IFN-β signalling regulates RAW 264.7 macrophage activation, cytokine production, and killing activity

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Abstract
Type I IFN holds a critical role in host defence, providing protection against pathogenic organisms through coordinating a pro-inflammatory response. Type I IFN provides additional protection through mitigating this inflammatory response, preventing immunopathology. Within the context of viral infections, type I IFN signalling commonly results in successful viral clearance. Conversely, during bacterial infections, the role of type I IFN is less predictable, leading to either detrimental or beneficial outcomes. The factors responsible for the variability in the role of type I IFN remain unclear. Here, we aimed to elucidate differences in the effect of type I IFN signalling on macrophage functioning in the context of TLR activation. Using RAW 264.7 macrophages, we observed the influence of type I IFN to be dependent on the type of TLR ligand, length of TLR exposure and the timing of IFN-β signalling. However, in all conditions, IFN-β increased the production of the anti-inflammatory cytokine IL-10. Examination of RAW 264.7 macrophage function showed type I IFN to induce an activated phenotype by up-regulating MHC II expression and enhancing killing activity. Our results support a context-dependent role for type I IFN in regulating RAW 264.7 macrophage activity.

Keywords
Interferon-β, macrophages, Toll-like receptors, type I IFN

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Introduction
The type I IFN multigene cytokine family occupies integral roles in combating the pathogenic organisms of vertebrate hosts.1,2 The most well-characterised gene products encoded within this set of genes include the 13 IFN-α subtypes and the single IFN-β.1-3 Initially discovered by Isaac and Lindenmann in the context of viral infection,4 type I IFN cytokines have become distinguished as antiviral mediators of infection.5 Over the years, increasing attention has been given to the diverse functions of these cytokines beyond their antiviral activity.3,6,7 Type I IFN has been observed to be actively involved in the engagement of immune cells during not only viral, but also bacterial, fungal and parasitic infections.7 Throughout the course of infection, type I IFN functions as both pro- and anti-inflammatory cytokines stimulating and dampening the immune response.8,9 Interestingly, within the context of bacterial infections, the consequence of type I IFN has been shown to be unpredictable, as type I IFN signalling can lead to both beneficial and detrimental outcomes for the host. However, the factors underlying this variability in the effect of type I IFN signalling during bacterial infection remain unclear.6,9

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The induction of type I IFN occurs as a host response to the recognition of invading pathogenic organisms.\textsuperscript{3,10} The body interprets the presence of micro-organisms through recognising molecular characteristics uniquely expressed by pathogens. The presence of these microbial characteristics, often referred to as PAMPs, provide specific ligands for host PRRs to trigger the engagement of an immune response.\textsuperscript{1,11,12} Several different families of PRRs exist, such as the TLR family. In humans, 10 TLRs (TLR1-10) have been identified that localise either to the surface or intracellular compartments of the cell.\textsuperscript{13} The various TLRs are engaged by different PAMPs. For example, the bacterial LPS and the adhesion portion of type I fimbria, FimH, are both ligands for TLR\textsuperscript{4,14,15}; the nucleic acid PAMPs CpG and PolyI:C engage TLR9 and TLR3, respectively; and the triacylated lipopeptide, PAM3CSK4, is a ligand for TLR2.\textsuperscript{16} The association of TLRs with their respective PAMPs results in TLR activation. This activation induces the mobilisation and binding of adaptor proteins, such as myeloid differentiation factor 88 (MyD88), TRIF, TRAM or TIRAP to the TLR. The presence of the adaptor proteins facilitates the induction of the downstream signalling cascade, ultimately leading to the activation of transcription factors, including NF-kB and IFN regulatory factors (IRFs), which modulate the expression of cytokines, chemokines and the production of type I IFN.\textsuperscript{13,16} This includes the induction of pro-inflammatory cytokines such as TNF-\(\alpha\), IL-6,\textsuperscript{12,17} the chemokine RANTES\textsuperscript{18} and the anti-inflammatory cytokine IL-10.\textsuperscript{19}

