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Commensal microbiota modulation of natural resistance to virus infection

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Summary

IFN-Is are crucial mediators of antiviral immunity and homeostatic immune system regulation. However, the source of IFN-I signaling under homeostatic conditions is unclear. We discovered that commensal microbes regulate the IFN-I response through induction of IFN β by colonic DCs. Moreover, the mechanism by which a specific commensal microbe induces IFN β was identified. OM-associated glycolipids of gut commensal microbes belonging to the Bacteroidetes phylum induce expression of IFN β . Using *Bacteroides fragilis* and its OM-associated polysaccharide A, we determined that IFN β expression was induced via TLR4-TRIF signaling. Antiviral activity of this purified microbial molecule against infection with either VSV or influenza was demonstrated to be dependent on the induction of IFN β . In a murine VSV infection model, commensal-induced IFN β regulated natural resistance to virus infection. Due to the physiological importance of IFN-Is, discovery of an IFN β - inducing microbial molecule represents a potential approach for the treatment of some human diseases.

Introduction

The human body is colonized by trillions of microbial organisms collectively referred to as the commensal microbiota.(Erturk-Hasdemir and Kasper, 2013; Human Microbiome Project, 2012) With advances in culture-independent DNA sequencing technologies and bioinformatics, there has been a rapid increase in our knowledge of the diversity and dynamics of the microbiota over the past two decades.(Human Microbiome Project, 2012;

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Author Contributions

Conceptualization, K.L.S. and D.L.K.; Methodology, K.L.S, M.V.K., A.I., and D.L.K.; Investigation, K.L.S. and M.V.K.; Resources, M.V.K. and A.I.; Writing-original draft, K.L.S.; Writing-review & editing, D.L.K. and A.I.; Supervision, D.L.K. and A.I.; Funding Acquisition, D.L.K.

Declaration of Interests

Two patent applications have been filed by Harvard University with relation to this work. D.L.K. and K.L.S. are listed as inventors on both.

Qin et al., 2010) Current estimates suggest that there are roughly as many bacterial cells as human cells in the body, the vast majority of which reside in the lower gastrointestinal (GI) tract, which is colonized by an estimated 10^{14} bacteria.(Qin et al., 2010; Savage, 1977; Sender et al., 2016; Turnbaugh et al., 2007) Despite these advances, relatively little is understood about the molecular mechanisms by which specific commensal microbes interact with their mammalian host. A growing body of research has revealed that commensal microbes are not just passive residents, but they play an important role in modulating vital host physiological systems, both locally and systemically.(Lin et al., 2014; Surana and Kasper, 2014) Understanding how microbes interact with their host is therefore not only biologically interesting, but it provides a platform for discovering potential therapeutic targets for a variety of human diseases.

One mechanism by which microbes can modulate the host immune system is through regulation of cytokine signaling. Type I interferons (IFN-Is) are a family of structurally similar cytokines, consisting primarily of two different classes of proteins, interferon- α (IFN α) and interferon- β (IFN- β), all of which signal through the common type I interferon receptor (IFNAR).(Theofilopoulos et al., 2005) IFN-I expression is regulated at the transcriptional level, and can be induced at high levels in all nucleated cells in response to sensing of pathogens by pattern recognition receptors (PRRs).(Ivashkiv and Donlin, 2014) Indeed, IFN-Is play a critical role in the response to the majority of virus infections through induction of a restrictive anti-viral state in cells, induction of apoptosis in infected cells, and regulation of immune cell subsets crucial to the antiviral response.(McNab et al., 2015; Muller et al., 1994; Stetson and Medzhitov, 2006; Yan and Chen, 2012) Several recent studies have demonstrated that IFN-Is are not just present during infection, but are expressed constitutively at low-levels and possess vital homeostatic functions.(Gough et al., 2012; Kole et al., 2013; Schaupp et al., 2020) Indeed, IFN-Is have the capacity to regulate the development or function of virtually every immune effector cell, contributing to the anti-inflammatory and anti-tumor responses.(Gough et al., 2012) Despite this importance, the mechanism by which IFN-Is are induced and regulated in the absence of infection has yet to be elucidated.

Due to the importance of IFN-Is to the health of the organism, it is of critical importance to gain a mechanistic understanding of how the constitutive IFN-I response is regulated in healthy individuals and might be disrupted leading to disease. We hypothesized that the commensal microbiota supplies PRR signaling to regulate the IFN-I response in the absence of infection. In this study, we discovered that the commensal microbiota regulates the IFN-I response both locally and systemically through induction of IFN β by colonic lamina propria (LP) dendritic cells (DCs), enhancing resistance to virus infection. Moreover, the molecular mechanism by which a specific commensal microbe induces IFN β was identified. Indeed, we found that outer membrane-associated glycolipids of human gut commensal microbes belonging to the *Bacteroidetes* phylum induce expression of IFN β . Using *Bacteroides fragilis* (*B. fragilis*) as the paradigm, we determined that signaling to initiate IFN β expression was through the toll-like receptor 4 (TLR4)-TIR-domain-containing adapter-inducing interferon- β (TRIF) pathway.

Results

The commensal microbiota regulates host ISG expression through IFN β

To investigate the role of the commensal microbiota in the host IFN-I response, the effect of microbiota depletion on downstream interferon stimulated gene (ISG) expression in various murine tissues was analyzed. The vast majority of commensal microbes reside in the GI tract.(Savage, 1977; Turnbaugh et al., 2007) Analysis was therefore performed to determine effects of the microbiota on ISG expression in the intestinal immune compartment, in the mesenteric lymph nodes (mLN), and systemically, in the spleen. Age- and gender-matched wild type (WT) specific pathogen free (SPF) C57BL/6 mice were administered either vehicle control or a cocktail of broad-spectrum antibiotics (ABX) in the drinking water for 7 days to deplete the microbiota. The relative expression levels of a panel of ISGs were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). A reduction in ISG expression was observed in both the mLN and spleen of WT ABX mice compared to WT SPF mice (Figures 1A–D, Tables S1–S2), demonstrating a role for microbiota regulation of the IFN-I response both locally and systemically.

Due to the potential for off-target effects of antibiotics, a second method of microbiota depletion was used to confirm a direct role of the commensal microbiota on the observed phenotype.(Morgun et al., 2015) Splenic and mLN ISG expression was analyzed in WT C57BL/6 germ free (GF) mice devoid of all microbes compared to WT C57BL/6 SPF mice. Consistent with the ABX mice, a reduction in mLN and splenic ISG expression was observed (Figures S1A–B, Tables S1–2).

Of the mammalian IFN-Is, IFN β is unique with respect to its homeostatic functions and expression pattern.(Schreiber and Piehler, 2015) It was therefore hypothesized that the commensal microbiota induces the constitutive IFN-I response, as evidenced by ISG expression, specifically through regulation of IFN β . ISG expression levels were compared by qRT-PCR between WT and IFN β deficient (*Ifnb1*^{-/-}) SPF mice. A significant reduction in ISG expression was observed in the mLN and spleen of *Ifnb1*^{-/-} mice compared to WT mice (Figures 1A–D, Tables S1–2), confirming a role for IFN β in the homeostatic IFN-I response. Interestingly, the magnitude of reduction was comparable to that observed upon microbiota depletion, with no significant difference between WT ABX and *Ifnb1*^{-/-} SPF expression levels. To further test whether commensal-induced IFN β is required for ISG expression, the microbiota of *Ifnb1*^{-/-} mice was depleted by ABX administration. ABX treatment had no additional effect on mLN or splenic ISG expression in *Ifnb1*^{-/-} mice (Figures 1A–D, Tables S1–2), demonstrating that commensal microbes regulate the IFN-I response specifically through IFN β .

IFN β is expressed by colonic dendritic cells

To investigate the source of commensal-induced IFN β under homeostatic conditions, flow cytometric analysis was performed on tissues harvested from IFN β -yellow fluorescent protein (YFP) reporter mice, in which cells activated to express IFN β express YFP.(Scheu et al., 2008) By analyzing IFN β -YFP expression in the colon LP, mLN, and spleen, IFN β expression was found to be localized to the intestinal milieu, with significantly higher

percentages of IFN β -YFP⁺CD45⁺ leukocytes in the colon LP compared to the spleen or mLN (Figures 2A, Figure S2). The colon was chosen as a representative region of the GI tract because it is home to the greatest percentage of commensal organisms, both in terms of number and abundance.(Turnbaugh et al., 2007) Having identified the location of IFN β expression, the cellular source of this cytokine was next investigated. Due to their role as sentinel cells in the immune system, dendritic cells (DCs) were hypothesized to be the main source of IFN β under homeostatic conditions. It was found that out of the IFN β -YFP⁺CD45⁺ cells in the colonic LP, the majority (~74%) expressed CD11c, a molecule expressed on the surface of DCs and commonly used as a DC marker (Figure 2B).(Singh-Jasuja et al., 2013) These findings demonstrate that colon LP DCs are a major source of IFN β under homeostatic conditions.

As in other tissues, conventional DCs (cDCs) in the colon LP represent a heterogeneous population that can be classified into two subsets based on surface marker expression. cDC₁ cells are CD103⁺CD11b⁻ while cDC₂ cells comprise both CD103⁻CD11b⁺ and CD103⁺CD11b⁺ cells.(Stagg, 2018) To determine which DCs express IFN β , expression of CD103 and CD11b by the IFN β -YFP⁺CD11c⁺CD45⁺ colonic dendritic cells was analyzed using flow cytometry. IFN β expression was specific to the cDC₂ subset of cells, including both CD103⁺CD11b⁺ and CD103⁻CD11b⁺ cells (Figure 2C).

To confirm these findings, a second experimental approach was used in which DCs were isolated from the colon LP, mLN, and spleens of WT SPF mice using a magnetic bead-based cell separation method. Expression of *Ifnb1* was analyzed in the whole tissue single cell suspension as well as both the DC⁺ and DC⁻ fractions of these tissues by qRT-PCR. There was no detectable *Ifnb1* expression in the whole tissue or the DC⁻ fraction of the majority of colon LP samples (Figure 2D) or in the mLN (Figure S3A) and spleen (Figure S3B). However, DCs in both the colon LP (Figure 2D–E) and the mLN (Figure 2E, Figure S3A) but not the spleen (Figure 2F, Figure S3B) expressed *Ifnb1*. Interestingly, DC expression of *Ifnb1* is significantly higher in the colon LP than in the mLN (Figure 2E). These findings confirm that IFN β is predominantly expressed by colon LP DCs.

