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Salmonella Typhimurium Co-opts the Host Type I IFN System To Restrict Macrophage Innate Immune Transcriptional Responses Selectively

Darren J. Perkins,* Rajesh Rajaiah,* Sharon M. Tennant,† Girish Ramachandran,† Ellen E. Higginson, † Tristan N. Dyson,* and Stefanie N. Vogel*

Innate immune inflammatory responses are subject to complex layers of negative regulation at intestinal mucosal surfaces. Although the type I IFN system is critical for amplifying antiviral immunity, it has been shown to play a homeostatic role in some models of autoimmune inflammation. Type I IFN is triggered in the gut by select bacterial pathogens, but whether and how the type I IFN might regulate innate immunity in the intestinal environment have not been investigated in the context of Salmonella enterica serovar Typhimurium (ST). ST infection of human or murine macrophages reveals that IFN-β selectively restricts the transcriptional responses mediated by both the TLRs and the NOD-like receptors. Specifically, IFN-β potently represses ST-dependent innate induction of IL-1 family cytokines and neutrophil chemokines. This IFN-β-mediated transcriptional repression was independent of the effects of IFN-β on ST-induced macrophage cell death, but significantly dependent on IL-10 regulation. We further evaluated ST pathogenesis in vivo following oral inoculation of mice lacking IFN-β. We show that IFN-β−/− mice exhibit greater resistance to oral ST infection and a slower spread of ST to distal sterile sites. This work provides mechanistic insight into the relationship between ST and type I IFN, and demonstrates an additional mechanism by which IFN-β may promote spread of enteric pathogens. The Journal of Immunology, 2015, 195: 000–000.

Salmonella enterica serovar Typhimurium (ST) is a gram-negative enteric pathogen most often acquired from contaminated food or water that is associated clinically with severe gastroenteritis in humans and a systemic disease resembling typhoid fever in mice. A robust innate immune response in the gut is required to provide early control of ST infection, slow dissemination to distal sterile sites, and mitigate subsequent morbidity and mortality (1). Over 30 y ago, it was recognized that innate immunity is important in controlling ST (2). Specifically, the TLR system is critical, as animals deficient in the key bacterial recognition sensors TLR4 and TLR2 are significantly more susceptible to ST (3–6).

Despite the clear importance of innate immune responses in controlling infection, ST displays a remarkable capacity for subversion and co-opting of these same responses. Among the evasion mechanisms employed by ST are the type III secretion system effectors encoded by the Salmonella Pathogenicity Island 2 locus that blunt the antimicrobial oxidative burst driven by inducible NO synthase (iNOS) and NADPH oxidase in phagocytes (7). The ability of type III secretion system effectors to subvert endogenous host NOD-like receptor (NLR) chaperones to regulate NLR activity (8), as well as the ability to reprogram transcriptional responses in epithelial cells (9), have also been identified as mechanisms of ST subversion of the immune response.

However, it has recently become abundantly clear that evasion of innate responses is not the only strategy employed by ST, but that ST actively utilizes, and in some instances requires, host innate immune responses. Indeed, the process of gut inflammation itself can promote the pathogenesis of ST by enabling competition with microflora (10) and by generating novel energy sources (11). Additionally, ST takes advantage of TLR-governed phagosomal acidification to induce pH-dependent expression of Salmonella Pathogenicity Island effectors in macrophages that facilitate intracellular ST survival (3, 12, 13).

Significantly, it has recently been reported that type I IFN receptor-null macrophages (IFNAR−/−) are refractory to ST-initiated necrotic cell death (14). The type I IFNs are pleiotropic cytokines produced downstream of many innate receptors that function in both autocrine and paracrine fashions and possess remarkable antiviral properties (15, 16). However, the relationship between type I IFNs and bacterial pathogens is far more complex, and can be deleterious or protective, depending on the specific pathogen (17–19). In nearly all cases examined, however, the molecular mechanisms by which IFN might promote pathogenesis are poorly defined. We postulated that type I IFN may have a significant capacity to govern innate responses to ST. To investigate this hypothesis, we examined the response to infection by ST in IFN-β−/− macrophages, and found that the autocrine or paracrine action of IFN-β profoundly shapes the ST-induced TLR and NLR transcriptional responses in murine macrophages. This selective type I IFN sculpting of the TLR inflammatory response most likely dampens cell-mediated innate immunity to ST by repressing neutrophil chemotaxis and IL-1 cytokine family activity, thereby enabling bacterial dissemination.
Materials and Methods

Ethics statement

Animal work performed for this study complied with all applicable provisions of the Animal Welfare Act; U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; Public Health Services Policy on the humane Care and Use of Laboratory Animals; and Guide for the Care and Use of Laboratory Animals (8th edition). The protocol for this work was approved by the Institutional Animal Care and Use Committee of the University of Maryland, School of Medicine. Clinical ST strains isolated from pediatric patients were obtained from the Salmonella culture collection stored at the Center for Vaccine Development, University of Maryland (Baltimore, MD). These isolates are labeled with a specimen identifier only and lack information that can link them to individual patients. These isolates were collected as part of a previous study, and Institutional Review Board approval information is contained therein (20).

