which inhibits CD4⁺ T cell proliferation *in vitro*. We further tested the importance of TGF- β signaling in EPS-induced M2 macrophage inhibition of T cells by using a TGF- β receptor kinase inhibitor. This inhibitor, SB-431542, blocks phosphorylation of Smad proteins, thereby inhibiting TGF- β signaling. In accordance with TGF- β inhibition by antibody, we find a dose-dependent restoration of T cell proliferation in the presence of SB-431542. Inhibition using this small molecule also restores T cell activation, as expected; however, this restoration occurs at all concentrations of the inhibitor (Figure 3.32C, D). These data suggest that TGF- β mediates CD4⁺ T cell inhibition by EPS-induced M2 macrophages.

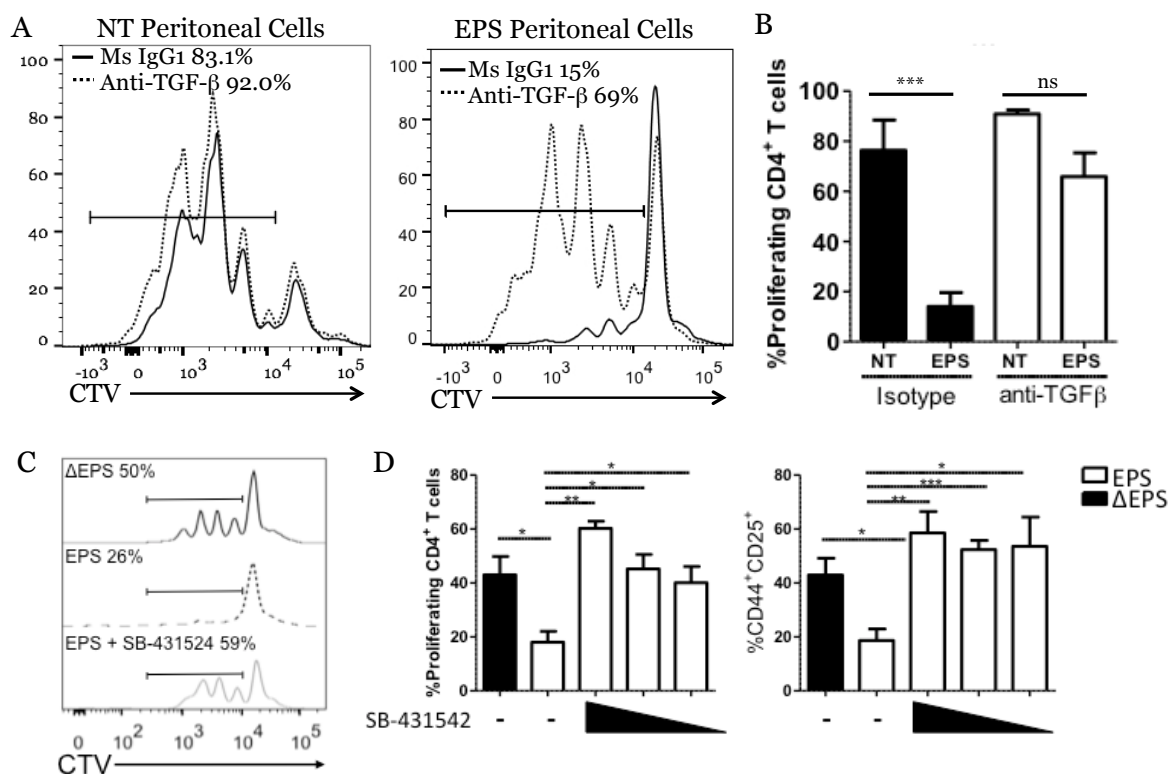


Figure 3.32. Inhibition of TGF- β in co-cultures of proliferating T cells and peritoneal cells. **A.** Proliferation of CD4⁺ T cells of anti-CD3 stimulated splenocytes cultured with peritoneal cells from untreated mice (left) or EPS-treated mice (right) in the presence of neutralizing anti-TGF- β antibody or mouse (Ms) IgG1 isotype control. Horizontal bar = % proliferation. **B.** Average proliferation in three independent experiments. **C and D.** Proliferation and activation of CD4⁺ T cells of anti-CD3 stimulated splenocytes cultured with peritoneal cells from untreated mice (left) or EPS-treated mice (right) and SB-431542 (C); data from 4 independent experiments (D) using decreased amounts of SB-431542. Average proliferation (left) and activation (CD44⁺CD25⁺) (right) of T cells. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

In contrast to TGF- β inhibition, addition of neutralizing antibodies to PD-L2 (Figure 3.33A), PD-L1 (Figure 3.33B), and IL-10 (Figure 3.33C) did not restore CD4⁺ T cell proliferation (Figure 3.34). Further, inhibition of Arg-1 activity by Nor-NOHA, or addition of exogenous L-arginine, did not restore T cell

proliferation (Figure 3.33D, E and Figure 3.34), even though we found increased expression of Arg-1 in EPS-induced M2 macrophages (Figure 3.3). The addition of NS-398, a COX2 inhibitor that prevents PGE₂ production also had no effect on T cell inhibition by EPS-induced M2 macrophages (Figure 3.33F). These data suggest that TGF- β is responsible for the inhibitory effect of M2 macrophages on CD4⁺ T cells (Figure 3.34).

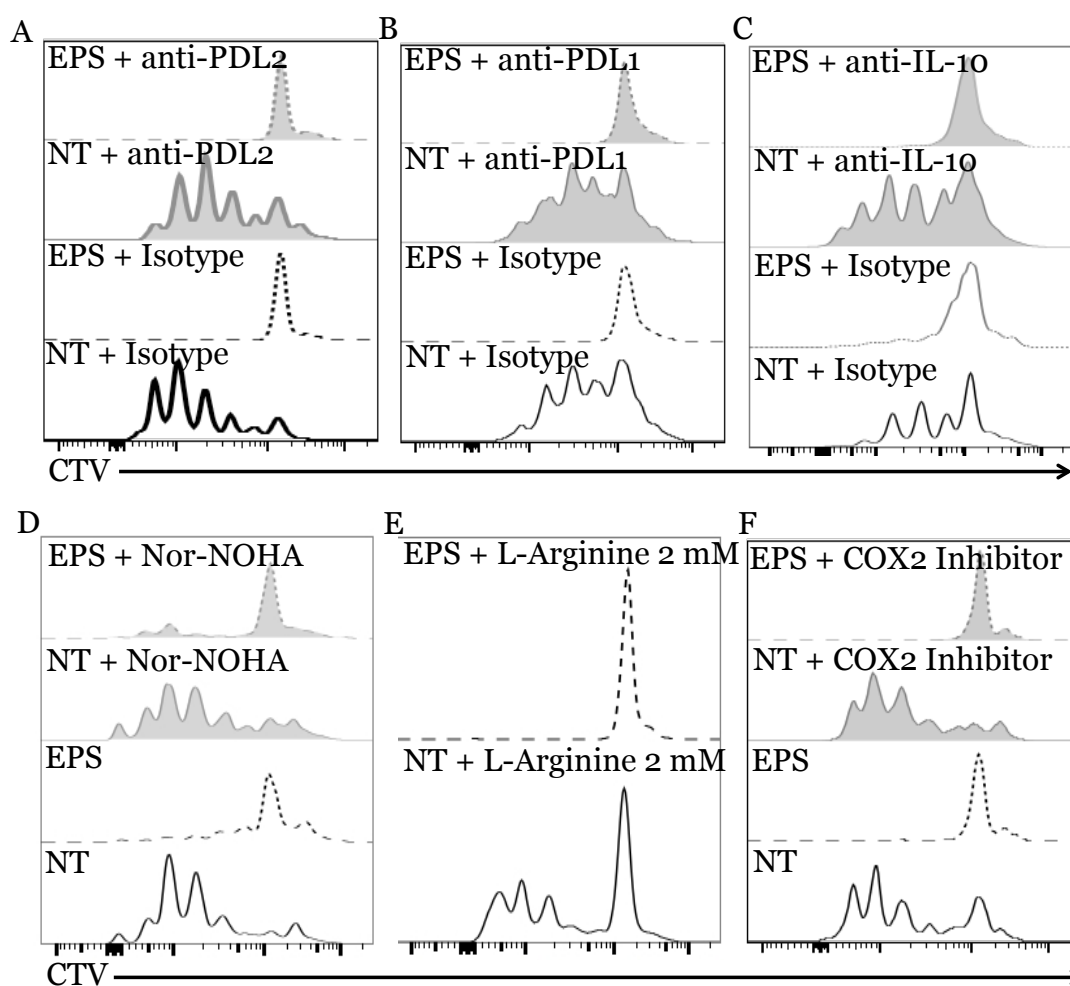


Figure 3.33. Restoration of T cell proliferation by inhibitors of M2 macrophage function. Proliferation of CD4⁺ T cells cultured with peritoneal cells from EPS-treated mice and containing inhibitors of M2 macrophage function: blocking anti-PD-L2(A); blocking anti-PD-L1(B); neutralizing anti-IL-10 (C); Nor-NOHA (D); L-Arginine (E); NS-398, a COX2 inhibitor (F). Representative flow cytometric profiles are from 3 independent experiments each, N = 6 mice total per group.

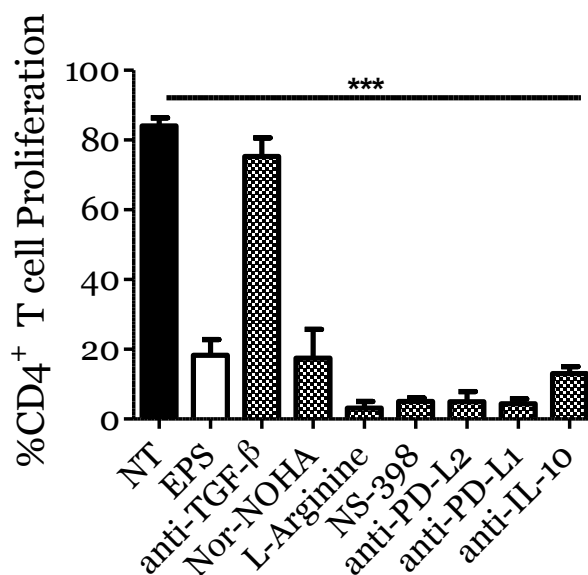


Figure 3.34. Restoration of T cell proliferation by inhibitors of M2 macrophage function. % Proliferation of CD4⁺ T cells cultured with peritoneal cells from EPS-treated mice and containing inhibitors of M2 macrophage function. (■) NT peritoneal cells alone (negative control); (□) peritoneal cells from EPS-treated mice (positive control); (▣) cultures with inhibitors of M2 macrophages, anti-TGF-β, Nor-NOHA, L-Arginine, NS-398, anti-PD-L2, anti-PD-L1, and anti-IL-10. Data are from 3 independent experiments each, N = 6 mice total per group. Statistical significance determined by one-way ANOVA in combination with Bonferroni's test for multiple comparisons. *** denotes $p < 0.0001$ by ANOVA.

Restoration of CD8⁺ T cell proliferation by inhibitors of M2 macrophage function. Since TGF-β acts on all T cells, we expected TGF-β to mediate EPS-induced M2 macrophage inhibition of CD8⁺ T cells, as well of CD4⁺ T cells. Indeed, anti-TGF-β also partially restored CD8⁺ T cell proliferation (Figure 3.35A). Interestingly, blocking anti-PD-L1 antibody also partially restored CD8⁺ T cell proliferation (Figure 3.35B). The addition of both anti-PD-L1 and anti-TGF-β antibodies resulted in complete restoration of CD8⁺ T cell proliferation (Figure 3.35C), indicating that both TGF-β and PD-L1 contribute to

the inhibition of CD8⁺ T cells. We conclude that the EPS-induced M2 macrophages inhibit T cell responses through TGF- β and PD-L1.