To test the role of the microbiota in colonic DC IFN β expression, two methods of microbiota depletion were employed. ABX were administered in the drinking water of IFN β -YFP mice for 7 days, followed by flow cytometric analysis of colon LP cells. A significant reduction in the frequency of IFN β -YFP⁺CD11c⁺ cells was observed in IFN β -YFP mice after ABX treatment (Figure 2F). In addition, *Ifnb1* transcript levels in colon LP DCs of WT GF mice were analyzed by qRT-PCR. A significant reduction in *Ifnb1* expression in WT GF mice compared to WT SPF mice was observed (Figure 2G). These results confirm that the microbiota induces IFN β expression by colon LP DCs

Having identified effects of commensal-induced IFN β on ISG expression both locally and systemically, we investigated IFN β levels in systemic circulation, through measurement of serum IFN β levels using an enzyme-linked immunosorbent assay (ELISA). Consistent with reports that IFN-I expression is extremely low under homeostatic conditions, we were unable to detect IFN β in the serum of WT SPF or ABX mice (Figure S3C).

***B. fragilis* induces expression of IFN β by colon LP DCs**

Of the trillions of commensal bacteria that inhabit the mammalian gut, one of the most prominent phyla represented is the *Bacteroidetes*, consisting of obligate anaerobic, Gram-negative rods. An estimated 25% of all colonic anaerobes belong to the genus *Bacteroides* within this phylum. (Salyers, 1984; Wexler, 2007) Recently an association was reported between several species of the genus *Bacteroides*, pDC numbers, and an IFN-I response gene signature. (Geva-Zatorsky et al., 2017) One of the *Bacteroides sp.* investigated in this study was *B. fragilis*, a common human colonic commensal bacterial species with previously demonstrated immunomodulatory properties. (An et al., 2014; Dasgupta et al., 2014; Mazmanian et al., 2005; Mazmanian et al., 2008) Due to its demonstrated ability to influence the immune system, as well as the association with pDCs and IFN-I, we hypothesized that *B. fragilis* regulates colonic DC IFN β expression and thus regulates the IFN-I response.

GF mice were colonized with *B. fragilis* strain NCTC 9343 at 4 weeks of age, followed by isolation of colon LP DCs after 2 weeks and analysis of *Ifnb1* expression. As a colonization control, GF mice were colonized with a different bacterial species with demonstrated immunomodulatory properties, *Clostridium ramosum* (*C. ramosum*), thus controlling for any non-specific effects due to bacterial colonization. (Geva-Zatorsky et al., 2017; Sefik et al., 2015) Colonization with *B. fragilis* significantly enhanced *Ifnb1* expression in colon LP DCs, while *C. ramosum* colonization had no effect (Figure 3A). While *B. fragilis* predominantly colonizes the lower intestinal tract, it is possible that it might also regulate *Ifnb1* expression in other parts of the intestine. We therefore also examined *Ifnb1* expression by DCs isolated from the small intestine (SI) LP of *B. fragilis* monocolonized mice. In contrast to the colon, colonization with *B. fragilis* had no observable effect on SI LP DC *Ifnb1* expression (Figure S4), demonstrating that regulation of DC IFN β by this commensal organism is location specific.

***B. fragilis* capsular polysaccharide A induces IFN β expression**

The outer membrane (OM) of Gram-negative bacteria, serves as the site of interaction between the bacterial cell and its environment and comprises several classes of potential immunomodulatory molecules. (Kasper and Seiler, 1975; May and Grabowicz, 2018) To test whether an OM component is responsible for IFN β expression, an *in vitro* model was developed in which bone marrow-derived dendritic cells (BMDCs) isolated from WT mice were treated with OM complexes isolated from *B. fragilis*, followed by ELISA analysis of secreted IFN β in the supernatants. *B. fragilis* OM complexes significantly induced IFN β secretion by BMDCs (Figure 3B). Capsular polysaccharides can be attached to the OM through covalent linkage to lipid anchors, which are inserted into the OM lipid bilayer. (Cress et al., 2014) One capsular polysaccharide of *B. fragilis*, polysaccharide A (PSA) has been demonstrated to have immunomodulatory properties. (Dasgupta et al., 2014; Mazmanian et al., 2005; Mazmanian et al., 2008) To test whether PSA is an IFN β -inducing component of the *B. fragilis* OM, BMDCs were treated with purified PSA and secretion of IFN β in the supernatants was analyzed by ELISA. Indeed, PSA significantly induced IFN β secretion by BMDCs (Figure 3C).

To confirm the ability of PSA to induce a productive IFN-I response through IFN β , expression of downstream ISGs was analyzed by qRT-PCR in BMDCs treated for 24 hrs with PSA or vehicle control. An increase in ISG expression was observed in WT BMDCs treated with PSA (Figure 3D). Expression levels of the surface-expressed ISG, bone marrow stromal cell antigen 2 (BST2), were determined by flow cytometric analysis of mean fluorescence intensity (MFI) and also found to be increased in PSA-treated compared to vehicle control WT BMDCs (Figure 3E). Surface BST2 levels after PSA treatment were significantly reduced in IFNAR1 deficient (*Ifnar1*^{-/-}) and *Ifnb1*^{-/-} BMDCs, confirming that signaling of IFN β through IFNAR1 is necessary for the observed increase in levels of a representative ISG by PSA. Together, these data demonstrate that PSA induces secretion of IFN β and downstream ISG expression *in vitro* by BMDCs.

To confirm the relevance of these findings *in vivo*, *Ifnb1* expression by colon LP DCs was analyzed in WT SPF mice administered 150 ug of PSA by oral gavage. A significant increase in *Ifnb1* expression was observed 1.5 hrs after treatment (Figure 3F), suggesting that PSA is capable of signaling to colon LP DCs *in vivo* to induce *Ifnb1* expression.

B. fragilis lipooligosaccharide signals through TLR4 to induce IFN β

PSA has been previously demonstrated to signal via TLR2/1 heterodimers and dectin-1 on DCs to induce secretion of IL-10 by CD4⁺ T cells. (Dasgupta et al., 2014; Erturk-Hasdemir et al., 2019; Round and Mazmanian, 2010) The role of TLR2 and dectin-1 in PSA induction of IFN β was therefore investigated by comparing the response of WT, dectin-1 (*Clec7a*^{-/-}), and TLR2 (*Tlr2*^{-/-}) deficient BMDCs to PSA using ELISA to detect IFN β secreted in the supernatants. Much to our surprise, loss of dectin-1 or TLR2 had no effect on IFN β secretion (Figure 4A), suggesting that PSA signals through a different and previously unidentified pathway to regulate IFN β expression.

The majority of IFN-I inducing PRRs sense viral nucleic acid, with the bacteria-sensing TLR2, dectin-1, and TLR4 pathways representing some of the few exceptions. TLR4 senses microbial glycolipids and of the IFN-Is predominantly induces IFN β , consistent with the signaling observed by PSA. (Lu et al., 2008; Sheikh et al., 2014) TLR4 deficient (*Tlr4*^{-/-}) BMDCs completely lost the ability to secrete IFN β in response to PSA treatment (Figure 4A), confirming that PSA signals through TLR4 to induce IFN β secretion.

TLR4 signaling proceeds through two different signaling adapter molecules, myeloid differentiation primary response protein (MyD88) and TRIF. MyD88 canonically activates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) to induce proinflammatory gene expression while TRIF activates interferon regulatory factor 3 (IRF3) to mediate IFN β expression. (Maeshima and Fernandez, 2013) Consistent with these well-described signaling events, PSA induction of IFN β was entirely dependent on TRIF, as evidenced by the lack of increased IFN β secretion in TRIF deficient (*Trif*^{-/-}) BMDCs in response to PSA (Figure 4A). A significant decrease in IFN β secretion was also observed in PSA-treated MyD88 deficient (*Myd88*^{-/-}) BMDCs (Figure 4A), potentially due to regulatory effects that MyD88 expression has on BMDC function.

The canonical TLR4 ligand is LPS, the primary component of the OMs of Gram-negative bacteria.(Maeshima and Fernandez, 2013) In contrast to the archetypal Gram-negative bacterial species *Escherichia coli* (*E. coli*), the OM of *B. fragilis* lacks LPS but instead consists of a lipooligosaccharide (LOS), comprising a lipid A domain linked to a short oligosaccharide.(Lindberg et al., 1990) We recently identified that PSA contains a glycolipid portion, which structurally resembles *B. fragilis* LOS, and now believe that the polysaccharide domain of PSA is covalently linked to LOS as a means of attachment to the OM.(Erturk-Hasdemir et al., 2019) The lipid A component of the LOS of *Bacteroides* has structural and biological features which distinguish it from the much studied lipid A of *E. coli* (Erturk-Hasdemir et al., 2019; Kasper, 1976). It was therefore hypothesized that the LOS portion of PSA is responsible for induction of IFN β . Consistent with this hypothesis, purified *B. fragilis* LOS was observed to induce IFN β in a dose-dependent manner through TLR4 (Figure 4B–C). In contrast, PSA that was delipidated using acid hydrolysis followed by chloroform/methanol extraction to remove the lipid induced no detectable levels of IFN β (Figure 4B), confirming that the LOS domain of PSA is responsible for IFN β signaling.

IFN β induction is a shared function of *Bacteroides* sp.

LOS-like molecules are not unique to *B. fragilis*, and multiple bacterial species of the genus *Bacteroides* have been demonstrated to have LPS or LOS molecules consisting of glycolipids that are structurally similar to *B. fragilis* LOS and distinct from classical *E. coli* LPS.(Cullen et al., 2015; Kasper, 1976; Vatanen et al., 2016) We therefore hypothesized that induction of IFN β by *Bacteroides* LOS/LPS represents a broader mechanism by which commensal microbes are capable of influencing the host immune system. OM complexes were isolated from several different species of *Bacteroides* to test their effect on cytokine secretion by BMDCs. OMs extracted from all of the *Bacteroides* sp. tested induced IFN β by BMDCs in a TLR4-dependent manner (Figure 4D), suggesting that the LPS/LOS species of each of these *Bacteroides* is indeed capable of signaling through TLR4 to induce IFN β .

