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ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways

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Abstract

The interferon-inducible protein Z-DNA binding protein 1 (ZBP1, also known as DNA-dependent activator of IFN-regulatory factors (DAI) and DLM-1) was identified as a dsDNA sensor, which instigates innate immune responses. However, this classification has been disputed and whether ZBP1 functions as a pathogen sensor during an infection has remained unknown. Herein, we demonstrated ZBP1-mediated sensing of the influenza A virus (IAV) proteins NP and PB1, triggering cell death and inflammatory responses via the RIPK1–RIPK3–Caspase-8 axis. ZBP1 regulates NLRP3 inflammasome activation as well as induction of apoptosis, necroptosis and pyroptosis in IAV-infected cells. Importantly, ZBP1 deficiency protected mice from mortality during IAV infection owing to reduced inflammatory responses and epithelial damage. Overall, these findings indicate that ZBP1 is an innate immune sensor of IAV and highlight its importance in the pathogenesis of IAV infection.

Keywords

ZBP1; DAI; NLRP3; inflammasome; caspase-1; pyroptosis; necroptosis; apoptosis; influenza virus; IFNs; caspase-8; RIPK3

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The authors declare no competing financial interests.

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The microarray dataset was deposited under the accession code GSE77611.

Authors Contributions:

T.K. and T.D.K. conceptualized the study; T.K., R.K.S.M., K.S., S.M.M., R.K., D.P., G.N designed the methodology, performed the experiments and conducted the analysis; P.V. performed histopathological analysis; T.K. performed statistical analysis; T.K., S.M.M., and T.D.K. wrote the manuscript; T.D.K. acquired the funding and provided overall supervision.

INTRODUCTION

The influenza virus infects millions of people annually and causes significant morbidity and up to half a million deaths (1). Type A Influenza virus (IAV) is also associated with epidemics and pandemics owing to high mutation rates and genetic reassortment. Pattern-recognition receptors, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and nucleotide and oligomerization domain, leucine-rich repeats-containing proteins (NLRs) have a central role in the recognition of IAV infection (2). Virus sensing by these receptors initiates an innate immune response aimed at controlling virus replication and eliminating the infectious virus.

Innate sensing of IAV triggers multiple intracellular signaling cascades that coordinately regulate induction of type I interferons (IFNs) and proinflammatory cytokines (2). In addition, virus sensing also induces cell death in order to destroy the replicative niche necessary for survival and propagation of these intracellular pathogens (3). While epithelial cell death facilitates control of virus replication by eliminating infected cells, uncontrolled cell death can exacerbate tissue injury and compromise lung function (4). Previous studies have demonstrated an inherent link between orchestration of various programmed cell death pathways and pathogenesis of IAV (5). Consistent with this, apoptosis in lung epithelial cells was shown to exacerbate pneumonia and mortality during IAV infection (6). Similarly, uncontrolled necroptosis in airway epithelial cells also increases morbidity and mortality during IAV infection (7). Unlike the detrimental effects of apoptosis and necroptosis, NLRP3 inflammasome activation leading to pyroptosis and release of proinflammatory cytokines IL-1 β and IL-18 is protective during acute IAV infection (8, 9). Although all these cell death pathways are known to be activated during IAV infection, the innate immune sensors initiating cell death and the intracellular signaling cascades regulating this response are largely unknown and are defined in this study.

RESULTS

The IFN-inducible protein ZBP1 mediates cell death in response to IAV infection

To delineate the virus sensing pathways initiating cell death responses during IAV infection, cell death was assessed in primary murine BMDMs infected with Influenza virus A/Puerto Rico/8/34 (PR8; H1N1). BMDMs lacking the TLR adaptor proteins MyD88 or TRIF, or the RIG-I adaptor MAVS undergo cell death during infection with this mouse-adapted virus, probably due to functional redundancy of TLR and RIG-I pathways in IAV sensing in these cells (2) (fig. S1A and C). Furthermore, BMDMs and fibroblasts lacking the adaptor STING (central for DNA sensing), TNF receptor 1 (TNFR1), TNFR2 or the adaptor molecule TNFR1-associated death domain protein (TRADD) were also susceptible to IAV-induced cell death (Fig. 1A to D and fig. S1B and C). In contrast, cells lacking type I IFN receptor 1 (IFNAR1) or its downstream signaling proteins STAT1 and IRF9 were fully resistant to IAV-induced cell death (Fig. 1A to D and fig. S1D). Increased levels of M1 and NS1 proteins in IAV-infected *Ifnar1*^{-/-} BMDMs confirmed proper virus entry and replication in these cells (fig. S1E). These results demonstrated that cell death during IAV infection is initiated through a pathway mediated by type I IFN signaling.

