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IRF1 is a transcriptional regulator of ZBP1 promoting NLRP3 inflammasome activation and cell death during influenza virus infection

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Abstract

Innate immune sensing of influenza A virus (IAV) induces activation of various immune effector mechanisms including the NLRP3 inflammasome and programmed cell death pathways. Although type I IFNs are identified as key mediators of inflammatory and cell death responses during IAV infection, the involvement of various IFN-regulated effectors in facilitating these responses are less studied. Here, we demonstrate the role of interferon regulatory factor 1 (IRF1) in promoting NLRP3 inflammasome activation and cell death during IAV infection. Both inflammasome-dependent responses and induction of apoptosis and necroptosis are reduced in cells lacking IRF1 infected with IAV. The observed reduction in inflammasome activation and cell death in IRF1-deficient cells during IAV infection correlates with reduced levels of Z-DNA binding protein 1 (ZBP1), a key molecule mediating IAV-induced inflammatory and cell death responses. We further demonstrate IRF1 as a transcriptional regulator of ZBP1. Overall, our study identified IRF1 as an upstream regulator of NLRP3 inflammasome and cell death during IAV infection and further highlights the complex and multilayered regulation of key molecules controlling inflammatory response and cell fate decisions during infections.

Keywords

IRF1; ZBP1; NLRP3; inflammasome; caspase-1; influenza virus; cell death

Introduction

Influenza A virus (IAV) causes acute respiratory tract infection in mammals and avian species and is associated with substantial morbidity and mortality (1). The virus infects and replicates in the epithelial cells of upper and lower respiratory tract and disease severity is regulated by both virus-encoded as well as host factors (2). Lung inflammation and epithelial damage are regarded as the major factors modulating pathogenesis of IAV and

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dampening of these responses offers an attractive treatment option especially in patients infected with highly pathogenic IAV (3–7). Recent studies have identified several host factors and intracellular signaling cascades important in regulating inflammatory and cell death responses during IAV infection (8–10). Despite being recognized as the hallmark feature of an antiviral response, type I IFNs and several IFN-inducible effector proteins have been shown to enhance epithelial cell death, inflammatory response and lung damage during acute IAV infection (11–13). Although multiple studies have demonstrated the pathogenic effects of type I IFNs, the effector proteins and the mechanisms by which IFN signaling mediate these responses have not been fully characterized.

IFN regulatory factor 1 (IRF1) was the first member of the IRF family of transcription factors and was initially identified as a regulator of IFNs and IFN-stimulated genes (ISGs) (14,15). The antiviral functions of IRF1 were revealed after generation of *Irf1*$^{-/-}$ mice and IRF1 was shown to selectively restrict replication of certain classes of viruses (16). IRF1 is necessary for host protection and restricting replication of encephalomyocarditis virus (EMCV). IRF1 also has a minor role in cellular resistance to herpes simplex virus (HSV), however it is not involved in restricting replication of vesicular stomatitis virus (VSV) in embryonic fibroblasts (16). Antiviral functions of IRF1 were also reported against hepatitis C virus, human immunodeficiency virus (HIV), tick-borne encephalitis virus, gammaherpes virus, Japanese encephalitis virus, non-myocarditic reovirus and chikungunya virus (17–23). IRF1 has a critical role in counteracting neuropathology of VSV and is essential for host protection during West Nile virus (WNV) infection (24,25). IRF1 is also important in regulating IFNλ production suggesting antiviral functions for IRF1 during viral infections at mucosal sites (26–28). Although the role for IRF1 in mediating absent in melanoma 2 (AIM2) inflammasome activation during cytosolic bacterial infections was reported (29), it is not known whether IRF1 is involved in inflammasome activation during viral infections. Moreover, the role of IRF1 in antiviral immune responses during IAV infection is also not explored in detail. In this study, we investigated the role of IRF1 in regulating nucleotide and oligomerization domain, leucine-rich repeat-containing protein family, pyrin domain containing 3 (NLRP3) inflammasome activation and cell death during IAV infection and demonstrated a key role for IRF1 in promoting these responses in BMDMs and lung fibroblasts infected with IAV. The reduction in cell death and inflammasome activation in IRF1-deficient cells correlates with reduced levels of Z-DNA binding protein 1 [ZBP1; also known as DLM1 and DNA-dependent activator of IFN regulatory factors (DAI)], a key regulator of cell death, inflammasome activation and proinflammatory responses during IAV infection (8,30). We further identified IRF1 as a transcriptional regulator of ZBP1 and therefore provide additional insights regarding the regulation of ZBP1.

Materials and Methods

Mice

C57BL/6J mice [wide type (WT)] were purchased from The Jackson Laboratory and bred at St. Jude Children's Research Hospital (SJCRH). *Ifnar1*$^{-/-}$ mice, *Irf1*$^{-/-}$ mice, *Irf9*$^{-/-}$ mice, *Nlrp3*$^{-/-}$ mice, *Nlrc4*$^{-/-}$ mice and *Aim2*$^{-/-}$ mice were generated as described previously (29). For *in vivo* infections, WT *and Irf1*$^{-/-}$ mice were anesthetized with 250 mg/kg Avertin

followed by intranasal infection with a sublethal dose of PR8 virus in 50 μl PBS. Infected mice were monitored over a period of 14 days for survival study. Lungs harvested on day 5 after infection were homogenized in 1ml PBS and viral titers were estimated by plaque assays. All mice were bred at the Animal Resource Center at SJCRH and animal studies were conducted according to protocols approved by the SJCRH Animal Care and Use Committee.

