TYPE I INTERFERONS IN INFLAMMATORY AND INFECTIOUS DISEASE

by

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Type I interferons were discovered based on their capacity to induce a potent antiviral response in the face of viral challenge. They are now known to induce a variety of outcomes, which greatly depend on immune context. Immune context is shaped by viral, bacterial, autoimmune or other immune-altering scenarios. The role of type I interferons varies greatly from being beneficial to the host in some infectious contexts and detrimental in others. Additionally, type I interferons are currently used as treatments for some conditions including infection with hepatitis C virus and the autoimmune condition, multiple sclerosis (MS). These treatments however, come with a host of side effects and problematic outcomes including production of antibodies that neutralize type I interferons. There is a clear need for better understanding of the roles of type I interferons in various disease settings and for treatments that can complement or negate the use of type I interferons as treatments so as to avoid undesirable consequences of treatment. An exciting new area of therapeutic investigation is researching natural compounds like oligomeric procyanidins which we have shown to have immunomodulatory and type I interferon-related effects. Through the use of in vitro experiments with human peripheral blood mononuclear cells and in vivo experiments with murine models, we demonstrated a role for type I interferon signaling in the emerging disease, *Clostridium difficile* infection, and propose the use of oligomeric procyanidins derived from apple polyphenols as a potential treatment.
CHAPTER 1

INTRODUCTION

Induction of Type I Interferons

Originally discovered for their ability to induce an antiviral state, type I interferons (IFNs) are now known to have broad capacity to impact a variety of outcomes, which greatly depend on immune context. Type I IFNs are one of three types of interferons. Type I, II and III IFNs are classified based on activity, structure and receptor type. These IFNs are made in different immune settings and are produced by various cell types. There are two groups of type I IFNs: they are IFN-β and 13 subtypes of IFN-α. There is only one type of type II IFN, IFN-γ, which has very different downstream effects compared to type I IFNs. Finally, there are up to three types of type III IFNs, called IFN-λ 1, 2 or 3 in humans and IFN-λ 2 and 3 in mice. Type III IFNs are structurally distinct from type I IFNs. Thus, they bind different receptors, but cause nearly indistinguishable signaling events and gene induction profiles compared to type I IFNs and are related to the IL-10 cytokine family (1)(2). Regardless of type, all IFNs are produced in response to upstream signaling cascades that are due to recognition of host- or microorganism-derived molecules. This review focuses on type I IFNs only.

Classically, pathogen-associated molecular patterns (PAMPs) are sensed by their cognate pattern recognition receptors (PRRs), which leads to downstream transcription of many gene products including type I IFNs. These PAMPs can be bacterial or viral. Host-derived molecules with similar activity are called damage-associated molecular patterns
(DAMPs). PAMPs and DAMPs are sensed by different receptors depending on cellular location, cell type and tissue. Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) are examples of PRRs. TLRs and CLRs are transmembrane receptors whereas RLRs and NRLs reside in the cytoplasm (3). Multiple PRRs can be engaged during an immune response to an invading pathogen. This leads to a variety of outcomes resulting in diverse innate and adaptive immune responses that are specific to a pathogen’s repertoire of PAMPs and induced DAMPs.

TLRs sense PAMPs including nucleic acids and lipoproteins from invading pathogens and are expressed on the cell surface or present in endosomes and lysosomes in immune cells. Different TLRs lead to different outcomes depending on what cell type they are expressed in or on. For example, when TLR2 is expressed on dendritic cells or macrophages and bound by bacterial ligands, type I IFNs are not produced. However, when TLR2 is expressed on inflammatory monocytes, defined as Ly6C high monocytes, and engaged by viral non-nucleic acid PAMPs, type I IFN production occurs (3). TLRs 3, 7, 8 and 9 recognize viral and bacterial nucleic acids inside infected cells, which leads to type I IFN production. Endosomal and cell surface TLR3 signal through the adaptor protein TRIF while the other TLRs utilize MyD88. TLR4, however, can use both TRIF and MyD88 with type I IFN production downstream of TRIF (4). Type I IFN production is redundant in response to various PAMPs and is thus a key component of innate immune recognition of and response to, pathogens.
C-type lectin receptors (CLR)s are transmembrane innate receptors but rather than recognizing nucleic acids and lipoproteins, CLRs have a domain that allows them to recognize carbohydrate motifs. CLRs are known for signaling production of pro-inflammatory cytokines or inhibiting TLR activity. In fact, a particular CLR called CD303 can inhibit TLR9-induced type I IFN production by plasmacytoid dendritic cells (pDCs) (5). Even though CLRs cannot lead to direct production of type I IFNs, functionally, they can indirectly effect type I IFNs.

RLRs include RIG-I, MDA5, and LGP2, all of which recognize different types of viral RNA. Recognition of viral RNA by RLRs leads to type I IFN production. RIG-I recognizes short viral dsRNA or ssRNA with a 5’-triphosphate group (6). MDA5 recognizes long dsRNA and its synthetic analog, polyI:C. In one study, polyI:C was converted from an MDA5 ligand into a RIG-I ligand by simply shortening the dsRNA segment (7). LGP2 is unique from MDA5 and RIG-I in that it does not contain a CARD (caspase activation and recruitment domain) region, a domain critical for interacting with downstream proteins. LGP2 serves to regulate MDA5 and RIG-I signaling and activity. This regulation is achieved by the ability of LGP2 to bind to and sequester dsRNA, preventing it from interacting with the other RLRs (8). LGP2 has also been shown to directly interact with RIG-I and prevent it from undergoing the necessary conformational changes that lead to type I IFN production (9). In contrast, a recent study using LGP2 knockout mice revealed that LGP2 is required for RIG-I and MDA5 to signal appropriate and robust type I IFN production in response to viral infections (10). Thus, production of type I IFNs is induced and tightly regulated by intracellular RLRs.
NLRs share many similarities with TLRs. They are important for regulation and signaling of inflammatory and apoptotic events and recently have been shown to play an important role in antiviral responses (6). These receptors are expressed in both immune and non-immune cells, such as epithelial cells. Similar to TLRs, PAMPs and DAMPs bind NLRs and cause a variety of downstream effects (11). More specifically, PAMP ligation leads to activation of MAPKs (mitogen activated protein kinases), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), or caspase-1 which each cause distinct inflammatory outcomes. There are several NLRs, each of which recognize various viral and bacterial PAMPs. For example, NLRP3 can recognize danger signals such as ATP and bacterial toxins and activate caspase-1 whereas NLRC4 recognizes flagellin on bacterial pathogens like *Listeria monocytogenes* and *Pseudomonas aeruginosa* (12). Though both of these pathogens can activate type I IFN production, this production is likely not through NLRs. These receptors serve as important sensors of intracellular invaders and critical inducers of innate immune inflammation.

It is clear that sensing of DAMPs and PAMPs by various receptors can lead to different outcomes. These outcomes vary due to different PRRs sensing different ligands, PRRs interacting with various adaptor proteins, and the overall disease context. Bacterial, viral and autoimmune diseases all have distinct immune signatures which will shape the impact of type I IFNs. Once the receptors are engaged and the signaling cascade unfolds, type I IFN gene expression and protein production occurs. Whether pathogens are sensed via TLRs in endosomes or on cell surfaces, or via intracellular sensors, the pathways share common proteins. For example, cytosolic sensing of nucleic acids leads to a
pathway in which the kinase, TBK-1 (TANK binding kinase) phosphorylates and activates IRF3 (interferon regulatory factor). Activated IRF3 can then translocate to the nucleus and cause type I IFN production. Furthermore, when dsRNA binds endosomal TLR3 or LPS binds cell surface located TLR4, these interactions lead to TBK-1/IRF3-induced type I IFN signaling. Though some of these signaling pathways converge at TBK-1, upstream and downstream adaptor proteins differ. TLRs 3 and 4 interact with adaptor protein TRIF and utilize transcription factor IRF3 while endosomal TLRs 7 and 9, interact with adaptor protein MyD88 and transcription factor IRF7. However, cytosolic sensors RIG-I and MDA5 do not utilize the common TLR adaptor proteins MyD88 or TRIF. The variety of innate immune receptors expressed on immune and non-immune cells coupled with unique adaptor proteins produce finely tuned innate responses which include type I IFN expression.

Type I Interferon Signaling

Once type I IFNs are produced and released, they can interact with the same cell that produced them in an autocrine manner or bind to other cells in a paracrine fashion. All type I IFNs signal through a common receptor composed of two chains, IFNAR1 and IFNAR2 (1). This signal then transcends down a chain of interacting proteins and is called the JAK-STAT pathway. Upon receptor engagement, JAK1 and TYK2 can phosphorylate STAT1 and STAT2, which then dimerize and form a complex with IRF9. This complex, called interferon-stimulated gene factor 3 (ISGF3), translocates to the nucleus. Nuclear translocation of transcription factors like ISGF3 to interferon-stimulated
response elements (ISREs) leads to expression of interferon stimulated genes (ISGs), which will be discussed later (13). In addition to STAT1 and STAT2 heterodimers, depending on the cell type in which type I IFN signaling occurs, different STAT dimers may be formed downstream of IFNAR receptor engagement. For example, STAT1 homodimers can form and bind to IFN-γ-activated sites, adding even more complexity and diversity to type I IFN signaling (14).

Most, if not all, cells can respond to and produce type I IFNs, but it is well known that pDCs are capable of producing significant amounts of type I IFNs. pDCs were characterized fairly recently. Their main attributes are their ability to produce a large amount of type I IFNs, their plasma cell-like phenotype, and the fact that, in the right conditions, they can differentiate into classical dendritic cells (15). They are made in the bone marrow, found mostly in lymphoid organs during steady state conditions, and can sense viral PAMPS via TLRs expressed both endosomally and extracellularly (16).

Human pDCs express TLRs 7, 8 and 9, while it has not yet been determined what the exact panel of TLR expression is in mice. There is evidence suggesting that mouse pDCs respond robustly to TLR7 and 9 agonists and weakly to TLR2, 3 and 4 agonists. This argues in favor of expression of TLRs 7 and 9 on mouse pDCs, similar to human pDCs (17). What makes these cells unique and robust producers of type I IFNs is that pDCs constitutively express IRF7 (1,18). This allows for rapid induction of type I IFN expression in response to various stimuli, most importantly to viral and bacterial nucleic acids (15). Type I IFNs produced by pDCs affect many cell types including NK cells and plasma cells thus bridging the gap between innate and adaptive immunity (15). In fact,
type I IFN production by other cell types cannot compensate in the absence of pDCs. In studies in which pDCs were depleted or conditionally knocked out in mice, it was concluded that pDCs are not only necessary for controlling acute viral infections but also in preventing development of self-reactive T cells (19),(20). Consequently, pDCs are critical producers of type I IFNs in response to viral insult in both an innate and adaptive sense.

Various signaling molecules in conjunction with the pleiotropic effects of type I IFNs leads to a wide array of host responses that can positively or negatively affect both innate and adaptive immunity. Additionally, type I IFNs have broader reaching effects beyond aiding in innate immune response against pathogens. For example, they can modulate cell proliferation, apoptosis, and other cellular functions (1). During viral infections, type I IFNs induce an antiviral state in surrounding cells that may result in viral clearance. Type I IFN responses to bacterial infections occur in a similar manner by means of extracellular and intracellular recognition of bacterial products. Finally, in autoimmune diseases, such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE), type I IFNs can have contrasting actions. The role of type I IFNs in viral, bacterial and autoimmune disease is further complicated by the fact that type I IFNs can have negative or positive effects on the host. In the following sections, the multifaceted effects that type I IFNs have on the host in various disease settings are described.

