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Distinct regulatory mechanisms control proinflammatory cytokines IL-18 and IL-1β

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Abstract

IL-18 and IL-1β, which are cytokines of the IL-1 family, are synthesized as precursor proteins and activated by the inflammasome via proteolytic processing. IL-1β is induced only in response to inflammatory stimuli, but IL-18 is constitutively expressed. However, how IL-18 and IL-1β expression is regulated by different inflammatory signals remains poorly studied. In this study, we found that IL-18 and IL-1β are differentially regulated. Despite being constitutively expressed, IL-18 expression was increased and sustained after stimulation of Toll-like receptors. In contrast, IL-1β was induced but not sustained after chronic treatment. Furthermore, type I IFN signaling was essential for induction of IL-18 and macrophages lacking type I IFN signaling were impaired in their ability to promote IL-18 induction. Thus, our findings reveal a fundamental difference in IL-18 and IL-1β regulation and uncover novel mechanisms that are relevant to the inflammatory settings where these proinflammatory cytokines play a critical role.

Keywords

IL-18; IL-1β; type I IFN; IFNAR1; IFNAR2; IRF9; STAT1

Introduction

IL-18 and IL-1β, members of the IL-1 cytokine family, are important mediators of inflammatory diseases and play critical roles in infection and cancer (1–4). Unlike other cytokines, cellular IL-18 and IL-1β are synthesized as precursor proteins and need to be cleaved to generate their biologically active forms. Inflammasome, a multimeric protein complex, is a central regulator of this process by which bioactive IL-18 and IL-1β are generated. The inflammasome comprises an innate immune sensor that includes the nucleotide-binding domain, leucine-rich-repeat–containing protein (NLR), AIM2-like receptor, and pyrin; the adaptor protein ASC; and the cysteine protease caspase 1 (5). Inflammasome assembly induces the activation of caspase-1, which mediates the proteolytic

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processing of pro-IL-18 and pro-IL-1β to generate their bioactive forms and their release from the cell (5).

IL-18 is constitutively expressed in blood monocytes and intestinal epithelial cells of healthy humans (6, 7). It is also expressed in murine macrophages, dendritic cells, endothelial cells, intestinal epithelial cells, and keratinocytes under steady state (8–10). However, IL-1β is not constitutively expressed under homeostasis. IL-1β expression is induced in blood mononuclear cells, macrophages, and dendritic cells during stimulation with Toll-like receptor (TLR) ligands and other cytokines (e.g., tumor necrosis factor) (1, 2, 4). Unlike IL-1β, a cellular pool for IL-18 already exists before an inflammatory stimulus, and is ready to be activated and released by the inflammasome (11). It must however be noted that LPS-mediated priming of NLRP3 is required for inflammasome activation and subsequent release of both IL-18 and IL-1β (12). However, several studies suggest that despite being constitutively expressed, IL-18 can also be induced under certain circumstances. Treatment with LPS (TLR4 agonist) has been shown to upregulate the mRNA levels of *Il18* (7, 13). Incubation of CpG oligonucleotides (TLR9 agonist) with dendritic cells or infection of monocytes with the Sendai virus can also increase the expression of *Il18* (14, 15).

The mechanisms by which IL-18 and IL-1β levels are regulated by different inflammatory signals remain unclear. In this study, we showed that expression of IL-18 is induced and that the increased level is sustained during TLR4, TLR2, or TLR7 ligands stimulation. In contrast, IL-1β expression declines soon after reaching its peak level. The TLR3 and cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathways induced IL-18 but were modest at inducing IL-1β expression. Importantly, type I IFN signaling was required to upregulate IL-18 in response to all stimuli tested. Thus, in macrophages lacking components of type I IFN signaling, IL-18 was not induced by any inflammatory stimuli. Together, our findings demonstrate that IL-18 and IL-1β expression are differentially regulated. Expression of these inactive proinflammatory cytokines is an essential step for their maturation into bioactive forms by caspase-1 inflammasomes. Overall, our data demonstrating differential regulation of IL-18 and IL-1β will be fundamental to understanding several inflammatory disease settings.