The type I IFN receptor, IFNAR, is expressed by all nucleated cells within the body.\textsuperscript{20} The ubiquitous expression of IFNAR therefore permits type I IFN to modify the cellular activity of a variety of cells, including those involved in the immune response.\textsuperscript{3,5,21} The outcome of type I IFN signalling varies depending on the context in which it is acting. For example, the type of pathogen is understood to have an influence on how type I IFN coordinates the cellular response to infection and ultimately the health of the organism.\textsuperscript{3} In addition, the timing and duration of type I IFN signalling has been suggested to hold an important role in determining the outcome of infection.\textsuperscript{5}

The purpose of our study was to gain a more comprehensive understanding of the ways in which type I IFN influences macrophage cell function under different conditions. Macrophages are one of the vital components in the innate cellular response to infection whose activity is modulated by type I IFN. Through TLR signalling, macrophages are capable of inducing type I IFN.\textsuperscript{22-24} Type I IFN can then act to alter macrophage activity via autocrine or paracrine signalling.\textsuperscript{24,25} Using RAW 264.7 macrophages as an in vitro model, we examined how type I IFN signalling can influence the macrophage response to TLR activation by a variety of PAMPs. Our results show the type I IFN, IFN-\(\beta\), to differentially modulate the TLR-induced cytokine production of macrophages depending on the specific PAMP to which the cells have been exposed. The effect of type I IFN signalling on cytokine production also changed with different lengths of PAMP exposure. In addition, the timing and duration of IFN-\(\beta\) exposure resulted in changes in macrophage cytokine production which also varied among PAMPs. Further examination into the functional changes induced by IFN-\(\beta\) signalling showed that macrophages display an activated phenotype, with elevated levels of MHC II-expressing cells and enhanced bacterial killing. Our results support a role for type I IFN as a regulator of macrophage TLR-induced cytokine production and as an activator of macrophage activity.

**Materials and methods**

**Reagents**

LPS derived from *Escherichia coli* and PolyI:C was purchased from Sigma–Aldrich (St Louis, MO). For FimH isolation, the FimH gene from *E. coli* strain EC99 (O78) was cloned into the pQE-30 and expressed in BL-21 competent *E. coli*. FimH expression and purification was performed as previously described.\textsuperscript{26} The initial nickel-column-purified recombinant FimH protein was further purified using gel filtration via fast protein liquid chromatography. The level of contaminating LPS in the FimH preparations was determined to be no greater than 40 pg/\(\mu\)g. The level of LPS contamination was determined using a Limulus Amebocyte Lysate assay (Associates of Cape Cod, East Falmouth, MA) as directed. CpG and PAM3CSK4 were purchased from Invivogen (San Diego, CA). ELISA kits for TNF-\(\alpha\), IL-10, IL-12p40, IL-6 and RANTES were purchased from R&D systems (Minneapolis, MN), and plates were read using the SpectraMax i3 from Molecular Devices (San Jose, CA).

**Cell culture**

RAW 264.7 macrophages (TIB-71; ATCC, Manassas, VA) were cultured in Roswell Park Memorial Institute medium. Medium was supplemented with 5% FBS, 1% l-glutamine, 1% penicillin and streptomycin and 1% HEPES.

**IFN-\(\beta\) pretreatment assay**

RAW 264.7 macrophages were seeded on a 96-well plate with \(1 \times 10^5\) cells/well. Cells were pre-treated
with either 100 IU/ml of IFN-β or media for 16 h, followed by treatment with LPS (100 ng/ml), FimH (10 µg/ml), PolyI:C (10 µg/ml), CpG (10 µg/ml), PAM3CSK4 (100 ng/ml) or media. Following 24 h of incubation, supernatants were collected, and protein levels of TNF-α, IL-10, IL-6, RANTES and IL-12p40 were examined via ELISA. When examining the influence of IFN-β timing, RAW 264.7 macrophages were pretreated with 100 IU/ml of IFN-β or media for either 6 or 18 h prior to adding the TLR ligands or received IFN-β treatment and TLR ligands simultaneously. Supernatant was collected, and protein levels of TNF-α and IL-10 were measured via ELISA. To examine the effect of different lengths of PAMP exposure on type I IFN signalling, macrophages received the standard 16 h of IFN-β pretreatment. Then PAMPs were added at the appropriate concentrations, as previously mentioned, and incubated for 6, 12 or 24 h prior to supernatant collection. Levels of IL-10 within the supernatant were then measured via ELISA.