**Viral Infections**

The originally described mode of action for type I IFNs is their antiviral effect and ability to instill an antiviral state in surrounding cells. Type I IFNs were originally
discovered in the context of influenza virus infection and are critical to host survival. The response to a viral infection will depend on the tissue and cell infected and the type of virus. RNA and DNA viruses have different infection and replication processes and therefore stimulate through different extracellular and intracellular pattern recognition receptors. In cases of acute viral infections, type I IFNs are critical for host protection before adaptive immunity takes effect. This is because initial type I IFN production serves as an alarm to other cells, signaling the presence of a virus. An arsenal of interferon-stimulated genes (ISGs) are induced upon type I IFN engagement with the type I IFN receptor. The protein products of these genes exhibit a multitude of antiviral effects. These ISGs include OAS1, MX1, IFIT1/2/3 and others. OAS1 activates RNase L which can break down viral RNA genomes and MX1 is a GTPase which can block viral transcription and replication. Notably, common inbred mouse strains like C57s do not have functional MX1 genes. This could explain some strains’ increased susceptibility to influenza (21). IFIT1/2/3 can bind helicases of particular viruses, interacts with eIF3 which greatly decreases cellular translation, and can also directly bind viral nucleic acids (22-24). Without the type I IFN system inducing these antiviral products, the host is not able to mount a sufficient antiviral response and clear the infection.

Before discussing chronic viral infections and the role of type I IFNs, it is necessary to note that some viruses, like rhinovirus, cause infections that fall between acute and chronic. Rhinovirus can persist in the host for weeks, but does not necessarily become chronic. It is now known that rhinovirus achieves this by blocking production of IFN-β via interception of IRF-3 activation, suggesting that this persistence may be due to
the lack of a robust type I IFN response (25). On the other hand, some viruses can persist for years or a lifetime in the host. This is the result of the evolutionary forces that allow viruses to mitigate innate and adaptive immune responses. Type I IFNs can have a negative impact on the host during persistent or chronic viral infections depending on the virus. Human immunodeficiency virus (HIV) becomes chronic and can lead to detrimental outcomes for the host. It has been shown that treatment with IFN-α is beneficial early in infection to control viral burden during HIV infection. However, once chronic infection is established, IFN-α can contribute to disease by causing dysfunctional T cell activity, increased apoptosis in uninfected cells and general immune dysregulation (26). Given that pDCs produce large amounts of type I IFNs, these cells are a new target for HIV therapy (27).

Another virus that can become chronic is hepatitis B. Once chronic infection is established, immune-mediated liver damage occurs and can result in cirrhosis or hepatocellular carcinoma (28). The cause of immune-mediated liver damage is still under investigation but a strong correlation exists between liver damage and type I IFN production. There is an association between IL-8 and IFN-α production and hepatic flares in patients with chronic hepatitis B. These flares are due to IFN-α-induced NK cell activity and expression of TRAIL on NK cells, causing hepatocyte apoptosis (28). A virus with similar tissue tropism and downstream effects on the host is hepatitis C virus. Similar to hepatitis B, it is very prone to establishing chronic infection in the host, causing severe liver damage. Interestingly, there are chronic hepatitis C patients who do not respond to the typical IFN-α and ribavirin treatment. This patient population
represents 40-50% of treated patients. These non-responders have elevated type I IFN signatures compared to those who do respond to IFN-\(\alpha\) and ribavirin. In this case, the virus is resistant and persists despite elevated type I IFN signatures whereas in responders the virus is cleared (29). These findings suggest that host genetics may influence effectiveness of treatments for chronic viral infection.

Lymphocytic choriomeningitis virus in mice behaves similar to HIV and hepatitis B/C. The immune system cannot surmount a type I IFN response that is great enough to clear the initial infection, leading to chronic infection. In addition to failure to initiate the appropriate innate immune response for viral clearance, an immunosuppressive program that solidifies persistence is established. The immunosuppression is driven by type I IFNs. It is associated with production of IL-10, an increase in PD-L1 (programmed death ligand), which promotes cytotoxic T lymphocyte exhaustion, and increased production of other suppressive factors by antigen presenting cells (30)(31). There are also reports that counter these results and show that type I IFNs derived from pDCs are critical in containing chronic LCMV. These studies determined that pDCs are a necessity in chronic LCMV infection due to their importance in adaptive immunity (15). Clearly, type I IFNs function uniquely during chronic viral infections in comparison with the response initiated during acute viral infections. This information can be used to appropriately treat chronic versus acute viral infections.

**Bacterial Infections**

The type I IFN response is not normally considered in the context of bacterial infections. Historically, type I IFNs were overshadowed by IFN-\(\gamma\), a cytokine whose
importance in bacterial infections has been known for some time. However, it is intuitive to take type I IFNs into account when considering intracellular bacteria and possibly even extracellular bacteria. Intracellular bacteria replicate in host cells, allowing for many bacterial PAMPS to engage TLRs and other receptors that reside inside the cell. Many of these receptors are directly capable of inducing type I IFNs. With respect to extracellular bacteria, a major bacterially-derived ligand for TLR4 that can and does induce type I IFNs, is lipopolysaccharide (LPS). LPS is present in large amounts on Gram negative bacterial cell surfaces. The actions of type I IFNs in the context of bacterial infections is dependent on the pathogen and can favor the host or the pathogen.

The initial finding that type I IFNs had an anti-bacterial effect was discovered with the pathogen *Chlamydia trachomatis*. When both human and mouse cell lines were infected with *C. trachomatis*, treatment with type I IFN before infection decreased infectivity of the pathogen (32)(33). This does not hold true *in vivo*, however. In a murine model of *C. trachomatis* (infection with *C. muridarum*), mice lacking the type I IFN receptor had reduced bacterial burden and cleared the infection quicker than wild type mice. This result was attributed to an increased chlamydial-specific CD4 T cell response in the mice lacking the type I IFN receptor. The exact molecular mechanisms underlying this outcome in type I IFN receptor knockout mice are still under investigation but may be related to increased CXCL9, a cytokine responsible for T cell recruitment (34).

Type I IFNs are also induced during infection with the Gram positive pathogen, *Listeria monocytogenes*. In this situation, their production is detrimental to the host (35). *L. monocytogenes* is engulfed by macrophages/monocytes, but rather than being
destroyed, the organism survives and replicates. Survival is due to Lysteriolysin O production. Lysteriolysin O is a haemolysin produced by *L. monocytogenes* that causes rupture of the phagosome in which *L. monocytogenes* resides and bacterial translocation into the cytoplasm (13)(36). Type I IFN induction is predicted to occur through an intracellular receptor and may correlate with expression of lysteriolysin O. The mode of action by which type I IFNs act against the host in this infection is believed to occur via cross talk between type I IFNs and IFN-γ. When macrophages are infected with *L. monocytogenes*, type I IFNs are secreted, causing down regulation of IFN-γ receptor on these cells. As a result, macrophages are less responsive to IFN-γ, which typically triggers macrophage resistance (36). Therefore, type I IFNs are harmful to the host because their signaling alters macrophages to become more permissive to infection.

Similar to *L. monocytogenes*, type I IFN production favors survival and replication of the pathogens *Mycobacterium tuberculosis* and *M. bovis*. *M. tuberculosis* is an intracellular bacterium which can infect macrophages and persist in the host for life. Among many other virulence factors, this organism has a secretion system called ESX1. ESX1 is part of the RD-1 gene locus and is responsible for secreting *M. tuberculosis* factors into the cytosol. These factors can activate type I IFN production and IL-1β activation via the inflammasome. However, *M. tuberculosis* has evolved to counter the effects of IL-1β by inducing type I IFNs. This only occurs with virulent strains of *M. tuberculosis* which contain the RD-1 gene locus (37). Treatment with type I IFNs greatly increased susceptibility to *M. tuberculosis* and also decreases macrophages’ ability to control *M. bovis* once they are infected (38)(39). Clearly, type I IFNs can have
deleterious effects in many bacterial infections but as with *Mycobacterium*, how and why this occurs is still under investigation. One current hypothesis is that the type I IFNs produced in response to infection with *Chlamydia, Mycobacterium*, or *Listeria* increase expression of caspases and other pro-apoptotic proteins, making lymphocytes and macrophages more sensitive to apoptosis (1). Though, there are likely a multitude of other mechanisms that drive the host type I IFN response to act in favor of the pathogen.

Contrary to the above mentioned bacterial pathogens that utilize type I IFNs to their advantage, in both *Salmonella enterica* serovar Typhimurium and *Legionella pneumophila* infections, type I IFNs protect the host (1). *L. pneumophila* infection of macrophages, in mice, is followed by production of type I IFNs that signal in an autocrine manner. This signaling induces macrophages to differentiate into classically activated, or inflammatory, macrophages which can produce nitric oxide. This coordinated response is dependent on the TBK-1/IRF3 pathway but surprisingly occurs independently of STAT1 and STAT2. Despite evidence of intracellular bacteria being detected via their nucleic acids, this is not the case for *L. pneumophila* and the intracellular sensor of this pathogen remains to be determined (40). A more recent study contradicts the role that STAT1/2 play in *L. pneumophila* infection. It showed that the protective effects of type I IFNs during *L. pneumophila* infection are lost in STAT1/2 double knockout macrophages but remain intact when either or both STAT1/2 are expressed (41). Any number of reasons could explain discrepant results but regardless, type I IFNs are key players in response to *L. pneumomphila*.
Salmonella enterica serovar Typhimurium is a Gram negative gastrointestinal pathogen, which induces the production of type I IFNs. In response to Gram negative pathogens like S. enterica serovar Typhimurium in mice, STAT4-dependent production of IFN-γ can be induced when both IFN-α/β and IL-18 are present. STAT4 activation is dependent on type I IFNs while full induction of IFN-γ involves both IL-18 and IL-12 (42). These experiments suggest a positive role for type I IFNs in S. enterica serovar Typhimurium infection. The role of type I IFN-induced necroptosis in macrophages and the subsequent negative impact on the host has been recently investigated. Mice lacking the receptor for type I IFNs infected with S. enterica serovar Typhimurium survived better than wild type mice. This result was determined to be an effect of increased resistance of type I IFN-deficient macrophages to cell death induced by S. enterica serovar Typhimurium infection despite similar inflammasome activation and cytokine expression (43). Though these results are contradictory, it is evident that type I IFNs impact the host immune response in this context.

Finally, type I IFNs are critical for bacterial clearance during infection with extracellular bacteria, Streptococcus pneumoniae and Pseudomonas aeruginosa. Type I IFN production by airway epithelial cells and resident innate immune cells, particularly macrophages and pDCs, create a milieu of cytokines and chemokines that aid in the innate immune response. This response includes inflammasome activation. For the Gram positive pathogen, S. pneumonia, the type I IFN-inducing ligand is a common virulence factor among strains called Ply. Ply is a pneumolysin, which forms pores in epithelial cells. Ply in conjunction with a streptococcal autolysin allows bacterial DNA to enter the
cell and bind intracellular receptors like DAI and STING (44). *P. aeruginosa* on the other hand is a Gram negative bacterium and therefore, its cell surface is rich in LPS, a major TLR4 ligand. It is through TLR4 and adaptor protein, TRIF, that type I IFNs are induced during infection. Interestingly, type I IFNs in this instance are partially responsible for neutrophil recruitment because downstream transcription factor, IRF3, is critical. It was shown that mice lacking IRF3 have dampened type I IFN production and do not clear the infection as well. This was attributed to reduced neutrophil and macrophage recruitment to the lung (45)(44).

