Commensal Microbiota-Induced Interferon-Beta Mediates Host Immune System Modulation and Natural Resistance to Virus Infection

Commensal microbiota-induced interferon-beta mediates host immune system modulation and natural resistance to virus infection

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Abstract

The type I interferon (IFN-I) response is a crucial mediator of antiviral immunity and homeostatic immune system regulation. Despite this importance, the source of IFN-I signaling under homeostatic conditions has yet to be identified. We have found that one of the IFN-Is, IFNβ, is expressed by colonic lamina propria dendritic cells in response to the commensal microbiota. Moreover, commensal-induced IFNβ was found to be required for local and systemic expression of interferon-stimulated genes, and thus for regulation of the IFN-I response, under steady-state conditions. Having identified that the IFN-I response is regulated by the commensal microbiota, we proceeded to investigate the mechanism of this interaction and identified a single commensal microbial molecule, the lipooligosaccharide (LOS) of the human intestinal commensal *Bacteroides fragilis*, that is capable of inducing IFNβ secretion by dendritic cells *in vitro* and *in vivo* through the TLR4 signaling pathway. Since *Bacteroidetes* are a very prominent bacterial phylum in the gut microbiota, we examined outer membrane extracts from several different *Bacteroides* species that are rich in LOS reported to have similar structure to the LOS of *B. fragilis* and found that they are also potent IFNβ inducers. The physiological relevance of commensal-induced IFNβ in immunoregulation and in virus infection was next investigated. We discovered that IFNβ signaling is important for induction of

tolerogenic mechanisms by *B. fragilis* capsular polysaccharide A, which is also comprised in part by an LOS-like molecule, and for protection in an experimental autoimmune disease model, EAE. Importantly, we found using vesicular stomatitis virus infection as a model, that commensal-induced IFNβ regulates the natural resistance of the host to virus infection.

To my husband Eric, my parents Jim and Janelle, and my sisters Hannah and Sara for their love and support.

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Abbreviations

°C	degrees Celsius
ABX	antibiotics
Actb	beta-actin
APC(s)	antigen presenting cell(s)
B. fragilis	*Bacteroides fragilis*
BMDC(s)	bone marrow-derived dendritic cell(s)
BST2	bone marrow stromal cell antigen 2
C. ramosum	*Clostridium ramosum*
CD	cluster of differentiation
cDC(s)	conventional DC(s)
CFA	complete Freund's adjuvant
CFU	colony-forming unit
cGAS	cyclic GMP-AMP synthase
Clec7a$^{-/-}$	dectin-1 deficient
CO_2	carbon dioxide
Ctrl	control
d.p.i.	days post infection
DC(s)	dendritic cell(s)
E. coli	*Escherichia coli*
EAE	experimental autoimmune encephalomyelitis
EC_{50}	half-maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid

ELISA	enzyme-linked immunosorbent assay
Flt3L	FMS-related tyrosine kinase 3 ligand
FOXP3	forkhead box p3
g	gram
GAS	IFNγ-activated site
GF	germ free
GFP	green fluorescent protein
GI	gastrointestinal
GLA	glycolipid anchor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome-wide association studies
h.p.i.	hours post infection
hr(s)	hour(s)
IAV	influenza A virus
IBD	inflammatory bowel disease
ICOSL	inducible costimulatory ligand
IEC(s)	intestinal epithelial cell(s)
IFIT	interferon-induced proteins with tetratricopeptide repeats
IFN-I(s)	type I interferon(s)
IFNAR	interferon-α/β receptor
Ifnar1$^{-/-}$	*Ifnar1* deficient
Ifnb1$^{-/-}$	*Ifnb1* deficient
IFNα	interferon-α

IFNβ	interferon-β
IL-	interleukin-
Il10$^{-/-}$	*Il10* deficient
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IRF	interferon regulatory factor
ISG	interferon stimulated gene
ISGF3	interferon-stimulated gene factor 3
ISRE(s)	interferon-stimulated response element(s)
IU	international units
Jak	Janus kinase
L	liter
LAL	*Limulus* amoebocyte lysate
LOS	lipooligosaccharide
LP	lamina propria
LPS	lipopolysaccharide
M	molar
MAPK	mitogen-activated protein kinase
MDA5	melanoma differentiation-associated gene 5
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
mL	milliliter

mLN(s)	mesenteric lymph node(s)
MOG	myelin oligodendrocyte glycoprotein
MOI	multiplicity of infection
MS	multiple sclerosis
MyD88:	myeloid differentiation primary response protein
Myd88$^{-/-}$	*Myd88* deficient
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NK	natural killer
NLRP	NACHT, LRR, and PYD domains-containing protein
OASL2	2'-5'-oligoadenylate synthetase-like protein 2
OG	oral gavage
OM	outer membrane
PBS	phosphate buffered saline
pDC(s)	plasmacytoid dendritic cell(s)
PDCA-1	plasmacytoid dendritic cell antigen-1
PFU	plaque-forming units
pg	picogram
PI3K	phosphoinositide 3-kinase
pM	picomolar
PRR	pattern recognition receptor
PSA	polysaccharide A
qRT-PCR	quantitative reverse transcription polymerase chain reaction

RA	rheumatoid arthritis
RIG-I	retinoic acid-inducible gene I
RNase	ribonuclease
RPM	revolutions per minute
RSAD2	radical SAM domain-containing 2, also known as viperin
SEM	standard error of the mean
SH	siglec H
sp.	species
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
Tlr2$^{-/-}$	*Tlr2* deficient
Tlr4$^{-/-}$	*Tlr4* deficient
TNFα	tumor necrosis factor α
T_R1	type 1 regulatory
$T_{reg(s)}$	regulatory T cell(s)
TRIF	TIR-domain-containing adapter-inducing interferon-β
Trif$^{-/-}$	*Trif* deficient
Tyk2	non-receptor tyrosine-protein kinase 2
ug	microgram
uL	microliter

VSV	vesicular stomatitis virus
WT	wild type

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Chapter 1

Introduction

1.1. The commensal microbiota

1.1.1. Composition

The human body, like that of other mammalian organisms, is colonized by trillions of microbial organisms, including bacteria, viruses, fungi, and archaea, collectively referred to as the commensal microbiota.(1, 2) With advances in culture-independent DNA sequencing technologies and bioinformatics, there has been a rapid increase in our knowledge of the diversity and dynamics of the microbiota over the past two decades.(2, 3) The initial colonization of the human body occurs at birth and the composition of the neonatal microbiota is strongly influenced by the method of delivery, and remains dynamic throughout the first years of human life, altered by feeding method and antibiotic use.(4-8) The composition of the adult microbiota is more stable but can be altered by environmental factors such as diet composition, medical intervention, and diseased states.(9-11)

Current estimates suggest that there are roughly as many bacterial cells as human cells in the body, and approximately a hundred times more bacterial genes than human genes.(3, 12) The vast majority of commensal bacteria reside in the gastrointestinal (GI) tract, and particularly in the colon, which is colonized by an estimated 10^{14} bacteria.(13, 14) The small intestine has a lower density of commensal microbes, ranging from 10^{3}-10^{8} bacteria per gram of luminal content, depending on which region is sampled.(15) While there is significant diversity in terms of microbiota composition at the bacterial species level, with hundreds of different bacterial species represented in an individual's microbiome, there is a high level of conservation at the phylum level, with the majority of mammalian commensal bacteria belonging to only eight phyla.(14, 16)

1.1.2. Physiological role

The advances in characterization of the structure of the microbiome have opened up the possibility for studies of the interactions between the host and its microbiome and the functional outcomes of these interactions. The shear magnitude of bacterial cells and genes in the human body, and specifically in the intestine, begins to hint at the potential impact gut commensal organisms might have on their host.(3, 12-14) Indeed, commensal bacteria are not passive bystanders, but they have evolved to live in a symbiotic relationship with their mammalian host, forming a dynamic ecosystem in which both humans and microbes carry out physiological processes essential for the overall health of the host. Studies over the past two decades in humans and other mammals have begun to elucidate this mutually beneficial relationship, in which the bacteria receive nutrients and a specialized ecological niche in which to reside, while in turn playing a role in vital host physiological processes including intestinal and immune system development and homeostasis, metabolism, and protection against pathogens.(17-19)

One of the most important functions of the gut microbiota is in the development and regulation of the immune system. The importance of commensal microbes to immune system regulation has been demonstrated by studies in germ-free (GF) mice, which are completely devoid of all microbial life and thus have no commensal microbiota.(20) GF mice exhibit an underdeveloped immune system, with decreased total lymphocyte numbers as well as aberrant regulation of these lymphocytes, evidenced by skewed ratios of T helper (Th) cell subsets compared to conventionally colonized mice.(21, 22) This commensal regulation is not only vital to the local, mucosal immune

system, but also contributes to immune homeostasis at distal sites including the central nervous system (CNS), joints, and lungs.(19, 23-29)

The importance of immunomodulation by the GI microbiota to human health is highlighted by the impact commensal microbes have been shown to have in experimental models of human diseased states. Indeed, gut commensal bacteria have demonstrated effects in both local and systemic inflammatory disorders as well as a growing list of diseases in which aberrant inflammation plays a role in pathogenesis. Through numerous studies, the gut microbiota has been linked among others to inflammatory bowel disease (IBD), colon cancer, diabetes, asthma, cardiovascular disease, autism spectrum disorder, and both bacteria and viral infections.(30-36) These findings highlight the potential for manipulation of the microbiota to impact human health and disease outcome. A mechanistic understanding of how microbes interact with their host is therefore not only biologically interesting, but it provides a platform for discovering potential therapeutic targets for the treatment and prevention of a variety of human diseases.

Despite this importance, the study of the precise molecular mechanisms by which specific commensal microbes interact with their mammalian host is still in its infancy. There is an increasing number of specific commensal bacterial species that have been discovered to modulate the immune system, including *Clostridium* species (sp.), *Bacteroides fragilis (B. fragilis)*, *Faecalibacterium prausnitzii, Akkermansia muciniphila,* and *Bifidobacterium breve*.(37-42) However, work from our lab with *B. fragilis* still remains one of the only examples in which a specific bacterial molecule has been isolated and identified to possess immunomodulatory properties. Specifically, the zwitterionic capsular polysaccharide A (PSA) of *B. fragilis* was found to regulate immune system

homeostasis by restoring the Th1/Th2 balance in GF mice and inducing IL-10 secreting regulatory T cells (T_{regs}) in the context of inflammation.(22, 39, 43) Considering that there are hundreds of bacterial species known to reside in the human GI tract with the potential to interact with the host, the majority of mechanisms by which commensal bacteria impact immune homeostasis likely remain undiscovered.

The cells of the immune system rely on secretion of cytokines to communicate with one another and orchestrate or suppress an inflammatory response.(44) One possible mechanism of immunomodulation by commensals is therefore through regulation of cytokine secretion, including the secretion of type I interferons (IFN-Is).

1.2. The type I interferon response

IFN-Is are a family of structurally similar cytokines with potent immunomodulatory functions consisting predominantly of two different classes of proteins, interferon-α (IFNα) and interferon-β (IFNβ). In mammals, multiple genes encode for several different variants of IFNα, with 14 in mice and 13 in humans, but only one IFNβ gene, all clustered in a locus on chromosome 9 in humans and 4 in mice.(45)

1.2.1. Induction of type I interferons

IFN-I expression is regulated at the transcriptional level in response to sensing of microbial products through pattern recognition receptors (PRRs) or by signaling from other cytokines.(46) The PRR initiates a signaling cascade that activates the interferon regulatory factor (IRF) transcription factors which then translocate to the nucleus where they bind to regulatory elements in the IFN-I gene promoter regions to initiate expression

of the IFN-I genes.(47) The IFN-I-inducing PRRs can be broadly divided into two classes. The first are the cytosolic nucleic acid sensors, including the RNA-sensing retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) and the DNA-sensing cyclic GMP-AMP synthase (cGAS), which respond to viral nucleic acids in the cytosol.(48, 49) The second are the interferon-inducing toll-like receptors (TLRs) which include the nucleic acid-sensing TLRs 3, 7, 8, and 9 as well as TLR2 and TLR4 which canonically recognize microbial cell membrane components.(48, 50) Interestingly, all nucleated cells are capable of expressing IFN-Is in response to cytosolic nucleic acid sensing, while induction of IFN-I downstream of TLRs is predominantly a function of specialized innate immune cells, suggesting the potential for TLR-induced IFN-I to play a greater role in immune system modulation.(48)

1.2.2. Type I interferon signaling pathways

Paradoxically, IFN-Is have been demonstrated to have numerous immunomodulatory functions, in some cases enhancing protective immunity to pathogens while in others suppressing the immune response. Reflecting their pleiotropic and often contradictory functions, IFN-Is activate a multitude of intracellular signaling pathways. To initiate these signaling cascades, all of the IFN-I species bind to the same site on, and signal uniquely through, the heterodimeric transmembrane interferon-α/β receptor (IFNAR), which is composed of two subunits-IFNAR1 and IFNAR2- and is expressed by most mammalian cell types.(46)

The canonical IFN-I signaling pathway is the Janus kinase (Jak)- signal transducer and activator of transcription (STAT) pathway. Upon binding of IFN-Is to

IFNAR, non-receptor tyrosine-protein kinase 2 (Tyk2) and Jak1 are activated and phosphorylate STAT1 and STAT2. Activated STAT1 and STAT2 heterodimerize and translocate to the nucleus where they form the heterotrimeric interferon-stimulated gene factor 3 (ISGF3) complex through binding of the IRF9 transcription factor. The ISGF3 binds to interferon-stimulated response elements (ISREs) to induce expression of hundreds of genes, collectively termed interferon stimulated genes (ISGs), responsible for many of the antiviral functions associated with IFN-I signaling.(51)

Depending on the cell-type and stimulus involved, IFNAR signaling can also promote homodimerization of other STATs, including STATs 1-6. STAT1 homodimers bind to IFNγ-activated site (GAS) enhancer elements, leading to the expression of proinflammatory mediators. On the other hand, activation of STAT3 homodimers induces secretion of interleukin (IL)-10, a potent anti-inflammatory cytokine. It is thought that the relative abundance of STAT1 and STAT3 in cells at the time of signaling by IFN-I might contribute to the functional outcome of the IFN-I signaling event, whether it will enhance or suppress inflammation.(52)

In addition to the classical Jak-STAT signaling pathway, IFN-Is have been more recently demonstrated to signal through the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) signaling pathways in a stimulus and cell-type dependent manner.(53) MAPK signaling has been demonstrated to contribute to the antiviral activity of IFN-Is, again reflecting a more proinflammatory outcome of IFN-I signaling.(54) Activation of PI3K by IFNAR and Jak1 represents another anti-inflammatory mechanism of IFN-Is, leading to IL-10 production.(55) The ability of IFN-Is to activate multiple signaling pathways leading to distinct outcomes helps to elucidate

how signaling through a single receptor can lead to the numerous, context-specific, and opposing functions of IFN-Is.

1.2.3. Functions of type I interferons

1.2.3.1. Antiviral response

The canonical role of IFN-Is, and the role for which these cytokines were first discovered, is to protect the host during virus infection.(56) The vital nature of the IFN-I response in viral defense is exemplified by the extreme susceptibility to virus infection of mice lacking IFNAR1.(57) This protective role is accomplished by several different actions of IFN-Is, the first of which is the ability of IFN-Is to confer an antiviral state to cells, rendering them non-permissive to virus infection. Upon release of IFN-Is by an infected cell, they signal in an autocrine and paracrine fashion to induce expression of hundreds of ISGs in the infected and neighboring cells. These genes include intrinsic antiviral factors capable of directly blocking the different stages of the virus life cycle, thus restricting the ability of a virus to replicate and spread to neighboring cells.(58)

IFN-I signaling also helps to limit virus spread through regulation of apoptosis. Viruses are entirely dependent on the host cell for replication and completion of their life cycle. As such, killing a cell that is infected prevents the virus from replicating and is an effective strategy to prevent viral spread. When IFN-Is signal to uninfected cells, there is generally no effect on cell viability, just the induction of the antiviral state. However, if IFN-I signaling is concurrent with cytosolic nucleic acid sensing, apoptosis can be triggered through one of several cellular pathways depending on the type of virus infection. In this way, infected cells are eliminated, while damage to healthy cells is

limited.(48) The role of apoptosis in control of virus infection is so important that it has exerted selective pressure on viruses. Indeed, many viruses have evolved mechanisms to block host cell apoptosis.(59) Together, apoptosis and induction of an antiviral state represent immediate responses induced by IFN-Is at the onset of infection. In the case of most acute pathogenic virus infections, this rapid response is thought to be essential to limit initial virus replication before the onset of effective anti-viral humoral or cellular adaptive immune responses.(60)

The above cell-intrinsic functions of IFN-Is represent ways in which IFN-I signaling directly suppresses virus infection. In addition, the immunomodulatory functions of IFN-Is indirectly limit viral infection through coordination of the innate and adaptive immune response. Before immune cells can respond to a pathogen, they must arrive at the site of infection. IFN-I signaling induces chemokine expression, which facilitates recruitment of both innate and adaptive immune cells to the site of infection. Once immune cells are present, IFN-Is have also been linked to the activation and expansion of specific immune cell subsets that are important for the control of virus infection. Specifically, they have been demonstrated to regulate the activation of natural killer (NK) cells and to enhance the expansion, activation, and survival of cluster of differentiation (CD)-8^+ T cells both directly and indirectly through increased cross-presentation of viral antigens by dendritic cells (DCs). The cytotoxic response of both of these cell types effectively targets and eliminates infected cells, contributing to clearance of virus infection. IFN-Is additionally regulate $CD4^+$ Th cell subsets and promote the activation and antibody responses of B cells, ultimately contributing to viral clearance and resolution of infection.(61)

1.2.3.2. Homeostatic functions

Most early research focused on IFN-Is in the context of infection, during which they are induced at very high levels in response to pathogens. It is now recognized that there is extremely low-level, constitutive expression of IFN-I even in the absence of infection.(62, 63) Recent studies have therefore begun to elucidate the important homeostatic functions of these cytokines. Indeed, current research suggests that IFN-Is can regulate the development and/or activation of virtually every immune effector cell, including both innate and adaptive immune cells. This homeostatic regulation of the immune response has imparted IFN-Is with important anti-inflammatory, anti-tumor, and cytokine signaling regulatory functions, with important consequences to the health and homeostasis of the host, described below.(63)

1.2.3.3. Anti-inflammatory functions

The immunoregulatory role of IFN-Is and their potential to suppress pathologic inflammation has been demonstrated in animal models of several inflammatory disorders in which they were shown to have a protective effect, including experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (IBD), and rheumatoid arthritis (RA).(64-71) Genome-wide association studies (GWAS) have linked alterations in the *Ifnar1* gene to susceptibility for IBD, providing evidence to support a protective role of IFN-Is against intestinal inflammation in humans.(72, 73) Furthermore, while there has been limited success in human clinical trials for IBD or RA, administration of recombinant IFNβ is used therapeutically in the treatment of patients with relapsing-remitting multiple sclerosis (MS), linking IFN-Is to protection against

neuroinflammation.(74) Several recent studies have begun to elucidate the mechanism by which IFN-Is might regulate immune system homeostasis and thus contribute to protection in inflammatory disorders.

Prevention of aberrant immune responses is especially challenging in the GI tract, where the large mucosal surface area is in direct contact with the environment, and thus constantly exposed to microbes. The first line of defense at this site is the physical barrier separating the internal tissue from the external environment, the single-cell layer of intestinal epithelial cells (IECs). Proper barrier function not only protects against pathogens, but also is important to maintain physical separation with and properly regulate the immune response to the commensal microbiota. Indeed, dysfunction of the IEC barrier is thought to increase susceptibility to IBD.(52) IFN-Is have been demonstrated to promote the barrier function of IECs through STAT3-mediated regulation of tight-junction molecule expression, as well as to regulate epithelial cell proliferation and turnover, thus indirectly contributing to intestinal immune homeostasis.(52, 72).

Direct signaling of IFN-Is to innate immune cells also contributes to immune system homeostasis, through reduction of proinflammatory cytokine and increase in anti-inflammatory cytokine expression, thus maintaining a healthy cytokine balance. IFN-Is reduce secretion of IL-1β, a proinflammatory cytokine implicated in the pathogenesis of many diseases, by both reducing expression of inactive pro-IL-1β and inhibiting NACHT, LRR and PYD domains-containing protein (NLRP) 1 and NLRP3 inflammasome activation to reduce cleavage of pro-IL-1β to its active form.(75) IFN-I

signaling also promotes an anti-inflammatory milieu by increasing expression of the regulatory cytokines IL-10, IL-27, and IL-1RA by macrophages and DCs.(76)

Disruption of the balance between different $CD4^+$ Th cell subsets, and specifically the Th17/T_{reg} balance is thought to play a major role in the development of autoimmune disease.(77) While important in the protective response against bacterial infection, the proinflammatory Th17 lineage of $CD4^+$ T cells has been implicated in the pathogenesis of several autoimmune disorders, including MS, RA, and psoriasis.(78) In contrast, T_{regs} possess immunosuppressive properties, crucial to regulation of the adaptive immune response and maintenance of tolerance to self and to commensals.(79) The ability of IFN-Is to regulate the Th17/T_{reg} balance through effects on both Th17 and T_{reg} cells is therefore thought to contribute to the anti-inflammatory properties of these cytokines. IFN-Is suppress differentiation of Th17 cells through the IFNAR-osteopontin signaling axis, and also reduce expression of IL-17 by these cells.(80, 81) While reducing inflammatory $CD4^+$ T cell subsets, IFN-Is also augment immunoregulatory T cell responses. IFN-Is enhance secretion of the regulatory cytokine IL-10 by both murine and human $CD4^+$ T cells.(82, 83) In addition, IFN-Is regulate the activity of T_{regs}, by driving constitutive expression of forkhead box P3 (FOXP3), enhancing T_{reg} suppressive activity, and promoting T_{reg} differentiation.(67, 84) Through regulation of the Th17/T_{reg} balance, IFN-Is therefore exert control over immune system homeostasis and prevention of inflammatory disorders.

1.2.3.4. Calibration of cytokine signal transduction

An interesting observation was made that mice deficient in IFNβ or in IFNAR1 exhibited not just loss of IFN-I signaling, but also reduced responses to diverse, unrelated cytokines, such as IFNγ, CSF-1, and IL-6.(63, 85, 86) These findings demonstrated the potential for IFN-I to exert broad influence over the outcome of the immune response and of immune homeostasis by calibrating the response of cells to other cytokines. This priming effect is due to regulation of the expression of STATs 1, 2, 3 and 6, which are signaling intermediates used by many different cytokines, by low-level, constitutive IFN-I secretion. It is thought that the ratio of expression levels of the different STAT transcription factors determines the outcome of the response downstream of the Jak-STAT signaling pathways.(63) In this way, by altering expression of different STAT proteins, IFN-Is can sculpt the quality and the magnitude of the cellular response to different cytokines, thus modulating the immune response.

1.2.3.5. Antitumor response

The cells of the tumor microenvironment, including infiltrating innate and adaptive immune cells, stromal cells, as well as the tumor cells themselves are all capable of producing IFN-Is. Evidence for an antitumor role of endogenous IFN-I has been provided by numerous studies. Disruption of IFN-I signaling through antibody-mediated inhibition of IFNα/β or loss of either IFNβ or IFNAR1 in knockout animals increased tumor development, growth, and disease progression in both carcinogen-induced and tumor-implantation murine cancer models.(87, 88) In humans, genetic analyses of human cancers revealed a diverse set of malignancies with mutations causing defects in

IFN-I secretion or signaling, suggesting that the ability to evade IFN-I might confer a survival advantage to tumor cells.(63) Moreover, expression levels of IFN-I or ISGs in tumors correlated with favorable disease outcome in patients affected by several different types of cancer, including breast carcinoma and melanoma.(88, 89)

Mechanistically, IFN-I signaling induces antitumor responses through both tumor cell intrinsic and extrinsic mechanisms. Signaling directly to tumor cells, IFN-Is regulate expression of genes involved in the growth, proliferation, survival, and migration of tumor cells.(87) IFN-Is have long been recognized to possess antiproliferative activity. Indeed, treatment with IFN-I reduced proliferation of breast and prostate cancer cell lines through stalling of cell cycle progression.(90, 91) IFN-Is further reduce survival of tumor cells through their ability to regulate apoptosis. Indeed, treatment of various cancer cell lines with IFN-Is increased apoptosis and reduced survival.(87)

Surveillance and response by the immune system is required to restrict development of malignancies.(92) Through their ability to signal to and regulate virtually all of the cells of the immune system, IFN-Is have been demonstrated to enhance the antitumor immune response. Similar to the context of virus infection, IFN-Is promote the activity of $CD8^+$ T cells and NK cells, whose cytotoxic functions are important for destruction of malignant cells.(87) Indeed, in preclinical mouse cancer models, IFN-I signaling was required for priming and activation of tumor-specific $CD8^+$ T cells, and is therefore thought to contribute to activation of the natural antitumor immune response.(93) Furthermore, IFN-Is promote the antitumor response in the tumor microenvironment by inhibiting the suppressor function of myeloid-derived suppressor

cells as well as the proliferation and function of T_{regs}, both of which accumulate in cancer patients and interfere with the antitumor response.(87, 94)

Highlighting the clinical relevance of IFN-I research, the observed antitumor functions of endogenous IFN-Is have led to their adaptation for therapeutic use. IFNα is currently used in the treatment of a variety of both solid and hematological malignancies, including chronic myeloid leukemia, hairy cell leukemia, and melanoma.(60, 87)

1.2.4. IFNβ – a unique type I interferon family member

The observation that natural selection has favored the evolution of multiple IFN-I species, which have been retained over time, correctly suggests the possibility for distinct functionalities of the different variants. Despite all binding to the same site on IFNAR to form highly similar ligand-receptor complexes, there are numerous reports of functional differences among the variants, especially between IFNβ and the other IFN-I family members.(95) Interestingly, this seems to apply specifically to the homeostatic functions of IFN-Is. While all IFN-Is are capable of inducing antiviral activity in all cell types, only IFNβ induces immunomodulatory and antiproliferative responses at physiological concentrations, making IFNβ the homeostatic IFN-I.(95, 96)

The different IFNα variants share approximately 80% or greater homology at the sequence level, but only 50% homology with IFNβ suggesting a structural basis for the differential functionality.(95) Indeed, biochemical analyses have revealed that IFNβ has a substantially higher combined binding affinity to the two IFNAR receptor subunits compared to all other naturally occurring IFN-I species.(97, 98) This high receptor binding affinity is hypothesized to be the source of the distinct functionalities of IFNβ.

Indeed, mutation of IFNα2, which has the lowest natural affinity for IFNAR, to increase its receptor affinity to levels similar to IFNβ, conferred it with the immunomodulatory and antiproliferative functions of IFNβ.(99)

Not only is IFNβ distinct from the other IFN-Is in its interaction with IFNAR, but also in its expression pattern. Initial evidence came from studies using mouse embryo fibroblasts, which demonstrated that in the absence of priming levels of IFNβ, the cells were not able to produce the other type I interferons, indicating that IFNβ might serve as a regulator of IFN-I induction and the downstream ISG response.(100) Mechanistically, this is due to the expression of the transcription factors that turn on IFN-I gene expression, the IRFs. The expression of all the IFNα variants and of IFNβ is induced by IRF7, while IRF3 preferentially induces IFNβ. Interestingly, only IRF3 is expressed constitutively by most cell types, while IRF7 is expressed constitutively only by plasmacytoid DCs (pDCs). Therefore, in response to initial PRR signaling, most cells, with the exception of pDCs, produce only IFNβ. IRF7 expression is induced by IFN-I signaling, creating a positive feedback loop by which IFNα is produced subsequently in cells that have already been primed by IFNβ.(47) IFNβ is therefore not just the homeostatic IFN-I based on its functionality, but it is also the master regulator of the IFN-I response.

The nucleic acid sensing PRRs can induce both IFNα and IFNβ, but IFNβ is preferentially induced by the bacterial cell wall sensing TLRs 2 and 4.(50, 101) Therefore, in the absence of viral infection, it can be hypothesized that commensal bacteria might preferentially induce IFNβ expression, further underscoring the unique role of IFNβ in homeostasis.