Cell culture and stimulation

Primary bone marrow–derived macrophages (BMDMs) were grown for 6 days and were seeded in antibiotic–free media at a concentration of 1×10^6 cells onto 12-well plates and incubated overnight. Lung fibroblasts were generated as described previously (31) and were seeded at a concentration of 0.2×10^6 cells onto 12-well plates and incubated overnight. The influenza A/Puerto Rico/8/34 H1N1 virus (PR8) was generated by an eight-plasmid reverse genetics system. Stocks were propagated in allantoic cavity of 9- to 11-day-old embryonated chicken eggs and viral titers were enumerated by plaque assays. Cells were infected with PR8 virus (MOI, 25) for 2 h in DMEM media without L-glutamine and sodium pyruvate (Sigma). DMEM media containing 20% FBS was added to the infection media 2 h after infection and samples were collected at indicated timepoints. Real time assessment of cell death was performed using IncuCyte and sytox green nucleic acid staining. Cells were also treated with IFNβ (100 U/ml) or poly I:C (10μg/ml) or LPS (500ng/ml). For some cell death experiments, BMDMs were treated with zVAD-FMK (50μM; Calbiochem) or GW806742X (1μM; SYNkinase) at the time of infection.

Microarray

Transcript profiling was performed using two biological replicate samples of unprimed BMDMs infected with PR8 virus. Total RNA (100 ng) was converted to biotin-labeled cRNA using the Ambion WT expression kit (Life Technologies) and hybridized to a Mouse Gene 2.0 ST GeneChip (Affymetrix, Inc). After staining and washing, array signals were normalized and transformed into $\log_2$ transcript expression values using the Robust Multi-array Average algorithm (Partek Genomics Suite v6.6). Differential expression was defined by applying a 0.5 $\log_2$ (signal) difference between conditions. Lists of differentially expressed transcripts were analyzed for functional enrichment using the DAVID bioinformatics databases (http://david.abcc.ncifcrf.gov/) and Ingenuity Pathways Analysis software (www.qiagen.com/ingenuity). The microarray data is deposited under GEO accession ID GSE103059 and is available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103059.

qRT-PCR analysis

RNA was extracted using TRIzol (Life Technologies). Isolated RNA was reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Life Technologies). Real-time PCR was performed on an ABI 7500 real-time PCR instrument with 2× SYBR Green (Applied Biosystems). Relative gene expression levels were calculated by normalizing the Ct levels of the target gene to both endogenous GAPDH levels and an unstimulated WT control using the ΔΔCt method. Sequences for qRT-PCR primers used are: *Irf1*: 5′- ATG CCA ATC ACT CGA ATG CG-3′; 5′-TTG TAT CGG CCT GTG TGA ATG-3′; *Nlrp3*: 5′-TGC AGA AGA

CTG ACG TCT CC-3′, 5′CGT ACA GGC AGT AGA ACA GTT C-3′; *Il1b*: 5′-GAT CCA CAC TCT CCA GCT GCA-3′, 5′-CAA CCA ACA AGT GATATTCTCCATG-3′; *Il6*: 5′-GAC AAA GCC AGA GTC CTT CAG AGA G-3′, 5′-CTA GGT TTG CCG AGT AGA TCT C-3′, *Ifnb*: 5′-GCC TTT GCC ATC CAA GAG ATG C-3′, 5′-ACA CTG TCT GCT GGT GGA GTT C-3′; *Zbp1*: 5′-AAG AGT CCC CTG CGA TTA TTT G-3′, 5′- TCT GGA TGG CGT TTG AAT TGG-3′; *Nos2*: 5′- GTT CTC AGC CCA ACA ATA CAA GA-3′, 5′- GTG GAC GGG TCG ATG TCA C-3′; *Irf7*: 5′- GAG ACT GGC TAT TGG GGG AG-3′, 5′- GAC CGA AAT GCT TCC AGG G-3′.

Cytokine measurement by ELISA

IFNβ and IL-18 were measured using ELISA kits (Biolegend (IFNβ); eBioscience (IL-18)). All other cytokines were measured using multiplex ELISAs (Millipore).

Immunoblotting analysis

For caspase-1, caspase-8, caspase-3 and IL-1β immunoblotting, cells and supernatant were lysed in cell lysis buffer. For immunoblotting other proteins, cells were lysed using RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Protein samples were separated by SDS-PAGE and transferred to PVDF membranes. The primary antibodies used are: caspase-1 (AG-20B-0042, Adipogen), ZBP-1 (AG-20B-0010-C100, Adipogen), Influenza A virus NS1 (sc-130568, Santa Cruz Biotechnology), NLRP3 (AG-20B-0014, Adipogen), ASC (04–147, Millipore), caspase-8 (#4927, CST), cleaved caspase-8 (#8592, (CST), cleaved caspase-3 (#9664, CST), Caspase-3 (bs-0081R, Bioss), pIRF3 (clone 4D4G, CST), IRF3 (D83B9, CST), and pSTAT1 (D4A7, CST); IL-1β (AF-401-NA; R&D systems) and GAPDH (#5174, CST) followed by secondary anti-rabbit or anti-mouse HRP antibodies (Jackson Immuno Research Laboratories). Quantification of blots was performed using ImageJ software.