Enrichment of the microarray gene expression dataset from IAV-infected WT and *Ifnar1^{-/-}* BMDMs for nucleic acid sensing pathways followed by qRT-PCR revealed a significant reduction in the expression of a number of genes encoding proinflammatory cytokines and nucleic acid sensors in *Ifnar1^{-/-}* BMDMs compared to WT BMDMs (Fig. 1E and F and fig. S2A). Interestingly, one of the most downregulated nucleic acid sensors in *Ifnar1^{-/-}* BMDMs was the gene encoding Z-DNA binding protein 1 (ZBP1; also called DLM-1 and DAI) (Fig. 1E and F). Expression of ZBP1 was robustly upregulated in WT BMDMs infected with IAV, through a mechanism that required IFNAR1, STAT1 and IRF9 (Fig. 1G and H and fig. S2B). ZBP1 was initially identified as a cytosolic sensor of double-stranded (ds)DNA which drives type I IFN responses (10). However, this classification has been disputed after generation of *Zbp1^{-/-}* mice (11–13). ZBP1 was of potential interest because of its unknown role in innate immune sensing and its possible role in the regulation of cell death (10–14).

The IFNAR-dependent cell death observed in IAV-infected cells prompted us to hypothesize that ZBP1 is an innate sensor of IAV driving cell death responses. Remarkably, *Zbp1^{-/-}* BMDMs and fibroblasts were completely resistant to IAV-induced cell death (Fig. 1I to L). Real time analysis of ZBP1-dependent cell death in IAV-infected fibroblasts using IncuCyte and SYTOX green nucleic acid staining demonstrated that cell death was initiated at 8 to 10 h post infection (pi) which corresponds to one replication cycle of IAV (Fig. 1M). Comparable levels of the viral protein NS1 and IFN β in *Zbp1^{-/-}* BMDMs infected with IAV confirmed proper virus entry, replication and IFN β secretion in these cells (fig. S2C and D). Together, these data demonstrate a critical role for ZBP1 in the regulation of cell death during IAV infection.

ZBP1 regulates NLRP3 inflammasome activation and proinflammatory cytokine production during IAV infection via the RIPK1–RIPK3–Caspase-8 axis

Distinct forms of programmed cell death, including pyroptosis, necroptosis and apoptosis, are initiated by context-specific stimuli encountered by the cell (15). Previous studies have demonstrated NLRP3 inflammasome-mediated activation of the pyroptosis-inducing cysteine protease, caspase-1, during IAV infection (16–19). While robust activation of caspase-1 was observed in WT BMDMs infected with IAV, this response was abrogated in *Zbp1^{-/-}*, *Ifnar1^{-/-}* and *Nlrp3^{-/-}* BMDMs (Fig. 2A). Furthermore, the levels of the inflammasome-dependent cytokines, IL-1 β and IL-18, were significantly reduced in *Zbp1^{-/-}* and *Ifnar1^{-/-}* cells compared to WT BMDMs (Fig. 2B to C), confirming a requirement for ZBP1 and type I IFN signaling in the activation of the NLRP3 inflammasome during IAV infection. ZBP1 was dispensable for the activation of caspase-1 and the release of IL-1 β and IL-18 in response to the RNA virus vesicular stomatitis virus (VSV), the canonical NLRP3 activator LPS plus ATP, and to the non-canonical NLRP3 activators *Escherichia coli* and *Citrobacter rodentium* infection (Fig. 2D and fig. S3A to C). Notably, NLRP3 inflammasome activation during IAV infection did not require caspase-11 (Fig. 2E). These data identified a unique, type I IFN- and ZBP1-dependent pathway of NLRP3 inflammasome activation, which differ from the canonical and non-canonical NLRP3 inflammasome pathways.

ZBP1 is dispensable for activation of the NLRC4 and AIM2 inflammasomes since infection by *Salmonella enterica* serovar Typhimurium, *Francisella novicida* and murine cytomegalovirus (MCMV), as well as transfection of poly(dA:dT) induced normal caspase-1 activation and secretion of IL-1 β or IL-18 in *Zbp1*^{-/-} BMDMs (fig. S3D to G). Although ZBP1 is a key regulator of IAV-induced NLRP3 inflammasome activation, ZBP1-dependent cell death during IAV infection occurred normally in *Nlrp3*^{-/-}, *Casp1*^{-/-} and *Gsdmd*^{-/-} BMDMs (fig. S4A to D). These data suggest activation of other complimentary cell death pathways in IAV-infected cells.

ZBP1 contains two receptor-interacting protein homotypic interaction motif (RHIM) domains that can interact with other RHIM-containing proteins including the receptor interacting protein kinase-3 (RIPK3) (20, 21). Indeed, immunoprecipitation experiments revealed interaction of ZBP1 with RIPK3 in IAV-infected cells (Fig. 2F). Consistent with previous studies demonstrating RIPK3-mediated activation of the NLRP3 inflammasome (19, 22, 23), a substantial reduction in caspase-1 cleavage and the levels of IL-1 β and IL-18 were observed in *Ripk3*^{-/-} BMDMs infected with IAV (Fig. 2G and H). The residual activation of caspase-1 observed in *Ripk3*^{-/-} BMDMs was abolished in *Ripk3*^{-/-} *Casp8*^{-/-} BMDMs (Fig. 2G). BMDMs lacking RIPK1 kinase activity (*Ripk1*^{KD/KD}) showed normal activation of caspase-1 and release of IL-1 β and IL-18 (Fig. 2G and H).

