Published in final edited form as:

Immunobiology. 2019 July ; 224(4): 565–574. doi:10.1016/j.imbio.2019.04.004.

Interferon Regulatory Factor 3 plays a role in macrophage responses to Interferon-γ

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Abstract

IFN-γ produced during viral infections activates the IFN-γ receptor (IFNGR) complex for STAT1 transcriptional activity leading to expression of Interferon Regulatory Factors (IRF). Simultaneous activation of TBK/IKKε via TLR3 during viral infections activates the transcription factor IRF3. Together these transcription factors contributes to expression of intracellular proteins (e.g. ISG49, ISG54) and secreted proteins (e.g. IFN- β, IP-10, IL-15) that are essential to innate antiviral immunity. Here we examined the role of IRF3 in expression of innate anti-viral proteins produced in response to IFN-γ plus TLR3 agonist. Wild-type (WT) and IRF3KO RAW264.7 cells, each with ISG54- promoter-luciferase reporter vectors, were stimulated with IFN-γ, poly I:C, or both together. ISG54 promoter activity was significantly reduced in IRF3KO RAW264.7 cells responding to IFN-γ, poly I:C, or IFN-γ plus poly I:C, compared with WT RAW264.7 cells. These data were confirmed with western blot and qRT-PCR. Primary macrophages and dendritic cells (DCs) from IRF3KO mice also showed decreased ISG54 in response to IFN-γ, poly I:C, or IFN-γ plus poly I:C compared with those from WT mice. Moreover, pharmacological inhibition of TBK/IKKε significantly reduced ISG54 promoter activity in response to IFN-γ, poly I:C, or IFN-γ plus poly I:C. Similarly, expression of ISG49 and IL-15, but not IP-10, was impaired in IRF3KO RAW264.7 cells responding to IFN-γ or poly I:C, which also had impaired STAT1 phosphorylation and IRF1 expression. These data show that IRF3 contributes to IFN-γ/IFNGR signaling for expression of innate anti-viral proteins in macrophages.

Graphical abstract

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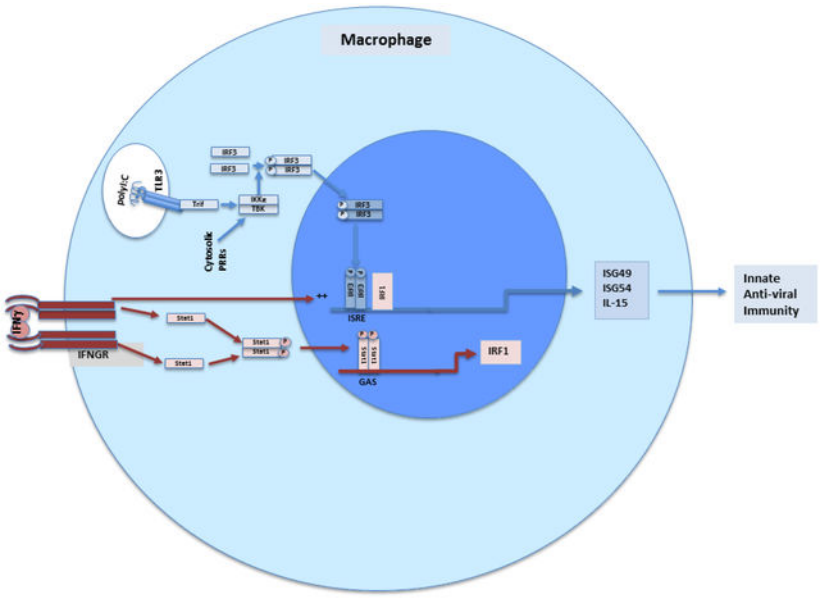
Contributions

T. Petro and Z. Guinn designed, executed and analyzed the data from the experiments.

T. Petro designed the project and supervised the study. T. Petro and Z. Guinn wrote the paper.

Conflicts of Interest

The authors declare no conflicts of interest.



Keywords

IRF3; poly I:C; Interferon-gamma; Macrophages; ISG54; TLR3; ant-viral immunity

1. Introduction

Viruses, such as HIV, Ebola virus, Respiratory syncytial Virus, and Influenza A virus infect macrophages causing phenotypic changes in these cells that contribute to disease (Mercer and Greber, 2013). Moreover, these viruses can persist in macrophages resulting in virus dissemination, thereby causing recurring pathogenesis (Rahman, et al., 2011). IFN-γ secreted by T cells and NK cells during virus infections triggers innate antiviral immune responses through the IFN-γ receptor (IFNGR) of macrophages. In addition, macrophage Pattern Recognition Receptors (PRRs) respond to viral macromolecules, such as dsRNA, to trigger innate antiviral responses. Together the IFNGR and PRR pathways help control viruses in infected macrophage populations to prevent viral persistence and dissemination (Nathan, et al., 1983). In contrast, ineffective responses from IFNGR and PRRs are factors in the pathology of many autoimmune and inflammatory diseases brought about by persistent viral infection of macrophages (Lucey, et al., 1996). Therefore, improved insights in the response of macrophages from IFNGR and PRRs is needed to prevent persistent infection of macrophages with viruses.

Activation of multiple Interferon regulatory Factors (IRFs) through IFNGR or PRRs pathways is an essential component of innate anti-viral responses of macrophages. In these responses, IRF transcription factors induce Type I Interferons and Interferon stimulated gene (ISG) proteins (Osterlund, et al., 2007), which are key effector proteins that control virus. Binding of IFN-γ to IFNGR1 and IFNGR2, triggers phosphorylation of Janus kinases (JAK), JAK1 and JAK2 leading to subsequent recruitment of signal transducer and activator of transcription 1 (STAT1) to IFNGR and its STAT1-Tyr-701 phosphorylation. STAT1

homodimers translocate to the nucleus for induction of IRF1. Induction of IRF1 also occurs through TLR7 and TLR9 PRR pathways during responses to viral ssRNA and DNA, respectively (Osterlund, et al., 2007).

IRF3, which is constitutively expressed in macrophages, is also activated through PRRs during viral infection of macrophages. PRRs that activate IRF3 include TLR2 (Aubry, et al., 2012), TLR3, TLR4 (Fitzgerald, et al., 2003) and STimulator of Interferon Genes (STING) (Tanaka and Chen, 2012). IRF3 activation occurs after PRR pathways activate Tank binding kinase 1 (TBK1)/Inhibitor of Kappa Kinase (IKK)ε that then phosphorylates IRF3 at multiple serine residues. IRF3 then hetero- or homo- dimerizes with other IRFs, including IRF3, IRF5 and IRF7, which translocate to the nucleus for transcriptional activity (Barnes, et al., 2003; Schmid, et al., 2014; Yang, et al., 2004). Target genes for IRF3 transcriptional activity include IFN-β (Wathelet, et al., 1998), IRF7, and IFN-induced proteins with tetratricopeptide repeats (IFIT) family of antiviral proteins (Nakaya, et al., 2001), IFIT1, IFIT2, IFIT3 and IFIT5 (aka Interferon Stimulated Gene (ISG)56, ISG54, ISG60 and ISG58, respectively.) (Zhou, et al., 2013) ISG54, whose induction depends on IRF3 (Nakaya, et al., 2001), induces apoptosis, inhibits cell migration, and inhibits translation, all of which curtail virus infection and dissemination (Zhou, et al., 2013). Therefore, ISG54 helps prevent persistent virus infection of macrophages and pathologies associated with persistently infected macrophages (Butchi, et al., 2014). Therefore, agonists of the IRF3/ISG54 nexus should stimulate these innate antiviral responses (Bedard, et al., 2012).

Recently, we showed that stimulation at both TLR3 with poly I:C and IFNGR with IFN-γ induced ISG54 in an IRF3 dependent fashion (Guinn, et al., 2017). These results were interesting because, TLR3 primarily activates IRF3 through TBK1 (Meurs and Breiman, 2007), whereas IFN-γ through the IFNGR is not known to activate IRF3. We report here that ISG54 is induced in an IRF3 dependent manner downstream of both TLR3 and IFNGR. IRF3 activity from the IFNGR/IFN-γ pathway appears to be independent of IRF1 but dependent on TBK1/IKKε. Additionally, we found that IRF3 contributes to induction of other genes in response to IFN-γ, including IL-15 and ISG49.