TGF- β and PD-L1 inhibit T cells by inducing apoptosis, among other

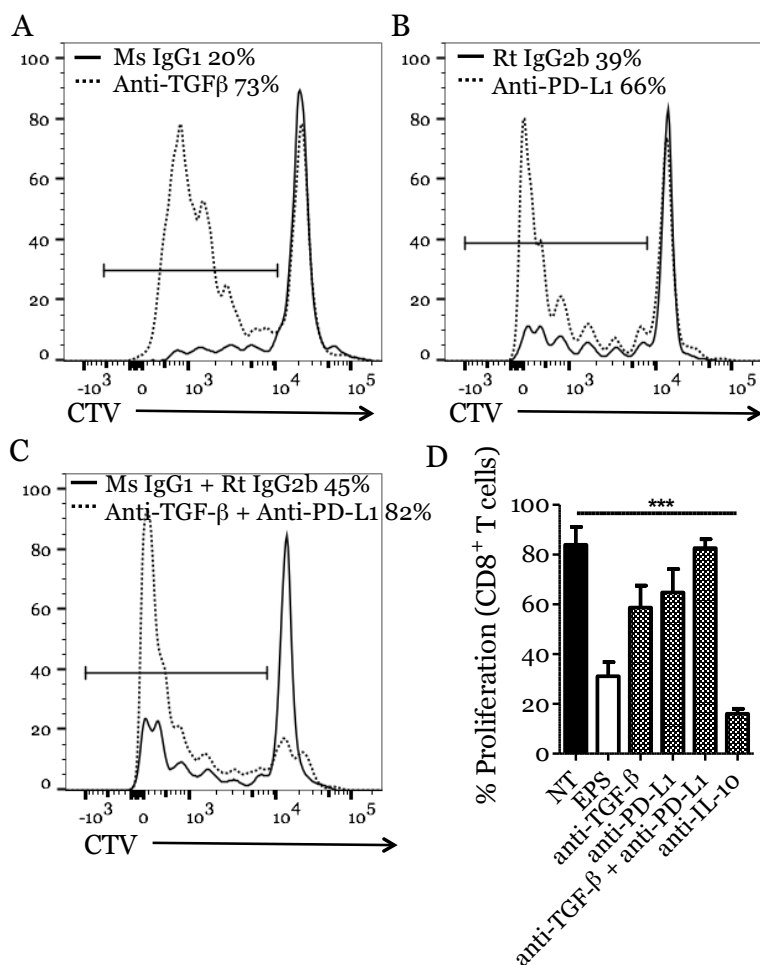


Figure 3.35. Restoration of CD8⁺ T cell proliferation by inhibitors of M2 macrophages. A-C. Proliferation of CD8⁺ T cells of anti-CD3 stimulated splenocytes cultured with peritoneal cells from EPS-treated mice in the presence of neutralizing anti-TGF- β antibody or mouse (Ms) IgG1 isotype control (A); blocking anti-PD-L1 or rat (rt) IgG2b isotype control (B); both inhibitory antibodies or isotype controls combined (C). Horizontal bar = % proliferation. D. Average % proliferation from 3 independent experiments each, N = 6 mice total per group. (■) NT peritoneal cells alone (negative control); (□) peritoneal cells from EPS-treated mice (positive control); (▣) cultures of EPS peritoneal cells with inhibitors of M2 macrophages. Statistical significance determined by one-way ANOVA in combination with Bonferroni's test for multiple comparisons. *** denotes $p < 0.0001$ by ANOVA.

mechanisms (Gorelik & Flavell 2002; Zou et al. 2016). We tested if peritoneal cells from EPS-treated mice induce apoptosis in co-cultures of proliferating T cells by staining for Annexin V and 7-AAD. Annexin V binds to phosphatidylserine that is exposed on the cell surface in cells undergoing apoptosis. During the early stages of apoptosis, cells bind Annexin V, but exclude the viability dye, 7-AAD. Once membrane integrity is lost, cells take up 7-AAD and are positive for both stains. We expected to see an increase in apoptosis in CD4⁺ and CD8⁺ T cells co-cultured with peritoneal cells from EPS-treated mice compared to untreated mice. After one day of culture with peritoneal cells from EPS-treated mice, 37% of CD4⁺ T cells and 16% of CD8⁺ T cells are apoptotic, compared to only 7.9% and 7.2% of CD4⁺ and CD8⁺ T cells, respectively, in co-cultures with peritoneal cells from untreated mice (Figure 3.36). These data suggest that EPS-induced M2 macrophage induce apoptosis in T cells through TGF- β and PD-L1. The increase in Annexin V and 7-AAD double positive cells could possibly due to necrosis, and additional, earlier time points need to be examined to address this possibility. Future experiments will determine if induction of apoptosis is dependent on these molecules and if T cells are also rendered anergic by EPS-induced M2 macrophages.

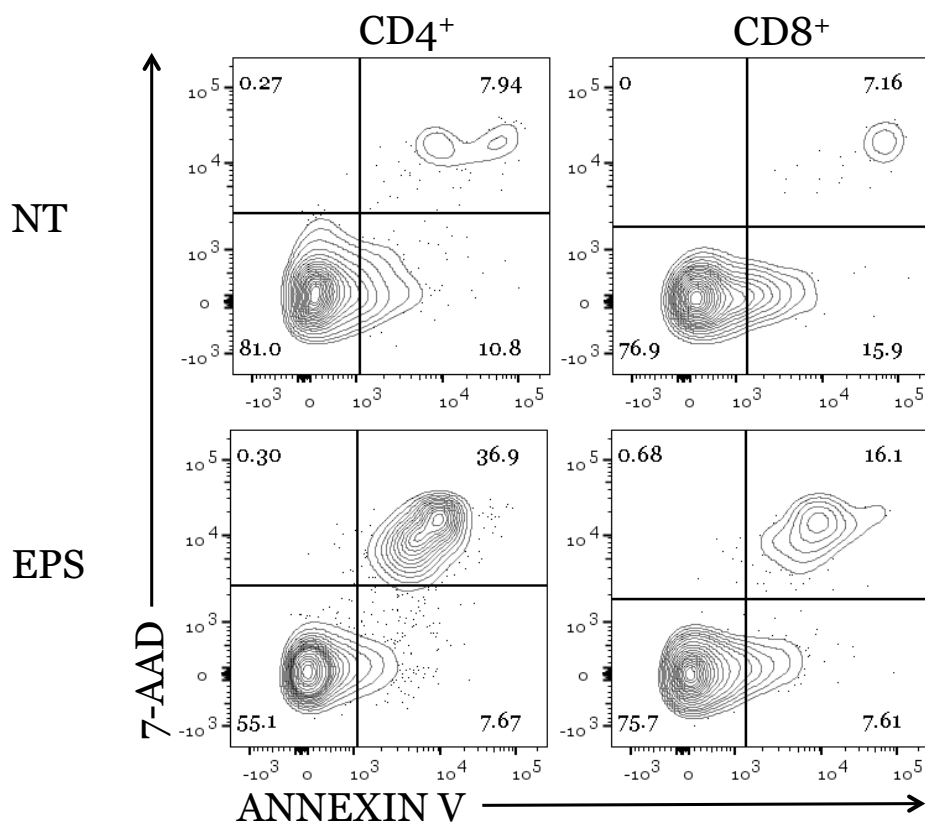


Figure 3.36. Apoptosis staining in co-cultures of proliferating T cells and peritoneal cells from EPS-treated mice. CTV-labeled spleen cells were stimulated with anti-CD3 and co-cultured with peritoneal cells from untreated or EPS-treated mice for one day. Annexin V and 7-AAD were assessed in CD4⁺ and CD8⁺ T cells by flow cytometry. Representative data from 3 independent experiments, 1-3 mice in each group per experiment.

Conclusions from Section 2.

EPS-induced M2 macrophages inhibit T cell responses both *in vitro* and *in vivo*. This inhibition is mediated by TGF- β and PD-L1, in a cell contact-dependent manner.

Section 3. Induction of Regulatory T cells by EPS-induced M2

Macrophages

Induction of T Regulatory Cells *in vivo*.

Naïve CD4⁺ T cells polarize into effector T cell subsets according to environmental conditions. In the presence of TGF- β , CD4⁺ T cells upregulate expression of Foxp3 and become inducible Treg cells (iTreg). Additionally, PD-1–PD-L1 interactions not only maintain peripheral tolerance by inhibiting effector T cells, but also help to maintain iTreg cells. Since M2 macrophages produce TGF- β and PD-L1, which inhibit T cell responses, we hypothesize that production of TGF- β could also induce regulatory T cells. We examined Foxp3 expression in CD4⁺CD25⁺ T cells in the peritoneal cavity 3 days post EPS-treatment and found an increase in the percentage of Foxp3⁺ cells in EPS-treated mice (average of 12%) compared to untreated mice (average of 52%), although the percentage of activated T cells (CD4⁺CD25⁺) in the peritoneal cavity is reduced (Figure 3.37). We find little difference in the percentage of Treg cells in the spleen (27% in untreated vs. 22% in EPS-treated) (Figure 3.38). The percentage of Treg cells is so small, however, that small changes may be difficult to quantify by flow cytometry. To efficiently assess the development and impact of EPS in the induction and development of Treg cells in spleen as well as in the MLN and GIT, a Foxp3 reporter mouse maybe a useful tool.

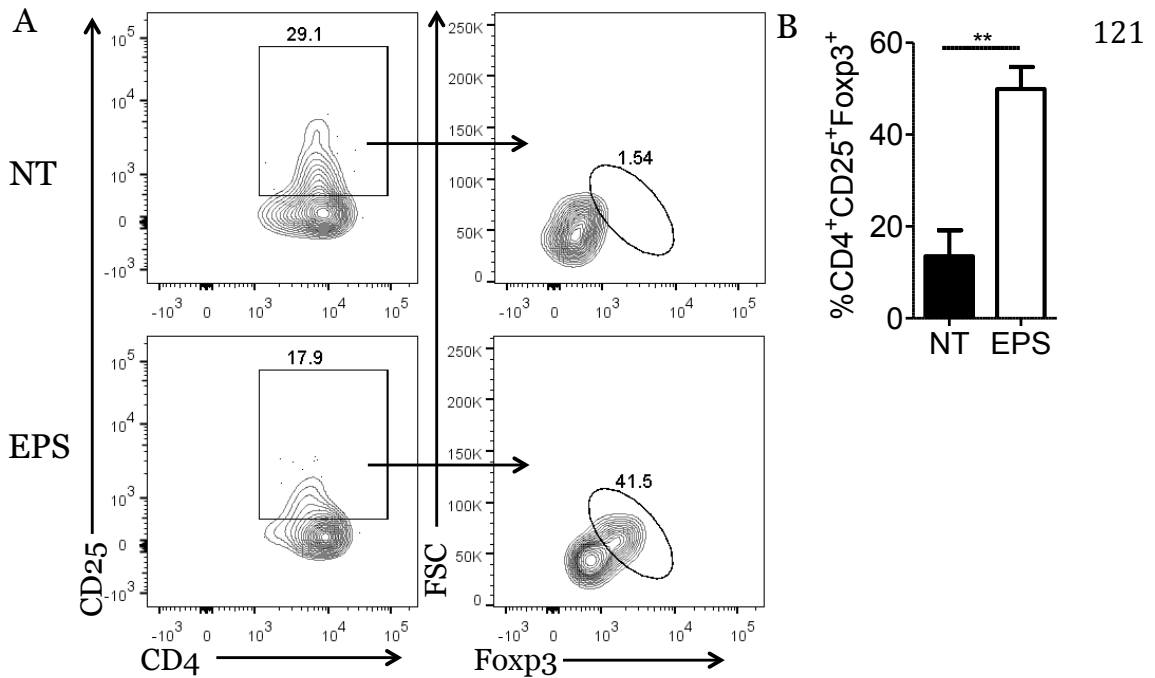


Figure 3.37. Induction of Treg cells in the peritoneum of EPS-treated mice. **A.** Representative example of Foxp3 expression in CD4⁺CD25⁺ T cells in the peritoneal cavity of untreated (NT, top) or EPS-treated (EPS, bottom) mice 3 dpt. **B.** % CD4⁺CD25⁺Foxp3⁺ cells in 5 independent experiments, N = 7 mice per group. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

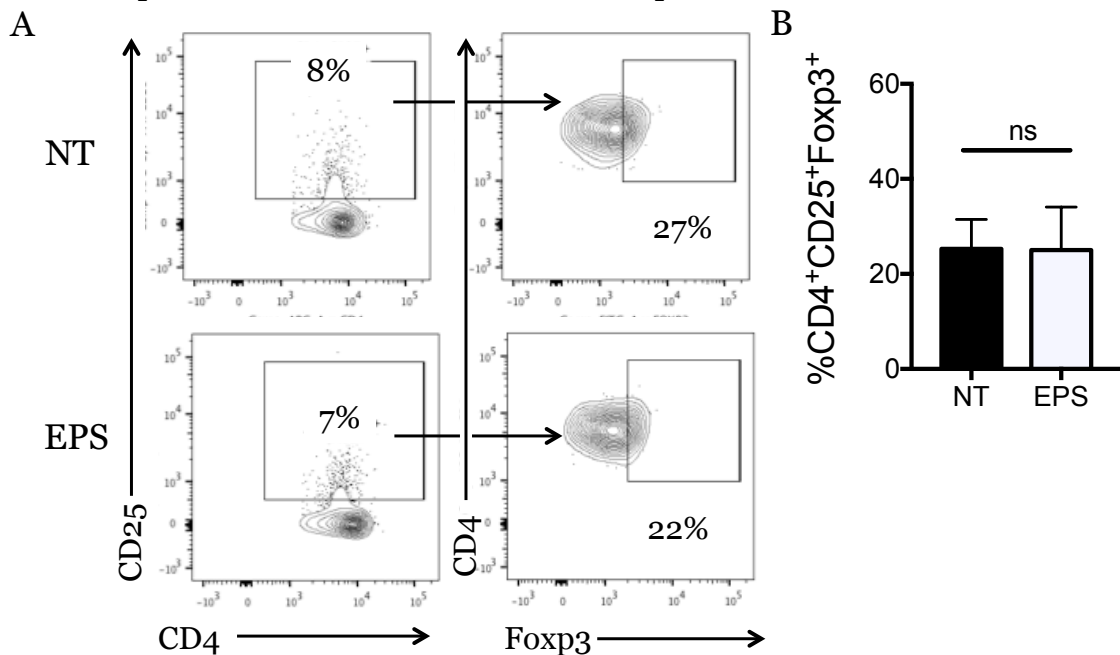


Figure 3.38. Induction of Treg cells in spleen of EPS-treated mice. Representative example of Foxp3 expression in CD4⁺CD25⁺ T cells in the spleen of untreated (NT, top) or EPS-treated (EPS, bottom) mice 3 dpt. **B.** % CD4⁺CD25⁺Foxp3⁺ cells in 5 independent experiments, N = 7 mice per group. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

Induction of T Regulatory Cells *in vitro* by M2 Macrophages.