Cell lines and mice

Primary peritoneal macrophages were prepared, as described previously (21). Briefly, 3 ml 3% sterile thioglycolate was injected i.p. into 6- to 8-wk-old, wild-type (WT) C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Four days later, macrophages were harvested by peritoneal lavage with sterile saline. IFN-β null mice (IFN-β−/−), MyD88-null mice (MyD88−/−), and TLR4-null mice (TLR4−/−) backcrossed onto a C57BL/6J background (n ≥ 9) were bred in house, as described previously (22). Peritoneal macrophages from IL-10-null (IL-10−/−) mice were a gift of J. Bream (Johns Hopkins School of Public Health). Peritoneal macrophages from caspase-11 null (caspase-11−/−) mice were a gift of J. Pedra (University of Maryland, Baltimore, MD) that were obtained under a Materials Transfer Agreement with Genentech (South San Francisco, CA). The THP-1 cell line was maintained in RPMI 1640 supplemented with 10% FBS. Prior to infection, cells were plated and differentiated using a Materials Transfer Agreement with Genentech (San Francisco, CA). The THP-1 cell line was maintained in RPMI 1640 supplemented with 10% FBS. Prior to infection, cells were plated and differentiated using PMA (50 ng/ml) for 48 h. The MODEK cell line was a gift of D. Philpott (University of Toronto, Toronto, Canada), and these cells were maintained in DMEM supplemented with 10% FBS.

Abs and reagents

Abs against total p38, phospho-p38, total p-38, phospho-ERK1/2, total ERK 1/2, total p65, phospho-p65, and total IL-1β were purchased from Cell Signaling (Danvers, MA). Anti–IL-10 and anti–IL-27 neutralizing Abs were purchased from eBioscience (San Diego, CA). All Abs used in flow cytometry were purchased from BioLegend (San Diego, CA). Recombinant murine IL-10 and IL-27 were purchased from BioLegend. Protein-free phenol/water–extracted Escherichia coli K235 LPS was prepared, as described elsewhere (23). S-(2,3-Bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-Ser-Lys4-OH (Pam3Cys) was purchased from InvivoGen (San Diego, CA). Purified mammalian-expressed rIFN-β was purchased from PBL (Piscataway NJ). Murine rIFN-γ was provided by Genentech (South San Francisco, CA). Necrottin was purchased from Santa Cruz (Santa Cruz, CA). L18-MDP and C12-TE-DAP were purchased from InvivoGen.

Bacterial strains

Salmonella Typhimurium strain SL1344 was the gift of R. Ernst (University of Maryland, Baltimore, MD). Strains ST7 (ST19) and D65 (ST313) are clinical isolates of ST isolated from blood of febrile pediatric patients in Bamako, Mali, and have been previously described (20).

Quantitative real-time PCR

Total mRNA was isolated from peritoneal macrophages using TRizol (Invitrogen, Carlsbad, CA) reagent, according to manufacturer’s instructions. A total of 1 μg RNA was used in oligo(dT) cDNA synthesis (Bio-Rad RT system). Quantitative RT-PCR (qRT-PCR) was carried out using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) utilizing SYBR Green reagent (Applied Biosystems) and transcript-specific primers. mRNA expression profiles were normalized to levels of housekeeping gene mRNA expression profiles were normalized to levels of housekeeping gene mRNA expression using the 2−ΔΔCt method (24).

Western blot analysis

Whole-cell lysates from primary murine macrophages were obtained by the addition of lysis buffer (20 mM HEPES, 1.0% Triton X-100, 0.1% SDS, 150 mM NaCl, 10 mM NaF, 1 mM PMSF) and subsequent incubation at 4˚C. Cell lysates were separated by electrophoresis in a denaturing SDS-PAGE gel and subsequent transfer to polyvinylidene difluoride membrane.

Blots were incubated overnight in relevant primary Abs at 4˚C, washed three times with PBS, and then incubated with appropriate HRP-conjugated secondary Ab (Jackson Immunoreagents, West Grove, PA). Blots were developed following incubation in ECL PLUS Western blotting detection reagent (Amersham Bioscience, Piscataway, NJ).

In vitro bacterial infections

A single colony of ST strain SL1344 was inoculated into 5 ml Hestrin–Schramm media and grown overnight at 37˚C with shaking. The following morning, an additional 10 ml Hestrin–Schramm media was inoculated with non-LPS–stimulated peritoneal macrophage infected cultures until OD600 reached 1.5. A total of 1 ml culture was pelleted by centrifugation and resuspended into 1 ml sterile PBS. A quantity amounting to 400 μl bacterial suspension was mixed with 600 μl sterile PBS to obtain a concentration of ~4 × 106 bacteria per 10 μl culture. An appropriate volume of this resuspension was mixed with 37˚C antibiotic-free RPMI 1640 to obtain the desired multiplicity of infection (MOI). Bacteria were added to macrophages in culture plates, and infections were synchronized by centrifugation for 5 min at 700 rpm. Infected cultures were incubated at 37˚C for 30 min, and the infection media was replaced and removed with RPMI 1640 containing 50 μg/ml gentamicin and incubated for an additional 45 min at 37˚C. Following gentamicin incubation, media was removed, the cultures were washed twice with sterile PBS, and the media was replaced with antibiotic-free RPMI 1640. For infections involving IFN-β priming, cells were pretreated for 1 h (unless otherwise stated) with 100 IU/ml murine or human IFN-β (in mouse or human cells, respectively) in antibiotic-free RPMI 1640, and subsequently washed twice in PBS prior to addition of infection medium.

In vivo Salmonella infections

Transfer Agreement with Genentech (South San Francisco, CA) reagent, according manufacturer’s instructions. For IL-10 neutralizing experiments, non-LPS–stimulated cells. For oral infection model, mice were inoculated by oral gavage utilizing 100 μl PBS inoculum. For the sepsis infection model, animals were given a single i.p. injection of 1 × 109 ST SL1344 in 100 μl PBS. Animals were sacrificed 8 h later, and livers were harvested for RNA expression analysis by qRT-PCR.