Materials and Methods

Mice

Ifnar1$^{-/-}$ mice (16), *Ifnar2*$^{-/-}$ mice (17), *Irf1*$^{-/-}$ mice (18), *Irf3*$^{-/-}$ mice (19), *Irf7*$^{-/-}$ mice (18), and *Irf9*$^{-/-}$ mice (20) were generated as described previously. C57BL/6J mice [wide type (WT)], *Irf4*^{tm1Rdf}*/J* mice (Stock Number 009380), *Irf8* $^{tm1.2Hm}$/J mice (also known as *Irf8*$^{-/-}$; Stock Number 018298) and *Irf5*^{tm1Ppr}/J mice (Stock Number 017311) were purchased from The Jackson Laboratory. *Irf4*^{tm1Rdf}*/J* mice and *Irf5*^{tm1Ppr}/J mice were crossed with mice expressing LsyM-Cre and Cre recombinase, respectively, to generate *Irf4*$^{fl/fl}$-*Lysm-Cre* mice and *Irf5*$^{-/-}$ mice. *Stat1*$^{-/-}$ mice were provided by Dr. Abhay Satoskar (Ohio State University). All mice were bred at the Animal Resource Center at St. Jude. Animal studies were conducted according to protocols approved by the St. Jude Animal Care and Use Committee.

Cell culture and stimulation

Bone marrow–derived macrophages were prepared as described previously (21). Cells were stimulated with LPS (500 ng/mL; InvivoGen), Pam3CSK4 (1 μg/mL), gardiquimod (1 μg/mL; InvivoGen), Poly(I:C) (10 μg/mL; InvivoGen), or IFNβ (400 U/mL; PBL Assay Science). For transfection of DNA or 2′3′-cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), 0.25 μg of poly(dA:dT) (InvivoGen) or 1 μg of 2′3′-cGAMP (InvivoGen) was mixed with 0.3 mL of Xfect polymer in Xfect reaction buffer (Clontech Laboratories) for 10 min and then added to BMDMs in Opti-MEM (ThermoFisher Scientific).

Quantitative real-time PCR

cDNA was prepared as described previously (22). Primers used were *Hprt*-forward 5′-CTC ATG GAC TGA TTA TGG ACA GGA C-3′ and *Hprt*-reverse 5′-GCA GGT CAG CAA AGA ACT TAT AGC C-3′; *Il18*-forward-F 5′-GCC TCA AAC CTT CCA AAT CA-3′ and *Il18*-reverse-R 5′-TGG ATC CAT TTC CTC AAA GG-3′; *Il1b*-forward-F 5′-GAC CTT CCA GGA TGA GGA CA-3′ and *Il1b*-reverse-R 5′-AGC TCA TAT GGG TCC GAC AG-3′. Gene expression levels were normalized to *Hprt*.

Western blotting

Protein samples for western blotting were prepared as previously described (21). Primary antibodies used were anti-IL-18 (#D046-3, MBL International), anti-IL-1β (#AF-401-NA, R&D Systems), and anti-β-actin (#8457, Cell Signaling Technology).

Results and Discussion

IL-18 and IL-1β are differentially induced in response to inflammatory stimuli

TLRs, one of the major types of pattern recognition receptors in the cell (23), induce expression of pro-inflammatory cytokines and type I IFNs in response to pathogen-associated molecular patterns and damage-associated molecular patterns (23). We first confirmed that IL-18 was constitutively expressed in untreated WT BMDMs, similar to previous reports (7, 8). Interestingly, we observed that its expression was upregulated by the TLR4 ligand LPS (Fig. 1A). As expected, *Il1b* was not expressed in untreated BMDMs but was induced shortly after LPS treatment (Fig. 1A). Interestingly, although gene and protein expression of IL-18 was sustained 24 h after stimulation, that of IL-1β was induced initially but reduced substantially at 24 h (Fig. 1A). These results suggest that the expressions of IL-18 and IL-1β are differentially regulated after LPS stimulation.