2. Results

2.1 IRF3 dependent IFN-γ induction of ISG54

IFN-γ, a cytokine produced by T cells and NK cells during viral infections, activates and stimulates anti-viral immunity in macrophages, including production of ISG54 (Butchi, et al., 2014; Cho, et al., 2013; Davis, et al., 2017). Therefore, we investigated IRF3's role in IFN-γ induced ISG54 expression in macrophages using WT RAW264.7 and the IRF3KO RAW264.7 cell lines, each expressing secreted luciferase (Lucia) under control of an ISG54 promoter. Significant ISG54 promoter activity was observed in WT RAW- Lucia cells in response to IFN-γ (Fig. 1A). ISG54 promoter activity in IRF3KO RAW- Lucia cells was significantly diminished compared with WT RAW264.7 cells at all time points after stimulation, suggesting that IRF3 is activated during stimulation with IFN-γ. To confirm this observation, WT and IRF3KO RAW-Lucia cells were stimulated for 6 or 12 h with IFN-γ or the TLR3/IRF3 agonist, poly I:C, and ISG54 expression was evaluated by qPCR (Fig. 1B). As seen with ISG54 promoter activity, IFN-γ stimulated significantly more ISG54 mRNA in

WT RAW-Lucia cells compared with IRF3KO RAW- Lucia cells (Figure 1B). As expected, poly I:C induced ISG54 in WT but not IRF3KO RAW-Lucia cells (Fig. 1C). Western blots also confirmed that IFN-γ stimulated production of ISG54 protein in WT RAW cells, but not IRF3KO RAW cells (Fig. 2A). To evaluate ISG54 protein expression in primary myeloid lineage cells, we utilized WT and IRF3KO mice to generate macrophages and BMDCs, and stimulated these cells with

2.2 IRF3 deficiency impairs IFN-γ activation of STAT1

Phosphorylation of STAT1 at Tyr 701 (Y701-pSTAT1) occurs following IFN-γ stimulation. Activated STAT1 facilitates transcription of anti-viral genes, such as *IRF1*. IRF1 influences induction of type 1 IFNs, which then contributes to expression of ISG54 (Sakamoto, et al., 2004). Therefore we evaluated Y701-pSTAT1 and IRF1 by western blot. IRF3KO RAW cells and IRF3KO macrophages exhibited a small but clear reduction in Y701-pSTAT1 compared with WT cells (Fig. 2A, 2B). The reduction in Y701-pSTAT1 in IRF3KO RAW cells in response to IFN-γ was associated with measurable reduction of IRF1 levels. Overall, these data indicate that IRF3 contributes to IFN-γ-induced ISG54 expression, to some extent through STAT1 and IRF1 activation.

2.3 IFN-γ/poly I:C synergism for induction of ISG54.

RNA virus infections stimulate both the TLR3 pathway by viral RNA and the IFNGR pathway by IFN-γ. TLR3 and IFNGR co-stimulation has been shown synergize for induction of certain ISGs (Kajita, et al., 2015). To determine if TLR3 and IFNGR co-stimulation synergistically increases ISG54 expression, we incubated WT and IRF3KO RAW-Lucia cells with PBS, poly I:C (a TLR3 agonist), IFN-γ, or both together, and determined ISG54 promoter activity at multiple time points. At all time-points following stimulation with IFN-γ plus poly I:C, ISG54 promoter activity was significantly impaired in IRF3KO RAW-Lucia cells (Fig. 3A). Moreover, a synergistic increase in ISG54 promoter activity was confirmed in WT cells stimulated with IFN-γ plus poly I:C (Fig. 3A). Using qRT-PCR and western blots, expression of ISG54 mRNA and protein was greater with IFN-γ plus poly I:C than with either one alone. Moreover ISG54 expression significantly decreased in IRF3KO RAW cells compared with WT RAW cells responding to IFN-γ alone or with IFN-γ plus poly I:C (Fig. 3B, C). These results strengthen the notion that IRF3 contributes to ISG54 expression by macrophages in response to both IFN-γ or IFN-γ plus poly I:C and that there is synergism between IFN-γ and poly I:C in this expression.

2.4 IFN-γ induces IFN-β for IRF3 dependent induction of ISG54

While IRF3 contributes to IFN-γ-induced ISG54, this may occur indirectly through IFN-γ-induced Type I Interferon (Berchtold, et al., 2008). To assess this possibility, we incubated supernatants of IFN-γ- stimulated WT and IRF3KO RAW cells with IFNGRKO B16-F1 cells, which produce SEAP under the control of the ISG54 promoter. Using this approach, the effects of secreted factors and not IFN-γ could be assessed. Supernatants from RAW cells stimulated with IFN-γ induced ISG54 promoter activity in IFNGRKO ISG54-SEAP-B16 cells (Fig. 4A). Moreover, supernatants from IFN-γ- stimulated WT RAW induced significantly more ISG54 promoter activity compared with those from IRF3KO RAW cells (Fig. 4B), suggesting that differences in IFN-γ-stimulated Type I IFNs could be responsible

for differences in ISG54 expression in WT RAW264.7 cells compared with IRF3KO RAW264.7 cells.

Previously we reported that IFN-β stimulates ISG54 promoter activity in RAW- Lucia cells (Guinn, et al., 2017). To determine if IFN-γ induced IFN-β, we evaluated IFN-β expression in WT and IRF3KO RAW cells during responses to IFN-γ, poly I:C, or both together. The results show that IFN-γ alone induces expression of IFN-β in WT RAW cells (Fig. 4C). Interestingly, expression of IFN-β in response to IFN-γ in IRF3KO RAW cells was less than that of WT RAW cells at 6 h post stimulation, but this difference was not significant. However, IFN-β expression in response to poly I:C alone or IFN- γ plus poly I:C was significantly less from IRF3KO RAW cells (Fig.4D, C). These results suggest that IRF3 indirectly contributes to IFN-γ-induced ISG54 through induction of soluble factors, one of which is IFN-β.

2.4 TBK1 inhibition reduces ISG54 promoter activity in response to IFN-γ.

Previous reports have demonstrated that TBK1 and IKKε, which activate IRF3 and IRF7 in response to TLR3 and TLR7 agonists, are also activated downstream of IFNGR (Farlik, et al., 2012). To assess TBK1/IKKε involvement in induction of ISG54 through IFNGR, WT and IRF3KO RAW-Lucia cells were pretreated with the TBK1/IKKε inhibitor, MRT67307 (Clark, et al., 2011), before stimulation with IFN-γ or poly I:C. Pretreatment of WT RAW-Lucia cells with the MRT67307 significantly reduced ISG54 promoter activity in response to IFN-γ, poly I:C or both together (Fig. 5A). As expected ISG54 promoter activity in response to IFN-γ, polyI:C or both together in IRF3KO RAW-Lucia cells was barely detectable (Fig. 5B). These data imply that TBK1/IKKε is essential in the IFN-γ/IFNGR pathway for optimal ISG54 expression.

2.6 IRF3 deficiency impairs IFN-γ induced ISG49 and IL-15, but not IP-10

Besides ISG54, IRF3 contributes to expression of other proteins, such as ISG49 (Zhang, et al., 2013), IL-15 (Soudja, et al., 2012), and IP-10 (McWhirter, et al., 2004), that are part of early anti-viral immunity (Brownell, et al., 2014; Ebihara, et al., 2010; Fensterl, et al., 2008; Zhang, et al., 2013). Utilizing qRT-PCR, WT RAW cells expressed significantly more ISG49 and IL-15 than IRF3KO RAW cells in response to IFN-γ, poly I:C, or IFN-γ plus poly I:C (Fig. 6A–F). In contrast, expression of IP-10 was not significantly different between WT compared with IRF3KO RAW cells stimulated with IFN-γ or IFN-γ plus poly I:C (Fig. 6 G, I). However, IP-10 expression was significantly less in IRF3KO RAW cells compared with WT RAW cells in response to poly I:C (Fig. 6H). Overall, these data suggest that IRF3 contributes to some, but not all, anti-viral factors in macrophages in response to IFN-γ.