The ZBP1-RIPK1 complex transduces NF κ B activation signals (20, 21). Consistent with this, secretion of proinflammatory cytokines, IL-6 and TNF was abrogated in *Ripk3*^{-/-} *Casp8*^{-/-} *Ripk1*^{-/-} as well as *Zbp1*^{-/-} BMDMs (Fig. 2I and J). A modest reduction in IL-6 and TNF production was observed in *Ripk3*^{-/-} and *Ripk3*^{-/-} *Casp8*^{-/-} BMDMs (Fig. 2I). Regulation of IL-6 and TNF production is dependent on RIPK1 scaffolding function, but occurs independently of its kinase activity since the levels of these cytokines were comparable in WT and *Ripk1*^{KD/KD} cells (Fig. 2I). Collectively, these data identified ZBP1 as an upstream regulator mediating NLRP3 inflammasome activation via the RIPK3–Caspase-8 axis and proinflammatory responses via RIPK1.

ZBP1 mediates RIPK3-dependent induction of apoptotic and necroptotic cell death pathways during IAV infection

Previous studies identified RIPK3 as a critical regulator determining cell death via necroptotic or apoptotic pathways (24, 25). BMDMs and fibroblasts lacking RIPK3 or both RIPK3 and caspase-8 or RIPK3 and FADD were fully resistant to IAV-induced cell death, confirming a critical role of RIPK3 in mediating ZBP1-dependent cell death (Fig. 3A to C and fig. S5A). Necroptosis is executed by the RIPK3 substrate mixed lineage kinase like (MLKL) (26). However, cell death comparable to WT levels was observed in *Mkl1*^{-/-} BMDMs and fibroblasts infected with IAV, demonstrating that MLKL is dispensable for ZBP1- and RIPK3-dependent cell death (Fig. 3A to C and fig. S5A). ZBP1 also promotes apoptosis during IAV infection since activation of caspase-8, caspase-3 and caspase-7 was abrogated in *Zbp1*^{-/-} BMDMs compared to WT BMDMs infected with IAV (Fig. 3D). Robust activation of both caspase-1 and caspase-8 was observed in *Mkl1*^{-/-} BMDMs infected with IAV and inhibition of these caspase activities using zIETD-FMK prevented IAV-induced cell death in *Mkl1*^{-/-} BMDMs (Fig. 3E and fig. S5B to D). Activation of

multiple, complementary cell death pathways in IAV-infected cells was further confirmed by simultaneous inhibition of apoptosis, pyroptosis and necroptosis in WT BMDMs with the caspase-8 inhibitor zIETD-FMK plus MLKL inhibitor GW806742X (27). Treatment with these inhibitors prevented WT BMDMs from undergoing cell death during IAV infection (Fig. 3E). Inhibition of either apoptosis or necroptosis in WT and *Casp1*^{-/-} BMDMs was unable to block IAV-induced cell death (Fig. 3E and fig. S5D). These results collectively demonstrate parallel contribution of pyroptosis, necroptosis and apoptosis in the execution of IAV-induced cell death governed by ZBP1.

ZBP1 regulates cell death in response to both mouse-adapted and seasonal strains of IAV, but not in response to RNA viruses belonging to *Paramyxoviridae* and *Rhabdoviridae* family

In addition to mouse adapted PR8 virus, ZBP1 broadly regulates cell death in response to IAV of different species- and strain-specificity since *Zbp1*^{-/-} fibroblasts were protected from cell death during infection with mouse adapted Influenza A/X31 (H3N2) as well as non-mouse adapted seasonal strains Influenza A/Brisbane/59/2007 (H1N1) and A/Switzerland/9715293/2013 (H3N2) (Fig. 4A to F). Unlike different strains of IAV, cell death and inflammatory cytokine production occurred independently of ZBP1 in BMDMs infected with other negative-sense RNA viruses, VSV, Sendai virus (SeV) and respiratory syncytial virus (RSV) (Fig. 4G and fig. S6). Moreover, transfection of synthetic, IAV or mammalian-derived ssRNA species as well as the dsRNA ligand poly(I:C) and dsDNA ligand poly(dA:dT) induced comparable levels of cell death in WT and *Zbp1*^{-/-} BMDMs (fig. S7A to C). Together, these data highlighted an IAV-specific role for ZBP1 in initiating cell death responses.