As a pleiotropic cytokine, IFN-I signaling can result in several different signaling outcomes all mediated through the same receptor. Importantly, the many different biological outcomes, whether pro- or anti-inflammatory, could be either beneficial or detrimental to the host depending on the context. The IFN response must therefore be regulated to maximize the benefits while minimizing detrimental effects to the host. Interestingly, the expression pattern of IFNβ is distinct from IFNα, suggesting the possibility that the body uses controlled expression of the distinct type I interferons to favor homeostatic expression only of the regulatory variant, IFNβ. We have hypothesized that the commensal microbiota might preferentially induce expression of IFNβ to regulate IFN-I signaling and in turn to mediate immune system homeostasis.

1.3. Regulation of the type I interferon response by the microbiota

Analysis of microbial regulation of the IFN-I response has been largely limited to diseased states or administration of exogenous, and often synthetic, microbial ligands. A limited number of studies have begun to elucidate how the homeostatic IFN-I response is regulated in healthy individuals, with implications for a role for commensal microbes. In light of the crucial biological functions of IFN-Is, it is important to understand the normal physiological regulation of this cytokine family.

IFN-Is are expressed at very low levels in the absence of infection, and are below the limit of detection for conventional protein-based cytokine analysis. As such, RNA based detection methods to look at transcript level expression of IFN-Is and downstream ISGs are commonly used for *in vivo* IFN-I analysis.(63) While there have not been studies directly addressing the role of commensal microbes in regulation of constitutive

IFNβ, several groups have indirect evidence suggesting the possibility. The first evidence comes from the location of IFNβ expression. When looking at expression of IFNβ by $CD11c^+$ DCs, IFNβ was expressed specifically in the intestinal (LP) (both small and large intestine). Interestingly, expression was restricted to the intestinal LP, and was notably absent not only from the spleen, a systemic immune organ, but also from gut associated lymphoid tissues including the Peyer's patches and mesenteric lymph nodes (mLNs).(102) This tight locational restriction of IFNβ expression by DCs to the intestinal LP, a site in close contact to the majority of the bodies trillions of commensal microbes, strongly implies a role for the microbiota in constitutive IFNβ expression. Further support for this hypothesis came from using mice deficient for various PRR signaling adapters to examine their role in intestinal LP DC IFNβ expression. This work found that the signaling adapter TIR-domain-containing adapter-inducing interferon-β (TRIF), involved in the response to several TLRs, was required for IFNβ expression by intestinal LP DCs, further suggesting the requirement of activation of TLR signaling by a microbial molecule.(62) In the absence of infection, it can thus be predicted that the commensal microbiota provides the stimulus for this PRR signaling and therefore regulates IFNβ expression.

The aforementioned studies focused on the expression of the IFN-I cytokines themselves, and did not look at a direct connection to the microbiota. Looking at the downstream effect of these cytokines, ISG expression, the commensal microbiota has been more directly implicated in the homeostatic IFN-I response. Using broad-spectrum antibiotics treatment to deplete the microbiota of mice, Abt *et al* found that the commensal microbiota were required for low-level expression of ISGs in splenic

macrophages. Furthermore, this tonic ISG expression was physiologically important, as it primed the macrophages to respond robustly to subsequent respiratory virus infection, ultimately providing resistance and improved disease outcome to the host.(103) Interestingly, the antibiotics used in this study were delivered orally, with three of the four known to be poorly absorbed, suggesting that depletion was limited to the intestinal microbiota and thus that gut commensals are capable of functionally influencing the systemic IFN-I response.(104-107)

Another recent study provides evidence for regulation of the IFN-I response by specific commensal microbes, through demonstration of an association between colonization of GF mice with certain human gut commensals and an interferon response gene expression signature by microarray analysis.(108) However, interpretation of these findings is confounded by the multitude of effects these microbes had on the host. GF mice have an underdeveloped immune system, with reduced numbers and altered frequencies of many immune cell types.(21, 22) The bacterial species associated with an increased IFN-I gene signature compared to GF mice also regulated immune cells, including pDCs, which are potent producers of IFN-I.(109) It is therefore unclear whether the microbes were directly inducing the IFN-I response or whether their effects on restoring different immune cell subsets and the overall maturation of the immune system indirectly led to the enhanced IFN-I gene expression signature. Further work must therefore be done to dissect the regulation of the homeostatic IFN-I response by the commensal microbiota.

1.4. Summary

It is increasingly evident that IFN-Is are not only constitutively expressed, but are required for maintenance of homeostasis, and the overall health of the host. As such, it is important to understand how constitutive IFN-I signaling is regulated in healthy individuals. A mechanistic understanding of this regulation, as well as how it might be disrupted leading to disease, has the potential to lead to new therapeutic strategies. Current IFN-I based therapies rely on the administration of exogenous IFN-Is, which are not under regulatory control and can have unwanted side effects.(110, 111) A clinical approach based on manipulation of the microbiota to restore homeostatic regulation of IFN-Is might therefore have the potential to provide the beneficial aspects of IFN-I signaling, while still maintaining a homeostatic balance, thus reducing undesired adverse effects.

We hypothesized that the commensal microbiota is required for IFNβ induction and regulation of the homeostatic IFN-I response and that IFNβ is an important mediator of commensal immunoregulation. Our findings demonstrate that the commensal microbiota is required for intestinal LP DC IFNβ expression and further identify a specific human gut commensal microbe capable of regulating this response. Furthermore, we have identified a specific commensal microbial molecule and the mechanism by which it signals to induce IFNβ regulation. Finally, we demonstrate functional importance of commensal-induced IFNβ in virus infection and in maintenance of tolerance.

Chapter 2

Regulation of the type I interferon response by the commensal microbiota

2.1. Introduction

There is increasing evidence that the IFN-I signaling pathway is active not just during infection, but also under steady-state conditions.(62, 63) IFN-Is are induced by PRR sensing of microbial products.(46) In the absence of pathogen associated molecular products, we hypothesized that the commensal microbiota might supply PRR signaling, and regulate the consititutive IFN-I response.

2.2. The commensal microbiota regulates tissue ISG expression

During infection, IFN-Is are induced at high levels and can be readily detected in the systemic circulation. However, under homeostatic conditions, IFN-I expression is extremely low and the half-life of IFN-Is is short, making them difficult to detect.(63, 112) It is therefore common to use expression of downstream ISGs as an assay for IFN-I activity. To begin to probe the role of the intestinal microbiota in IFN-I regulation, we therefore sought to see how microbiota depletion affects ISG expression in various murine tissues. The relative expression levels of a select panel of ISGs in adult wild type (WT) GF mice devoid of all microbes compared to age- and gender-matched WT specific pathogen free (SPF) mice were analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The vast majority of commensal microbes reside in the GI tract.(13, 14) Analysis was therefore performed to determine effects of the microbiota on ISG expression in the intestinal immune compartment, in the mesenteric lymph nodes (mLN), and systemically, in the spleen. A reduction in ISG expression was observed in both the mLN (Figure 1A-D) and spleen (Figure 2A-D) in GF mice compared to SPF mice, supporting a role for microbiota regulation of the IFN-I response.

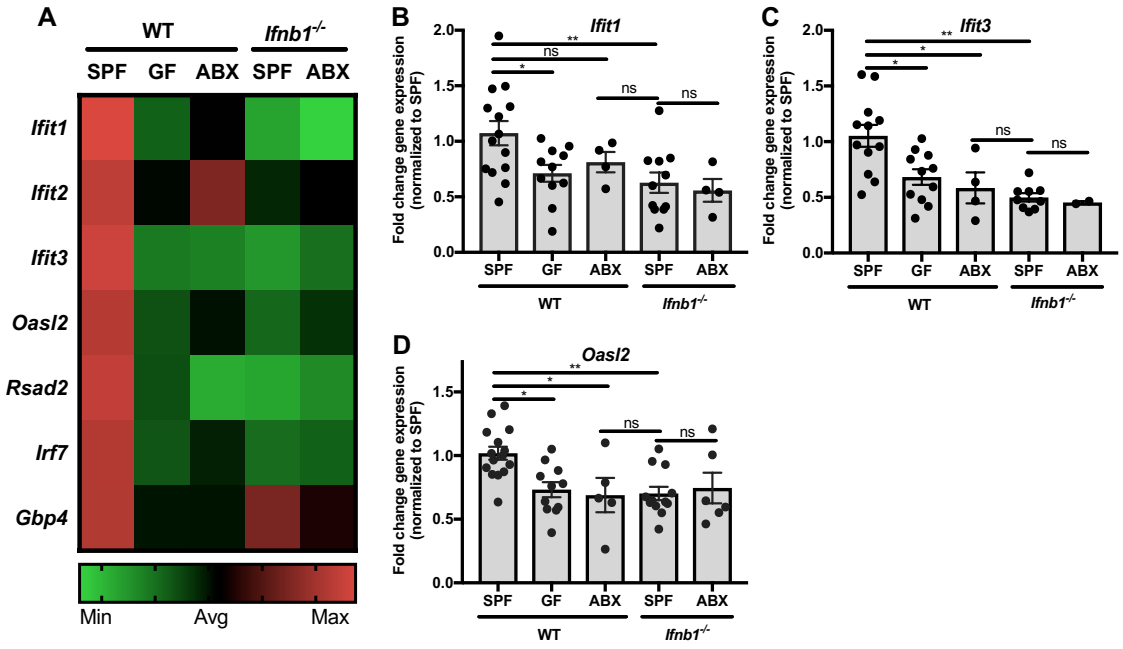

Figure 1 **Microbiota depletion reduces mLN ISG expression.** mLNs were harvested from age- and gender-matched WT GF mice and from WT or *Ifnb1*$^{-/-}$ SPF mice with and without broad-spectrum antibiotics (ABX) treatment. RNA was isolated from whole tissue samples. qRT-PCR was performed to analyze ISG expression levels. Fold change gene expression was calculated compared to WT SPF mice using the delta-delta CT method, with *ActB* as the reference gene and depicted as (A) a heat map of all ISGs tested or representative bar graphs of mean +/- SEM with each point representing one mouse for (B) *Ifit1* (C) *Ifit3*, and (D) *Oasl2*. One-way ANOVA statistical analysis (B) p=0.0066, (C) p=0.0002, and (D) p=0.0021, followed by Tukey's multiple comparisons test. ns=not significant, *p<0.05, **p<0.01.

As aforementioned, GF mice have numerous abnormalities, especially with respect to immune system development.(21, 22) To confirm that the observed phenotype was due to a direct role of commensal microbes rather than an indirect developmental defect, a second method of microbiota depletion was used. Age- and gender-matched WT SPF mice were orally administered either vehicle control or a broad-spectrum antibiotics cocktail consisting of vancomycin, gentamicin, metronidazole, and ampicillin for 7 days. Vancomycin, gentamicin, and ampicillin were given in the drinking water.

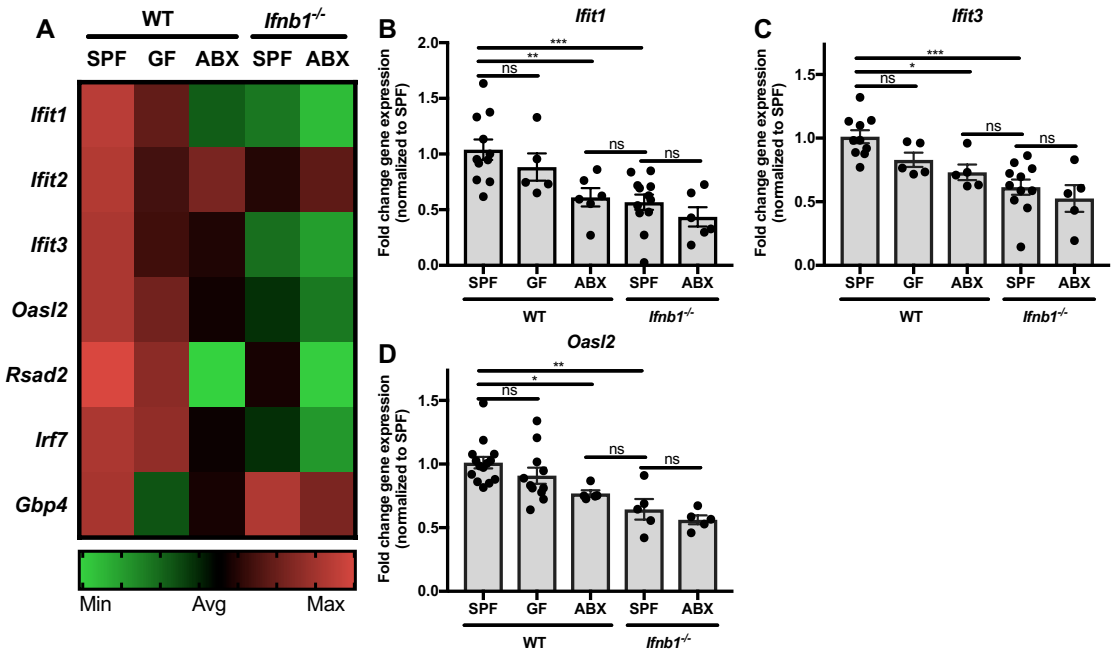

Figure 2 **Microbiota depletion reduces splenic ISG expression.** Spleens were harvested from age- and gender-matched WT GF mice and from WT or *Ifnb1*$^{-/-}$ SPF mice with and without broad-spectrum antibiotics (ABX) treatment followed by RNA isolation from whole tissue samples. qRT-PCR was performed to analyze ISG expression levels. Fold change gene expression was calculated compared to WT SPF using the delta-delta CT method, with *ActB* as the reference gene and represented as (A) a heat map of all genes or representative bar graph of mean +/- SEM with each point representing one mouse for (B) *Ifit1* (C) *Ifit3*, or (D) *Oasl2*. One-way ANOVA statistical analysis (B) p=0.0001, (C) p<0.0001, and (D) p=0.0017, followed by Tukey's multiple comparisons test. ns=not significant, *p<0.05, **p<0.01, ***p<0.001.

The bitter taste of metronidazole causes severe dehydration of mice due to avoidance of drinking metronidazole-containing water. Metronidazole or vehicle control was therefore administered once daily by oral gavage (OG). Amphotericin B was included to prevent fungal outgrowth when bacterial loads are markedly decreased. Fecal samples were plated both aerobically and anaerobically to confirm microbiota depletion. There was a complete absence of culturable organisms in the stool 48 hrs after beginning antibiotics treatment, which continued through day 7 of treatment (data not shown), at which point mice were euthanized and tissues were harvested for RNA isolation and qRT-PCR

analysis. Using this method, depletion of the microbiota can be temporally controlled, allowing for depletion of microbes in adult SPF mice, which are physiologically and immunologically normal, thus controlling for effects caused by absence of microbes at birth and throughout development. It was found that antibiotics treated mice (ABX) had significantly reduced ISG expression in the mLN (Figure 1A-D) and spleen (Figure 2A-D), further supporting a role for the commensal microbiota in induction of IFN-I signaling and ISG expression.

As can be observed in Figures 1 and 2, microbiota depletion by antibiotics suppressed ISG expression in both the spleen and the mLN. With the exception of metronidazole, the administered antibiotics are poorly absorbed by the oral route.(104-107) Furthermore, similar oral antibiotics regimens successfully depleted the intestinal microbiota but had no effect on the microbiota composition of the respiratory tract, a distal commensal-colonized tissue.(113) It is therefore likely that complete microbiota depletion in our experiments was limited to the GI tract. Our findings therefore specifically implicate the intestinal microbiota in ISG regulation and suggest that it is capable of regulating ISG expression not just locally, in the mLN, but also systemically in the spleen.

Interestingly, in the spleen (Figure 2), there was a more significant reduction in ISG expression compared to SPF mice in antibiotics treated mice than in GF mice. This discrepancy could be due to the abnormal physiology of GF mice, which could have indirect effects on ISG expression.

2.3. IFNβ mediates the microbiota-induced constitutive IFN-I response

The observed decrease in ISG expression upon microbiota depletion suggests that commensal microbes induce IFN-I expression, which in turn induces ISG expression. As discussed previously, the mammalian IFN-I family consists of multiple structurally similar cytokines.(45) IFNβ is unique among the IFN-Is with respect to its homeostatic functions and regulation of its expression.(95) We therefore hypothesized that the commensal microbiota might specifically induce expression of IFNβ and that microbiota-induced IFNβ is required for the observed constitutive IFN-I response, as evidenced by ISG expression.

To address the importance of IFNβ in constitutive ISG expression, we compared ISG expression levels by qRT-PCR in either WT or IFNβ deficient (*Ifnb1*$^{-/-}$) SPF mice. A significant reduction in ISG expression was observed in the mLN (Figure 1A-D) and spleen (Figure 2 A-D) of *Ifnb1*$^{-/-}$ mice compared to WT mice, confirming a role for IFNβ in the homeostatic IFN-I response. Interestingly, the magnitude of reduction was comparable to that observed upon microbiota depletion.

To further test whether specifically commensal-induced IFNβ is required for ISG expression, the microbiota of *Ifnb1*$^{-/-}$ mice was depleted with antibiotics administration. Antibiotics treatment had no additional effect on mLN (Figure 1A-D) or splenic (Figure 2A-D) ISG expression in *Ifnb1*$^{-/-}$ mice, supporting the hypothesis that commensal microbes regulate ISG expression, and therefore the IFN-I response, through induction of IFNβ.

2.4. IFNβ is expressed by intestinal dendritic cells

Based on the importance of IFNβ for homeostatic ISG expression, we next investigated the source of commensal-induced IFNβ. Having identified effects not just locally, in the mLN, but also systemically in the spleen, we first sought to confirm whether IFNβ could be identified in the systemic circulation, by measuring serum IFNβ levels using an enzyme-linked immunosorbent assay (ELISA). Consistent with reports that IFN-I expression is extremely low under homeostatic conditions, we were unable to detect IFNβ in the serum of WT SPF or ABX treated mice (Figure 3A).

Analysis of gene expression using qRT-PCR provides a more sensitive detection method compared to ELISA.(114) Using this technique, we therefore proceeded to investigate whether IFNβ could instead be detected at the transcript level in WT SPF mice. To identify which tissues express IFNβ under homeostatic conditions, we analyzed *Ifnb1* expression in the colon lamina propria (LP), mLN, and spleen of WT SPF mice using qRT-PCR. Interestingly, there were no detectable *Ifnb1* transcripts in the colon LP (Figure 3B), mLN (Figure 3C), or spleen (Figure 3D).

Based on the observed decreased ISG expression in *Ifnb1*$^{-/-}$ mice (Figures 1 and 2), it is evident that IFNβ must be expressed in order to exert its functionality. We hypothesized that IFNβ might only be expressed in a subset of cells in each of these tissues, and that enrichment of this cell type might also enhance the levels of *Ifnb1* transcripts and thus enable detection of *Ifnb1* expression.

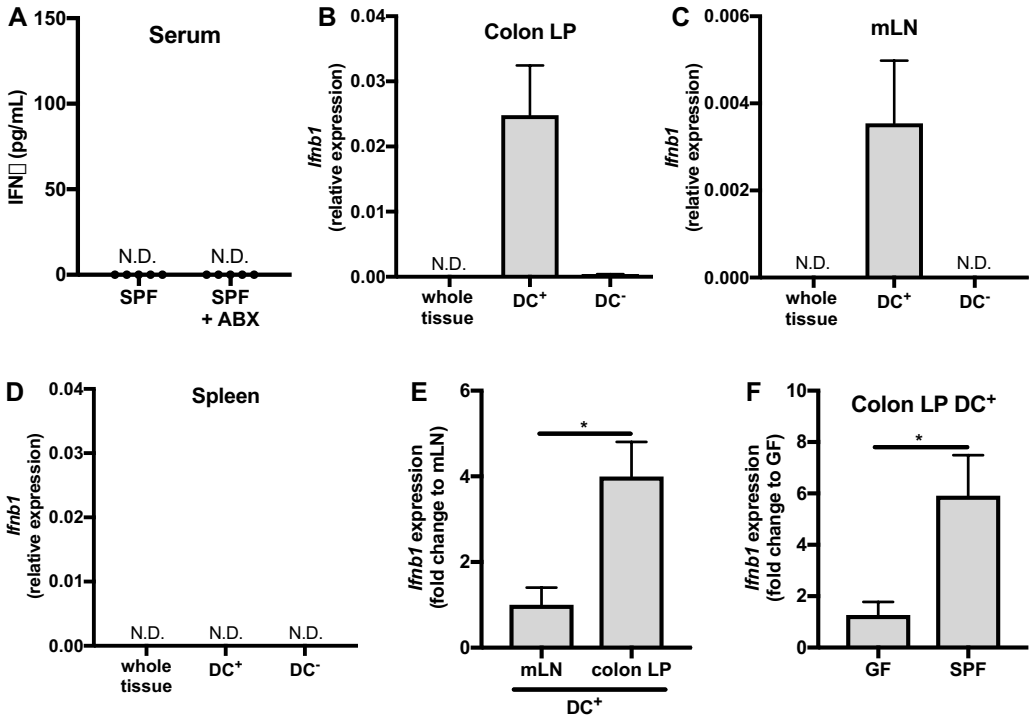

Figure 3 **The commensal microbiota regulates *Ifnb1* expression by dendritic cells in the colon LP and mLN.** (A) ELISA analysis of IFNβ in the serum of WT SPF or antibiotics treated mice (SPF + ABX). (B-F) Single cell suspensions were prepared from spleens, mLNs and colon LP harvested from WT SPF and GF mice. Dendritic cell isolation was performed, yielding dendritic cell positive (DC^+) and dendritic cell negative (DC^-) fractions. *Ifnb1* expression was analyzed by qRT-PCR. Relative expression of *Ifnb1* normalized to *Actb* in the (B) colon LP, (C) mLN, and (D) spleen of WT SPF mice. (E) Fold change *Ifnb1* expression in WT SPF DC^+ fraction of colon LP and mLN, normalized to mLN. (F) *Ifnb1* expression in colon LP DC^+ cells from WT SPF or GF mice, fold change normalized to GF. Fold change calculated using the delta-delta CT method, with *Actb* as a reference gene. Bars represent average +/- SEM. Statistical analysis by unpaired t-test. N.D.=not detected, *$p<0.05$.

DCs are important sentinel cells of the immune system, serving to detect microbes and initiate and shape the immune response.(115) Because IFNβ is induced in response to microbial signals and PRR signaling, we hypothesized that DCs might be the source of IFNβ under homeostatic conditions.(46) DCs were isolated from the colon LP, mLN, and spleens of WT SPF mice using a magnetic bead-based cell separation method to isolate both cDCs and pDCs based on expression of CD11c or pDC antigen-1 (PDCA-1).

Expression of *Ifnb1* was analyzed in both the DC^+ and DC^- fractions by qRT-PCR. There was no detectable *Ifnb1* expression in the DC^- fraction of the colon LP of four out of the five mice tested (Figure 3B). In addition, *Ifnb1* transcript levels were undetectable in the DC- fractions of the mLN (Figure 3C) and spleen (Figure 3D). However, DCs in both the colon LP (Figure 3B) and the mLN (Figure 3C) expressed *Ifnb1*. Interestingly, DC expression of *Ifnb1* is significantly higher in the colon LP than in the mLN (Figure 3E). Intestinal DCs continuously sample the intestinal luminal content, where they are exposed to commensal microbial antigens. DCs that are loaded with commensal antigen are restricted to the mucosal-associated lymphoid tissue, where they traffic to the mLN but do not enter systemic immune circulation.(116) Our finding that *Ifnb1* expression is detected most strongly in colon LP DCs, but also in mLN DCs is consistent with a model by which commensal microbes signal to colon LP DCs to induce IFNβ, and that some of these DCs then traffic to the mLN.

2.5. The commensal microbiota regulates colonic DC IFNβ expression

Our results suggest a role for the commensal microbiota in regulation of the homeostatic IFN-I response, specifically through regulation of IFNβ. To further test the role of the microbiota in intestinal DC *Ifnb1* expression, we analyzed DCs from the colon LP of GF and SPF mice. The colonic LP DCs of GF mice exhibited significantly reduced *Ifnb1* expression compared to SPF mice (Figure 3F), confirming that the commensal microbiota is required for colon LP DC IFNβ expression.

Several studies have reported biological effects of oral administration of low dose IFN-I.(117, 118) We therefore sought to investigate whether intestinal-derived IFNβ,

such as that which might be induced by the intestinal microbiota, could also affect the homeostatic IFN-I response. IFNβ was therefore administered by OG to antibiotics-treated mice to determine whether delivery of IFNβ via the GI tract could restore the deficient IFN-I response. Following daily administration of 100 international units (IU) of IFNβ for 4 days to ABX-treated mice, there was a trend towards increased ISG expression in the spleen (Figure 4A) and mLN (Figure 4B), confirming that IFNβ of intestinal origin can mediate systemic changes, and specifically regulation of ISG expression. Of note, the effect on ISG expression was much less than in SPF mice. This is potentially due to an inadequate dose and/or frequency of oral IFNβ and it would be interesting to further optimize this dosing regimen to determine the effects on ISG expression. Alternatively, ISG expression might depend on multiple microbiota derived signals, and thus IFNβ alone might not be sufficient to completely restore ISG expression.

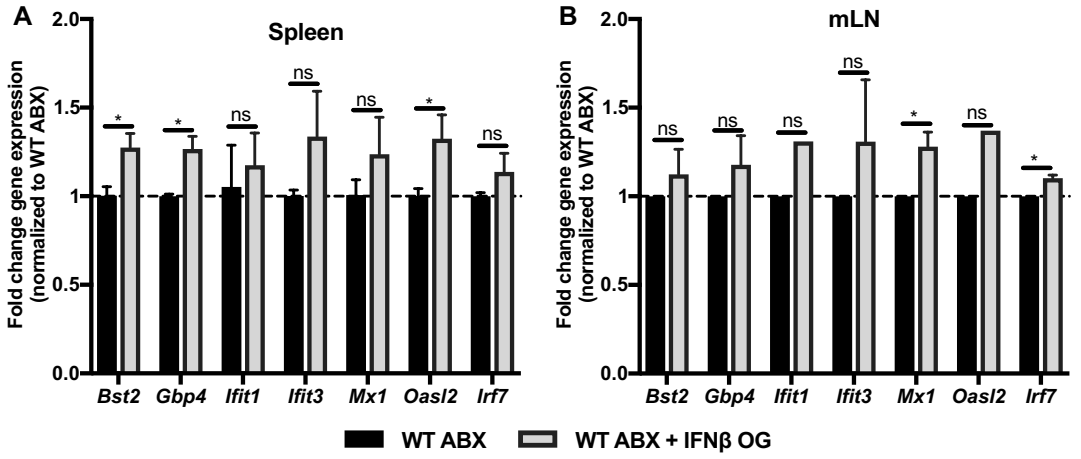

Figure 4 **Oral IFNβ treatment partially restores splenic and mLN interferon stimulated gene expression.** Spleens (A) and mLN (B) were harvested from age- and gender-matched antibiotics treated wild type mice treated by oral gavage with vehicle (WT ABX) or 100 IU IFNβ daily for 4 days (WT ABX + IFNβ OG). RNA was isolated from whole tissue samples. qRT-PCR was performed to analyze ISG expression levels. Fold change gene expression was calculated compared to WT ABX vehicle control using the delta-delta CT method, with *ActB* as the reference gene. Bars represent average +/- SEM. Statistical analysis with unpaired t-test. ns=not significant, *p<0.05.

Based on the observation that low levels of orally administered IFNβ can modulate the systemic IFN-I response, it is therefore plausible that commensal-induced IFNβ expression by intestinal LP DCs can mediate systemic regulation of the homeostatic IFN-I response and its functions.

2.6. Summary

In this chapter, we demonstrated a novel mechanism by which the commensal microbiota influences the immune system, through regulation of IFNβ expression specifically by colonic LP DCs. The commensal-induced IFNβ response was further demonstrated to be required for the homeostatic expression of ISGs both locally, in the mLN and systemically, in the spleen.