We also tested the capacity of EPS-induced M2 macrophages to induce iTreg cells *in vitro*. Dendritic cells and M2 macrophages co-cultured with naïve CD4⁺ T cells have the capacity to polarize cells to Treg cells *in vitro*. We hypothesized that in our co-cultures of EPS peritoneal cells and proliferating T cells, the M2 macrophages would provide the TGFβ necessary for polarization. IL-2 is a growth factor required for Treg cell survival, therefore we also added in IL-2 to the co-cultures. Following co-culture of peritoneal cells from EPS-treated mice with anti-CD3 stimulated splenocytes and IL-2, we find an increase in the percentage of CD4⁺CD25⁺Foxp3⁺ T cells compared to co-culture with peritoneal cells from untreated mice (Figure 3.39). Taken together, these data suggest that M2 macrophages have the capacity to induce Treg cells.

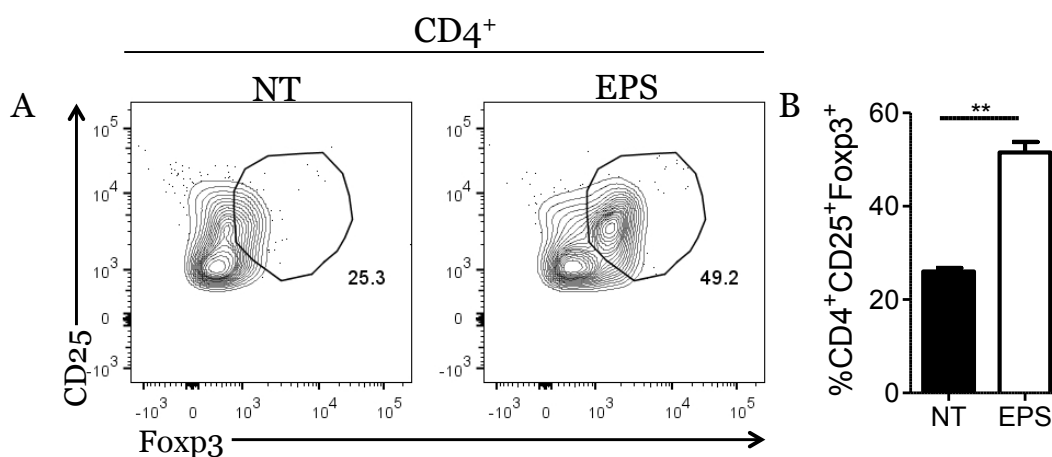


Figure 3.39. Induction of Treg cells by EPS-induced M2 macrophages. **A.** CD4⁺CD25⁺Foxp3⁺ T cells found in co-cultures of anti-CD3 stimulated splenocytes, IL-2 (50 ng/mL), and peritoneal cells from EPS-treated or untreated (NT) mice. **B.** % CD4⁺CD25⁺Foxp3⁺ T cells (of total CD4⁺CD25⁺ T cells) in 4 independent experiments with N = 6 mice total per group. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

Conclusions from Section 3.

EPS-induced M2 macrophages produce TGF- β and PD-L1, which inhibit T cell responses and contribute to the development and maintenance of peripheral T regulatory cells (Gandhi et al. 2007; Francisco et al. 2009). We find a slight increase in the percentage of Treg cells in the peritoneal cavity of EPS-treated mice, and *in vitro*, EPS-induced M2 macrophages directly induce Treg cells. Future experiments will determine if EPS-induced M2 macrophages induce Treg cells in other locations in the body.

Section 4: Potential of *B. subtilis* to Treat Human Disease

Purified EPS has been used to determine the mechanism by which *B. subtilis* spores prevent inflammatory disease induced by an enteric pathogen. As a therapeutic, EPS would be an ideal way to transiently treat different diseases, especially in immunosuppressed individuals. However, since *B. subtilis* spores are already available as a food and as a probiotic in many countries, *B. subtilis* could easily be used therapeutically without the timely regulatory processes required for use of purified EPS in humans. Although we hypothesize that *B. subtilis* spores that protect in an EPS-dependent manner utilize that same mechanism as EPS, we sought to determine if spores also induce anti-inflammatory M2 macrophages.

Induction of M2 Macrophages by *B. subtilis* Spores.

We first tested if like EPS, *B. subtilis* induces an M2 macrophage phenotype. Mice were treated with WT or *epsH* *B. subtilis* mutant spores by oral

gavage. The *epsH* spores do not produce EPS and do not protect mice from *C. rodentium*-induced disease (Jones & Knight 2012). On days 4 and 6 post-treatment, we isolated MLN and peritoneal cells from mice and assessed M2 macrophage phenotype by flow cytometry. In the peritoneal cavity at 4 dpt, we found an increase in the expression of IL-4R α , and a slight increase in CD206 on macrophages in mice treated with WT *B. subtilis* spores compared to macrophages from *epsH B. subtilis* spores (Figure 3.40A). The cells were not examined for expression of CD80, CD86 or PD-L1. At this time-point, we did not find changes M2 macrophage markers in the MLN (data not shown).

Interestingly, although the percentage of macrophages is only 1-4% of the MLN, by day 6, we found upregulation of several M2 macrophage markers on F4/80⁺ cells of mice orally gavaged with WT *B. subtilis* spores compared to mice gavaged with *epsH B. subtilis* spores (Figure 3.40B). CD206 and PD-L1 are upregulated and CD80 expression has a slightly larger peak, but no changes are seen in IL-4R α or CD86. At day 6, no changes are seen in macrophage phenotype in the peritoneum. These data suggest that *B. subtilis* induces M2 macrophages in the peritoneal cavity at 4 dpt and in the MLN by day 6. The location of M2 macrophage induction in *B. subtilis*-treated mice has not been exhaustively studied. Future studies are needed to determine the dynamics of induction and migration of M2 macrophages in these mice.

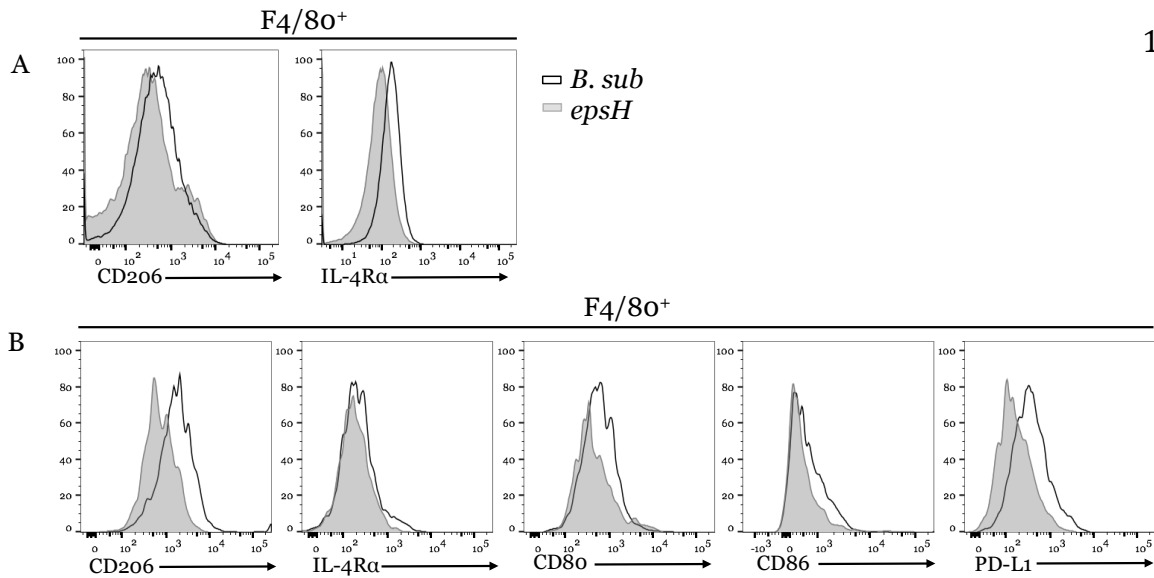


Figure 3.40. Induction of M2 macrophages in MLN and the peritoneum of *B. subtilis*-treated mice. Mice were gavaged with WT *B. subtilis* spores or *epsH B. subtilis* spores. Peritoneal cells (A) and MLN (B) were isolated 4 dpt and 6 dpt, respectively, and M2 macrophage markers were assessed on F4/80⁺ cells by flow cytometry. Data are representative of 5 independent experiments with 6-8 mice per group.

Inhibition of T cell Responses by *B. subtilis* Spores.

We further assessed the potential of cells from the peritoneal cavity and MLN of mice treated with *B. subtilis* spores for the capacity to inhibit T cell proliferation. Similar to purified EPS, administration of *B. subtilis* spores (by oral gavage) resulted in the generation of peritoneal cells (4 dpt) that potently inhibit T cell proliferation (Figure 3.41A). In contrast, peritoneal cells from mice gavaged with EPS-deficient *epsH B. subtilis* spores did not inhibit T cell proliferation. Since we hypothesize that macrophages within the MLN of *B. subtilis*-treated mice will inhibit T cells, and macrophages represent such a small percentage of the MLN, we purified F4/80⁺ cells using magnetic beads. F4/80⁺ MLN cells from WT- or *epsH B. subtilis*-treated mice isolated 6 dpt were co-

cultured with proliferating T cells and the capacity to inhibit proliferation was assessed. As seen in Figure 3.41B, in cultures with F4/80⁺ cells from WT *B. subtilis*-treated mice, we find reduced T cell proliferation (48% CD4⁺ T cells) compared to proliferation in co-cultures with F4/80⁺ cells from *epsH*-treated mice (94% CD4⁺ T cells). These reductions in proliferation are reproducible; however, they are not statistically significant ($p=0.06$), potentially due to variability in proliferation between experiments. From these data, we conclude that both *B. subtilis*, like EPS, induces cells that inhibit T cell proliferation, indicating that both *B. subtilis* and EPS induce systemic anti-inflammatory responses. Further studies are needed to determine if the inhibitory cells induced by *B. subtilis* are M2 macrophages, as is the case for EPS.

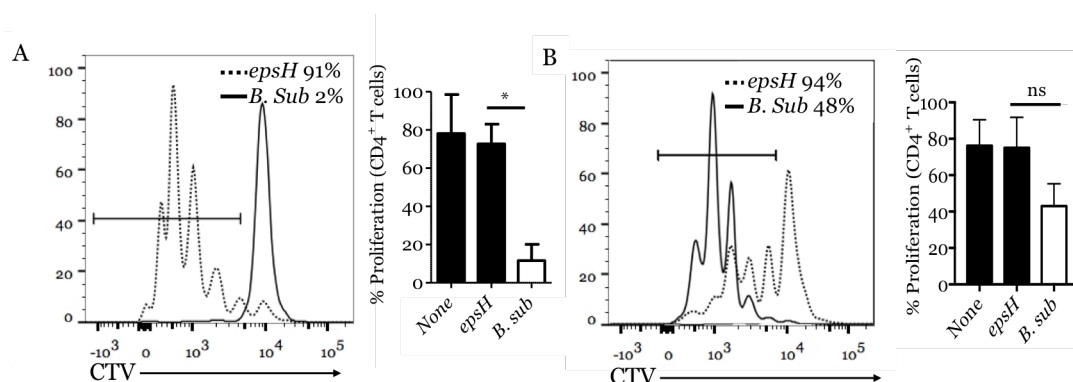


Figure 3.41. Inhibition of T cell proliferation by peritoneal and MLN cells from *B. subtilis* treated mice. Proliferation of CD4⁺ T cells in cultures of anti-CD3 stimulated splenocytes and peritoneal cells from mice gavaged with WT or *epsH* *B. subtilis* spores. **A.** Representative flow cytometric profile of co-cultures of peritoneal cells isolated 4 dpt (Left), and % proliferating T cells in 4 independent experiments; N = 6 mice per group (right). **B.** Magnetic bead-purified F4/80⁺ cells from MLN 6 dpt. Representative flow cytometric profile (Left); % proliferating CD4⁺ T cells in three independent experiments, with N = 5 mice total per group (right). Horizontal bar = % proliferating cells; none = splenocytes alone. Statistical significance determined by Student's *t* test. $p < 0.001$ denoted as ***, $p = 0.001$ to 0.01 denoted as **, and $p = 0.01$ to 0.05 denoted as *.