**Bacterial killing assay**

To measure killing of *Streptococcus pneumoniae*, 1 × 10⁶ RAW 264.7 macrophages were pre-incubated with a MOI of 250 bacteria per cell for 30 min at 37°C with gentle inversion to allow for internalisation of bacteria. Macrophages were washed three times with PBS to remove any unbound and uninternalised bacteria. Viable CFUs were determined by culturing the supernatants of lysed cells on tryptic soy agar plates (DF0370; Thermo Fisher Scientific, Ottawa, Canada) with 5% sheep’s blood (CL2581-500D; Cedarlane, Burlington, Canada) and 10 µg/ml of neomycin.

**Flow cytometric staining and Abs**

RAW 264.7 macrophages were treated with IFN-β or media for 16 h then stained for flow cytometry. Extracellular surface staining of MHC II was completed using Alexa Fluor 700 conjugated anti-mouse MHC II Abs (clone M5/114.15.2; eBioscience, San Diego, CA). RAW 264.7 macrophages were stained with MHC II Ab for 30 min at 4°C followed by three washes in FACs buffer, PBS supplemented with 0.2% BSA, then fixed with 1% paraformaldehyde for 1 h. Samples were run on the BD LSR II flow cytometer (BD Biosciences, San Jose, CA). For analyses of the flow plots, FlowJo software (v10.0.6 2; FlowJo LLC, Ashland, OR) was used.

**Statistical analysis**

Means were compared using two-way ANOVA analysis, with Bonferroni post hoc tests. For all tests, a P value < 0.05 was used to indicate statistical significance. All statistical analyses were carried out using GraphPad Prism v5 (GraphPad Software, La Jolla, CA).

**Results**

**Treatment of macrophages with IFN-β alone is not sufficient to induce the production of IL-6, 10, 12, TNF-α or RANTES**

Prior to establishing the effect of type I IFN within the context of TLR ligand stimulation, we first examined the influence of IFN-β on RAW 264.7 macrophages in the absence of PAMPs. The RAW 264.7 macrophages were treated with either media as a negative control or IFN-β prior to cytokine analysis. The supernatant was collected, and the various pro- and anti-inflammatory cytokine levels were examined via ELISA. Untreated RAW 264.7 macrophages showed no detectable levels of TNF-α, IL-6, IL-12 or IL-10. The levels of RANTES in the untreated RAW 264.7 macrophages, although detectable, were quite low. Treatment with IFN-β was shown to have no effect on the cytokine profile expressed by RAW 264.7 macrophages (Figure 1). Our observations indicate that treatment with exogenous IFN-β does not modulate the expression of IL-10, IL-12, IL-6, TNF-α or RANTES in macrophages in the absence of TLR stimulation.