2.7 Other PRR pathways that activate IRF3 synergize with IFN-γ to induce ISG54

In addition to viral RNA through TLR3, LPS through TLR4 activates TBK1/IKKε and then IRF3 (Clark, et al., 2011). Moreover, viral DNA activates TBK1/IKKε and IRF3 through the cGAS/cGAMP/STING axis (Fremond, et al., 2017), while TLR2 ligands activate IRF3, presumably through TBK1/IKKε (Nilsen, et al., 2015). In contrast, viral DNA and viral ssRNA through TLR9 and TLR7, respectively, do not activate IRF3. To determine if these PRRs can synergize with IFN-γ for ISG54 expression, we stimulated WT and IRF3KO

RAW-Lucia cells in the presence IFN-γ with or without ODN 1585 (TLR9), Loxoribine (TLR8), cGAMP (STING), Zymosan (TLR2), or LPS (TLR4) over a range of concentrations. Previously, we showed that RAW264.7 cells constitutively express TLR2, TLR3, TLR4, TLR7, and TLR9 (Al-Salleeh and Petro, 2007). Stimulation of RAW264.7 cells with ODN 1585 or Loxoribine induced very low ISG54 promoter activity in WT RAW cells. In contrast, Zymosan induced greater ISG54 promoter in WT RAW cells that was not detected in IRF3KO RAW cells (Fig.7A, C, G). IFN-γ synergized with ODN1585, Loxoribine, or Zymosan to enhance ISG54 promoter activity in WT RAW cells. On the other hand, cGAMP or LPS alone induced significant ISG54 promoter activity in WT RAW cells, but not in IRF3KO RAW cells (Fig. 7E and 7F). IFN-γ synergized with cGAMP or LPS in increasing ISG54 promoter activity. Overall, IRF3 contributed to ISG54 expression in response to TLR2, TLR4, and STING agonists, and was a significant factor in PRR synergism with IFN-γ to enhance ISG54 promoter activity.

3. Discussion

Macrophages encounter and are infected by many different types of viruses during the lifetime of an individual (Benkahla, et al., 2018; Kolli, et al., 2014; Mercer and Greber, 2013). Because macrophages efficiently take up viruses by phagocytosis, prompt induction of anti-viral proteins is needed to prevent persistent viral infection of macrophages. It is well-known that ISG54 expression, which is critical for innate antiviral immunity in different host cell types, occurs in an IRF3 specific manner (Butchi, et al., 2014; Cho, et al., 2013; Davis, et al., 2017; Nakaya, et al., 2001). Additionally, ISG54 expression in oral squamous cell carcinomas is correlated with enhanced five year survival rates in patients (Lai, et al., 2008; (Lai, et al., 2016). This suggests that ISG54 is beneficial in anti-tumor immunity, as well as anti-viral immunity. Many viruses target ISG54 expression by inhibiting IRF3 activation, thereby promoting early phases of viral infections (Xue, et al., 2018; Zhu, et al., 2011). Interestingly however, IFN-γ, and not IFN-β, was first described as a key inducer of ISG54 (Mirkovitch, et al., 1992). Therefore, a more complete understanding of ISG54 expression in the context of IRF3 activation and IFN-γ stimulation could help uncover antiviral treatments that prevent persistent virus infection of macrophages.

Here, we show IRF3 to be a significant factor in ISG54 expression during the response of macrophages to IFN-γ. Without IRF3 in macrophages, ISG54 expression in response to IFN-γ is significantly diminished but not eliminated. IRF3KO cells were able to express ISG54 in response to IFN-γ at significantly reduced levels and with slower kinetics. This suggests that other transcription factors besides IRF3 are activated in response to IFN-γ at a later time for expression of ISG54. In this regard, type I Interferons induce ISG54 expression in responses to poly I:C and IFN-γ (Berchtold, et al., 2008; Siegfried, et al., 2013). As expected, we observed here decreased IFN-β expression in IRF3KO RAW cells compared to WT RAW cells. The decline in IFN-β expression in IRF3KO RAW cells correlated with the decline in ISG54 promoter activity. This strongly suggests that a significant portion of IRF3's contribution to ISG54 expression in response to IFN-γ is due IFN-β.

While IRF3 is a key transcription factor for expression of IFN-β, IRF1, IRF5 and IRF7 can also contribute to expression of IFN-β and other type I IFNs (Zhao, et al., 2015). Moreover, IFN-γ can induce IRF1, via STAT1, which can then promote expression of other IRFs, like IRF7 (Farlik, et al., 2012). Although we did see reduced activation of STAT1 in our IRF3KO RAW264.7 cells stimulated with IFN-γ, WT and IRF3KO PEC primary macrophages did not have any observable differences in IRF1 expression when stimulated with IFN-γ. Thus, it is likely that in the absence of IRF3, other IRFs offset the loss of IRF3 for delayed expression of ISG54.

The key serine/threonine kinases activated downstream of TLR3 that contribute to IRF3 activation are TBK1/IKKε (Fitzgerald, et al., 2003). We observed here that pharmacological inhibition of TBK1/IKKε significantly reduces ISG54 promoter activity in response to IFN-γ stimulation, suggesting that IFN-γ via IFNGR goes through TBK1/IKKε for activation of IRF transcription factors. In fact one report showed TBK1/IKKε activation occurring in response to IFN-γ (Farlik, et al., 2012). Moreover, TBK1/IKKε also activate IRF5 and IRF7 through several PRR pathways, including TLR3, RIG-I/MDA5, and STING (Zhao, et al., 2015). While IRF3 is constitutively expressed in most cell types, expression of IRF5 and IRF7 is mainly inducible. IFN-γ has been shown to induce expression of IRF7 (Guarda, et al., 2011). This suggests that in the absence of IRF3, induction of anti-viral factors such as ISG54 will be much slower than that which occurs in the presence of IRF3. Another consideration is the synergism between IRF3 and IRF7 for transcriptional activity. Dimers of IRF3 and IRF7 have greater transcriptional activity than each transcription factor alone (Schmid, et al., 2014). Thus, it is possible that formation of IRF3/IRF7 heterodimers during costimulation with IFN-γ plus poly I:C is the basis for IFN-γ/poly I:C synergism.

Previous reports also showed synergism between TLR3 and IFNGR signaling pathways (Taura, et al., 2010; Tissari, et al., 2005). However, in those reports IFN-γ induction of TLR3 expression was the proposed mechanism for synergy (Taura, et al., 2010; Tissari, et al., 2005). We propose here that synergy between TLR3 and IFNGR could also stem from activation of TBK1/IKKε, whereby IFN-γ induces IRF7 (Farlik, et al., 2012) and activates TBK1/IKKε, while poly I:C activates TBK1/IKKε for activation of IRF3.

Our results also indicate that the contribution of IRF3 in IFNGR signaling also influences expression of ISG49 and IL-15, but not IP-10. Previous reports have found that stimulation of TLR3 can promote expression of ISG49, IL-15, and IP-10 following induction of Type I Interferons (Pantel, et al., 2014). However, IRF3 binds directly to IL- 15 and IP-10 promoters (Azimi, et al., 2000; (Brownell, et al., 2014; Courreges, et al., 2012). Others have shown that IRF1 (Azimi, et al., 2000) and we have seen that IRF3 (Moore, et al., 2014) are important transcription factors for IL-15 expression. Our data here suggest that both IRF1 and IRF3 in response to IFN-γ contribute to optimal expression of IL-15.