Our findings are consistent with previous reports of commensal regulation of the IFN-I response, which identified that ISG expression is reduced in splenic macrophages by antibiotics treatment.(103) However, our work builds on the existing literature by using two methods of microbiota depletion as well as analysis of whole tissue ISG expression levels instead of just one cell type in both the mLN and the spleen. Our work therefore demonstrates a broader effect of the microbiota on the IFN-I response, which affects multiple tissues and cell types. In addition, we provide direct evidence for the involvement of the IFN-Is in the regulation of ISGs by the commensal microbiota by identifying which of the IFN-I family cytokine members is required for regulation of homeostatic ISG expression. Indeed, ISG expression was reduced in *Ifnb1*$^{-/-}$ mice, with no additional reduction upon antibiotics treatment, confirming the role of commensal-induced IFNβ in the homeostatic IFN-I response.

A specific cellular source of commensal-induced IFNβ was also identified. Specifically, we determined that commensal microbes induce IFNβ expression by colonic LP DCs, with lower levels of IFNβ expression also detected in MLN DCs but not in splenic DCs. Intestinal DCs have previously been established to carry commensal antigen to the mLN, where they are restricted from entering into systemic immune circulation, and thus remain in the intestinal immune compartment.(116) Our results suggest that IFNβ is expressed in response to detection of microbial antigens by colonic LP DCs, which then remain in the intestinal LP or traffic to the mLN.

Interestingly, while splenic DCs do not express IFNβ, we observed decreased splenic ISG expression in *Ifnb1*$^{-/-}$ and ABX treated mice. It is possible that IFNβ that is secreted in the mucosal immune compartment might enter the systemic circulation, thus inducing ISG expression. However, there was no detectable level of IFNβ in the serum of WT SPF mice when analyzed by ELISA (Figure 3A). IFNβ is extremely potent, capable of inducing ISG expression with a half-maximal effective concentration (EC_{50}) of 0.2 pM.(95) It is therefore possible that even a minute quantity of IFNβ, below the limits of detection of ELISA, might enter the circulation from the mucosal immune compartment and induce systemic effects on ISG expression. Alternatively, secretion of IFNβ by intestinal DCs might induce downstream gene expression changes locally, in neighboring cells, which can then traffic to distal body sites. Further investigation will be required to determine which of these mechanisms accounts for systemic ISG expression under homeostatic conditions.

Chapter 3

Mechanism of IFNβ induction by the commensal microbiota

3.1. Introduction

While numerous associations between microbes and disease or host physiology have been reported, very few studies have identified specific microbes and the molecular mechanism by which they modulate the host. We therefore sought to identify a commensal organism capable of regulating IFNβ and the constitutive IFN-I response.

3.2. *Bacteroides fragilis* induces expression of IFNβ by colon LP DCs

Of the trillions of commensal bacteria which inhabit the mammalian colon, one of the most prominent phyla represented is the phylum *Bacteroidetes,* consisting of obligate anaerobic, Gram-negative rods.(119) Indeed, approximately 25% of all colonic anaerobes belong to the genus *Bacteroides* within the phylum *Bacteroidetes.*(119, 120) An association was recently reported between several species of the genus *Bacteroides*, pDC numbers, and a IFN-I response gene signature.(108) One of the *Bacteroides sp.* investigated in this study was *B. fragilis,* a common human colonic commensal bacterial species. In addition to the association with pDCs and a IFN-I signature, *B. fragilis* has been demonstrated to have important immunomodulatory properties, capable of restoring the Th cell balance in GF mice, regulating NK T cells, and inducing IL-10 producing T_{regs} in the context of inflammation.(22, 39, 43, 121) Due to its demonstrated ability to interact with and influence the immune system, as well as the association with pDCs and IFN-I, we hypothesized that *B. fragilis* might be capable of inducing IFNβ in colonic DCs and thus regulating the IFN-I response.

GF mice were colonized with *B. fragilis* strain NCTC 9343 at 4 weeks of age, followed by isolation of colon LP DCs and analysis of *Ifnb1* expression by qRT-PCR

after 2 weeks. As a colonization control, GF mice were colonized with a different bacterial species with demonstrated immunomodulatory properties, *Clostridium ramosum* (*C. ramosum*), thus controlling for any non-specific effects due to colonization.(108, 122) We observed that colonization with *B. fragilis* significantly enhanced *Ifnb1* expression in colon LP DCs, while *C. ramosum* colonization had no effect (Figure 5).

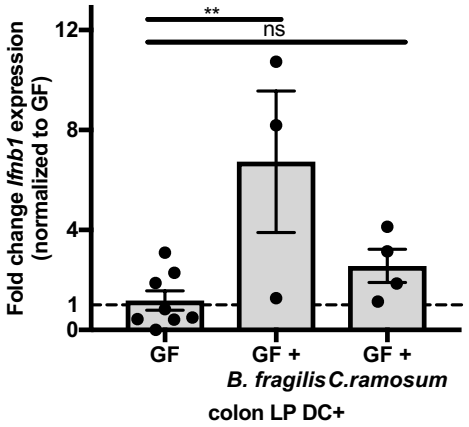

***Figure 5 B. fragilis* colonization induces *Ifnb1* expression by colon LP dendritic cells.** Wild type germ free mice were gavaged with vehicle (GF) or colonized at 4 weeks of age with *B. fragilis* or *C. ramosum.* Two weeks post colonization, dendritic cells were isolated from single cell suspensions of colon LP cells. RNA was isolated and *Ifnb1* expression was analyzed by qRT-PCR. Fold change gene expression compared to GF was calculated using the delta-delta CT method, with *Actb* as the reference gene. Each point represents one mouse, each bar the average +/- SEM. Statistical analysis with one-way ANOVA (p=0.0153) followed by Tukey's multiple comparisons test. **p<0.01, ns=not significant.

3.3. *B. fragilis* capsular polysaccharide A regulates IFNβ expression

Having identified a human commensal microbe capable of regulating IFNβ signaling, we next sought to identify the mechanism responsible for this immunomodulation. The outer membrane (OM) of Gram-negative bacteria, such as *B. fragilis*, serves as the site of interaction between the bacterial cell and its environment, in this case the mammalian intestine.(123) In addition, the OM comprises several classes of

potentially immunomodulatory molecules, including glycolipids, phospholipids and proteins.(123, 124) We therefore hypothesized that an OM component might be responsible for *B. fragilis* induction of IFNβ. To test this hypothesis, an *in vitro* model was developed in which bone marrow-derived dendritic cells (BMDCs) isolated from WT mice were treated with OM complexes isolated from *B. fragilis* as previously described, followed by ELISA analysis of secreted IFNβ in the supernatants.(123) It was found that *B. fragilis* OM complexes significantly induced IFNβ secretion by BMDCs (Figure 6A).

The capsular polysaccharide A (PSA) of *B. fragilis* has been demonstrated to have immunomodulatory properties, capable of inducing IL-10 producing T cells and protecting against inflammatory disorders.(22, 39, 43) In Gram-negative bacteria, capsular polysaccharides can be attached to the OM through covalent linkage to lipid anchors, which are inserted into the OM lipid bilayer.(125) We therefore hypothesized that PSA might be an IFNβ-inducing component of the *B. fragilis* OM. To test this hypothesis, we treated BMDCs with purified PSA for 6 hours (hrs) and secretion of IFNβ in the supernatants was analyzed by ELISA. Indeed, PSA significantly induced IFNβ secretion by BMDCs (Figure 6B). Interestingly, secretion of the other predominant IFN-I species, IFNα, was not induced by PSA (Figure 6B), suggesting that PSA preferentially induces IFNβ.

To further confirm the ability of PSA to induce a productive IFN-I response through IFNβ, expression of downstream ISGs was analyzed by qRT-PCR in BMDCS treated for 24 hrs with PSA or vehicle control. An increase in expression of ISGs was observed in WT BMDCs treated with PSA (Figure 6C). Furthermore, expression levels

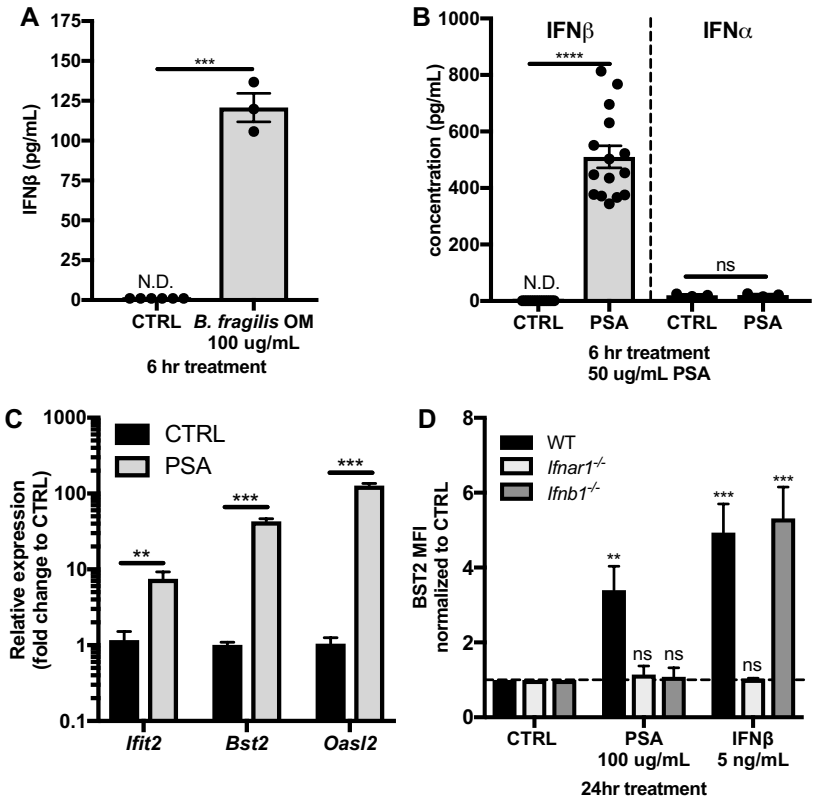

***Figure 6 B. fragilis* polysaccharide A induces IFNβ in vitro in bone marrow dendritic cells.** BMDCs from WT, *Ifnb1*$^{-/-}$, or *Ifnar1*$^{-/-}$ mice were differentiated in the presence of GM-CSF. IFNβ or IFNα levels in the cell free supernatants of WT BMDCs treated with (A) 100 ug/mL *B. fragilis* OM extract or (B) 50 ug/mL PSA for 6 hrs were analyzed by ELISA. (C) RNA was isolated from WT BMDCs 24 hrs post treatment with 50 ug/mL PSA and ISG expression was analyzed by qRT-PCR. Fold change gene expression was calculated compared to vehicle control samples using the delta-delta CT method with *Actb* as the reference gene. (D) After 24 hrs of treatment with 100 ug/mL PSA or 5 ng/mL IFNβ, surface expression of BST2 gated on live CD11c^{+} cells was measured by flow cytometry and reported as fold change MFI compared to CTRL. Data represents average +/- SEM. Statistical analysis with (A-C) unpaired t-test and (D) Two-way ANOVA followed by Dunnett's multiple comparisons test comparing each group to the CTRL group for that genotype. N.D.= not detected, ns=not significant, **p<0.01, ***p<0.001, ****p<0.0001.

of the surface-expressed ISG, bone marrow stromal cell antigen 2 (BST2), were determined by flow cytometric analysis of mean fluorescence intensity (MFI) and found to be increased in PSA treated compared to vehicle control WT BMDCs (Figure 6D). PSA failed to enhance surface BST2 levels in IFNAR1 deficient (*Ifnar1*$^{-/-}$) or *Ifnb1*$^{-/-}$

BMDCs, while treatment of WT BMDCs with exogenous IFNβ alone was sufficient to increase surface levels of BST2 (Figure 6D), confirming that IFNβ is both necessary and sufficient for the observed increase in levels of a representative ISG by PSA. Together, these data demonstrated that PSA induces secretion of IFNβ and downstream ISG expression *in vitro* by BMDCs.

Having established that PSA is capable of inducing IFNβ *in vitro*, we next sought to investigate the *in vivo* IFNβ-inducing capabilities of PSA, through analysis of *Ifnb1* expression by colon LP DCs. WT SPF mice were administered 150 ug of PSA by OG and euthanized after 1.5 hrs for harvest of colons, preparation of colon LP single cell suspensions, and DC isolation. RNA was isolated from colon LP DCs and *Ifnb1* expression was analyzed by qRT-PCR. A significant increase in *Ifnb1* expression was observed 1.5 hrs after treatment (Figure 7), suggesting that PSA is capable of signaling to colon LP DCs *in vivo* to induce *Ifnb1* expression.

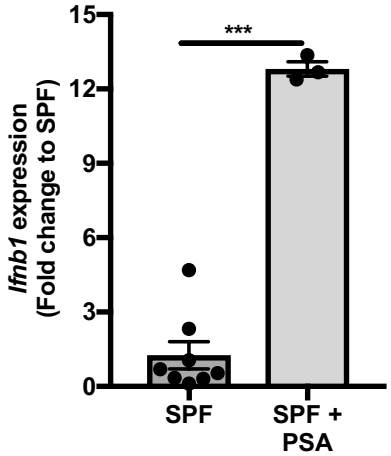

Figure 7 **PSA induces *Ifnb1* expression *in vivo* by colon LP dendritic cells.** Wild type SPF mice were gavaged with vehicle (SPF) or 150 ug of PSA then rested for 1.5 hrs. Colons were harvested for isolation of dendritic cells from single cell suspensions of colon LP cells. RNA was isolated and *Ifnb1* expression was analyzed by qRT-PCR. Fold change *Ifnb1* expression compared to SPF mice was calculated using the delta-delta CT method, with *Actb* as the reference gene. Each point represents one mouse, each bar the average +/- SEM. Statistical analysis with unpaired t-test. ***$p<0.001$.

3.4. *B. fragilis* lipooligosaccharide signals through TLR4 to induce IFNβ

Having identified an IFNβ-inducing commensal microbial molecule, the mechanism of signaling was investigated next. PSA has been previously demonstrated to signal through the PRRs TLR2 and dectin-1 (unpublished data) to induce secretion of IL-10 by T cells.(43, 126) Interestingly, signaling through both the TLR2 and dectin-1 pathways has been reported previously to induce IFNβ in a context-dependent manner.(50, 127) The role of TLR2 and dectin-1 in PSA induction of IFNβ was therefore investigated by comparing the response of WT, dectin-1 (*Clec7a*$^{-/-}$) and TLR2 (*Tlr2*$^{-/-}$) deficient BMDCs to PSA using ELISA to detect IFNβ in the cell free supernatants. Much to our surprise, loss of dectin-1 or TLR2 had no effect on IFNβ secretion (Figure 8A), suggesting that PSA signals through a previously unidentified pathway to regulate IFNβ expression.

IFN-I expression is generally attributed to nucleic acid, virus-sensing PRRs.(48, 50) The bacteria sensing TLR2, dectin-1, and TLR4 pathways represent some of the few exceptions.(50) TLR4 senses microbial glycolipids, with the Gram-negative bacterial OM component lipopolysaccharide (LPS) serving as the canonical TLR4 ligand.(128) Importantly, IFNβ is the predominant IFN-I induced downstream of TLR4, consistent with the signaling observed by PSA (Figure 6B).(129) We therefore investigated whether PSA signals through TLR4 to induce IFNβ secretion. TLR4 deficient (*Tlr4*$^{-/-}$) BMDCs completely lost the ability to secrete IFNβ in response to PSA treatment (Figure 8A), confirming that PSA signals through TLR4 to induce IFNβ secretion.

TLR4 signaling proceeds through two different signaling adapter molecules, myeloid differentiation primary response protein (MyD88) and TRIF, to mediate

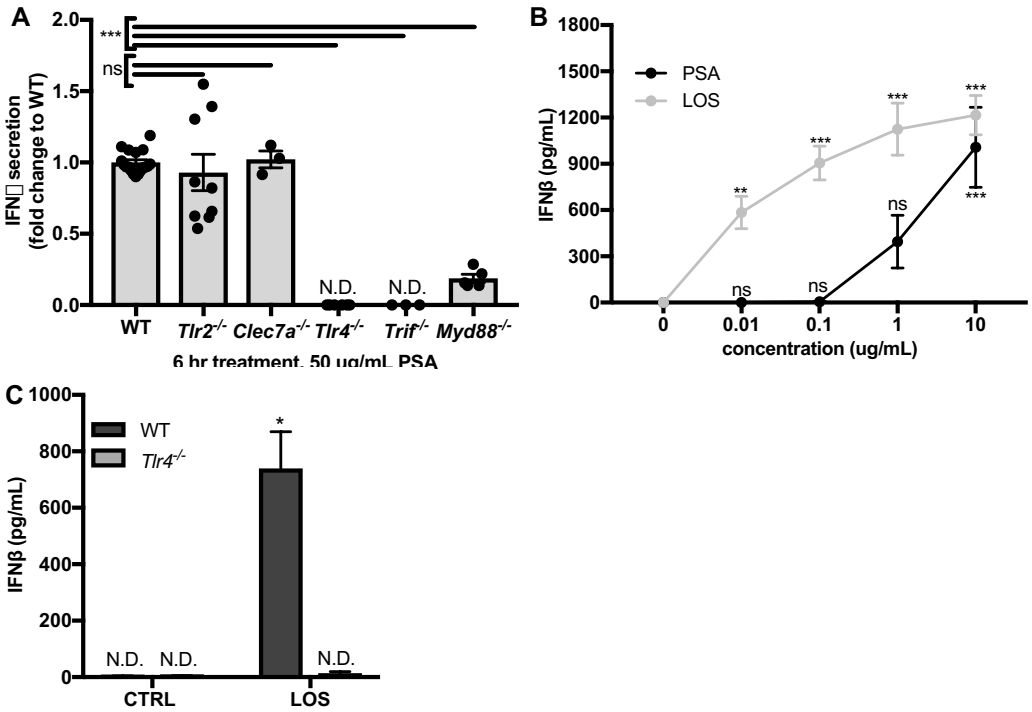

***Figure 8 B. fragilis* glycolipids signal through TLR4 on BMDCs to induce IFNβ.** BMDCs from WT, *Tlr2*$^{-/-}$, *Clec7a*$^{-/-}$, *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, or *Myd88*$^{-/-}$ mice were differentiated in the presence of GM-CSF. WT or knockout BMDCs were treated with (A) 50 ug/mL *B. fragilis* PSA, (B) a dose response of PSA or LOS, or (C) 1 ug/mL LOS. After 6 hrs, cell free supernatants were harvested and IFNβ was analyzed by ELISA. Data represents average +/- SEM. Statistical analysis with (A) one-way ANOVA ($p<0.0001$) followed by Dunnett's multiple comparisons test, (B) two-way ANOVA followed by Dunnett's multiple comparisons test comparing each group to 0 ug/mL control sample for that genotype, or (C) unpaired t-test. N.D.=not detected, ns=not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

downstream gene expression changes. These two branches activate divergent pathways, with MyD88 canonically activating the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) to induce proinflammatory gene expression while TRIF is reported to activate IRF3 to mediate IFNβ expression and the IFN-I response.(130) Consistent with these well-described signaling events, PSA induction of IFNβ was entirely dependent on TRIF, as evidenced by the lack of increased IFNβ secretion in TRIF deficient (*Trif*$^{-/-}$) BMDCs in response to PSA (Figure 8A). A significant decrease in IFNβ secretion was also observed in PSA treated MyD88 deficient

(*Myd88*$^{-/-}$) BMDCs (Figure 8A), potentially due to regulatory effects that MyD88 expression might have on BMDC function rather than direct induction of IFNβ by MyD88.

The canonical TLR4 ligand is LPS, the primary component of the OMs of gram-negative bacteria.(130) We recently identified that PSA contains a glycolipid portion hypothesized to anchor the capsular polysaccharide to the OM, which will hereafter be referred to as the *B. fragilis* glycolipid anchor (GLA). On the basis of molecular mass, it is estimated that GLA represents approximately 1% of the PSA structure. Interestingly, GLA resembles an LPS species, with the repeating tetrasaccharide unit of PSA extending from a diglucosamine backbone linked to three, four, or five acyl chains. In contrast to the archetypal Gram-negative bacterial species *Escherichia coli* (*E. coli*), the OM of *B. fragilis* lacks LPS but instead consists of a lipooligosaccharide (LOS), comprising a lipid A domain linked to a short oligosaccharide.(131) Recent work in our laboratory has identified that the GLA portion of PSA structurally resembles *B. fragilis* LOS. In fact, it is now believed that PSA is covalently linked to LOS to anchor it to the OM. We therefore hypothesized that the LOS portion of PSA might be responsible for induction of IFNβ and that purified *B. fragilis* LOS might be capable of inducing the TLR4-TRIF-IFNβ signaling pathway. LOS was isolated from an acapsular mutant strain of *B. fragilis*, which lacks all capsular polysaccharides including PSA, but has an intact LOS-containing OM. WT and *Tlr4*$^{-/-}$ BMDCs were treated with LOS and it was discovered that LOS alone induces IFNβ in a dose-dependent manner through TLR4 (Figures 8B and 8C).

3.5. *B. fragilis* LOS is less inflammatory than *E. coli* LPS

In addition to being an essential structural component of the Gram-negative bacterial OM, LPS is an important mediator of host-microbe interactions. Indeed, the lipid A portion of LPS is the primary Gram-negative bacterial molecule that is recognized by the immune system.(132) Activation of TLR4 by lipid A signals not only through TRIF to induce IFN-I signaling, but also through MyD88 to induce proinflammatory mediators including tumor necrosis factor α (TNFα), inducible nitric oxide synthase (iNOS), and IL-12.(130) Through activation of a robust and uncontrolled immune response, LPS exposure can cause severe systemic inflammation, leading to sepsis and in some cases potentially lethal septic shock and multi-organ failure.(132, 133) As a result of its endotoxicity and the associated adverse effects, the clinical applications of LPS as an immunomodulator have been limited. However, most research on LPS has focused on *E. coli*.(130)

Using various assays including the *Limulus* amoebocyte lysate (LAL) assay, rabbit pyrogenicity test, chick embryo lethality, and the Swartzman reaction, *B. fragilis* LOS has previously been demonstrated to have low endotoxic activity, suggesting the potential to be a less inflammatory LPS species.(134, 135) This difference in activity can be attributed to several structural differences between the two molecules. Whereas *E. coli* LPS is hexa-acylated, *B. fragilis* LOS represents a mixture of penta-, tetra-, and tri-acylated molecules.(136) In addition, the acyl-chains of *B. fragilis* LOS are longer, with C15-C17 fatty acid chains compared to C12-C14 fatty acids used by *E. coli* LPS.(136, 137) Finally, the diglucosamine backbone of *B. fragilis* LOS is mono- or non-phosphorylated compared to the bis-phosphorylated lipid A of *E. coli*.(132, 136)

Numerous studies have revealed that the number and length of the acyl-chains as well as the phosphorylation state of the disaccharide backbone in LPS are critical factors affecting the interaction with TLR4 and the downstream function of these molecules.(130)

Based on its previously demonstrated low endotoxicity and structural differences, we therefore hypothesized that *B. fragilis* LOS signals differently through TLR4 than *E. coli* LPS to induce lower levels of proinflammatory mediators while still inducing the immunomodulatory cytokine IFNβ. Induction of the cytokine TNFα, which is thought to be a major contributor to the adverse effects during septic shock, was used as a measure of proinflammatory activity.(138) WT BMDCs were treated with a dose response of *B. fragilis* LOS or *E. coli* LPS and both TNFα and IFNβ secretion were measured by ELISA. While *B. fragilis* LOS did induce TNFα, it was less potent than *E. coli* LPS, requiring a greater concentration in order to observe detectable levels (Figures 9A and 9B). Indeed, the EC_{50} of TNFα induction by *E. coli* LPS was calculated to be 0.3 ng/mL, 232 times more potent than the 69.52 ng/mL EC_{50} of *B. fragilis* LOS. Interestingly, there was a significant difference in the dose response curve of *E. coli* LPS for induction of TNFα compared to IFNβ (Figure 9A). Indeed, TNFα was induced at lower concentrations of *E. coli* LPS than IFNβ. In contrast, the dose response curves for induction of TNFα and IFNβ by *B. fragilis* LOS were statistically indistinguishable (Figure 9B). These findings suggested that *E. coli* LPS preferentially induces the proinflammatory, MyD88-dependent pathway, while *B. fragilis* LOS induces both the MyD88- and TRIF-dependent pathways equally.

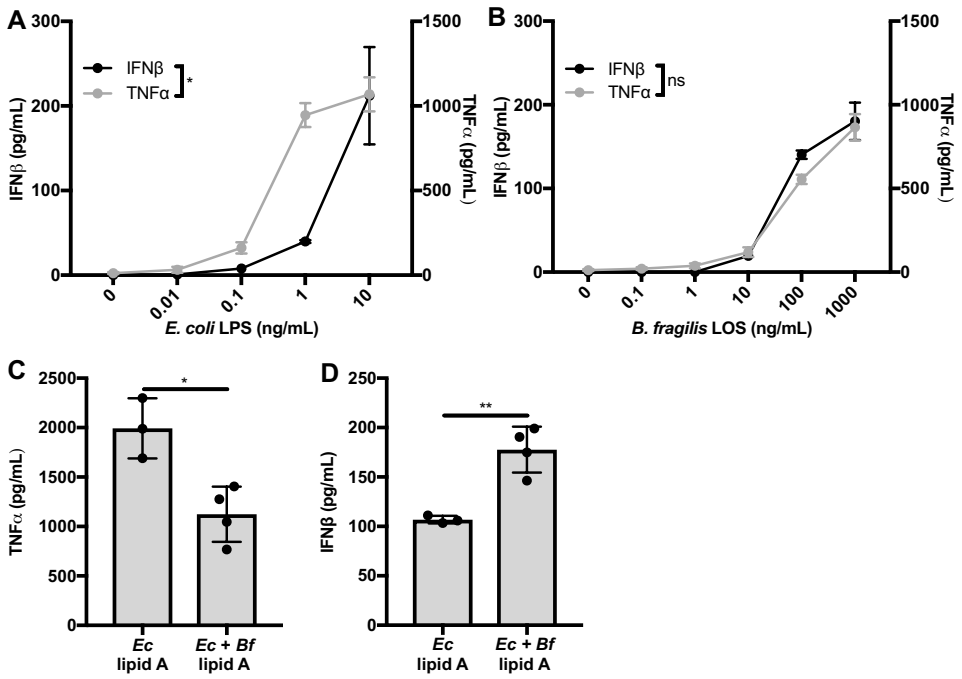

***Figure 9 B. fragilis* LOS is less inflammatory than *E. coli* LPS.** BMDCs from WT mice were differentiated in the presence of GM-CSF. ELISA analysis of TNFα and IFNβ levels in the cell free supernatants of WT BMDCs treated with a dose response of (A) *E. coli* LPS or (B) *B. fragilis* LOS for 6 hr. ELISA analysis of (C) TNFα or (D) IFNβ in the supernatants of WT BMDCs treated with 50 ng/mL *E. coli* (*Ec*) lipid A alone or in combination with 250 ng/mL *B. fragilis* (*Bf*) lipid A for 6 hr. Data represents average +/- SEM. Statistical analysis with (A,B) nonlinear regression analysis or (C,D) unpaired t-test. ns=not significant, *p<0.05, **p<0.01

Because both of these molecules bind to and signal through the same receptor, we hypothesized that *B. fragilis* LOS might not only induce less proinflammatory mediators itself, but that it might also reduce induction of these molecules by other more inflammatory microbes, through competitive inhibition. To test this, lipid A was isolated from *B. fragilis* LOS by acid hydrolysis and added to WT BMDC cultures in combination with synthetic *E. coli* hexa-acylated lipid A. We discovered that addition of *B. fragilis* lipid A significantly reduced TNFα secretion by BMDCs in response to *E. coli* lipid A (Figure 9C). Interestingly, the reverse effect was observed when looking at IFNβ secretion, in which addition of *B. fragilis* lipid A had an additive effect, significantly

increasing IFNβ secretion compared to *E. coli* lipid A alone (Figure 9D). These results suggested that the LOS of the human gut commensal *B. fragilis* is able to preferentially induce the immunoregulatory IFNβ signaling pathway while reducing induction of the proinflammatory MyD88-dependent pathway by other microbes.