Induction of M2 Macrophages in Human Cells.

B. subtilis appears to induce M2 macrophages in an EPS-dependent manner, similar to the effects seen with purified EPS, although we have not determined if the inhibitory peritoneal cell type is a macrophage. To further demonstrate if the model could be relevant to treat human disease, we asked if EPS induces an M2 macrophage phenotype in human macrophages. Many of the M2 macrophage markers we utilize, Arg-1, Ym-1 and FIZZ-1 are expressed in mouse, and not human macrophages. The scavenger receptor, CD163, is routinely used as a human M2 marker, and we used this marker to assess the capacity of EPS to induce M2 macrophages. We also assessed CD80 and PD-L1 expression, because these markers are upregulated in mouse macrophages, and they have homologs in humans. We generated human macrophages from human cord blood by first allowing monocytes to adhere to plastic and washing away non-adherent cells. We then treated the enriched monocyte population with M-CSF (10 ng/mL) for 5 d. After treating the macrophages with 5 μ g EPS or an equal volume of Δ EPS for 3 d, the expression of CD80, PD-L1 and CD163 was assessed on CD68⁺ cells, the human macrophage marker, by flow cytometry. If EPS induces M2 macrophages in humans, we expect to find an increase in CD163 expression in CD68⁺ cells treated with EPS compared to untreated or Δ EPS-treated cells. Indeed, we found an increase in CD80⁺CD163⁺ cells within CD68⁺ cells from EPS-treated cultures compared to CD68⁺ cells from untreated cultures, or cultures treated with Δ EPS (Figure 3.42). Further, EPS-treated cells

upregulate PD-L1 (Figure 3.42, right), consistent with observations in mouse macrophages. These data suggest that EPS induces M2 macrophages in human cells. Future studies will be needed to determine if these M2 macrophages generated in human also inhibit T cell responses.

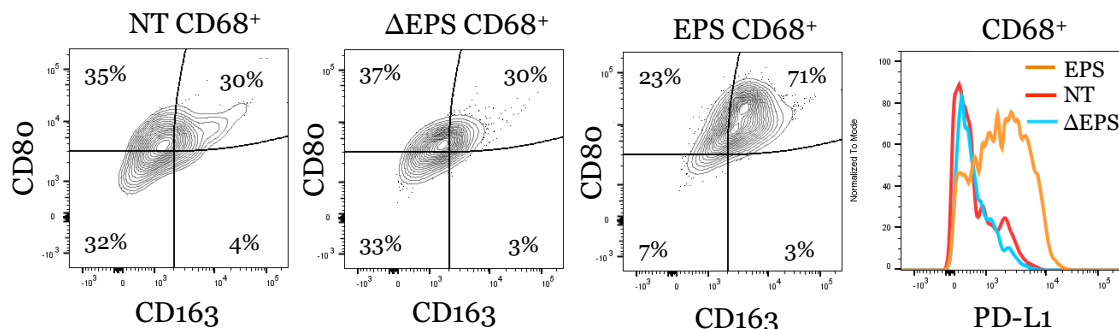


Figure 3.42. M2 macrophage induction in human cells. Macrophages were generated from cord blood monocytes using M-CSF (10ng/mL) for 5 d. Cells were treated with 5 μ g/mL EPS or an equal volume of Δ EPS for 3 d; CD68⁺ macrophages were assessed for CD80, CD163 and PD-L1 expression by flow cytometry. Representative of 3 independent experiments, each from a different cord blood donor.

Conclusions from Section 4.

These studies show that *B. subtilis*, like EPS, induces an anti-inflammatory state and is a potential treatment for inflammatory disorders in humans until we identify the structure of EPS. Additionally, we found that EPS induces an M2 macrophage-like phenotype in human macrophages.

CHAPTER FOUR

DISCUSSION

Introduction.

Bacteria contribute to the development of many organ systems and physiological processes. The microbiota is essential to train the immune system to tolerate foreign and self-antigen and develop regulated immune responses. Our health relies on maintaining a robust and diverse microbiota within our gastrointestinal tract. Failure to establish a healthy microbiome at birth leaves individuals with increased susceptibility to numerous diseases.

Not only are bacteria required for the proper development of the immune system, but specific bacterial molecules appear to be essential to mediate these effects. Studies of bacterial products that modulate the immune system have, until recently, focused primarily on pathogenic molecules that elicit pro-inflammatory responses or contribute to evasion of the immune system. These studies identified host pattern recognition receptors, such as TLRs and NODs and their cognate ligands (e.g. LPS, lipoteichoic acid and flagellin) along with the downstream signaling pathways. Less understood are the mechanisms by which commensal bacteria and commensal-derived products circumvent pro-inflammatory responses, allowing select bacteria to live in homeostasis with the host. The work in this thesis seeks to understand how commensal bacteria protect the host from disease.

We found that a single oral dose of the probiotic *B. subtilis* or a single i.p. injection of *B. subtilis* EPS prevents inflammatory responses against the enteric pathogen, *C. rodentium*. Additionally, we have identified that EPS from *B. subtilis* exerts this anti-inflammatory response by inducing M2 macrophages. This is the first demonstration of a probiotic molecule exerting anti-inflammatory effects through M2 macrophages.

Mechanism by which *B. subtilis* Induces Anti-inflammatory Response.

EPS binding to macrophages. Peritoneal macrophages bind and rapidly internalize EPS (Figure 4.1). Macrophages throughout the body and especially peritoneal macrophages highly express pattern recognition receptors. Since, EPS does not protect *TLR4*^{-/-} mice from disease caused by *C. rodentium*, we suspected that EPS binds to TLR4, however, we find that EPS binds to peritoneal macrophages in mice deficient in TLR4 and TLR2. We hypothesize that EPS utilizes a co-receptor of the TLR4 signaling complex, similar to LPS, which binds to the co-receptors CD14 and MD2 (Da Silva Correia et al. 2001). In addition, EPS could bind C-type lectins or scavenger receptors, which serve as carbohydrate-binding pattern recognition receptors, and can associate with TLR4 (Stewart et al. 2009; Mukhopadhyay et al. 2011; Amiel et al. 2009; Yu et al. 2012).

Based on binding, we think that EPS acts strictly on macrophages, however, EPS may be internalized or phagocytosed by other cell types and elicit a response. Some efforts to biotinylate EPS have been successful, however, the

process seems to render the EPS inactive and may alter the structure. Once we understand the composition of EPS, we can more rationally determine a way to label EPS based on the polysaccharide chemistry, and effectively explore the effect of EPS on other cell types that perhaps bind EPS at low levels. In fact, we have observed alterations in dendritic cells function following EPS treatment.

Dendritic cells pulsed with ovalbumin stimulate CD4⁺ T cell proliferation in OVA-specific T cells. Following EPS treatment, OVA-pulsed dendritic cells no longer stimulate T cells to proliferate. Although we don't observe many alterations in the expression of co-stimulatory or co-inhibitory molecules, we find increased expression of IL-10 and TGF- β . Whether this is due to the upregulation of IL-10 and TGF- β following EPS treatment, or if EPS alters antigen presentation and co-stimulation has not been determined. Further studies will determine the mechanism by which EPS modulates dendritic cells. Dendritic cells and macrophages express many of the same PRRs and therefore, the signaling cascade induced following EPS binding and internalization may be similar in both cell types. In both cases, EPS seems to induce a regulatory phenotype.

What is EPS? Our lack of understanding of the basic biology of EPS complicates our search for the 'EPS receptor'. EPS is essential for *B. subtilis* biofilm formation. Within the *eps* operon are four genes, *epsH-K*, required for synthesis of β -1,6-linked *N*-acetylglucosamine, identified to be a major component of EPS and thereby the *B. subtilis* biofilm. The *epsH* and *epsJ* genes encode glycosyltransferases. The *B. subtilis* mutant used as a negative control for

EPS production in all of our experiments is an *epsH* mutant, suggesting that N-acetylglucosamine may comprise some of our immunomodulatory EPS. If an EPS mutant outside of the *epsH-K* locus does not protect mice from disease, EPS is likely made up of an additional carbohydrate component.

In initial analyses of EPS composition, we determined that EPS purified from *B. subtilis* grown in LB medium was comprised of 88.0% mannose, 11.9% glucose, and 0.1% N-acetylglucosamine (Complex Carbohydrate Research Center, University of Georgia). In collaboration with Dr. Neil Price at the USDA, we have determined that high amount of mannose in our EPS preparations was due to yeast extract mannan present in LB broth that co-purifies with EPS. A small amount of EPS preparation purified from LB broth, although active *in vivo*, is actual EPS. To obtain a higher and more pure yield of EPS, we grew *B. subtilis* in MSgg minimal medium to minimize polysaccharide background contamination. Using EPS purified from MSgg medium, we find the same immunomodulatory effects *in vivo* that we see with EPS purified from LB broth or BHI media, which we had used in early binding experiments (Table 4.1). Interestingly, we can use 10-fold less EPS from MSgg to induce the same response observed with EPS purified from LB broth. The only functional difference identified thus far between EPS purified from LB and MSgg is that EPS from MSgg (and from BHI) induces M2 macrophages *in vitro*, whereas no *in vitro* changes were ever detected using the less-pure EPS from LB broth (Table 4.1). Although we have yet to determine the composition of EPS prepared from MSgg, it is possible that

the mannose and N-acetylglucosamine observed to be a small percentage previously are the actual, functional EPS. Once we determine the composition and structure of EPS, we can identify macrophage receptors that bind to the identified polysaccharide.

Table 4.1. Functional properties of EPS purified from different culture conditions

Experiment	Growth Medium		
	BHI	LB	MSgg
EPS binding	+	+/-	+
M2 induction (<i>in vivo</i>)	+	+	+
M2 induction (<i>in vitro</i>)	+	-	+
T cell inhibition by EPS-induced M2 macrophages	+	+	+
Anti-CD3 challenge <i>in vivo</i>	n.d.	+	+
Splenic T cell responses	n.d.	+	+
Induction of Treg cells	n.d.	+	+

Key: (+): EPS purified from specified medium functions in this assay; (-): EPS purified from this medium does not function in this assay; (+/-): Variable results. List of abbreviations: BHI – brain heart infusion media; EPS – exopolysaccharide; LB – Luria-bertani; n.d. – not determined.

Induction of M2 macrophages. Even though EPS does not bind directly to TLR4, induction of M2 macrophages requires this PRR. Canonical TLR4 signaling through MyD88 leads to NF- κ B activation, however this pathway

is often thought of as an inflammatory pathway. M1 macrophage induction often occurs by LPS signaling through TLR4. How then, does EPS drive an anti-inflammatory response and induce anti-inflammatory M2 macrophages?

The dependency on TLR4 is not likely due to LPS because *B. subtilis* is Gram (+) and produces little to no LPS. In contrast to other bacterial polysaccharides, notably PSA from *B. fragilis* that signals through TLR2, EPS is unlikely to function through TLR2 because EPS induces M2 macrophages in

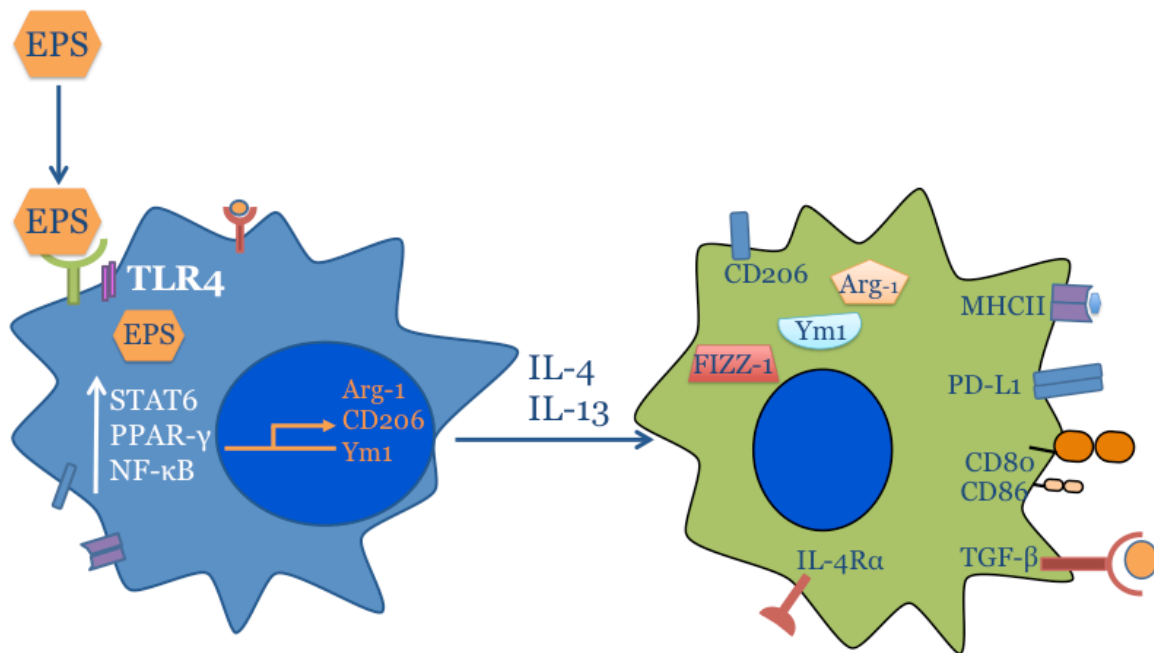


Figure 4.1. Model of induction of M2 macrophages by EPS. EPS binds to macrophages and is rapidly internalized. EPS upregulates expression of M2 macrophage markers in a TLR-4 dependent manner. These cells also upregulate IL-4 and IL-13 that further enhances activation of M2-dependent genes. STAT6, PPAR- γ , or NF- κ B potentially drive this process.