Besides Poly I:C, our results indicate that other PRR agonists can induce ISG54 expression. Not surprisingly, LPS and cGAMP, agonists for TLR4 and STING, respectively, were able to induce ISG54 promoter activity and synergize with IFN-γ for ISG54 expression, most likely due to activation of TBK1/IKKε by these PRR pathways (Tanaka and Chen, 2012; Youn, et al., 2005). Interestingly, zymosan also induced ISG54 promoter activity and synergized with

IFN-γ in ISG54 promoter activity. There is increasing evidence that indicates that TLR2 agonists can activate TBK1/IKKε (Kulsantiwong, et al., 2017; (Nilsen, et al., 2015). In contrast, loxoribine and ODN1585, which are TLR7 and TLR9 agonists that do not activate IRF3, respectively, did not induce robust ISG54 promoter activity by themselves. Nevertheless, they did synergize with IFN-γ to augment ISG54 expression. This latter synergy is likely due to TLR7 and TLR9 activation of IRF5 and IRF7 (Moynagh, 2005; Shrivastav and Niewold, 2013) and IFN-γ induction of IRF5 and/or IRF7 (Farlik, et al., 2012). Overall, it is clear that IFN-γ can synergize with several PRR agonists to induce expression of key anti-viral genes, such as ISG54 and IRF3 has a significant role in these expressions. Future work will need to focus on whether IRF3 contributes to gene expression downstream of the IFNGR by heterodimerizing with other IFN-γ-induced IRFs.

4. Materials and Methods

4.1 Animals, Cells, and Cultures

Female C57BL/6 mice were purchased from Harlan Sprague Dawley and used at 10–12 weeks of age. Age-matched female IRF3 deficient mice (IRF3KO) were offspring of breeder pairs obtained from Dr. Karen Mossman (McMaster University), originally produced by Dr. Tadatsugu Taniguchi from the University of Tokyo (Sato, et al., 2000). Experimental animal procedures using mice were approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center. Bone marrow derived dendritic cells (BMDCs) and thioglycollate elicited peritoneal exudate cell macrophages (PECs) from these mice were generated as originally described (Guinn, et al., 2016; Moore, et al., 2011). WT and IRF3KO RAW 264.7 cells, both of which express secreted luciferase (Lucia) under the control of the ISG54 promoter-5x ISRE were obtained from InVivogen (#rawl-isg and #rawl-koirf3, respectively). RAW264.7-Lucia cells were maintained in DMEM with 10% FBS and 50 μg/mL gentamycin. IFNGRKO B16F1 cells, which do not express IFNGR but express secreted embryonic alkaline phosphatase (SEAP) under the control of the ISG54 promoter-5x ISRE (IFNGRKO ISG54-SEAP-B16 cells) were also obtained from InVivogen (#bb-ifnt1). B16 cells were maintained in RPMI1640 with 10% FBS and 50 μg/mL gentamycin.

4.2 PRR Agonists and IFN-γ

All PRR agonists, poly I:C (# vac-pic), ODN 1585 (#tlrl-1585), cGAMP (#tlrl-nacga23), Zymosan (#29–329-ZYN), LPS (#27–02-PELPS), were obtained from InVivogen (San Diego, CA), reconstituted in water, aliquoted and frozen until use. Recombinant IFN- γ was obtained from BioLegend (# 575306), diluted in cell culture media, aliquoted, and frozen until use.

4.3 Quantitative RT-PCR

For evaluation of mRNA expression, 2×10^5 RAW264.7 cells were incubated overnight in culture media. Cells were treated with PBS, IFN-γ (20 ng/mL), Poly I:C (10 μg/mL) or both for 6 or 12 h. At those times, RNA was isolated using PureLink® RNA Mini Kit (ThermoFisher 12183018A) and quantified. cDNA was made using EasyScript Reverse Transcriptase (Midwest Scientific BERTCDNA-100) with 500 ng of RNA. cDNA was

incubated with EvaGreen qPCR MasterMix (Midwest Scientific #BEQPCR-R) and the following primer pairs: ISG49 sense 5' ATGAGTGAGGTCAACCGGGAAT 3' and antisense 5' TGTTGCCTTCTCCTCAGAGTTT 3', IL-15 sense 5' TTAACTGAGGCTGGCATTCATG 3' and antisense 5' ACCTACACTGACACAGCCCAAA 3', IP-10 sense 5' ATGAACCCAAGTGCTGCCGTCA 3' and antisense 5' CACTGGCCCGTCATCGATATGG 3', ISG54 sense 5' ATGAGTACAACGAGTAAGGAGT 3' and antisense 5' GGCTTTAAACTCACTGTTCTGA 3', or GAPDH sense 5′ TTGTCAGCAATGCATCCTGCAC 3′ and antisense 5′ ACAGCTTTCCAGAGGGGCCATC 3′. Quantitative PCRs were run on an ABI Prism 7000 thermal cycler at 50°C for 2 minutes and 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Cycle thresholds (CT) of samples were normalized to the CT of GAPDH for each sample and then normalized to the average CT of the 6 hour WT unstimulated (PBS) sample, after which the data was expressed as relative levels of mRNA using $2^{\Delta\Delta}$CT.

4.4 ISG54 promoter activity

For evaluation of ISG54 promoter activity, 2×10^5 WT or IRF3KO RAW-Lucia cells were incubated overnight in 500 μL cell culture media in 24-well plates. Cells were then incubated with fresh culture media containing PolyI:C (10 μg/mL) or IFN-γ (20 ng/mL), or a mixture of both. At 3, 6, 12, and 24 h, 10 μL of supernatant was removed, mixed with 40 μL of QuantiLuc ™ (InVivogen #repl-qlc1), after which luciferase luminescence was measured using a Turner Biosystems Luminometer, model TD-20/20. Alternatively, 1×10^5 WT or IRF3KO RAW-Cells were incubated in 200 μL cell culture media on 96-well plates and stimulated with agonists for TLR2, TLR4, TLR9, and STING. After 24 h, secreted luciferase was measured. To inhibit TBK1, 2×10^5 WT or IRF3KO RAW-Lucia cells were incubated overnight in 500 μL culture media in 24-well plates and then incubated with PBS or MRT67307 (2 μM) (Sigma Chem. Co.; SML0702) for 1 h followed by PBS, Poly I:C (25 μg/mL), IFN-γ (50 ng/mL), or both IFN-γ and Poly I:C. After 24 h, luciferase was measured as previously described.

4.5 Western Blot

1×10^6 WT and IRF3KO RAW-Lucia, PECs, or BMDMs were incubated overnight in 3 mL cell culture media in 6-well plates. Cells were then incubated in fresh media with PBS, Poly I:C, IFN-γ, or both Poly I:C and IFN-γ. At indicated times cell lysates were obtained using Cell Lysis Buffer (Cell Signaling Cat #9803) containing 2 mM PMSF. Protein was quantified using Bio-Rad DC Protein Assay (Bio-Rad Cat # 500–0116). RAW-Lucia protein (10 μg) and PECs and BMDCs protein (5 μg) in Tris-Glycine SDS Sample Buffer (Invitrogen #LC2676) were electrophoresed using 10% Tris-Glycine PAGE gels and then electrophoretically transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-pSTAT1 Y701 (Cell Signaling #7649), rabbit anti-pSTAT1 S727 (Cell Signaling #8826), rabbit anti-IRF-1 (Santa Cruz #sc-640), rabbit anti-ISG54 (Thermo-Fisher #PA3–845), or mouse anti-tubulin E7 (Developmental Studies Hybridoma Bank, University of Iowa). Membranes were then incubated with goat anti-mouse-IgG-Alexa Fluor680- (1/5000)

or donkey anti-rabbit-IgG-IRDye® 800CW. The membranes were then washed three times and scanned with a LICOR Odyssey® Infrared Imaging System. For some blots pixel densities of imaged proteins were analyzed using Image Studio Ver. 5.2 (LICOR). To determine relative protein levels on western blots, median pixel densities of protein bands were divided by median pixel densities of tubulin bands from treated cells, all of which were divided by net median pixel densities of protein bands/tubulin bands from unstimulated cells.