After binding to TLR4, LPS signals through both the MyD88 and TRIF pathways (23). To determine the specific roles of MyD88 and TRIF pathways in regulating IL-18 and IL-1β expression, we treated cells with ligands that signal through either the MyD88 or TRIF pathway. Interestingly, Pam3CSK4, which signals through the MyD88 pathway downstream of TLR2 induced sustained expression of IL-18 over the treatment period in WT BMDMs (Fig. 1B). On the other hand, IL-1β was upregulated 4 h after treatment but diminished dramatically at later time points (Fig. 1B). Gardiquimod induces the MyD88 pathway downstream of TLR7. Induction kinetics of mRNA expression and protein levels of IL-18

and IL-1β in WT BMDMs treated with gardiquimod were similar to those treated with Pam3CSK4 (Supplemental Fig. 1A). Poly(I:C) is sensed by TLR3 to activate the TRIF pathway. Poly(I:C) treatment induced *Il18* expression in WT BMDMs. It also induced *Il1b* expression, although at a much lower fold induction than by LPS, Pam3CSK4, and gardiquimod (Fig. 1C). These findings suggest that the TRIF pathway moderately induces *Il1b* expression, and both the MyD88 and TRIF pathways induce *Il18* expression. Taken together, our results show that IL-18 and IL-1β can be induced by various TLR ligands. Further, during chronic TLR stimulation, IL-18 expression is sustained but IL-1β expression is downregulated. These data suggest distinct regulatory pathways that control the expression of pro-IL-18 and pro-IL-1β levels during chronic TLR stimulation.

One of the prominent mediators induced by the TRIF pathway are type I IFNs, including IFNβ (23). Poly(I:C) induced the expression of *Il18* comparable to LPS stimulation, which led us to hypothesize that the induction of *Il18* requires type I IFNs. To determine the role of type I IFNs in modulating the expressions of *Il18* and *Il1b*, we treated WT BMDMs with recombinant IFNβ and measured *Il18* and *Il1b* expression. IL-18 mRNA and protein levels were induced after IFNβ treatment, but IL-1β mRNA and protein levels were only modestly induced at levels similar to those seen for Poly(I:C) stimulation (Fig. 1C and 1D). Overall, these data suggest that treatment of type I IFNs upregulates IL-18 expression.

Induction of IL-18 but not IL-1β is dependent on type I IFN signaling

To test whether type I IFN signaling was required for induction of *Il18* expression by inflammatory stimuli, we treated WT BMDMs and cells lacking the subunit of type I IFN receptor IFNAR1 (*Ifnar1*$^{-/-}$) with LPS and monitored the expression of *Il18* and *Il1b* over the treatment period. LPS treatment led to upregulation of IL-18 in WT BMDMs as early as 4 h after treatment. IL-18 expression further increased and was sustained during chronic LPS treatment (Fig. 2A). Surprisingly, this induction of IL-18 by LPS was impaired in *Ifnar1*$^{-/-}$ cells (Fig. 2A), indicating that type I IFN signaling is crucial to induce IL-18 during LPS treatment. In WT BMDMs, IL-1β expression increased 4 h and 8 h after treatment but diminished at later time points (Fig. 2A). Unlike *Il18*, induction of IL-1β remained largely intact in *Ifnar1*$^{-/-}$ cells (Fig. 2A). Moreover, expression of *Il1b* mRNA and protein was higher in *Ifnar1*$^{-/-}$ BMDMs than in WT BMDMs 16 h and 24 h after stimulation (Fig. 2A), suggesting that type I IFN signaling may negatively regulate *Il1b* expression during chronic LPS stimulation.

To further test whether the induction of IL-18 by stimuli that trigger only the MyD88 or TRIF pathway also depends on type I IFN signaling, we treated WT and *Ifnar1*$^{-/-}$ BMDMs with Pam3CSK4, gardiquimod, or Poly(I:C). Induction of IL-18 was abolished in *Ifnar1*$^{-/-}$ BMDMs compared with WT BMDM when MyD88 signaling was triggered by Pam3CSK4 or gardiquimod treatment (Fig. 2B and 2C). However, IL-1β expression remained unchanged in *Ifnar1*$^{-/-}$ BMDMs after either stimulation (Fig. 2B and 2C). Further, during treatment with Poly(I:C), which signals via the TRIF pathway, IL-18 induction was impaired in *Ifnar1*$^{-/-}$ BMDMs compared with WT BMDMs (Fig. 2D). Recognition of cytosolic DNA by the cGAS-STING pathway results in the production of type I IFNs (24). We hypothesized that stimuli that induce the production of type I IFNs will promote IL-18 expression that is