Despite the stark differences in the outcome of type I IFN production between various bacteria, a fascinating similarity exists in how some Gram negative bacteria are sensed. It is required in every case that the bacteria be metabolically active in order to successfully induce production of type I IFNs. During metabolically active states and replication within the cell, many metabolites and other cellular factors are being released into the cytosol where they bind to receptors, leading to type I IFN production (46). Given the fact that each type I IFN-inducing bacterial pathogen has a unique outcome post type I IFN induction, these infections need to be considered individually and handled on a case by case basis.

**Autoimmune Disease**

The connection between some viral and bacterial infections and autoimmunity has long been known. More recently, type I IFNs have been investigated as a possible bridge between infection and autoimmunity. Not surprisingly, in the context of autoimmunity, both infectious and of unknown etiology, type I IFNs can be either detrimental or
beneficial to the host. When type I IFNs act against the host, it is due to overt activation of dendritic cells. This can be amplified by unnecessary antigen presentation and response to host antigens, likely derived from apoptotic cells and production of damaging autoantibodies (1). It has been suggested that type I IFNs may have antitumor activity in mouse models of cancer because mice injected with type I IFN displayed long lasting antitumor effects. This may be attributed to the ability of type I IFNs to induce differentiation of monocytes into IFN-producing dendritic cells which cause T and B cell immunity (47). Though the action of type I IFNs in cancer will not be discussed, this information is relevant to autoimmune diseases like systemic lupus erythematosus (SLE), insulin-dependent diabetes mellitus (IDDM/type I diabetes), and multiple sclerosis (MS). Once again, this cytokine’s pleiotropic effects govern many different outcomes depending on autoimmune context.

SLE is an autoimmune disease that is associated with the generation of immune complexes composed of host cell nuclear material. These immune complexes, in conjunction with degeneration of self-tolerance and declined ability to clear apoptotic cellular debris, lead to major immune dysfunction and autoimmune consequences. A characteristic feature in SLE patients is the elevation of genes regulated by type I IFNs and type I IFNs themselves. Elevated levels of IFN-α specifically have been noted in SLE patients for many years, but increases in IFN-β do not necessarily occur in conjunction. These higher levels of IFN-α can cause maturation of circulating monocytes into dendritic cells. Though pDCs are thought to be sentinels that protect against autoimmunity, they are a major source of type I IFNs and contribute greatly to SLE
disease (1)(48). The exact cause of elevated IFN-α levels in SLE patients is still somewhat elusive, but it is hypothesized that there are both endogenous and exogenous inducers. Exogenous inducers would be bacterial or viral infections and endogenous inducers are host-derived. Host-derived immune complexes made up of nucleic acids signal through TLR7 and TLR9. This leads to type I IFN production and signaling through these TLRs on pDCs and causes the cells to become unresponsive to glucocorticoids. This response prevents treated patients from benefiting from the drug (49). Since type I IFNs are important for plasma cell production, this is a potential point of overlap between production of autoantibodies and elevated levels of IFN-α (50).

Similar to SLE, multiple sclerosis (MS) is caused by a dysfunctional immune response that leads to autoimmunity. MS is a disease of the central nervous system. The end result is host axonal damage, demyelination and general neurodegeneration. The etiology of MS is complex and somewhat unknown. It is believed that a combination of host genetics and environment lead to the generation of autoreactive T cells. These cells destroy the myelin sheath, causing a high degree of damage to the central nervous system of the affected individual. Contrary to SLE in which type I IFNs are a damaging agent, treatment with type I IFNs in some individuals with MS relieves symptoms. IFN-β is one of the most effective treatments available however, other perspectives are currently being investigated. These include targeting the main autoreactive T helper cell subset in patients, which must be addressed on a per person basis, and targeting myeloid cells and factors important for their activation, trafficking, and survival (51)(52). In regards to IFN-β treatment for MS and in the mouse model of MS, experimental autoimmune
encephalomyelitis (EAE), it acts by suppressing the activity of NLRP1 and NLRP3 inflammasomes, in turn decreasing IL-1β production. These mechanisms have been effective in particular for patients that are in the relapsing-remitting phase of MS. In addition to the dampening effect on IL-1β production, IFN-β also works by aiding in the maintenance of the blood brain barrier and affects innate immune cells such as DCs and macrophages that can inhibit T helper 17 cells via IL-27 (53)(54). In addition, mice lacking the gene for IFN-β have exacerbated disease in EAE. These knockout mice had more severe damage to the central nervous system and had higher rates of chronic EAE development. The study attributed more severe disease in IFN-β knockout animals to increased microglia activation which led to greater cytokine production, tissue damage and general inflammation (55). Despite contrasting evidence in support of IFN-β treatment in MS and EAE, the function of type I IFNs in these circumstances is clearly important.

Insulin-dependent diabetes mellitus (IDDM, type I, or juvenile diabetes) is an autoimmune disease in which autoreactive T cells attack insulin-producing pancreatic β cells. This results in hyperglycemia and ketosis among other symptoms of diabetes mellitus (56). Many studies have confirmed both beneficial and damaging effects of type I IFNs in IDDM patients and animal models of the disease. As with SLE, higher levels of IFN-α have been observed in the serum of IDDM patients, but in the case of IDDM, they are also elevated in the pancreas. Additionally, when individuals with chronic viral infections are treated with type I IFN, IDDM-like symptoms have been observed as a side effect. Other studies involving transgenic mice expressing increased levels of IFN-α or -
β, or mice treated with type I IFN-inducing agent, polyI:C, have confirmed a negative effect of type I IFNs in this setting (57). Even though many studies support this angle of type I IFNs in IDDM, different studies state otherwise. In two different rodent models of the disease, BB rats and NOD mice, administration of IFN-α ameliorated symptoms. In BB rats, treatment decreased insulitis but was more beneficial when administered at an earlier age (58). In NOD mice, another rodent model of human IDDM, IFN-α treatment decreased the activity of anti-islet effector cells. This action was observed without the induction of general immunosuppression and surprisingly, according to these investigators, treatment of non-diabetic mice with IFN-α itself did not alter glucose tolerance (59). Similar results were observed in NOD mice treated with polyI:C as well, the opposite of what occurred in other studies (57). Though it is not obvious in what context of IDDM type I IFNs are beneficial or detrimental, it is clear that they are involved in this disease.

Justification and Relevance of Thesis

Treatment options for bacterial and viral infections and autoimmune disorders exist but often come with challenging side effects. It is widely known that there is an ongoing race between researchers’ ability to generate new antibiotics and antivirals and bacterial and viral pathogens’ ability to evolve mechanisms against these drugs. With the discovery of penicillin in the 1920’s came an era of rapid antibiotic development. Unfortunately, after time, use of each of these novel antibiotics led to antibiotic resistance, which is still a problem today. All infectious and pathogenic microorganisms
are capable of evolving mechanisms that subvert or prevent the actions of their respective treatment. As viral and bacterial infections and autoimmune diseases are the focus of this thesis, fungal and parasitic infections will not be discussed. While some bacteria are naturally resistant to antibiotics, others are not but can acquire this resistance via plasmids, for example. In addition, some bacteria contain the genes necessary to produce efflux pumps that pump the antibiotic out of the cell (60)(61). Antivirals including nucleoside analogs like acyclovir work by terminating chain elongation in viral DNA. In this instance, resistance occurs when thymidine kinase, a viral enzyme that adds monophosphates to nucleosides, mutates or when viral DNA polymerase mutates (62). In conjunction with antivirals, type I IFNs can also be used as treatments for chronic viral infections. As mentioned earlier, chronic hepatitis C is often treated with a combination of antivirals and IFN-α. However, IFN-α can have a number of side effects including flu-like symptoms, decreased granulocytes and thrombocytes in the bone marrow, and autoimmune disorders like thyroiditis (63). Similar side effects can occur with IFN-β therapy used for MS (64). Additionally, treating patients with type I IFNs poses the risk of the host immune system developing type I IFN-neutralizing antibodies (65). Clearly, current treatment options for bacterial, viral and autoimmune diseases have their drawbacks.

Difficulties like these necessitate the development of novel therapeutic treatments. A unique field of study for developing new treatments revolves around the immunomodulatory properties of natural compounds that are derived from plants. One group of compounds, polyphenols, are plant metabolites which are chemically distinct
because they have many phenol rings. They are unique to the plant kingdom and can be found in many plants, including grapes, pomegranates, teas and apple peels (66)(67). Plants produce a large variety of polyphenols with various structures and have a variety of functions from regulation of hormones to protection from herbivores (67). Polyphenols are antioxidants and are thus best known for their antioxidant properties. However, recent work has shown that specific types of polyphenols can, in fact, impact the immune system and have immunomodulatory properties. By currently unknown mechanisms, pomegranate-derived polyphenols successfully blocked influenza virus replication, had virucidal properties and worked synergistically with the antiviral, oseltamivir (68). Additionally, other publications have been able to hone in on a class of polyphenols with more potent anti-viral properties. This class, oligomeric procyanidins (OPCs), is composed of large procyanidins and has been described to block hepatitis C replication (69). Recently, our group reported that OPCs derived from unripe apple peels (APP) have direct anti-viral effects in in vitro dengue virus infection. It was determined that APP not only decreased dengue virus titers in vitro but they also had broader immunomodulatory effects. Not surprisingly, the pathway affected was the type I IFN pathway. Treating high MOI dengue virus-infected human PBMCs with APP increased expression of MX1 and IFN-β, two key players in the anti-viral response. In addition, treating human PBMCs with APP and type I IFN increased STAT2 phosphorylation. This observation pointed to a possible mechanism for this effect. Finally, in low MOI infection of human PBMCs treated with OPCs and infected with dengue virus, increases in STAT1 gene expression and MHC I and TNFα protein expression were observed. Altogether, this study
highlighted the innate immune-stimulating capacity of OPCs, which is a new potential area of therapeutic investigation (66).

This thesis will address the work that our group published in response to the initial publication by Kimmel et al (66) and the following work that developed from my knowledge of the type I IFN pathway and applied to Clostridium difficile (Chapter 3). Chapter 2 will consist of the publication that stemmed from a follow up project and delineates the effects of OPCs on type I IFN signaling in vivo. This work led to investigation of other diseases in which type I IFNs may play a role, in which case APP or other OPCs could be used as therapy. I then investigated type I IFN signaling in Clostridium difficile infection and was able to show from multiple angles that this signaling pathway likely plays a critical role in host immunity in mice. Thus, APP or other OPCs could be applied to this disease model in the future. Type I IFN signaling in C. difficile infection will be discussed in Chapter 3.
CHAPTER TWO

ORAL DELIVERY OF OLIGOMERIC PROCYANIDINS IN APPLEPOLY® ENHANCES TYPE I INTERFERON RESPONSE IN VIVO

Contribution of Authors and Co-Authors

Manuscripts in Chapter 2

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Running title: Supplement enhances type I IFN responses in vivo

Summary sentence: Responses to type I IFN are augmented following ingestion of
Applepoly® and may have therapeutic application to enhance antiviral immunity or
suppress inflammation.