Stimulation of macrophages

For TLR ligand experiments, macrophages were stimulated with 50 ng/ml purified E. coli LPS or 250 ng/ml P3C for the indicated times prior to harvest for RNA expression analysis. For IL-10 neutralizing experiments, 10 μg/ml anti–IL-10 or an isotype-matched control Ab was added prior to IFN-β priming and maintained in media throughout stimulation with LPS. For NOD ligand experiments, primary peritoneal macrophages were treated overnight with either 20 μg/ml NOD1 ligand (C12-IEDAP) or NOD2 ligand (L18-MDP) directly added to culture medium. Cell supernatants were harvested the following day for analysis of CXCL1 protein levels by ELISA.

Macrophage cytotoxicity assays

Primary peritoneal macrophages were infected with ST strain SL1344 at a MOI of 4, and samples of culture supernatant were harvested at the indicated time points for analysis of lactate dehydrogenase (LDH) levels. LDH assay was carried out utilizing the CytoTox 96 (Promega, Madison, WI) LDH release assay, according to manufacturer’s directions.

Flow cytometry/TLR4 internalization

Peritoneal macrophages were pretreated with or without IFN-β, washed twice with PBS, and stimulated with E. coli LPS (100 ng/ml) for indicated times. Cells were subsequently washed with FACS buffer (PBS with 2% FBS), blocked using anti CD16/32 Ab (BioLegend) for 20 min, and specifically stained with anti–TLR4-PE (BioLegend) or isotype control (BioLegend) Ab for 40 min. Cells were washed, and staining was assessed with BD FACS Canto. All Ab staining was carried out on ice. Isotype control staining was used to calculate TLR4 internalization based on mean fluorescence intensity and expressed as a percentage of surface TLR4 staining on non-LPS–stimulated cells.

Myeloperoxidase activity assay

Excised tissues were weighed and then homogenized using an electric homogenizer in 0.5% hexa-decyl-trimethyl-ammonium bromide in 50 mmol/L potassium phosphate buffer (pH 6) and centrifuged at 12,000 rpm for 10 min. Aliquots of the supernatant were mixed with a chromogenic peroxidase substrate (BM blue; Roche) and allowed to react
for 20 min. Absorbance was measured by spectrophotometry at 450 nm. Human purified myeloperoxidase enzyme was used to create standard curves. Myeloperoxidase activity was determined by calculating OD/mass of tissue sample.

Statistical analysis
Statistical analysis, when applied, was done using the Prism software v5.0. All t tests were done using a two-tailed analysis.

Results
Paracrine IFN-β selectively suppresses TLR-dependent inflammatory responses to ST

As previous work had described an increase in ST-induced cell death in IFNAR−/− macrophages, but did not describe altered innate receptor responses in this background (14), we sought to quantify the effect of IFN-β on the innate transcriptional response.

**FIGURE 1.** Early neutrophil chemokine and IL-1 family cytokine responses to ST infection of macrophages are TLR4 dependent and suppressed by paracrine IFN-β. (A) MyD88−/− or MyD88+/+ peritoneal macrophages were infected with ST SL1344 at a MOI of 4 for the indicated times, and total RNA was used for mRNA analysis by qRT-PCR. (B) TLR4−/− and TLR4+/+ peritoneal macrophages were infected as in (A), and total RNA was used for cytokine mRNA analysis by qRT-PCR. (C–E) WT C57BL6/J macrophages were pretreated for 60 min with media alone or 100 U/ml murine IFN-β and infected with ST SL1344 for indicated times prior to mRNA analysis. Data are presented as mean ± SEM. (F) WT C57BL6/J macrophages were infected with strain SL1344 at a MOI of 4 for 6 h. The cells were lysed, and recoverable bacteria were counted after plating on Luria–Bertani agar. (G) Human THP-1 monocytic cells were differentiated for 48 h in PMA prior to infection with SL1344 at a MOI of 4 for 6 h. RNA was harvested, and expression of IL-8 was analyzed by qRT-PCR. Data in (A)–(G) are representative of at least three independent experiments. *p = 0.003.
to ST in primary peritoneal murine macrophages. To confirm the primacy of TLRs in governing the early transcriptional responses of macrophages, WT or MyD88−/− peritoneal macrophages were infected with ST SL1344 at a MOI of 4, followed by examination of classic proinflammatory gene transcripts by qRT-PCR (Fig. 1A). We observed a complete dependence on MyD88 for ST-induced expression of inflammatory mediators (e.g., IL-1β, IL-6) within the first 6 h postinfection (p.i.) (Fig. 1A). As TLR4 has

FIGURE 2. Neutrophil chemokine and IL-1 family cytokine expression are augmented following ST infection of IFN-β−/− macrophages. WT C57BL/6J or IFN-β−/− peritoneal macrophages were infected with SL1344 at a MOI of 4 for 6 h, and RNA was harvested for analysis by qRT-PCR. Data are representative of three independent experiments and shown as mean ± SEM. *p < 0.05, #p = 0.0001. ND, not detectable.