ZBP1 is a sensor of IAV nucleoprotein (NP) and polymerase subunit PB1

To investigate potential interaction of ZBP1 with IAV proteins, endogenous ZBP1 was immunoprecipitated from IAV-infected cells and probed for interacting IAV proteins. Immunoblotting for IAV M1, NS1 and HA proteins did not show any detectable levels of protein interaction (Fig. 5A). Interestingly, the IAV nucleoprotein (NP) and RNA polymerase subunit PB1 were co-precipitated from WT, but not *Zbp1*^{-/-} fibroblasts infected with IAV (Fig. 5B). Conversely, immunoprecipitation of NP or PB1 from lysates of IAV-infected fibroblasts also showed co-precipitation of these proteins with ZBP1, confirming interaction of endogenous ZBP1 with NP and PB1 (Fig. 5C). ZBP1, PB1 and NP were observed in both nuclear and cytoplasmic fractions from infected cells at 8hr post infection indicating that ZBP1 interaction with viral proteins can occur in either or both of these compartments (Fig. 5D). Overexpression of PB1 or NP in ZBP1-expressing 293T cells also demonstrated interaction of these viral proteins with ZBP1 (Fig. 5E).

In order to map the domains with which ZBP1 interacts with IAV proteins, various deletion mutants of ZBP1 with HA tag were generated and overexpressed in 293T cells (Fig. 5F). Efficient co-immunoprecipitation of PB1 or NP proteins with WT ZBP1 and ZBP1 lacking the RHIM, but not with ZBP1 lacking the C-terminal domain, demonstrated the importance of the C-terminal domain of ZBP1 for its interaction with IAV proteins (Fig. 5G and H). In addition to the C-terminal domain, the Z α domain of ZBP1 was also required for interaction

with PB1 (Fig. 5G). Collectively, we identified ZBP1 as a sensor of influenza virus proteins that can trigger both cell death and inflammatory responses during infection.

ZBP1 promotes inflammatory responses and epithelial damage during IAV infection in vivo

The physiological relevance of ZBP1 in regulating pathogenesis of IAV infection was assessed in WT and *Zbp1*^{-/-} mice infected with approximately one LD₅₀ of PR8 virus. Remarkably, *Zbp1*^{-/-} mice were protected from mortality during IAV infection while 30% of WT mice succumbed to infection (Fig. 6A). *Zbp1*^{-/-} mice also showed a significant reduction in weight loss and morbidity during the early phase of infection compared to WT mice (Fig. 6B). However, viral titers were significantly higher in *Zbp1*^{-/-} mice at day 7 pi consistent with the notion that cell death destroys the niche necessary for virus replication (Fig. 6C). The defective viral clearance observed in *Zbp1*^{-/-} mice led to increased weight loss at later stages of infection and these mice showed delayed recovery from infection compared to WT animals (Fig. 6B). In agreement with our *in vitro* findings, inflammatory responses as well as epithelial damage was markedly reduced in *Zbp1*^{-/-} mice infected with IAV (Fig. 6D and E). While severe and extensive inflammatory responses characterized by diffuse intra-alveolar infiltrates of neutrophils and macrophages and perivascular accumulations of lymphocytes and granulocytes were observed in the lungs of WT mice, these responses were reduced in *Zbp1*^{-/-} mice on day 7 pi (Fig. 6D and E). Collectively, these data demonstrated a critical role of ZBP1 in regulating pathogenesis and immunopathology during acute IAV infection by controlling both cell death and inflammatory responses.

DISCUSSION

Cell fate decisions are well integrated into antiviral immune responses during an infection and are critical for elimination of replicating viruses. Although cell death constitutes a major antiviral host defense mechanism during IAV infection, exaggerated responses often leads to severe clinical disease and mortality as exemplified by the substantial damage to the lungs and destruction of respiratory epithelium observed in autopsy samples from the 1918 influenza pandemic (28). Despite the importance of epithelial cell death in determining disease outcome during acute IAV infection, the host factors regulating cell death responses are less characterized.

The critical role of type I IFN signaling in potentiating IAV-induced apoptosis in MEFs via activation of the FADD–caspase-8 signaling axis has been reported (29). Induction of IFNAR1–RIPK3-dependent necroptosis was also demonstrated in both MEFs and macrophages when FADD–caspase-8 signaling was inactivated (30, 31). Although IFN Stimulated Gene Factor 3 (ISGF3) complex was recognized as a critical promoter of necroptosis, the IFN-stimulated gene mediating necroptosis and inflammatory responses in macrophages remained elusive (31). In addition to these *in vitro* studies, pathogenic potential of type I IFN signaling in mediating uncontrolled inflammatory response and epithelial cell death was also demonstrated during IAV infection *in vivo* (32). Our study has now identified ZBP1 as the IFN-inducible protein regulating both inflammatory responses and cell death during IAV infection *in vitro* and *in vivo*.