dependent on type I IFN signaling. Therefore, we activated the cGAS-STING pathway by transfecting double-stranded DNA, poly(dA:dT), or the STING ligand 2′3′-cGAMP in BMDMs. Expression of *Il18* was increased by activation of the cGAS-STING pathway (Fig. 2E and 2F), but that of *Il1b* was not robustly induced (Fig. 2E and 2F). As expected, the induction of *Il18* was lower in *Ifnar1*$^{-/-}$ BMDMs in response to poly(dA:dT) or 2′3′-cGAMP than in WT BMDMs (Fig. 2E and 2F). Collectively, these results suggest that type I IFN signaling is critical for induction of IL-18, but not IL-1β after inflammatory stimulations.

Components of type I IFN signaling are essential to induce IL-18

The type I IFN receptor is a heterodimeric complex formed by IFNAR1 and IFNAR2 (25). Binding of type I IFN to IFNAR1 and IFNAR2 induces the formation of interferon stimulating gene factor 3 (ISGF3) by signal transducer and activator of transcription (STAT) members STAT1, STAT2, and IRF9, which function as transcriptional factors to induce the expression various interferon-stimulated genes (ISGs) (25). To test whether other components of type I IFN signaling are also required to induce IL-18, we first measured IL-18 and IL-1β levels in WT BMDMs and cells lacking IFNAR2, IRF9, or STAT1 (*Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$ and *Stat1*$^{-/-}$, respectively) after LPS treatment. Similar to *Ifnar1*$^{-/-}$ BMDMs, induction of IL-18 was also impaired in *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, and *Stat1*$^{-/-}$ BMDMs (Fig. 3A). However, expression of *Il1b* mRNA and protein was not compromised but showed a slight increase at 16 h and 24 h after stimulation in *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, and *Stat1*$^{-/-}$ BMDMs (Fig. 3A). Moreover, pro-IL-18 was not upregulated in *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, and *Stat1*$^{-/-}$ BMDMs treated with Pam3CSK4 or Poly(I:C) compared with WT BMDMs (Fig. 3B and 3C). ISGF3 directly binds to promoters of ISGs to induce their expression, some of which include IFN regulatory factors (IRFs) that function as transcriptional factors to drive the expression of additional genes (26). To test whether IL-18 was induced via ISGF3 or other IRFs downstream of the type I IFN receptor, we stimulated *Ifnar1*$^{-/-}$, *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, and *Stat1*$^{-/-}$ BMDMs with recombinant IFNβ and observed that IFNβ–induced expression of *Il18* was compromised in *Ifnar1*$^{-/-}$, *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, and *Stat1*$^{-/-}$ BMDMs compared with WT cells (Supplemental Fig. 2A). On the other hand, the induction of *Il18* by IFNβ was not affected in BMDMs lacking other IRFs, including IRF1, IRF3, IRF4, IRF5, IRF7, or IRF8 (*Irf1*$^{-/-}$, *Irf3*$^{-/-}$, *Irf4*$^{fl/fl}$-*Lysm-Cre*, *Irf5*$^{-/-}$, *Irf7*$^{-/-}$ and *Irf8*$^{-/-}$, respectively; Supplemental Fig. 2B and 2C), suggesting that induction of *Il18* is dependent on ISGF3 but not other IRFs.

We have established that although both IL-18 and IL-1β belong to the IL-1 family and are activated by the inflammasome, IL-18 and IL-1β are differentially regulated (Supplemental Fig. 2D). Indeed, previous report has shown that secretion of IL-1β but not IL-18 from murine dendritic cells in response to Listeria monocytogenes p60 protein requires ROS production and caspase 11 (27). Distinctive expression patterns of IL-18 and IL-1β are observed in the intestine. IL-18 is expressed at high level in colon and confers protection against inflammation by promoting epithelial cell proliferation and tissue repair (28). IL-1β, on the other hand is expressed at lower levels basally, but enhanced during acute inflammation (28). Given that type I IFNs are protective against DSS-induced colitis (29), it is possible that the type I IFNs contribute to protection by upregulating the expression of *Il18* in the colon. Activation of the cGAS-STING pathway leads to the production of type I