**Pre-treatment with IFN-β alters the cytokine production of RAW 264.7 macrophages in response to TLR stimulation**

We next examined how the presence of type I IFN signalling is able to modify the cytokine production
of RAW 264.7 macrophages when responding to different PAMPs. In the presence of PAMPs, the pretreatment of RAW 264.7 macrophages with IFN-β was observed to have a significant effect on cytokine production. Treatment with different PAMPs elicited a different cytokine response (Figure 2). Among the pro-inflammatory cytokines examined, all of the PAMP conditions showed a common trend in which the levels of TNF-α decreased in the presence of IFN-β. With the exception of Poly I:C, this decrease in TNF-α was statistically significant (Figure 2a). The effect of IFN-β on the production of the pro-inflammatory cytokines IL-6, RANTES and IL-12p40, varied depending on the type of PAMP introduced into culture (Figure 2b–d). The levels of IL-6 significantly decreased in the IFN-β pretreated group in response to LPS. The same trend was seen with FimH. However, this was not statistically significant. When exposed to Poly I:C, the IFN-β pre-treatment had an opposite effect in which there was a significant increase in IL-6 production. Finally, in the presence of CpG or PAM3CSK4, pre-treatment with IFN-β showed no alteration in IL-6 production compared to non-treated RAW 264.7 macrophages (Figure 2b). Pretreatment with IFN-β resulted in a significant decrease in RANTES expression by RAW 264.7 macrophages in the presence of LPS. Conversely, when exposed to Poly I:C and CpG, IFN-β pre-treatment resulted in a significant increase in RANTES expression (Figure 2c). The levels of IL-12p40 significantly increased in the IFN-β pre-treated group in response to FimH and CpG. However, the levels of IL-12p40 detected via ELISA were quite low (Figure 2d). IFN-β pre-treatment resulted in an increase in the expression of the anti-inflammatory cytokine IL-10 in response to all tested PAMPs. Although FimH showed a similar trend, it was not statistically significant. Overall, the ways in which the pre-treatment of IFN-β modulated the cytokine expression of RAW 264.7 macrophages were unique to the type of PAMP to which the cells were exposed. Our findings support the role of type I IFN in modulating macrophage functioning to be highly variable and dependent on the type of PAMP encountered. Table 1 provides a summary of all TLR ligands and their cytokine induction in response to IFN-β.

**Figure 2.** Cytokine production of IFN-β pretreated RAW 264.7 macrophages when exposed to TLR ligands. RAW 264.7 macrophages were pre-treated with IFN-β (100 IU/ml) for 16 h, followed by treatment with media, LPS (100 ng/ml), FimH (10 μg), Poly I:C (10 μg/ml), CpG (10 μg/ml) or PAM3CSK4 (100 ng/ml) for 24 h. Supernatant TNF-α, IL-6, IL-12p40, IL-10 and RANTES levels were examined via ELISA. *P < 0.05. Bars represent means ± SEM for four experiments.

**Regulatory effect of type I IFN signalling on the RAW 264.7 macrophage TLR response is dependent on the timing of IFN-β signalling**

To examine how the timing of type I IFN signalling modifies cytokine production, RAW 264.7 macrophages were pre-treated with IFN-β for 18 or 6 h prior to introducing PAMPs into cell culture or treated with IFN-β and PAMPs simultaneously. Regardless of the presence of IFN-β, RAW 264.7 macrophages produced TNF-α in response to all PAMPs (Figure 3a–c). However, we did observe type I IFN signalling to modify the levels of TNF-α production. The influence
of IFN-β signalling varied depending on the PAMP and timing of IFN-β exposure. When macrophages were simultaneously exposed to PAMPs and IFN-β, the presence of IFN-β signalling resulted in a significant decrease in the production of TNF-α when stimulated by LPS, FimH and PolyI:C. The remaining PAMPs showed no significant changes in macrophage cytokine production in response to IFN-β when signalling was concurrent with PAMP exposure (Figure 3a). Following 6 h of IFN-β pre-treatment, a significant decrease in TNF-α production was seen in the presence of LPS. Within the presence of FimH, a similar decline in TNF-α production was observed in the IFN-β-pre-treated macrophages. However, this was not statistically significant. Conversely, macrophages showed a significant increase in the levels of TNF-α production following 6 h of IFN-β pre-treatment in the presence of PolyI:C and CpG. No clear trend or significant changes were observed in the presence of PAM3CSK4 when macrophages were pre-treated with IFN-β for 6 h (Figure 3b). In the presence of LPS, FimH and PAM3CSK4, the long duration of IFN-β pre-treatment (18 h) resulted in a significant decrease in TNF-α production. However, in the presence of PolyI:C and CpG, there was no significant decrease in TNF-α production within the context of IFN-β signalling (Figure 3c).