3.6. Regulation of IFNβ by *Bacteroides sp.*

Several recent publications have revealed that the LPS molecules of other species of *Bacteroides* consist of glycolipids that are structurally similar to *B. fragilis* LOS.(139, 140) Importantly, the genus *Bacteroides* makes up a large proportion of the human GI microbiome and is widespread in prevalence across the human population.(141) Indeed, the *Bacteroides* LOS/LPS might be one of the most abundant microbial molecules in the GI tract. We therefore hypothesized that induction of IFNβ by *Bacteroides* LOS/LPS might represent a broader mechanism by which commensal microbes are capable of influencing human health.

To test this hypothesis, we isolated OM complexes from several different species of *Bacteroides* to test their *in vitro* activity in stimulating IFNβ secretion from BMDCs. It was observed that OMs of all of the *Bacteroides sp.* tested were able to induce IFNβ by BMDCs in a TLR4-dependent manner (Figure 10), suggesting that the LPS species of each of these *Bacteroides* is indeed able to signal through TLR4 to induce IFNβ. OMs comprise numerous bacterial molecules, including glycolipids, phospholipids and proteins, all of which might interact with immune cells and influence their response.(123, 124) Isolation of LPS from each of these bacterial species will therefore be required to further assess their IFNβ-inducing capabilities.

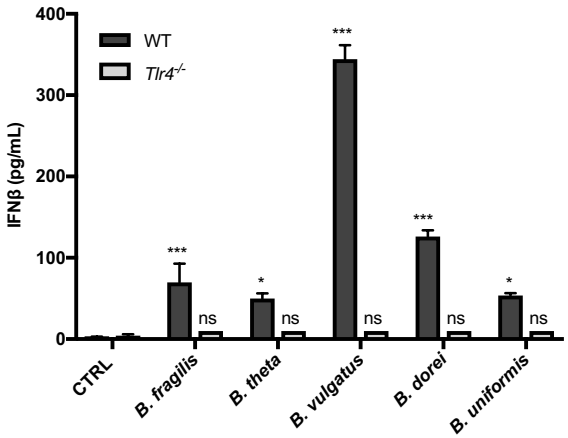

Figure 10 **Outer membrane extracts of *Bacteroides sp.* signal through TLR4 to induce IFNβ secretion.** BMDCs from WT or *Tlr4*$^{-/-}$ mice were differentiated in the presence of GM-CSF followed by treatment with 100 ug/mL of OM extracts from the indicated *Bacteroides sp.* After 6 hrs, cell free supernatants were harvested and IFNβ was analyzed by ELISA. Data represents average +/- SEM. Two-way ANOVA statistical analysis followed by Dunnett's multiple comparisons test comparing each group to the CTRL group for each genotype. ns= not significant, *p<0.05, ***p<0.001.

3.7. Summary

In summary, we have demonstrated a mechanism by which a specific human commensal bacterial molecule modulates the immune response. Indeed, we found that OM glycolipids of *B. fragilis,* both the capsular PSA and LOS, induce IFNβ signaling through the TLR4-TRIF pathway. Importantly, this mechanism of immunoregulation seems to be shared by an entire class of microbial molecules, *Bacteroides* LOS/LPS. IFN-I signaling plays a role in many functions essential to host physiology. Because the *Bacteroides sp.* are important components of the human GI microbiota, both in terms of prevalence and abundance, induction of IFNβ by this taxa of bacteria might represent a ubiquitous and crucial mechanism by which the immune system and other physiological processes are regulated to maintain human health.(141) Compared to *E. coli*, the LOS of *B. fragilis* is less endotoxic and also a weaker inducer of proinflammatory cytokines, a feature likely shared with the other *Bacteroides sp.* due to the structural similarities

between their LPS molecules. As a result, the LPS species of these organisms are likely to have less adverse effects at their effective concentrations than *E. coli* LPS, and thus might be able to be used clinically for their ability to induce IFNβ and modulate the immune response.

3.8. Acknowledgements

We would like to acknowledge the work of Barbara Reinap for purification of PSA, Jason Daugherty for purification and chemical analysis of LOS, Dr. Sungwhan Oh for purification and chemical analysis of *B. fragilis* purified lipid A, and Kareem Mosaheb for help preparing *Bacteroides* OM extracts.

Chapter 4

Immunoregulatory role of commensal-induced type I interferon

4.1. Introduction

Having discovered a commensal bacterial species and the mechanism by which it regulates the IFN-I response, we next sought to identify the physiological importance of commensal-induced IFN-I signaling, using *B. fragilis* as the model IFNβ-inducing microbe.

As introduced above, IFN-Is are pleiotropic cytokines, modulating many different vital host physiological processes.(52, 63, 76) To maintain the health of an organism, the immune system must be kept in a homeostatic balance, able to respond efficiently to eliminate pathogens or tumor cells while also maintaining tolerance to self and non-pathogenic environmental antigens, including commensal microbes.(142) To maintain tolerance, cells of the innate and the adaptive immune system actively calibrate the immune response in a dynamic process that depends on the constant integration of signals such as cytokines.(143) IFN-Is are one such family of immunoregulatory cytokines. Work from several groups has described the ability of IFN-Is to regulate secretion of the immunoregulatory cytokine IL-10 and $FOXP3^+$ T_{regs} both *in vitro* and *in vivo*, thus supporting a tolerogenic environment.(62, 67, 82-84) Furthermore, the anti-inflammatory functions of IFN-Is have been demonstrated to be physiologically relevant, providing protection in several preclinical murine inflammatory disease models, including both chemically-induced and T cell transfer colitis, EAE, and RA.(64-68) Importantly, IFNβ is also used therapeutically in the treatment of relapsing-remitting MS, confirming that IFNβ might also be important in regulation of the human immune response and prevention of inflammatory and autoimmune disorders.(74)

In addition to IFN-I signaling, the commensal microbiota has also been described to be vital for maintaining immune system homeostasis and thus limiting excessive inflammatory responses.(144) T_{regs} have well-described immunosuppressive properties and play an important role in immunoregulation and maintenance of tolerance. The microbiota is required for the proper development, maintenance, and function of T_{regs} in the colon, as well as regulation of numbers of T_{regs} in the gut associated lymphoid tissues.(144-146) The tolerogenic role of the microbiota is further demonstrated by observations that GF mice fail to establish tolerance to orally ingested antigens and by the connection between disruption of the microbiota and inflammatory disorders.(144, 147, 148) Interestingly, *B. fragilis* PSA is one of the few specific microbial molecules that have been demonstrated to possess immunoregulatory functions, capable of inducing IL-10 producing T_{regs} and reducing severity of several inflammatory murine disease models.(39, 43, 126)

Based on the observation that the commensal microbiota, and specifically *B. fragilis* PSA, regulates IFN-I signaling, we hypothesized that signaling through IFNβ might represent one mechanism by which the commensal microbiota mediates immunoregulation and contributes to a tolerogenic environment.

4.2. IFNβ signals through dendritic cell IFNAR1 to induce IL-10 secretion by $CD4^+$ T cells

Previous reports have demonstrated the ability of different IFN-I family members to regulate IL-10 secretion by T cells, each using a different experimental system.(76, 82, 83, 149) We first sought to confirm the immunoregulatory potential of IFNβ using a

mouse splenic DC-T cell co-culture system. Briefly, $CD4^+$ T cells were isolated by negative selection and DCs were isolated by positive selection based on CD11c expression from the spleens of WT mice using magnetic bead-based cell separation kits. Isolated splenic $CD4^+$ T cells and DCs were cultured together in the presence of anti-CD3 and increasing concentrations of recombinant mouse IFNβ. After 5 days, IL-10 levels in the supernatants were analyzed by ELISA. It was observed that IFNβ induces IL-10 secretion in the co-culture system of WT DCs and WT T cells in a dose-dependent manner (Figure 11A), confirming that IFNβ induces IL-10 and that the splenic DC-T cell co-culture system can be used to analyze the immunoregulatory function of IFNβ.

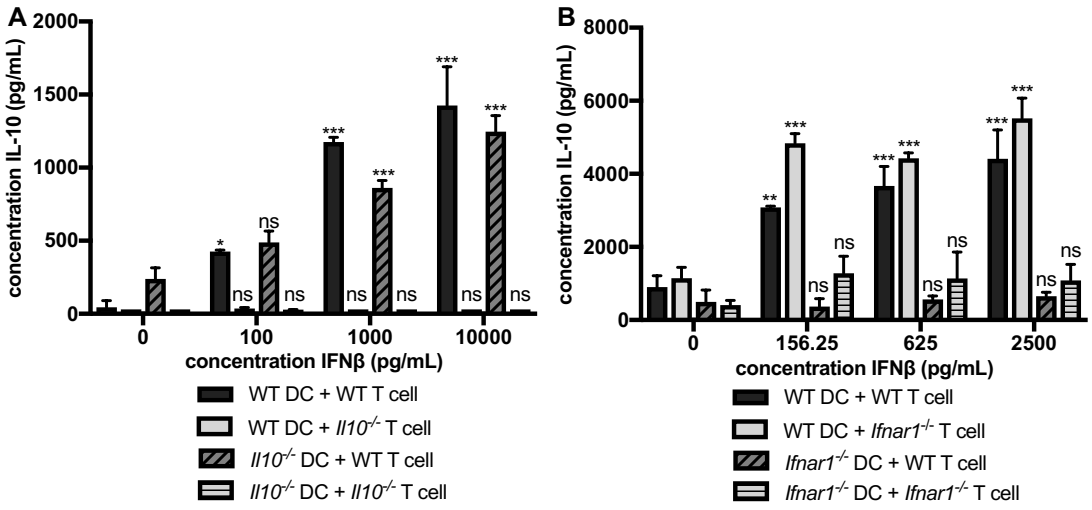

Figure 11 **Signaling of IFNβ through IFNAR1 on DCs induces secretion of IL-10 by splenic T cells *in vitro.*** DCs and $CD4^+$ T cells were isolated from (A) WT and *Il10*$^{-/-}$ or (B) WT and *Ifnar1*$^{-/-}$ spleens and cultured with IFNβ and 1 ug/mL anti-CD3. After 5 days, supernatants were harvested and IL-10 levels were determined by ELISA. Data represents average +/- SEM. Two-way ANOVA statistical analysis followed by Dunnett's multiple comparisons test comparing each concentration of IFNβ to the 0 pg/mL vehicle control for each genotype. ns=not significant. *p<0.05, **p<0.01, ***p<0.001.

To determine the cellular source of the secreted IL-10 in this system, DCs and $CD4^+$ T cells were isolated from the spleens of IL-10 deficient (*Il10*$^{-/-}$) mice and cultured

either together or in combination with WT DCs or WT $CD4^+$ T cells. When *Il10*$^{-/-}$ T cells were included in the culture, either in combination with WT or *Il10*$^{-/-}$ DCs, IFNβ failed to induce IL-10 secretion (Figure 11A). In contrast, loss of IL-10 in DCs had no effect on IFNβ-induced IL-10 (Figure 11A), confirming that IFNβ enhances secretion of IL-10 by $CD4^+$ T cells in the DC-T cell co-culture system.

While several earlier reports have corroborated the ability of different IFN-Is to enhance IL-10 secretion by T cells, the mechanism of this induction is a subject of debate. Specifically, depending on the experimental system used, there is a discrepancy over which cell IFN-Is signal to in order to induce their anti-inflammatory effects- either directly to the T cell or indirectly to APCs.(76) To determine whether IFNβ signals to the DC or the $CD4^+$ T cell in this system, both cell types were isolated from spleens lacking the receptor for IFNβ, IFNAR1, and cultured either together or with WT DCs or $CD4^+$ T cells. Loss of IFNAR1 on $CD4^+$ T cells had no effect, while cultures with *Ifnar1*$^{-/-}$ DCs were unable to secrete IL-10 in response to IFNβ (Figure 11B), thus revealing that IFNβ signals through IFNAR1 on DCs to induce T cell IL-10 secretion. Taken together, these results suggested that it is the APC, in this case the splenic DC, which receives the regulatory signal from IFNβ and communicates with the $CD4^+$ T cell to induce IL-10 secretion.

4.3. IFNβ signaling is required for induction of T cell IL-10 by *B. fragilis* PSA

As mentioned above, *B. fragilis* PSA has well-described immunoregulatory properties. Indeed, PSA induces IL-10-secreting T_{regs}, limiting pathologic inflammation locally in the gut and distally, in the central nervous system.(25, 39, 126) Having

established that IFNβ signals to DCs to induce T cell IL-10, we next sought to investigate the role of DC IFNβ signaling in induction of T cell IL-10 secretion by PSA. Splenic DCs were isolated from WT, *Ifnar1*$^{-/-}$, or *Ifnb1*$^{-/-}$ mice and cultured with WT CD4^{+} T cells and either PSA or PBS as a vehicle control for 5 days followed by IL-10 ELISA analysis. Secretion of IL-10 in response to PSA treatment was significantly reduced when T cells were co-cultured with either *Ifnar1*$^{-/-}$ or *Ifnb1*$^{-/-}$ DCs (Figure 12A), confirming that secretion of IFNβ by DCs and autocrine signaling through IFNAR1 on DCs is required for optimal induction of T cell IL-10 by PSA. These findings are consistent with the observed tolerogenic function of treatment with IFNβ alone, in which IFNβ acts directly on the APC in the co-culture system to indirectly influence the T cell phenotype.

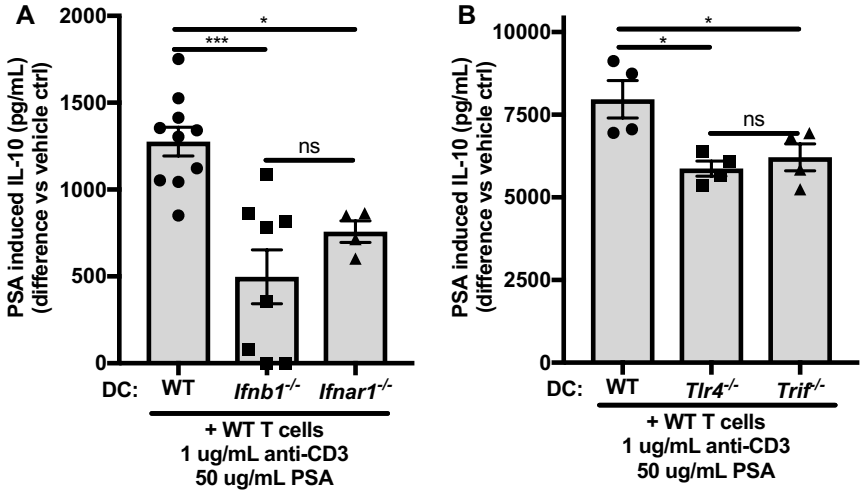

***Figure 12 B. fragilis* PSA signals through the TLR4-TRIF-IFNβ-IFNAR signaling axis on DCs to induce IL-10 secretion by CD4^{+} T cells.** CD4^{+} T cells were isolated from WT spleens and cultured with DCs isolated from (A) WT, *Ifnb1*$^{-/-}$, and *Ifnar1*$^{-/-}$ or (B) WT, *Tlr4*$^{-/-}$, and *Trif*$^{-/-}$ spleens in the presence of 50 ug/mL *B. fragilis* PSA or vehicle control and 1 ug/mL anti-CD3. After 5 days, supernatants were harvested and IL-10 levels were determined by ELISA. Data represents average +/- SEM. One-way ANOVA statistical analysis (A) p=0.0003 and (B) p=0.0002, followed by Tukey's multiple comparisons test. ns=not significant, *p<0.05, ***p<0.001.

Our work has demonstrated that PSA induces IFNβ through the TLR4-TRIF signaling pathway (Figure 8A). We therefore hypothesized that expression of TLR4 and TRIF by DCs would also be required for PSA induction of IL-10 by T cells. Using the splenic DC-T cell co-culture system, we found that PSA-induced secretion of IL-10 was significantly reduced in cultures of WT $CD4^+$ T cells with either *Tlr4*$^{-/-}$ or *Trif*$^{-/-}$ DCs (Figure 12B). Together, these results demonstrated that PSA interacts with DCs by signaling through the TLR4-TRIF pathway to induce secretion of IFNβ, which in turn activates IFNAR1 on DCs to induce IL-10 secretion by T cells.

Co-culture of WT $CD4^+$ T cells with *Ifnb1*$^{-/-}$, *Ifnar1*$^{-/-}$, *Tlr4*$^{-/-}$, or *Trif*$^{-/-}$ DCs resulted in only a partial loss of IL-10 induction by PSA (Figures 12A and 12B). These results were consistent with previous work from our lab which has demonstrated that other IFNβ-independent innate immune signaling pathways on DCs- specifically TLR2 and dectin-1 (unpublished data)- are also required for PSA IL-10 induction.(43) The emerging picture is that the complex activation of multiple signaling pathways by PSA is required for optimal induction of IL-10 and protection from pathologic inflammation.

4.4. IFNβ induces tolerogenic dendritic cell properties

Our findings suggested that induction of IFNβ signaling is an important mechanism of generation of tolerogenic T cell responses by commensal microbial antigens. Interestingly, IFNβ does not communicate directly with the T cell, but likely acts indirectly by influencing how the APC interacts with the T cell. During activation of an antigen-specific, $CD4^+$ T cell response, the APC generally provides three signals to the T cell. Signal 1 dictates the specificity of the response, through presentation of

antigen on major histocompatibility complex (MHC) class II molecules to the cognate T cell receptor. Signals 2 and 3 dictate the intensity and the nature of the T cell response, with signal 2 coming from expression of costimulatory molecules and signal 3 from cytokine secretion by APCs.(150, 151) We hypothesized that commensal-induced IFNβ might support a tolerogenic dendritic cell phenotype, through enhancing signals 2 and 3 associated with a tolerogenic T cell response.

To investigate regulation of signal 2, we looked at expression of inducible costimulatory ligand (ICOSL). Interaction between ICOSL on APCs with ICOS on T cells has been demonstrated to promote T_{reg} development and function as well as to enhance the development of IL-10-producting, $FOXP3^-$ type 1 regulatory (T_R1) cells.(152-154) Indeed, the ICOSL-ICOS signaling axis has been associated with reduced disease severity in several mouse inflammatory disease models, including non-obese diabetes, EAE, and airway hyperreactivity.(152, 155-157)

The tolerogenic functions of PSA are dependent on pDCs. Interestingly, PSA has been demonstrated to enhance expression of ICOSL on pDCs and ICOSL was found to be required for the anti-inflammatory effects of PSA both *in vitro* and *in vivo*.(43) We therefore sought to investigate whether IFNβ mediates PSA-induced ICOSL expression. WT, *Ifnb1*$^{-/-}$, or *Ifnar1*$^{-/-}$ BMDCs were derived in the presence of FMS-related tyrosine kinase 3 ligand (Flt3L) using a well-established protocol to favor differentiation of pDCs as well as conventional DCs (cDCs).(158) Following 10 days in culture, Flt3L-derived BMDCs represent a mixed population, containing approximately 10% pDCs, distinguished from cDCs by expression of the I-type-lectin receptor siglec H (SH), which is expressed with high fidelity on pDCs (Figure 13A).(159, 160) Flt3L BMDCs were

treated with PSA for 24 hrs and ICOSL expression on pDCs was analyzed by flow cytometry (Figure 13A). We observed that treatment of WT but not *Ifnb1*$^{-/-}$ or *Ifnar1*$^{-/-}$ BMDCs with PSA for 24 hrs increased expression of ICOSL on pDCs (Figure 13B). Treatment of WT or *Ifnb1*$^{-/-}$ BMDCs with IFNβ alone for 24 hrs also enhanced ICOSL expression on pDCs, to similar levels as PSA (Figure 13B). Together these results demonstrated that IFNβ is both necessary and sufficient for enhanced expression of ICOSL downstream of PSA signaling. Because ICOSL was previously demonstrated to be required for the tolerogenic effects of PSA *in vitro* and *in vivo*, we can infer that

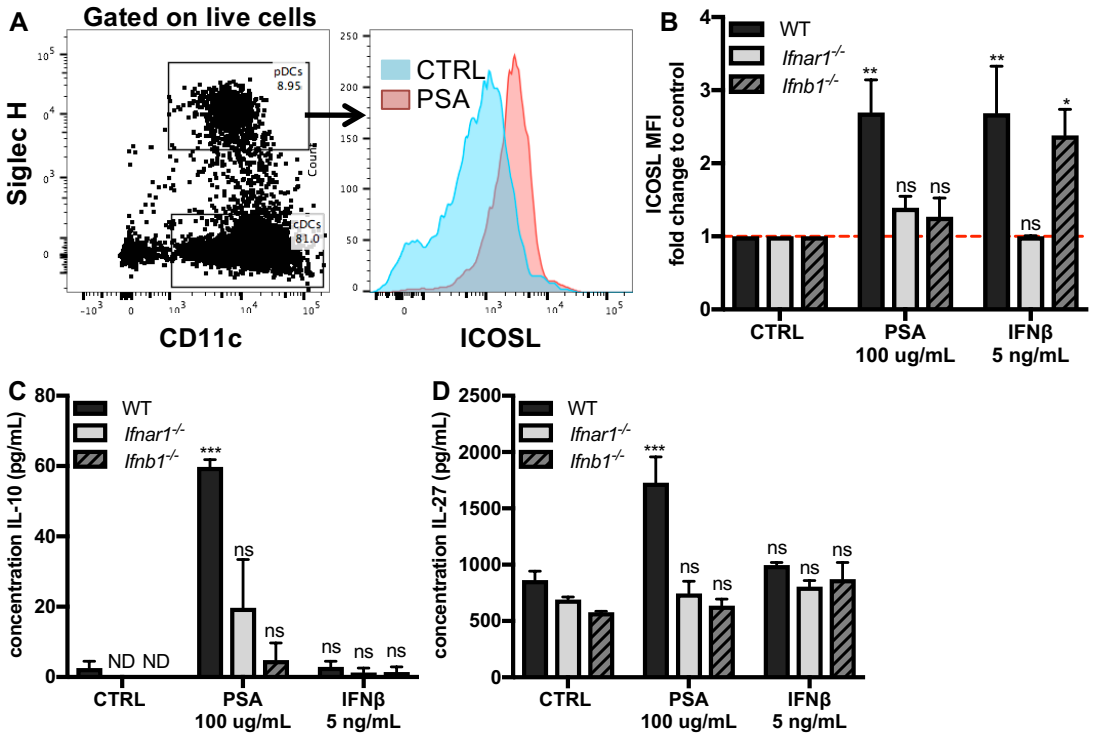

***Figure 13* PSA-induced IFNβ signaling enhances the immunoregulatory phenotype of DCs.** BMDCs from WT, *Ifnb1*$^{-/-}$, or *Ifnar1*$^{-/-}$ mice were differentiated in the presence of Flt3L and treated with 100 ug/mL PSA or 5 ng/mL IFNβ for 24 hrs. Surface expression of ICOSL (A) gated on live CD11c^{+}SH^{+} pDCs was measured by flow cytometry and (B) reported as fold change MFI compared to CTRL. ELISA analysis of (C) IL-10 and (D) IL-27 levels in the cell free supernatants. Data represents average +/- SEM. Two-way ANOVA statistical analysis followed by Dunnett's multiple comparisons test comparing each group to the CTRL group for each genotype. ND=not detected, ns=not significant, *p<0.05, **p<0.01, ***p<0.001.

regulation of ICOSL might represent a mechanism by which PSA-induced IFNβ regulates T cell IL-10 secretion.

To determine whether PSA-induced IFNβ also regulates signal 3 to promote a tolerogenic environment, we investigated the role of PSA and IFNβ in enhancing secretion of the tolerogenic cytokines IL-10 and IL-27 by DCs. IL-10 and IL-27 are both involved in regulation of the T cell response to promote an anti-inflammatory environment.(161) IL-10 has been shown to enhance differentiation and function of T_{regs} in the periphery as well as differentiation of IL-10 producing T_R1 cells.(161-163) IL-27 has also been shown to promote T_{reg} function, differentiation of T_R1 cells, and suppression of pathologic Th17 responses.(164) Treatment of WT BMDCs with PSA resulted in increased secretion of both IL-10 (Figure 13C) and IL-27 (Figure 13D), confirming that PSA does enhance tolerogenic cytokine expression by APCs. Loss of IFNβ or IFNAR1 disrupted the ability of PSA to increase secretion of IL-10 and IL-27, while treatment of BMDCs with IFNβ alone had no effect on levels of these cytokines (Figures 13C and 13D). We therefore determined that IFNβ is necessary but not sufficient for PSA to increase DC secretion of tolerogenic cytokines.

To determine whether PSA also induces tolerogenic cytokine expression by DCs *in vivo*, we isolated colon LP DCs from GF, SPF, or GF mice that were colonized with *B. fragilis* at 4 weeks of age for 2 weeks. Using qRT-PCR analysis, it was found that *Il10* expression by colon DCs was significantly reduced in GF mice compared to SPF mice (Figure 14), confirming that commensal microbial derived signals are important *in vivo* for DC *Il10* expression. Interestingly, colonization of mice with *B. fragilis* enhanced expression of *Il10* by colon DCs, even surpassing the DC *Il10* expression levels of SPF

mice (Figure 14). Together, these data reveal that *B. fragilis* induces expression of the tolerogenic cytokine, IL-10, by colon LP DCs. Further investigation will be required to determine the requirement of IFNβ for the *in vivo* induction of IL-10 by *B. fragilis*.

By increasing expression of the costimulatory molecule ICOSL and secretion of IL-10 and IL-27, our results indicated that IFNβ mediates induction of a tolerogenic DC phenotype by PSA, likely contributing to the ability of PSA to induce IL-10 secretion by T cells.

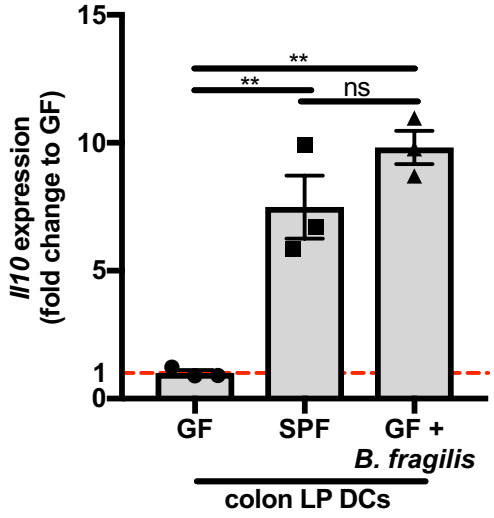

***Figure 14 B. fragilis* colonization restores *Il10* expression by colon LP dendritic cells.** Colons were harvested from WT GF mice, GF mice colonized for 2 weeks with *B. fragilis*, or SPF mice. DCs were isolated from single cell suspensions of colon LP cells, followed by RNA isolation and qRT-PCR analysis of *Il10* expression. Each point represents one mouse, each bar the average +/- SEM. Statistical analysis with one-way ANOVA (p=0.0006) followed by Tukey's multiple comparisons test. **p<0.01, ns=not significant.

4.5. The therapeutic effects of PSA in EAE are dependent on IFN-I signaling

The tolerogenic effects of PSA are not limited to *in vitro* settings, but have been confirmed *in vivo* in several animal disease models. In mouse models of human IBD, including both chemically-induced and T cell transfer-mediated colitis, PSA was shown

to reduce disease severity.(39) Interestingly, these effects were not limited to the local, intestinal milieu, but PSA was also found to be protective in an inflammatory condition of the CNS, EAE.(165) IFN-Is have also been shown to reduce severity in these same mouse disease models.(52) We therefore hypothesized that IFNβ might be required for PSA-mediated protection *in vivo*. To test this hypothesis, we used the mouse EAE model, in which mice are immunized subcutaneously with the immunogenic epitope of myelin oligodendrocyte glycoprotein (MOG_{35-55}) emulsified in complete Freund's adjuvant (CFA) along with intraperitoneal (IP) injection of pertussis toxin on the day of and 2 days after immunization. Using this EAE model, mice develop a self-limited, monophasic disease characterized by ascending paralysis, which is evaluated daily using a clinical scoring system.(166)

Age- and gender-matched WT or *Ifnar1*$^{-/-}$ mice were treated with PSA or PBS as a vehicle control by oral gavage 3 times per week, starting 7 days prior and continuing 14 days after immunization with MOG_{35-55}. WT mice that were treated with PSA were protected from EAE, based on the observation of reduced daily (Figure 15A) and cumulative (Figure 15E) disease scores and a reduction in incidence of disease (Figure 15C). Interestingly, this protection was completely lost in *Ifnar1*$^{-/-}$ mice, with no significant difference in daily disease score (Figure 15B), incidence of disease (Figure 15 D), or cumulative disease score (Figure 15E) between PBS and PSA treated mice. These results demonstrated that IFN-I signaling is required for PSA mediated protection in EAE, and thus for the tolerogenic effects of PSA.