Statistical analysis

GraphPad PRISM software was used for statistical analysis. Statistical significance was determined by a two-tailed *t* test; $P < 0.05$ was considered statistically significant where $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Results

IRF1 promotes activation of the NLRP3 inflammasome during IAV infection

The NLRP3 inflammasome regulates both innate and adaptive immune responses and functions as a major antiviral host protective mechanism during IAV infection (32–35). A multitude of host and viral factors were identified as regulators of IAV-induced assembly and activation of the NLRP3 inflammasome (36). Both TLR-MyD88/TRIF and RIG-I-MAVS signaling have redundant roles in IAV-induced inflammasome activation (37,38). Although the critical role of type I IFN signaling in facilitating NLRP3 inflammasome activation is well established (8,37), the involvement of various IFN-regulated transcription factors in mediating this response in IAV-infected cells has not been investigated in detail. Microarray analysis of WT and *Ifnar1*$^{-/-}$ BMDMs infected with IAV confirmed IFN-dependent induction of the IRF family transcription factors (Fig. 1A). Among various IRFs, IRF3,

IRF7 and IRF5 regulate induction of IFNs whereas IRF9 transduces IFN signaling as part of the ISGF3 complex (14). IRF1 acts as a transcriptional activator of many ISGs whereas IRF2 suppresses IRF1 activity (14). We have previously shown that IFN-dependent induction and transcriptional activity of IRF1 is critical for AIM2 inflammasome activation during *Francisella novicida* infection (29). Similar to *F. novicida* infected cells, IRF1 was robustly upregulated in BMDMs infected with IAV (Fig. 1 B–C). The levels of IRF1 were abrogated in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ cells confirming type I IFN-dependent induction of IRF1 in IAV-infected BMDMs (Fig. 1B–C). To investigate whether IRF1 regulates NLRP3 inflammasome activation during IAV infection, we infected unprimed WT, *Irf1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs with mouse-adapted Influenza virus A/Puerto Rico/8/34 (PR8; H1N1) and assessed inflammasome responses 16 hours after infection. Interestingly, a substantial reduction in NLRP3-mediated caspase-1 activation was observed in *Irf1*$^{-/-}$ BMDMs compared with WT cells infected with IAV (Fig. 1D–E). We also measured the levels of inflammasome-dependent cytokines and observed a significant reduction in IL-1β and a modest, but consistent reduction in IL-18 levels in *Irf1*$^{-/-}$ cells compared with WT BMDMs (Fig. 1F–G). Activation of NLRP3 inflammasome is a two-step process where upregulation of inflammasome components (priming) occurs in the first step after recognition of various pathogen-associated molecules (39). The observed reduction in inflammasome activation was not due to impaired priming because induction of *Nlrp3*, *Il1b* and *Il6* occurred normally in *Irf1*$^{-/-}$ cells infected with IAV (Fig. 1H). Moreover, the levels of NLRP3 and ASC as well as inflammasome-independent cytokines, IL-6, TNF and KC were also comparable between WT and IRF1-deficient cells (Fig. 1I–J). In addition to BMDMs, IRF1 is also required for inflammasome activation in lung fibroblasts infected with IAV. Whereas levels of NLRP3 and ASC were comparable, processing of pro-IL-1β to bioactive IL-1β was substantially reduced in IRF1-deficient lung fibroblasts compared with WT cells (Fig. 1K–M). Collectively, these data demonstrate the role of IRF1 in regulating activation of NLRP3 inflammasome during IAV infection independently of inflammasome priming in both BMDMs and lung fibroblasts.

IRF1 specifically regulates canonical NLRP3 inflammasome in IAV-infected cells

Activation of NLRP3 inflammsome during IAV infection was demonstrated in multiple studies (36). Notably, caspase-11 is dispensable for inflammasome activation in IAV-infected cells indicating activation of the canonical NLRP3 inflammasome in response to IAV infection (8). Although IRF1 has a role in facilitating AIM2 inflammasome activation during *F. novicida* infection, it is dispensable for activation of NLRP3, NLRC4 and AIM2 inflammasomes in response to other stimuli (29). To confirm the role of IRF1 in regulation of IAV-induced inflammasome activation, we infected WT and *Irf1*$^{-/-}$ BMDMs with different strains of IAV (X31 and WSN) and assessed caspase-1 cleavage. Consistent with our observations using PR8 strain of IAV, a substantial reduction in caspase-1 cleavage was observed in *Irf1*$^{-/-}$ cells infected with both X31 and WSN viruses (Fig. 2A). We also confirmed the role of IRF1 in inflammasome activation in *F. novicida*-infected cells, but not in response to LPS + ATP treatment or during infection with *S.* Typhimurium or murine cytomegalovirus (MCMV) (Fig. 2B–E). Moreover, caspase-1 cleavage occurred normally in both NLRC4- and AIM2- deficient cells infected with IAV further demonstrating that these sensors are not involved in inflammasome responses during IAV infection (Fig. 2F). These

data collectively demonstrate a specific role for IRF1 in promoting canonical NLRP3 inflammasome activation during IAV infection.