In the presence of IFN-β, RAW 264.7 macrophages produced significantly elevated levels of IL-10 within the context of all PAMP ligands, regardless of timing, in comparison to the untreated macrophages. Although FimH did not show statistical significance following the 18 h of IFN-β pre-treatment, nor did PolyI:C show significance during the condition with concurrent IFN-β and PAMP exposure. Similar trends were observed in IL-10 production in the presence of IFN-β (Figure 3d–f). Interestingly, IFN-β signalling had the most significant effect on IL-10 production by macrophages in response to FimH when IFN-β signalling occurred at the time of TLR-stimulation (Figure 3d). Conversely, PolyI:C showed a slightly opposite trend in IL-10 production in which the RAW 264.7 macrophages pre-treated with IFN-β for 6 h or 18 h showed elevated levels of IL-10 production (Figure 3e and f). However, following immediate exposure to IFN-β, RAW 264.7 macrophages did not produce significantly elevated levels of IL-10 in the presence of PolyI:C (Figure 3d). Our data suggest an influential role for the timing of IFN-β signalling in determining the macrophage cytokine production in response to TLR-stimulation.

### Levels and timing of IL-10 production by IFN-β is dependent on the length of PAMP exposure and type of PAMP

Next, we examined how the length of PAMP exposure may change the influence of IFN-β signalling on macrophage IL-10 cytokine production. RAW 264.7 macrophages were treated with bacterial and viral PAMPs for 6, 12 or 24 h. By introducing PAMPs for different lengths of time, we are able to see how type I IFN signalling modifies the cellular response over longer and shorter periods of TLR stimulation. Our results show IFN-β signalling to enhance IL-10 production as the length of TLR stimulation increases (Figure 4a–c). During short periods of TLR stimulation, IFN-β signalling resulted in a significant increase in IL-10 production in the presence of LPS, CpG and

### Table 1. The effect of IFN-β on the TLR response of RAW 264.7 macrophages.a

| TLR  | TLR ligand | Effect of IFN-β on TLR cytokine production |
|------|------------|------------------------------------------|
| TLR 2 | PAM3CSK4   | Pro-inflammatory cytokines:                |
|      |            | i. TNF-α significant decrease             |
|      |            | ii. IL-6 no significant change            |
|      |            | iii. RANTES no significant change         |
|      |            | iv. IL-12 not significant                 |
|      |            | Anti-inflammatory cytokines:              |
|      |            | i. IL-10 significant increase             |
| TLR 3 | Poly I:C   | Pro-inflammatory cytokines:                |
|      |            | i. TNF-α no significant change            |
|      |            | ii. IL-6 significant increase             |
|      |            | iii. RANTES significant increase          |
|      |            | iv. IL-12 significant increase            |
|      |            | Anti-inflammatory cytokines:              |
|      |            | i. IL-10 significant increase             |
| TLR 4 | i. LPS     | Pro-inflammatory cytokines:                |
|      |            | i. TNF-α significant decrease             |
|      |            | ii. IL-6 significant decrease             |
|      |            | iii. RANTES significant decrease          |
|      |            | iv. IL-12 not significant                 |
|      |            | Anti-inflammatory cytokines:              |
|      |            | i. IL-10 significant increase             |
|      | ii. FimH   | Pro-inflammatory cytokines:                |
|      |            | i. TNF-α significant decrease             |
|      |            | ii. IL-6 not significant                  |
|      |            | iii. RANTES increase not significant      |
|      |            | iv. IL-12 significant increase            |
|      |            | Anti-inflammatory cytokines:              |
|      |            | i. IL-10 significant increase             |
| TLR 9 | CpG        | Pro-inflammatory cytokines:                |
|      |            | i. TNF-α significant decrease             |
|      |            | ii. IL-6 not significant                  |
|      |            | iii. RANTES significant increase          |
|      |            | iv. IL-12 significant increase            |
|      |            | Anti-inflammatory cytokines:              |
|      |            | i. IL-10 significant increase             |

aValues are relative to the levels of cytokines produced by untreated RAW 264.7 macrophages.
**Figure 3.** The influence of IFN-β timing on the RAW 264.7 macrophage TLR-induced cytokine response. RAW 264.7 macrophages were either pre-treated with IFN-β (100 IU/ml) for 18 h or 6 h prior to the addition of PAMPs or received IFN-β in conjunction with media or PAMPs (LPS (100 ng/ml), FimH (10 μg), Poly1:C (10 μg/ml), CpG (10 μg/ml) and PAM3CSK4 (100 ng/ml)). Supernatant TNF-α (a–c) and IL-10 (d–f) levels were examined via ELISA. *P < 0.05. Bars represent means ± SEM for three experiments.