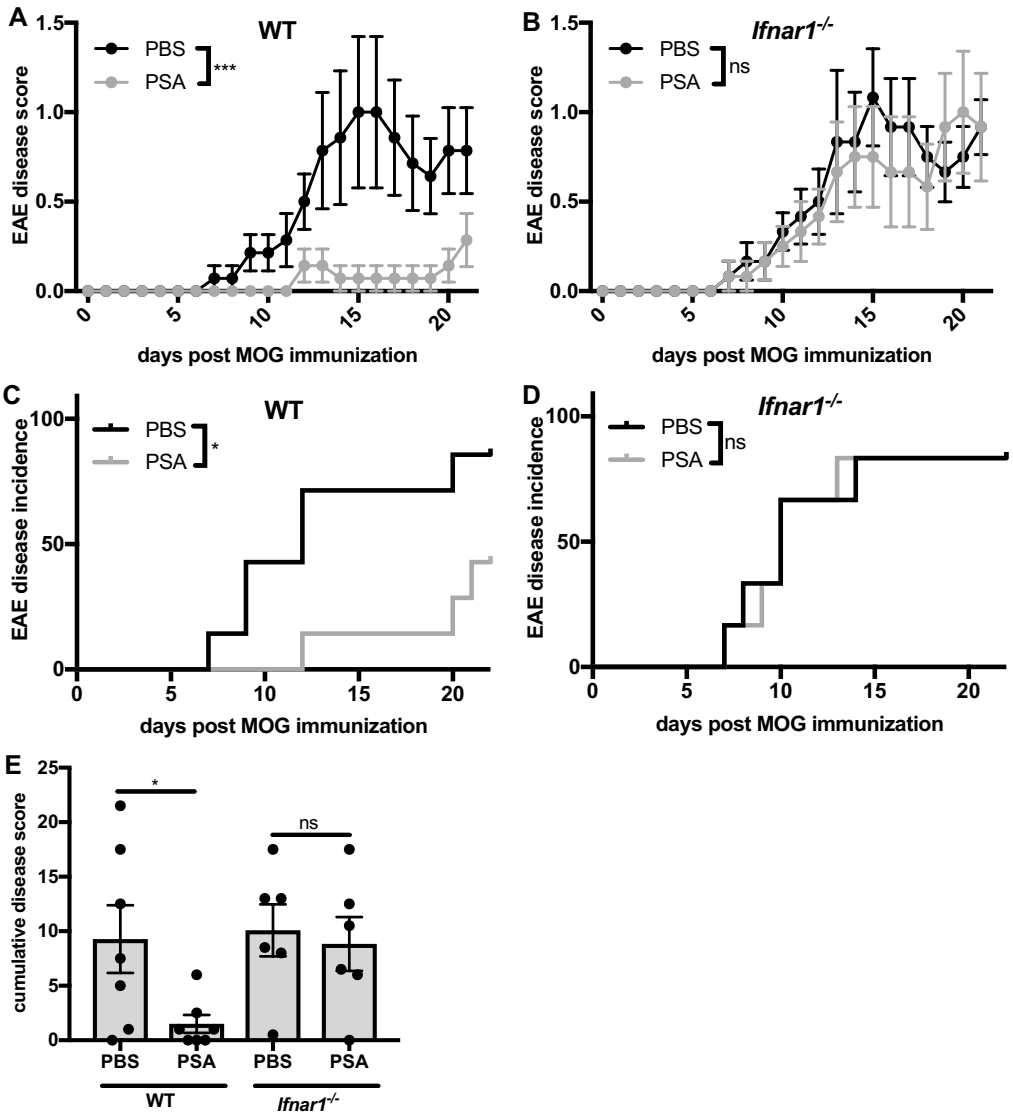

Figure 15 **Type I interferon signaling is required for protection from EAE by *B. fragilis* PSA.** Age- and gender-matched WT or *Ifnar1*$^{-/-}$ mice were treated with 75 ug PSA or PBS as a vehicle control by oral gavage 3 times per week, starting 7 days prior and continuing 14 days after subcutaneous immunization with MOG_{35-55} emulsified in CFA to induce EAE. Disease symptoms were quantified as daily disease score based on ascending paralysis (A,B), percentage of mice with disease symptoms (incidence of disease; C,D) and the sum of the disease score for each mouse for the duration of the experiment (cumulative disease score; E). Statistical analysis with (A,B) linear regression analysis, (C,D) log-rank test and (E) unpaired t-test comparing the PBS and PSA groups for each genotype. ns=not significant, *p<0.05, ***p<0.001.

4.6. IL-10 expression by $CD4^+CD25^-$ T cells is regulated by commensal-induced IFNβ

Our results had demonstrated the importance of IFNβ and IFN-I signaling in the induction of tolerogenic mechanisms by the specific commensal microbe *B. fragilis.* We then sought to investigate whether commensal-induced IFNβ signaling might represent a broader mechanism of maintaining a tolerogenic environment and promoting immune system homeostasis. As mentioned previously, $FOXP3^+$ T_{regs} are immunosuppressive cells that are crucial for maintaining tolerance. We therefore sought to investigate the effect of commensal-induced IFNβ on this tolerogenic cell type. $FOXP3^+$ T_{regs} were analyzed in the mLN and spleen of WT and *Ifnb1*$^{-/-}$ littermates using flow cytometry (gating strategy, Figure 16A). There was no difference in the frequency of $FOXP3^+$ T_{regs} out of the total $CD4^+$ T cell population in either the spleen (Figure 16B) or mLN (Figure 16C) of *Ifnb1*$^{-/-}$ mice compared to WT littermate control mice, which suggested that IFNβ signaling does not regulate T_{reg} proliferation or differentiation.

Because the frequency of T_{regs} was unaltered by IFNβ deficiency, we hypothesized that IFNβ might enhance the immunosuppressive function of T cells, by regulating secretion of the anti-inflammatory cytokine IL-10. Prior to analysis of *Il10* expression, mLN T_{regs} were separated from other mLN $CD4^+$ effector T cells by magnetic bead-based separation on the basis of CD25 expression, a commonly used surface marker for identification of T_{regs} (167). $CD4^+CD25^+$ T_{regs} and $CD4^+CD25^-$ T cells were isolated from the mLNs of WT or *Ifnb1*$^{-/-}$ mice followed by RNA isolation and qRT-PCR analysis of *Il10* transcript levels. There was no change in *Il10* expression levels in $CD4^+CD25^+$ T_{regs}

of *Ifnb1*$^{-/-}$ mice compared to WT mice (Figure 16D). However, *Ifnb1*$^{-/-}$ CD4^{+}CD25^{-} T cells exhibited significantly reduced *Il10* expression compared to WT cells (Figure 16D).

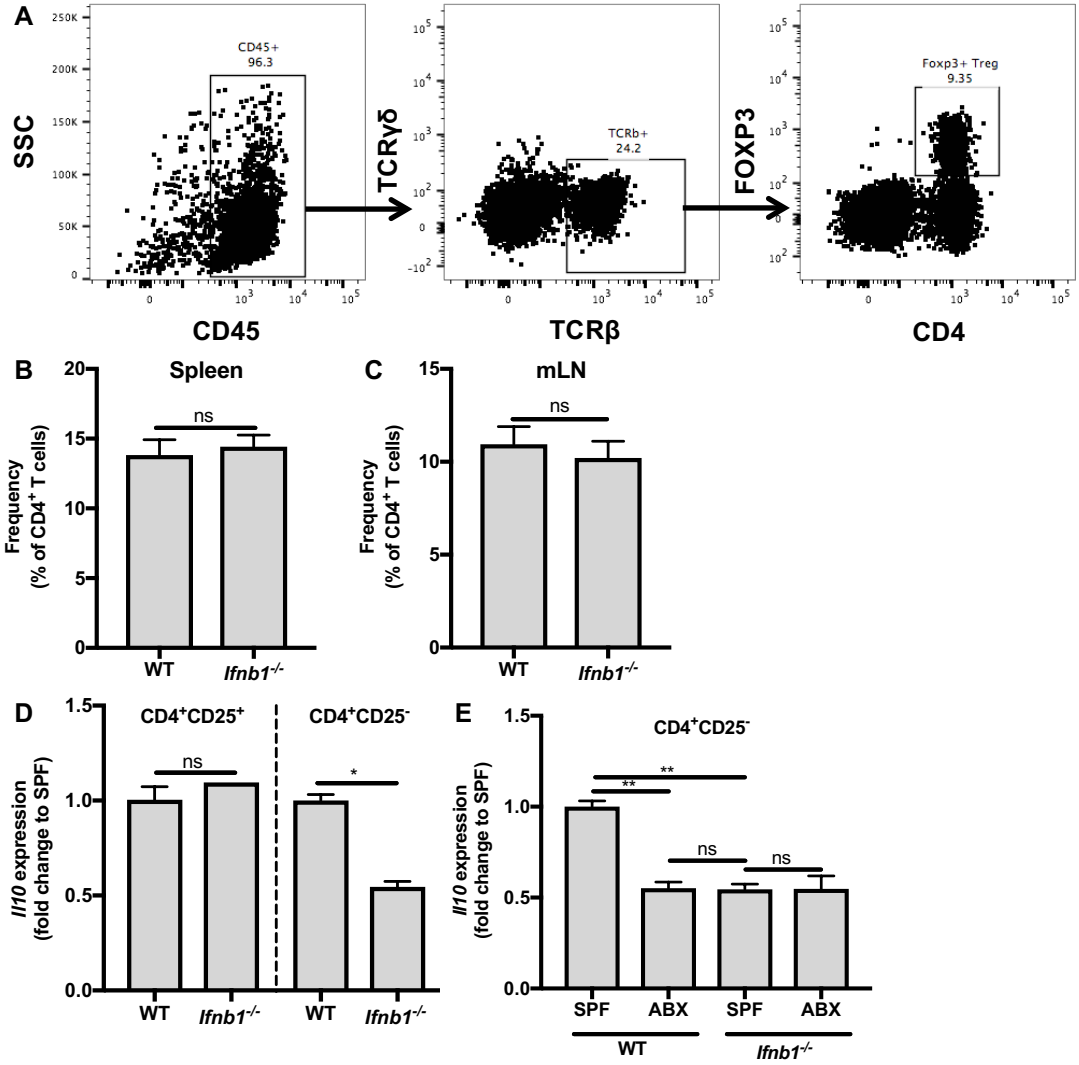

Figure 16 **Commensal-induced IFNβ regulates IL-10 expression by CD4^{+}CD25^{-} T cells.** (A) Gating strategy for flow cytometric analysis of T_{reg} frequencies out of CD4^{+} T cells in the (B) spleens and (C) mLNs of littermate WT and *Ifnb1*$^{-/-}$ mice. (D,E) CD4^{+}CD25^{+} and CD4^{+}CD25^{-} T cells were isolated from mLNs of WT and *Ifnb1*$^{-/-}$ SPF or ABX treated mice using magnetic bead-based separation followed by RNA isolation and qRT-PCR analysis of *Il10* expression. Fold change gene expression compared to SPF was calculated using the delta-delta CT method, with *Actb* as the reference gene. Bars represent the average +/- SEM. Statistical analysis with (B-D) unpaired t-test, (E) One-way ANOVA (p=0.0029) followed by Tukey's multiple comparisons test. ns=not significant, *p<0.05, **p<0.01.

Therefore, IFNβ signaling enhances expression of the immunoregulatory cytokine IL-10 by $CD4^+$ T cells other than T_{regs}. One possibility is that the source of IL-10 in this system is from T_R1 cells, a regulatory $CD4^+$ T cell subset characterized by high levels of IL-10 secretion and the absence of FOXP3 or CD25 expression.(168) Interestingly, the *in vitro* tolerogenic mechanisms that we observed in response to IFNβ signaling, including ICOSL, IL-27, and IL-10 signaling by APCs, have all been reported to positively regulate T_R1 cells, either through effects on their function or development.(168) It is therefore possible that IFNβ signaling drives tolerogenic DC mechanisms *in vivo* leading to regulation of T_R1 cells.

To investigate the role of the commensal microbiota in the observed IFNβ-dependent *Il10* expression by $CD4^+CD25^-$ T cells, age-and gender-matched WT or *Ifnb1*$^{-/-}$ mice were subjected to antibiotics depletion of the microbiota, followed by isolation of mLN $CD4^+CD25^-$ T cells for qRT-PCR analysis of *Il10* levels. After microbiota depletion, a significant reduction in $CD4^+CD25^-$ T cell *Il10* expression was observed in WT but not *Ifnb1*$^{-/-}$ mice (Figure 16E). In addition, when comparing antibiotics-treated WT mice to *Ifnb1*$^{-/-}$ mice there was no difference between *Il10* levels in $CD4^+CD25^-$ T cells (Figure 16E). The lack of effect of antibiotics on *Ifnb1*$^{-/-}$ mice and the statistically indistinguishable expression levels observed between SPF *Ifnb1*$^{-/-}$ mice and WT antibiotics treated mice suggested that the effects of the microbiota on *Il10* expression are indeed mediated by IFNβ signaling and thus that commensal-induced IFNβ is required for induction of IL-10 secretion by mLN $CD4^+CD25^-$ T cells.

Taken together, analysis of mLN T cell *Il10* expression provides initial evidence of an important tolerogenic mechanism by IFNβ-inducing commensal microbes, resulting in

regulation of IL-10 expression by T cells. Further work will be required to confirm whether T_R1 cells are the source of IL-10 in this system, and thus whether commensal-induced IFNβ regulates T_R1 cells. Additional investigation will also be required to address whether this phenotype is present in other tissues and to investigate the effects on proinflammatory Th cell subsets and ultimately the consequences for host health.

4.7. Summary

An imbalance in the composition of the microbiota, known as dysbiosis, is thought to play a major role in many human inflammatory disorders.(148) In other words, a balance between beneficial, tolerance-promoting bacteria and potentially pathogenic, proinflammatory microbes must be maintained to promote host homeostasis and prevent excessive, pathologic inflammation. Our findings suggest that the beneficial, symbiotic microbes might use IFNβ signaling to promote tolerance, thus preventing disease. We discovered that multiple *Bacteroides* species possess the ability to induce secretion of IFNβ and have confirmed using one of these species, *B. fragilis*, the ability of microbial-induced IFNβ to induce tolerogenic mechanisms and protect against pathologic inflammation. *Bacteroides* are a prominent member of the phylum *Bacteroidetes*.(141) Interestingly, a reduction in *Bacteroidetes* has been associated with human IBD,(169) suggesting that a loss in potentially beneficial *Bacteroidetes*, such as the IFNβ-inducing *Bacteroides*, might play a role in human disease. The identification of commensal bacterial molecules capable of inducing IFNβ might therefore provide a novel therapeutic approach to treating human inflammatory disorders.

Chapter 5

Commensal-induced IFNβ is protective against virus infection *in vitro* and *in vivo*

5.1. Introduction

In addition to their more recently discovered immunoregulatory role, IFN-Is have long been appreciated for their critical contribution to the antiviral response. Indeed, IFN-Is were first discovered as a factor secreted by virus-infected cells that when applied to uninfected cells rendered them non-permissive to virus infection, thus "interfering" with infection.(56) IFN-Is directly block virus infection through induction of an antiviral state in cells as well as through induction of apoptosis in virus-infected cells.(48, 58) In addition, IFN-Is indirectly restrict virus infection through regulation of various immune cell subsets involved in the antiviral immune response.(61) The crucial role of IFN-Is in antiviral immunity is highlighted by the strong susceptibility to virus infection of mice lacking IFNAR signaling.(57)

Interestingly, the commensal microbiota has also been demonstrated to contribute to the antiviral immune response. Initial evidence came from studies which found that GF mice are more susceptible than conventionally colonized mice to several virus infections, including Friend leukemia virus, Coxsackie B virus, and influenza A virus (IAV). (170-172) Microbiota depletion in adult mice has also been shown to increase susceptibility to IAV and lymphocytic choriomeningitis virus.(103, 173) Recent work has focused on addressing mechanisms by which this modulation might occur. One such study found that in mice treated with broad-spectrum antibiotics, there was deficient activation of the NLRP3 inflammasome, leading to increased susceptibility to IAV infection.(173) Another study reported that activation of TLR5 signaling by oral treatment of mice with flagellin induced IL-22- and IL-18-mediated protection against rotavirus infection, representing another potential mechanism of microbial modulation of the antiviral

response.(174) However, this study relied on flagellin isolated from a pathogen, *Salmonella typhimurium*, and flagellin levels are low in the healthy GI tract.(175) Thus, TLR5 signaling might not represent an antiviral mechanism shared with commensal microbes. In addition, both of these studies focused on one virus infection model and a mechanism of immunity specific to each of these viruses. Due to the demonstrated importance of the commensal microbiota in multiple and diverse virus infection models, it is likely that other mechanisms of commensal antiviral immune regulation remain to be discovered.

Interestingly, it was recently reported that constitutive, low-level induction of ISG expression by the commensal microbiota is required to prime macrophages to respond robustly to subsequent respiratory virus infection, ultimately providing resistance and improved disease outcome to the host.(103) Due to the important role of IFN-I signaling in the context of many different virus infections, such a finding might represent a broad-spectrum, and therefore universally important, antiviral activity of the commensal microbiota.(57) Despite this importance, the specific commensal microbes and the mechanism by which they regulate the IFN-I response have not been addressed. We therefore sought to investigate whether commensal induction of IFNβ might prime the IFN-I response to reduce susceptibility to virus infection, using *B. fragilis* PSA and LOS as model IFNβ-inducing commensal microbial molecules.

5.2. *B. fragilis* LOS induces expression of intrinsic antiviral factors *in vitro*

One of the first lines of defense against virus infection is the ability of IFN-Is to confer an antiviral state to cells, rendering them non-permissive to virus infection.

Infection of a cell stimulates release of IFN-Is, which signal to infected and neighboring cells to induce expression of hundreds of ISGs, including intrinsic antiviral factors, which directly inhibit the different stages of the virus life cycle, thus limiting virus replication, assembly, and spread.(58) Because *B. fragilis* LOS induces IFNβ, we hypothesized that LOS might enhance intrinsic antiviral factor expression, thus priming cells to be resistant to virus infection

Using qRT-PCR, we analyzed the expression of several well-described intrinsic antiviral factors by BMDCs in response to treatment with LOS. BST2 blocks the release of enveloped viruses from infected cells by tethering them to the cell surface and targeting them for degradation.(176, 177) The Interferon-Induced proteins with tetratricopeptide repeats (IFIT) family of proteins inhibits translation of viral mRNA, and has been demonstrated to play a role in limiting replication of RNA and DNA viruses, including IAV and vesicular stomatitis virus (VSV).(58, 178) 2'-5'-Oligoadenylate synthetase-like protein 2 (OASL2) activates ribonuclease (RNase) L to degrade viral RNA in the cytoplasm, thus broadly targeting many RNA viruses.(58, 179) After 24 hrs of treatment, we observed that LOS strongly and significantly increased the expression of *Bst2* (Figure 17A), *Ifit1* (Figure 17B), *Ifit2* (Figure 17C), *Ifit3* (Figure 17D), and *Oasl2* (Figure 17E) in BMDCs.

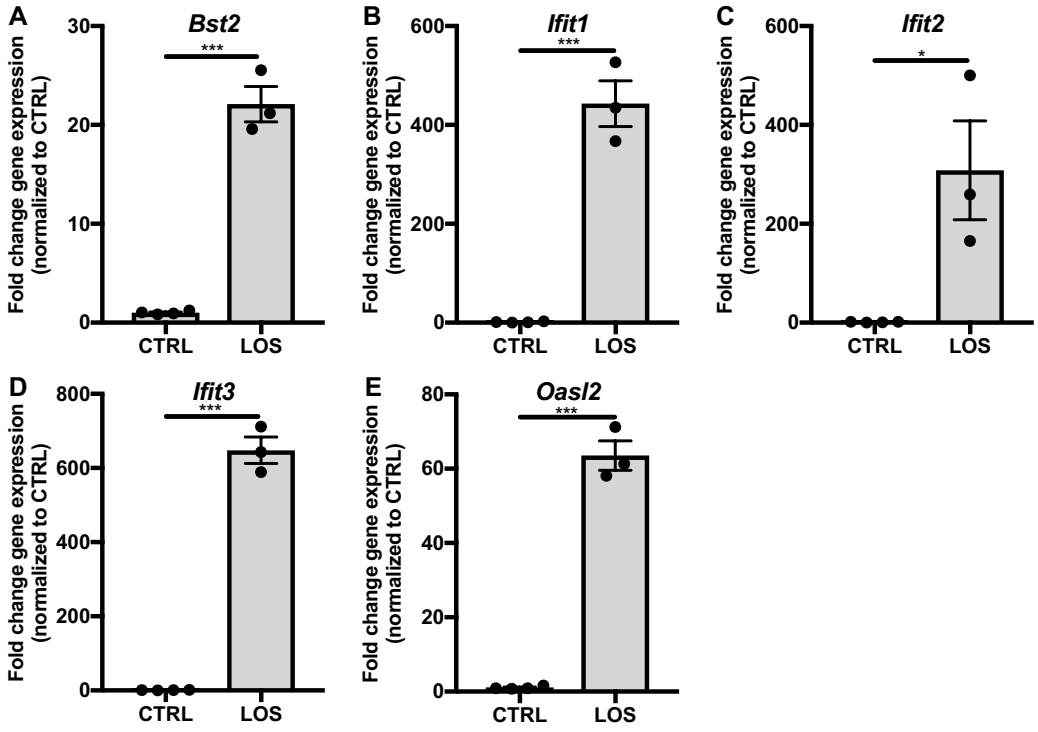

***Figure 17 B. fragilis* LOS induces intrinsic antiviral factor expression in BMDCs.** WT BMDCs were treated with 100 ng/mL of *B. fragilis* LOS for 24 hrs followed by RNA isolation and qRT-PCR analysis of gene expression of (A) *Bst2*, (B) *Ifit1*, (C) *Ifit2*, (D) *Ifit3*, and (E) *Oasl2*. Data represents average +/- SEM of fold change gene expression compared to vehicle control using the delta-delta CT method with *Actb* as the reference gene. Unpaired t-test statistical analysis. *$p<0.05$, ***$p<0.001$.

5.3. *B. fragilis* glycolipids exhibit antiviral activity *in vitro* through induction of IFNβ

Having observed that signaling of LOS to BMDCs results in increased intrinsic antiviral factor expression (Figures 17A-E), we next sought to address whether this increased expression is functional, and thus whether priming of BMDCs with LOS or PSA can render them non-permissive to virus infection. To test this, we developed an *in vitro* virus infection model in collaboration with the laboratory of Dr. Akiko Iwasaki at Yale University using VSV strain Indiana. VSV is an enveloped, negative-sense, single-stranded RNA virus of the family *Rhabdoviridae*, of which the human pathogen rabies

virus is also a member. VSV displays wide host and cell tropism.(180) While only rarely infecting humans, it is an important livestock pathogen, infecting cows, horses and pigs, and is also capable of infecting and causing disease in mice.(181-183) Importantly, VSV is highly sensitive to IFN-Is and is thus commonly used as a model pathogen to assess the antiviral activity of IFN-I signaling.(184, 185) Indeed, VSV is targeted by a multitude of different interferon-stimulated intrinsic antiviral factors including BST2 and IFIT2.(183, 186, 187)

To first confirm whether IFN-Is are involved in the control of VSV infection in murine BMDCs, we infected WT or *Ifnar1*$^{-/-}$ BMDCs with a recombinant green fluorescent protein (GFP) expressing strain of VSV strain Indiana (VSV-GFP) at a multiplicity of infection (MOI) of 1 and then analyzed infection 24 hrs later through flow cytometric analysis of the percentage of infected (GFP^{+}) live cells. A significant increase in the percentage of infected *Ifnar1*$^{-/-}$ compared to WT BMDCs was observed (Figure 18A), confirming that *Ifnar1*$^{-/-}$ BMDCs are more susceptible to VSV infection.

To test whether priming of BMDCs with LOS or PSA could enhance resistance to VSV infection, BMDCs were treated with a dose response of these molecules for 24 hrs prior to infection with VSV-GFP, followed by analysis of infection by flow cytometry at 24 hours post infection (h.p.i.). Priming with either LOS or PSA inhibited virus infection in a dose-dependent manner (Figure 18B), resulting not only in a lower percentage of infected cells but also increased cell viability (Figure 18D). Indeed, treatment with 0.01 ug/mL of LOS or 10 ug/mL of PSA completely inhibited virus infection to levels statistically indistinguishable from the background fluorescence observed in uninfected

control cells (Figure 18C). Based on these results, it was concluded that pretreatment of cells with either PSA or LOS is protective against VSV infection.

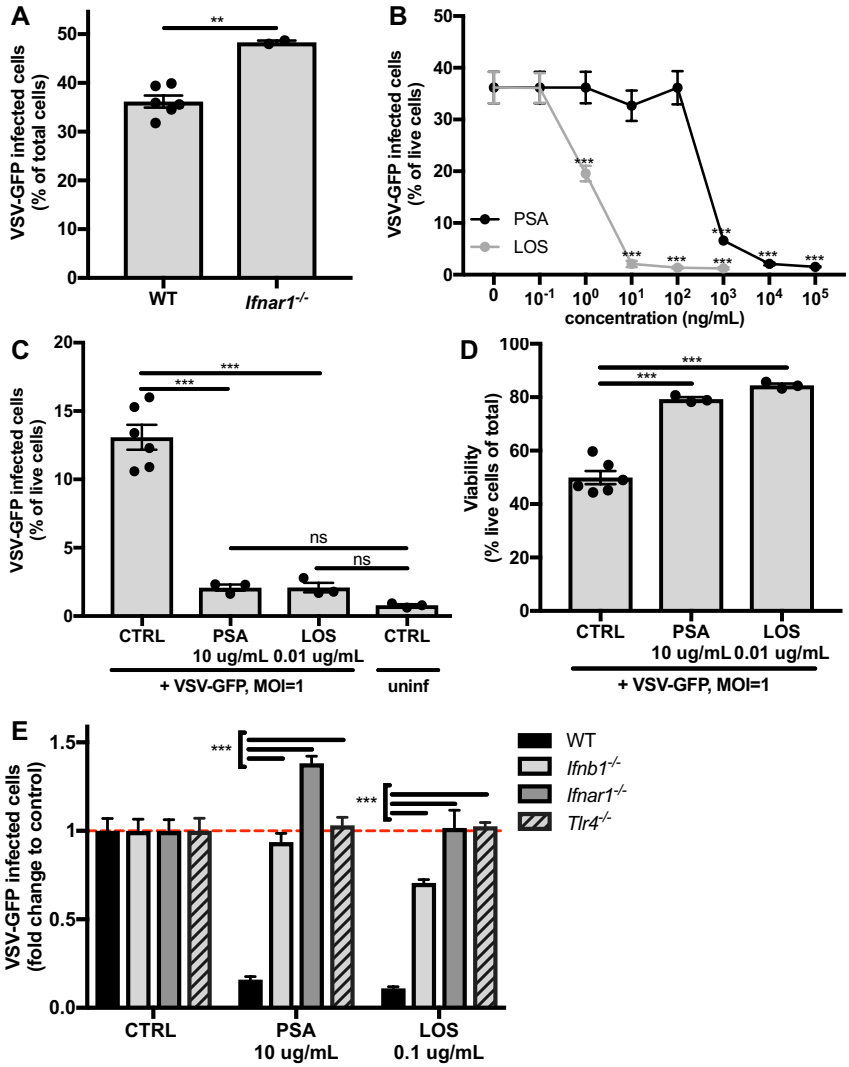

***Figure 18 B. fragilis* glycolipids reduce vesicular stomatitis virus infection of BMDCs by signaling through TLR4 to induce IFNβ.** BMDCs were infected with VSV-GFP (MOI=1). GFP^+ virus-infected cells were analyzed by flow cytometry 24 h.p.i. in (A) untreated WT and *Ifnar1*$^{-/-}$ BMDCs or WT BMDCs treated with (B) a dose response of PSA or LOS or (C) 0.01 ug/mL LOS or 10 ug/mL PSA for 24 hours prior to infection. (D) Flow cytometric analysis of viability of WT BMDCs pretreated with 10 ug/mL PSA or 0.01 ug/mL LOS 24 h.p.i. with VSV-GFP (MOI=1). (E) Flow cytometric analysis of GFP^+ virus-infected WT, *Ifnb1*$^{-/-}$, *Ifnar1*$^{-/-}$, or *Tlr4*$^{-/-}$ BMDCs 24 h.p.i with VSV-GFP following priming with vehicle control, 10 ug/mL PSA, or 0.1 ug/mL LOS. average +/- SEM. Statistical analysis with (A) unpaired t-test, (B, E) two-way ANOVA followed by Dunnett's multiple comparisons test comparing each group to (B) 0 ng/mL control or (E) WT, or (C, D) one-way ANOVA (C: $p<0.0001$, D: $p<0.0001$) followed by Tukey's multiple comparisons test. ns=not significant, **p<0.01, ***p<0.001.