4.6 Evaluation of ISG54-inducing activity in supernatants

WT or IRF3KO RAW-Lucia cells were incubated in cell culture media at 2×10^5 cells in 500 μL in 24-well plates, after which aliquots of PBS or IFN-γ (20 ng/mL) were added. After 24 h, supernatants were collected (PBS- WT RAW-Lucia, IFN-γ stimulated WT RAW-Lucia, PBS IRF3KO RAW-Lucia, and IFN-γ stimulated IRF3KO RAW-Lucia). To evaluate ISG54-inducing activity in each supernatant, supernatants were then added to cell cultures containing IFNGRKO ISG54-SEAP-B16 cells at 2×10^5 cells per well at 1:1 supernatant:fresh culture media. For positive controls, IFNGRKO ISG54-SEAP-B16 were incubated with recombinant IFN-γ (20 ng/mL) or Poly I:C (10 μg/mL). After 24h, SEAP was quantified using QuantiBlue™ (InVivogen) at 630nm using a Biotek ELx808 microplate reader.

4.7 Statistical analyses

Student's two-tailed t test was used to determine the significance of differences between means; $p < 0.05$ was considered significant. A two-way ANOVA was used to determine the significance of synergism between IFN-γ and PRR agonists; $p < 0.05$ was considered significant for interactions.

Acknowledgements

The authors convey their thanks to Marian Schmid for her excellent animal care. This work was supported by funding from the University of Nebraska Medical Center College of Dentistry and University of Nebraska-Lincoln, School of Biological Sciences, and supported by a subcontract award to (T.M.P) from Award Number 5P30GM106397 from the National Institute of General Medicine, a component of the National Institutes of Health (NIH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

Abbreviations:

ISG	Interferon Stimulated Gene
IRF	Interferon Regulatory Factor
TBK	Tank Binding Kinase
IKK	Inhibitor of Kappa Kinase
STING	STimulator of Interferon Genes
STAT	signal transducer and activator of transcription
IFNGR	IFN-γ receptor

PRR	Pattern Recognition Receptors
IFIT	IFN-induced proteins with tetratricopeptide repeats
PEC	thioglycollate elicited peritoneal exudate cell macrophages (PECs) BMDCs, Bone marrow derived dendritic cells
SEAP	secreted embryonic alkaline phosphatase
Lucia	secreted luciferase
Poly I:C	Polyinosinic-polycytidylic acid
cGAS	cyclic GAMP synthase
WT	wild-type
KO	Knockout

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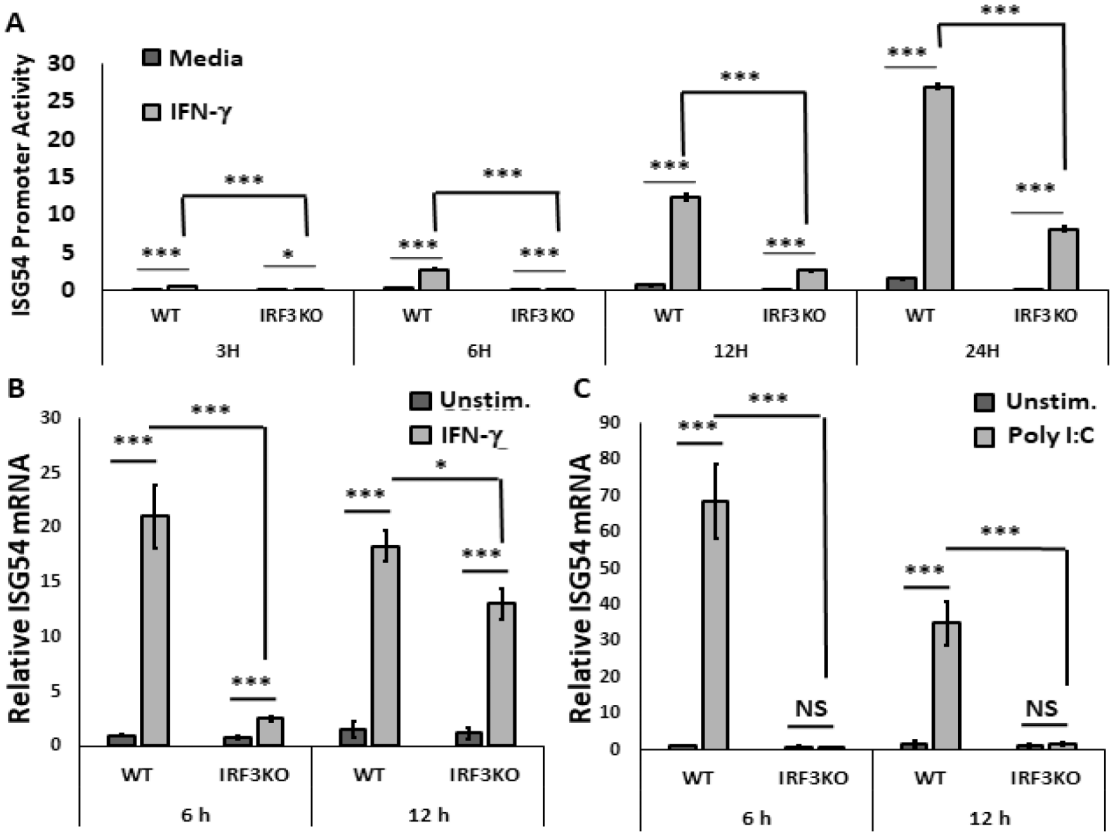

FIGURE 1. IFN-γ induces ISG54 expression in an IRF3 dependent manner.

2×10^5 WT or IRF3KO RAW-Lucia cells were unstimulated or stimulated with IFN-γ (20 ng/mL) **(A, B)** or Poly I:C (10 μg/mL) **(C)**. At indicated times, **(A)** luciferase activity in 10 μL of supernatant was used to determine relative ISG54 promoter activity or **(B, C)** ISG54 expression was determined using qRT-PCR. Data are means ± S.E.M and **(A)** represent two independent experiments, each with n=3 or **(B, C)** data are combined from two independent experiments, each with n=2–3 (Total n=5–6). *, **, *** indicate statistical significance ($p < 0.05$, 0.005, 0.0005, respectively) and NS indicates no significance ($p > 0.05$) using two-tailed student T-test.

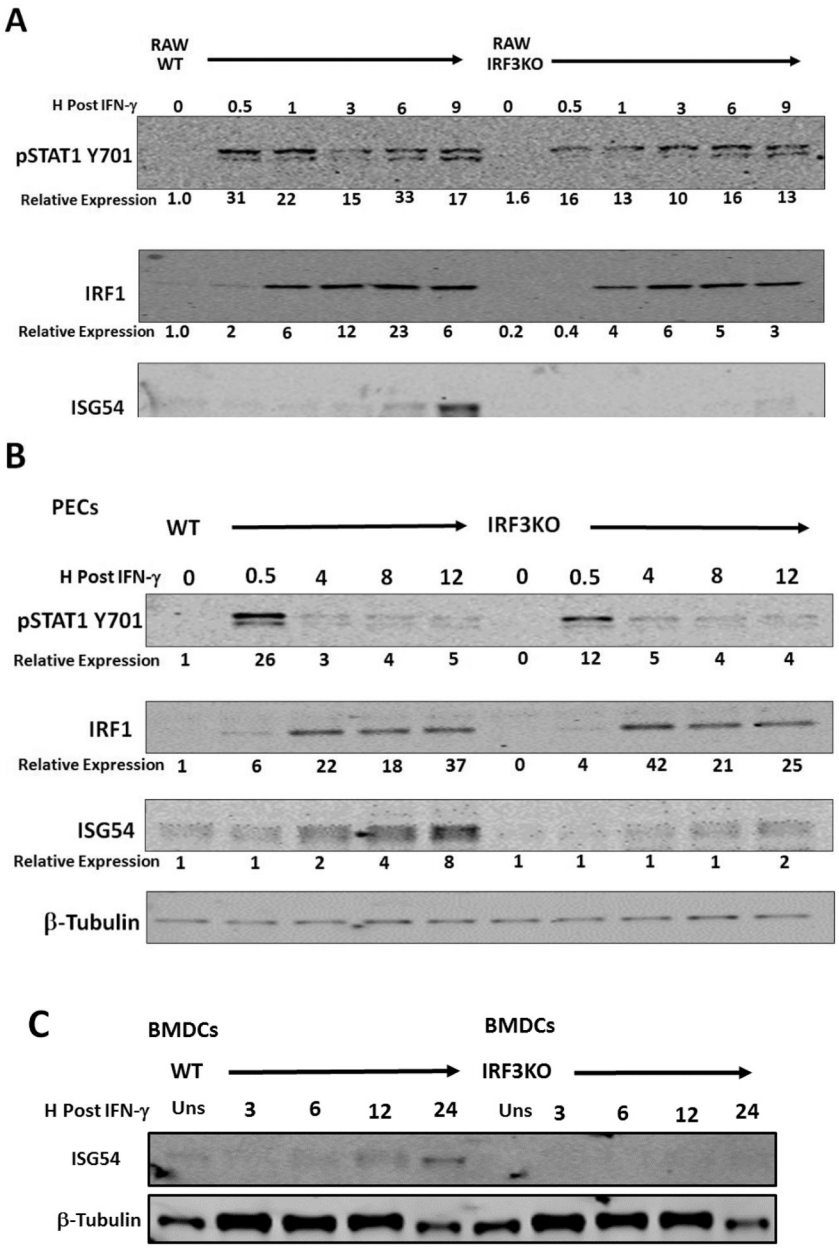

FIGURE 2.