IFN (24). Colon tissues from mice lacking STING also have decreased *Il18* mRNA and protein expression after DSS treatment (22, 30). Therefore, type I IFN signaling can be explored as a therapeutic target in IL-18–associated diseases. We further demonstrated that *Il1b* gene and protein expression was reduced during chronic TLR stimulation in WT BMDMs, indicating that the transcription of *Il1b* or stability of *Il1b* mRNA may be compromised. Reduced translational activity or protein stability can also contribute to decreased levels of pro-IL-1β. Indeed, there is translational inhibition of *Il1b* expression in LPS-stimulated macrophages (31). Moreover, pro-IL-1β is ubiquinated by E2 conjugase UBE2L3 and subsequently degraded during chronic LPS stimulation (32). This observation is in line with several studies showing that when macrophages are treated with TLR ligands, pro-IL-1β is sequestered in autophagosomes for degradation (33, 34). Of note, we found that although increases in mRNA levels were similar between *Il18* and *Il1b* in response to Poly(I:C) in WT BMDMs, upregulation of IL-1β protein levels was much less robust compared with that of IL-18 levels (Fig. 1C). This could be resulted from decreased translational activity of *Il1b* mRNA or lower stability of pro-IL-1β due to proteasome or autophagy-mediated degradation, revealing another layer of differential regulation between IL-18 and IL-1β. In addition, the modest expression of IL-1β in response to Poly(I:C) is supported by a previous study showing that pro-IL-1β is much less induced by Poly(I:C) than by LPS or gardiquimod (35). Our study also found that downregulation of both *Il1b* mRNA and protein expression was ameliorated in cells lacking type I IFN signaling at later time points of LPS stimulation. An intriguing theory is that type I IFN signaling may be involved in the negative control of *Il1b* expression. Indeed, macrophages lacking tyrosine kinase 2, which is downstream of the type I IFN receptor, have increased translation of *Il1b* and hence high levels of pro-IL-1β in response to LPS (36). Previous study has also demonstrated that type I IFN signaling suppresses IL-1β expression via IL-10-STAT3 signaling axis and inhibits proteolytic processing of IL-1β by NLRP3 inflammasome (37). Interestingly, downregulation of IL-1β expression was not affected in BMDMs lacking type I IFN signaling compared with WT cells at later time points of Pam3CSK4 or gardiquimod stimulation (Fig. 2B, 2C, 3B and 3C). The MyD88 pathway might induce lower production of type I IFNs and consequently the negative control on IL-1β is less robust as compared to the TRIF pathway that is activated by LPS.

IL-1 family cytokines have pleiotropic functions and are involved in several inflammatory and autoinflammatory disease settings. Interestingly, IL-1α, IL-1β and IL-18 have specific functions in regulating disease outcomes (5). Autoinflammatory models associated with overt pyrin inflammasome activation are differentially mediated through IL-18 (38) or IL-1β (39) production. While levels of circulating IL-1β and IL-18 in the serum are upregulated in patients bearing activating NLRC4 or NLRP3 mutation, IL-18 is induced to a greater extent in patients harboring mutation in NLRC4 (40). IL-18 is also chronically elevated in several of the autoinflammatory diseases and inflammasomepathies (41). However, the cause and effect of this chronically sustained elevated level of IL-18 is not studied well. Our current studies demonstrate that targeting type I IFN signaling pathway could be a way to regulate the pathologic level of IL-18 in the affected patients. In summary, our study demonstrated that during chronic stimulation, IL-18 expression is induced and sustained. In contrast, during chronic stimulations IL-1β is robustly induced but not sustained. Finally, we show

that type I IFN signaling is necessary for the induction of IL-18, but not IL-1β, which points to a critical and differential role for type I IFN in regulating IL-18 signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMDMs	bone marrow–derived macrophages
cGAMP	cyclic guanosine monophosphate–adenosine monophosphate
cGAS	cyclic GMP–AMP synthase
DSS	dextran sulfate sodium
IFNAR	interferon-α/β receptor
ISGs	interferon-stimulated genes
IRF	interferon regulatory factor
ISGF3	interferon stimulated gene factor 3
STING	stimulator of interferon genes
STAT	signal transducer and activator of transcription
TRIF	TIR-domain-containing adapter-inducing interferon-β
WT	wild type