TLR2^{-/-} mice. We hypothesize that EPS binding to TLR4 co-receptors recruits TLR4 and initiates a signaling cascade, directly modifying macrophages and induces M2 macrophage-specific transcription factors (Figure 4.1). EPS binding to these receptors could upregulate M2 macrophage-inducing transcription factors such as STAT6, IRF4 and peroxisome proliferator-activated receptor gamma (PPAR- γ), which regulate transcription of Arg-1 and CD206 (Lawrence & Natoli 2011; Shirey et al. 2010). IL-4 and IL-13 activate STAT6 signaling through IL-4R α to induce M2 macrophages. Since we see increased IL-4 and IL-13 production by macrophages following EPS treatment, EPS may activate the STAT6 pathway. An intriguing study by Shirey et al. determined that respiratory syncytial virus (RSV) activates PPAR- γ to induce M2 macrophages. Similar to EPS-induced M2 macrophages, RSV-induced M2 cells upregulate FIZZ-1, Ym1, CD206 and Arg-1 in a TLR4-dependent manner, that also requires STAT6 (Shirey et al. 2010). This study identifies TLR4-dependent transcription factors that drive M2 induction that EPS could potentially utilize.

We have preliminary evidence that EPS treatment upregulates NF- κ B signaling in RAW264.7 cells that stably express an NF- κ B luciferase reporter (Wonbeom Paik). IL-10 activates STAT3 signaling in macrophages, leading to M2 macrophage generation in a process that also activates NF- κ B, but EPS does not upregulate IL-10 in the conditions tested thus far. Interestingly, STAT3 activation in dendritic cells upregulates PD-L1 expression, similar to what we see in macrophages in response to EPS (Wölfle et al. 2011). We have not yet

determined the timing of NF- κ B activation, or if it occurs in primary macrophages. It is possible that NF- κ B activation occurs downstream of the initial signaling events induced by EPS. Further, the phenotype of RAW264.7 cells following EPS treatment is somewhat different than primary macrophages treated with EPS. Studies of the signaling cascades induced by EPS will have to be completed in both cell types to verify that the correct signaling cascade is identified.

An intriguing alternate hypothesis is that EPS antagonizes TLR4 signaling to induce an anti-inflammatory response. In adipose tissue, the absence of TLR4 drives macrophages towards an M2 phenotype (Orr et al. 2012). M2 macrophages present in adipose tissue highly express suppression of tumorigenicity 2 (ST2), a negative regulator of TLR4 signaling (Westcott et al. 2009). Other negative regulators of TLR4 signaling, such as AP-1 (activator protein 1), are stimulated by TLR4 activation (Patel et al. 2006). EPS could activate M2 macrophage polarization by upregulating a negative regulator of the inflammatory TLR4 signaling cascade.

EPS acts directly on macrophages to upregulate M2 macrophage markers. Other cell types, however, potentially contribute to the maintenance of M2 macrophages within the peritoneal cavity. Type 2 immune responses are characterized by IL-4, IL-5, IL-10 and IL-13 production. ILC2s, Th2 cells, B cells and eosinophils are all activated by these cytokines, which leads to even greater production of these cytokines (Figure 4.2). Although M2 macrophages may

initiate the response to EPS, these other type 2 immune cells are present in the dynamic environment of the peritoneal cavity and may be activated by macrophage cytokines.

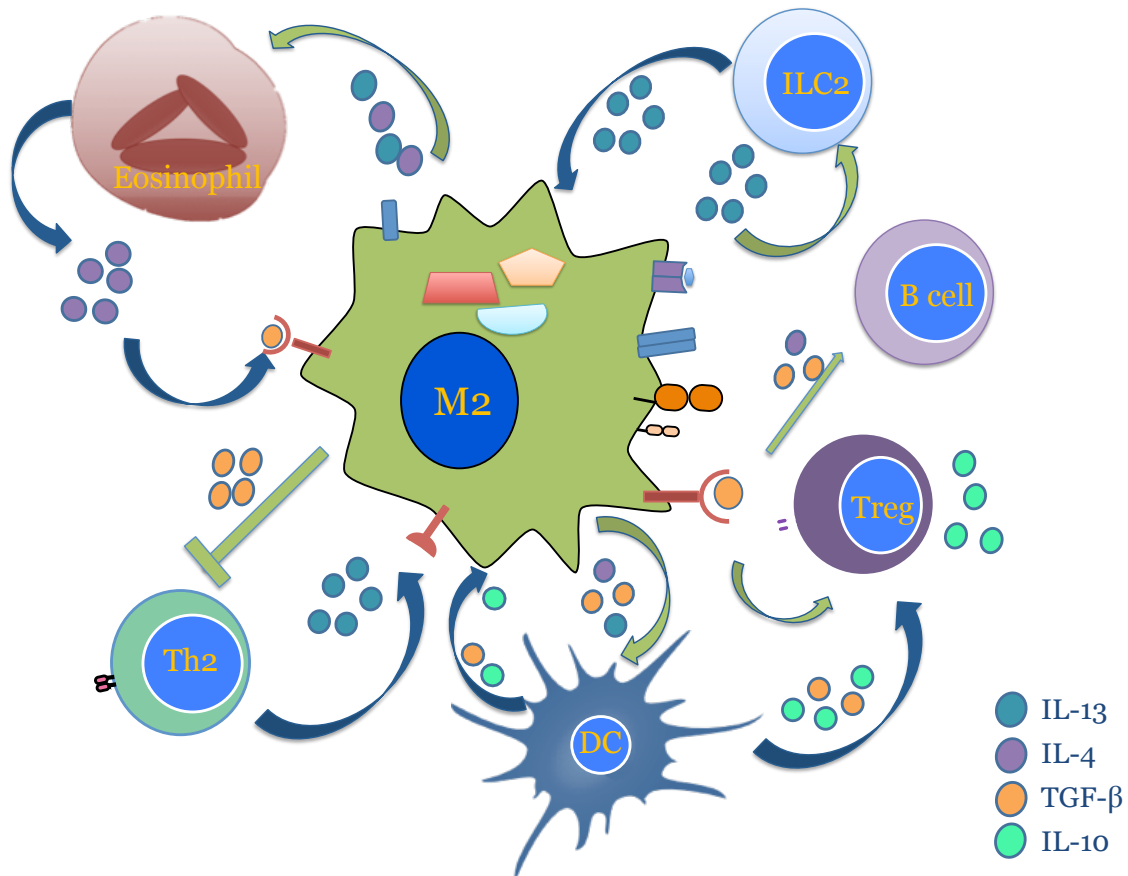


Figure 4.2. Dynamics of EPS-induced type 2 Immunity within the peritoneal cavity.

Induction of M2 macrophages by other probiotics. Our work is the first to identify a probiotic molecule that suppresses pro-inflammatory responses through M2 macrophages. Many bacterial species have been shown to induce the generation of Treg cells to suppress inflammation, however it is

unclear how much M2 macrophages facilitate this induction. The Mazmanian lab identified DCs as the cell type driving Treg induction by PSA, and DCs likely induce Treg cells in other probiotic models. Other bacteria potentially induce anti-inflammatory macrophages and these cells may induce Treg cells in many models, as well as DCs. One study on *Clostridium butyricum* identified a population of IL-10⁺ macrophages in the GIT of mice treated with this probiotic. This mechanism of induction is unique to that of *B. subtilis* EPS, given that *C. butyricum* utilizes TLR2 signaling and upregulates IL-10 production. These cells are required for protection from DSS-induced colitis, and Treg cells seem to be partially required, however a direct connection between macrophages and Treg cells in this model has not yet been established.

Trafficking of EPS-induced M2 macrophages. EPS induces M2 macrophages within the peritoneal cavity following intraperitoneal injection of EPS. Increases in M2 macrophages are found both in the peritoneum after i.p. or i.v. injection of EPS, as well as after oral administration of *B. subtilis* spores. Does EPS travel systemically following injection, and/or do M2 macrophages traffic to other locations in the body? Macrophages are highly prevalent in the peritoneum and because they bind and internalize EPS, we expect a limited amount of EPS leaves the peritoneal cavity. Once we can label EPS, we will be able to track where EPS travels after injection.

The peritoneum contains two populations of macrophages: LPMs and SPMs. These cells differ in size and CD11b expression, and other markers, but

EPS binds to both LPMs and SPMs. We hypothesize that EPS induces an M2 phenotype in LPMs and SPMs. Following non-specific i.p. injection, the majority of LPM traffic to milky spots in the omentum, but cytokines still interact with peritoneal cells (Okabe & Medzhitov 2016). Meanwhile, monocytes infiltrate the peritoneal cavity and differentiate into macrophages (Ghosn et al. 2010). Although these infiltrating cells may not directly interact with EPS, the cytokine environment induced by initial EPS binding to LPMs and SPMs polarizes these new macrophages to an M2 macrophage phenotype. In fact, supernatants from EPS-induced M2 macrophages polarize naïve macrophages to an M2 macrophage phenotype (unpublished observations). F4/80⁺ cells proliferate in response to EPS, but we have not determined if this population of proliferating cells is LPMs, SPMs, or both. Further, these studies were done by adoptive transfer of EPS-induced M2 macrophages into naïve mice. An important study will be to determine if the macrophages of mice fed Bromodeoxyuridine, BrdU, (in order to label proliferating cells) proliferate in response to EPS.

In our *in vitro* experiments, EPS-induced M2 macrophages inhibit T cell responses via direct cell contact, suggesting that M2 macrophages traffic to inhibit T cell responses during disease. We do not know if *in vivo*, these cells can produce soluble factors to inhibit cells. TGF- β activation is not well understood and could occur differently *in vivo* compared to our *in vitro* co-culture conditions. We do find increased serum levels of total TGF- β , suggesting that TGF- β may be cleaved from the surface of M2 macrophages *in vivo*. We often

find increases in M2 macrophages in MLN and spleen following EPS treatment where they may interact with and inhibit T cells. These T cells are recruited to the colon following epithelial damage induced by *C. rodentium* in the colon. Since we do not see infiltration into the mucosa in mice treated with EPS prior to *C. rodentium* infection, we hypothesize that M2 macrophages render the T cells inactive or anergic in the spleen or MLN, blocking recruitment to the colon. Additionally, damage caused by *C. rodentium* may enhance recruitment of M2 macrophages to the spleen, MLN, or the colon where they directly inhibit inflammation. We have preliminary evidence that an increase in Arg-1⁺ cells occurs in the colon of mice treated with EPS prior to infection compared to mice infected with *C. rodentium* alone. We have not established if the Arg-1⁺ cells are macrophages, but these data suggest that M2 macrophages are recruited to the colon during enteric disease.

Peritoneal cells administered by i.p. injection traffic to the spleen and MLN, and we expect that some EPS-induced M2 macrophages traffic to other organs following EPS injection. In different diseases, damage in other organs could recruit EPS-induced M2 macrophages and lead to protection from other sources of inflammation. The mechanisms of macrophage egress from the peritoneal cavity are not well defined. A recent study identified a CD44-dependent mechanism by which peritoneal macrophage are recruited to the liver following sterile inflammation (Wang & Kubes 2016). This may be a relevant mechanism for direct influx into other organs from the peritoneum.

Interestingly, peritoneal macrophages upregulate CD44 following EPS treatment, suggesting EPS-induced M2 macrophage may use a similar mechanism to travel to the liver and perhaps other organs (Wonbeom Paik, unpublished observations).

Alterations in T cell Responses by EPS-induced M2 Macrophages.