FIGURE 3. Clinical isolates of ST exhibit IFN-β–dependent suppression of inflammatory responses, and cooperativity with IFN-β is independent of cell death. (A) WT C57BL/6J peritoneal macrophages were pretreated for 60 min with media alone or 100 U/ml IFN-β and subsequently infected with either ST strain I77 or strain D65 for 6 h. RNA was harvested, and expression of CXCL1 and CXCL2 mRNA was analyzed by qRT-PCR. (B) WT C57BL/6J peritoneal macrophages were pretreated for 60 min with media alone or 100 U/ml IFN-β. Additionally, half of the cells were pretreated with the cell death inhibitor Necrostatin-1. Macrophages were infected with SL1344 at a MOI of 4 for 6 or 24 h. At each time point, samples of cell supernatants were taken and the levels of LDH in the supernatant were quantified by colorimetric assay. (C) WT C57BL/6J or caspase-11−/− peritoneal macrophages were pretreated for 60 min with media alone or 100 U/ml IFN-β and subsequently infected with either ST strain SL1344 for 6 h. RNA was harvested, and expression of CXCL1 mRNA was analyzed by qRT-PCR. Data are representative of three independent experiments. Data are shown as mean ± SEM. *p < 0.05.
been specifically ascribed the largest role in mediating the early inflammation (3, 25), we similarly infected WT and TLR4−/− macrophages. Like MyD88−/− macrophages, TLR4−/− macrophages showed a nearly complete loss of induction of IL-1β and IL-6 mRNA (Fig. 1B) as well as other proinflammatory genes (data not shown). In vivo, type I IFN secreted by infected tissue acts on and establishes antimicrobial states in adjacent uninfected cells in advance of infection in a paracrine mode of action. We therefore sought to model the paracrine effects of IFN-β on macrophages. WT peritoneal macrophages were pretreated with murine rIFN-β (100 IU/ml) for 60 min prior to infection with ST SL1344 (MOI = 4). Remarkably, exposure of macrophages to IFN-β selectively suppressed inflammatory cytokine responses to ST (Fig. 1C). Induction of IL-1β and IL-18 was significantly suppressed in IFN-β–primed macrophages over the first 6 h of infection (Fig. 1C). Additionally, we found the neutrophil chemokines, that is, CXCL1, CXCL2, and CXCL5, were suppressed by pretreatment of macrophages with IFN-β. IFN-β–mediated suppression of ST-induced cytokine/chemokine mRNA expression was not a global phenomenon, as no significant suppression of either IL-6 or TNF-α induction was observed (Fig. 1D). Interestingly, prior exposure to IFN-β markedly enhanced subsequent ST-induced production of IFN-β mRNA (Fig. 1E), consistent with previous findings showing that type I IFN priming of macrophages increased induction of type I IFN in response to LPS (26). Each of these observations made at the mRNA level was independently confirmed at the protein level (Supplemental Fig. 1A). As reduced proinflammatory gene induction might have resulted from differential infection of media- and IFN-β–pretreated macrophages, the potential effects of IFN-β on the phagocytosis or early survival of ST in macrophages were ruled out by recovering equivalent numbers of intracellular bacteria from media- or IFN-β–pretreated macrophages 6 h p.i. (Fig. 1F). To determine whether the IFN-mediated cytokine suppression we observed was generalizable to a human system, the human macrophage-like cell line, THP-1, was also infected with a MOI of 4 and human CXCL1 homolog, IL-8

![Image of a graph showing the expression of various cytokines and chemokines in response to IFN-β pretreatment.](http://www.jimmunol.org/)

**FIGURE 4.** IFN-β suppresses transcriptional responses to purified ligands across multiple innate receptor systems. (A) WT C57BL6/J macrophages were pretreated for 60 min either with media alone or 100 U/ml IFN-β. Macrophages were stimulated with 50 ng/ml purified *E. coli* LPS for the indicated times. RNA was harvested, and transcript levels were assayed by qRT-PCR. (B) Macrophages were pretreated as in (A) and stimulated with 50 ng/ml LPS for 6 h. Whole-cell lysates were probed by Western blot for pro–IL-1β protein. (C) Samples of cell supernatants from macrophages treated as in (A) were collected at a 24-h time point and assayed for chemokine content by ELISA. (D) WT C57BL6/J peritoneal macrophages were pretreated with 100 U/ml mammalian IFN-β for the indicated times and subsequently restimulated with 50 ng/ml *E. coli* LPS for 6 h. RNA was harvested, and IL-1β transcript expression was quantified by qRT-PCR. (E) WT C57BL6/J peritoneal macrophages were pretreated with 100 U/ml mammalian IFN-β for 60 min prior to stimulation for the indicated times with the TLR2 ligand Pam3Cys (250 ng/ml). (F) Peritoneal macrophages pretreated with media alone or IFN-β were subsequently treated overnight with the NOD-1 ligand C12-iE-DAP (20 μg/ml) or the NOD-2 ligand L18-MDP (20 μg/ml). Samples of cell supernatant were harvested, and CXCL1 levels were assayed by ELISA. Data in (A)–(F) are representative of three independent experiments. Data are shown as mean ± SEM. *p < 0.05.
mRNA, measured. A similar ST suppression of IL-8 mRNA was observed, as seen with murine CXCL1 (Fig. 1G). To address a second relevant tissue type in ST infection, we examined the suppressive effects of type I IFN on small intestinal epithelia. IFN-β–mediated suppression of ST-induced CXCL2 was not observed in the ileal epithelial cell line, MODE-K, suggesting that this effect may be limited to innate myeloid cells (Supplemental Fig. 1B).