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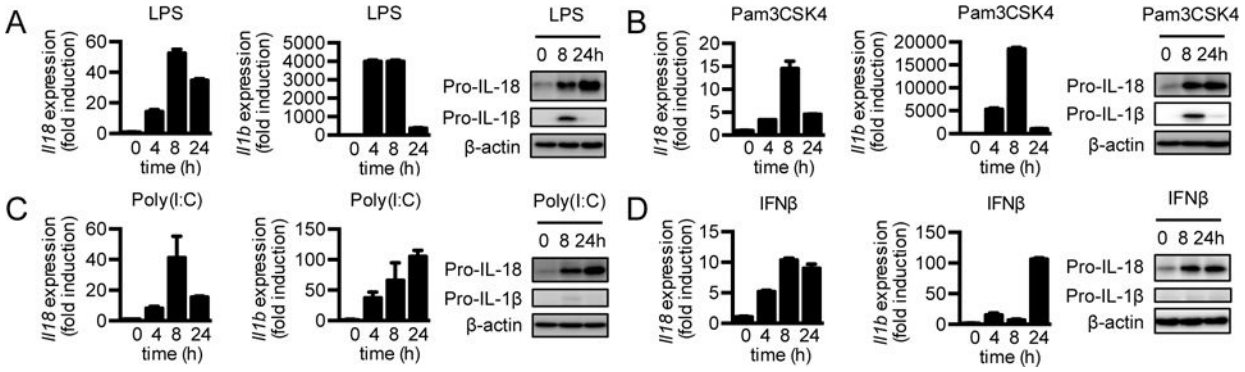

Figure 1. IL-18 and IL-1β are differentially expressed in response to inflammatory stimuli

(A–D) Real-time qPCR ana1ysis of genes encoding IL-18 and IL-1β and immunoblot analysis of pro-IL-18, pro-IL-1β, and β-actin (loading control) in WT BMDMs at various time points after LPS (A), Pam3CSK4 (B), Poly(I:C) (C), or IFNβ (D) treatment. Data are representative of 3 independent experiments.