**Figure 4.** The levels of IL-10 produced by IFN-β pretreated macrophages following various lengths of PAMP exposure. RAW 264.7 macrophages were pretreated with IFN-β (100 IU/ml) or media for 16 h, followed by treatment with media or PAMPs (LPS (100 ng/ml), FimH (10 μg), Poly1:C (10 μg/ml), CpG (10 μg/ml) and PAM3CSK4 (100 ng/ml)). Supernatant was collected 6, 12 or 24 h following TLR stimulation (a–c). *P < 0.05. Bars represent means ± SEM for three experiments.
PAM3CSK4 in comparison to macrophages without IFN-β pretreatment (Figure 4a). As the length of PAMP exposure increased to 12 h, macrophages exposed to PolyI:C began to show a significant increase in IL-10 production in the presence of IFN-β signalling. The influence of IFN-β signalling on TLR-induced cytokine production by LPS, CpG and PAM3CSK4 continued to increase the levels of IL-10 production significantly. Furthermore, the overall levels of IL-10 produced in response to IFN-β were higher following the 12 h of PAMP exposure compared to 6 h (Figure 4b). After 24 h of PAMP exposure, macrophages showed a significant increase in IL-10 production in response to IFN-β in all PAMP conditions, now including FimH. Additionally, the overall levels of IL-10 production by the macrophages receiving IFN-β were higher than the levels produced following 6 or 12 h of PAMP exposure (Figure 4c). Our data support a heightened regulatory function for type I IFN as the length of time exposed to PAMPs increases. Moreover, our results show the levels and timing of IL-10 production by IFN-β differs in response to different PAMPs.

**Treatment with IFN-β increases the level of MHC II expression and enhances the killing activity of macrophages**

To gain a more comprehensive understanding of how type I IFN is modifying the function of macrophages, we examined the receptor expression and functional phenotype of macrophages in response to IFN-β signalling. RAW 264.7 macrophages were treated with 100 IU/ml of IFN-β for 16 h prior to examining MHC II expression or bacterial challenge. In macrophages, the expression of MHC II is often representative of an activated phenotype and enhanced Ag presentation. Macrophage activation and the up-regulation of MHC II is commonly understood to be in response to IFN-γ signalling. In response to this signalling, macrophages have heightened bactericidal and anti-pathogenic activity. Our data show IFN-β signalling to result in a significant increase in the percentage of MHC II expressing RAW 264.7 macrophages. This supports a role for IFN-β in the activation of macrophages (Figure 5a and b).

Since type I IFN was able to modify the TLR-induced cytokine production, it is likely to be altering additional functions of these macrophages, such as bacterial killing. To examine how type I IFN signalling is modifying macrophage killing, we used *S. pneumoniae* as a model. Infection with *S. pneumoniae* is sensed through the binding of *S. pneumoniae* PAMPs to TLR2, TLR4 and TLR9. Therefore, we are able to further examine how type I IFN signalling modulates the TLR response in macrophages. RAW 264.7 macrophages were challenged with *S. pneumoniae*, and the percentage of live bacteria remaining following incubation was measured. Our results show IFN-β signalling to enhance the killing activity of the RAW 264.7 macrophages in comparison to the non-treated controls. Over time, this difference in killing ability became significant between the two groups (Figure 5c). The elevated MHC II expression and decrease in live bacteria by the IFN-β treated RAW 264.7 macrophages support a role for IFN-β in activating macrophages and enhancing their bactericidal activity.

**Discussion**

Type I IFN has been shown to be both an essential and a detrimental component for successful bacterial clearance and host survival. However, the outcome of type I IFN signalling appears to be contingent on the specific bacterium. The conditions determining whether type I IFN signalling will result in a favourable or harmful outcome for the host remain unclear. In an effort to tease apart the factors influencing the role of type I IFN during bacterial infections, we examined how the presence of IFN-β modifies the response of RAW 264.7 macrophages to various viral and bacterial PAMPs. Our study suggests type I IFN modulates macrophage cytokine expression and that it has different roles depending on the timing of its signalling, the type of PAMP the cell is responding to and length of PAMP exposure.