M2 macrophages mediate an anti-inflammatory response not only by TGF- β and PD-L1, but also by arginase-1, IL-10, and PD-L2 (Huber et al. 2010; Gobert et al. 2004; Pesce et al. 2009; Yue et al. 2015). Such molecules protect from colitis, promote tissue repair and metabolic homeostasis, and provide protective immunity to helminth infections (Little et al. 2014). Even though EPS-induced M2 macrophages produce multiple anti-inflammatory molecules, in our *in vitro* co-cultures of EPS-induced M2 macrophages and proliferating T cells, we find restoration of CD4⁺ T cell proliferation only by interfering with TGF- β . In contrast, CD8⁺ T cell restoration occurs through PD-L1, as well as TGF- β . Inhibition of other molecules including arginase-1, PGE₂, PD-L2 and IL-10 had no effect on T cell inhibition. We conclude that TGF- β and PD-L1 are the main inhibitory factors of the EPS-induced M2 macrophages (Figure 4.3). We cannot rule out the contribution of other factors, potentially contributing to protection *in vivo*. Arginase-1 inhibits TCR signaling, but also promotes wound healing and fibrosis (Pesce et al. 2009), effects we have not examined following EPS treatment. EPS-induced M2 macrophages increase expression of CD80, but decrease expression of CD86. These molecules interact with CD28 to provide co-

stimulation to T cells, but can also interact with CTLA-4 to strongly inhibit T cell responses (Figure 4.3). Although these molecules don't contribute in our *in vitro* T cell proliferation assay, further studies are needed to determine the importance of these molecules in our system with T cells and other cells.

Programmed death-ligand 1. T cells upregulate PD-1 following TCR stimulation. Its ligands, PD-L1 and PD-L2 are expressed on dendritic cells and macrophages. These ligands serve as co-inhibitory receptors that are part of the

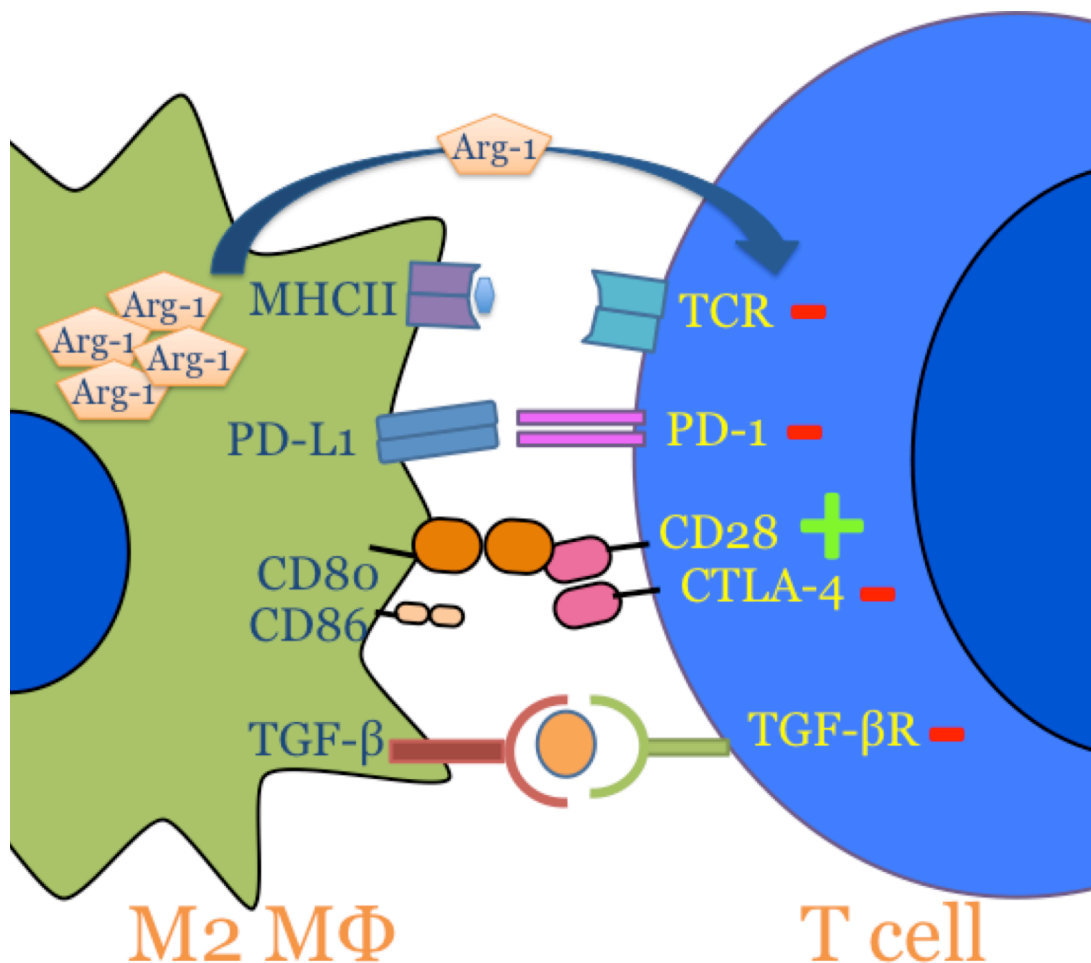


Figure 4.3. Interactions between effector molecules of EPS-induced M2 macrophages and T cells. M2 macrophages produce multiple factors to inhibit T cell responses (-), including arginase-1, PD-L1 and TGF-β. CD80 provides co-stimulation when it interacts with CD28 (+), but inhibits following interactions with CTLA-4.

immune checkpoint during T cell activation (Freeman et al. 2000). PD-1 pathway interactions limit the positive signals that T cells receive from CD28 and CD80/CD86. PD-1/PD-L1 signaling reduces cytokine production and halts T cell proliferation by inducing T cell apoptosis and anergy (Freeman et al. 2000; Liang et al. 2003). In studies of chronic viral infection, PD-1 is highly expressed on exhausted T cells, suggesting that this pathway also promotes T cell exhaustion (Barber et al. 2006). CD8⁺ T cells are more sensitive to PD-1 ligation (Carter et al. 2002), which may explain why, in our system, that PD-L1 blockade increases proliferation of CD8⁺ T cells, but not of CD4⁺ T cells. The levels of co-stimulatory and co-inhibitory molecules determine if the interaction leads to inhibition or activation. EPS-induced M2 macrophage upregulate CD80 and downregulate CD86, both co-stimulatory molecule, and increase expression of inhibitory PD-L1. In our *in vitro* T cell proliferation assay, inhibition of CD8⁺ T cells occurs in an antigen-independent manner, but potentially inhibition could occur in an antigen-specific interaction. EPS-induced M2 macrophages inhibit antigen-specific CD4⁺ T cell proliferation, presumably through TGF- β , but we have not tested inhibition of CD8⁺ T cells in an antigen-specific system. Understanding the balance of these signals from M2 macrophages will help us determine how EPS could be used in other disease states to inhibit CD4⁺ and CD8⁺ T cell responses.

Transforming growth factor- β . TGF- β inhibits T cell responses by inducing apoptosis and T cell anergy, but also promotes immune responses in

other cells (Gorelik & Flavell 2002). As discussed above, although we see cell contact-dependent inhibition via TGF- β signaling *in vitro*, activation may occur differently *in vivo*. Integrins, matrix metalloproteinases (MMPs), and other proteases present in the extracellular matrix activate TGF- β (Hyytiäinen et al. 2004). Peritoneal macrophages express integrins, including CD11b, and MMPs that may activate TGF- β , but some macrophage functions require tissue-specific signals for expression (Okabe & Medzhitov 2016). It is unclear if TGF- β -expressing macrophages interact with cells other than T cells *in vivo*. If TGF- β is cleaved and activated from macrophages *in vivo*, it could act on a variety of downstream cells. TGF- β signaling in DCs decreases maturation and antigen presentation and decreases cytokine production and cytotoxicity of NK cells. Additionally, it increases chemotaxis of eosinophils, granulocytes, mast cells, and monocytes and macrophages. TGF- β also decreases activation, proliferation and survival in B cells, but interestingly, it promotes class switching to IgA (Gorelik & Flavell 2002). This would be beneficial in *C. rodentium* infection, where IgA production is required for clearance of the pathogen. This also supports the use of EPS treatment for disease in other mucosal tissues, such as the lung. Additionally, if EPS can promote class switch to IgA, it could prevent development of unwanted isotypes, such as IgE in the case of allergic responses, from being generated. In certain diseases, decreased effector cell function as a result of EPS treatment would be detrimental and increase disease pathology.

Contribution of TGF- β signaling *in vivo*. TGF- β and PD-L1 mediate the inhibitory effects of EPS-induced M2 macrophages *in vitro*, but do these molecules contribute to protection *in vivo*? Mice deficient in TGF- β signaling die before birth and due to the wide-range of effects mediated by TGF- β , selective inhibition can greatly exacerbate disease, making studies of the *in vivo* relevance of the cytokine technically difficult. To determine if TGF- β also mediates T cell inhibition *in vivo*, we utilized the anti-CD3 model, where i.p administration of anti-CD3 causes a robust increase in serum TNF α and IL-2 within 2 hours of treatment. We first tested if anti-TGF- β alleviates the protective effects of EPS, using mouse IgG1 as the isotype control. Although neutralization of TGF- β removes EPS-induced reduction in T cell cytokine production, the isotype control alone also increases cytokine production. Therefore, it is difficult to determine the effect of anti-TGF- β on EPS inhibitory effects. In contrast, mice administered mouse IgG1 isotype control prior to infection with *C. rodentium* had reduced disease, making it difficult to discern the effect of TGF- β neutralization following EPS treatment in this model. This may be due to interactions between the mouse IgG1 and Fc receptors that generate some sort of protective immune response. Unfortunately, neutralizing antibodies to TGF- β raised in a different species are not available.

We next tried inhibiting TGF- β through the TGF- β receptor kinase inhibitor SB-423512, or the vehicle control DMSO. Unfortunately, this inhibitor is highly insoluble in anything but DMSO, and like the isotype control, the DMSO

control also abolished the protective effects of EPS in the anti-CD3 model. Similarly in the *C. rodentium* model, mice administered the TGF- β inhibitor had exacerbated disease, and control mice administered EPS and DMSO were no longer protected from disease. These experiments demonstrate that inhibiting TGF- β *in vivo* is technically difficult and requires further optimization. We have not tested the relevance of PD-L1 signaling *in vivo*, but this would require a CD8⁺ T cell-specific model since TGF- β also partially restores EPS-induced M2 macrophage inhibition of CD8⁺ T cells.

Regulatory T cell induction by *B. subtilis* EPS. One of the well-studied effects of TGF- β is the induction of Treg cells. We do see an increase in Treg cells within the peritoneal cavity of EPS treated mice and in co-cultures of EPS-induced M2 macrophages, but how do CD4⁺ T cells contribute to protection from *C. rodentium*-induced disease? Often times iTreg cells upregulate IL-10 to suppress immune responses. In preliminary experiments, we do find upregulation of IL-10 in co-cultures of EPS-induced M2 macrophages and proliferating T cells, where we also see an increased percentage of Treg cells. We have not yet found upregulation of IL-10 *in vivo*, or determined if EPS-induced Treg cells contribute to protection from *C. rodentium*-induced inflammation.

Early in the search for the protective cell-type, we tested if CD4^{-/-} mice are protected by EPS from disease. CD4⁺ T cells drive much of the pathology of *C. rodentium*-induced inflammation, but CD4^{-/-} mice still develop disease, including diarrhea and damage dependent on MyD88 signaling, but goblet cell-depletion

and extensive hyperplasia do not occur. We found that CD4^{-/-} mice are not protected by EPS from disease, suggesting that CD4⁺ T cells are required for EPS-mediated protection (Dr. Sara Jones-Burrage, unpublished observations). EPS-induced M2 macrophages are present around 2-4 days post-treatment. Day 4 post-EPS treatment, peritoneal cells still display an M2 macrophage phenotype and inhibit T cell proliferation compared to peritoneal cells from mice treated with ΔEPS. *C. rodentium* first colonizes the cecal patch, then the colon and begins to induce inflammation 4-7 dpi, however, peak disease doesn't occur until 10 dpi. EPS-induced M2 macrophages possibly inhibit early T cell recruitment to the colon, but Treg cells may limit later stages of disease around days 7-10 since the pathogen is still present at levels comparable to ΔEPS-treated mice. This could be what occurs in CD4^{-/-} mice that develop M2 macrophages 3 dpt, but still develop diarrhea by day 10 post-infection. Reconstitution of CD4^{-/-} mice with CD4⁺ T cells restores the protective benefits of EPS treatment during infection (unpublished observations), but since these experiments use total CD4⁺ T cells and make the equivalent of a wild-type mouse, we do not know if iTreg cells mediate this benefit. In order to directly test the contribution of iTreg cells to EPS-mediated protection from inflammation, we will need to specifically deplete iTreg with anti-CD25 or use mice in which Treg cells can be inducibly-depleted with diphtheria toxin.

Potential of Probiotics to Treat Human Diseases.