IRF1 regulates cell death in response to IAV infection

We and others have recently reported parallel and complementary activation of various programmed cell death pathways in IAV-infected cells (8–10,38). Although NLRP3 inflammasome is necessary for induction of pyroptosis during IAV infection, cell death occurs normally in cells lacking NLRP3, caspase-1 or gasdermin D (8). Whereas pyroptosis is mediated by the effector protein gasdermin D, induction of apoptosis and necroptosis is mediated by effector caspases and MLKL, respectively (40–42). Common upstream molecules and signaling cascades have been shown to regulate induction of cell death pathways and inflammasome activation during IAV infection (8,38). Therefore, we sought to determine whether IRF1 regulates cell death in addition to NLRP3 inflammasome in IAV-infected cells. We performed real time analysis of cell death in primary lung fibroblasts and BMDMs from WT and *Irf1*$^{-/-}$ mice in response to IAV infection using IncuCyte live cell analysis system and sytox green nucleic acid staining. We observed reduction in cell death in *Irf1*$^{-/-}$ fibroblasts and BMDMs infected with IAV compared to WT cells (Fig. 3A–D). Activation and cleavage of caspase-8 and caspase-3 were also significantly decreased in *Irf1*$^{-/-}$ cells demonstrating IRF1 regulation of apoptosis during IAV infection (Fig. 3E–F). Consistent with our previous studies (8), activation of apoptotic caspases occurred independently of NLRP3 in IAV-infected cells (Fig. 3E–F). In addition to pyroptosis and apoptosis, IRF1 regulates necroptosis in IAV-infected cells and phosphorylation of MLKL was reduced in IRF1-deficient cells compared with WT cells (Fig. 3G). To further investigate the cell death pathways regulated by IRF1 in IAV-infected cells, we used either pan-caspase inhibitor (zVAD-FMK) or MLKL inhibitor (GW806742X) or combination of both along with IAV infection. Consistent with parallel and complementary activation of all programmed cell death pathways during IAV infection, cell death was inhibited only when cells were treated with a combination of both inhibitors (Fig. 3H). Inhibition of all cell death pathways, but not individual ones, also inhibited IAV-induced cell death in *Irf1*$^{-/-}$ cells confirming IRF1 regulation of parallel cell death pathways in IAV-infected cells (Fig. 3H). Together, these data demonstrate IRF1 as a central regulator of programmed cell death pathways during IAV infection.

IRF1 upregulates Zbp1 expression independently of type I IFN production and signaling

IRF1 was initially identified as a transcriptional activator of type I IFNs (15). Because type I IFNs are critical regulators of inflammasome activation and cell death during IAV infection (8), we assessed whether IFN responses are impaired in *Irf1*$^{-/-}$ cells infected with IAV. Similar levels of IRF3 phosphorylation in IRF1-deficient cells confirmed that signaling pathways regulating IFN expression are intact in *Irf1*$^{-/-}$ cells (Fig. 4A). Moreover, *Ifnb* expression as well as secretion of IFNβ occurred normally in *Irf1*$^{-/-}$ cells demonstrating that IRF1 is not involved in IFN production during IAV infection (Fig. 4B–C). We also assessed phosphorylation of STAT1 in WT and *Irf1*$^{-/-}$ cells infected with IAV and found comparable levels of STAT1 phosphorylation in IRF1-deficient cells (Fig. 4D). Together, these data demonstrate that IRF1 is dispensable for IFN production and signaling in IAV-infected cells.

IRF1 functions as a transcriptional activator of many ISGs and other genes involved in antimicrobial immunity (15). To investigate the IRF1-dependent transcriptional signature induced in IAV-infected cells, we performed microarray analysis of *Irf1*$^{-/-}$ BMDMs infected with IAV. Enrichment of the microarray gene expression dataset for nucleic acid sensing pathways revealed a significant reduction in the expression of *Zbp1* in *Irf1*$^{-/-}$ BMDMs compared with WT cells (Fig. 4E). Of note, ZBP1 is recently identified as a central regulator of inflammasome activation and cell death during IAV infection (8,10,30,38). We confirmed the microarray data by RT-PCR and found significant reduction in the expression of *Zbp1* in *Irf1*$^{-/-}$ cells compared with WT cells (Fig. 4F). The levels of ZBP1 were also substantially reduced in both BMDMs and lung fibroblasts infected with IAV (Fig. 4G) further demonstrating the requirement of IRF1 for ZBP1 induction in IAV-infected cells. To confirm whether IRF1 acts as a common transcription factor regulating *Zbp1* expression, we treated BMDMs with IFNβ, poly I:C or LPS and assessed induction of *Zbp1*. *Zbp1* expression was reduced in *Irf1*$^{-/-}$ cells whereas induction of *Irf7* was comparable to that of WT cells in response to all these stimuli (Fig. 4H). As expected, levels of *Nos2*, a well-known IRF1-regulated gene, was also reduced in *Irf1*$^{-/-}$ cells (Fig. 4H). IRF1 regulation of ZBP1 in response to these stimuli was also confirmed by Western blotting (Fig. 4I). Collectively, these data identified IRF1 as a transcriptional regulator of ZBP1. The reduced levels of ZBP1 in *Irf1*$^{-/-}$ cells correlates with the observed reduction in inflammasome activation and cell death during IAV infection suggesting that IRF1 functions as an upstream regulator of NLRP3 inflammasome and cell death in IAV-infected cells by regulating transcriptional induction of *Zbp1*.

Deficiency of IRF1 does not significantly alter susceptibility to IAV infection

To investigate whether IRF1 regulates susceptibility to IAV infection, we infected WT and *Irf1*$^{-/-}$ mice with a sublethal dose of IAV (PR8 strain) and monitored clinical signs and weight loss over a period of 14 days. Consistent with a previous report (24), we did not observe any significant difference in morbidity due to deficiency of IRF1 (Fig. 5A). However, a modest increase in weight loss and delay in recovery was observed in *Irf1*$^{-/-}$ mice compared to WT mice (Fig. 5A). Lung viral titers were also comparable between WT and *Irf1*$^{-/-}$ mice (Fig. 5B). These results demonstrate that deficiency of IRF1 does not significantly alter susceptibility to IAV infection.