The results of our study demonstrate that the treatment of RAW 264.7 macrophages with IFN-β alone, in the absence of TLR ligands, does not induce the release of pro- or anti-inflammatory cytokines. In the presence of PAMPs, IFN-β signalling drastically altered the cytokine production of macrophages. The level of IL-10 production was significantly enhanced with the introduction of LPS, PolyI:C, CpG and PAM3CSK4. Previous work by McNab et al. examining *Mycobacterium tuberculosis* showed that the introduction of IFN-β during infection resulted in a significant increase in IL-10. This increase in IL-10 was shown to be enhanced with increasing concentrations of IFN-β. These findings and our own suggest IFN-β to have a role in enhancing IL-10 production during infection and inducing an anti-inflammatory state in response to both bacterial and viral PAMPs. Our findings contrast with previous work by Thomas et al., investigating how the pre-treatment of peritoneal macrophages from C56BL/6 mice with exogenous IFN-β modifies the cytokine response to LPS stimulation. In this study, the peritoneal macrophages were pre-incubated with recombinant IFN-β for 16 h, followed by a 0, 1, 3 or 5 h stimulation with LPS. Their findings show that the LPS-induced gene expression
response of wild type macrophages was up-regulated for some pro-inflammatory genes, including IFN-β, monocyte chemotactic protein 5 (Mcp5), IFN-γ-induced protein 10, Mcp1 and Il-6. However, the pre-treatment with IFN-β showed no change in the steady-state levels of IL-1β, IL-12p40 and MyD88 mRNA in the wild type macrophages. However, our findings show IFN-β pre-incubation of RAW 264.7 macrophages leads to an increase in the anti-inflammatory cytokine IL-10 in response to LPS stimulation, and a decrease in pro-inflammatory cytokine expression of IL-6, RANTES and IL-12p40. Our study stimulated the RAW 264.7 macrophages with LPS for 24 h following IFN-β pre-incubation. The differences in our findings may be due to a difference in the timing of LPS stimulation, further highlighting the importance of how context can modify the effect of IFN-β on the cellular response.

The influence of IFN-β signalling on the production of IL-10 in response to the bacterial PAMP, FimH, was more significant following shorter lengths of IFN-β pre-treatment. These levels appear to decline with longer exposure times to IFN-β. However, in response to LPS, CpG and PAM3CSK4, IFN-β signalling was observed to elevate the levels of IL-10, regardless of the amount of IFN-β exposure time. For the ligand PolyI:C, the increase in the levels of IL-10 occurred only following prolonged exposure to IFN-β. These variances in the timing of the IL-10 production may be very critical in determining how detrimental or beneficial type I IFN signalling may be during infection. It should be noted that previous work has shown that the presence of IFN-β does not alter the TLR expression of RAW 264.7 cells. Therefore, the observed changes in cytokine production following IFN-β stimulation does not appear to be a result of changes in TLR expression.

Figure 5. MHC II expression and killing activity of RAW 264.7 macrophages in response to IFN-β signalling. RAW 264.7 macrophages were pretreated with IFN-β (100 IU/ml) for 16 h. The expression of MHC II was measured via flow cytometry (a and b, control and IFN-β, respectively). The percentage of MHC II-positive cells are shown in the bar graph and pseudo-colour plot (a). The overlaid histogram of the control (red) and IFN-β treated (blue) RAW 264.7 macrophages show the number of MHC II-positive cells (b). The killing activity of RAW 264.7 macrophages was measured via a bacterial killing assay with S. pneumoniae (c). *P < 0.05. Error bars represent means ± SEM for three replicates.
The timing of type I IFN signalling has been previously examined within the context of *M. tuberculosis* infection in bone marrow-derived macrophages. Pre-treatment of macrophages with IFN-β was shown to enhance the levels of IL-10 production. However, the presence of IFN-β had the greatest impact on IL-10 production when added at or shortly before the time of *M. tuberculosis* infection. FimH was the only PAMP in our study that was observed to follow this same trend, in which the initial presence of IFN-β signalling had the most significant increase in IL-10 production. As type I IFN signalling appears to have a presence throughout the entirety of infection, if the initial response to type I IFN is anti-inflammatory, such as IL-10 production, type I IFN signalling will likely result in an unproductive immune response at a crucial point during infection. In fact, this detrimental effect is observed during the *M. tuberculosis* infection. Type I IFN signalling impedes on the critical antimicrobial immune response by inhibiting macrophage activation and the production of protective pro-inflammatory cytokines TNF-α, IL-12 and IL-1β.