To address the role of *Bacteroides* sp. and TLR4 signaling in regulation of the IFN-I response *in vivo*, ISG expression was compared in WT SPF mice, *Tlr4*^{-/-} SPF mice, and WT SPF mice 7 days post treatment with metronidazole, a bactericidal antibiotic which specifically targets anaerobic bacteria, including *Bacteroides* sp.(Edwards, 1979; Salyers, 1984) WT mice treated with metronidazole alone and *Tlr4*^{-/-} SPF mice both exhibited significant decreases in ISG expression in the mLN (Figure 4E, Table S3) and spleen (Figure 4F, Table S4). These findings confirm the potential for anaerobic commensal bacteria, of which *Bacteroides* are one of the most dominant taxa, to regulate the host IFN-I response through TLR4 signaling.

Commensal-induced IFN β enhances resistance to VSV infection

It was recently reported that constitutive, low-level induction of ISG expression by the commensal microbiota is required to prime macrophages to respond robustly to subsequent respiratory virus infection.(Abt et al., 2012) It was therefore hypothesized that commensal-induced IFN β is functionally important for the priming of the antiviral response and resistance to virus infection. A murine vesicular stomatitis virus (VSV) infection model, which causes meningoencephalitis and fatal paralytic disease, was used to investigate the

role of commensal-induced IFN β . To test the contribution of IFN β to protection in this disease model, cohoused *Ifnb1*^{+/+} and *Ifnb1*^{-/-} littermates were infected by subcutaneous injection into the footpad with 10⁶ plaque-forming units (PFU) of VSV strain Indiana. *Ifnb1*^{-/-} mice exhibited increased susceptibility to disease based on all parameters tracked, with increased daily and cumulative disease scores (Figures 5A and 5D), incidence of disease (Figure 5B), and reduced survival (Figure 5C). These findings demonstrate a strong role for IFN β in protection against subcutaneous VSV infection.

VSV infection itself induces IFN β expression through PRR signaling, which likely contributes to ISG expression after infection and antiviral immunity.(Kato et al., 2005) Therefore, the increased susceptibility observed in the *Ifnb1*^{-/-} mice likely reflects virus infection-induced IFN β in addition to any contributions of priming levels of IFN β induced by commensal microbes prior to infection. To investigate the effects of priming of the antiviral response by constitutive, microbiota-induced IFN β , an IFN β neutralizing antibody (anti-IFN β) was used to selectively inhibit IFN β prior to infection.(Sheehan et al., 2015). WT mice were administered two doses of anti-IFN β or mouse IgG2a isotype control antibody by IP injection, 72 hrs and 24 hrs prior to infection with 10⁶ PFU of VSV. Anti-IFN β treated mice had increased severity of disease (Figure S5) compared to isotype control treated mice. These data reveal that blockade of IFN β prior to infection enhances susceptibility to subsequent VSV infection, supporting a role for commensal-induced, homeostatic IFN β .

To further investigate the impact of the commensal microbiota on VSV infection, WT mice were administered ABX for 7 days prior to infection, stopping on the day of infection. Increased susceptibility to disease was observed in WT ABX mice compared to WT SPF mice, with significantly increased paralysis scores (Figures 6A,G), incidence of disease (Figure 6B), and reduced survival (Figure 6C). These data reveal a novel role for the commensal microbiota in natural resistance to VSV infection. *Ifnb1*^{-/-} mice were also subjected to 7 days of ABX treatment prior to infection with VSV Indiana. Interestingly, ABX treatment had no effect on the susceptibility of *Ifnb1*^{-/-} mice to VSV infection (Figures 6D–G). In the absence of IFN β , depletion of the microbiota therefore does not alter the response to VSV, demonstrating that the microbiota exerts its protective effect specifically through IFN β .

***B. fragilis* PSA possesses IFN β -mediated antiviral activity**

To further investigate the antiviral activity of commensal-induced IFN β , we sought to identify whether signaling of a purified IFN β -inducing commensal microbial molecule, *B. fragilis* PSA, is sufficient to protect against virus infection. While LOS and the LOS domain of PSA are responsible for IFN β induction, whole PSA was used in this system due to its enhanced solubility. An *in vitro* virus infection model was developed in which BMDCs were treated with a dose response of PSA for 24 hrs prior to infection with a green fluorescent protein (GFP) expressing strain of VSV Indiana (VSV-GFP) at a multiplicity of infection (MOI) of 1, followed by flow cytometric analysis of the percentage of infected (GFP⁺) live cells 24 hrs post infection (h.p.i.) (Figure S6A).(Dalton and Rose, 2001) Priming with PSA inhibited virus infection in a dose-dependent manner, resulting not only in a lower

percentage of infected cells (Figure 7A) but also increased cell viability (Figure 7C). To investigate the mechanism of antiviral activity, infection with VSV-GFP was compared in WT, *Ifnb1*^{-/-}, or *Tlr4*^{-/-} BMDCs that were primed with PSA for 24 hrs. Protection by PSA was completely lost in the absence of TLR4 or IFN β signaling (Figure 7E), confirming that the observed antiviral function of PSA is indeed mediated by IFN β signaling.

To identify whether the observed *in vitro* protective effects of PSA are limited to VSV infection, a second virus infection model was developed, using the murine adapted influenza A virus strain PR8 (IAV/PR8). WT, *Ifnb1*^{-/-}, or *Tlr4*^{-/-} BMDCs were primed with PSA followed by infection with a GFP-expressing IAV/PR8 strain (PR8-GFP) at an MOI of 1, followed by flow cytometric analysis 24 h.p.i. (Figure S6B). (Manicassamy et al., 2010) Priming of WT BMDCs with PSA inhibited subsequent IAV infection, with a significant reduction in the percentage of infected cells (Figure 7B) and increase in cell viability (Figure 7D). This antiviral effect was completely abrogated in the absence of IFN β or TLR4 (Figure 7F), revealing that signaling of PSA through the same pathway protects cells from infection with two different viruses. These results establish a novel *in vitro* broad-spectrum antiviral function of *B. fragilis* PSA.

To investigate the *in vivo* antiviral activity of PSA, WT or *Ifnb1*^{-/-} ABX mice were administered PSA daily by oral gavage starting 4 days prior to and continuing to the day of infection with VSV. PSA treatment significantly reduced disease severity in WT ABX mice, with decreased daily and cumulative paralysis scores (Figures 6A,G), incidence of disease (Figure 6B), and enhanced survival (Figure 6C), to levels statistically indistinguishable from WT SPF mice. In *Ifnb1*^{-/-} ABX mice, PSA treatment had no observable effect on disease severity (Figures 6D–G), supporting the hypothesis that the protective effects of PSA are mediated through IFN β . These findings demonstrate that not only is commensal induction of IFN β necessary for protection against VSV infection, but treatment with a single IFN β -inducing commensal microbial molecule is sufficient to restore the protective effects of the whole microbiota in this model.

Discussion

We have described a novel mechanism of immunomodulation by the commensal microbiota through IFN β -mediated regulation of the homeostatic IFN-I response. Furthermore, we identified the mechanism by which a specific species of commensal bacteria regulates this response, signaling of *B. fragilis* glycolipids through TLR4 to induce expression of IFN β by colon LP DCs. Importantly, this commensal-mediated regulation of IFN β and the IFN-I response was found to play a critical role in maintaining health of the host by enhancing resistance to virus infection.

Our findings are consistent with previous reports of commensal regulation of the IFN-I response, which identified that the microbiota induces constitutive IFN-I production and basal levels of ISG expression. (Abt et al., 2012; Schaupp et al., 2020) However, our work builds on the existing literature by identifying which IFN-I family cytokine member is required for regulation of homeostatic ISG expression, a specific cellular source of commensal-induced IFN β , and the molecular mechanism of IFN β induction by a

commensal microbial species. Interestingly, among the IFN-I family members, IFN β has been demonstrated to possess unique functionality, with increased antiproliferative and regulatory function. IFN-I signaling can result in several different signaling outcomes all mediated through the same receptor. Importantly, the many different biological outcomes, whether pro- or anti-inflammatory, could be either beneficial or detrimental to the host depending on the context. The observed specific induction of IFN β by the microbiota suggests controlled expression of the distinct IFN-Is to favor homeostatic expression only of the more regulatory variant, IFN β .

Intestinal DCs have previously been established to carry commensal antigen to the mLN, where they are restricted from entering into systemic immune circulation, and thus remain in the intestinal immune compartment.(Macpherson and Uhr, 2004) Our results are consistent with a model in which IFN β is expressed in response to detection of microbial antigens by colon LP DCs, which then remain in the intestinal LP or traffic to the mLN. Despite this tight locational restriction of IFN β expression, we observed systemic decreases in ISG expression in both *Ifnb1*^{-/-} and ABX mice as well as functional effects of commensal induced-IFN β during virus infection at an extra-intestinal site. It is possible that IFN β secreted in the intestinal environment enters the systemic circulation to mediate these effects. IFN β is extremely potent, capable of inducing ISG expression with a half-maximal effective concentration (EC₅₀) of 0.2 pM.(Schreiber and Piehler, 2015) While there were no detectable levels of IFN β in the serum of WT SPF mice, it is possible that a minute quantity of IFN β might enter the circulation from the intestinal immune compartment and induce systemic effects on ISG expression. Alternatively, secretion of IFN β by intestinal DCs might induce gene expression changes locally, in neighboring cells, which traffic to distal body sites. Further investigation will be required to determine which of these mechanisms accounts for systemic ISG expression under homeostatic conditions.

We discovered that the glycolipids of *B. fragilis*, PSA and LOS, signal through TLR4 to induce expression of IFN β by DCs. Canonically, activation of TLR4 by bacterial LPS signals not only through TRIF to induce IFN-I signaling, but also through MyD88 to induce proinflammatory mediators.(Maeshima and Fernandez, 2013) Through activation of a robust and uncontrolled immune response, LPS exposure can cause severe systemic inflammation, leading to potentially lethal septic shock.(Bohannon et al., 2013; Molinaro et al., 2015) As a result of its endotoxicity, the clinical applications of LPS as an immunomodulator have been limited. However, most research on LPS has focused on *E. coli*.(Maeshima and Fernandez, 2013) Using numerous assays including proinflammatory cytokine secretion, the *Limulus* amoebocyte lysate (LAL) assay, rabbit pyrogenicity test, chick embryo lethality, and the Shwartzman reaction, *B. fragilis* LOS has previously been demonstrated to have low endotoxic activity, suggesting it is a less inflammatory LPS species.(Erturk-Hasdemir et al., 2019; Kasper, 1976; Sveen et al., 1977) This difference in activity can be attributed to several structural differences between the two molecules. Whereas *E. coli* LPS is hexa-acylated, *B. fragilis* LOS represents a mixture of penta-, tetra-, and tri-acylated molecules. (Weintraub et al., 1989) In addition, the acyl-chains of *B. fragilis* LOS are longer, with C15-C17 fatty acid chains compared to C12-C14 fatty acids used by *E. coli* LPS.(Raetz et al., 2007; Weintraub et al., 1989) Finally, the diglucosamine backbone of *B. fragilis* LOS is mono- or non-phosphorylated compared to the bis-phosphorylated lipid A of *E. coli*.