Discussion

In this study, we have identified IRF1 as one of the upstream regulators of NLRP3 inflammasome and cell death during IAV infection. Whereas IRF1 induction is mediated by IFN signaling and ISGF3 complex, IRF1 subsequently functions as a transcriptional regulator of ZBP1. These findings suggest that IRF1 comes downstream of the RIG-I/TLR-IFNAR axis, but upstream of ZBP1 in the hierarchy of proteins regulating inflammasome activation and cell death in IAV-infected cells (8,38). The data from this study also identified IRF1 as a regulator of NLRP3 inflammasome specifically during IAV infection in addition to its role in mediating AIM2 inflammasome activation during *F. novicida* infection (29). This further demonstrates the role of distinct effector molecules in facilitating inflammasome assembly and activation in a stimulus-specific manner. The findings from our

study also confirm the key role of IFN signaling in mediating inflammasome activation and cell death during IAV infection via induction of various effector molecules. It is well established that multiple signaling cascades and transcription factors can independently or cooperatively regulate IFN response and induction of ISGs. We have previously demonstrated the critical role for IFN signaling and ISGF3 complex in induction of ZBP1 and ZBP1 levels were abrogated in *Ifnar1*$^{-/-}$, *Stat1*$^{-/-}$ and *Irf9*$^{-/-}$ cells (8). The observed reduction in ZBP1 in IRF1-deficient cells suggests that IRF1 cooperates with ISGF3 complex for transcriptional induction of *Zbp1*. The identification of IRF1 as a regulator of inflammasome activation and cell death also adds an upstream transcription factor to the different classes of proteins controlling these responses.

Except for the initial characterization as an IFN-inducible gene (43), the regulatory mechanisms controlling ZBP1 has not been studied in detail so far. Before the recent interest in ZBP1 as a pathogen sensor and regulator of inflammatory and cell death responses (8,10,30,44,45), ZBP1 was shown to regulate DNA-mediated activation of IRF3 and IFN production and therefore the alternative name DNA-dependent activator of IFN-regulatory factors (DAI) was proposed for the protein (46). However, its role as a DNA sensor was questioned because later studies failed to show the involvement of ZBP1 in DNA-mediated IFN and immune responses (47,48). Our study has now identified IRF1 as a previously unknown transcriptional regulator of ZBP1 and also demonstrates IFN and IRF regulation of ZBP1 rather than ZBP1 regulating activation of IRF transcription factors.

Despite its role in regulating inflammasome and cell death responses during IAV infection, IRF1 deficiency did not significantly alter susceptibility to IAV infection. Unlike ZBP1-deficient cells where secretion of proinflammatory cytokines is impaired in response to IAV infection (8), the levels of IL-6, TNF and KC were comparable to WT levels in *Irf1*$^{-/-}$ cells infected with IAV. This suggests that the levels of ZBP1 present in IRF1-deficient cells are sufficient to mediate the proinflammatory responses, which is a critical factor controlling pathogenesis of IAV infection (4). The residual levels of ZBP1 might also be affecting the overall phenotype in *Irf1*$^{-/-}$ mice infected with IAV compared with *Zbp1*$^{-/-}$ mice. It is also possible that various IRF1-regulated effectors might function differently or in a compensatory manner to modulate the pathogenesis and disease outcome during IAV infection.

IRF1 has been previously characterized as a central mediator of immune cell function and induction of innate and adaptive immune responses (15). Although IRF1 is reported to be important in antiviral activity against many viruses, there is no consensus regarding the mechanisms by which IRF1 regulates these responses. The identification of IRF1 as a transcriptional regulator of ZBP1 suggests a potential mechanism by which IRF1 mediates inflammatory and cell death responses to facilitate antiviral host defense. Further studies are warranted to investigate whether IRF1 is also involved in mediating these responses during infection with other classes of viruses. Overall, our study delineates the role of IRF1 in inflammasome activation and cell death during IAV infection and further demonstrates the complex regulation of key molecules controlling inflammatory response and cell death during infections.

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Abbreviations:

AIM2	absent in melanoma 2
BMDMs	bone marrow–derived macrophages
IAV	Influenza A virus
IFNAR	interferon-α/β receptor
ISGs	interferon-stimulated genes
IRF	interferon regulatory factor
ISGF3	interferon stimulated gene factor 3
MLKL	Mixed lineage kinase-like
NLRP3	nucleotide and oligomerization domain, leucine-rich repeat–containing protein family, pyrin domain containing 3
RIG-I	Retinoic acid-inducible gene
STAT	signal transducer and activator of transcription
WT	wild type
ZBP1	Z-DNA binding protein 1