Within the context of *Listeria monocytogenes*, type I IFN signalling results in IL-10 production early on during infection, leading to increased host susceptibility to infection *in vivo*. Throughout the course of *M. tuberculosis* infection, the production of IL-10 by type I IFN has been shown to be severely detrimental to the host, inhibiting macrophage activation through disrupting their responsiveness to IFN-γ and inhibiting the production of the pro-inflammatory cytokines TNF-α, IL-12p40 and IL-1β. Work examining *Mycobacterium leprae* has shown that IFN-β-associated IL-10 production by monocytes infected with *M. leprae* is significantly enhanced in disseminated and progressive lepromatous lesions. However, the production of IL-10 within the context of influenza A infection is an important factor in containing the immune response. Restoring IL-10 within IFNAR knockout mice during a lethal influenza A infection resulted in decreased immune-mediated lung pathology.

Our investigation to understand the influence of type I IFN on macrophages further revealed IFN-β signalling to up-regulate the percentage of MHC II expressing cells significantly. The expression of MHC II normally occurs in activated macrophages in response to IFN-γ stimulation. Previous work has shown IL-10 to inhibit the IFN-γ-induced up-regulation of MHC II in macrophages. However, in the absence of IFN-γ, our results suggest that the presence of IL-10 does not appear to have this same inhibition on MHC II expression.

Additionally, our results suggest type I IFN holds a role in macrophage activation, which corresponds with our observations of enhanced *S. pneumoniae* killing by the IFN-β-treated RAW 264.7 macrophages. Previous work has shown type I IFN signalling to render the host more susceptible to *M. tuberculosis* infection via inhibiting macrophage pro-inflammatory cytokine production and IFN-γ-induced macrophage activation. A follow-up study, however, revealed that in the absence of IFN-γ signalling, type I IFN is capable of modulating macrophage activation and can confer the host with protection against *M. tuberculosis*. Interestingly, type I IFN was shown to inhibit macrophage polarisation towards an alternatively activated phenotype and sustained their protective, classically activated phenotype. Although our results do not demonstrate inhibition of alternative macrophage activation, our findings corroborate the observations of a classically activated, bactericidal and protective macrophage induced by type I IFN signalling in the absence of IFN-γ signalling. A recent study by Müller et al. showed a similar phenomenon in which both type I IFNs, IFN-β and IFN-α, are capable of inducing antitumor M1 macrophages in the absence of IFN-γ. Wild-type bone marrow-derived macrophages treated with type I IFNs exhibited enhanced cytotoxic and cytostatic ability against Lewis lung carcinoma cancer cells. This further corroborates our results showing a role for type I IFN in macrophage activation.

The function of type I IFN has shown to be highly context dependent. Therefore, obtaining a broader understanding of type I IFN signalling and the potential patterns or predictors of a detrimental or beneficial effect within the context of infection is a critical component in developing antimicrobial therapies. Our study has helped to provide a broader understanding of how type I IFN modulates the cytokine profile and functioning of macrophages in response to TLR stimulation. Our findings highlight the importance of the timing of IFN-β signalling and the timing and type of PAMPs exposed to the cell. The cytokine profile of macrophages in response to TLRs in the presence of type I IFN was observed to be highly dynamic over time. It is likely that how the pro- and anti-inflammatory cytokines are coordinated by type I IFN is a significant factor in influencing the outcome of infection.

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