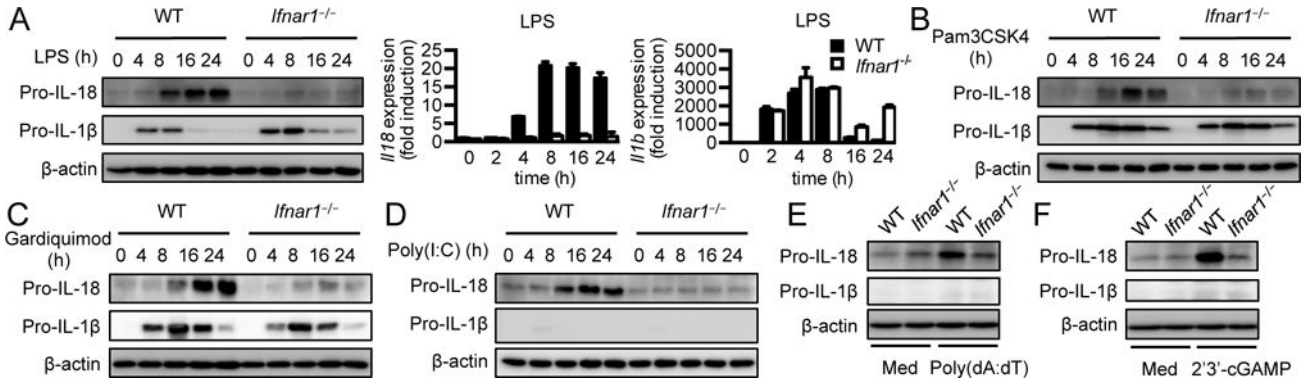

Figure 2. Type I IFN signaling is essential for induction of IL-18 but not IL-1β

(A) Immunoblot analysis of pro-IL-18, pro-IL-1β, and β-actin (loading control) and real-time qPCR analysis of genes encoding IL-18 and IL-1β in WT or *Ifnar1*$^{-/-}$ BMDMs at various time points after LPS treatment. (B–D) Immunoblot analysis of pro-IL-18, pro-IL-1β, and β-actin (loading control) in WT or *Ifnar1*$^{-/-}$ BMDMs at various time points after Pam3CSK4 (B), gardiquimod (C), or Poly(I:C) (D) treatment. (E and F) Immunoblot analysis of pro-IL-18, pro-IL-1β, and β-actin (loading control) in untreated WT or *Ifnar1*$^{-/-}$ BMDMs (medium alone [Med]) or BMDMs at 8h after transfected with poly(dA:dT) (E) or 2′3′-cGAMP (F). Data are representative of 3 independent experiments.

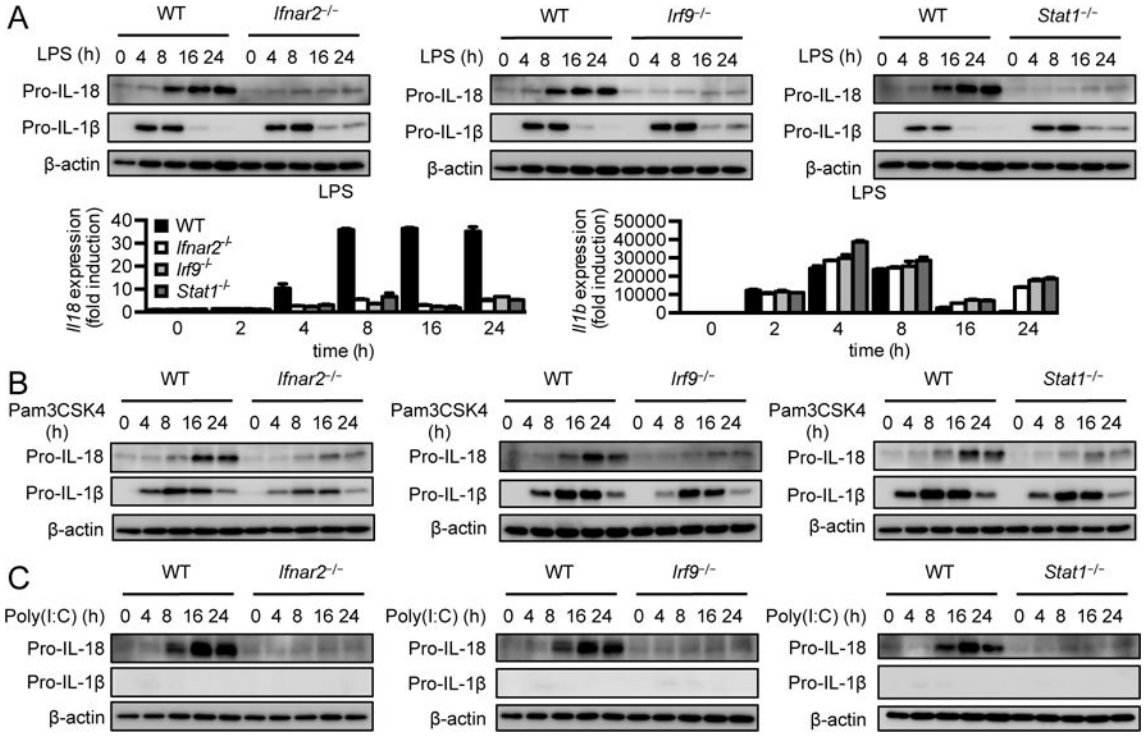

Figure 3. Components of type I IFN signaling are required for induction of IL-18

(A) Immunoblot analysis of pro-IL-18, pro-IL-1β, and β-actin (loading control) and real-time qPCR analysis of genes encoding IL-18 and IL-1β in WT, *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, or *Stat1*$^{-/-}$ BMDMs at various time points after LPS treatment. (B and C) Immunoblot analysis of pro-IL-18, pro-IL-1β, and β-actin (loading control) in WT, *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, or *Stat1*$^{-/-}$ BMDMs at various time points after Pam3CSK4 (B) or Poly(I:C) treatment (C). Data are representative of 3 independent experiments.