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Abbreviations: IFN-interferon, APP-Applepoly®, OPC-oligomeric procyanidin, RES-
Resveratrol, AS-Acai Seed
Abstract

Type I interferon (IFN) signaling is a central pathway that provides critical innate protection from viral and bacterial infection, and can have regulatory outcomes in inflammatory settings. We previously determined that oligomeric procyanidins (OPCs) contained in the dietary supplement Applepoly® (APP) enhanced responses to type I IFN in vitro. Here we confirm that OPCs from two different sources significantly increased phosphorylation of STAT1, whereas a monomeric form of procyanidin did not. We hypothesized that similar responses could be induced in vivo following ingestion of APP. Ingestion of APP prior to injection of polyI:C enhanced in vivo responses to type I IFNs in mice. When human subjects ingested APP, enhanced responses to type I IFN and enhanced phosphorylation of STAT1 ex vivo were detected, whereas ingestion of Resveratrol, a monomeric polyphenol, induced minimal such changes. Polyphenols are best known for induction of anti-inflammatory and antioxidant responses, however our findings suggest a unique non-antioxidant aspect of OPCs that is broadly applicable to many disease settings. The capacity of oral OPCs to enhance type I IFN signaling in vivo can augment innate protection and may, in part, contribute to the noted anti-inflammatory outcome of ingestion of OPCs from many sources.
Introduction

Type I IFN signaling is a central cellular process with a broad range of downstream effects. Type I IFNs are immediately induced upon sensing viral molecular patterns and signal to protect both infected and uninfected cells, thus are a major component of innate antiviral immunity (70). As such, recombinant type I IFNs are used to treat chronic viral infections (71), and have been shown to be effective therapies for a broad range of viral threats (72-75). Type I IFN can also result in anti-inflammatory signaling and has long been used as a therapy for Multiple Sclerosis (MS) patients (76), but only recently have specific mechanisms of protection been discovered (77,78). Type I IFNs also have a critical role in early anti-tumor immunity by stimulating dendritic cells (79) and have been used as cancer therapy (80). Additionally, type I IFN signaling contributes to optimum type II IFN (IFN-γ) responses (81) and promotes hematopoiesis (82). Type I IFN signaling is clearly important in multiple disease settings and is a clinical target. An orally delivered novel immunotherapy that enhances this signaling pathway may have broad therapeutic application as a combination therapy, or could potentially obviate IFN injections.

Polyphenols are chemical compounds found in varying concentrations and types in many dietary sources, such as pomegranates, grapes, and tea. Polyphenols have widely been shown to have antioxidant and anti-inflammatory capacity (83,84). Applepoly® (APP) is an extract from unripe apple peel that contains high concentrations of oligomeric procyanidins [OPCs, (85)]. This supplement has notable anti-inflammatory effects in a mouse colitis model that are dependent on αβ T cells (83). OPCs are
composed of 2 to 10 repeats of hydroxylated Flavan-3-ol building blocks, known as catechin or epicatechin (Fig. 1A). OPCs from apple peels and other sources such as grape seed and marine pine bark (Pycnogenol®) are already known to be safe to ingest (67,86). We have recently defined innate immune enhancing capacity of OPCs from APP that is clearly distinct from antioxidant activity (66,67,85,87,88). Using dengue virus infected human cells in vitro we discovered that treatment with OPCs derived from APP decreased viral titers and exhibited a unique antiviral mechanism: enhanced responses to type I IFN (66). Here, these results were confirmed by demonstrating that two robust sources of OPCs enhanced the phosphorylation of STAT1, which occurs following type I IFN receptor interaction, whereas a monomeric procyanidin did not. The primary goal of this investigation was to demonstrate that enhanced type I IFN signaling could also be measured in vivo following ingestion of APP. Ingestion of APP enhanced type I IFN signaling in mice injected with the double stranded RNA mimetic polyI:C, and optimal responses were detected a week after APP ingestion. Underscoring the medical relevance of these data, APP ingestion by human subjects induced enhanced responses to type I IFN in blood cells cultured ex vivo. These data indicate that minimal oral doses of APP enhance type I IFN responses in vivo. The capacity for a simple, safe oral supplement to enhance responses to type I IFN may have broad potential for enhancing innate protection from virus infection or for use as a combination therapy for patients receiving recombinant type I IFN for chronic viral infection, autoimmune conditions or cancer.
Materials and Methods

Human cells in vitro. To assess the effects of OPCs and catechin on the phosphorylation of STAT1, total PBMCs were isolated using Histopaque (Sigma), as previously described (66), and cultured for 24 hours with OPCs derived from APP (66), water extract of APP, a newly identified source of OPCs [methanol extract Acai seed (AS)], catechin, or medium alone. The cells were then stimulated for 15 minutes with rIFN-α (100U/ml, Human Interferon-Alpha A/D, PBL Interferon Source), fixed, permeabilized, and stained with Phosflow™ pSTAT1 PE antibody (BD Biosciences) using manufacturer’s protocols, and analyzed using a FACS Caliber cytometer and FlowJo software. In additional experiments, cells were treated for 3 or 24 hours with APP, then washed and incubated an additional 48 hours in culture followed by the addition of rIFN-α and similar analyses.

Induction of type I IFN responses by polyI:C injection. All animal studies were carried out in compliance with the Montana State University Institutional Animal Care and Use Committee. Six week old BALB/c mice were fed either water or APP extract doses (5 mg per 100ul, based on approximate equivalent to 1g dose in humans (89) at varying intervals then 1ug of polyI:C was delivered by intraperitoneal injection. Mice were sacrificed after 4 hours and livers (1 lobe) and whole spleens were homogenized, RBCs were water lysed, and cells were lysed for RNA extraction using RNeasy Mini Kit (Qiagen).

Human subjects studies. Studies involving human subjects were carried out in compliance with the Montana State University Institutional Review Board as such, each subject signed informed consent documentation. In the first study, blood was collected
from volunteers having ingested nothing (NEG), or from the same subjects ($n=11$) 2 weeks later, 90 minutes to 2 hours after ingestion of 3g APP (from applepoly.com, 6 capsules). This alternating pattern was repeated 4 times (Rounds 1-4) and continued in the absence of APP ingestion for 3 additional cycles (Rounds 5-7). In the second study, ingestion of APP was compared to ingestion of Resveratrol (RES) in a randomized and double blind setting. Starting with ten subjects per group, groups either ingested 3g APP or 3g RES (Biotivia Transmax) and blood was collected 2 hours later (Round 1). Round 2 was a repeat performed 3 weeks later. Round 3 in which subjects did not ingest supplements, was performed 4 weeks later and the same parameters were compared. In both studies, subject groups were alternated and mixed such that blood from a maximum of 6 subjects was analyzed per day to diminish the possible effects of day to day variation biasing the results. Diets and activities of volunteers were not otherwise restricted. Total PBMCs were isolated as previously described (66) and stimulated ex vivo with medium or 100U/ml rIFN-α. Cells in separate wells were stimulated 4 hours then lysed for RNA extraction and qPCR or cultured for 24 hours for analyses by flow cytometry. Fold induction was calculated by dividing the IFN treated value by the medium only value from cells from the same donor.

Flow cytometry with human cells. Human PBMCs were stained with antibodies specific for CD11b (to identify mononuclear cells, Clone ICRFF44, BD Pharmingen), and MHC I (Clone W6/32, Biolegend) and CD86 (Clone IT2.2 Biolegend), which increase in expression in response to type I IFNs (90,91). Cells were stained using standard
protocols for flow cytometry and analyzed using a FACS Caliber cytometer and analyzed using FlowJo Software, MS Excel and GraphPad (Prism).

RNA Extraction and RT-qPCR. RNA from both mouse tissues and human cells was extracted, reverse transcribed, transcript levels assessed and analyzed as previously described (66).

Statistical analyses. Statistical significance was determined using both parametric and non-parametric tests. Data presented was analyzed using the unpaired one-tailed t test, unless otherwise noted.

Results and Discussion

To confirm earlier in vitro data and investigate whether APP’s effect on the type I IFN pathway was specific to compounds containing OPCs, an in vitro assay measuring STAT1 phosphorylation was performed. Human PBMCs were incubated for 24 hours with either a water extract of APP, a water extract of another recently identified robust source of OPCs (Acai seed, AS), OPCs derived from APP as previously described (66), catechin, or medium only. Figure 1.1B demonstrates that the lower concentrations of both APP and AS augmented phosphorylation of STAT1 on CD11b+ cells in response to rIFN-α. After 24 hours incubation, AS at 1ug/ml, and all OPC sources at 10ug/ml, including OPCs isolated from APP (Fig. 1.1B inset), also induced STAT1 phosphorylation in the absence of rIFN-α. Incubations for 1, 3 or 6 hours with 10ug/ml APP did not induce any such changes (data not shown). Catechin, a monomeric
Figure 1.1 OPCs, and not monomeric procyanidins, augmented STAT1 phosphorylation. A. Chemical structures of oligomeric procyanidin (OPC) and monomeric procyanidins. B. Incubation with robust sources of OPCs [APP and Acai Seed (AS) extracts], but not catechin, in culture resulted in direct augmentation of STAT1 phosphorylation (on CD11b+ cells) with and without exogenous type I IFN. Inset is a representative plot comparing the phosphorylation of STAT1 in human PBMCs treated with either OPCs derived from APP (dark line), catechin, and media only (light lines). On the right side of the graph, cells were initially incubated for 24 or 3 hours with APP, then were washed, Figure 1 continued and incubated for an additional 48 hours, for a total of 72 hours. Longer term incubation enhanced the responses to exogenous type I IFN, but eliminated direct responses of APP on pSTAT1. Asterisks denote significant difference from similarly treated media only samples. Students t test, unpaired (*p<0.05, **p<0.01).
procyanidin, induced responses similar to medium only controls. These data suggest that OPCs, and sources of OPCs, but not monomeric procyanidins, enhanced STAT1 phosphorylation in vitro, and that monomeric polyphenols might be utilized as negative controls. Additional experiments addressed the necessary interval for pretreatment with APP. To investigate a longer term response, cells were treated with APP for 3 or 24 hours then washed, and incubated for an additional 48 hours before treatment with rIFN-α. In this case, direct phosphorylation of STAT1 in the absence of rIFN-α was not affected, but in response to rIFN-α, pSTAT1 was greatly increased by APP treatment over medium only controls. These data suggest that APP treatment primes cells to better respond to type I IFN, and maximal priming response takes days to fully develop. Anti-inflammatory outcomes are the most commonly described effect of polyphenol ingestion (83,84), which may be generally related to their antioxidant capacity. OPCs, but not monomeric forms, reduce dengue virus titers in vitro (66) and enhanced phosphorylation of STAT1, even though these polyphenols have similar antioxidant capacity. Collectively, our results show that OPCs have a novel and unique effect on responses to type I IFN signaling that is distinct from the well characterized antioxidant effects.