IFN-β−/− macrophages are selectively hyperresponsive to ST infection

To confirm our findings, we also sought to establish a model for autocrine IFN action, in which IFN produced by infected cells acts back on these same cells to slow infection in the absence of exogenous IFN-β. To this end, primary peritoneal macrophages from IFN-β−/− mice were infected with ST SL1344 at MOI = 4 for 4 h. Remarkably, in the IFN-β−/− macrophages, significantly enhanced induction of IL-1β and the neutrophil chemokines CXCL1, CXCL2, and CXCL5 mRNA was observed (Fig. 2). The specificity of the IFN-β effect was supported by examining the induction of IL-6, TNF-α, and the monocyte chemokine, CCL5, none of which were enhanced in the absence of IFN-β (Fig. 2), a pattern of gene induction that was also observed at multiple time points (data not shown). Each of the changes observed at the mRNA level was recapitulated by measuring protein levels (Supplemental Fig. 2).

IFN-β suppression of ST-induced transcriptional responses is dissociable from macrophage cell death

The ST SL1344 strain has been used extensively for studies on the innate response to ST; however, this strain has been passaged for a prolonged period under laboratory conditions (27), and we considered the possibility that this laboratory passage had influenced the relationship of ST SL1344 with IFN-β. To rule out this possibility, we obtained clinical isolates from Mali North Africa in two sequence type variants (20, 28). Both the I77 and the D65 clinical strains exhibited an IFN-β–dependent suppression of IL-1β and neutrophil chemokines, confirming the potential clinical relevance of our findings (Fig. 3A).

**FIGURE 5.** IFN-β governs *S. typhimurium* production of anti-inflammatory IL-10, and IL-10 contributes to IFN-β–mediated suppression. (A) WT C57BL/6J peritoneal macrophages were pretreated with 100 U/ml IFN-β and subsequently infected with ST at a MOI of 4 for 6 h. mRNA and protein levels of IL-10 were assayed. (B) WT and IFN-β−/− macrophages were infected with SL1344 for 6 h, and mRNA and IL-10 protein levels were assayed. (C) IL-27 mRNA expression assayed following 6 h of SL1344 infection, as in (B). (D) WT peritoneal macrophages were pretreated with rIL-10 for 60 min prior to stimulation with 50 ng/ml *E. coli* LPS for 4 h. (E) WT peritoneal macrophages were pretreated with rIL-27 for 60 min prior to stimulation with 50 ng/ml *E. coli* LPS for 4 h. (F and G) Macrophages were pretreated with IFN-β in the presence or absence of neutralizing IL-10 (10 μg/ml) (F) or IL-27 (10 μg/ml) (G) Ab, followed by subsequent stimulation with 50 ng/ml *E. coli* LPS for 4 h. Levels of IL-1β mRNA were assayed by qRT-PCR. (H) Treatment as in (F) with analysis of pro–IL-1β levels in whole-cell lysate by Western blot. Data in (A)–(G) are representative of three independent experiments. *p < 0.05.
Although we observed striking alterations of innate inflammatory transcriptional responses following ST infection of IFN-β–primed macrophages, a previous study found that type I IFN enhanced ST killing of macrophages in a manner consistent with necroptosis (14). As it remained formally possible that the IFN-dependent altered transcriptional responses we observed were related to the onset of a cell death program, we examined ST-induced cytotoxicity in media- or IFN-β–treated macrophages by LDH release assay. Macrophages were treated with media alone or IFN-β for 4 h prior to infection for 6 or 24 h (MOI = 4) in the absence or presence of the necroptosis inhibitor necrostatin-1 (33 μM). Samples of cell supernatant were analyzed for the release of LDH. Direct lysis of the cell monolayer by detergent was used as a positive control. We did not observe significant cytotoxicity at 6 h p.i. (the time point at which we made our mRNA observations) (Fig. 3B, left panel). However, there was significant ST-induced cytotoxicity observable by 24 h that could be substantially inhibited by necrostatin-1 (Fig. 3B, right panel). This result strongly argues that early IFN-β–mediated transcriptional suppression is dissociable from its effects on macrophage viability. Recently, it has been reported that caspase-11 is an IFN-β–inducible executioner caspase capable of triggering macrophage necroptosis in response to ST (29). As an additional control for IFN-regulated cell death pathways, we infected WT C57BL/6J or caspase-11−/− macrophages with ST following treatment with IFN-β. The absence of caspase-11 did not diminish the ability of IFN-β to suppress TLR4 responses (Fig. 3C).