(Molinaro et al., 2015; Weintraub et al., 1989) The number and length of the acyl-chains as well as the phosphorylation state of the disaccharide backbone in LPS are critical factors affecting the interaction with TLR4 and the downstream function of these molecules. (Maeshima and Fernandez, 2013) In this way, *B. fragilis* glycolipids might be able to induce the protective beneficial effects of IFN β without the consequences of uncontrolled, pathologic inflammation.

Our findings suggest that induction of IFN β through TLR4 signaling is not limited to *B. fragilis* but is a shared function of an entire class of commensal microbial molecules, *Bacteroides* LPS/LOS. Indeed, OM extracts from all of the *Bacteroides sp.* tested were able to induce IFN β . OMs comprise numerous bacterial molecules, all of which have the potential to interact with immune cells and influence their response. (Kasper and Seiler, 1975; May and Grabowicz, 2018) Isolation of LPS/LOS from each of these bacterial species will therefore be required to further assess their IFN β -inducing capabilities. Importantly, the genus *Bacteroides* makes up a large proportion of the human GI microbiome and is widespread in prevalence across the human population. (Salysers, 1984; Wexler, 2007) Indeed, *Bacteroides* LOS/LPS species might be the most abundant microbial molecules in the GI tract. As such, it is plausible that *Bacteroides*-induced IFN β represents a ubiquitous mechanism by which commensal microbes communicate with their host to regulate the immune system and other physiological processes, ultimately contributing to human health.

Low-level induction of ISG expression under homeostatic conditions primes cells of the immune system so that they can respond effectively to subsequent virus infection and ultimately reduces severity of disease. (Abt et al., 2012) It is therefore plausible that the observed antiviral effect of commensal-induced IFN β is mediated through priming of constitutive expression of ISGs, thus arming the immune system to respond immediately and robustly to virus infection. Due to the important role of IFN-I signaling and ISG expression in the context of the majority of mammalian virus infections, such a finding might represent a broad-spectrum, and therefore universally important, antiviral activity of the commensal microbiota. (van den Broek et al., 1995) In addition to antiviral immunity, IFN-Is have been described to influence numerous other vital aspects of host physiology, including immune tolerance, antitumor immunity, cytokine signaling regulation, bone homeostasis, and hematopoiesis. (Gough et al., 2012) It is therefore possible that the commensal microbiota influences each of these processes as well, through the ability to regulate IFN β . Future studies will be required to determine the importance of commensal-induced IFN β in each of these physiological processes and the potential therapeutic use of IFN β -inducing commensal microbial molecules, such as PSA, in a wide array of human diseases. Current IFN-I based therapies rely on the administration of non-physiologic amounts of exogenous IFN-Is, which can have unwanted side effects. (Gottberg et al., 2000; Sleijfer et al., 2005) Delivery of an IFN β -inducing microbial molecule thus represents a novel IFN-I-based therapeutic approach, which could enhance the IFN-I response while still being subjected to homeostatic regulatory mechanisms, reducing the potential for undesired side effects.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Dennis Kasper (dennis_kasper@hms.harvard.edu).

Materials Availability—This study did not generate new unique reagents

Data and Code Availability—This study did not generate new datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—Details of mouse strains used in this study can be found in the Key Resources Table. Conventional SPF mice were housed under SPF conditions in the Harvard Medical School mouse vivarium. All genetically deficient mice and their respective controls were gender- and age-matched (typically 5–10 weeks) and both males and females were used in this study. Mice were assigned randomly to experimental groups. Germ free C57BL/6 mice were originally purchased from the National Gnotobiotic Rodent Resource Center of the University of North Carolina at Chapel Hill, and were bred and maintained in sterile vinyl isolators in the animal facility at Harvard Medical School. Sterility was checked weekly by aerobic and anaerobic microbial culture. For monocolonization studies, experimental manipulation of mice was performed in autoclaved cages in which animals received autoclaved food and water. All experiments on animals were approved by the Harvard Medical Area Standing Committee on Animals (animal protocol number IS00000187–3).

Bacteria—The bacteria strains used in this study are described in the Key Resources Table. All bacteria used in this study were grown on *Brucella* blood agar plates or in liquid culture in rich Brain Heart Infusion Broth (BD) supplemented with Hemin (0.01%), vitamin K (0.1 mg/mL), glucose (0.5%), Monopotassium phosphate (0.5%), and L-cysteine (0.1%) in an anaerobic chamber (Coy Industries) at 37° Celsius (C).

METHOD DETAILS

Antibiotics Treatment—For studies involving depletion of the microbiota, SPF mice of the indicated strains were treated with a broad-spectrum antibiotics cocktail for 7 days. Vancomycin hydrochloride (0.5 g/L, Alfa Aesar), gentamicin sulfate (0.5 g/L, Sigma-Aldrich), and ampicillin (1 g/L, MP Biomedicals) were administered in the drinking water. Metronidazole (8 g/L, Alfa Aesar) and amphotericin B (0.1 g/L, Amresco Inc) were administered in 200 uL by OG once daily. Fecal samples were collected and plated under aerobic and anaerobic conditions to confirm microbiota depletion. For studies involving treatment with metronidazole alone, Metronidazole (0.5g/L, Alfa Aesar) was administered in the drinking water for 7 days.

qRT-PCR gene expression analysis—RNA was isolated from whole tissues or the DC⁺ and DC⁻ fractions of spleens, mLN_s, and colons following manufacturer's instructions with the RNeasy mini kit (Qiagen). cDNA was prepared with the Quantitect Reverse

Transcription Kit (Qiagen). qRT-PCR was performed on a QuantStudio7 Flex or Pro Real-Time PCR system (Applied Biosystems) with RT² SYBR Green ROX qPCR Mastermix (Qiagen). Amplification was achieved using an initial cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression was normalized using *Actb* as a reference gene. The forward (f) and reverse (r) primers used to detect expression of the corresponding murine genes are described in Table S5.

Preparation of tissue single-cell suspensions—Single-cell suspensions of the colonic lamina propria were prepared as previously described.(Dasgupta et al., 2014) Briefly, the tissue was cut transversely into approximately 2 cm pieces and then longitudinally to expose the lumen. To extract epithelial cells, tissue pieces were incubated in 1mM dithiothreitol (Sigma) in PBS for 10 minutes, washed, and incubated in 30mM Ethylenediaminetetraacetic acid (EDTA) three times for 8 minutes each. To digest the tissue, colon pieces were treated with RPMI medium supplemented with 5% fetal bovine serum (FBS) and 1 mg/mL collagenase type IV for 45 minutes at 37°C in an atmosphere of 5% carbon dioxide (CO₂) and then passed through a 70 µM filter. For single-cell suspensions of mLNs and spleens, tissues were harvested, treated with 1mg/mL collagenase type IV in RPMI for 30 minutes at 37°C in an atmosphere of 5% CO₂, and passed through a 70 µm filter. Splenic samples were treated with red blood cell lysis buffer (Gibco).

Dendritic cell isolation—Single-cell suspensions of the indicated tissues were prepared as described. DC positive and negative fractions were isolated with mouse pan-DC microbeads (Miltenyi), following the manufacturer's protocol.

Monocolonization of GF mice—GF WT C57BL/6 mice were administered *B. fragilis* strain NCTC 9343 or *C. ramosum* strain AO31 by oral gavage [200 µL, approximately 10⁸-10⁹ colony-forming units (CFU) per mouse]. Mice were housed in autoclaved cages and maintained on a diet of autoclaved food and water for 2 weeks and then euthanized for analysis.

IFNβ ELISA analysis—For serum analysis, WT SPF or antibiotics treated mice were euthanized and blood was collected into 1.1 ml Z-Gel Micro Tubes (Sarstedt). Blood was left at room temperature for 30 minutes, centrifuged to remove coagulated cells [12,000 revolutions per minute (RPM), 10 minutes], and levels of IFNβ in the serum were quantified with the High Sensitivity Mouse IFN Beta ELISA kit (PBL Assay Science) according to the manufacturer's protocol. For cell culture supernatant analysis, cell free supernatants were collected from BMDC cultures and IFNβ levels were quantified with the Mouse IFN Beta ELISA kit (PBL Assay Science) according to the manufacturer's protocol.

BMDC culture—DCs were derived from bone marrow of the indicated mice as previously described.(Helft et al., 2015) Briefly, bone marrow was collected from femurs, treated with red blood cell lysis buffer (Gibco), and cultured in 10 cm tissue culture (TC)-treated dishes at a density of 10×10⁶ cells in 10 mL per plate in complete RPMI medium (cRPMI: RPMI 1640 supplemented with 10 mM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin, and 50 µM 2-mercaptoethanol) with 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). Cells were cultured for 6

days at 37°C in an atmosphere of 5% CO₂ with replenishment of medium on day 3. On day 6 the non-adherent and loosely adherent cells were harvested and plated at a density of 10⁶ cells/mL in 200 uL per well of a 96-well round-bottom TC-treated cell culture plate for use in the indicated experimental procedures.

Bacteroides OM extraction—OM complexes were isolated from the indicated *Bacteroides sp.* as previously described.(Kasper and Seiler, 1975) Briefly, mid-log phase bacteria were pelleted, washed twice in 0.15 M sodium chloride (NaCl) and resuspended in a buffer of 0.15 M NaCl, 0.05 M sodium phosphate, and 0.01 M EDTA. The resuspended organisms were incubated for 30 minutes at 60°C then subjected to mild shearing pressure by passing through a 25-gauge hypodermic needle. Cells were pelleted from the suspension and the supernatants were harvested and subjected to two rounds of centrifugation at 33,000 RPM for 2 hrs at 4° C to pellet the OM extracts.

B. fragilis glycolipid and polysaccharide purification—PSA was purified from *B. fragilis* mutant strain 44 and LOS was purified from an acapsular *B. fragilis* mutant strain by phenol-water extraction as previously described.(Tzianabos et al., 1992) To obtain the purified delipidated polysaccharide domain of PSA, PSA was subjected to acid hydrolysis with 2% acetic acid at 90 °C for 2 h. The sample was neutralized the lipid was removed using chloroform/methanol extraction.