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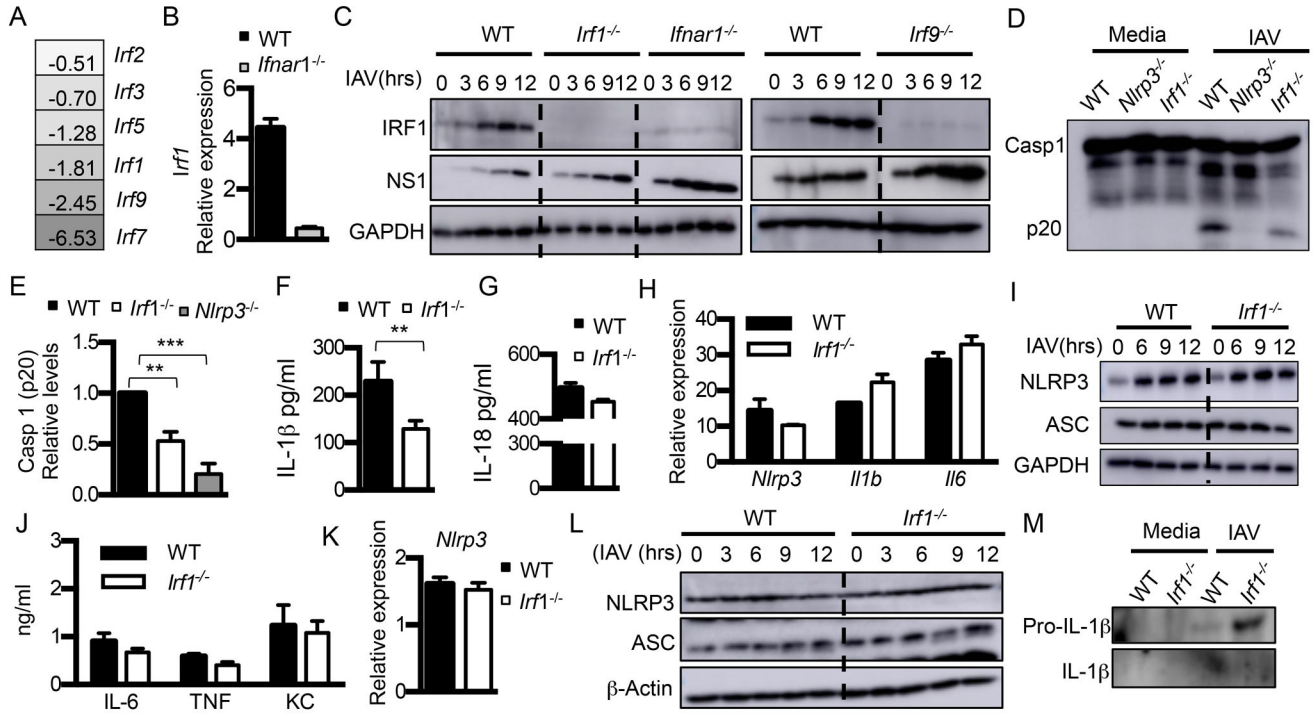

Figure 1. IRF1 promotes activation of NLRP3 inflammasome during IAV infection.

(A) Microarray analysis of IRF family transcription factors differentially regulated in *Ifnar1*$^{-/-}$ BMDMs infected with IAV compared with WT BMDMs. (B) Transcript levels of *Irf1* in WT and *Ifnar1*$^{-/-}$ BMDMs infected with IAV for 9 h. (C) Immunoblot analysis of IRF1, IAV-NS1 and GAPDH in WT, *Irf1*$^{-/-}$, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMDMs at various time points after IAV infection. (D) Immunoblot analysis of caspase-1 activation in uninfected or IAV-infected WT, *Irf1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs 16 h after infection. (E) Quantification of caspase-1 p20 in IAV-infected WT, *Irf1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs. (F - G) Levels of IL-1β and IL-18 in cell culture supernatants from WT and *Irf1*$^{-/-}$ BMDMs infected with IAV for 16 h. (H) Transcript levels of *Nlrp3*, *Il1b* and *Il6* in WT and *Irf1*$^{-/-}$ BMDMs infected with IAV for 9 h. (I) Immunoblot analysis of NLRP3, ASC and GAPDH in WT and *Irf1*$^{-/-}$ BMDMs infected with IAV at various time points. (J) Levels of IL-6, TNF and KC in cell culture supernatants from WT and *Irf1*$^{-/-}$ BMDMs infected with IAV for 16h. (K) Transcript levels of *Nlrp3* in WT and *Irf1*$^{-/-}$ lung fibroblasts infected with IAV for 9h. (L) Immunoblot analysis of NLRP3, ASC and β-actin in lung fibroblasts infected with IAV. (M) Immunoblot analysis of pro-IL-1β and IL-1 β (p17) in lung fibroblasts infected with IAV for 16 h. Data are representative (B-D, G-H, (K-M)) or pooled (E-F, I) from 3–4 independent experiments. ** denotes $P<0.01$, *** denotes $P<0.001$.

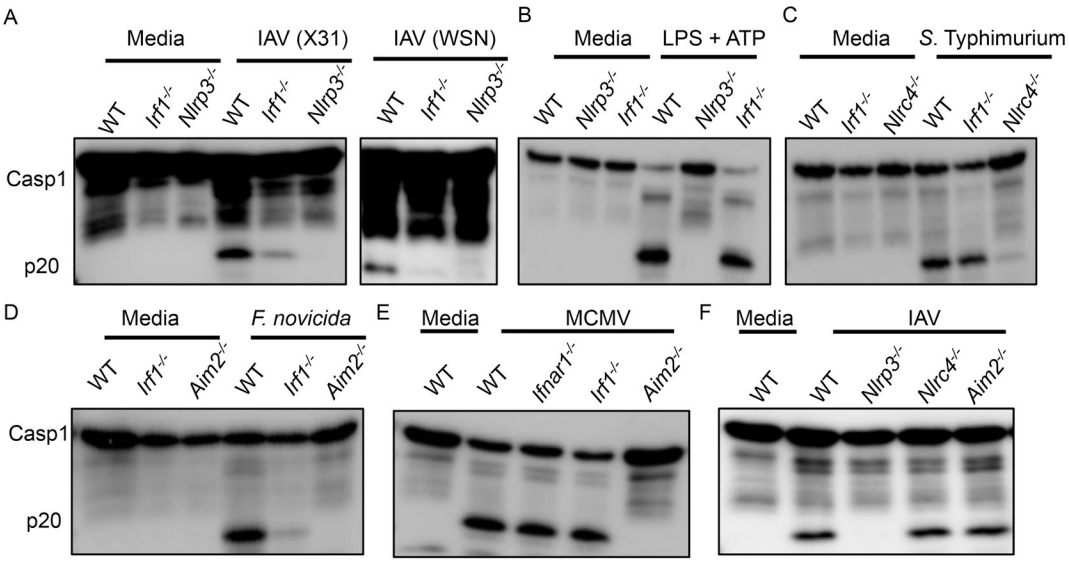

Figure 2: IRF1 regulation of canonical NLRP3 inflammasome is specific for IAV infection

Immunoblot analysis of caspase-1 cleavage in BMDMs infected with X31 or WSN strains of IAV (A); treated with LPS followed by ATP (B); infected with *S.* Typhimurium (C); *F. novicida* (D); MCMV (E); IAV PR8 strain (F).