1×10^6 WT or IRF3KO **(A)** RAW-Lucia, **(B)** Thioglycollate Elicited Intraperitoneal Macrophage (PECs), or **(C)** Bone Marrow Derived Dendritic Cells (BMDCs) were unstimulated or stimulated with IFN-γ (20 ng/mL **(A, B)**, 2 ng/mL **(C)**). Protein lysates were collected at the indicated times and western blotted for pSTAT1 (Y701 and S727), IRF1, ISG54, and β-Tubulin where indicated.

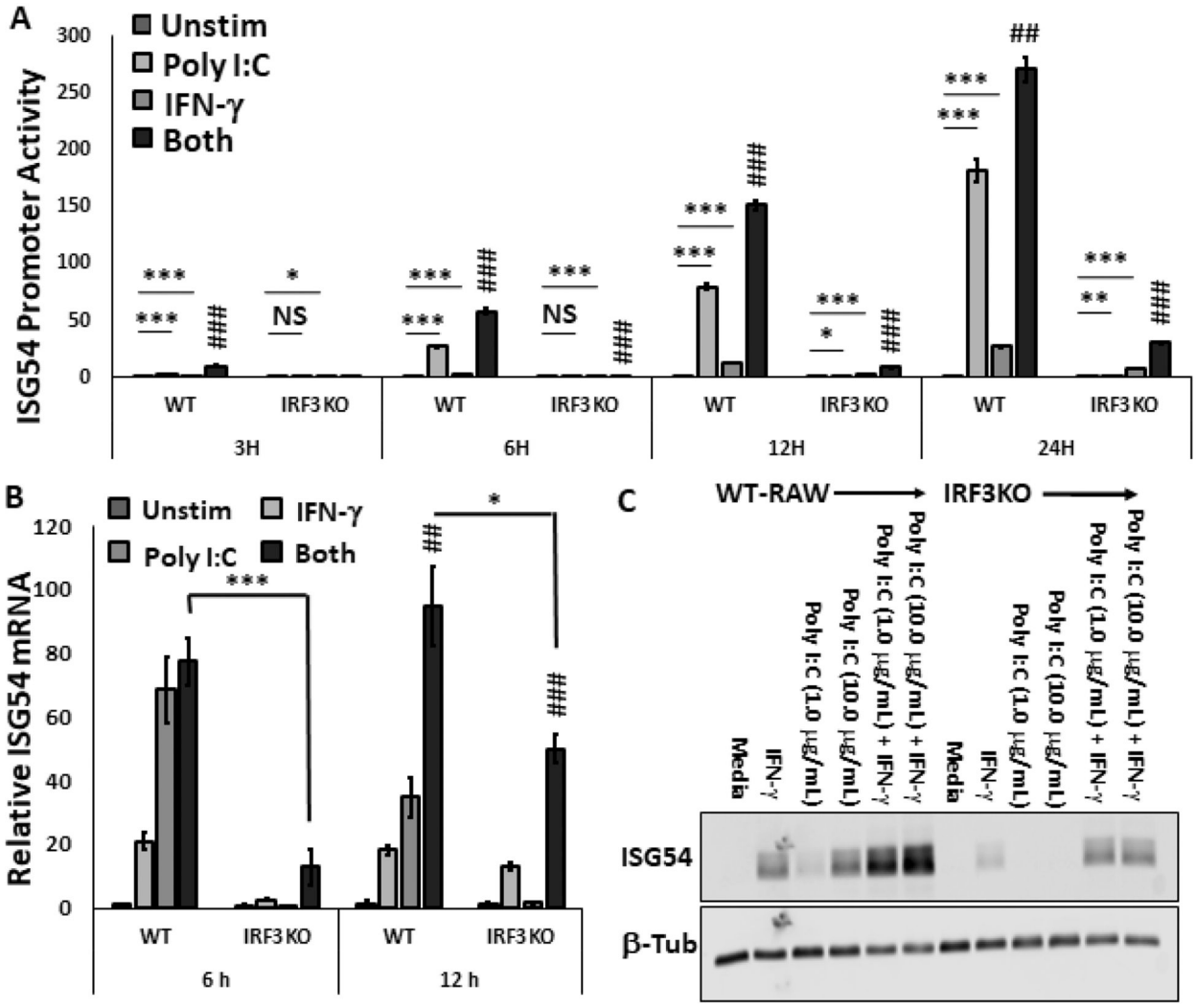

FIGURE 3. IFN-γ and Poly I:C co-stimulation synergistically induces ISG54.

(A, B) 2×10^5 WT or IRF3KO RAW-Lucia cells or **(C)** 1×10^6 WT or IRF3KO RAW-Lucia cells were unstimulated or stimulated with IFN-γ (20 ng/mL), Poly I:C (10 mg/mL), or both IFN-γ and Poly I:C. At indicated times, (A) luciferase activity in 10 μL of supernatants was used to determine relative ISG54 promoter activity or **(B)** ISG54 expression was determined using qRT-PCR or (C) protein lysates were collected and ISG54 and β-Tubulin (β-Tub) were evaluated by western blot. **(A)** two independent experiments, each with n=3. **(B)** Data are means ± S.E.M and are combined from two independent experiments, each with n=2–3 (Total n=5–6). *, **, *** indicate statistical significance ($p < 0.05$, 0.005, 0.0005, respectively) and NS indicates no significance ($p > 0.05$) using two-tailed student T-test. #, ##, ### indicate statistically significant interaction ($p < 0.05$, 0.005, 0.0005, respectively) between IFN-γ and Poly I:C using two-way ANOVA analysis.