The RHIM domains of ZBP1 associate with RHIM domains of both RIPK1 and RIPK3 to mediate NF κ B activation and cell death responses (14, 20, 21). Consistent with these findings, we observed a critical role for ZBP1–RIPK1 axis in mediating proinflammatory responses during IAV infection. Moreover, ZBP1 regulation of cell death in IAV-infected cells is mediated via RIPK3. Of note, while our manuscript was in revision Nogusa et al. reported RIPK3-dependent activation of parallel necroptotic and apoptotic pathways in IAV-infected cells (33). Although this study demonstrate the importance of RIPK3 in driving cell death during IAV-infection, the upstream receptors and signaling pathways regulating RIPK3 were not identified.

Despite being identified as a DNA sensor, it is still unclear whether ZBP1 functions as a pathogen sensor during an infection. Our study has now identified a role for ZBP1 in the recognition of an RNA virus, and linked it to both immune and cell death responses to clinically relevant IAV infection. Innate sensors of IAV have been well characterized, however all known IAV-receptors recognize viral RNA in infected cells (2). Notably, our study demonstrates ZBP1 as an innate sensor of IAV proteins regulating antiviral innate immune responses.

After our discovery of IAV-induced NLRP3 inflammasome activation almost a decade ago (16), multiple studies have investigated the molecular and cellular mechanisms regulating inflammasome assembly in response to IAV infection (17–19, 34–36). The importance of IAV M2 ion channel protein, RIG-I, type I IFN signaling, RIPK3 and RNaseL in mediating inflammasome activation during IAV infection have been demonstrated (18, 19, 34, 36). Our data demonstrating an IAV-specific role for IFNAR–ZBP1 axis in the regulation of NLRP3 inflammasome activation will help to reconcile these findings. While both RIG-I and RNaseL mediate type I IFN production necessary for induction of ZBP1, RIPK3 associates with ZBP1 to transduce downstream signals. The interconnected and complimentary nature of IAV-induced cell death pathways demonstrated in our study also helps to explain PB1-F2-mediated NLRP3 inflammasome activation since PB1-F2 is regarded as the viral effector mediating cell death in IAV-infected cells (35, 37). Therefore, the identification of ZBP1 as an IAV-specific sensor helps to further resolve the molecular mechanisms regulating NLRP3 inflammasome assembly during IAV infection.

The seemingly paradoxical observations of reduced mortality in *Zbp1*^{-/-} mice in spite of increased viral titers are not unexpected, since previous studies in both humans and animal models reported exaggerated inflammatory response, substantial loss of pulmonary epithelia and acute lung injury as the major factors contributing to morbidity and mortality during pathogenic IAV infection (38, 39). An extensive systems analysis study also demonstrated elevated activation of inflammatory signaling networks as a signature that distinguishes lethal from sublethal IAV infections (40). Moreover, transcriptome analysis of lung samples from infected mice identified immune and cell death responses as the major factor distinguishing mild from highly pathogenic infections (41). Our data are in agreement with these studies demonstrating exaggerated immune response rather than direct viral damage as the key trigger potentiating mortality during IAV infection.

Although BMDMs and fibroblasts do not represent the major cell types infected during IAV infection, the *in vitro* data obtained from these cells are validated by *in vivo* infections. However, further studies are warranted to identify the host and viral effectors as well as the precise molecular mechanism by which ZBP1-dependent cell death is executed in IAV-infected cells. The relevance of human ZBP1 during IAV infection was also not assessed in our study. *In vitro* studies investigating the role of human ZBP1 during IAV infection are less feasible since most of the lung epithelial cell lines that support productive virus replication do not undergo IAV-induced cell death (37). Notably, proteomics analysis of human innate immunity interactome for type I IFN identified ZBP1 as one of the interacting proteins with antiviral activity (42). Moreover, human ZBP1 restricts replication of both human cytomegalovirus and herpes simplex-1 demonstrating functional importance of ZBP1 in antiviral responses (43, 44). Since cell death, inflammatory responses and virus replication are intricately associated with each other, various host factors modulating susceptibility to infection as well as dosage of infection will be critical in determining the role of ZBP1 in disease progression. Identification of genetic lesions in *ZBP1* locus in patient populations will be of great value and further studies in this direction are necessary to determine the importance of ZBP1 in pathogenesis of IAV in humans. Nevertheless, the insights gained from this study improve our understanding about the mechanisms regulating pathogenesis and prognosis of acute influenza virus infection and may lead to improved disease intervention strategies for the prevention and treatment of IAV infection.

Materials and Methods

Study Design

Animal studies were conducted under protocols approved by the St. Jude Children's Research Hospital on the Use and Care of Animals. Age- and sex-matched, 6- to 8-weeks old WT and *Zbp1*^{-/-} mice bred at the Animal Research Center at SJCRH were used for *in vivo* experiments. Infected mice were monitored and body weights were recorded daily, and mice exhibiting severe signs of disease or more than 30% weight loss relative to pre-infection body weight were euthanized. Animals were euthanized at the indicated time points for lung harvest. Lung sections were processed at Veterinary Pathology Core at SJCRH and histopathological analysis was conducted by a pathologist blinded to experimental groups. No other blinding or randomization was performed.