Regulatory T cell induction by other commensals. Several

commensal bacteria other than *B. subtilis* prevent inflammatory diseases by modulating the immune response (Table I). The beneficial effects of commensals are described mostly for models of inflammatory bowel disease (IBD), where, with a few exceptions (Pagnini et al. 2010; Ivanov et al. 2009; Fanning et al. 2012), protection appears to be mediated, in large part, by TLR2 signaling, iTreg cells, and IL-10. For example, *B. fragilis*, *Bifidobacteria infantis*, and *Clostridia* species induce Treg cells that ameliorate disease in chemically-induced colitis models (Shen et al. 2012; Atarashi et al. 2011; O'Mahony et al. 2008; Round & Mazmanian 2010) and VSL#3 and *Bifidobacterium breve* induce IL-10-producing Tr1 cells (Di Giacinto et al. 2005; Jeon et al. 2012). *B. subtilis* EPS induces M2 macrophages that inhibit T cell responses through PD-L1 and TGF- β and have the capacity to induce Treg cells. We conclude that *B. subtilis* EPS induces an anti-inflammatory response distinct from that of all previously described probiotics.

With the exception of *B. subtilis* EPS, *B. fragilis* PSA and sphingolipids, and *Faecalibacterium prausnitzii* MAM protein (Mazmanian et al. 2008; Jones et al. 2014; Sokol et al. 2008; Quévrain et al. 2015; An et al. 2014), most of the bacterial molecule(s) responsible for protection have not yet been identified or purified.

Therapeutic potential of probiotics outside the intestine. While most known probiotics target gastrointestinal disease, many probiotics will likely be useful for treating or preventing other inflammatory diseases, including

diabetes, allergy and experimental autoimmune encephalomyelitis (EAE) (Stefka et al. 2014; Cao et al. 2014; J et al. 2010; Hu et al. 2015). In fact, SFB-induced Th17 cells prevent the spontaneous development of type-1 diabetes in NOD mice (Kriegel et al. 2011). *Clostridia* species alter innate lymphoid cells and prevent development of food allergy (Stefka et al. 2014), and *B. fragilis* PSA prevents development of EAE (J et al. 2010). As our understanding of the mechanisms by which specific commensal bacteria modulate the immune system increases, we will likely identify more diseases for which these probiotics will be beneficial.

Therapeutic Potential of *B. subtilis* EPS.

Therapeutic administration of *B. subtilis* and EPS. Because EPS and *B. subtilis* can elicit an anti-inflammatory response when administered through several different routes, we think that EPS and/or *B. subtilis* are good candidates as therapeutic agents for treating inflammatory diseases. *B. subtilis* can be administered as spores that easily transit unharmed through the harsh conditions of the stomach and upper GIT to a nutrient-rich location in the colon where the spores can germinate express EPS. Bacterial molecules such as EPS are attractive therapeutics given that many immunosuppressed individuals cannot tolerate the whole bacterium. EPS induces M2 macrophages when administered by intraperitoneal or intravenous injection, but this would be a cumbersome way to treat humans outside of the clinic. Ideally, EPS would be administered orally. Administration of EPS by oral gavage did not protect mice from disease caused by *C. rodentium* (Dr. Sara Jones, unpublished observations).

We expect that stomach acid breaks down and inactivates EPS. From early studies in our lab, we also know that *B. subtilis* needs to localize to a specific niche within the GIT to induce a protective response, as an immobile, flagella mutant strain does not prevent disease. Encapsulating EPS would provide a vehicle for safe passage through the stomach, but might not ensure that it would reach the ideal location within the GIT. Investigations of other types of administration and the elicited immune responses will be vital to translating this work to humans.

Relevance of *B. subtilis* in humans. Although purified EPS is our ideal therapeutic, until we determine the composition and have a more comprehensive purification process without contamination, EPS is unlikely to pass any sort of regulatory process for use in humans. Probiotics in a number of countries already contain *B. subtilis* and it is regularly consumed in Natto and miso soup, proving the safety of spore consumption; but is it efficacious? A member of our lab, Alexander Argianas, found that *B. subtilis* spores purified from Natto prevented *C. rodentium*-induced disease in mice (unpublished observations). This study suggests that the *B. subtilis* strains used in Natto produces EPS. We have shown that EPS induces an M2 macrophages phenotype in human macrophages similar to what we see in mouse, with increased expression of CD80 and PD-L1. The M1/M2 macrophage paradigm has not been well characterized in humans. Part of this is due to differences in marker expression, but regardless of phenotype, the functions are somewhat conserved

between mouse and human (Martinez et al. 2008). We have not yet determined if these cells produce TGF- β or if they are functional, but these studies suggest that EPS may be beneficial in humans.

Autoimmune disorders. EPS promotes a balance following the inflammatory Th1 and Th17 insult that occurs during *C. rodentium* infection. EPS also decreases T cell responses following i.p. challenge with anti-CD3. We hypothesize that EPS can suppress other T cell-mediated diseases, specifically autoimmune disorders. Th1 and Th17 cells that target the CNS drive EAE pathology; and, both M2 macrophages and TGF- β -bearing CD4⁺ T cells alleviate disease symptoms, suggesting EPS may be a potential treatment for disease. Many other autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus, are driven by T cells or can be treated by blocking T cell responses (Raphael et al. 2015), and are attractive candidates to test if EPS is useful in other disease states.

Graft-versus-host disease. Following allogeneic bone marrow transplants, recipients are at a high risk for acute and chronic graft-versus-host disease (GVHD). During GVHD, T cells from the donor attack host tissues, often in an immunosuppressed patient (Hoffmann et al. 2002). Macrophages are resistant to irradiation and we hypothesize that EPS treatment of the recipient patient prior to and following receiving the transplant may reduce pathology associated with GVHD. Suppression of T cell activation by EPS would be an attractive therapy for preventing the T cell attack of host tissues.

All of these diseases discussed thus far are driven by Type 1 immune responses, and we hypothesize that EPS balances this environment to create a homeostatic environment. M2 macrophages are canonically thought to contribute to Type 2 immunity, but EPS-induced M2 macrophages inhibit Th2, as well as Th1 cytokine responses. We hypothesize that EPS can contribute to the homeostatic balance of preventing hyper-th1 as well as hyper-Th2 responses.

Allergic disease. Children not exposed to certain bacteria early in life do not develop a proper Th1/Th2 balance resulting in allergy and asthma (La Rosa et al. 2003). Allergic disease is caused by the immune system inappropriately reacting to innocuous substances. The process of allergic sensitization is initiated by APCs (such as dendritic cells) that capture and internalize allergen. APCs present antigen, especially to T cells which secrete cytokines, IL-4, IL-5 and IL-13 that help drive the inflammatory response (Galli & Tsai 2012). IL-4 is critical for class switch to IgE, the immunoglobulin that mediates allergic symptoms. Allergen-specific IgE is produced by B cells after encounter with helper Th2 cells. Once allergen-specific antibodies are generated, they bind to mast cells and upon subsequent exposure, the allergen cross-links IgE, causing mast cell degranulation and rapid release of chemical mediator such as histamine, that recruit of Th2 cells, basophils and eosinophils that exacerbate the immune response.

Dr. Julie Swartzendruber asked if EPS prevents allergic sensitization, a hyper-Th2 response, in a mouse model of IgE-mediated allergic sensitization to

ovalbumin (OVA). In mice pre-treated with EPS, we find a decrease in OVA-specific IgE induced by OVA/alum sensitization, suggesting that EPS prevents allergic sensitization. A potential mechanism for this suppression is that EPS-induced M2 macrophages inhibit the initial interaction between APCs and T cells, limiting production of IL-4 and production of IgE. Additionally, TGF- β produced by M2 macrophages could induce class switch to IgA, instead of IgE. Alternatively, EPS-induced alterations in DCs mentioned earlier could alter the generation of a detrimental Th2 response. This study demonstrates that EPS can prevent allergic sensitization but it is unclear if EPS can prevent allergy symptoms after sensitization has already occurred.

It is possible that EPS may exacerbate symptoms through the induction of M2 macrophages. For example, TGF- β increases chemotaxis of eosinophils and other granulocytes, suggesting that even though TGF- β inhibits Th2 cells, it may enhance disease by recruitment of other detrimental cells. Future studies need to be done to test this hypothesis.

Cell targets of EPS. In our current studies, we identified modulation of macrophages by EPS and have hypothesized DCs may also respond EPS. Both of these cells are, or can be, myeloid lineage cells (certain DC subsets have a lymphoid lineage), and are known to phagocytose antigens. Since many of the signaling pathways overlap between macrophages and DCs, it's reasonable that EPS acts exclusively on these cells types. Much of this expectation is based on binding of EPS to macrophages and low levels of EPS binding to DCs. We do not

see alterations in T cells following co-culture with CD4⁺ T cells and have not observed any changes directly on B cells. We have not extensively ruled out effects of EPS on other cell types, which would be necessary for future studies in humans.

M2 macrophages in health and disease. M2 Macrophages promote wound healing and tissue repair. Further, they provide immunity to helminth infections. Arginase-1 and TGF- β mediate protective effects in both tissue homeostasis and immunity to helminths, suggesting that EPS treatment may enhance anti-helminth immunity or promote tissue repair.

M2 macrophages also help maintain metabolic homeostasis by secreting anti-inflammatory cytokines and utilizing oxidative metabolism. During obesity, M1 macrophages promote chronic inflammation in adipose tissue, which leads to insulin resistance (Castoldi et al. 2016). A shift to an M1 macrophage phenotype is characterized by aerobic glycolysis. We have not assessed the metabolic profile of EPS-induced M2 macrophages. One interesting hypothesis is that macrophages metabolize EPS, driving M2 macrophage development. The benefits of *B. subtilis* and EPS and their anti-inflammatory properties could extend to promoting a lean (non-obese) metabolism. Future experiments will discern these possibilities.

Treatment vs. prevention. In many of the diseases discussed, we have used EPS as a preventative measure. We do know that *B. subtilis* administered up to 3 dpi with *C. rodentium* still prevents disease (Figure 4.4). *B. subtilis*-

induced M2 macrophages are present at 4 dpt in the peritoneal cavity and 6 dpt in the MLN, suggesting the anti-inflammatory response induced by *B. subtilis* takes about 4 days to develop. When mice were infected with *C. rodentium* 3 days prior to *B. subtilis* treatment, the anti-inflammatory environment developed after inflammation had already begun (Figure 4.4). In this study, therefore, *B. subtilis* treats, rather than prevents, *C. rodentium*-induced inflammation, but it is unclear if this occurs in other diseases with EPS.

In humans, it’s not practical for EPS to be administered as a pretreatment for an unknown or unexpected disease. In certain cases of autoimmunity, such as MS, the disease is diagnosed early, before much damage has occurred, but for the majority of diseases, we would use EPS to treat inflammation. Future studies

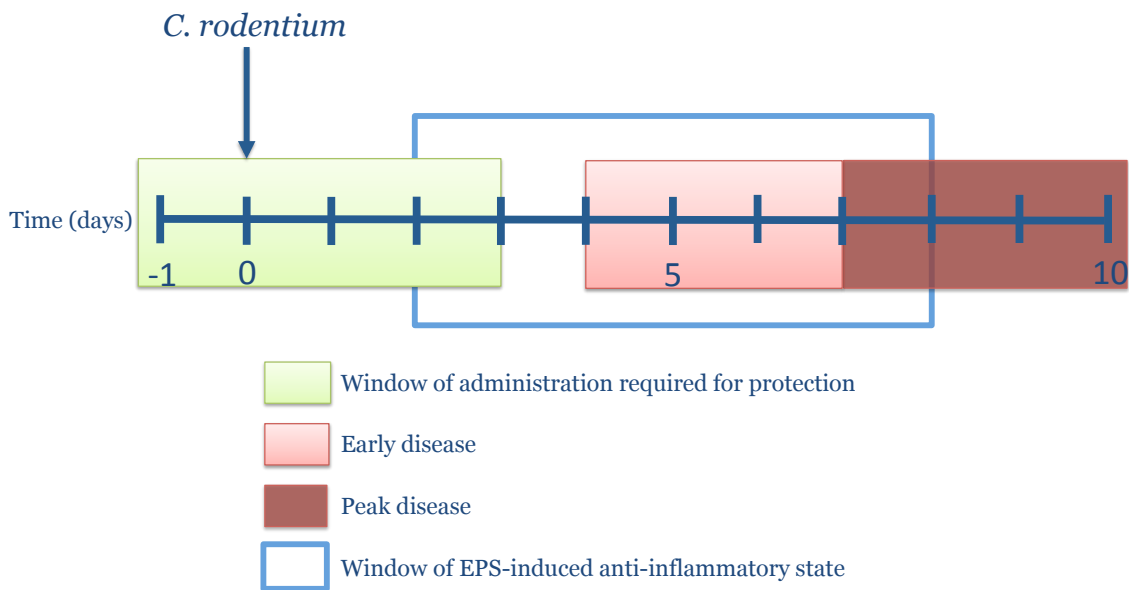


Figure 4.4. Therapeutic window of *B. subtilis* EPS-induced anti-inflammatory response.

testing the capacity of EPS to dampen down T cell responses once they have already been initiated are necessary to determine the true efficacy of EPS in humans.