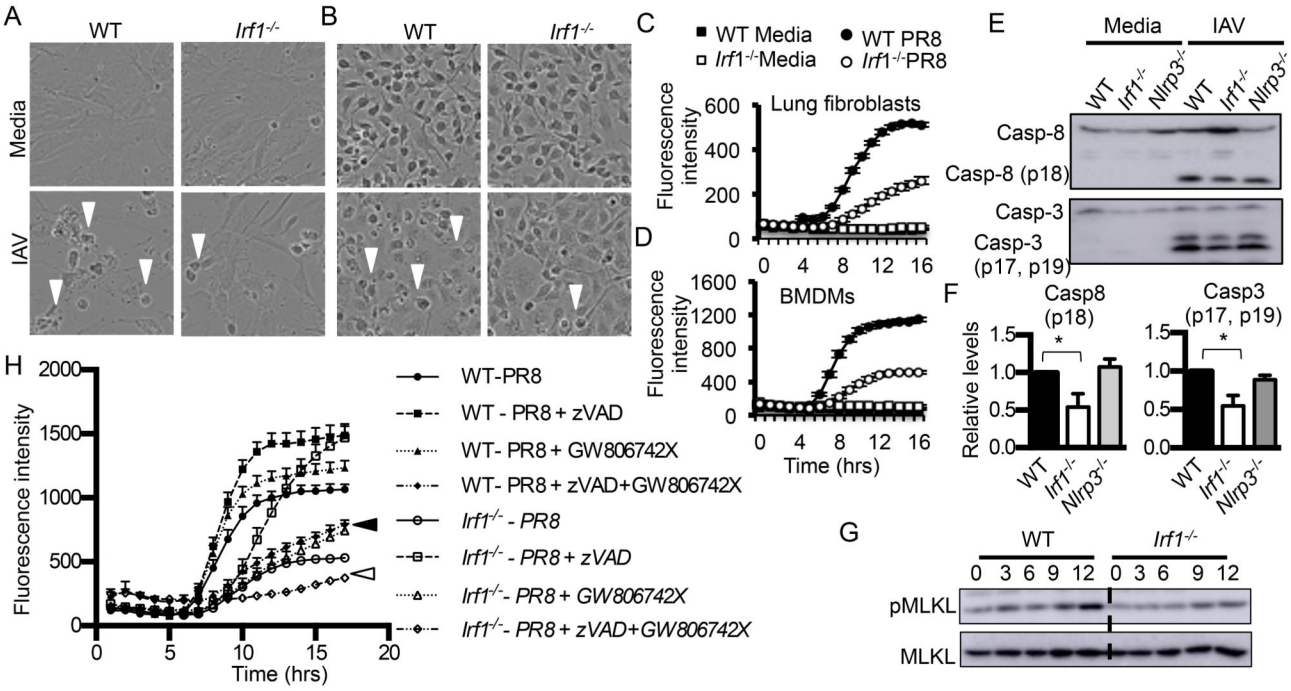

Figure 3. IRF1 regulates cell death in response to IAV infection.

(A-D) Real time analysis of cell death using incuCyte system and sytox green nucleic acid staining in lung fibroblasts and BMDMs infected with IAV. Images shown are acquired 15 h after infection. (E) Immunoblot analysis of the pro- and cleaved-forms of caspase-8 and caspase-3 in BMDMs 16 h after IAV infection. (F) Quantification of cleaved caspase-8 and caspase-3 in BMDMs infected with IAV. (G) Immunoblot analysis of phosphorylated and total MLKL in WT and *Irf1*$^{-/-}$ lung fibroblasts infected with IAV at various time points. (H) Real time analysis of cell death using incuCyte and sytox green staining of BMDMs treated with inhibitors and infected with IAV. Data are representative of 2–3 independent experiments. ** denotes P<0.01.