Mice

Zbp1^{-/-}, *Ifnar1*^{-/-}, *Stat1*^{-/-}, *Irf9*^{-/-}, *Aim2*^{-/-}, *Nlrp3*^{-/-}, *Nlrc4*^{-/-}, *Casp1*^{Null}, *Casp11*^{-/-}, *Gsdmd*^{-/-}, *Mavs*^{-/-}, *Myd88*^{-/-}, *Trif*^{-/-}, *Tradd*^{-/-}, *Sting*^{Gt/Gt}, *Ripk3*^{-/-}, *Ripk3*^{-/-} *Casp8*^{-/-}, *Ripk3*^{-/-} *Casp8*^{-/-} *Ripk1*^{-/-}, *Ripk1*^{KD/KD} and *Mkl1*^{-/-} mice have been described previously (13, 45–49). *Tnfr*^{-/-} mice (Stock #003243) were purchased from The Jackson Laboratory.

Cell culture and stimulation

Cells were cultured overnight in antibiotic-free media before infection. The influenza A/Puerto Rico/8/34 virus (PR8) generated by an eight-plasmid reverse genetics system was propagated in allantoic cavity of 9- to 11-day old embryonated SPF chicken eggs and viral titers were enumerated by standard plaque assays. BMDMs (MOI 25) and fibroblasts (MOI

10) were infected with PR8 virus for 2 h. DMEM media containing 20% FBS was added after 2 h and samples were collected at indicated time points. For pharmacological inhibition, BMDMs were treated with 50 μ M Z-IETD-FMK (Millipore), 50 μ M Z-VAD-FMK (Calbiochem) and/or 1 μ M GW806742X (SYNkinase) at the same time as IAV infection. RSV Line 19F was grown in HEp-2 cell line.

Cytokine measurement

IFN β and IL-18 were measured using ELISA kits (BioLegend (IFN β); eBioscience (IL-18)). All other cytokines were measured by multiplex ELISAs (Millipore).

Generation of ZBP1 overexpressing cells

pVSVg, pEQ-Pam3(-E) and pMIGII plasmids encoding the *Zbp1* gene are transfected into 293T cells to generate retroviral stocks. Retroviral supernatants were harvested after 48 h of transfection and filtered through 0.4 μ m sterile filters. 293T cells were infected with the corresponding retroviral stocks in the presence of polybrene to generate cells stably expressing the respective ZBP1 proteins.

Co-immunoprecipitation

For immunoprecipitation, cell lysates were incubated with 3 μ g of indicated primary antibodies on a rocking platform for 12–16h at 4°C. Protein A/G PLUS-Agarose (Santa Cruz) was added to the samples and incubated for another 2 h on the rocking platform. Agarose was centrifuged and washed 3 times with the lysis buffer. Immunoprecipitates were eluted in sample buffer after three washes in lysis buffer and then subjected to immunoblotting analysis.

Animal infection

WT and *Zbp1*^{-/-} mice were anesthetized with 250 mg/kg Avertin followed by intranasal infection with 1000pfu (approximately one LD₅₀) of PR8 virus in 30 μ l PBS. Infected mice were observed over a period of 18 days for survival study. Lungs were harvested on day 7 pi and left lobe of the lungs was used for histopathological analysis. Formalin-preserved lungs were processed and embedded in paraffin according to standard procedures. Sections were stained with hematoxylin and eosin (H&E) or IAV nucleoprotein. Lung viral titers were enumerated by standard plaque assays after homogenizing lungs in 1 ml PBS using a bead mill homogenizer (Qiagen).