Flow cytometric analysis—The antibodies used for flow cytometric analysis are described in the Key Resources Table. Single-cell suspensions were stained with a suitable combination of fluorochrome-conjugated antibodies and fixable viability dye. Cells were fixed in 2% paraformaldehyde in PBS, and examined with an LSR II flow cytometer (BD). The data were analyzed with FlowJo software.

Mouse VSV infection—Mice were anesthetized by IP injection of ketamine and xylazine. 1×10⁶ PFU of VSV strain Indiana was delivered in 10 uL per mouse by subcutaneous injection into the footpad as previously described.(Fensterl et al., 2014) Body weight and clinical disease score were monitored daily for 14 days following infection. VSV-infected mice exhibit an ascending paralytic disease that was scored as follows: 0 = no symptoms, 1= tail paralysis, 2= hind-limb weakness, 2.5= paralysis of one hind limb, 3= paralysis of both hind limbs, 3.5= paralysis of both hind limbs and forelimb weakness, 4=forelimb and hind-limb paralysis, 5= moribund). Mice with a body condition score 2 or a disease score 4 were humanely euthanized.

in vitro virus infections—On day 6 of culture, GM-CSF BMDCs were plated at a density of 10⁶ cells/mL in 200 uL of cRPMI medium per well of a 96-well round-bottom TC-treated cell culture plate with 20 ng/mL GM-CSF and the indicated treatments for 24 hrs, washed, and resuspended in cRPMI medium containing either IAV/PR8-GFP or VSV-GFP at an MOI of 1. Cells were harvested for flow cytometric analysis 24 hrs post infection.

IFNβ neutralization—Mice were administered two doses of 250 ug IFNβ neutralizing antibody (anti-IFNβ, clone HDβ-4A7; Leinco Technologies) or Mouse IgG2a isotype

control antibody (Leinco Technologies) by IP injection, 72 hrs and 24 hrs prior to infection with 10^6 PFU of VSV strain Indiana.

QUANTIFICATION AND STATISTICAL ANALYSIS

Information regarding statistical details and methods for each experiment can be found in the figure legends. Data is presented as mean \pm SEM unless otherwise indicated. Statistical analysis was performed using GraphPad Prism 7.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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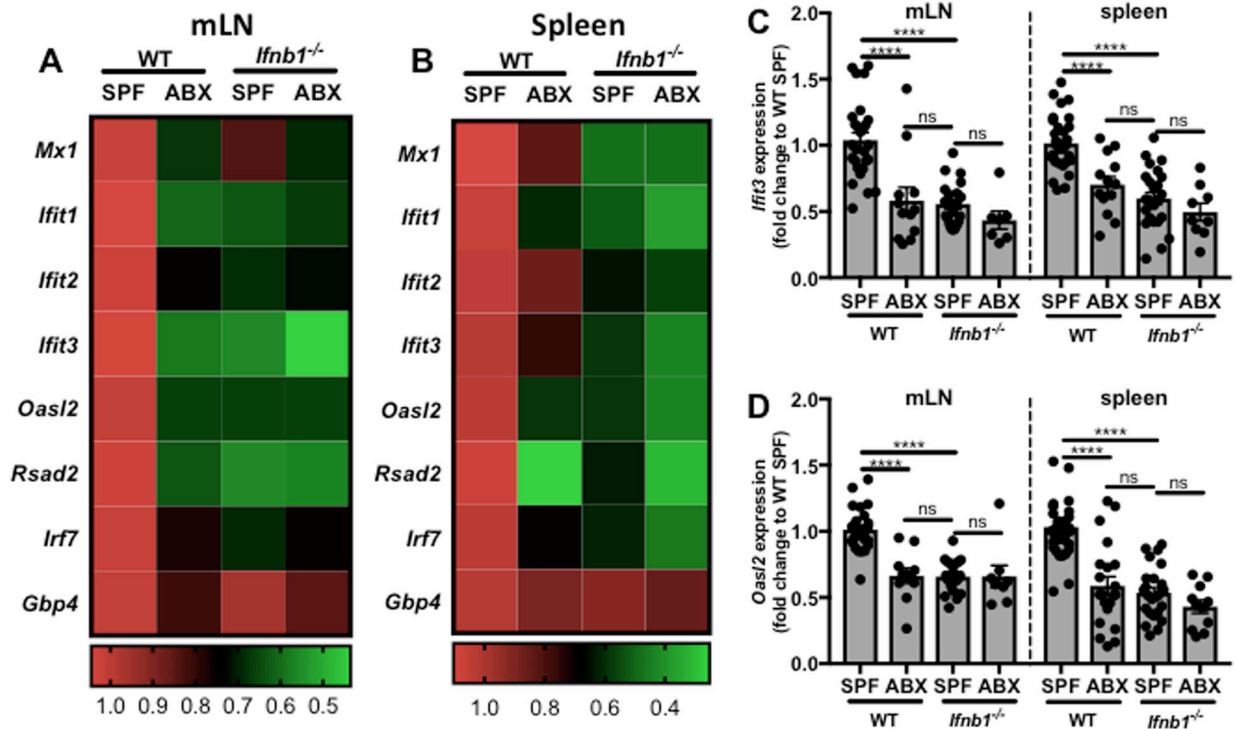


Figure 1. Microbiota depletion reduces local and systemic ISG expression.

mLNs and spleens were harvested from WT or *Ifnb1^{-/-}* SPF mice with and without broad-spectrum antibiotics (ABX) treatment. RNA was isolated from whole tissue samples followed by qRT-PCR to analyze ISG expression levels. Fold change gene expression in the mLN (A,C-D) or spleen (B,C-D) was calculated compared to WT SPF mice using the $2^{-\Delta\Delta CT}$ method, with *ActB* as the reference gene and depicted as (A,B) a heat map of all ISGs tested or representative bar graphs of mean \pm SEM with each point representing one mouse for (C) *Ifit3* and (D) *Oasl2*. One-way ANOVA statistical analysis followed by Tukey's multiple comparisons test. Details of statistical analyses can be found in Tables S1–2. ns=not significant, **** $p < 0.0001$.

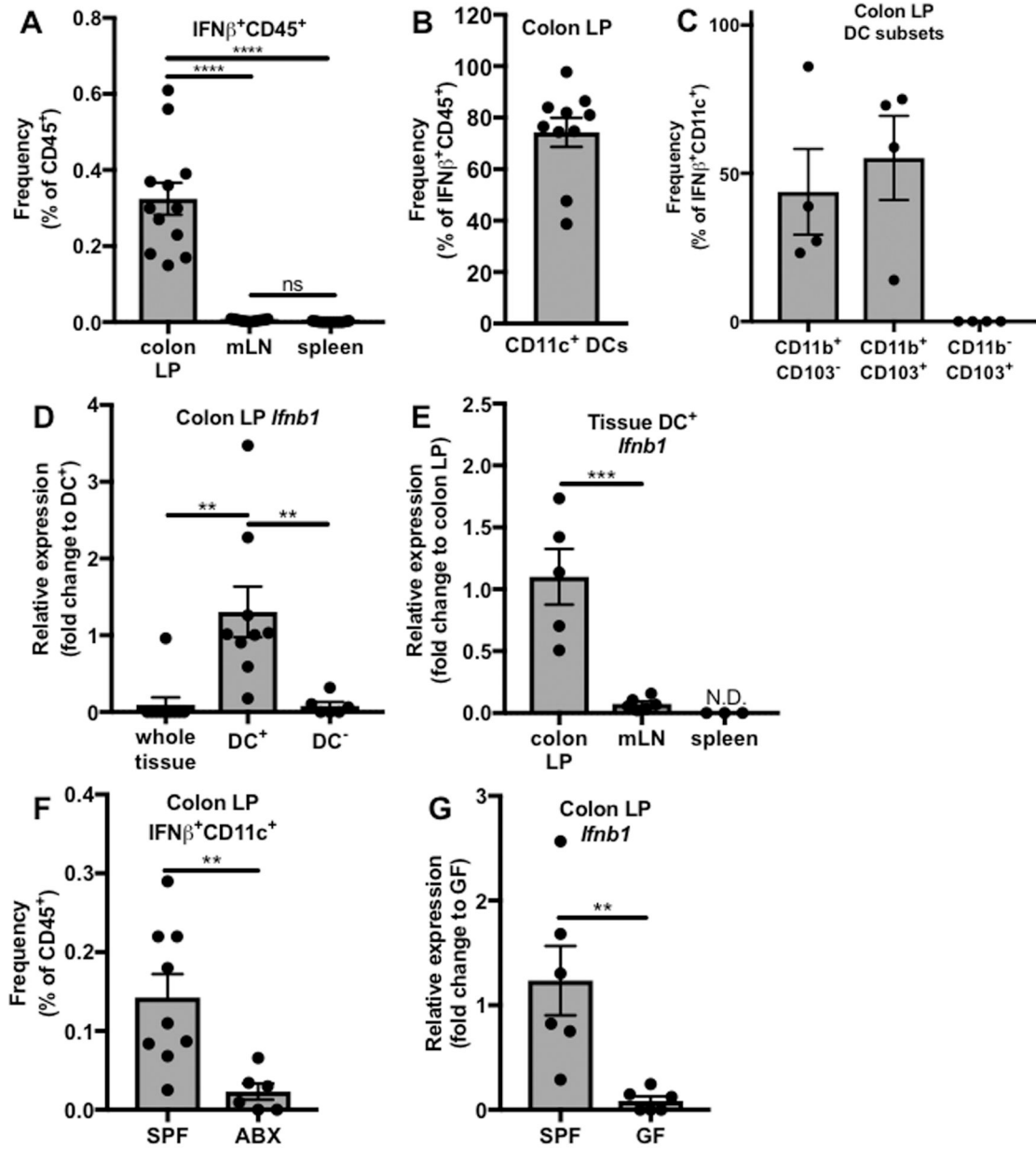


Figure 2. The commensal microbiota regulates IFNβ expression by dendritic cells in the colon LP.