**Multiple innate sensing systems are suppressed by IFN-β**

As ST expresses a number of microbe-associated molecular patterns and is capable of simultaneously ligation of multiple pattern recognition receptors, we sought to determine whether the IFN-β–mediated cytokine and chemokine suppression seen with live bacteria could be recapitulated using a single purified TLR ligand. As the early inflammatory cytokine responses to ST in peritoneal macrophages were highly TLR4 dependent (Fig. 1A, 1B), peritoneal macrophages were stimulated with *E. coli* LPS (50 ng/ml) after pretreatment with medium or IFN-β, and cytokine mRNA was assayed over a 6-h time course. The previously observed pattern of selective gene suppression seen when macrophages were infected with live ST (Fig. 1) was reproduced by LPS stimulation (Fig. 4A). Similar trends were observed when cytosolic pro–IL-1β (Fig. 4B) and secreted levels of CXCL1 and CXCL2 protein (Fig. 4C) were measured by Western blot and ELISA, respectively. To define further the kinetics of IFN-β action, peritoneal macrophages were pretreated with IFN-β for time periods ranging from 30 min to 24 h and then stimulated with *E. coli* LPS for 4 h, at which time IL-1β mRNA was examined by qRT-PCR. As little as 30-min exposure to IFN-β was sufficient to achieve selective inhibition of LPS-induced genes, whereas inhibition could be observed as late as 24 h post-IFN treatment (Fig. 4D). As our experiments had therefore been concentrated on TLR4 responses, we sought to determine whether the suppressive effects of IFN-β were generalizable to other TLRs, as well as other potentially relevant innate sensing systems. We stimulated media- or IFN-β–primed macrophages with the synthetic TLR2 ligand Pam3CSK4 (250 ng/ml) for 4 h and assayed pro–IL-1β gene induction by qRT-PCR. IFN-β priming effectively suppressed IL-1β mRNA induced via TLR2 (Fig. 4E). Although TLRs are the most significant innate sensing system contributing to cytokine induction within the initial hours of ST infection of macrophages, there is significant literature documenting a role for the NLRs in contributing to responses to ST, and, in particular, in contributing to the production of neutrophil chemokines (30, 31). We therefore assayed for the ability of IFN-β to govern NLR chemokines by stimulating medium- or IFN-β–primed macrophages with the synthetic NOD1 ligand (C12-iE-DAP; 20 μg/ml) or a synthetic NOD2 ligand (L-18-MDP; 20 μg/ml). CXCL1 secretion derived from either NOD1 or NOD2 ligand was also diminished by IFN-β priming (Fig. 4F).

Having established an important role for IFN-β in shaping the TLR- and NO-invariant-dependent response to ST, we wanted to address the question of underlying molecular mechanism. Although the regulation of IFN-β induction by upstream TLR ligation has been studied in great detail, the effect of prior exposure to type I IFN on the biology of TLRs themselves has not been systematically investigated. We therefore characterized TLR expression, trafficking, and signaling following LPS stimulation of media- or IFN-β–treated macrophages. We did not observe a reduction in steady-state mRNA levels of TLRs 2 or 4 or the adaptors MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β, but did observe IFN-dependent enhanced transcription of TLR3 and TLR9. TLR4 protein surface expression was slightly enhanced by IFN-β. LPS-induced signal transduction, as judged by MAPK activation (Supplemental Fig. 3), as well as NF-κB and IFN regulatory factor 3 activation (data not shown), was not diminished by IFN-β. TLR4 internalization and endosomal trafficking were similarly unaffected (Supplemental Fig. 3).

**IFN-β governs ST production of anti-inflammatory cytokines**

Having found no IFN-β–mediated suppression of TLR4 expression, trafficking, or signaling, we speculated that IFN-β may act on innate immune receptor stimulation by augmenting production of secreted anti-inflammatory mediators that act as a negative feedback loop. The best-characterized secreted anti-inflammatory mediator induced by TLRs is IL-10. Therefore, we examined IL-10 production in medium- or IFN-β–treated macrophages during ST infection. Strikingly, we found that IL-10 mRNA and protein production was dramatically

![Figure 6](http://www.jimmunol.org/) Loss of IFN-β–mediated transcriptional suppression in IL-10−/− macrophages. WT and IL-10−/− macrophages were pretreated with media alone or 100 U/ml IFN-β for 60 min prior to infection with SL1344 for 4 h. IL-1β and CXCL1 mRNA were assayed by qRT-PCR.
FIGURE 7. Oral ST infection elicits differential inflammatory responses in WT and IFN-β−/− mice. (A and B) WT C57BL6/J or IFN-β−/− were inoculated with ∼2×10⁷ or 5×10⁶ CFU ST strain SL1344 in PBS by oral gavage. Animals were monitored twice daily for morbidity and mortality. Data are representative of three experiments. Significance determined by Mantel–Cox test, p = 0.0012. (C) WT C57BL6/J or IFN-β−/− animals were infected as in (A), and sections of small bowel were harvested at 48 h. RNA was prepared from sections, and mRNA levels of CXCL2 were analyzed by qRT-PCR. p = 0.0211. (D) WT C57BL6/J or IFN-β−/− animals were infected with 2×10⁷ CFU of Sl1344 by oral gavage. The livers were removed 48 h later. Livers were weighed, and homogenates were plated on Luria–Bertani agar. p = 0.002. (E) WT C57BL6/J or IFN-β−/− animals were infected as in (A), and sections of small bowel were harvested at 48 h. Tissue homogenates were analyzed for peroxidase activity by colorimetric assay (p = 0.0255). Data from (B)–(D) representative of three independent experiments and shown as mean ± range. Significance determined by Student’s t test. (F–J) (Figure legend continues)
enhanced in macrophages primed with IFN-β (Fig. 5A). We complemented this result by examining IL-10 production, as well as production of another reportedly immunosuppressive cytokine, IL-27, in IFN-β−/− macrophages. In the absence of IFN-β, ST-infected macrophages produced extremely low levels of IL-10 mRNA and protein (Fig. 5B) and IL-27 (Fig. 5C). Conversely, we tested the ability of rIL-10 and rIL-27 to suppress cytokines downstream of TLR4 with the kinetics observed in our system. rIL-10 and rIL-27 potently suppressed IL-1β and KC mRNA expression even when added 24 h prior to LPS treatment (Fig. 5D, 5E). Finally, to test the hypothesis that IL-10 underlies the observed IFN-β–mediated suppression, WT macrophages were stimulated with IFN-β in the presence of anti–IL-10 or anti–IL-27 neutralizing Ab, followed by 4 h of LPS treatment. Neutralization of IL-10 significantly, but incompletely, reversed the suppression of IL-1β mRNA caused by IFN-β (Fig. 5F), whereas neutralization of IL-27 had no effect (Fig. 5G). Reversal of IL-1β suppression by anti–IL-10 was also observed at the protein level (Fig. 5H). As the incomplete reversal of IFN-β suppression of IL-1β may have been due to the incomplete neutralization of IL-10 by Ab, we tested the capacity of IFN-β to inhibit TLR4-mediated gene expression in IL-10−/− peritoneal macrophages. WT and IL-10−/− macrophages were pretreated with medium or IFN-β for 60 min prior to infection with ST SL1344 for 4 h. Substantial rescue of CXCL1 mRNA expression was seen in the IL-10−/− background (Fig. 5I). In agreement with our Ab neutralization data, lack of IL-10 significantly, but incompletely, reversed the suppression of IL-1β by IFN-β (Fig. 6 at this point time. These results argue for an important role for autocrine IL-10 in mediating the transcripational repression due to IFN-β.