We next sought to investigate the mechanism of antiviral activity of these microbial molecules. Specifically, we asked whether the observed protection is mediated by the TLR4-IFNβ-IFNAR1 signaling axis that we discovered is activated by both PSA and LOS. WT, *Ifnb1*$^{-/-}$, *Ifnar1*$^{-/-}$, or *Tlr4*$^{-/-}$ BMDCs were primed with PSA or LOS for 24 hrs, washed, infected with VSV-GFP, and analyzed by flow cytometry at 24 h.p.i. Protection by either PSA or LOS was completely lost in the absence of TLR4, IFNβ, or IFNAR1 signaling (Figure 18E), confirming that the observed antiviral function of these microbial molecules is indeed mediated by IFN-I, and specifically IFNβ signaling. These findings suggested that PSA or LOS induction of IFNβ and downstream ISG expression induces an antiviral state in cells, thus protecting them from subsequent virus infection.

Viruses represent a vastly diverse taxonomic group in terms of their structure, genetic material, and replication strategy and mammals have in turn evolved multiple strategies for inhibiting virus infection.(58, 188) As such, control of different viruses might require different host antiviral mechanisms. We therefore investigated whether the observed *in vitro* protective effects of PSA and LOS are limited to VSV infection or whether they represent a broad-spectrum antiviral functionality of these commensal molecules. A second *in vitro* virus infection model was developed, using the murine adapted IAV strain PR8 (IAV-PR8).(189) IAV is also an enveloped, negative-sense, single-stranded RNA virus, but belongs to a different family, the *Orthomyxoviridae* family, and is thus not closely related to VSV.(190) IAV is a major causative agent of severe respiratory infection in humans, contributing to significant morbidity and mortality worldwide.(191) It primarily infects epithelial cells of the respiratory tract, but can also infect airway macrophages and DCs, which contribute to the immune response

against this virus.(192) Importantly, IAV is also sensitive to IFN-I signaling, having been demonstrated to be inhibited by numerous ISGs, including BST2, Mx1, and the IFIT family of proteins.(58, 193, 194) Indeed, *Ifnar1*$^{-/-}$ mice exhibit increased susceptibility to IAV infection, confirming the relevance of IFN-I signaling in control of this infection.(195)

As mentioned above, IAV has previously been reported to infect APCs of the airway, with important implications for the antiviral immune response.(192) We therefore sought to establish an *in vitro* IAV infection model using BMDCs. To first confirm that IFN-I signaling is important in the control of IAV in this model system, WT or *Ifnar1*$^{-/-}$ BMDCs were infected with a recombinant GFP-expressing IAV-PR8 strain (PR8-GFP) at an MOI of 1, followed by flow cytometric analysis 24 h.p.i. *Ifnar1*$^{-/-}$ mice exhibited increased susceptibility to PR8-GFP infection, as evidenced by a significant increase in the percentage of live cells that were GFP^{+} (Figure 19A), thus confirming that IFN-Is are important for control of IAV infection in BMDCs.

To test the effect of pretreatment with either PSA or LOS in this model, we incubated WT BMDCs with increasing doses of PSA or LOS for 24 hrs, washed, and infected with PR8-GFP. The percentage of infected, GFP^{+} cells was analyzed at 24 h.p.i. by flow cytometry. Similar to what was observed for VSV infection, priming of BMDCs with either PSA or LOS inhibited subsequent IAV infection, with a significant reduction in the percentage of infected cells (Figure 19B) and increase in cell viability (Figure 19C). Through priming of *Ifnb1*$^{-/-}$, *Ifnar1*$^{-/-}$, or *Tlr4*$^{-/-}$ BMDCs with PSA or LOS followed by PR8-GFP infection, we observed that the antiviral effect of these microbial glycolipids was completely abrogated in the absence of IFNβ, IFNAR1, or TLR4 (Figure

19D). These results revealed that signaling of PSA or LOS through the same pathway protects cells from infection with two different viruses.

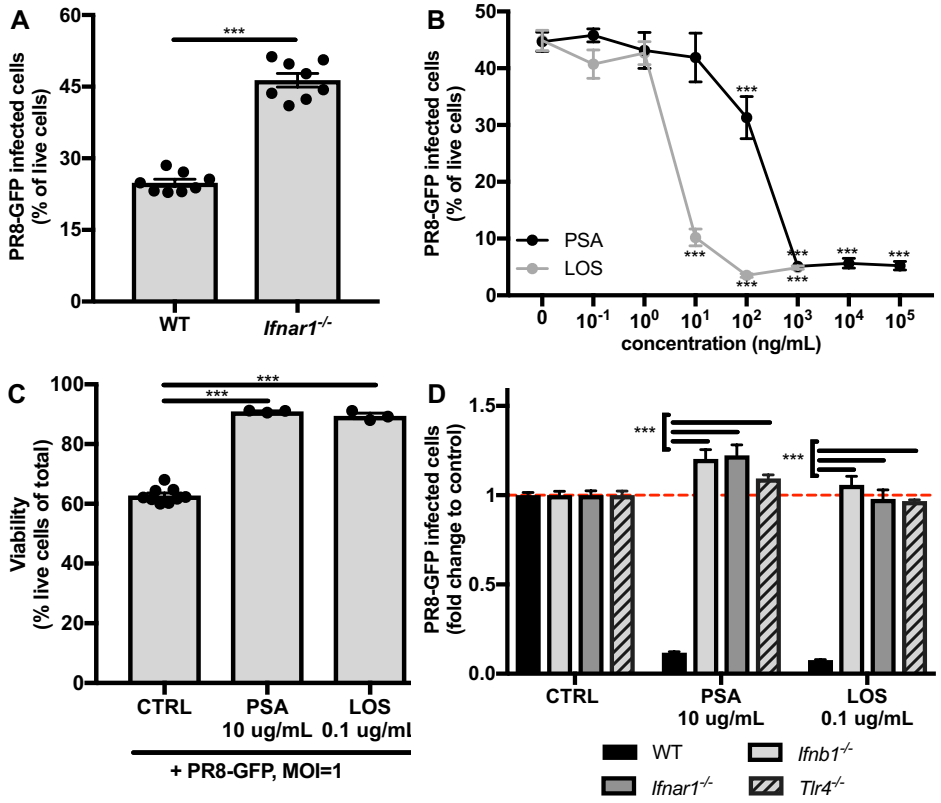

***Figure 19 B. fragilis* glycolipids reduce influenza A virus infection of BMDCs through the TLR4-IFNβ pathway.** BMDCs were infected with IAV/PR8-GFP (MOI=1). GFP^+ virus-infected cells were analyzed by flow cytometry 24 h.p.i. in (A) untreated WT and *Ifnar1*$^{-/-}$ BMDCs or WT BMDCs treated with (B) a dose response of PSA or LOS. (C) Flow cytometric analysis of viability 24 h.p.i with PR8-GFP of WT BMDCs pretreated with 10 ug/mL PSA or 0.1 ug/mL LOS. (E) Flow cytometric analysis of GFP^+ virus infected WT, *Ifnb1*$^{-/-}$, *Ifnar1*$^{-/-}$, or *Tlr4*$^{-/-}$ BMDCs 24 hrs post PR8-GFP infection following priming with vehicle control, 10 ug/mL PSA, or 0.1 ug/mL LOS. average +/- SEM. Statistical analysis with (A) unpaired t-test, (B, D) two-way ANOVA followed by Dunnett's multiple comparisons test comparing each group to (B) 0 ng/mL control or (D) WT, or (C) one-way ANOVA ($p<0.0001$) followed by Tukey's multiple comparisons test. **$p<0.01$, ***$p<0.001$.

Taken together, these results established a novel *in vitro* broad-spectrum antiviral function of *B. fragilis* glycolipids. Pre-incubation of BMDCs with either PSA or LOS protected cells from subsequent infection with either VSV or IAV. Using BMDCs from

genetic knockout mice, the mechanism of protection was confirmed to depend on the ability of these microbial molecules to induce IFNβ through TLR4 signaling. Based on our observation that LOS increases expression of ISGs known to be intrinsic antiviral factors (Figure 17), it can be predicted that PSA and LOS prime cells with an antiviral state, so that they can respond rapidly to block the virus life cycle and thus prevent establishment of a productive virus infection.

5.4. IFNβ is protective in a murine subcutaneous VSV infection model

As aforementioned, numerous studies have established critical roles for both IFN-I signaling and the commensal microbiota in the antiviral immune response, but there is a paucity of research mechanistically linking these two factors. IFN-Is are well known for their ability to induce expression of intrinsic antiviral factors that directly block virus replication, assembly, and spread to other host cells and exhibit broad antiviral activity against classes of viruses with similar structural features.(58) Previous publications as well as our own results (Figures 1 and 2) have revealed that the commensal microbiota is required for constitutive, low level expression of these antiviral ISGs across several different tissue sites.(103) Indeed, our findings specifically identified commensal-induced IFNβ as a homeostatic regulator of antiviral ISG expression. We therefore asked whether commensal-induced IFNβ is required *in vivo* for protection from virus infection.

Due to the strong susceptibility of VSV to IFN-I signaling, we proceeded to establish a murine VSV infection model, which would allow us to investigate the role of commensal-induced IFNβ.(184, 185) VSV is a mammalian neurotropic virus, spread by an arthropod vector. In addition to being a significant livestock pathogen, VSV is a model

pathogen for studies of the closely related and clinically important rabies virus as well as of other arthropod-borne, neurotropic viruses, such as West Nile virus.(182, 196, 197)

In mice, infection with VSV strain Indiana causes meningoencephalitis, leading to a fatal paralytic disease. Several routes of infection are used for murine VSV studies, including the intravenous, intranasal, and subcutaneous routes.(198, 199) Subcutaneous injection most closely resembles the natural route of infection of this arthropod-borne virus and was therefore selected for use in this study. Following subcutaneous injection of the footpad, viral particles enter into the local lymphatics and are transported to the draining lymph nodes, where they first infect and replicate within APCs.(187, 199) From the draining lymph node, VSV proceeds to infect neurons, spreading through the sciatic nerve to the CNS, ultimately causing a lethal paralytic disease, with symptoms appearing approximately 1week post infection.(187) Importantly, IFN-Is are critical for control of VSV infection by this route, with mice lacking IFNAR1 succumbing to infection by 2-3 days post infection (d.p.i.), even before the onset of neurological symptoms.(187) Interestingly, the role of the microbiota has never been investigated in VSV infection by any route, and so this study represents the first analysis of the microbiota in this virus infection model.

The role of IFNβ in subcutaneous VSV infection has not been previously reported. To begin to address the role of commensal-induced IFNβ in VSV infection, we therefore first sought to investigate whether IFNβ contributes to protection in this model. To control for possible microbiota composition differences between WT and knockout mice, heterozygous *Ifnb1*$^{+/-}$ mice were bred and *Ifnb1*$^{+/+}$ and *Ifnb1*$^{-/-}$ littermate offspring were housed together and used for these studies. Mice were infected by subcutaneous

injection into the footpad with 10^6 plaque-forming units (PFU) of VSV strain Indiana and monitored daily for weight loss and ascending paralysis, which was monitored using a clinical scoring system. *Ifnb1*$^{-/-}$ mice exhibited increased susceptibility to disease based on all parameters tracked, with increased weight loss (Figure 20A), daily and cumulative

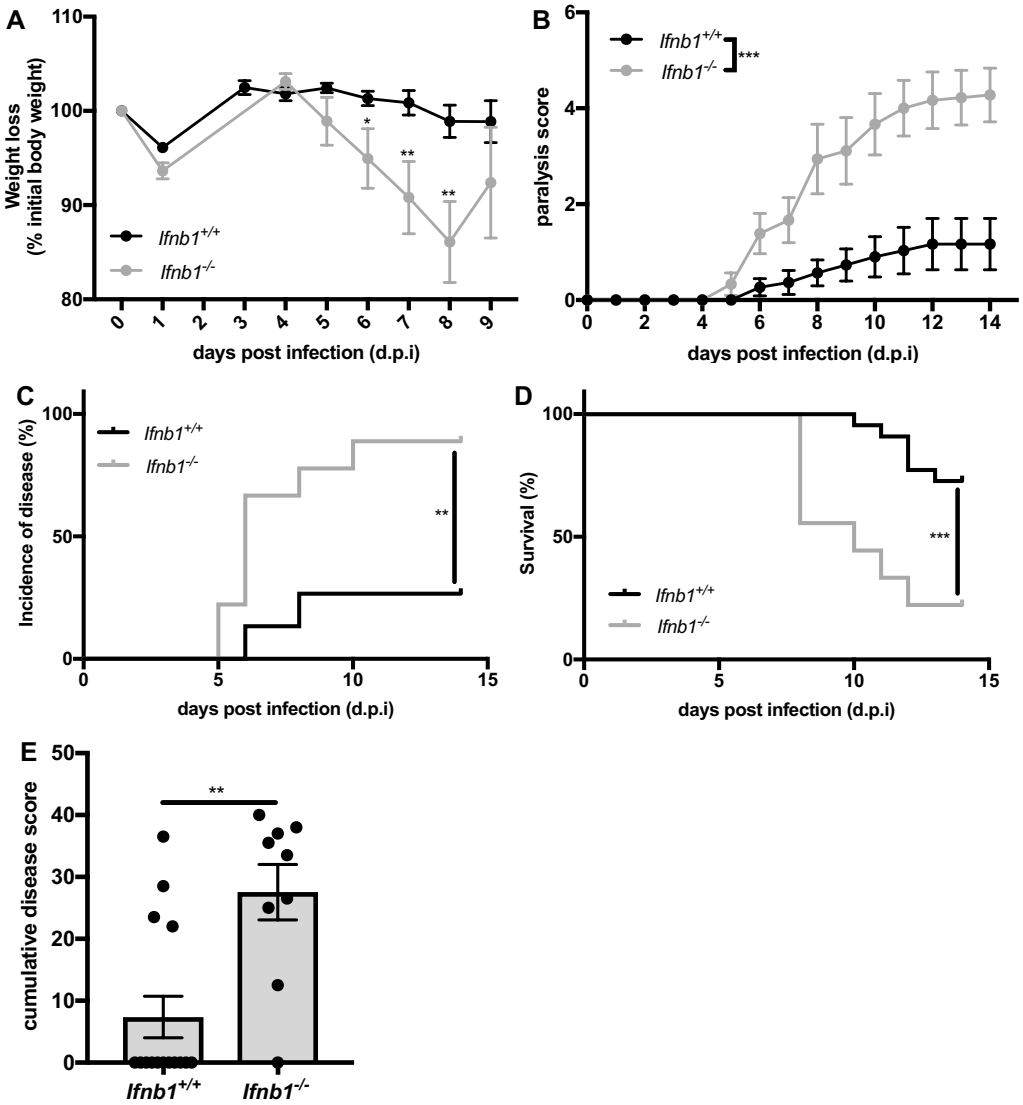

***Figure 20* IFNβ is required for protection from murine vesicular stomatitis virus infection.** Littermate *Ifnb1*$^{+/+}$ or *Ifnb1*$^{-/-}$ mice were infected with 10^6 PFU of VSV strain Indiana by subcutaneous injection into the footpad. Mice were monitored daily for (A) weight loss, (B) daily paralysis score based on ascending paralysis, (C) percentage of mice with paralysis (incidence of disease), and (D) survival. (E) The sum of the daily disease scores for each mouse for the duration of the experiment (cumulative disease score) was calculated 14 d.p.i. Statistical analysis with (A,E) unpaired t-test, (B) linear regression analysis, and (C,D) Log-rank test *p<0.05, **p<0.01, ***p<0.001.

disease scores (Figures 20B and 20E), incidence of disease (Figure 20C), and ultimately reduced survival (Figure 20D). These findings demonstrated a strong role for IFNβ in protection against subcutaneous VSV infection.

It is important to note that VSV infection itself induces IFNβ expression through PRR signaling, which likely contributes to ISG expression after infection and antiviral immunity.(200) Therefore, the increased susceptibility observed in the *Ifnb1*$^{-/-}$ mice likely reflects virus infection-induced IFNβ in addition to any contributions of priming levels of IFNβ induced by commensal microbes prior to infection. To investigate the effects of priming of the antiviral response by constitutive, microbiota-induced IFNβ, we therefore used an IFNβ neutralizing antibody (anti-IFNβ, clone HDβ-4A7).(201) Using this approach, instead of a complete absence of IFNβ, we could selectively inhibit IFNβ only prior to infection, thus limiting our effects to homeostatic, and not virus-induced, IFNβ.

WT mice were administered two doses of anti-IFNβ or mouse IgG2a isotype control antibody by IP injection, 72 hrs and 24 hrs prior to infection with 10^6 PFU of VSV strain Indiana. Indeed, anti-IFNβ treated mice had increased severity of disease, as observed by increased weight loss (Figure 21A), daily and cumulative paralysis scores (Figures 21B and 21D), and incidence of disease (Figure 21C) compared to isotype control treated mice. Of note, the severity of disease observed after treatment with anti-IFNβ was less than that of *Ifnb1*$^{-/-}$ mice in terms of paralysis score and incidence of disease (Figures 20 and 21). Together, our results revealed that blockade of IFNβ prior to infection enhances susceptibility to subsequent VSV infection, thus suggesting a role for commensal-induced, homeostatic IFNβ.

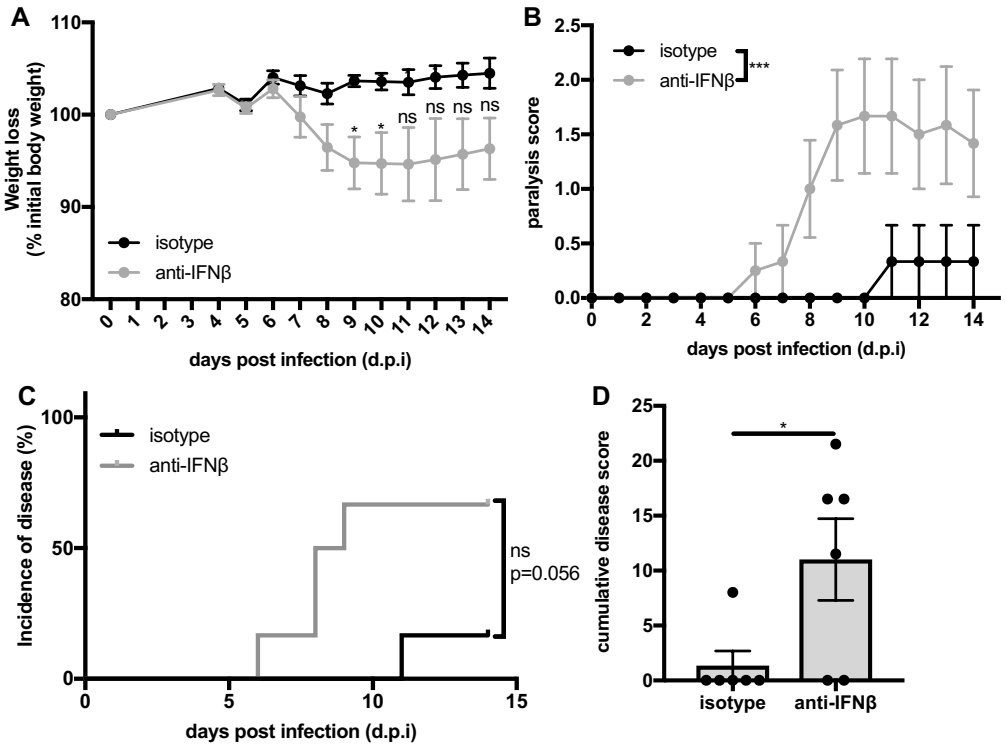

Figure 21 **Inhibition of IFNβ prior to infection increases susceptibility to vesicular stomatitis virus infection.** Age- and gender-matched WT SPF mice were treated with 250 ug anti-IFNβ or mouse IgG2a isotype control antibody by intraperitoneal injection 72 and 24 hours prior to subcutaneous infection with 10^6 PFU VSV strain Indiana. Mice were observed daily for (A) weight loss, (B) paralysis score based on ascending paralysis, and (C) percentage of mice with paralysis (incidence of disease). (D) The sum of the daily disease scores for each mouse for the duration of the experiment (cumulative disease score) was calculated 14 d.p.i. Statistical analysis with (A,D) unpaired t-test, (B) linear regression analysis, and (C) log-rank test. ns=not significant, *p<0.05, **p<0.01, ***p<0.001.

5.5. Commensal-induced IFNβ enhances resistance to VSV infection

To further investigate the impact of the commensal microbiota on VSV infection, we used treatment of WT mice with broad-spectrum antibiotics to deplete the microbiota prior to infection. Following this methodology, we have the ability to exert exquisite control over the timing and length of antibiotics treatment. For these studies, mice were treated with antibiotics for 7 days prior to infection, stopping on the day of infection. Microbiota depletion can have numerous effects on the host immune system, all of which

could potentially impact VSV infection. Our short dosing regimen was designed with the intent to favor effects on the innate immune system, including the IFN-I response, which should change more rapidly than the adaptive immune response. In addition, because we are specifically interested in the priming effects of the microbiota and not its effects during the course of infection, we stopped antibiotics exposure at the onset of infection. Partial restoration of the culturable microbiota is observed by 24 hrs after cessation of antibiotics (unpublished data), and so microbiota depletion should start to reverse during the initial phase of infection. Finally, this dosing regimen should also reduce the occurrence of direct effects of the antibiotics themselves on the virus, such as those which have been reported for the aminoglycoside antibiotics in the context of IAV.(202)

WT mice were subjected to vehicle control (SPF) or antibiotics treatment (ABX) for 7 days, with vancomycin, neomycin, and gentamicin administered in the drinking water and metronidazole and amphotericin B administered by daily oral gavage. Increased susceptibility to disease was observed in ABX mice compared to SPF mice, with significantly increased daily (Figure 22A) and cumulative (Figure 22G) paralysis scores, incidence of disease (Figure 22C), and reduced survival (Figure 22E). These data suggested a role for the commensal microbiota in natural resistance to subcutaneous VSV infection.

Having implicated both IFNβ and the microbiota in protection against VSV infection, we next sought to link these two immunomodulatory mediators mechanistically, to determine whether the antiviral effects of the microbiota are mediated through IFNβ signaling. *Ifnb1*$^{-/-}$ mice were treated with ABX for 7 days prior to infection

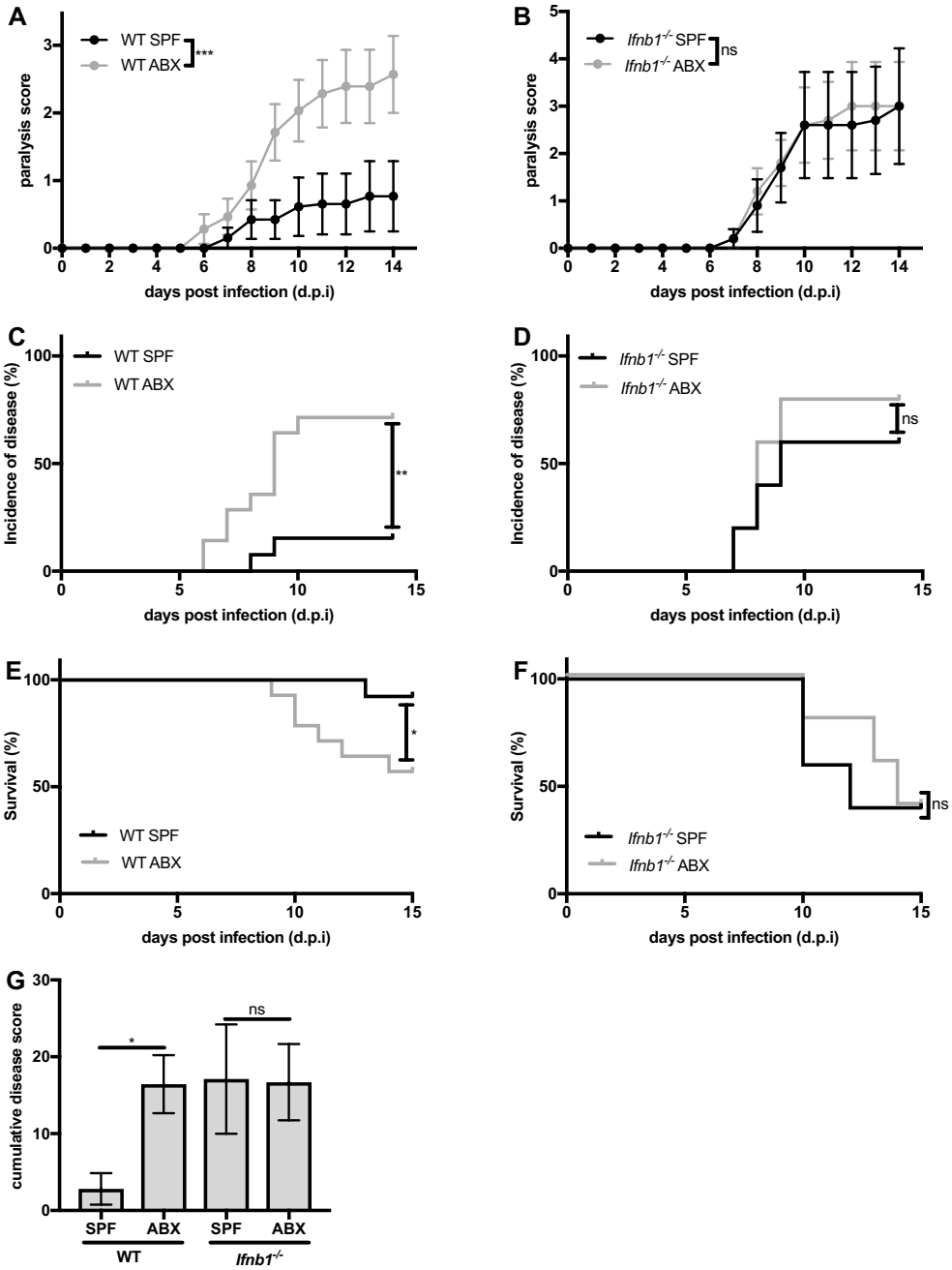

Figure 22 **Microbiota depletion of WT but not *Ifnb1*$^{-/-}$ mice increases susceptibility to vesicular stomatitis virus infection.** Age- and gender-matched WT or *Ifnb1*$^{-/-}$ SPF mice were treated with vehicle control or broad spectrum antibiotics for 7 days prior to infection with 10^6 PFU VSV strain Indiana. Mice were observed daily for (A,B) daily paralysis score based on ascending paralysis, (C,D) percentage of mice with paralysis (incidence of disease), and (E,F) survival. (G) The sum of the daily disease scores for each mouse for the duration of the experiment (cumulative disease score) was calculated 14 d.p.i. Statistical analysis with (A,B) linear regression analysis, (C-F) log-rank test, and (G) one-way ANOVA (p=0.0132) followed by Tukey's multiple comparisons test. ns=not significant, *p<0.05, **p<0.01, ***p<0.001.

with VSV. Interestingly, ABX treatment had no effect on the susceptibility of *Ifnb1*$^{-/-}$ mice to VSV infection (Figures 22B, 22D, 22F, 22G). In the absence of IFNβ, depletion of the microbiota therefore does not alter the antiviral immune response to VSV, suggesting that the microbiota exerts its protective effect specifically through IFNβ.