Statistical analysis

GraphPad Prism 6.0 software was used for data analysis. Statistical significance was determined by a *t* tests (two-tailed) or Mann Whitney test for two groups or one-way ANOVA for three or more groups; *P* < 0.05 was considered statistically significant where **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Mean \pm SEM of the data is presented.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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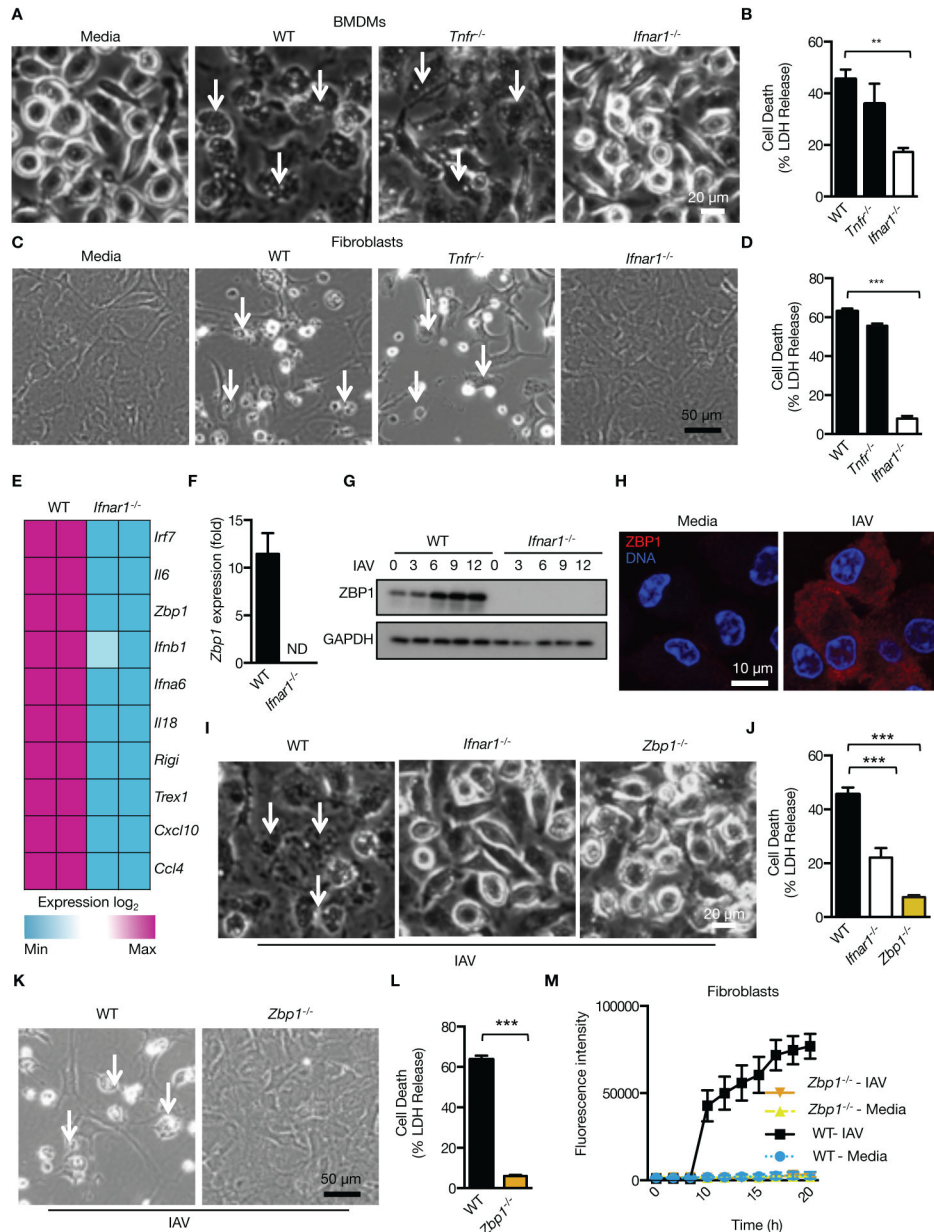


Figure 1. The IFN-inducible protein ZBP1 mediates cell death in response to IAV infection (A and B) Microscopic analysis and quantification of cell death by LDH release in BMDMs infected with IAV 16 h pi ($p = 0.0131$; $n = 4$; two-tailed t test). (C and D) Microscopic analysis and quantification of cell death by LDH release in ear fibroblasts infected with IAV 16 h pi ($p = 2.92096 \times 10^{-6}$; $n = 4$; two-tailed t test). (E) Microarray gene expression dataset enriched for nucleic acid sensing pathways with higher or lower expression in WT and *Ifnar1*^{-/-} BMDMs 9 h pi with IAV (one experiment with two biological replicates per genotype). (F) Real-time quantitative RT-PCR analysis of *Zbp1* expression in WT and *Ifnar1*^{-/-} BMDMs 9 h pi with IAV (one experiment with two biological replicates per genotype). (G) Immunoblot analysis of ZBP1 and GAPDH (loading control) in unprimed BMDMs (0–12) h pi with IAV ($n = 3$). (H) Immunostaining for ZBP1 in WT BMDMs

infected with IAV for 16 h (n=2). **(I and J)** Microscopic analysis and quantification of cell death by LDH release in BMDMs infected with IAV 16 h pi (p= 0.0001; n=5; one-way ANOVA). **(K and L)** Microscopic analysis and quantification of cell death by LDH release in fibroblasts infected with IAV 16 h pi (p= 0.000460657; n=3; two-tailed *t* test). **(M)** Real time analysis of the kinetics of cell death in primary ear fibroblasts infected with IAV (MOI 10) (one experiment with three biological replicates per genotype).