The effects of ingestion of APP extracts on responses to type I IFN in vivo were assessed by measuring expression of type I IFN stimulated gene (ISG) transcripts following injection of mice with polyI:C. To determine the optimal oral dose of APP, mice were divided into 4 treatment groups and received, by oral gavage, one 5mg APP dose 36 hours prior to injection, four 5mg APP doses or water only at 36, 24, 12 and 0
hours prior to injection, or the same four doses every 12 hours given 1 week prior to injection. Mice were injected with polyI:C and tissues were collected 4 hours later and

Figure 1.2. Oral APP enhanced responses to type I IFN in vivo. Mice (n=5) were fed 5mg doses of APP or water at various intervals as noted on x axis then injected with 1ug polyI:C. A. Four doses of APP one week prior to injection with polyI:C was the most affective dose for enhancing expression of ISGs in the spleen and liver, although other dosing intervals were effective for enhanced expression of some ISGs. B. After four doses of APP one week prior to injection with polyI:C, type I IFN transcripts were more detectible in the liver than the spleen, however, significant differences between APP fed mice and water fed mice were only detectable in the spleen for IFN-α (*p<0.05, **p<0.01 by unpaired t test, asterisks denote statistical significance of difference between indicated group and water treated group).
processed for RT-qPCR analyses. Four hours was empirically determined to be the optimal point for ISG transcript expression following polyI:C injection. ISG transcripts were not detected following ingestion of APP alone, nor were they detected 1 hour after polyI:C injection (data not shown). ISG transcripts SOCS1, OAS1 IFIT1 and IFIT2 were more robustly induced in the spleen, whereas expression of CXCL10 was greater in the liver (Figure 1.2A). Consistent with in vitro findings suggesting enhanced activity following multiple days (48 hours) of incubation, delivery of APP one week prior to polyI:C injection was the most effective dosing interval for enhanced type I IFN responses, as this dose significantly increased expression of SOCS1, IFIT1 and IFIT2 and CXCL10 compared to water-fed controls. Only the 4 doses within 36 hours of injection significantly altered OAS1 expression in the liver and feeding APP did not alter OAS1 expression in the spleen. The 4 doses of APP within 36 hours of injection also significantly affected IFIT1 and IFIT2 expression in the livers, as did one dose 36 hours prior affect IFIT2 expression. Delivery of APP a week prior to injection induced fold increases of 4.7 and 3.9 in CXCL10 expression in spleens and livers, respectively, relative to water treated mice. This increase was much greater than the induction of other ISGs, which was between 1.8 and 2.8 fold. These data indicate that oral APP affected ISG expression in vivo and the dosing interval that most affected gene expression was that delivered 1 week prior to injection of poly I:C. The relatively long term effect of minimal OPC ingestion was consistent with in vitro findings, and may indicate that OPCs prime cells for enhanced responses to type I IFN in vivo, and optimal detection of the primed state in a large fraction of cells requires days to fully develop.
Poly I:C injection induces type I IFN expression, however, a potential cause of enhanced ISG expression in the APP fed group may be further increased expression of type I IFNs themselves, so this possibility was investigated. Transcripts that encode type I IFNs were measured in spleens and livers of mice that received water, or APP at the most effective dosing interval, 1 week prior to polyI:C injection (Fig. 1B). Type I IFN transcripts were detectible following polyI:C injection, and levels were similar between the two groups. Relative to the livers, the type I IFN transcripts were only detectable at very low levels in spleens. The only significant difference between the water and APP-fed groups was a 1.65 fold increase in IFN-α1 expression in the spleens of the group that received APP relative to the water fed mice. No significant differences between groups were noted in the liver for either IFN-α1 or IFN-β transcripts (Fig. 1B). Similarly, and as expected, there were no differences in either tissue in expression of IFN-α transcripts (data not shown). The slightly greater expression of IFN-α1 in the spleens of APP fed mice offers a possible explanation for the increase in ISGs expression observed in this tissue, but this was clearly not the case in the liver, where ISGs increased independently of type I IFN expression. These data confirm our in vitro findings and suggest that ingestion of OPCs does not significantly change type I IFN expression, but enhances type I IFN signaling in vivo.

To maximize the clinical and translational potential of our findings, small-scale human subject experiments were performed to demonstrate that ingestion of APP increased responses to type I IFN by human PBMCs cultured ex vivo. In the first study, healthy subjects consumed nothing (NEG) and 2 weeks later consumed 3g of APP and
Figure 1.3. Ingestion of polyphenols by human subjects enhanced ex vivo responses to type I IFN. Human subjects (n=11) either ingested nothing (NEG) or, two weeks later, a single dose of 3g Applepoly® (APP). This was repeated for 4 Rounds. During Rounds 5-7, APP ingestion was discontinued and blood was collected from the same subjects at 2 week intervals. A. CD86 expression on PBMCs increased following ingestion of APP and remained elevated after multiple doses. B. Fold induction of MHCI (on CD11b+ cells, normalized to media only) by 24 hour treatment with rIFN-α increased during alternating 2 week intervals of nothing (NEG) and (APP) ingestion and remained elevated. C. Induction of RIGI transcripts by 4 hour incubation with IFN-α was increased in Round 1, and remained elevated after Round 2 (paired, *p<0.05, **p<0.01).
this pattern was repeated. Responses were compared to PBMCs from the same individual, thus, each individual served as their own negative control. After 24 hours in ex vivo culture and no other treatment, there was a significant increase in CD86 mean fluorescence following the first and second doses of APP compared to when the same subjects initially ingested nothing. At the beginning of Round 3, expression of CD86 remained significantly elevated compared to levels at the beginning of Round 1, and was not further increased in subsequent Rounds by ingestion of APP (Figure 1.3A). Thus, even despite the discontinuation of APP ingestion, expression of CD86 remained elevated for several weeks suggesting a very long lasting effect on CD86 expression induced by multiple doses of APP. A similar pattern was evident for MHC I induction in response to rIFN-α treatment ex vivo. The fold induction of MHC class I (on CD11b+ cells, Fig. 1.3B) was significantly greater following the first and second APP ingestion. Induction of MHC I at the start of Rounds 3 and 4 was significantly greater than at the start of Round 1 and was not further increased in these later Rounds by supplement ingestion. Using cells from the same subjects, parallel analyses of induced gene expression in response to rIFN-α indicated a similar trend as that seen with MHC I. We measured ISG transcripts; IFIT1, MX1, SOCS1, and RIGI after type I IFN treatment for four hours. ISGs were not expressed in the absence of type I IFN treatment. RIGI was consistently induced by type I IFN, and fold induction (over untreated cells from the same donor) was increased following APP ingestion (Fig. 1.3C). As with MHC I and CD86, subsequent Rounds did not further induce RIGI expression and its fold induction at the start of Round 3 and 4 without APP ingestion (NEG) were similarly elevated compared to the
start of Round 1. Induction of IFIT1, SOCS and MX1 was not as consistent as that of RIGI (data not shown). These data indicate that ingestion of minimal doses of a rich source of OPCs can enhance ex vivo expression of proteins and transcripts induced by low levels of type I IFN and that the effect of multiple doses may be long lasting.

In the second study, ingestion of APP was directly compared to ingestion of the same dose of RES and following two doses of each, with three weeks between the doses, there were significant differences between the groups. There was a significant increase in fold induction of MHC I in the cells derived from those who ingested two doses of APP compared to those that ingested the same amount of RES (Fig. 1.4A). However, in this study, there was no difference in CD86 expression between subjects that ingested APP or those that ingested RES, rather, CD86 mean fluorescence increased in both groups. These data suggest that increased CD86 expression may be enhanced by ingestion of polyphenols in general, or is increased by placebo effect. Similar to the findings for MHC I, fold induction of RIGI expression was significantly higher following APP ingestion compared to ingestion of RES (Fig. 1.4B). We expanded this study to include transcripts encoding IL-10 and IL-27, because these anti-inflammatory cytokines are likely involved in the benefit of type I IFN treatment in EAE, a mouse model of MS (77,78). Both of these transcripts were also induced to a significantly greater level in subjects that ingested APP compared to those that ingested RES (Fig. 1.4B). Anti-inflammatory responses following ingestion of some polyphenol containing supplements may be due, in part, to enhanced type I IFN responses through enhanced expression of anti-inflammatory cytokines IL-10 and IL-27. IL-27 receptor signaling, in particular, has
Figure 1.4. Ingestion of APP induced enhanced responses to type I IFN compared to ingestion of RES. Subjects either ingested two doses of 3g APP (n=7) or 3g RES (n=9) separated by 3 weeks. A. APP ingestion resulted in enhanced induction of MHC I expression in response to type I IFN compared to ingestion of RES. Expression of CD86 in media treated cells was increased in blood from both treatment groups. B. Following doses of each supplement, relative to RES, ingestion of APP by human subjects resulted in enhanced induction of the type I IFN-stimulated transcripts RigI, IL-10 and IL-27 in response to rIFN-α ex vivo. After the second ingestion of APP or RES, blood cells from a subset of human subjects were collected assessed for phosphorylation of STAT1. C. A representative FACS plot demonstrating enhanced baseline (media, left) and rIFN-α stimulated (right) phosphorylation of STAT1 in subjects that ingested APP (shaded) compared to subjects that ingested RES (lines). D. MFI of phospho-STAT1 indicated enhanced phosphorylation in subjects that ingested APP (n=4) compared to subjects that ingested RES (n=6). These data were compared using the unpaired, t test *p<0.05, **p<0.01.
recently been shown to specifically decrease inflammatory cytokine expression, primarily IFN-γ and trafficking by CD4+ T cells (92,93). These cells are a primary source of inflammation in the mouse model of colitis (94). APP-induced expression of IL-27 through enhanced type I IFN signaling may be, in part, responsible for the noted suppression of inflammation by APP that was dependent on αβ T cells in a colitis model (83). Enhancing type I IFN expression may be an unrecognized novel mechanism responsible for at least some of the noted beneficial and immunomodulatory outcomes following ingestion of OPC-containing dietary supplements. Because of the pleiotropic effects of type I IFNs, this response may be masked in other studies by the robust antioxidant and inhibitory responses common to polyphenols or, the responses truly are a unique aspect of OPCs from limited sources.

We also assessed differences in STAT1 phosphorylation ex vivo in human cells following two doses of APP or RES. PBMCs from a subset of subjects were cultured overnight, then stimulated with rIFN-α for 15 minutes, fixed, permeabilized, and stained with the antibody specific to phosphorylated STAT1. Similar to the in vitro findings (Figure 1B), both the basal and rIFN-α-stimulated levels of STAT1 phosphorylation were slightly increased in subjects that ingested APP compared to those that ingested RES (Fig 1.4C). When this response was compared in multiple donors, there was a statistically significant increase in IFN-α-induced phosphorylation of STAT1 in subjects that ingested APP compared to subjects that ingested RES (Fig. 1.4D). For each of the parameters shown in Figure 4, there were no significant differences between APP and RES groups following 1 dose, nor were the differences retained four weeks after the second dose (data
not shown), suggesting multiple occasional small doses of OPCs are necessary to retain the effects, and two doses is not sufficient for a long lasting change. These data indicate that ingestion of APP induced enhanced type I IFN responses in mice and human subjects. This response may be dependent on OPCs found in a limited set of dietary sources.

An orally delivered, broad-spectrum therapy that enhances type I IFN responses has not been previously described. Type I IFNs are immediately induced upon sensing viral molecular patterns and are a critical component of innate antiviral immunity. Our therapeutic defense against viruses is currently limited to a few antiviral drugs and treatment with recombinant type I IFN (73). Our data suggest that ingested APP has long lasting effects on expression patterns downstream of type I IFNs. These results support a novel biological effect of OPC sources in vivo that may be a viable anti-viral prophylactic approach. Recombinant type I IFN is used in antiviral therapies for human hepatitis C virus (71,95) and is effective in models of influenza infection (72,74). Thus, oral OPC sources could also be used as a combination therapy to augment the effects of type I IFN therapy for chronic viral infection (71,95), especially considering the detected responses in the liver following APP ingestion in mice. Anyone at increased risk of viral infection and complications, including the elderly, parents of small children, teachers and healthcare workers could greatly benefit from enhanced innate protection from viral infection.

Type I IFN has also long been used as a therapy for MS patients (65,76), but only recently have specific mechanisms of protection involving enhanced expression of anti-
inflammatory cytokines IL-10 and IL-27 been discovered (77,78). Responses to type I IFN therapy differ greatly between patients, thus an oral treatment that globally increases responses to type I IFN may increase efficacy of this therapy. A complication of type I IFN treatment is the necessity to administer large continuous doses resulting in IFN-specific neutralizing antibodies that render treatment less effective (65). Use of oral OPCs as a combination therapy with injected or ingested type I IFNs could potentially decrease the effective dose and thereby diminish side effects. Furthermore, enhancing responses to endogenously expressed type I IFNs could obviate the need for any additional therapy. Only palliative therapy is available for most inflammatory conditions, including MS, thus, inventive therapeutic approaches are clearly warranted.