5.6. Summary

Our findings demonstrated a novel function of the commensal microbial glycolipids *B. fragilis* PSA and LOS, through identification of their ability to protect against virus infection *in vitro* through TLR4 and IFNβ signaling. Using a combination of mice lacking IFNβ, IFNβ neutralizing antibody treatment, and antibiotics treatment we provide evidence that this antiviral mechanism is also important *in vivo*, for protection against subcutaneous VSV infection.

The significance of these findings is highlighted by the near universal role of IFN-I signaling and ISG activity in the response to many different virus infections. In these infections, the direct antiviral activity of IFN-I and its ISGs is thought to act rapidly to limit virus replication and spread, which retains control of the infection until adaptive immune response activation and subsequent clearance of infection can occur. Previous reports have suggested that without microbiota priming of the homeostatic IFN-I response, the host cells are not able to immediately mount an effective IFN-I response upon exposure to pathogenic viruses.(103) Through identification of a specific IFN-I-inducing microbial molecule and the host pathway it signals through, we therefore have identified a mechanism that might not just be important in the context of VSV infection, but in the response to interferon-sensitive viruses in general.

While our work has focused on the glycolipids of *B. fragilis*, we have demonstrated the ability of other *Bacteroides sp.* to activate the same signaling pathways (Figure 10). Due to the prominent role of *Bacteroides* in the human GI microbiota, it is likely that the glycolipids of these bacteria contribute to constitutive priming of the IFN-I response and thus to the susceptibility or resistance of individuals to virus infection.

5.7. Acknowledgments

We would like to thank the Iwasaki lab at Yale University for helping to develop *in vitro* virus infection assays, Dr. Adolfo Garcia Sastre for PR8-GFP, Dr. Jack Rose for VSV-GFP, and Dr. Sean Whelan for WT VSV Indiana used in animal studies.

Chapter 6

Conclusion

In summary, we have described a novel mechanism of immunomodulation by the commensal microbiota through IFNβ-mediated regulation of the homeostatic IFN-I response. Furthermore, we identified the mechanism by which a specific species of commensal bacteria regulates this response, signaling of *B. fragilis* glycolipids through TLR4 to induce expression of IFNβ by colonic DCs. Interestingly, this commensal-mediated regulation of IFNβ and the IFN-I response was found to be important to the health of the host in two different models. In the context of autoimmune disease, commensal-induced IFNβ promoted tolerance to protect against pathological inflammation. Specifically, induction of IFN-I signaling by *B. fragilis* PSA was required for protection of mice from EAE, the mouse model of MS. Based on *in vitro* observations, it can be predicted that this protective effect is mediated through the induction of a tolerogenic DC phenotype and IL-10 secreting T cells by IFNβ. In the context of virus infection, the same commensal-induced cytokine enhanced protective immunity, and was observed to reduce susceptibility to VSV infection *in vitro* and *in vivo*. Initial evidence suggests that this protective effect is due to the priming of low-level expression of antiviral ISGs, which enables host cells to respond rapidly to block virus infection. Commensal-mediated regulation of IFNβ is therefore not just biologically interesting, but also has the potential to contribute to host health.

The observed dichotomy of effects by one commensal-induced cytokine highlights one of the most significant challenges facing the immune system, that of maintaining a homeostatic balance in which cells are ready to respond rapidly to eliminate pathogenic invaders while simultaneously maintaining tolerance to host cells and commensal microbes.(142) Indeed, the same immunological mechanisms that are

protective against pathogens can be harmful if left uncontrolled or if directed towards self antigens, as evidenced by the numerous and significant human diseases now linked to pathological inflammation or autoimmunity, including IBD, RA, cardiovascular disease, and Alzheimer's disease.(203-206) The immune system must therefore be tightly regulated, constantly integrating and responding to signals from the host as well as the environment to maintain homeostasis and prevent aberrant and excessive activation.(143) The commensal microbiota is thought to provide crucial environmental signals to regulate the immune system and promote a tolerogenic balance under homeostatic conditions.(144) Indeed, alterations in the microbiota composition, and thus in the way in which it communicates with the host, have been linked to many inflammatory disorders.(148) In this study, we have identified a mechanism by which the microbiota uses one cytokine to regulate both sides of this homeostatic balance, depending on the context of the signaling. In the context of pathologic inflammation, commensal-induced IFNβ enhances tolerogenic mechanisms, and reduces disease severity. Upon exposure to virus infection, the opposite effect is observed, in which commensal-induced IFNβ appears to enhance protective immunity to reduce susceptibility to disease.

Our research joins a growing body of literature demonstrating that the intestinal microbiota is capable of regulating host physiology at distal sights. Indeed, the intestinal microbiota has been linked to disorders of the joints, cardiovascular system, the brain, and even to behavioral disorders.(27, 35, 36) While our results suggest that the source of IFNβ under homeostatic conditions is intestinal DCs, we have made several observations suggesting that intestinal-derived IFNβ can mediate systemic effects on host physiology. First, we demonstrated that expression of ISGs in the spleen as well as the mLN was

reduced by depletion of the intestinal microbiota. Second, delivery of IFNβ by the orogastric route was sufficient to increase ISG expression in microbiota-depleted mice in both of these tissues. Finally, functional effects of commensal-induced IFNβ were observed in two different disease models of extra-intestinal origin, subcutaneous VSV infection and EAE. Taken together, these findings suggest that IFNβ induction by the intestinal microbiota is capable of altering gene expression systemically and ultimately impacting host physiology and susceptibility to disease.

While our results indeed indicate the systemic effects of commensal-induced IFNβ, the mechanism by which IFNβ of intestinal origin can mediate systemic changes remains to be defined. One hypothesis is that IFNβ secreted by DCs in the intestine enters the systemic circulation and thus signals through IFNAR on cells at distal sites to regulate their gene expression and function. We were unable to detect IFNβ in the serum of conventionally colonized mice. However, IFNβ signaling is well established to occur at very low cytokine concentrations.(95) It is therefore plausible that IFNβ is present in the serum at concentrations capable of inducing a functional response, but still below detection limits of traditional cytokine detection assays. Alternatively, another hypothesis is that intestinal-derived IFNβ never leaves the intestinal milieu but instead signals to local immune cells which then traffic to distal sites of the body, carrying the interferon-induced gene signature with them to their new tissue location. Further investigation will be required to identify which of these mechanisms accounts for the systemic effects of commensal-induced IFNβ.

In addition to the two facets of IFN-I biology that were the focus of this study, tolerogenic mechanisms and antiviral immunity, IFN-Is have been described to influence

numerous other vital aspects of host physiology, including antitumor immunity, cytokine signaling regulation, bone homeostasis, and hematopoiesis.(63) It is therefore likely that the commensal microbiota might influence each of these processes as well, through the ability to regulate IFNβ. Future studies will be required to determine the importance of commensal-induced IFNβ in each of these physiological processes.

Our findings suggest that induction of IFNβ through TLR4 signaling is not limited to *B. fragilis* but is a function common to *Bacteroides sp*. As mentioned previously, microbes of the *Bacteroides* genus are significant members of the human GI microbiota, in both numerical abundance as well as prevalence throughout the population.(119, 120) As such, it is likely that *Bacteroides*-induced IFNβ might be an important immunomodulatory mechanism in most individuals, signaling to the immune system and regulating homeostasis. Alterations in the microbiota composition have been linked to numerous diseases.(148) It is possible that in disorders where IFN-Is play a protective role, a dysbiotic state involving reductions in the IFNβ-inducing *Bacteroides sp.* might increase disease susceptibility. Some evidence supporting this notion comes from studies that identified associations between reduced levels of either *Bacteroidetes* or *Bacteroides* in the gut microbiota and IBD, a spectrum of diseases in which IFN-I has also been suggested to be protective.(169, 207) New therapeutic strategies involving administration of IFNβ-inducing microbes or microbial molecules to counteract the effects of dysbiosis could therefore be envisioned.

In addition to contributing to homeostasis, the identification of IFNβ-inducing microbial molecules could be of therapeutic use. Numerous adverse effects have accompanied the clinical successes of IFN-Is, potentially because delivery of large

amounts of exogenous IFN-I subverts the normal immunoregulatory mechanisms.(110, 111) Delivery of an IFNβ-inducing microbial molecule might represent a novel IFN-I-based therapeutic approach, which would enhance the IFN-I response while still being subjected to homeostatic regulatory mechanisms, which might reduce the adverse side effects. Importantly, compared to a previously described IFNβ-inducing microbial molecule, *E. coli* LPS, the glycolipids of *Bacteroides,* as exemplified by *B. fragilis,* are less inflammatory and antagonize the proinflammatory mechanisms of more inflammatory bacterial species. In this way, *Bacteroides* glycolipids might be able to induce the protective beneficial effects of IFNβ without the consequences of uncontrolled, pathologic inflammation. As mentioned previously, *B. fragilis* LOS represents a mixture of several different structurally distinct molecules, including tri-, tetra-, and penta-acylated glycolipid species. Further work will be required to investigate whether specific structural forms of *Bacteroides* OM glycolipids preferentially induce the immunomodulatory IFNβ pathway downstream of TLR4 signaling, with implications for therapeutic use.

Chapter 7

Experimental Methods

Mice

Conventional SPF mice of strains WT (C57BL/6, stock number 000664), *Ifnar1*$^{-/-}$ (B6.129S2-*Ifnar1tm1Agt*/Mmjax, stock number 010830), *Tlr4*$^{-/-}$ (B6.B10ScN-*Tlr4*$^{lps-del}$/JthJ, stock number 007227), *Myd88*$^{-/-}$ (B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J, stock number 009088), *Trif*$^{-/-}$ (C57BL/6J-*Ticam1*^{Lps2}/J, stock number 005037), and *Il10*$^{-/-}$ (B6.129P2-*Il10*^{tm1Cgn}/J, stock number 002251) on a C57BL/6 background were purchased from Jackson Laboratory. Conventional SPF *Ifnb1*$^{-/-}$ (ifnb1tm1(komp)vlcg) mice were purchased from the UC Davis KOMP repository. Mice were housed under SPF conditions. All genetically deficient mice and their respective controls were gender- and age-matched (typically 5–10 weeks). All experiments on animals were approved by the Harvard Medical Area Standing Committee on Animals (animal protocol number IS00000187-3). Germ free C57BL/6 mice were bred and maintained in sterile vinyl isolators in the animal facility at Harvard University.

Antibiotics Treatment

SPF mice of the indicated strains were treated with an antibiotics cocktail for 7 days to deplete the microbiota. Vancomycin hydrochloride (0.5 g/L, Alfa Aesar), gentamicin sulfate (0.5 g/L, Sigma-Aldrich), and ampicillin (1 g/L, MP Biomedicals) were administered in the drinking water. Metronidazole (4 g/L, Alfa Aesar) and amphotericin B (0.1 g/L, Amresco Inc) were administered in 200 uL by OG once daily. Fecal samples were collected and plated under aerobic and anaerobic conditions to confirm microbiota depletion.

Bacteria

All bacteria used in this study were grown on *Brucella* blood agar plates or in liquid culture in rich Brain Heart Infusion Broth (BD) supplemented with Hemin (0.01%), vitamin K (0.1 mg/mL), glucose (0.5%), Monopotassium phosphate (0.5%), and L-cysteine (0.1%) in an anaerobic chamber (Coy Industries) at 37° Celsius (C). The following bacteria were used in this study *B. fragilis* (strain NCTC 9343, ATCC 25285), *B. thetaiotaomicron* (*B. theta*, ATCC 29741), *B. vulgatus* (ATCC 8482), *B. dorei* (DSM 17855), *B. uniformis* (ATCC 8492) and *C. ramosum* (strain AO31).

qRT-PCR gene expression analysis

RNA was isolated from whole spleens and mLNs or the indicated cells following manufacturer's instructions with the RNeasy mini kit (Qiagen). cDNA was prepared with the Quantitect Reverse Transcription Kit (Qiagen). qPCR was performed on a QuantStudio7 flex Real-Time PCR system (Applied Biosystems) with RT² SYBR Green ROX qPCR Mastermix (Qiagen). Amplification was achieved using an initial cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression was normalized using beta-actin as a reference gene. The forward (f) and reverse (r) primers used to detect expression of the corresponding murine genes were as follows: *Ifit1*(f 5'-CAGAAGCACACATTGAAGAA-3', r 5'-TGTAAGTAGCCAGAGGAAGG-3'), *ifit2* (5'-GGGAAAGCAGAGGAAATCAA-3', r 5'-TGAAAGTTGCCATACAGAAG-3'), ifit3 (f 5'-GCCGTTACAGGGAAATACTGG-3', r 5'-CCTCAACATCGGGGCTCT-3'), oasl2 (f 5'-GGATGCCTGGGAGAGAATCG-3', r 5'-TCGCCTGCTCTTCGAAACTG-3'), viperin (f 5'-

AACAGGCTGGTTTGGAGAAG-3', r: 5'-TGCCATTGCTCACTATGCTC-3'), Irf7 (f 5'-GCCAGGAGCAAGACCGTGTT-3', r 5'-TGCCCCACCACTGCCTGTA-3'), gbp4 (f 5'-TGGGGGACACAGGCTCTACA-3', r 5'-GCCTGCAGGATGGAACTCTCAA-3'), ifnb1 (f 5'-CCTACAGGGCGGACTTCAAG-3', r 5'-GGATGGCAAAGGCAGTGTAACT-3'), il10 (f 5'-ACAACATACTGCTAACCGACTCCTT-3', r 5'-AGGTAAAACTGGATCATTTCCGATA-3'), bst2 (f 5'-GAAGTCACGAAGCTGAACCA-3', r 5'-CCTGCACTGTGCTAGAAGTCTC-3'), *Actb* (f 5'-GATGCTCCCCGGGCTGTATT-3', r 5'-GGGGTACTTCAGGGTCAGGA-3')

Preparation of tissue single-cell suspensions

Single-cell suspensions of the colonic lamina propria were prepared as previously described.(208) For single-cell suspensions of mLNs and spleens, tissues were harvested, treated with collagenase type IV (1 mg/ml; Sigma) for 30 minutes at 37°C in an atmosphere of 5% carbon dioxide (CO_2), and passed through a 40 um mesh.

Magnetic bead-based cell separation

Single-cell suspensions of the indicated tissues were prepared as described. DC positive and negative fractions were isolated with mouse pan-DC microbeads (Miltenyi), $CD4^+$ T cells were isolated with the $CD4^+$ T cell isolation kit (Miltenyi), and $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ T cells were isolated with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi), each following the manufacturer's protocol.

Oral IFNβ treatment

Antibiotics treated mice were administered 100 IU of recombinant murine IFNβ (PBL Assay Science) by OG once daily starting on the 4th day of antibiotics treatment.

Monocolonization of GF mice

GF C57BL/6 mice were administered *B. fragilis* strain NCTC9343 or *C. ramosum* strain AO31 by oral gavage [200 uL, approximately 10^8-10^9 colony-forming units (CFU) per mouse]. Mice were housed in autoclaved cages and maintained on a diet of autoclaved food and water for 2 weeks and then euthanized for analysis.

Serum IFNβ quantification

WT SPF mice were euthanized and blood was collected into 1.1 ml Z-Gel Micro Tubes (Sarstedt). Blood was left at room temperature for 30 minutes, centrifuged to remove coagulated cells [12,000 revolutions per minute (RPM), 10 minutes], and serum was stored at -80°C until analysis. Levels of IFNβ were quantified with the High Sensitivity Mouse IFN Beta ELISA kit (PBL Assay Science) according to the manufacturer's protocol.

GM-CSF derived BMDCs

DCs were derived from bone marrow of the indicated mice as previously described.(209) Briefly, bone marrow was collected from femurs, treated with red blood cell lysis buffer (Gibco), and cultured in 10 cm tissue-culture-treated dishes at a density of $10x10^6$ cells in 10 mL per plate in complete RPMI medium (cRPMI: RPMI 1640 supplemented with 10

mM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, 50 units/mL penicillin, 50ug/mL streptomycin, and 50 uM 2-mercaptoethanol) with 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). Cells were cultured for 6 days at 37°C in an atmosphere of 5% CO_2 with replenishment of medium on day 3. On day 6 the non-adherent and loosely adherent cells were harvested and plated at a density of $1x10^6$ cells/mL in 200 uL per well of a 96-well round-bottom TC-treated cell culture plate for use in the indicated experimental procedures.

Bacteroides OM extraction

OM complexes were isolated from the indicated *Bacteroides sp.* as previously described.(123) Briefly, mid-log phase bacteria were pelleted, washed twice in 0.15 M sodium chloride (NaCl) and resuspended in a buffer of 0.15 M NaCl, 0.05 M sodium phosphate, and 0.01 M Ethylenediaminetetraacetic acid (EDTA). The resuspended organisms were incubated for 30 minutes at 60°C then subjected to mild shearing pressure by passing through a 25-gauge hypodermic needle. Cells were pelleted from the suspension and the supernatants were harvested and subjected to two rounds of centrifugation at 33,000 RPM for 2 hrs at 4° C to pellet the OM extracts.

Bacterial glycolipids

PSA was purified from *B. fragilis* mutant strain Δ44 and LOS was purified from an acapsular *B. fragilis* mutant strain by phenol-water extraction as previously described.(210) Purified *B. fragilis* lipid A was obtained by acid-hydrolysis (4% acetic acid, 95°C, 2 hrs) of LOS. *E. coli* LPS from *E. coli* 055:B5 was purchased from

InvivoGen. Synthetic hexa-acylated *E. coli* lipid A (Kdo2-Lipid A) was purchased from Avanti Polar Lipids.

Supernatant Cytokine Analysis

Cell free supernatants were collected for quantification of IFNβ with the Mouse IFN Beta ELISA kit (PBL Assay Science), IL-10 and TNFα with ELISA MAX kits (Biolegend), and IL-27 with the LEGENDplex mouse inflammation panel multiplex bead-based immunoassay kit (Biolegend) according to the manufacturer's protocol.

Flow cytometric analysis

The following mouse-specific IgG monoclonal antibodies were purchased from Biolegend: FITC-conjugated anti-BST2 and anti-CD4; biotin-conjugated anti-ICOSL; APC-conjugated streptavidin; Pacific Blue–conjugated anti-CD45; PE-conjugated anti-SH; PE-Cy7-conjugated anti-CD11c and anti-TCRβ; PerCP-Cy5.5-conjugated anti-TCRγδ; and BV605-conjugated anti-CD45. Fixable Viability Dye eFluor780 and APC-conjugated anti-FOXP3 were purchased from eBioscience. Single-cell suspensions were stained with a suitable combination of fluorochrome-conjugated antibodies and fixable viability dye. For BMDC analysis, cells were fixed in 2% paraformaldehyde in PBS, and examined with a LSR II flow cytometer (BD). For tissue T_{reg} analysis, cell fixation, permeabilization, and intracellular transcription factor staining were performed according to manufacturer's protocol with the FOXP3/transcription factor staining buffer set (eBioscience) and examined with a LSR II flow cytometer (BD). The data were analyzed with FlowJo software.

Splenic DC-T cell coculture

Isolated splenic DCs and $CD4^+$ T cells were cocultured in wells of a 96-well round-bottom TC-treated plate at a density of 100,000 $CD4^+$ T cells and 20,000 DCs per well in 200 uL complete RPMI medium with 1 ug/mL anti-CD3 (eBioscience) and the indicated experimental treatments. Cells were cultured at 37°C in an atmosphere of 5% CO_2 for 5 days prior to analysis.

Flt3L-derived BMDCs

DCs were derived from bone marrow from untreated mice as previously described.(158) Briefly, bone marrow was collected from mouse femurs followed by treatment with red blood cell lysis buffer (Gibco). The remaining cells were counted and cultured in supplemented RPMI-1640 medium (supplemented with 10 mM HEPES, 50 units/mL penicillin, 50 ug/mL streptomycin, 50 uM 2-mercaptoethanol, and 10% fetal bovine serum) at a density of 1.5×10^6 cells/mL with 100 ng/mL recombinant mouse Flt3L (Biolegend), at 37°C in an atmosphere of 5% CO2 for 10 days, with replenishment of media on day 5.

EAE induction and PSA treatment

Mice were administered 75 ug of PSA by OG 3 times per week starting 7 days prior to and continuing 14 days after disease induction. EAE was induced as previously described.(165) Briefly, mice were immunized subcutaneously with 250 ug $MOG_{35–55}$ (Peptides International) emulsified in 200 uL CFA (Sigma) with

Mycobacterium tuberculosis H37 Ra antigen (5 mg/ml; BD). On the day of and 2 days after MOG immunization, mice were administered 250 ng of *Bordetella pertussis* toxin (List Biological) by IP injection. Mice were evaluated daily for paralytic disease progression using a well-established clinical scoring system (0 = no symptoms, 0.5=partially limp tail, 1= complete paralysis of tail, 2= hind limb weakness, 2.5= paralysis of one hind limb, 3= paralysis of both hind limbs, 3.5= paralysis of both hind limbs and forelimb weakness, 4=forelimb and hind-limb paralysis, 5= moribund state).(211) Mice exhibiting a score of ≥4 were humanely euthanized.

***in vitro* virus infections**

On day 6 of culture, GM-CSF BMDCs were plated at a density of $1x10^6$ cells/mL in 200 uL of cRPMI medium per well of a 96-well round-bottom tissue culture-treated cell culture plate with 20 ng/mL GM-CSF and the indicated treatments for 24 hrs, washed, and resuspended in cRPMI medium containing either IAV strain PR8-GFP or VSV-GFP at an MOI of 1. Cells were harvested for flow cytometric analysis 24 hrs post infection.

Mouse VSV infection

Mice were anesthetized by IP injection of ketamine and xylazine. $1x10^6$ PFU of VSV strain Indiana was delivered in 10 uL per mouse by subcutaneous injection into the footpad as previously described.(187) Body weight and clinical disease score were monitored daily for 14 days following infection. VSV-infected mice exhibit an ascending paralysis similar to what is observed in EAE and so the same system for scoring paralysis was used (0 = no symptoms, 0.5=partially limp tail, 1= complete

paralysis of tail, 2= hind-limb weakness, 2.5= paralysis of one hind limb, 3= paralysis of both hind limbs, 3.5= paralysis of both hind limbs and forelimb weakness, 4=forelimb and hind-limb paralysis, 5= moribund). Mice with a body condition score ≤ 2 or a disease score ≥ 4 were humanely euthanized.

IFNβ neutralization

Mice were administered two doses of 250 ug IFNβ neutralizing antibody (anti-IFNβ, clone HDβ-4A7; Leinco Technologies) or Mouse IgG2a isotype control antibody (Leinco Technologies) by IP injection, 72 hrs and 24 hrs prior to infection with 10^6 PFU of VSV strain Indiana.

References

1. Erturk-Hasdemir D, Kasper DL. Resident commensals shaping immunity. Curr Opin Immunol. 2013;25(4):450-5.

2. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.

3. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.

4. Dominguez-Bello MG, Blaser MJ, Ley RE, Knight R. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. Gastroenterology. 2011;140(6):1713-9.

5. Neu J, Rushing J. Cesarean versus vaginal delivery: long-term infant outcomes and the hygiene hypothesis. Clin Perinatol. 2011;38(2):321-31.

6. Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr. 1999;69(5):1035s-45s.

7. Benno Y, Sawada K, Mitsuoka T. The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. Microbiol Immunol. 1984;28(9):975-86.

8. Cho I, Yamanishi S, Cox L, Methe BA, Zavadil J, Li K, et al. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature. 2012;488(7413):621-6.

9. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, et al. The long-term stability of the human gut microbiota. Science. 2013;341(6141):1237439.

10. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med. 2009;1(6):6ra14.

11. Rojo D, Mendez-Garcia C, Raczkowska BA, Bargiela R, Moya A, Ferrer M, et al. Exploring the human microbiome from multiple perspectives: factors altering its composition and function. FEMS Microbiol Rev. 2017;41(4):453-78.

12. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell. 2016;164(3):337-40.

13. Savage DC. MICROBIAL ECOLOGY OF THE GASTROINTESTINAL TRACT. Annu Rev Microbiol. 1977;31(1):107-33.

14. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804-10.

15. El Aidy S, van den Bogert B, Kleerebezem M. The small intestine microbiota, nutritional modulation and relevance for health. Curr Opin Biotechnol. 2015;32:14-20.

16. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.

17. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science. 2001;292(5519):1115-8.

18. van der Waaij D, Berghuis-de Vries JM, Lekkerkerk L-v. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg (Lond). 1971;69(3):405-11.

19. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 2012;149(7):1578-93.

20. Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin Immunol. 2007;19(2):59-69.

21. Gordon HA. Morphological and physiological characterization of germfree life. Ann N Y Acad Sci. 1959;78:208-20.

22. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005;122(1):107-18.

23. Shi N, Li N, Duan X, Niu H. Interaction between the gut microbiome and mucosal immune system. Military Medical Research. 2017;4(1):14.

24. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009;9(5):313-23.

25. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, et al. Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. J Immunol. 2010;185(7):4101-8.

26. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2011;108.

27. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.

28. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. Science. 2012;336(6080):489-93.

29. Noverr MC, Falkowski NR, McDonald RA, McKenzie AN, Huffnagle GB. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. Infect Immun. 2005;73(1):30-8.

30. Abt MC, Artis D. The dynamic influence of commensal bacteria on the immune response to pathogens. Curr Opin Microbiol. 2013;16(1):4-9.

31. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature. 2011;474(7351):298-306.

32. Shanahan F. The colonic microbiota and colonic disease. Curr Gastroenterol Rep. 2012;14(5):446-52.

33. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science. 2013;339(6123):1084-8.

34. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Rep. 2012;13(5):440-7.

35. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57-63.

36. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci. 2012;13(10):701-12.

37. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H. Treg induction by a rationally selected mixture of clostridia strains from the human microbiota. Nature. 2013;500.

38. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y. Induction of colonic regulatory T cells by indigenous clostridium species. Science. 2011;331.

39. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008;453(7195):620-5.

40. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008;105(43):16731-6.

41. Kang CS, Ban M, Choi EJ, Moon HG, Jeon JS, Kim DK, et al. Extracellular vesicles derived from gut microbiota, especially Akkermansia muciniphila, protect the progression of dextran sulfate sodium-induced colitis. PLoS One. 2013;8(10):e76520.

42. Jeon SG, Kayama H, Ueda Y, Takahashi T, Asahara T, Tsuji H, et al. Probiotic Bifidobacterium breve induces IL-10-producing Tr1 cells in the colon. PLoS Pathog. 2012;8(5):e1002714.

43. Dasgupta S, Erturk-Hasdemir D, Ochoa-Reparaz J, Reinecker HC, Kasper DL. Plasmacytoid dendritic cells mediate anti-inflammatory responses to a gut commensal molecule via both innate and adaptive mechanisms. Cell Host Microbe. 2014;15(4):413-23.

44. Zhang JM, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin. 2007;45(2):27-37.

45. Ng CT, Mendoza JL, Garcia KC, Oldstone MB. Alpha and Beta Type 1 Interferon Signaling: Passage for Diverse Biologic Outcomes. Cell. 2016;164(3):349-52.

46. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nat Rev Immunol. 2014;14(1):36-49.

47. Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. Immunity. 2006;25(3):349-60.

48. Stetson DB, Medzhitov R. Type I interferons in host defense. Immunity. 2006;25(3):373-81.

49. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science. 2013;339(6121):786-91.

50. Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol. 2009;10(11):1200-7.

51. Kalliolias GD, Ivashkiv LB. Overview of the biology of type I interferons. Arthritis Res Ther. 2010;12 Suppl 1:S1.

52. Gonzalez-Navajas JM, Lee J, David M, Raz E. Immunomodulatory functions of type I interferons. Nat Rev Immunol. 2012;12(2):125-35.

53. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol. 2005;5(5):375-86.

54. Ishida H, Ohkawa K, Hosui A, Hiramatsu N, Kanto T, Ueda K, et al. Involvement of p38 signaling pathway in interferon-alpha-mediated antiviral activity toward hepatitis C virus. Biochem Biophys Res Commun. 2004;321(3):722-7.

55. Wang H, Brown J, Garcia CA, Tang Y, Benakanakere MR, Greenway T, et al. The role of glycogen synthase kinase 3 in regulating IFN-beta-mediated IL-10 production. J Immunol. 2011;186(2):675-84.

56. Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci. 1957;147(927):258-67.

57. van den Broek MF, Muller U, Huang S, Zinkernagel RM, Aguet M. Immune defence in mice lacking type I and/or type II interferon receptors. Immunol Rev. 1995;148:5-18.

58. Yan N, Chen ZJ. Intrinsic antiviral immunity. Nat Immunol. 2012;13(3):214-22.

59. Seet BT, Johnston JB, Brunetti CR, Barrett JW, Everett H, Cameron C, et al. Poxviruses and immune evasion. Annu Rev Immunol. 2003;21:377-423.

60. Trinchieri G. Type I interferon: friend or foe? J Exp Med. 2010;207(10):2053-63.

61. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. Nat Rev Immunol. 2015;15(2):87-103.

62. Kole A, He J, Rivollier A, Silveira DD, Kitamura K, Maloy KJ, et al. Type I IFNs regulate effector and regulatory T cell accumulation and anti-inflammatory cytokine production during T cell-mediated colitis. J Immunol. 2013;191(5):2771-9.

63. Gough DJ, Messina NL, Clarke CJ, Johnstone RW, Levy DE. Constitutive type I interferon modulates homeostatic balance through tonic signaling. Immunity. 2012;36(2):166-74.

64. Abe K, Nguyen KP, Fine SD, Mo JH, Shen C, Shenouda S, et al. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. Proc Natl Acad Sci U S A. 2007;104(43):17022-7.

65. Guo B, Chang EY, Cheng G. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. J Clin Invest. 2008;118(5):1680-90.

66. Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest. 2005;115(3):695-702.

67. Lee SE, Li X, Kim JC, Lee J, Gonzalez-Navajas JM, Hong SH, et al. Type I interferons maintain Foxp3 expression and T-regulatory cell functions under inflammatory conditions in mice. Gastroenterology. 2012;143(1):145-54.

68. Rachmilewitz D, Karmeli F, Takabayashi K, Hayashi T, Leider-Trejo L, Lee J, et al. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. Gastroenterology. 2002;122(5):1428-41.

69. Tak PP, Hart BA, Kraan MC, Jonker M, Smeets TJ, Breedveld FC. The effects of interferon beta treatment on arthritis. Rheumatology (Oxford). 1999;38(4):362-9.

70. Teige I, Treschow A, Teige A, Mattsson R, Navikas V, Leanderson T, et al. IFN-beta gene deletion leads to augmented and chronic demyelinating experimental autoimmune encephalomyelitis. J Immunol. 2003;170(9):4776-84.

71. Yarilina A, DiCarlo E, Ivashkiv LB. Suppression of the effector phase of inflammatory arthritis by double-stranded RNA is mediated by type I IFNs. J Immunol. 2007;178(4):2204-11.

72. Kotredes KP, Thomas B, Gamero AM. The Protective Role of Type I Interferons in the Gastrointestinal Tract. Front Immunol. 2017;8:410.

73. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012;491(7422):119-24.

74. Annibali V, Mechelli R, Romano S, Buscarinu MC, Fornasiero A, Umeton R, et al. IFN-beta and multiple sclerosis: from etiology to therapy and back. Cytokine Growth Factor Rev. 2015;26(2):221-8.

75. Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Forster I, et al. Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity. 2011;34(2):213-23.

76. Cho H, Kelsall BL. The role of type I interferons in intestinal infection, homeostasis, and inflammation. Immunol Rev. 2014;260(1):145-67.

77. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmun Rev. 2014;13(6):668-77.

78. Yang J, Sundrud MS, Skepner J, Yamagata T. Targeting Th17 cells in autoimmune diseases. Trends Pharmacol Sci. 2014;35(10):493-500.

79. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133(5):775-87.

80. Shinohara ML, Kim JH, Garcia VA, Cantor H. Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin. Immunity. 2008;29(1):68-78.

81. Ramgolam VS, Sha Y, Jin J, Zhang X, Markovic-Plese S. IFN-beta inhibits human Th17 cell differentiation. J Immunol. 2009;183(8):5418-27.

82. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. J Immunol. 2001;166(9):5530-9.

83. Corre B, Perrier J, El Khouri M, Cerboni S, Pellegrini S, Michel F. Type I interferon potentiates T-cell receptor mediated induction of IL-10-producing CD4(+) T cells. Eur J Immunol. 2013;43(10):2730-40.

84. Piconese S, Pacella I, Timperi E, Barnaba V. Divergent effects of type-I interferons on regulatory T cells. Cytokine Growth Factor Rev. 2015;26(2):133-41.

85. Hamilton JA, Whitty GA, Kola I, Hertzog PJ. Endogenous IFN-alpha beta suppresses colony-stimulating factor (CSF)-1-stimulated macrophage DNA synthesis and mediates inhibitory effects of lipopolysaccharide and TNF-alpha. J Immunol. 1996;156(7):2553-7.

86. Takaoka A, Mitani Y, Suemori H, Sato M, Yokochi T, Noguchi S, et al. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. Science. 2000;288(5475):2357-60.

87. Parker BS, Rautela J, Hertzog PJ. Antitumour actions of interferons: implications for cancer therapy. Nat Rev Cancer. 2016;16(3):131-44.

88. Cheon H, Borden EC, Stark GR. Interferons and their stimulated genes in the tumor microenvironment. Semin Oncol. 2014;41(2):156-73.

89. Bidwell BN, Slaney CY, Withana NP, Forster S, Cao Y, Loi S, et al. Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape. Nat Med. 2012;18(8):1224-31.

90. Balkwill F, Watling D, Taylor-Papadimitriou J. Inhibition by lymphoblastoid interferon of growth of cells derived from the human breast. Int J Cancer. 1978;22(3):258-65.

91. Hobeika AC, Subramaniam PS, Johnson HM. IFNalpha induces the expression of the cyclin-dependent kinase inhibitor p21 in human prostate cancer cells. Oncogene. 1997;14(10):1165-70.

92. Candeias SM, Gaipl US. The Immune System in Cancer Prevention, Development and Therapy. Anticancer Agents Med Chem. 2016;16(1):101-7.

93. Fuertes MB, Kacha AK, Kline J, Woo SR, Kranz DM, Murphy KM, et al. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. J Exp Med. 2011;208(10):2005-16.

94. Gangaplara A, Martens C, Dahlstrom E, Metidji A, Gokhale AS, Glass DD, et al. Type I interferon signaling attenuates regulatory T cell function in viral infection and in the tumor microenvironment. PLoS Pathog. 2018;14(4):e1006985.

95. Schreiber G, Piehler J. The molecular basis for functional plasticity in type I interferon signaling. Trends Immunol. 2015;36(3):139-49.

96. Deonarain R, Verma A, Porter AC, Gewert DR, Platanias LC, Fish EN. Critical roles for IFN-beta in lymphoid development, myelopoiesis, and tumor development: links to tumor necrosis factor alpha. Proc Natl Acad Sci U S A. 2003;100(23):13453-8.

97. Jaks E, Gavutis M, Uze G, Martal J, Piehler J. Differential receptor subunit affinities of type I interferons govern differential signal activation. J Mol Biol. 2007;366(2):525-39.

98. Lavoie TB, Kalie E, Crisafulli-Cabatu S, Abramovich R, DiGioia G, Moolchan K, et al. Binding and activity of all human alpha interferon subtypes. Cytokine. 2011;56(2):282-9.

99. Jaitin DA, Roisman LC, Jaks E, Gavutis M, Piehler J, Van der Heyden J, et al. Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. Mol Cell Biol. 2006;26(5):1888-97.

100. Erlandsson L, Blumenthal R, Eloranta ML, Engel H, Alm G, Weiss S, et al. Interferon-beta is required for interferon-alpha production in mouse fibroblasts. Curr Biol. 1998;8(4):223-6.

101. Gessani S, Belardelli F, Pecorelli A, Puddu P, Baglioni C. Bacterial lipopolysaccharide and gamma interferon induce transcription of beta interferon mRNA and interferon secretion in murine macrophages. J Virol. 1989;63(6):2785-9.

102. Chirdo FG, Millington OR, Beacock-Sharp H, Mowat AM. Immunomodulatory dendritic cells in intestinal lamina propria. Eur J Immunol. 2005;35(6):1831-40.

103. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity. 2012;37(1):158-70.

104. Cheung RP, DiPiro JT. Vancomycin: an update. Pharmacotherapy. 1986;6(4):153-69.

105. Kunin CM, Chalmers TC, Leevy CM, Sebastyen SC, Lieber CS, Finland M. Absorption of orally administered neomycin and kanamycin with special reference to patients with severe hepatic and renal disease. N Engl J Med. 1960;262:380-5.

106. Lamp KC, Freeman CD, Klutman NE, Lacy MK. Pharmacokinetics and pharmacodynamics of the nitroimidazole antimicrobials. Clin Pharmacokinet. 1999;36(5):353-73.

107. Lafforgue G, Arellano C, Vachoux C, Woodley J, Philibert C, Dupouy V, et al. Oral absorption of ampicillin: role of paracellular route vs. PepT1 transporter. Fundam Clin Pharmacol. 2008;22(2):189-201.

108. Geva-Zatorsky N, Sefik E, Kua L, Pasman L, Tan TG, Ortiz-Lopez A, et al. Mining the Human Gut Microbiota for Immunomodulatory Organisms. Cell. 2017;168(5):928-43 e11.

109. Chatzidaki-Livanis M, Geva-Zatorsky N, Comstock LE. Bacteroides fragilis type VI secretion systems use novel effector and immunity proteins to antagonize human gut Bacteroidales species. Proc Natl Acad Sci U S A. 2016;113(13):3627-32.

110. Gottberg K, Gardulf A, Fredrikson S. Interferon-beta treatment for patients with multiple sclerosis: the patients' perceptions of the side-effects. Mult Scler. 2000;6(5):349-54.

111. Sleijfer S, Bannink M, Van Gool AR, Kruit WH, Stoter G. Side effects of interferon-alpha therapy. Pharm World Sci. 2005;27(6):423-31.

112. Wills RJ. Clinical pharmacokinetics of interferons. Clin Pharmacokinet. 1990;19(5):390-9.

113. Dumas A, Corral D, Colom A, Levillain F, Peixoto A, Hudrisier D, et al. The Host Microbiota Contributes to Early Protection Against Lung Colonization by Mycobacterium tuberculosis. Front Immunol. 2018;9:2656.

114. Plakokefalos E, Markoulatos P, Ktenas E, Spyrou N, Vamvakopoulos NC. A comparative study of immunocapture ELISA and RT-PCR for screening clinical samples from Southern Greece for human influenza virus types A and B. J Med Microbiol. 2000;49(11):1037-41.

115. Qian C, Cao X. Dendritic cells in the regulation of immunity and inflammation. Semin Immunol. 2018;35:3-11.

116. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science. 2004;303(5664):1662-5.

117. Bosio E, Beilharz MW, Watson MW, Lawson CM. Efficacy of low-dose oral use of type I interferon in cytomegalovirus infections in vivo. J Interferon Cytokine Res. 1999;19(8):869-76.

118. Bosio E, Cluning CL, Beilharz MW. Low-dose orally administered type I interferon reduces splenic B cell numbers in mice. J Interferon Cytokine Res. 2001;21(9):721-8.

119. Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. Clin Microbiol Rev. 2007;20(4):593-621.

120. Salyers AA. Bacteroides of the human lower intestinal tract. Annu Rev Microbiol. 1984;38:293-313.

121. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. Cell. 2014;156(1-2):123-33.

122. Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, et al. MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. Science. 2015;349(6251):993-7.

123. Kasper DL, Seiler MW. Immunochemical characterization of the outer membrane complex of Bacteroides fragilis subspecies fragilis. J Infect Dis. 1975;132(4):440-50.

124. May KL, Grabowicz M. The bacterial outer membrane is an evolving antibiotic barrier. Proc Natl Acad Sci U S A. 2018;115(36):8852-4.

125. Cress BF, Englaender JA, He W, Kasper D, Linhardt RJ, Koffas MA. Masquerading microbial pathogens: capsular polysaccharides mimic host-tissue molecules. FEMS Microbiol Rev. 2014;38(4):660-97.

126. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A. 2010;107.

127. del Fresno C, Soulat D, Roth S, Blazek K, Udalova I, Sancho D, et al. Interferon-beta production via Dectin-1-Syk-IRF5 signaling in dendritic cells is crucial for immunity to C. albicans. Immunity. 2013;38(6):1176-86.

128. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42(2):145-51.

129. Sheikh F, Dickensheets H, Gamero AM, Vogel SN, Donnelly RP. An essential role for IFN-beta in the induction of IFN-stimulated gene expression by LPS in macrophages. J Leukoc Biol. 2014;96(4):591-600.

130. Maeshima N, Fernandez RC. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol. 2013;3:3.

131. Lindberg AA, Weintraub A, Zahringer U, Rietschel ET. Structure-activity relationships in lipopolysaccharides of Bacteroides fragilis. Rev Infect Dis. 1990;12 Suppl 2:S133-41.

132. Molinaro A, Holst O, Di Lorenzo F, Callaghan M, Nurisso A, D'Errico G, et al. Chemistry of lipid A: at the heart of innate immunity. Chemistry (Easton). 2015;21(2):500-19.

133. Bohannon JK, Hernandez A, Enkhbaatar P, Adams WL, Sherwood ER. The immunobiology of toll-like receptor 4 agonists: from endotoxin tolerance to immunoadjuvants. Shock. 2013;40(6):451-62.

134. Sveen K, Hofstad T, Milner KC. Lethality for mice and chick embryos, pyrogenicity in rabbits and ability to gelate lysate from amoebocytes of Limulus polyphemus by lipopolysaccharides from Bacteroides, Fusobacterium and Veillonella. Acta Pathol Microbiol Scand B. 1977;85B(6):388-96.

135. Kasper DL. Chemical and biological characterization of the lipopolysaccharide of Bacteroides fragilis subspecies fragilis. J Infect Dis. 1976;134(1):59-66.

136. Weintraub A, Zahringer U, Wollenweber HW, Seydel U, Rietschel ET. Structural characterization of the lipid A component of Bacteroides fragilis strain NCTC 9343 lipopolysaccharide. Eur J Biochem. 1989;183(2):425-31.

137. Raetz CR, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem. 2007;76:295-329.

138. Ashkenazi A, Marsters SA, Capon DJ, Chamow SM, Figari IS, Pennica D, et al. Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin. Proc Natl Acad Sci U S A. 1991;88(23):10535-9.

139. Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, et al. Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. Cell. 2016;165(4):842-53.

140. Cullen TW, Schofield WB, Barry NA, Putnam EE, Rundell EA, Trent MS, et al. Gut microbiota. Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation. Science. 2015;347(6218):170-5.

141. Wexler AG, Goodman AL. An insider's perspective: Bacteroides as a window into the microbiome. Nat Microbiol. 2017;2:17026.

142. Iberg CA, Jones A, Hawiger D. Dendritic Cells As Inducers of Peripheral Tolerance. Trends Immunol. 2017;38(11):793-804.

143. Takenaka MC, Quintana FJ. Tolerogenic dendritic cells. Semin Immunopathol. 2017;39(2):113-20.

144. Belkaid Y, Harrison OJ. Homeostatic Immunity and the Microbiota. Immunity. 2017;46(4):562-76.

145. Cording S, Fleissner D, Heimesaat MM, Bereswill S, Loddenkemper C, Uematsu S, et al. Commensal microbiota drive proliferation of conventional and Foxp3(+) regulatory CD4(+) T cells in mesenteric lymph nodes and Peyer's patches. Eur J Microbiol Immunol (Bp). 2013;3(1):1-10.

146. Pandiyan P, Bhaskaran N, Zou M, Schneider E, Jayaraman S, Huehn J. Microbiome Dependent Regulation of Tregs and Th17 Cells in Mucosa. Front Immunol. 2019;10:426.

147. Wannemuehler MJ, Kiyono H, Babb JL, Michalek SM, McGhee JR. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. J Immunol. 1982;129(3):959-65.

148. Lee N, Kim WU. Microbiota in T-cell homeostasis and inflammatory diseases. Exp Mol Med. 2017;49(5):e340.

149. Dikopoulos N, Bertoletti A, Kroger A, Hauser H, Schirmbeck R, Reimann J. Type I IFN negatively regulates CD8+ T cell responses through IL-10-producing CD4+ T regulatory 1 cells. J Immunol. 2005;174(1):99-109.

150. Curtsinger JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. Curr Opin Immunol. 2010;22(3):333-40.

151. Chambers CA. The expanding world of co-stimulation: the two-signal model revisited. Trends Immunol. 2001;22(4):217-23.

152. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. Nat Med. 2002;8(9):1024-32.

153. Lee HJ, Kim SN, Jeon MS, Yi T, Song SU. ICOSL expression in human bone marrow-derived mesenchymal stem cells promotes induction of regulatory T cells. Sci Rep. 2017;7:44486.

154. Zheng J, Chan PL, Liu Y, Qin G, Xiang Z, Lam KT, et al. ICOS regulates the generation and function of human CD4+ Treg in a CTLA-4 dependent manner. PLoS One. 2013;8(12):e82203.

155. Herman AE, Freeman GJ, Mathis D, Benoist C. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. J Exp Med. 2004;199(11):1479-89.

156. Kornete M, Sgouroudis E, Piccirillo CA. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. J Immunol. 2012;188(3):1064-74.

157. Galicia G, Kasran A, Uyttenhove C, De Swert K, Van Snick J, Ceuppens JL. ICOS deficiency results in exacerbated IL-17 mediated experimental autoimmune encephalomyelitis. J Clin Immunol. 2009;29(4):426-33.

158. Gehrie E, Van der Touw W, Bromberg JS, Ochando JC. Plasmacytoid dendritic cells in tolerance. Methods Mol Biol. 2011;677:127-47.

159. Blasius AL, Cella M, Maldonado J, Takai T, Colonna M. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. Blood. 2006;107(6):2474-6.

160. Zhang J, Raper A, Sugita N, Hingorani R, Salio M, Palmowski MJ, et al. Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. Blood. 2006;107(9):3600-8.

161. Kushwah R, Hu J. Role of dendritic cells in the induction of regulatory T cells. Cell Biosci. 2011;1(1):20.

162. Hsu P, Santner-Nanan B, Hu M, Skarratt K, Lee CH, Stormon M, et al. IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1. J Immunol. 2015;195(8):3665-74.

163. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol. 2009;10(11):1178-84.

164. Meka RR, Venkatesha SH, Dudics S, Acharya B, Moudgil KD. IL-27-induced modulation of autoimmunity and its therapeutic potential. Autoimmun Rev. 2015;14(12):1131-41.

165. Ochoa-Reparaz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. Mucosal Immunol. 2010;3(5):487-95.

166. Miller SD, Karpus WJ, Davidson TS. Experimental autoimmune encephalomyelitis in the mouse. Curr Protoc Immunol. 2010;Chapter 15:Unit 15 1.

167. Rodriguez-Perea AL, Arcia ED, Rueda CM, Velilla PA. Phenotypical characterization of regulatory T cells in humans and rodents. Clin Exp Immunol. 2016;185(3):281-91.

168. Zeng H, Zhang R, Jin B, Chen L. Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance. Cell Mol Immunol. 2015;12(5):566-71.

169. Frank DN, St AA, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A. 2007;104.

170. Mirand EA, Grace JT, Jr. Responses of Germ-Free Mice to Friend Virus. Nature. 1963;200:92-3.

171. Schaffer J, Beamer PR, Trexler PC, Breidenbach G, Walcher DN. Response of germ-free animals to experimental virus monocontamination. I. Observation on Coxsackie B virus. Proc Soc Exp Biol Med. 1963;112:561-4.

172. Dolowy WC, Muldoon RL. Studies of Germfree Animals. I. Response of Mice to Infection with Influenza a Virus. Proc Soc Exp Biol Med. 1964;116:365-71.

173. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A. 2011;108(13):5354-9.

174. Zhang B, Chassaing B, Shi Z, Uchiyama R, Zhang Z, Denning TL, et al. Viral infection. Prevention and cure of rotavirus infection via TLR5/NLRC4-mediated production of IL-22 and IL-18. Science. 2014;346(6211):861-5.

175. Cullender TC, Chassaing B, Janzon A, Kumar K, Muller CE, Werner JJ, et al. Innate and adaptive immunity interact to quench microbiome flagellar motility in the gut. Cell Host Microbe. 2013;14(5):571-81.

176. Evans DT, Serra-Moreno R, Singh RK, Guatelli JC. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. Trends Microbiol. 2010;18(9):388-96.

177. Gustin JK, Douglas JL. BST-2/tetherin: viral tether, viral sensor or both? Future Virol. 2013;8(11).

178. Zhou X, Michal JJ, Zhang L, Ding B, Lunney JK, Liu B, et al. Interferon induced IFIT family genes in host antiviral defense. Int J Biol Sci. 2013;9(2):200-8.

179. Dhar J, Cuevas RA, Goswami R, Zhu J, Sarkar SN, Barik S. 2'-5'-Oligoadenylate Synthetase-Like Protein Inhibits Respiratory Syncytial Virus Replication and Is Targeted by the Viral Nonstructural Protein 1. J Virol. 2015;89(19):10115-9.

180. Hastie E, Cataldi M, Marriott I, Grdzelishvili VZ. Understanding and altering cell tropism of vesicular stomatitis virus. Virus Res. 2013;176(1-2):16-32.

181. Letchworth GJ, Rodriguez LL, Del cbarrera J. Vesicular stomatitis. Vet J. 1999;157(3):239-60.

182. Hubalek Z, Rudolf I, Nowotny N. Arboviruses pathogenic for domestic and wild animals. Adv Virus Res. 2014;89:201-75.

183. Fensterl V, Wetzel JL, Ramachandran S, Ogino T, Stohlman SA, Bergmann CC, et al. Interferon-induced Ifit2/ISG54 protects mice from lethal VSV neuropathogenesis. PLoS Pathog. 2012;8(5):e1002712.

184. Marcus PI, Sekellick MJ. Interferon action III. The rate of primary transcription of vesicular stomatitis virus is inhibited by interferon action. J Gen Virol. 1978;38(3):391-408.

185. Basu M, Maitra RK, Xiang Y, Meng X, Banerjee AK, Bose S. Inhibition of vesicular stomatitis virus infection in epithelial cells by alpha interferon-induced soluble secreted proteins. J Gen Virol. 2006;87(Pt 9):2653-62.

186. Brinkmann C, Hoffmann M, Lubke A, Nehlmeier I, Kramer-Kuhl A, Winkler M, et al. The glycoprotein of vesicular stomatitis virus promotes release of virus-like particles from tetherin-positive cells. PLoS One. 2017;12(12):e0189073.

187. Fensterl V, Wetzel JL, Sen GC. Interferon-induced protein Ifit2 protects mice from infection of the peripheral nervous system by vesicular stomatitis virus. J Virol. 2014;88(18):10303-11.

188. Simmonds P, Aiewsakun P. Virus classification - where do you draw the line? Arch Virol. 2018;163(8):2037-46.

189. Radigan KA, Misharin AV, Chi M, Budinger GS. Modeling human influenza infection in the laboratory. Infect Drug Resist. 2015;8:311-20.

190. Bouvier NM, Palese P. The biology of influenza viruses. Vaccine. 2008;26 Suppl 4:D49-53.

191. Eisfeld AJ, Neumann G, Kawaoka Y. Influenza A virus isolation, culture and identification. Nat Protoc. 2014;9(11):2663-81.

192. Short KR, Brooks AG, Reading PC, Londrigan SL. The fate of influenza A virus after infection of human macrophages and dendritic cells. J Gen Virol. 2012;93(Pt 11):2315-25.

193. Mangeat B, Cavagliotti L, Lehmann M, Gers-Huber G, Kaur I, Thomas Y, et al. Influenza virus partially counteracts restriction imposed by tetherin/BST-2. J Biol Chem. 2012;287(26):22015-29.

194. Verhelst J, Parthoens E, Schepens B, Fiers W, Saelens X. Interferon-inducible protein Mx1 inhibits influenza virus by interfering with functional viral ribonucleoprotein complex assembly. J Virol. 2012;86(24):13445-55.

195. Arimori Y, Nakamura R, Yamada H, Shibata K, Maeda N, Kase T, et al. Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. Antiviral Res. 2013;99(3):230-7.

196. Dietzgen RG, Kondo H, Goodin MM, Kurath G, Vasilakis N. The family Rhabdoviridae: mono- and bipartite negative-sense RNA viruses with diverse genome organization and common evolutionary origins. Virus Res. 2017;227:158-70.

197. Suthar MS, Diamond MS, Gale M, Jr. West Nile virus infection and immunity. Nat Rev Microbiol. 2013;11(2):115-28.

198. Sur JH, Allende R, Doster AR. Vesicular stomatitis virus infection and neuropathogenesis in the murine model are associated with apoptosis. Vet Pathol. 2003;40(5):512-20.

199. Iannacone M, Moseman EA, Tonti E, Bosurgi L, Junt T, Henrickson SE, et al. Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. Nature. 2010;465(7301):1079-83.

200. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity. 2005;23(1):19-28.

201. Sheehan KC, Lazear HM, Diamond MS, Schreiber RD. Selective Blockade of Interferon-alpha and -beta Reveals Their Non-Redundant Functions in a Mouse Model of West Nile Virus Infection. PLoS One. 2015;10(5):e0128636.

202. Kim H, Lee MK, Ko J, Park CJ, Kim M, Jeong Y, et al. Aminoglycoside antibiotics bind to the influenza A virus RNA promoter. Mol Biosyst. 2012;8(11):2857-9.

203. Golia E, Limongelli G, Natale F, Fimiani F, Maddaloni V, Pariggiano I, et al. Inflammation and cardiovascular disease: from pathogenesis to therapeutic target. Curr Atheroscler Rep. 2014;16(9):435.

204. Gardener SL, Rainey-Smith SR, Martins RN. Diet and Inflammation in Alzheimer's Disease and Related Chronic Diseases: A Review. J Alzheimers Dis. 2016;50(2):301-34.

205. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet. 2016;388(10055):2023-38.

206. Zhang YZ, Li YY. Inflammatory bowel disease: pathogenesis. World J Gastroenterol. 2014;20(1):91-9.

207. Zhou Y, Zhi F. Lower Level of Bacteroides in the Gut Microbiota Is Associated with Inflammatory Bowel Disease: A Meta-Analysis. Biomed Res Int. 2016;2016:5828959.

208. Couter CJ, Surana NK. Isolation and Flow Cytometric Characterization of Murine Small Intestinal Lymphocytes. J Vis Exp. 2016(111).

209. Helft J, Bottcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. Immunity. 2015;42(6):1197-211.

210. Tzianabos AO, Pantosti A, Baumann H, Brisson JR, Jennings HJ, Kasper DL. The capsular polysaccharide of Bacteroides fragilis comprises two ionically linked polysaccharides. J Biol Chem. 1992;267(25):18230-5.

211. Stromnes IM, Goverman JM. Active induction of experimental allergic encephalomyelitis. Nat Protoc. 2006;1(4):1810-9.