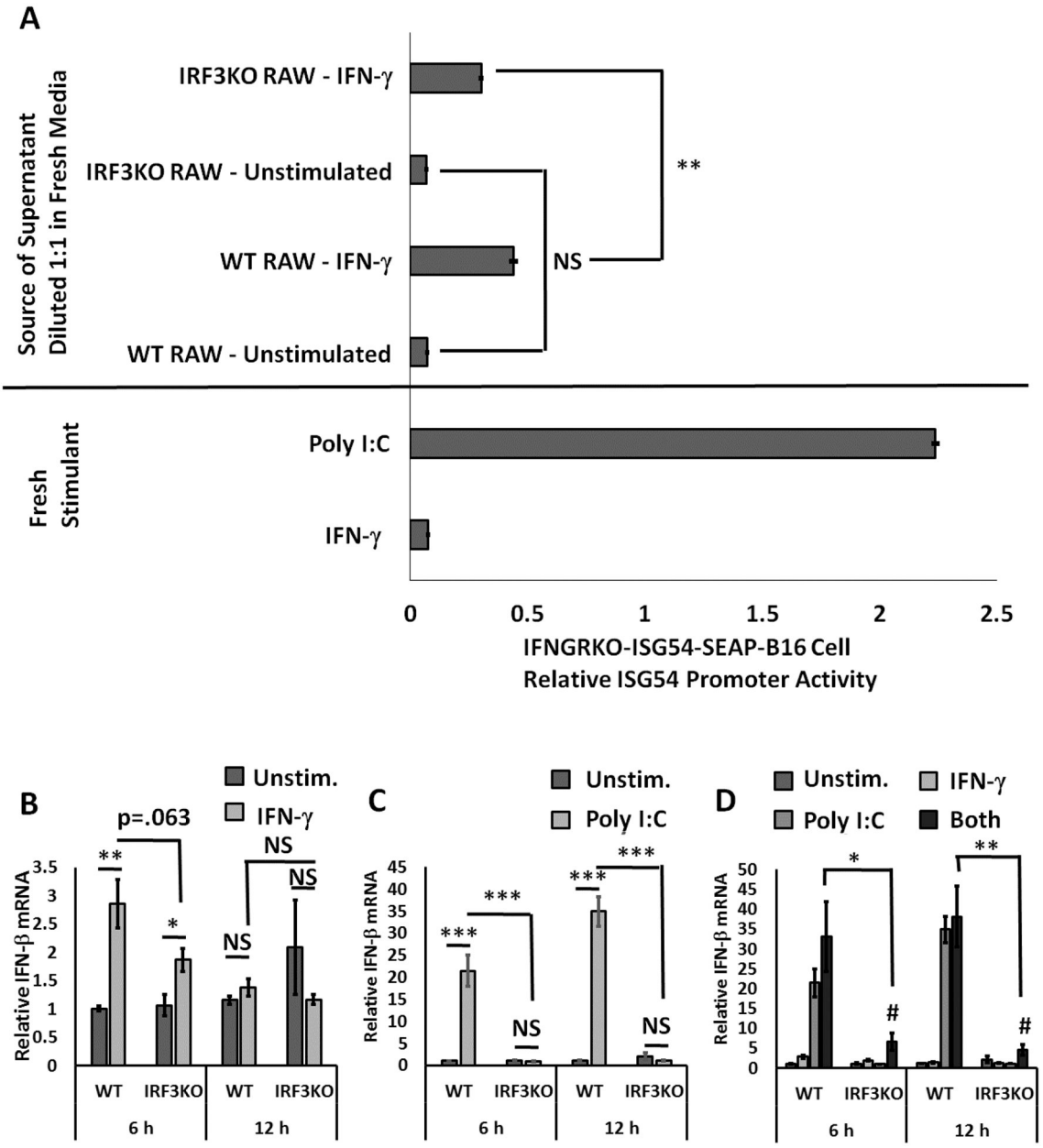

FIGURE 4. IFN-γ induces expression of ISG54-inducing secreted factors in an IRF3 dependent manner.

(A) IFNGRKO ISG54-SEAP-B16 cells were incubated at a 1:1 ratio with fresh culture media mixed with supernatant from WT or IRF3KO RAW-Lucia cells that were unstimulated or stimulated with IFN-γ (20 ng/mL) for 24 h. IFNGRKO ISG54-SEAP-B16 cells were also stimulated with IFN-γ (20 ng/mL) or Poly I I:C (10 μg/mL) in fresh media as positive controls. SEAP activity in 20 μL of supernatant was tested for SEAP using QuantiBlue to determine relative ISG54 promoter activity. **(B, C, D)** 2×10^5 WT or IRF3KO RAW-Lucia cells were stimulated with IFN-γ (20 ng/mL) **(B, D)**, Poly I:C (10 μg/mL) **(C, D)**, or both IFN-γ and Poly I:C **(D)**. At indicated time points, IFN-β expression was determined using qRT-PCR. **(A)** Data are means ± S.E.M and represent two independent

experiments, each with n=3. **(B, C, D)** Data are means ± S.E.M that are combined from two independent experiments (n=5–6). In **(A, B, C)** *, **, *** indicate statistical significance ($p < 0.05$, 0.005, 0.0005, respectively) and NS indicates no significance ($p > 0.05$) using two-tailed student T-test. In **(D)** #, ##, ### indicate statistically significant interaction ($p < 0.05$, 0.005, 0.0005, respectively) between IFN-γ and Poly I:C using two-way ANOVA analysis.

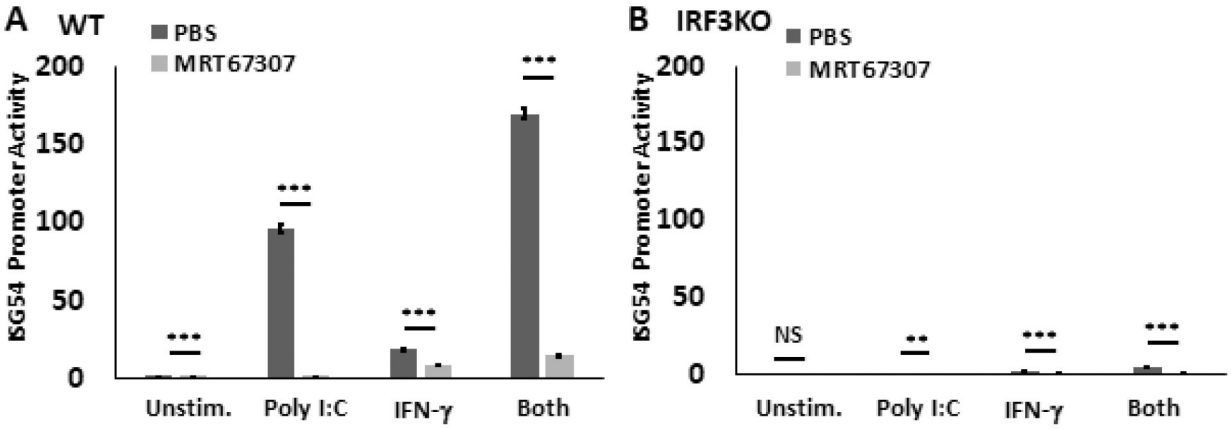

FIGURE 5. IFN-γ induces ISG54 expression in a TBK1/IKKε dependent manner.

(A, B) 2×10^5 WT **(A)** or IRF3KO **(B)** RAW-Lucia cells were incubated with PBS or MRT67307 (2 μM) for 1 hour. Cells were then unstimulated or stimulated with IFN-γ (50 ng/mL), Poly I:C (25 μg/mL), or both IFN-γ and Poly I:C. After 24 h, 10 μL of supernatant was tested for luciferase activity using QuantiLuc to determine relative ISG54 promoter activity. **(A, B)** Data is representative of two independent experiments, each with n=3. Data are means ± S.E.M. *, **, *** indicate statistical significance ($p < 0.05$, 0.005, 0.0005, respectively) and NS indicates no significance ($p > 0.05$) using two-tailed student T-test.