(A-C) Single cell suspensions were prepared from spleens, mLNs, and colon LP of SPF IFNβ-YFP reporter mice and analyzed by flow cytometry. (A) Frequency of IFNβ-YFP⁺ cells out of CD45⁺ cells (colon LP N=12, mLN N=13, spleen N=13). (B) Frequency of CD11c⁺ DCs out of total IFNβ-YFP⁺CD45⁺ cells in the colon LP (N=10). (C) Frequency of CD11b⁺CD103⁻, CD11b⁺CD103⁺, and CD11b⁻CD103⁺ DC subsets out of IFNβ-YFP⁺CD11c⁺ colonic LP dendritic cells. (D-E) DCs were isolated from single cell suspensions of different tissues from WT GF and WT SPF mice, yielding DC⁺ and DC⁻ fractions, and *Ifnb1* expression was analyzed by qRT-PCR. Relative expression of *Ifnb1* in (D) the whole tissue (N=10), DC⁺ (N=9), and DC⁻ (N=6) fractions of the colon LP or (E) the DC⁺ fraction of the colon LP (N=5), mLN (N=6), or spleen (N=3). (F) Flow cytometric analysis of

frequency of colon LP IFN β -YFP⁺CD11c⁺ cells out of CD45⁺ cells (ABX N=7, SPF N=10) in SPF or ABX treated IFN β -YFP reporter mice. (G) qRT-PCR analysis of *Ifnb1* expression in colon LP DC⁺ cells from WT SPF (N=6) or WT GF mice (N=6). (D-E,G) Fold change gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, with *Actb* as the reference gene, compared to the (D) DC⁺, (E) colon LP, or (G) SPF samples. Bars represent mean \pm SEM. Statistical analysis with (A,D,E) one-way ANOVA followed by Tukey's multiple comparisons test and (F,G) unpaired t-test. N= number of mice, N.D.=not detected, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.

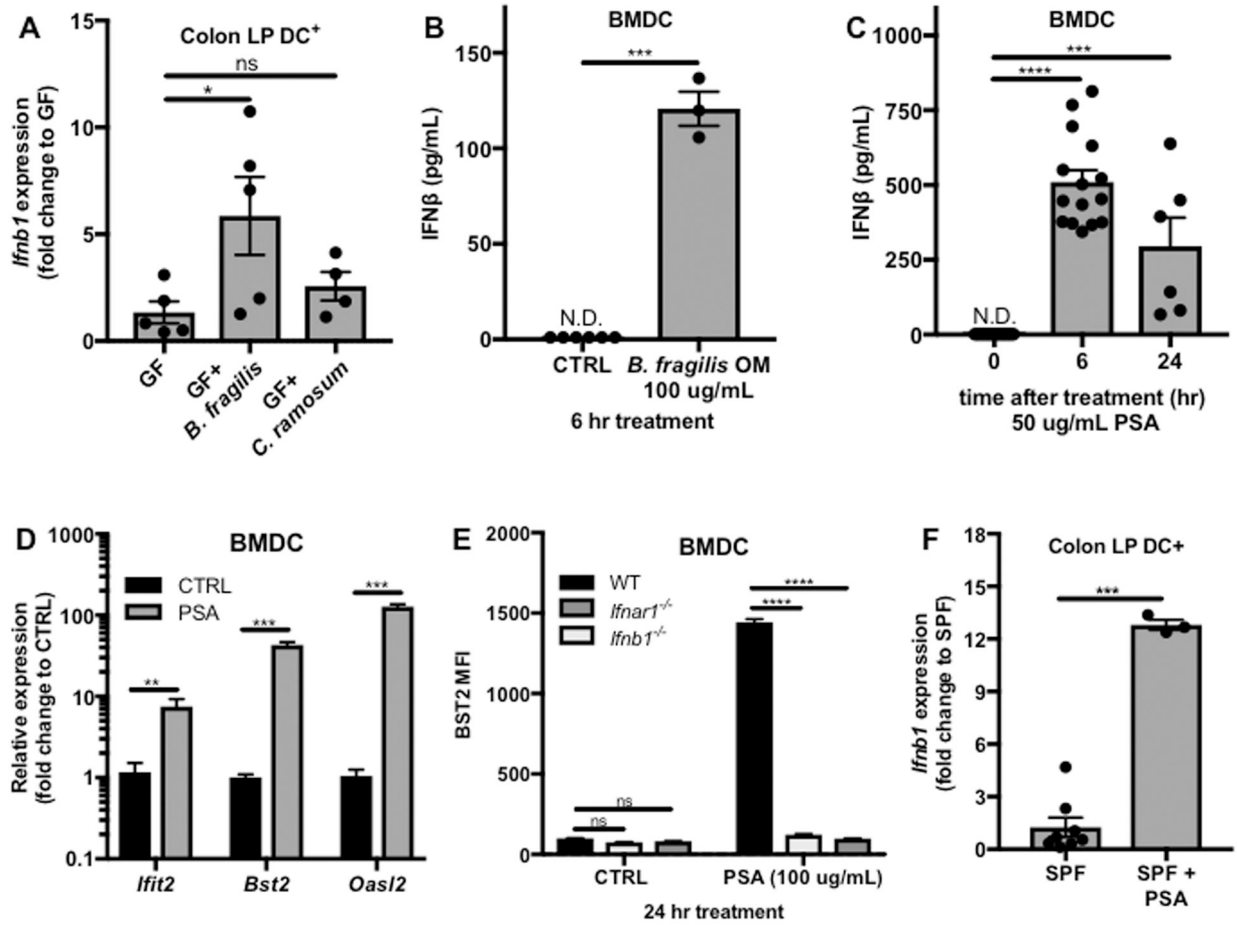


Figure 3. *B. fragilis* PSA induces IFN β *in vitro* and *in vivo*.

(A) WT GF mice were gavaged with vehicle (GF, N=5) or colonized at 4 weeks of age with *B. fragilis* (N=5) or *C. ramosum* (N=4). Two weeks post colonization, DCs were isolated from colon LP single cell suspensions, RNA was isolated, and *Ifnb1* expression was analyzed by qRT-PCR. Fold change gene expression to GF was calculated using the CT method with *Actb* as a reference gene. (B-E) BMDCs were cultured from WT, *Ifnb1*^{-/-}, or *Ifnar1*^{-/-} mice. (B) IFN β levels in the supernatants of WT BMDCs treated with vehicle control (CTRL, N=6) or 100 ug/mL *B. fragilis* OM extract (N=3). (C) ELISA analysis of IFN β levels in the supernatants of WT BMDCs treated with 50 ug/mL PSA for 0 (N=15), 6 (N=15), or 24 hrs (N=6). (D) RNA was isolated from WT BMDCs 24 hrs post treatment with vehicle control (CTRL, N=4) or 50 ug/mL PSA (N=3) and ISG expression was analyzed by qRT-PCR. Fold change gene expression to CTRL was calculated using the CT method with *Actb* as the reference gene. (E) After 24 hrs of treatment with 100 ug/mL PSA, BST2 mean fluorescence intensity (MFI) gated on live CD11c⁺ cells was measured by flow cytometry (N=4 for each condition). (F) WT SPF mice were gavaged with 150 ug PSA and after 1.5 hrs *Ifnb1* expression by colon LP DCs was analyzed by qRT-PCR (SPF, N=8; SPF+PSA, N=3). Fold change gene expression to SPF was calculated using the CT method with *Actb* as the reference gene. Data represents average \pm SEM. Statistical analysis with (A,C) one-way ANOVA followed by Dunnett’s multiple comparisons test, (B,D,F) unpaired t-test and (E) Two-way ANOVA followed by Sidak’s multiple comparisons

test to WT. N.D.= not detected, ns=not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,
**** $p < 0.0001$.

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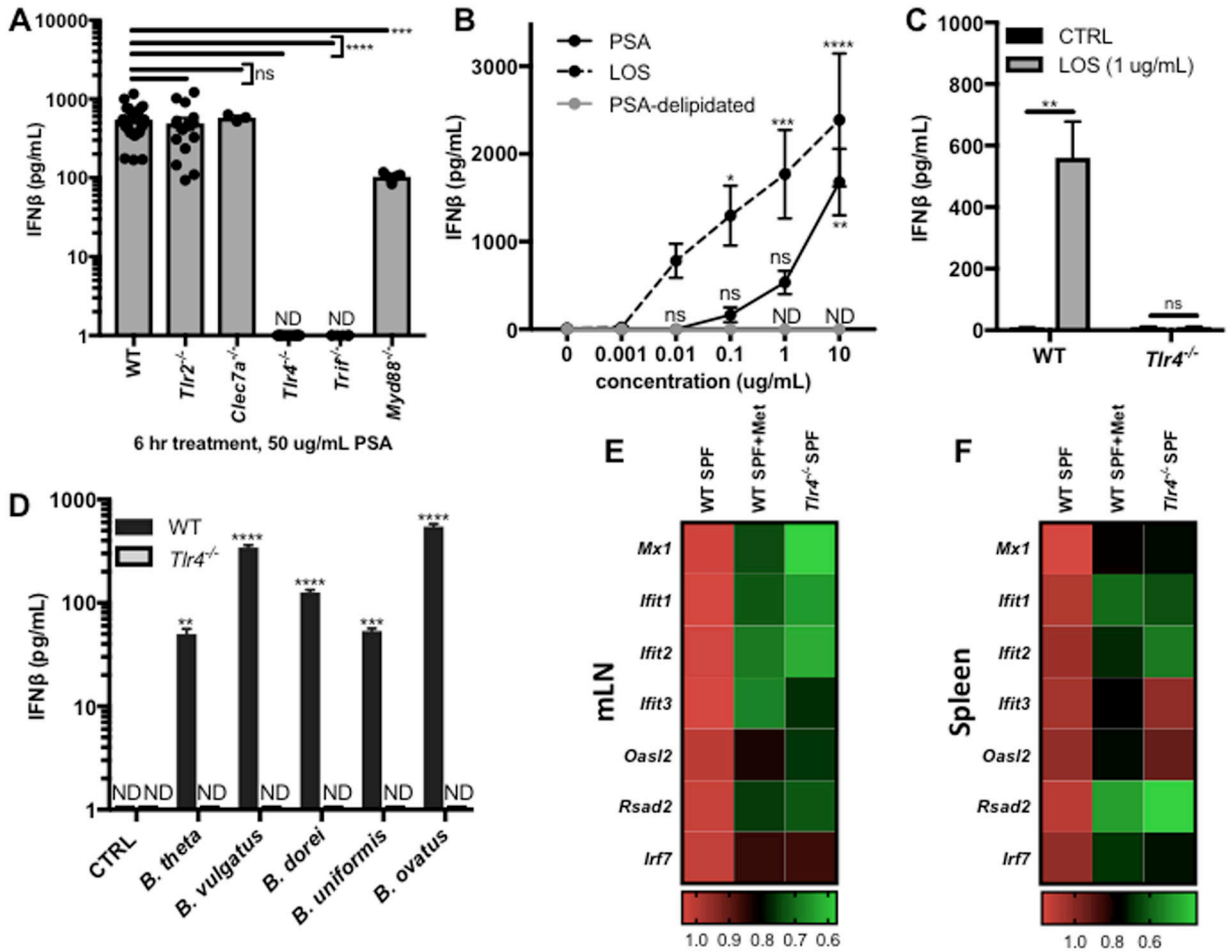