There are few aspects of disease that are not affected by type I IFN signaling. Type I IFNs are critical for immune responses to tumors (79), can impact hematopoiesis (96), and have recently been appreciated as critical for protection from some bacterial infections (44,97,98). Characterization of this novel effect of ingestion of OPCs on type I IFN signaling is likely to provide insights into in vivo mechanisms and strong rationale for its recommended application in an extensive range of disease conditions. Considering the paucity and complications of existing therapies, and the far-reaching implications of the type I IFN pathway, the capacity for a simple oral supplement to enhance responses to type I IFN expressed endogenously, during infection, or delivered as a therapeutic recombinant protein could strongly impact the field.
Acknowledgements

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CHAPTER THREE

TYPE I IFN DEPENDENT AND INDEPENDENT HOST RESPONSES DURING CLOSTRIDIUM DIFFICILE INFECTION

Contribution of Authors and Co-Authors

Manuscripts in Chapter 3

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Contributions: Co-PI. Designed, performed and analyzed experiments.

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Type I IFN dependent and independent host responses during *C. difficile* infection


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Running Head: Mechanisms of protection in *C. difficile* infection

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ABSTRACT

Nosocomial *C. difficile* infection (CDI) is increasingly prevalent among patients in diverse health care settings and is becoming a concern in food animals. *C. difficile* is a Gram-positive, anaerobic bacteria known for its ability to form spores that are resistant to many common disinfectants and antibiotics. Disruption of the normal gut microbiota allows *C. difficile* spores to germinate and produce toxins that compromise the intestinal epithelial barrier, resulting in extreme inflammation. Little is known about the innate immune response during *Clostridium difficile* infection (CDI). We hypothesized that type I IFNs may play a role in innate protection from CDI due to their diverse roles in inflammatory and infectious diseases. We found that IFN-α/β receptor (IFNAR)-deficient mice succumbed to CDI more rapidly compared to wild type mice. This effect was independent of weight loss. In addition, wild type mice treated with the type I IFN-inducing dsRNA mimetic, polyI:C, or exogenous type I IFN, had delayed onset of symptoms compared to sham-treated (saline) mice. In addition, polyI:C induced similar immune-altering effects in both wild type and IFNAR-deficient mice. Collectively, these findings suggest that type I IFN signaling may protect from CDI and that polyI:C may have the capability to do so by both IFNAR-dependent and -independent mechanisms. Considering that recombinant type I IFN and a polyI:C mimetic are already used clinically, investigation of downstream pathways, may represent new treatment options for this common infection.
INTRODUCTION

*Clostridium difficile* is a Gram positive, spore-forming, anaerobic, rod-shaped bacterium that is responsible for nearly all cases of antibiotic-associated pseudomembranous colitis. According to the Centers for Disease Control and Prevention (CDC), *Clostridium difficile* infection (CDI) is increasing in prevalence, and is responsible for > 14,000 deaths in the United States each year. Most cases are linked to spore exposure from the environment and person-to-person contact but foodborne transmission from agricultural animals is a growing concern (99). Patients on antibiotics often have a disturbed gut microbiota, which leads to changes in abundances of particular bile acids. These bile acids include taurocholate, which can cause *C. difficile* spores to germinate (100). Following germination, vegetative *C. difficile* cells produce at least one of two large exotoxins, *C. difficile* toxin B and/or A, that damage epithelial cells. Treatment options for mild *C. difficile* infections include discontinuation of current antibiotics and administration of different antibiotics like metronidazole or vancomycin, or intravenous fluids. More severe cases may require surgery (101)(102). However, other treatments are being investigated, including fecal microbiota transplants, vaccines to the toxins produced by *C. difficile*, and administration of probiotics (103).

Recurrent CDI is a significant problem, affecting 15-35% of CDI patients, and is responsible for the majority of CDI-associated healthcare costs (104). Similarly, certain patient populations are at increased risk for CDI, including the elderly, those undergoing gastrointestinal surgeries or procedures, inflammatory bowel disease patients, and immunosuppressed patients. Even more concerning is that CDI is becoming increasingly more common among previously unaffected patient populations, like cystic fibrosis
patients and young adults with no previous antibiotic treatment (105)(106). These community-acquired cases of CDI can range in severity from self-limiting infections to severe life-threatening cases and recurring episodes of disease (107). Some people carry C. difficile asymptomatically and may only experience disease upon antibiotic use. Alarmingly, it is estimated that as many as 55% of hospitalized patients are asymptomatically colonized by strains with toxin-producing potential, thus a large standing reservoir of C. difficile may exist in hospitals (108). Given the rising prevalence of CDI, increased efforts into developing new treatment options is warranted. One such intervention, and the focus of this study, is to enhance human innate immunity against C. difficile so that symptomatic infections are either prevented or less severe.

In addition to the large exotoxins, A and B, some strains of C. difficile produce another toxin called Clostridium difficile binary toxin (CDT), but its clinical significance is yet to be established (104)(109). Toxins A and B cause disruption of the epithelial cytoskeleton, leading to cell death, apoptosis and a robust inflammatory response. After toxins cross the epithelial barrier and come into contact with immune cells, a second wave of inflammation and tissue damage occurs, characterized by the presence of macrophages and mast cells (104). Additionally, neutrophil influx to the intestinal epithelium and an increase in neutrophils in peripheral blood are both characteristics of severe CDI. Early studies focused on downstream effects of toxin A and B, involving the NF-κB and MAP kinase pathways (107)(110). However, recent studies investigated the role of pro-inflammatory IL-1β release via the inflammasome pathway in CDI. It has been shown to be both protective and harmful to the host. One group observed that IL-1β
was important for containing commensal bacteria that breech the epithelial barrier due to intestinal epithelial damage while another deduced that massive intestinal inflammation and pathology was due to IL-1β production (110)(111). Recent work with Candida albicans, in which IL-1β is required for clearance, has shown that polyI:C-induced type I IFNs increased susceptibility of mice to infection by decreasing IL-1β (110)(54). While there is a lack of knowledge regarding type I IFNs and their importance in CDI, there is growing recognition that type I IFN signaling during bacterial infections can be both beneficial or deleterious. This depends on the cell types involved in the response and the in situ cytokine/chemokine milieu. For example, type I IFNs promote infections by Listeria monocytogenes, Mycobacterium tuberculosis, and Staphylococcus aureus (13)(97)(112). In contrast, type I IFNs are clearly beneficial and critical for host protection from Streptococcus pneumoniae and Pseudomonas aeruginosa infection (44).

Here, we investigate the role of type I IFNs and inflammasome activation in a murine model of CDI. We found that mice lacking the receptor for type I IFNs (IFNAR-/-) were more susceptible to C. difficile and that this may be due in part to elevated inflammasome activity in IFNAR-/- mice. Furthermore, we found that treating mice with recombinant IFN-α or polyI:C ameliorated disease and the effects of polyI:C on the host response to CDI were IFNAR-independent. Given the fact that type I IFNs and the polyI:C-like drug, Rintatolimod, are already clinically approved treatments for use in humans, our findings offer potential new treatments for CDI.
MATERIALS AND METHODS

Mice and infection All animal studies were carried out in compliance with the Montana State University Institutional Animal Care and Use Committee. Female C57BL/6 and IFNAR -/- mice on the C57BL/6 background age 6-8 weeks were bred and housed at the Animal Resource Center at Montana State University. In order to produce a C. difficile disease state similar to that in humans, the model established by Chen et al (113) was used. Briefly, mice were given sterilized MQ water containing kanamycin (0.4mg/mL), gentamicin (0.035mg/mL), colistin (850U/mL), metronidazole (0.215 mg/mL) and vancomycin (0.045mg/mL) from day -6 to day -3 before infection. Day -3, mice were put back on regular sterilized MQ water and on day -1 were given 200ug clindamycin in 100uL saline by intraperitoneal (i.p.) injection. On day 0, mice were infected with $10^1$ to $10^6$ C. difficile spores. In polyI:C studies, mice were injected i.p. with 15µg polyI:C (Invivogen) in 100uL saline or saline alone on either day 0 and day 1 post infection for infection studies with undefined endpoints or day -1 and day 0 for assessment of cytokine changes 24 hours post infection. In recombinant type I IFN studies, mice were injected i.p. with 4,000 U universal type I IFN-α (R&D Systems) in 100uL, days 0, 1 and 2 post infection. C. difficile spores were cultured as previously descibed (114). Spore enumeration was done by the drop plate method on TCCFA with horse blood and the strain used was VPI 10463.

RNA/bacterial DNA extraction and qRT-PCR. Upon sacrifice, the terminal centimeter of the colon was sonicated in lysis buffer RLT (Qiagen RNeasy Mini Kit) until homogenous. Mesenteric lymph nodes were removed at sacrifice and homogenized with
tissue douncers. Cells were spun, washed and lysed in buffer RLT. Nucleic acids in lysates were then shredded using Qiashredders and frozen at -80 °C until further processing. RNA extraction was carried out using the Qiagen RNeasy Mini Kit and Qiagen RNase-Free DNase Set according to manufacturer's instructions. RNA was then reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen). Cecal contents were collected, weighed and frozen at -80 °C. DNA was extracted using Qiagen’s QIAmp DNA Stool Mini Kit, according to the manufacturer’s instructions. Real time RT-qPCR was run using SYBR Green Mastermix (Bio-Rad) and read on the MYiQ PCR Detection System (Bio-Rad) both according to the manufacturer’s instructions. All primers were ordered from IDT and standard curves were generated for each primer pair. Transcripts were normalized to β actin levels in the tissues. Toxin B CT values were normalized to the weight of cecal contents.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product length</th>
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<tr>
<td>Murine β actin</td>
<td>5' - CTAAGGCCAACCGTGAA A-3'</td>
<td>5' - GAGGACTACAGGGACAGC ACA-3'</td>
<td>100 bp</td>
</tr>
<tr>
<td>Murine TREM-1</td>
<td>5' - TGCTGTGCCTGTCTTTTGT C-3'</td>
<td>5' - CCAGGGTGCAAGGTTCCTT C-3'</td>
<td>176 bp</td>
</tr>
<tr>
<td>Murine TGFβ</td>
<td>5' - CTGCTGACCCCCACTGAT AC-3'</td>
<td>5' - GTGAGCGCTGAATCGAAG C-3'</td>
<td>110 bp</td>
</tr>
<tr>
<td>Murine IGF</td>
<td>5' - GCAATGAGAGCTGCGCA ATG GA-3'</td>
<td>5' - GGTGGGCAAGGATATGAG GC-3'</td>
<td>110 bp</td>
</tr>
<tr>
<td>Murine VEGF</td>
<td>5' - ACTCGGATGCGACACGG GA-3'</td>
<td>5' - CCTGGCCTGTGCTGTCACCC -3'</td>
<td>99 bp</td>
</tr>
<tr>
<td>Murine IL-1β</td>
<td>5' - GCCACCTTTTGACAGTG TA-3'</td>
<td>5' - ATGTGCTGCTGCGAGATT G-3'</td>
<td>134bp</td>
</tr>
<tr>
<td>Primer</td>
<td>5' Sequences</td>
<td>3' Sequences</td>
<td>Length</td>
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<tr>
<td>Murine IFNγ</td>
<td>5'-TGCCATCGGCTGACCTAGAG-3'</td>
<td>5'-TCTCAGAGCTAGGCCGCA-3'</td>
<td>110bp</td>
</tr>
<tr>
<td>Murine IL-10</td>
<td>5'-AGCACTGCTATGCTGCTGCT-3'</td>
<td>5'-AGCATGTGGCTCTGCGCAG-3'</td>
<td>110bp</td>
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<tr>
<td>Murine IP-10</td>
<td>5'-TCTCAGAGCTAGGCCCGA-3'</td>
<td>5'-TCTCAGAGCTAGGCCGCA-3'</td>
<td>110bp</td>
</tr>
<tr>
<td>Murine GM-CSF</td>
<td>5'-AGCATTGCTATGCTGCTGCT-3'</td>
<td>5'-AGCATGTGGCTCTGCGCAG-3'</td>
<td>110bp</td>
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<tr>
<td>Murine IRF7</td>
<td>5'-CCTCAGAGCTAGGCCGCA-3'</td>
<td>5'-CCTCAGAGCTAGGCCGCA-3'</td>
<td>110bp</td>
</tr>
<tr>
<td>C. difficile Toxin B</td>
<td>5'-ACGGACAAGCAGTTGAATATAGTTGTTAGTTAGAG-3'</td>
<td>5'-ATTAAACCTTTGCTAGCCTTTAGTTTGTTGATT-3'</td>
<td>163 bp</td>
</tr>
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</table>

Table 1. Names of primer pairs used for RT-qPCR, sequences and length of product, if available.