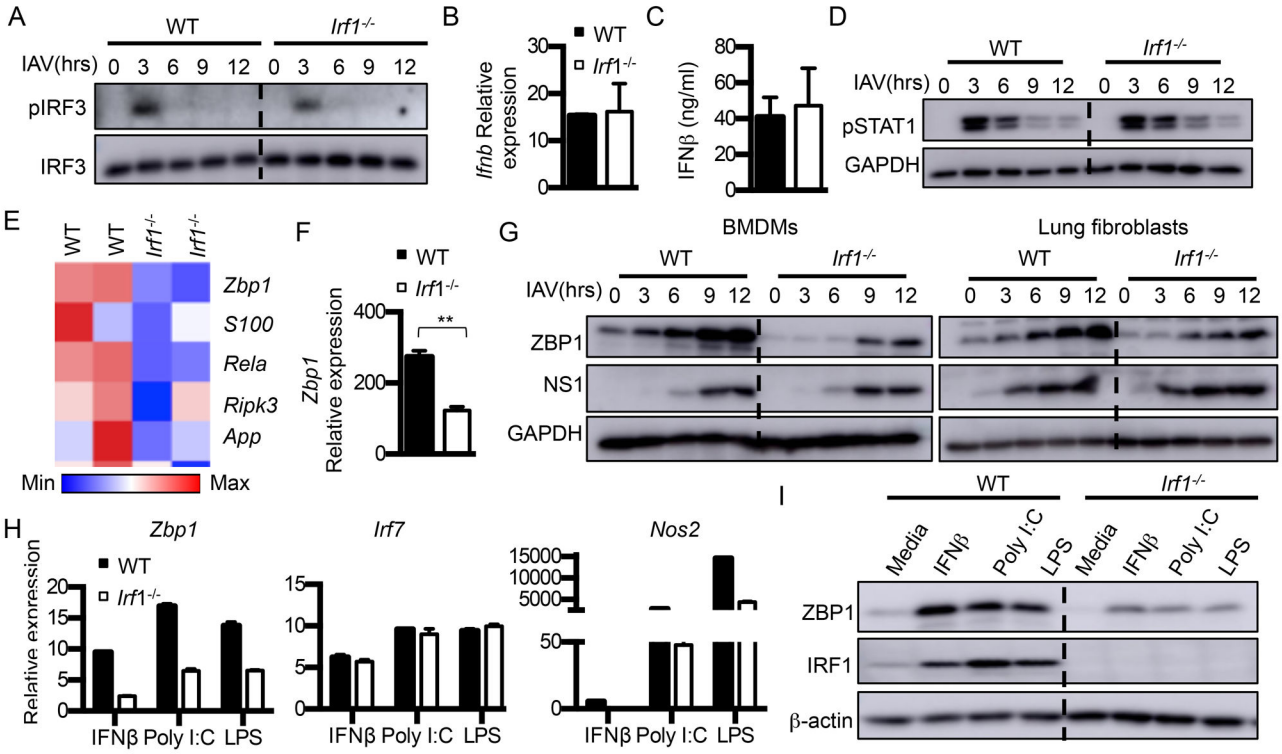

Figure 4. IRF1 induces ZBP1 expression independently of type I IFN production and signaling.

(A) Immunoblot analysis of phosphorylated and total IRF3 in WT and *Irf1*$^{-/-}$ BMDMs at various time points after IAV infection. (B) Transcript levels of *Ifnb* in WT and *Irf1*$^{-/-}$ BMDMs infected with IAV for 9 h. (C) Levels of IFNβ in cell culture supernatants from WT and *Irf1*$^{-/-}$ BMDMs infected with IAV for 16 h. (D) Immunoblot analysis of phosphorylated STAT1 and GAPDH in WT and *Irf1*$^{-/-}$ BMDMs at various time points after IAV infection. (E) Microarray analysis of nucleic acid sensors differentially regulated in *Irf1*$^{-/-}$ BMDMs infected with IAV compared with WT BMDMs. (F) Transcript levels of *Zbp1* in WT and *Irf1*$^{-/-}$ BMDMs infected with IAV for 9 h. (G) Immunoblot analysis of ZBP1, NS1 and GAPDH in WT and *Irf1*$^{-/-}$ BMDMs and lung fibroblasts at various time points after IAV infection. (H) Transcript levels of *Zbp1*, *Irf7* and *Nos2* in BMDMs treated with IFNβ, poly I:C or LPS for 8 h. (I) Immunoblot analysis of ZBP1, IRF1 and β-actin in BMDMs treated with IFNβ, poly I:C or LPS for 8 h. Data are representative (A-B, D, G, H-I) or pooled (C, F) from of 3–4 independent experiments. ** denotes $P<0.01$.

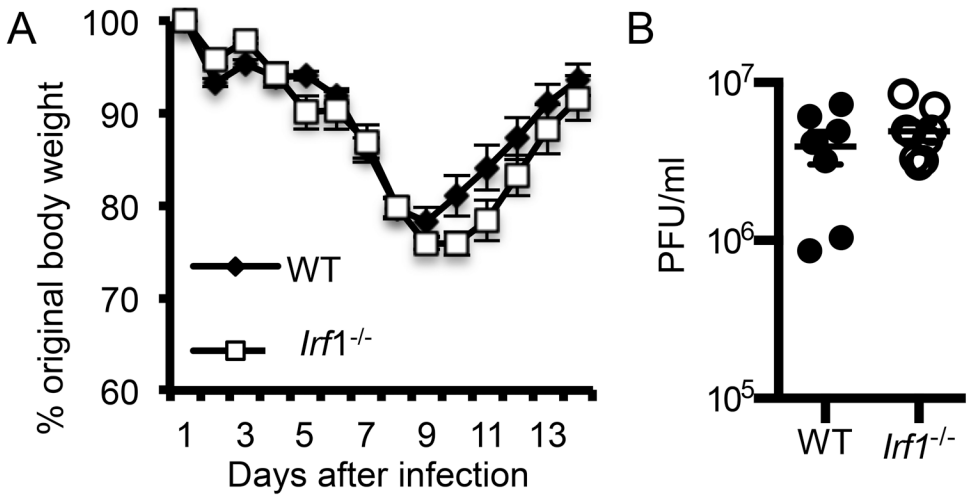

Figure 5: Deficiency of IRF1 does not significantly alter susceptibility to IAV infection.

(A) Body weight (%) of WT and *Irf1*$^{-/-}$ mice 0–14 days after infection compared with pre-infection body weight (set as 100%) (N= 10 (WT), 8 (*Irf1*$^{-/-}$) mice). (B) Lung viral titers in WT and *Irf1*$^{-/-}$ mice infected with IAV for 5 days.