Figure 4. *Bacteroides* OM glycolipids signal through TLR4 to induce IFN β . (A) BMDCs were differentiated from WT (N=27), *Tlr2*^{-/-} (N=15), *Clec7a*^{-/-} (N=3), *Tlr4*^{-/-} (N=12), *Trif*^{-/-} (N=6), or *Myd88*^{-/-} (N=5) mice and treated with 50 ug/mL *B. fragilis* PSA for 6 hrs. IFN β in the supernatants was measured by ELISA and normalized by subtracting vehicle control. (B) ELISA analysis of IFN β in the supernatants of WT BMDCs treated for 6 hrs with a dose response of PSA (N=9), the LOS lipid domain of PSA (LOS, N=14), or delipidated PSA (PSA-delipidated, N=3, ND for all concentrations). ELISA analysis of IFN β in the supernatants of WT or *Tlr4*^{-/-} BMDCs treated for 6 hrs with (C) vehicle control (CTRL, N=8), 1 ug/mL LOS (N=8) or (D) 100 ug/mL *Bacteroides* OM extracts (N=3 per condition). (A-D) Data represents average \pm SEM. (E,F) RNA isolation and qRT-PCR analysis of ISG expression was performed on mLNs and spleens harvested from WT SPF mice, WT SPF mice after 1 week of metronidazole (Met) treatment, or from *Tlr4*^{-/-} SPF mice. Fold change gene expression in the mLN (E) or spleen (F) was calculated compared to WT SPF mice using the $\Delta\Delta$ CT method, with *ActB* as the reference gene and depicted as a heat map. Statistical analysis with (A) one-way ANOVA followed by Dunnett’s multiple comparisons test to WT, (B,D) two-way ANOVA followed by Dunnett’s multiple comparisons test to 0 ug/mL CTRL, and (C) unpaired t-test. (E,F) Details of statistical analyses can be found in Tables S3–4. ND=not detected, ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

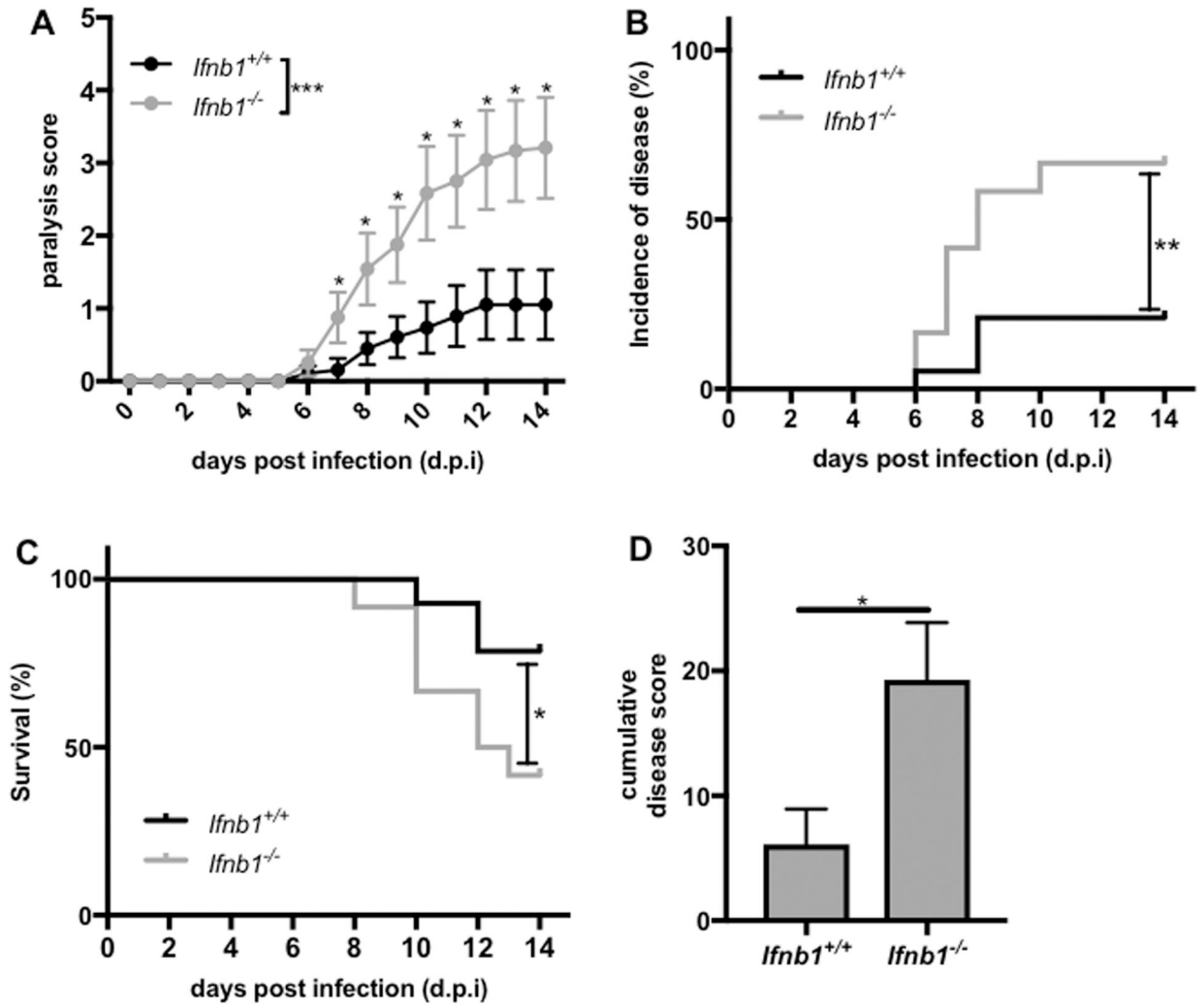


Figure 5. IFN β is required for protection from murine VSV infection.

Littermate *Ifnb1*^{+/+} (N=19) or *Ifnb1*^{-/-} (N=12) mice were infected with 10⁶ PFU of VSV strain Indiana by subcutaneous injection into the footpad. Mice were monitored daily for (A) paralysis score, (B) percentage of mice with paralysis (incidence of disease), and (C) survival. (D) The sum of the daily disease scores for each mouse for the duration of the experiment (cumulative disease score) was calculated 14 d.p.i. Statistical analysis with (A) linear regression analysis and unpaired t-test (for each day), (B,C) log-rank test, and (D) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.

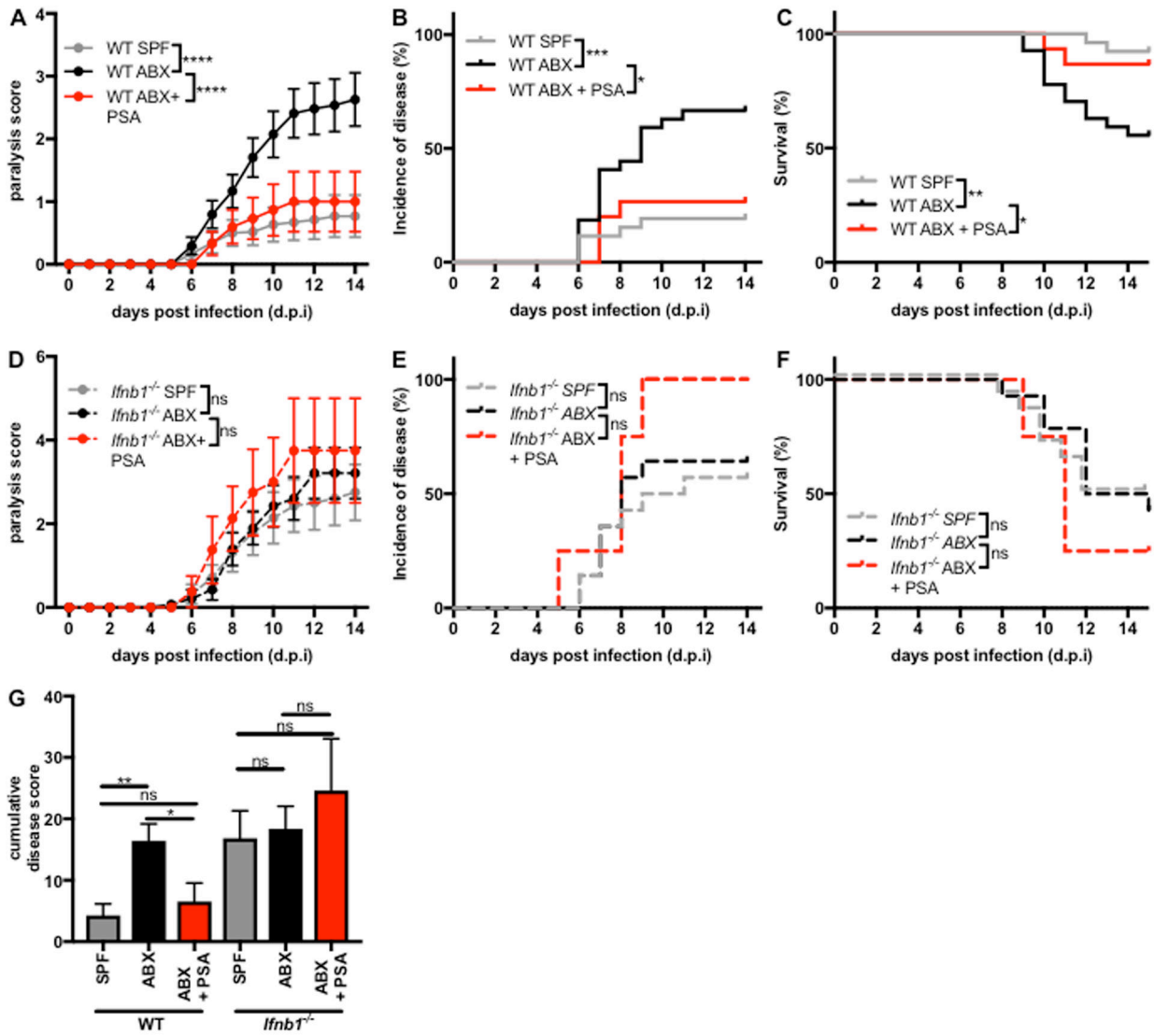


Figure 6. Microbiota-induced IFN β enhances resistance to VSV infection.