ELISAs on terminal serums and colon explant supernatant fluids. Upon sacrifice, terminal serum was collected using Sarstedt Microvettes. Blood was spun down at 10,000 rpm for 10 minutes at room temperature and serum was collected and frozen at -20°C until further use. For the colon explant, at sacrifice, the ceca-proximal centimeter was excised, washed thoroughly in RPMI containing 2X penicillin/streptomycin and 50μg/mL gentamicin and weighed. Prepared tissues were cultured in one mL of media. After 24 hours, supernatant fluids were spun down to remove cells and cell debris, and frozen at -20°C until further use. A minimum of 33μL of sample (1:3 dilution of sample into 77μL assay diluent) was used in all ELISAs and samples and standard curve controls were always incubated over night on the ELISA plate. The mIL-1β ELISA was from R&D, mIFN-γ and mIL-17 were from Mabtech and mTNFα was from Biolegend.
RESULTS

Differential innate immune response of IFNAR -/- mice contributes to disease. To investigate the potential role of type I IFNs in CDI, disease onset and progression in wild type (WT) and congenic type I IFN receptor knockout (IFNAR -/-) mice were compared. Mice were treated with a cocktail of antibiotics to predispose them to infection, which mimics CDI in humans, and exposed to *C. difficile* spores by oral gavage as previously described (113). IFNAR -/- and WT mice were exposed to $6.4 \times 10^4$, $6.5 \times 10^3$ or $9 \times 10^5$ spores and monitored daily for signs of disease (diarrhea, hunched posture, ruffled fur) and weight loss. For all three doses of *C. difficile*, IFNAR -/- mice had more severe disease compared to WT mice (Figure 2.1). The IFNAR -/- mice rapidly declined in health, were moribund and had to be euthanized, whereas WT mice either remained healthier overall or recovered more quickly from disease. Weight loss is the main outcome of CDI but despite this, there were no significant differences in weight loss between the two strains at any of the given doses. The lack of weight loss differences between strains may be explained by greatly differing innate immune responses that lead to more rapid morbidity and mortality in IFNAR -/- mice. These results suggested the importance of type I IFNs in CDI, and prompted additional experiments to determine how this pathway was contributing to protection.
To determine differences in innate immune response between WT and IFNAR--/- mice upon infection, gene and protein expression were analysed. We examined proteins in supernatant fluids from colon explants and serum and transcripts were measured in excised MLNSs and colons. Twenty four hours after infection, serum and a section of the proximal colon were collected. After 24 hours culture, supernatant fluids from colon explants were assessed by ELISAs measuring IL-1β, TNFα, IFN-γ, IL-17 and IL-10. Colon explants from IFNAR--/- mice produced significantly more IL-1β (Figure 2.2A)
than WT colon explants, while no consistent differences in production of any other measured cytokine were detected (data not shown).

Expression of inflammatory and anti-inflammatory mediators involved in intestinal immunity and inflammation were quantified in cDNA from reverse transcribed total RNA from mesenteric lymph nodes (MLNSs) and the terminal centimeter of the distal colon. In the MLNSs, transcripts encoding IL-1β were significantly increased in IFNAR-/- mice compared to WT (Figure 2.2B). There were no consistent differences in expression of innate immune genes IRF3, IL-17 or IP-10 in MLNSs or NRAMP, TGFβ, IL-1β or TREM-1 in the distal colon (data not shown). The fact that increased IL-1β secretion from colon explants was not supported by transcript expression data from the distal colon may have been due to the evaluation of different portions of the colon or differences in the timing of sample collection. Considering IL-1β secretion was increased in colon explants and MLNSs, peripheral IL-1β was measured in WT and IFNAR-/- serums by ELISA, upon sacrifice. Though there was not significantly more IL-1β detected in serum from IFNAR -/- mice compared to wild type mice after infection, there was a trend towards increased IL-1β in the periphery of IFNAR -/- mice (Figure 2.2C). In addition, there was a clear and significant increase in IL-1β in IFNAR -/- mice relative to antibiotic-treated controls. In contrast, the increase in IL-1β expression in wild type mice compared to the uninfected, but antibiotic-treated controls, was not significant (data not
Type I IFNs play a role in dampening inflammasome activity and subsequent IL-1β secretion. Since IL-1β contributes to disease severity in CDI these data support a possible link between IL-1β production and disease severity in IFNAR-/- mice (54,110).
In addition to examining the expression of inflammatory cytokines, mediators in intestinal health and development like IGF (insulin-like growth factor), VEGF (vascular endothelial growth factor) and GM-CSF (granulocyte macrophage colony-stimulating factor) were also considered. IGF, a mediator in intestinal health and development, was consistently decreased in the colon after infection in IFNAR -/- mice (Figure 2.2D). There were no consistent differences in expression between WT and IFNAR mice of VEGF in the colon or GM-CSF and IGF in MLNSs, in response to infection. While, the lack of type I IFN signaling resulted in increased production of IL-1β in MLNSs leading to exacerbated inflammation, decreased IGF expression in colonocytes could further compromise intestinal health. Together, differential expression of IL-1β and IGF in IFNAR -/- mice could have contributed directly to increased disease severity.

It is possible that expression of innate immune markers differed at baseline between WT and IFNAR -/- mice and may contribute to disease. To test this, uninfected WT and IFNAR -/- mice were exposed to the same course of antibiotics used for other experiments and euthanized at an equivalent time point (i.e. 24 hours post infection). The same tissues and samples were collected and analyzed as described above but no significant differences in IL-1β secretion from colon explants were detected between these mouse strains (Figure 2.3A). Additionally, no differences were detected in the expression levels of IL-1β, IFN-α, IFN-β, or IGF (Figure 2.3B, C, D and E). These results
Figure 2.3. Baseline expression levels of important innate immune markers are not different between WT and IFNAR mice without infection. Mice were treated with antibiotics as in Figure 2.1 and sacrificed 24 hours post mock infection. All tissues were collected and treated as in Figure 2.2. (A) Colon explants from WT and IFNAR mice did not secrete different amounts of IL-1β. Transcripts for (B) IL-1β, (C) IFN-α, (D) IFN-β and (E) IGF were not differentially expressed without infection. Results are representative of one, two or three replicate experiments with 5 to 10 mice per group. Student’s t test (*p<0.05, **p<0.01).
support our hypothesis that IGF and IL-1β were not different at baseline, and that both changes were the direct result of CDI.

Intestinal damage in CDI is partially mediated by *C. difficile* toxin-induced secretion of IL-1β via an ASC (apoptosis-associated speck-like protein containing CARD)-containing inflammasome. This cascade is independent of NLRP3, suggesting that *C. difficile* toxins can engage other NLRs that may not have been identified yet (110)(115). Given the fact that IL-1β is increased in multiple sites in response to infection, it was hypothesized that increased IL-1β was a direct effect of increased bacterial burden. In order to determine if bacterial burden correlated with increased morbidity and mortality in IFNAR -/- mice, intestinal content from the cecum was collected at sacrifice, and bacterial DNA was extracted. To indirectly estimate bacterial load, Toxin B DNA levels were measured by qRT-PCR. No significant difference was observed in the amount of toxin B based on qRT-PCR so bacterial burden in cecal contents could not explain the difference in morbidity and mortality between WT and IFNAR -/- mice (Figure 2.4). This is plausible because it has been noted before that intestinal damage and not bacterial burden, correlate with overall disease severity (104). Therefore, observed increases in inflammatory IL-1β and decreased expression of IGF in addition to increased mortality, are not a result of bacterial load.

**Treatment with exogenous IFN-α increases survival.** IFN-α therapy is already used in combination with ribavirin to treat chronic hepatitis C virus and IFN-β is an approved treatment for multiple sclerosis (29)(53). Though the mechanisms of action are unique in these very different disease contexts, treatment with type I IFNs is readily
available and may prove useful for bacterial infections like *C. difficile*. To explore this possibility, post-antibiotic regimen-treated mice were infected with 2×10⁴ *C. difficile* spores per mouse and injected i.p. with 4000 units rIFN-α. This treatment took place on days -1, 0 and 1 post infection and increased survival over sham-treated (saline) mice (Figure 2.5). As results were subtle but significant, they suggest that type I IFN administration and downstream signaling may partially protect mice from CDI and further investigation is warranted.

Figure 2.4. Differences in bacterial burden do not account for observed innate immune differences between WT and IFNAR -/- mice. Cecal content was collected from all mice at sacrifice and bacterial DNA was extracted to examine toxin B levels by qRT-PCR. There was no significant difference in the amount of toxin B based on qRT-PCR. Results are representative of one, two or three replicate experiments with 5 to 10 mice per group. Student’s t test (*p<0.05, **p<0.01).
PolyI:C treatment alters host response to CDI independently of IFNAR signaling. Polyinosinic-polycytidylic acid (polyI:C) is a dsRNA mimetic that signals through TLR3 and MDA5 (Melanoma differentiation-associated protein 5) to induce type I IFNs and expression of inflammatory genes downstream of the canonical NF-κB pathway (116)(117). Based on the potentially protective role of type I IFNs during CDI in mice, we hypothesized that treating mice with polyI:C would induce type I IFN production, which would reduce IL-1β production, and disease severity. WT mice were injected intraperitoneally with 15µg polyI:C day 0 and day 1 post infection with 8x10² C. difficile spores and monitored daily over the course of infection. Mice treated with saline
were euthanized due to disease severity as early as day 2 post infection, whereas those who were administered polyI:C remained healthier until days 3 and 5 post infection (Figure 2.6A). Thus, polyI:C injection appeared to provide some protection from infection.