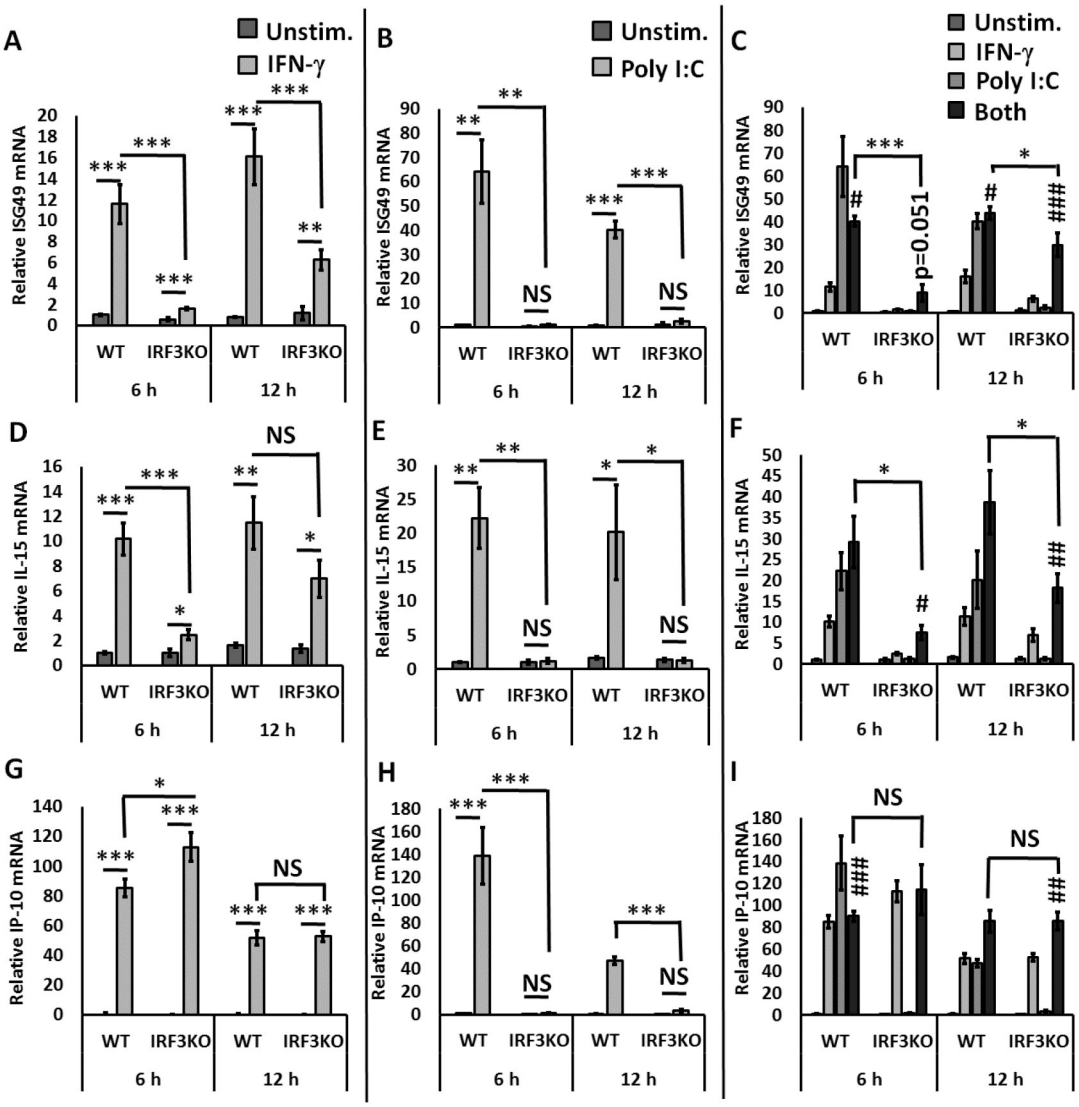

FIGURE 6. IFN-γ induces ISG49 and IL-15, but not IP-10, in an IRF3 dependent manner.

(A-I) 2×10^5 WT or IRF3KO RAW-Lucia cells were unstimulated or stimulated with IFN-γ (20 ng/mL) **(A, D, G)**, Poly I:C (10 μg/mL) **(B, E, H)**, or both IFN-γ and Poly I:C **(C, F, I)**. ISG49 **(A-C)**, IL-15 **(D-F)**, and IP-10 **(G-I)** expression was determined using qRT-PCR. **(A – I)** Data is combined from two independent experiments, each with n=2–3 (Total n=5–6). Data are mean ± S.E.M. *, **, *** indicate statistical significance ($p < 0.05$, 0.005, 0.0005, respectively) and NS indicates no significance ($p > 0.05$) using two-tailed student T-test. **(C, F, I)** #, ##, ### indicate statistically significant interaction ($p < 0.05$, 0.005, 0.0005, respectively) between IFN-γ and Poly I:C using two-way ANOVA analysis.

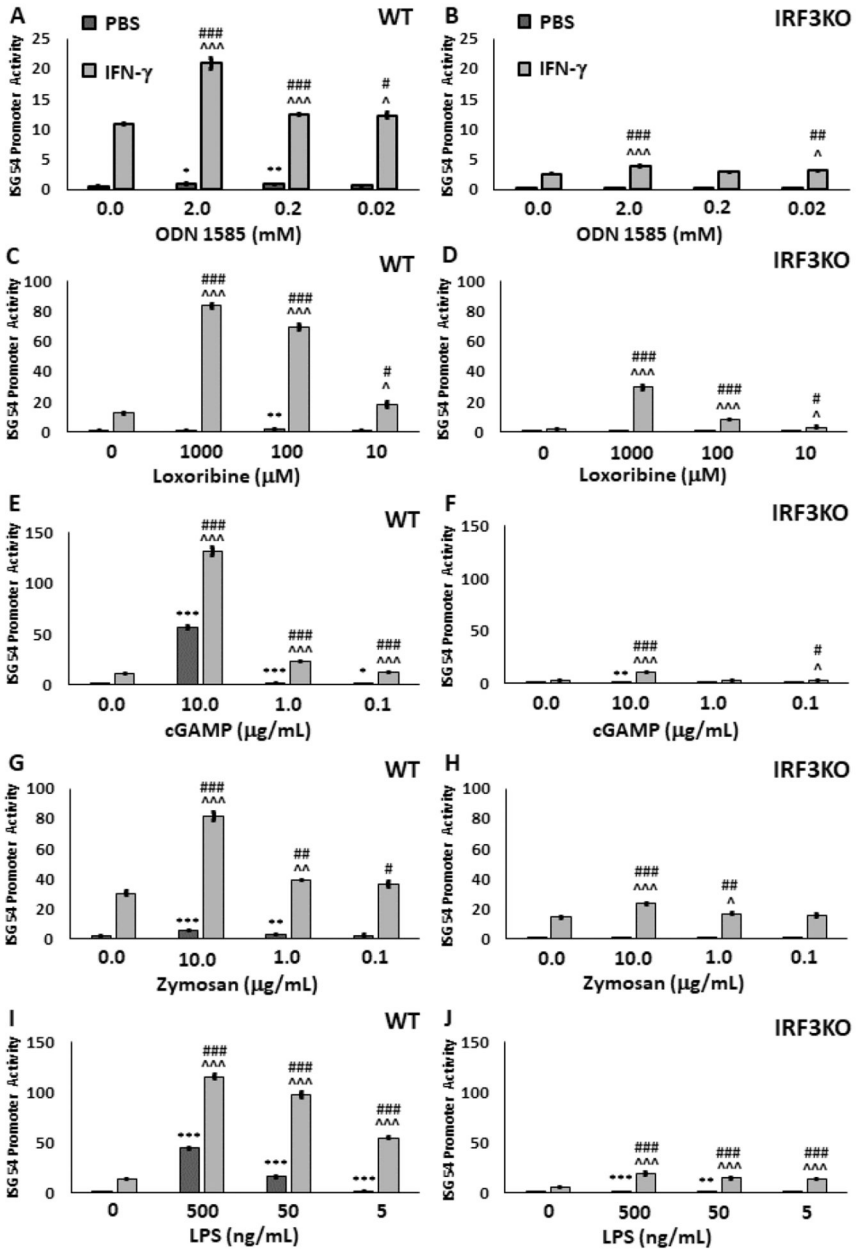

FIGURE 7. Several PRR agonists enhance ISG54 promoter activity in an IRF3 dependent manner.

1×10^5 WT **(A, C, E, G, I)** or IRF3KO **(B, D, F, H, J)** RAW-Lucia cells were unstimulated or stimulated with ODN1585, Loxoribine, cGAMP, Zymosan, or LPS at multiple 10 fold dilutions in the presence or absence of IFN-γ (20 ng/mL). After 24 h, luciferase activity in 10 μL of supernatant was determined to measure relative ISG54 promoter activity. Data are means ± S.E.M and represent two independent experiments with n=3. *, **, *** indicate statistical significance ($p < 0.05$, 0.005, 0.0005, respectively) between unstimulated cells and those stimulated with PRR agonist using two-tailed student T-test. ^, ^^, ^^^ indicate statistical significance ($p < 0.05$, 0.005, 0.0005, respectively) between cells stimulated with PRR agonist plus IFN-γ and those stimulated with IFN-γ alone. #, ##, ### indicate

statistically significant interaction ($p < 0.05$, 0.005, 0.0005, respectively) between IFN-γ and Poly I:C using two-way ANOVA analysis.