IFN-β promotes susceptibility to oral Salmonella infection

Although our investigations have established a clear capacity for IFN-β to regulate ST innate transcriptional responses in peritoneal macrophages, the inflammatory responses of intestinal macrophages are dependent on in vivo ST infection may not be identical. To interrogate further a role for IFN-β in the gut during ST infection, WT C57BL/6 or IFN-β−/− mice were inoculated with 1 × 10^8 CFU of ST strain SL1344 by oral gavage and were monitored for lethality over time (Fig. 7A). We observed a significant increase in mean time to death in the IFN-β−/− mice (p < 0.05). Additional experiments with lower doses of ST (∼5 × 10^9) revealed even greater differences (Fig. 7B). When we assessed organ burden from the livers of WT and IFN-β−/− mice 48 h p.i., in all cases, livers from ST-infected IFN-β−/− mice displayed a markedly reduced bacterial burden (Fig. 7D), with similar results at 24 h p.i. (data not shown). A similar difference in bacterial burden was also observed in the spleen (data not shown). We sought to determine whether the presence of IFN-β in vivo governed early innate inflammatory responses. Serial sections of small intestine proximal to the cecum were excised from PBS-treated or ST-infected WT and IFN-β−/− mice 48 h p.i., and mRNA was extracted for analysis of inflammation-related transcripts. At early time points (24 and 48 h), we did not detect an appreciable upregulation of classic proinflammatory genes (e.g., TNF-α, IL-6) in either strain (data not shown). The failure to detect significant inflammatory cytokine mRNA in whole gut tissue is not unexpected, as our infection model does not involve pretreatment of the animals with antibiotics to reduce levels of competing microflora, and therefore, to enhance ST colonization and attendant inflammation (32). We did, however, consistently observe a marked increase in the expression of the neutrophil chemoattractant, CXCL2 (MIP2), in the small intestine of IFN-β−/− compared with WT mice at 48 h p.i. (Fig. 7C). This difference in chemokine expression led us to speculate that there may be enhanced early neutrophil responses in the IFN-β−/− animals that may, in part, account for the early increased resistance to oral ST infection. To quantify the presence of infiltrating neutrophils in the small intestine, myeloperoxidase activity, a hallmark of neutrophil activity, was measured in homogenates from sections of WT and IFN-β−/− mice 48 h p.i. We observed significantly greater myeloperoxidase expression in the small intestine of IFN-β−/− mice at 48 h compared with WT animals (Fig. 7E), consistent with enhanced CXCL2 activity.

As we were unable to quantify levels of induced IFN-β or IL-10 in our model of oral infection, we used an additional model of systemic septic infection. WT or IFN-β−/− animals were infected i.p., and cytokine and chemokine levels as well as bacterial colony counts in the liver were determined after 8 h. We observed an induction of IFN-β mRNA in the liver of infected WT, but not the IFN-β−/− using this model (Fig. 7F). Importantly, we also observed a marked reduction in IL-10 levels in the livers of infected IFN-β−/− animals when compared with WT, in agreement with our in vitro studies (Fig. 7G). The levels of IL-6 were comparable in both infected genotypes, whereas CXCL1 and IL-1β were consistently elevated in infected IFN-β−/− mice (Fig. 7H-J). We did observe lower levels of recoverable bacteria in the livers of IFN-β−/− animals at 8 h p.i. (Fig. 7K).

**Discussion**

Among the mammalian IFNs, a common schema assigns antibacterial functions to the type II IFN (IFN-γ), whereas the type I IFN system is widely regarded as an inducer of antiviral responses. However, within the last decade, there has been a growing interest in the relationship in vivo between the type I IFN systems and bacterial infections. Salmonella enterica serovar Typhimurium is a significant human pathogen that induces an in vivo type I IFN response through its interactions with TLRs (25). Our current work argues that type I IFN plays a significant role in shaping the TLR-induced transcriptional response to ST (and most likely other enteric pathogens) and, in particular, in governing the cellular aspects of the innate response through regulation of IL-1 family and chemokine gene expression. A recent manuscript studying IFN-αβ and ST identified a role for type I IFN in regulating ST-induced macrophage cell death, an effect that may well be a result of IFN-induced caspase-11 (29, 33). However, it should be noted that a RIP3K−/− animal model that is defective in the induction of macrophage programmed cell death failed to recapitulate the dramatic resistance to ST seen in the IFNAR−/− mice (14), arguing for the existence of additional IFN-regulated pathways in addition to necroptosis.