A similar experiment was performed to assess polyI:C-induced immune changes. WT mice were given 15μg polyI:C or saline days -1 and 0 and sacrificed 24 hours post infection with 1 x 10⁶ *C. difficile* spores per mouse to evaluate innate immune responses.
Figure 2.7. PolyI:C induces immune changes in response to CDI that are independent of IFNAR signaling. WT and IFNAR mice were treated with either saline or polyI:C as in Figure 2.6B. (A) Colon explants were collected as described in previous figures for RNA and qRT-PCR. PolyI:C administration decreased IL-1β expression in response to polyI:C in colons of WT and IFNAR mice. (B) MLNSs were collected as described in previous figures for RNA and qRT-PCR. PolyI:C administration decreased IL-1β expression in MLNSs of WT and partially in IFNAR mice. (C) Colon explants were treated as in Figure 2. Colon explants from polyI:C-treated WT and IFNAR mice secreted less IL-1β protein. (D) PolyI:C administration alters IGF expression in colons of infected WT and IFNAR mice. (E) PolyI:C administration alters IGF expression in MLNSs of infected WT and IFNAR mice. Results are representative of one, two or three replicate experiments with 5 to 10 mice per group. Student’s t test was used for comparisons (*p<0.05, **p<0.01, ***p<0.001).
RNA was extracted from the terminal centimeter of the colon as described above. There was significantly less IL-1β transcript expressed in the colons of mice injected with polyI:C (Figure 2.6B). No consistent changes in other inflammatory, anti-inflammatory or intestinal markers were observed in this tissue (TREM-1, TGFβ, VEGF, IFN-γ, IFN-α and IFN-β). Nevertheless, there was a slight, but statistically insignificant increase in IGF transcript expression in response to polyI:C (data not shown). The fact that polyI:C is a potent inducer of type I IFNs and that neither IFN-α or IFN-β transcripts were elevated in the colon at 24 hours post infection suggests that perhaps, at the time of tissue collection, peak transcript expression had diminished. Nonetheless, these data suggest that the type I IFN induced by polyI:C may be dampening inflammasome activity, which has been seen in other models and may have a subtle effect on IGF expression in the distal colon (54).

PolyI:C has adjuvant effects independent of its ability to induce type I IFN production and depending on cell type and receptor engaged, has differing downstream consequences (116)(118). In fact, polyI:C is capable of signaling via its known receptors, but by utilizing different downstream adaptor proteins it can yield more than just type I IFN production. PolyI:C stimulation can lead to activation of TRAF6, degradation of Iкb and freeing of NF-κB to induce expression of an entirely different spectrum of genes. To test mechanisms of polyI:C independent of IFNAR signaling, WT and IFNAR -/- mice were treated as above and the same tissues and samples were collected at 24 hours post infection. It was observed that polyI:C treatment in WT mice decreased infection-induced IL-1β transripts in both the colon and MLNS (Figure 2.7A and B). This effect was also observed in IFNAR -/- mice in the colon and in the MLNS, though the trend in MLNSs
was not significant. Additionally, IL-1β protein measured in colon explant supernatant fluids was significantly decreased in both WT and IFNAR mice treated with polyI:C (Figure 2.7C). This experiment also corroborated previous experimental results. IL-1β transcript and protein was expressed at significantly higher levels in the colon of IFNAR -/- mice and a similar response was seen in MLNSs, a tissue that had not been evaluated in this context in previous experiments. In addition, IL-1β transcript expression in colons and MLNSs and secretion of IL-1β were significantly higher in polyI:C-treated IFNAR -/- mice than WT polyI:C-treated mice. Since IL-1β levels are high to begin with in response to infection in IFNAR -/- mice, this is not surprising. Further experimentation is necessary to verify this result, however. It is notable that polyI:C-treatment decreases both transcription of IL-1β mRNA and protein secretion. The IFNAR-/- independent mechanism of polyI:C could be due, in part, to an immediate immune response to polyI:C and its diverse downstream effects. The potent immunostimulatory effects of polyI:C could be enough to increase bacterial clearance and thus lead to decreased IL-1β expression at this time point. This hypothesis would require additional exploration to tease apart the various effects of polyI:C in this context and determine the effect of polyI:C treatment in IFNAR -/- mice in survival studies.

Results for IGF transcript expression were less revealing about the effects of polyI:C but partially confirmed previous results. There was a trend toward less IGF expression in the colons and significantly less IGF in MLNSs of IFNAR -/- mice in response to infection without polyI:C (Figure 2.7D and E). There were also trends in both MLNSs and colon samples for increased IGF in response to polyI:C in WT mice.
compared to sham-treated WT mice (Figure 2.7D and E). A similar trend was seen in the colon and MLNSs of IFNAR -/- mice. The change was not significant in the MLNSs but the increase in IGF in response to polyI:C was significant in the colon. These data support a potentially important function of IGF in protection from CDI in the colon in response to polyI:C and potentially in the MLNSs. Additionally, our group has recently determined that treatment with polyI:C proved beneficial in the context of *Coxiella burnetii* infection in both WT and IFNAR -/- mice (in press). Thus, it may not be surprising that polyI:C can act through multiple innate immune pathways during bacterial infection. Conclusively, these observations are in support of an IFNAR signaling-independent mechanism of polyI:C-induced immune changes in response to CDI and IFNAR-independent mechanisms of polyI:C reduction of IL-1β and induction of IGF.

As mentioned previously, polyI:C has pleiotropic effects and can influence the expression of many other genes besides type I IFNs. In fact, in human peripheral blood mononuclear cells, polyI:C induces a wide range of genes including IL-6, IL-1 receptor antagonist, FASL, and STAT3 among many others (117). Moreover, the effects of polyI:C stimulation on human fibroblasts are differential depending on whether polyI:C was administered intracellularly via transfection or extracellularly in tissue culture media. Stimulating cells intracellularly with polyI:C was independent of TLR3 and led to more robust IFN-β expression compared to TLR3-dependent extracellular stimulation. In addition, extracellular polyI:C stimulation depends on the NF-κB protein, RELB for regulation. When RELB expression was knocked down in human fibroblasts, inflammatory gene expression and IFN-β expression increased dramatically after
stimulation with polyI:C and RELB-deficient mice display overt mult-organ inflammation in general, without any immune stimulation (116). Fibroblasts are one of the only cell types that express TLR3 on their cell surface. Given the fact that stromal cells like fibroblasts lie directly below the epithelium in the small and large intestine, it is likely they play a key role in protection during CDI (119). Possibly, a combination of both intracellular and extracellular polyI:C stimulation could contribute to immune changes in WT mice both through type I IFN signaling-dependent and-independent mechanisms. Perhaps the effect of polyI:C in IFNAR -/- mice is due to the RELB-dependent suppression of inflammatory gene expression. The mechanism by which IL-1β expression is decreased in a manner independent of type I IFN signaling is unknown at this time in the context of CDI. Taken together, these data imply that polyI:C administration before CDI infection may be protective through multiple mechanisms.

**DISCUSSION**

Our results indicate that type I IFN signaling is partially protective during CDI in mice. Mice lacking the receptor for type I IFNs have increased mortality compared to WT mice. Additionally, treating WT mice with type I IFN-inducing agent, polyI:C, or rIFN-α, reduce disease. Cytokine analyses suggested that type I IFN signaling and production may be dampening inflammation by decreasing inflammasome activity in WT mice both in response to polyI:C and without polyI:C stimulation. Remarkably low levels of type I IFNs are constitutively expressed and subtle increases can have striking outcomes immunologically. Constitutive type I IFN production is important for not only priming additional type I IFN responses but also for maintenance of hematopoietic stem
cells (120). Type I IFNs are constitutively produced by lamina propria CD11c-expressing cells in the small and large intestine and expression of IFNAR on intestinal epithelial cells influences the microbial ecosystem, altering disease and immune responses (121). This supports an important role for type I IFN and IFNAR expression in these tissues and could, in part, explain increased survival in the face of CDI in WT mice (14). Additionally, innate signaling through other pathways may be protective in IFNAR -/- mice due to mechanisms independent of IFNAR signaling in response to polyI:C. Since polyI:C injection in IFNAR -/- mice causes a host response similar to the protective host response induced in WT mice, it is possible this effect is beneficial in IFNAR -/- mice as well. Further survival studies will be necessary to determine this.

Another relevant pathway to examine in the context of CDI in both WT and IFNAR -/- mice is the type III IFN, IFN-λ, pathway. IFN-λ is critical in protection from intestinal viruses like rotavirus and the receptor for IFN-λ expression is restricted to epithelial cells (1)(2). Type I and type III IFN pathways exert nearly identical effects. Therefore, IFN-λ may also be important in WT and IFNAR -/- mice during CDI.

The role of IL-1β in CDI has proven both protective and detrimental. One group proposed IL-1β was critical in containing commensal bacteria that breach the epithelial barrier due to intestinal epithelial damage while another attributed massive intestinal inflammation and pathology to IL-1β production (110)(111). These contrasting ideas could be a result of differences in infectious dose, C. difficile strain, and time points examined. Moreover, the observed decrease in IL-1β transcript and protein in IFNAR -/- mice in response to polyI:C could be a consequence, rather than a cause, of less severe
infection. PolyI:C can cause immediate innate responses that may aid in bacterial clearance. Perhaps there is an immediate-early burst of IL-1β and other inflammatory cytokines that are necessary to contain the pathogen which is followed by the observed decrease in IL-1β expression at 24 hours post infection. Earlier time points would have to be examined in order to determine the exact timing and action of IL-1β in both type I IFN signaling-dependent and -independent scenarios.

The effect of type I IFNs on inflammasome activity is well documented while the connection between type I IFNs and IGF expression is not clear (54). IGF has many functions in immune regulation and modulation as it can effect hematopoiesis, thymus development and even intestinal inflammation and epithelial barrier maintenance (122)(123). IGF is found in circulation and along with its receptor, IGFR, expressed by many cell types, during homeostatic conditions and in response to inflammatory insult (122). Increased IGF expression was observed both in WT and IFNAR-/- mice in response to polyI:C thus, increased IGF expression may be a result of both type I IFN signaling-dependent and -independent mechanisms. One possible explanation for increased IGF expression may simply be that mice with less intestinal damage because of less inflammation express higher levels of IGF. Alternatively, the increase in IGF could offer epithelial barrier healing or protection in the face of CDI.

Given the fact that type I IFNs have been used as treatment in both chronic viral infections and multiple sclerosis, it is possible they may be used to boost innate immunity in bacterial infections like *C. difficile*. In addition, Rintatolimid, a drug that like polyI:C, signals through TLR3, is clinically available for treating chronic fatigue syndrome (124).
Utilizing polyI:C or Rintatolimod to treat infectious diseases like CDI may prove beneficial. Currently, treatment options for mild CDI include discontinuation of current antibiotic and intravenous fluids, while more severe cases may require surgery. Other treatments under investigation include fecal microbiota transplants, vaccines to the toxins produced by *C. difficile*, and administration of probiotics (103). In addition to acute CDI, recurring *C. difficile* infections are a major financial burden on healthcare due to the cost of effective antibiotics and are becoming increasingly difficult to treat because of antibiotic resistance (104). Combining any of the above therapies with type I IFN treatment or treating with type I IFNs alone may limit the need or duration of antibiotics or decrease the number of recurrent episodes.

In response to increasing antibiotic resistance, extensive work on the development of new antibiotics and non-antibiotic treatments is ongoing. Interestingly, natural compounds with immunomodulatory and adjuvant properties are being explored as well. For example, we demonstrated that treating dengue virus-infected cells with a supplement rich in oligomeric procyanidins (OPCs) derived from unripe apple peels, augments the type I IFN pathway. In this model, OPCs reduced viral titers and increased expression of critical antiviral genes (66). We also determined that consumption of an OPC-rich source by both mice and humans resulted in long lasting immunomodulatory effects on the type I IFN pathway (125). These natural compounds also have the ability to act as adjuvants and some have the ability to prime γδ T cells, a critical cell type in intestinal homeostasis and wound healing (67). Considering the extreme need for new therapeutics or
prophylactic treatment for CDI and that our data suggests the importance of type I IFN signaling in the disease, OPCs may be a promising treatment option.

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