WT or *Ifnb1*^{-/-} SPF mice were treated with vehicle control or ABX for 7 days prior to infection as well as vehicle or 75 μ g PSA daily starting 4 days before until the day of infection with 10⁶ PFU VSV strain Indiana. Mice were monitored daily for (A,D) paralysis score, (B,E) percentage of mice with paralysis (incidence of disease), and (C,F) survival. (G) The sum of the daily disease scores for each mouse (cumulative disease score) was calculated 14 d.p.i. Statistical analysis with (A,D) linear regression analysis, (B,C,E,F) log-rank test, and (G) one-way ANOVA followed by Tukey's multiple comparisons test. WT SPF N=26, WT ABX N=27, WT ABX+PSA N=15, *Ifnb1*^{-/-} SPF N=14, *Ifnb1*^{-/-} ABX N=14, *Ifnb1*^{-/-} ABX+PSA N=4. ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

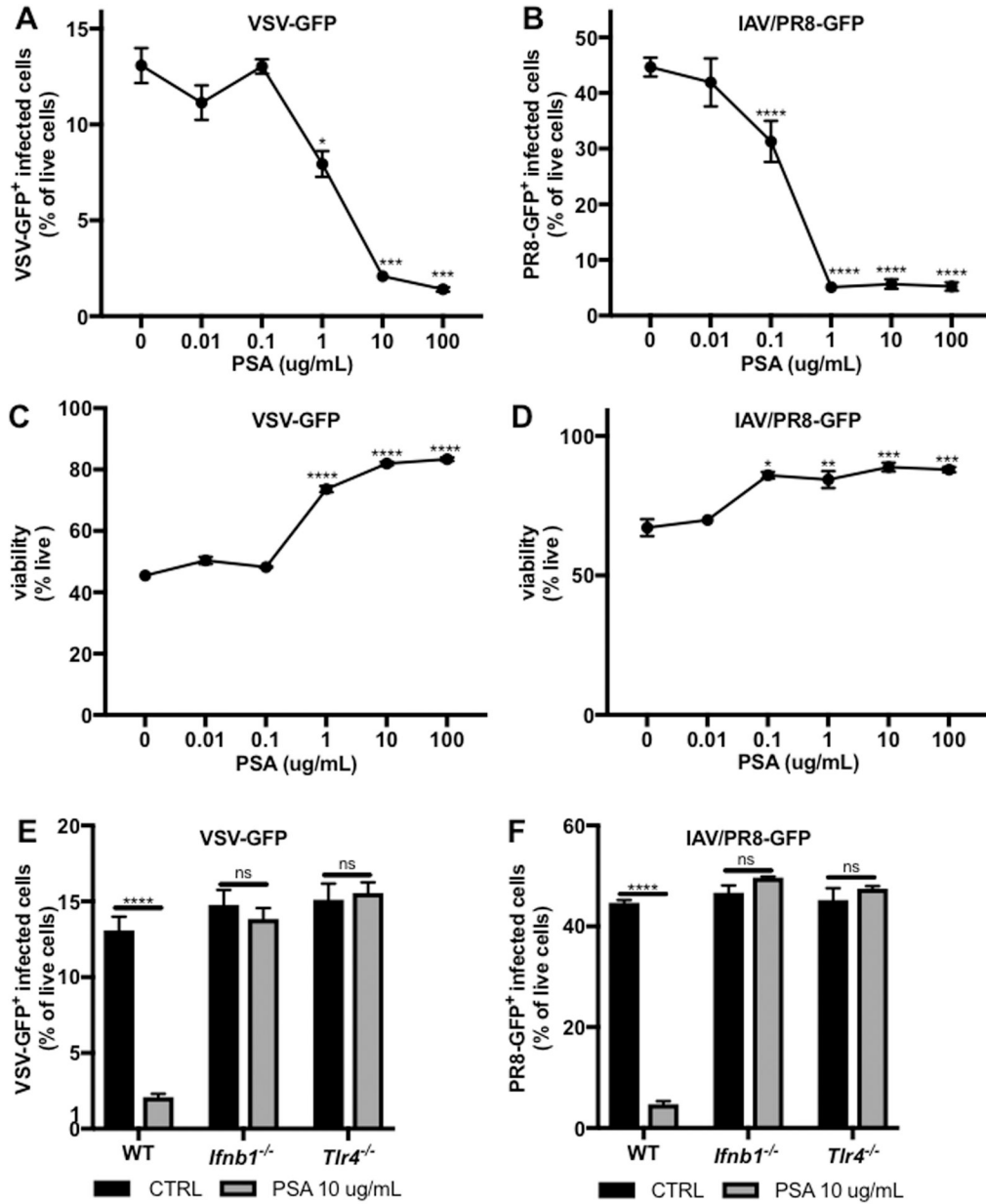


Figure 7. *B. fragilis* PSA reduces virus infection of BMDCs by signaling through TLR4 to induce IFN β .

BMDCs were infected with (A,C,E) VSV-GFP or (B,D,F) IAV/PR8-GFP (both MOI=1). GFP⁺ virus-infected cells were analyzed by flow cytometry 24 h.p.i. in WT, *Ifnb1*^{-/-}, or *Tlr4*^{-/-} BMDCs primed for 24 hours prior to infection with (A-D) a dose response of PSA or (E-F) 10 ug/mL PSA. Data represents mean \pm SEM. Statistical analysis with unpaired t-test to 0 ug/mL CTRL. VSV-GFP N=3–6 samples per condition. IAV/PR8-GFP N=3–12 samples per condition. ns=not significant, *p<0.05. **p<0.01, ***p<0.001, ****p<0.0001.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Pacific blue anti-mouse CD45 antibody	Biolegend	103126, clone 30-F11
PE anti-mouse CX3CR1	Biolegend	149005, clone SA011F11
PerCP-Cy5.5 anti-mouse CD11b	Biolegend	101228, clone M1/70
PE-Cy7 anti-mouse CD11c	Biolegend	117318, clone N418
APC anti-mouse CD103	Biolegend	121413, clone 2E7
Fixable Viability Dye eFluor780	eBioscience	65-0865-14
purified anti-mouse IFN β	Leinco Technologies	I-1182, clone HD β -4A7
Mouse IgG2a isotype control antibody	Leinco Technologies	I-118, clone C1.18.4
FITC anti-mouse BST2 (CD317, PDCA-1)	Biolegend	127007, clone 927
Bacterial and Virus Strains		
<i>Bacteroides fragilis</i>	Kasper laboratory (Erturk-Hasdemir et al., 2019; Mazmanian et al., 2008)	strain NCTC 9343
<i>Bacteroides thetaiotaomicron</i>	ATCC	29741
<i>Bacteroides vulgatus</i>	ATCC	8482
<i>Bacteroides dorei</i>	DSMZ-German Collection of Microorganisms and Cell Cultures	17855
<i>Bacteroides uniformis</i>	ATCC	8492
<i>Bacteroides ovatus</i>	ATCC	8483
<i>Clostridium ramosum</i>	Kasper laboratory	strain A031
Vesicular stomatitis virus (VSV)	Dr. Sean Whelan	strain Indiana
GFP-expressing Vesicular stomatitis virus strain Indiana (VSV-GFP)	Dr. Jack Rose	Dalton and Rose, 2001
GFP-expressing Influenza A virus strain PR8 (IAV/PR8-GFP)	Dr. Adolfo Garcia Sastre	Manicassamy et al., 2010
Chemicals, Peptides, and Recombinant Proteins		
Vancomycin hydrochloride	Alfa Aesar	J62790-06
Gentamicin sulfate	Sigma-Aldrich	G1914-25G
Ampicillin	MP Biomedicals	0219014680
Metronidazole	Alfa Aesar	H60258-22
Amphotericin B	Amresco, Inc.	97061-608
dithiothreitol (DTT)	Sigma-Aldrich	DTT-RO
collagenase type IV	Sigma-Aldrich	C4-28-100MG
Paraformaldehyde solution 4% in PBS	sc-281692	Santa Cruz Biotechnology
GM-CSF Recombinant Mouse Protein	ThermoFisher Scientific	PMC2013
ACK Red Blood Cell Lysing Buffer	Gibco	A1049201
Brucella 5% SB HEMIN VIT K1 plates	BBL, Becton, Dickinson and Company	6217793
Dehydrated Brain Heart Infusion Broth	BBL, Becton, Dickinson and Company	211059
Critical Commercial Assays		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNeasy mini kit	Qiagen	74106
Quantitect Reverse Transcription Kit	Qiagen	205313
RT ² SYBR Green ROX qPCR Mastermix	Qiagen	330523
Pan-DC Microbeads, mouse	Miltenyi Biotec	130-092-465
VeriKine-HS Mouse Interferon Beta Serum ELISA Kit	pbl Assay Science	42410-1
VeriKine Mouse Interferon Beta ELISA Kit	pbl Assay Science	42400-2
Experimental Models: Organisms/Strains		
WT SPF mice	Jackson Laboratory	C57BL/6J, stock# 000664
WT GF mice	GF C57BL/6 origin from the University of North Carolina, colony housed in Kasper lab animal facility	C57BL/6J
<i>Ifnar1</i> ^{-/-} mice	Jackson Laboratory	B6.129S2- <i>Ifnar1</i> ^{tm1Agt} /Mmjax, stock number 010830
<i>Tlr4</i> ^{-/-} mice	Jackson Laboratory	B6.B10ScN- <i>Tlr4</i> ^{ps-de1} /JthJ, stock number 007227
<i>Myd88</i> ^{-/-} mice	Jackson Laboratory	B6.129P2(SJL)- <i>Myd88</i> ^{tm1.1Defr} /J, stock number 009088
<i>Trif</i> ^{-/-} mice	Jackson Laboratory	C57BL/6J- <i>Ticam1</i> ^{Lps2} /J, stock number 005037
IFNβ-YFP mice	Jackson Laboratory	B6.129- <i>Ifnb1</i> ^{tm1Lky} /J, stock number 010818
<i>Ifnb1</i> ^{-/-} mice	created from ES cell clone 12782A-D4, generated by Regeneron Pharmaceuticals, Inc. and obtained from the KOMP Repository (www.komp.org)	<i>Ifnb1</i> ^{tm1(komp)vcg}
Oligonucleotides		
See Table S5 for qRT-PCR primers	N/A	N/A