The role we have identified for IFN-β in suppressing aspects of the TLR/NLR-driven innate inflammatory response is congruent with significant literature detailing a homeostatic as well as anti-inflammatory role for type I IFNs in a variety of inflammatory and autoimmune conditions (34–36), particularly in the intestine (37–39). Of distinct relevance for the current study is work showing that lactic acid bacteria, a common commensal bacterium, constitutively stimulate low-level production of IFN-β, but not IFN-α.
in the small intestine via dsRNA ligation of TLR3. This homeostatic production of IFN-β has significant protective effects in an induced colitis model, including reduction of infiltrating neutrophils (25). Importantly, however, the previous work does not address critical questions of molecular mechanism to explain the action of type I IFN in the gut. The ability of IFN-β to sculpt the TLR/NLR-induced transcriptional response in the intestinal environment may be critical to its palliative capacities.

Although low levels of type I IFN production are required to maintain homeostasis in the intestine, the present work reveals that induced production of type I IFN, and specifically, IFN-β, can be used by a pathogenic bacteria to suppress an innate inflammatory response. We have identified the immunosuppressive cytokine IL-10 as a significant mediator of IFN-β action. IL-10 and TLR action have been described as uniquely significant in the gut (40, 41), and elevated IL-10 has been shown to impair clearance of nontyphoidal ST in multiple infection models (42, 43). How the selective suppression of TLR4-induced genes is achieved by IFN-β requires further study. This question is made all the more interesting when considering that rIL-10 as a single agent is generally suppressive of TLR responses in macrophages (44). Thus, there appears to be a combinatorial effect of type I IFN and IL-10. This selective, rather than general, suppression of TLR/ NLR-mediated responses may be important for the biology of ST infection as ST has been shown to benefit in competition with intestinal microbiota from some aspects of the intestinal inflammation that it induces (10).

In assessing the likely biological consequences of IFN-β-mediated innate immune suppression, it is important to consider the roles of the individual genes that we have identified as being IFN sensitive. Both IL-1β and IL-18 have been shown in vivo to be important for the survival of mice following oral Salmonella infection, with IL-18 expression having a greater effect (45). The contributions of IL-1β and IL-18 to resistance are most likely manifold; however, it has been shown that IL-18, in particular, is a critical driver of IFN-γ expression in T cells and possibly neutrophils during Salmonella infection (46–48). The contributions of CXCL1, CXCL2, and CXCL5 to neutrophil chemotaxis have been extensively documented (49), as have the contributions of CXCL1, CXCL2, and CXCL5 to neutrophil chemotaxis in the gut (12, 50, 51), where neutrophil influx during infection is driven by production of CXC chemokines that are sensitive. Both IL-1β and IL-18 to resistance are most likely manifold; however, it has been shown that IL-18, in particular, is a critical driver of IFN-γ expression in T cells and possibly neutrophils during Salmonella infection (46–48). The contributions of CXCL1, CXCL2, and CXCL5 to neutrophil chemotaxis have been extensively documented (49), as have the contributions of CXCL1, CXCL2, and CXCL5 to neutrophil chemotaxis in the gut (12, 50, 51), where neutrophil influx during infection is driven by production of CXC chemokines that are largely governed at the mucosal surface by the IL-17/IL-23 axis (52, 53). Another point of note is that this present work may have implications for our understanding of the biology of ST and viral comorbidities. In particular, a substantial correlation between underlying HIV infection and invasive cases of nontyphoidal Salmonella has been demonstrated (54, 55). This may be notable as HIV infection induces abundant systemic type I IFN production (56). The promotion of ST dissemination from the gut in SVIinfected macaques has also been shown experimentally (53).

Although we show clearly that the production of IFN-β in vivo rapidly accelerates the spread of ST infection to secondary sites (Fig. 1B), this is clearly not the case for all pathogenic bacteria (19, 38) nor even all gram-negative bacteria. Indeed, Y. enterocolitica, which shares a common oral route of infection with ST, is suppressed by Toll/IL-1R domain-containing adapter-inducing IFN-β-dependent IFN production (57). It has long been known that the type I IFNs mediate the upregulation of hundreds of genes responsible for cell-intrinsic defense against viral infection and a select number of genes with direct antibacterial function, such as iNOS. To take advantage of the suppressive effects of IFN, however, bacteria must be able to evade the cell-intrinsic defenses. Such behavior has been demonstrated for ST in that it is particularly good at evading the iNOS-driven reactive nitrogen species prevalent in macrophages, while being significantly more susceptible to the neutrophil-derived reactive oxygen species (58, 59). We have shown that the suppressive effects of type I IFN on ST-induced cytokine transcription can be dissociated from the capacity of type I IFN to potentiate ST-induced macrophage pyroptotic cell death through caspase-11 (Fig. 3B, 3C). As pyroptotic cell death mediated by IFN-activated caspase-11 results in some mature IL-1β release, a legitimate question arises as to whether type I IFN has both a pro- and anti-inflammatory role during ST infection (33, 60). We believe that as caspase-11 does not directly process IL-1, but indirectly leads to auxiliary caspase-1 activation and IL-1β processing, the contribution of this caspase-11 pathway to total IL-1β release in vivo during ST infection is likely to be much smaller than that achieved by conventional inflammation and caspase-1 activation induced by ST-encoded pathogen-associated molecular patterns such as flagellin. Instead, the chief effect of caspase-11 regulation by IFN may be to kill ST-infected macrophages that, although depriving ST of an intracellular niche for replication, may also limit the duration of non–IL-1 inflammatory cytokine production by the infected cell.

Disclosures

The authors have no financial conflicts of interest.

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