EPS: is there a downside? EPS prevents inflammation in several different inflammatory conditions and is an exciting therapeutic option for many disorders. But can otherwise beneficial effects of EPS be detrimental in certain situations? The anti-inflammatory response induced by EPS is transient. By 5 days post-EPS treatment, peritoneal macrophages resemble those of an untreated mouse. Further, splenic T cell responses are no different between EPS-treated mice than untreated mice. We do not expect this transient anti-inflammatory response to induce detrimental effects. In cases of cancer, however, increased immunosuppression could lead to tumor growth. Tumor-associated macrophages (TAMs) bear many similarities to M2 macrophages, including TGF- β expression. TAMs produce anti-inflammatory cytokines and promote angiogenesis to bring nutrients to the tumor microenvironment, fostering tumor growth. PD-L1 expression within tumors also promotes tumor growth. Blockade of the PD-1 pathway has been a breakthrough treatment for many different types of cancers. Although EPS treatment would be harmful for an established tumor, we do not know if repeated EPS treatment and maintenance of M2 macrophages would promote tumor development.

Repeated doses of EPS might also generate EPS-specific antibodies. These antibodies might induce an inflammatory response or block the induction of M2

macrophages. Low dose inoculation of certain antigens has also been linked to induction of allergy. In certain situations, commensal specific antibodies promote retention of the bacterium within the GIT. Identification of the induced antibody isotypes, or the particular adaptive immune response elicited might lead to identification of other disease states in which this response could be beneficial. Additionally, in the case of *B. subtilis*, increased prevalence might block colonization of *C. rodentium* or other pathogenic bacteria.

In other cases of inflammation such as viral infection, inflammation is necessary to clear the pathogen. Inhibition of a viral specific response might render the host unable to clear the pathogen, allowing the virus to manifest in other organs and have deleterious effects. As with *C. rodentium* infection, much of the disease symptoms induced by some viral infections, such as the common cold, are an exacerbated response to assure clearance of the pathogen. A low dose of EPS may treat some of these symptoms and still allow the immune system to clear the infection.

Concluding Remarks.

***Bacillus subtilis* as a commensal.** Clearly, *B. subtilis* has evolved a complex interaction with the immune system; yet, *B. subtilis* is often overlooked and not considered a true commensal. During these early stages of identifying mechanisms of immune-modulation by the microbiota, much attention has been placed on highly prevalent bacterial species. Although *B. subtilis* is present at low levels in the GIT, it is detected in a many of the individuals surveyed (Benno et al.

1984; Benno et al. 1986; Qin et al. 2010). One possibility is that EPS is composed of similar polysaccharides to another highly prevalent commensal, but such a commensal has not been identified since no other commensals have been shown to induce M2 macrophages.

These studies emphasize the importance of organisms present at low levels and their important role in promoting a balance between the microbiota and the host. The gut microbiota requires balance with the immune system and many unavoidable factors, whether environmental, antibiotics or infections, cause fluctuations in the microbiota and the balance between type 1 and type 2 immune responses. The hygiene hypothesis suggests that reduced exposure to microorganisms early in life leads to abnormal development of the immune system, providing an explanation for the rise in allergic disease, inflammatory disorders and autoimmunity. The importance of commensal bacteria in shaping health is now widely recognized, and the potential therapeutic benefits of these bacteria are being explored in several diseases. Regardless of the prevalence of certain organisms during homeostasis, individual organisms with the capacity to induce tolerance possess strong potential to be used as a probiotic to restore homeostasis when dysbiosis occurs (Figure 4.5).

Probiotics are a multi-billion dollar industry world-wide. Yogurts, pills, energy drinks, the products and benefits of a healthy microbiota are all over the news, advertising and day-to-day life. But are these products actually eliciting a response? Our study highlighting that the mechanism by which *B. subtilis* limits

inflammation caused by an enteric pathogen indicates probiotics do actually prevent disease.

Many probiotics on the market are an undefined cocktail of bacteria; it is unclear what if any of the components are eliciting a positive effect. More mechanistic studies are needed to identify which products actually contribute to human health.

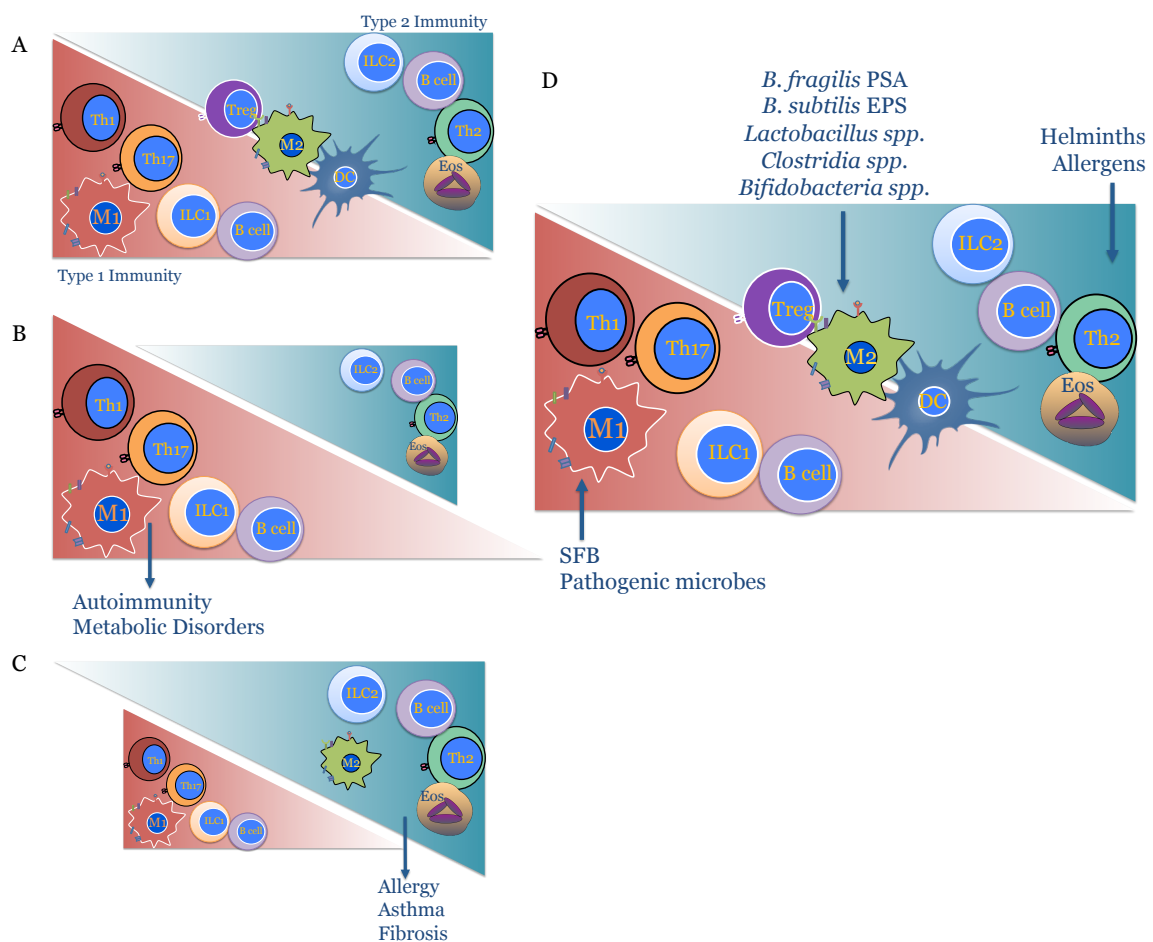


Figure 4.5. Probiotics maintain balance between type 1 and type 2 immunity. Balance between Type 1 and Type 2 immunity is maintained by Treg cells, M2 macrophages, and tolerogenic DCs (A). When dysbiosis occurs, a skewing towards type 1 immunity results in increased autoimmune diseases and metabolic disorders (B), whereas a detrimental type 2 immune response drives allergy, asthma and fibrosis (C). Probiotics and commensal bacteria maintain regulatory cells that drive homeostasis, allowing normal inflammatory processes to occur (D).

Trillions of bacteria inhabit the gastrointestinal tract; countless bacteria found in the environment exert beneficial effects as probiotics. The precise mechanism by which these bacteria modulate the immune system to benefit the host has been identified for around a dozen of these bacteria. Only four molecules (EPS, PSA, sphingolipids, and MAM) from three bacteria (*B. subtilis*, *B. fragilis*, and *F. prausnitzii*) have been identified to mediate these symbiotic effects. This study highlights a unique mechanism by which a Gram (+) commensal exopolysaccharide induces an anti-inflammatory environment, as modeled in Figure 4.6. EPS from *B. subtilis*, induces anti-inflammatory M2

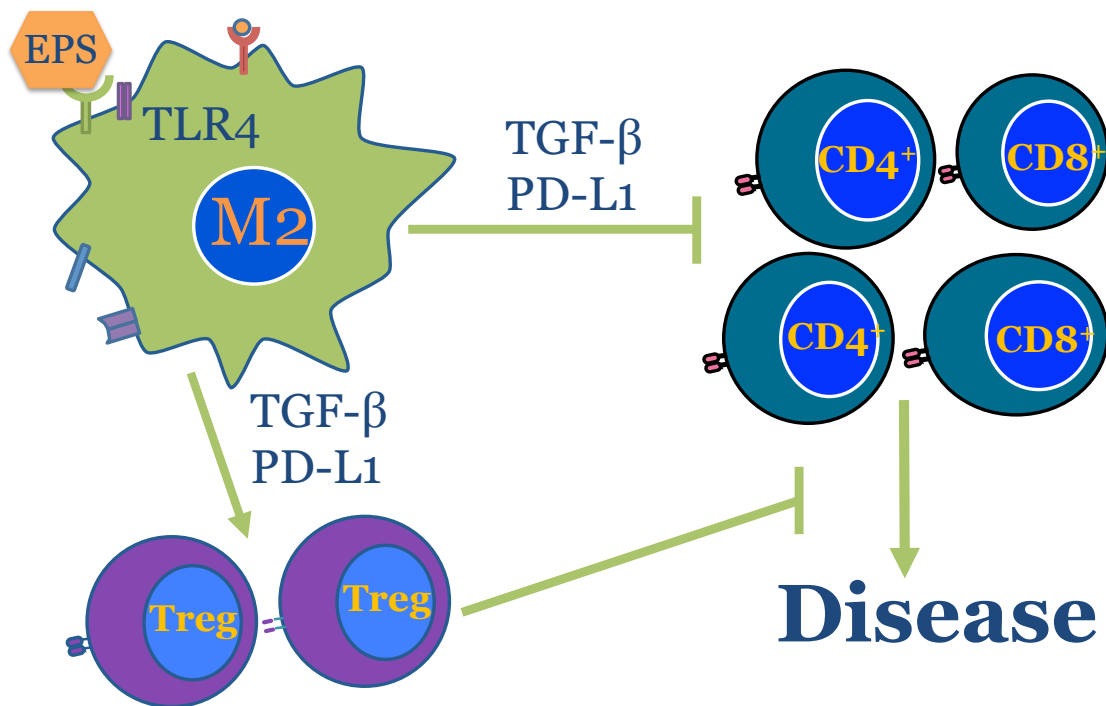


Figure 4.6. Model for EPS modulation of immune responses. *B. subtilis* and purified EPS induce M2 Macrophages which inhibit CD4⁺ and CD8⁺ T cells through production of TGF- β and PD-L1 and potentially through induction of regulatory T cells.

macrophages in a TLR4-dependent manner. These M2 macrophages produce multiple immune inhibitory molecules, including TGF- β , PD-L1 and arginase-1, all of which inhibit T cell responses and prevent inflammatory diseases.

Undoubtedly, additional molecules from a large number of commensal bacteria will be found to regulate immune responses. Understanding the mechanisms by which EPS and other molecules from commensal organisms regulate the immune system will lead to new rationally-designed and safe therapeutics for inflammatory diseases.

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VITA

The author, Mallory Lynn Paynich, was born on May 27, 1986 in Kalamazoo, Michigan to Michael and Susan Paynich. She is the middle of three daughters between older sister, Caitlin, and younger sister, Erin. In 2008, Mallory received her Bachelor of Science in Biochemistry and Molecular Biology/Biotechnology from Michigan State University. During her undergraduate studies, she worked in the Laboratory of Dr. Cindy Grove Arvidson studying the pathogenesis of *Neisseria gonorrhoeae*. After graduating, Mallory worked in the Global Biologics division of Pfizer Global Research and Development in St. Louis, MO.

In August of 2010, Mallory joined the Department of Microbiology and Immunology at Loyola University Chicago. Shortly thereafter, Mallory joined the laboratory of Dr. Katherine L. Knight, Ph.D., studying the benefits of the commensal microbiota on the immune system. Her doctoral work focused on the anti-inflammatory environment afforded by the probiotic *Bacillus subtilis* to prevent inflammatory diseases. While at Loyola, Mallory was awarded a Predoctoral fellowship from the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institute of Health.

After graduation, Mallory plans to continue pursuing her interest in scientific research and is currently in the process of applying for post-doctoral fellowships.