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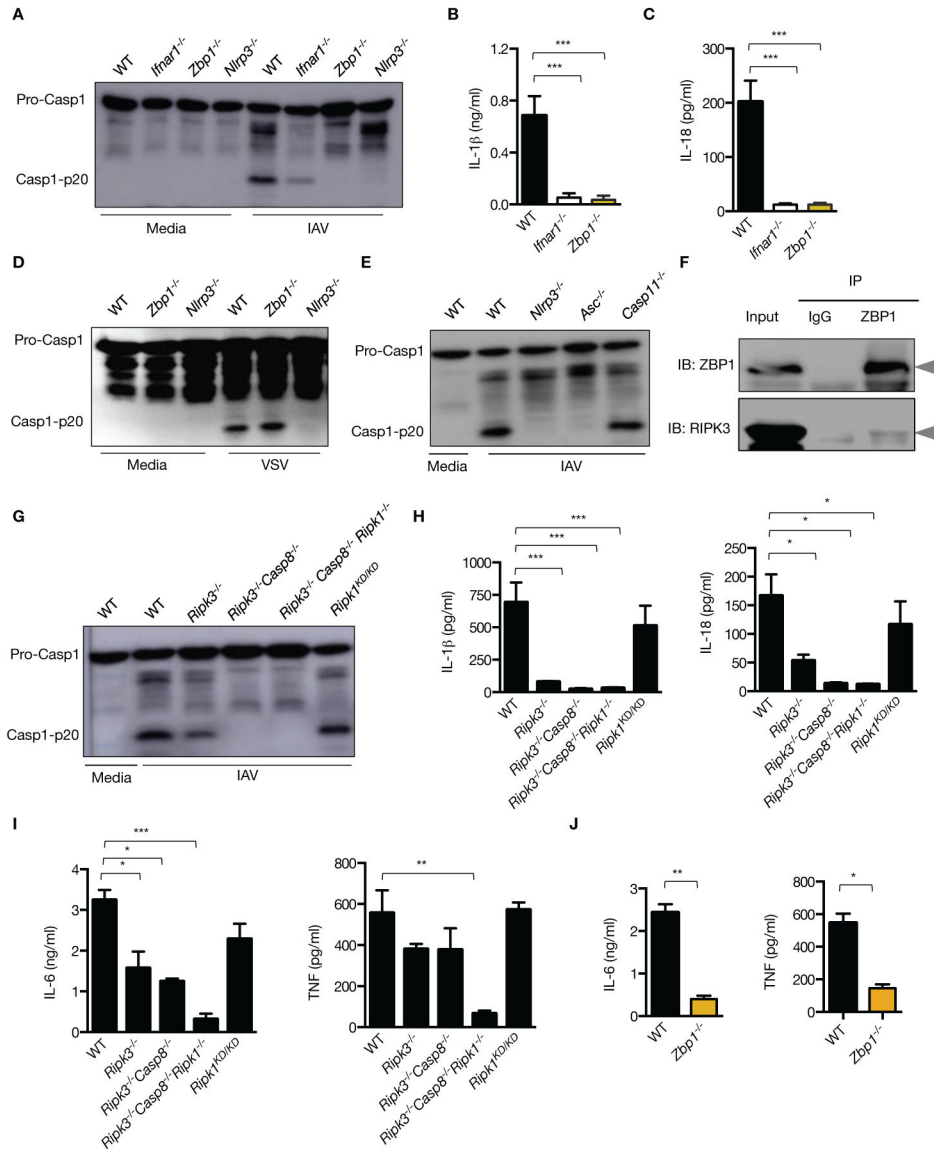


Figure 2. ZBP1 regulates NLRP3 inflammasome activation and proinflammatory cytokine production during IAV infection

(A) Immunoblot analysis of pro-caspase-1 and caspase-1 subunit p20 in BMDMs 16 h pi with IAV (n=4)..(B and C) Levels of IL-1 β and IL-18 in cell culture supernatants 16 h pi with IAV; (p= 0.0007 (IL-1 β); p = 0.0015 (IL-18); n=3; one-way ANOVA)..(D and E) Immunoblot analysis of pro-caspase-1 and caspase-1 subunit p20 in BMDMs 16 h pi with VSV (Indiana strain) or IAV (n=3)..(F) Immunoprecipitation of ZBP1 from lysates of WT BMDMs infected with IAV for 16 h, and immunoblotted for ZBP1 and RIPK3 (n=3)..(G) Immunoblot analysis of pro-caspase-1 and caspase-1 subunit p20 in BMDMs 16 h pi with IAV (n=3)..(H) Levels of IL-1 β and IL-18 in cell culture supernatants 16 h pi with IAV; (p=0.0003 (IL-1 β); p=0.0033 (IL-18); n=3; one-way ANOVA)..(I and J) Levels of TNF and IL-6 in cell culture supernatants 16 h pi with IAV;.(I) (p=0.0003 (IL-6; one way ANOVA);

p=0.04585299 (TNF; two-tailed *t* test) n=3); **(J)** (p=0.00427068 (IL-6); p=0.01547619 (TNF); n=3; two-tailed *t* test).

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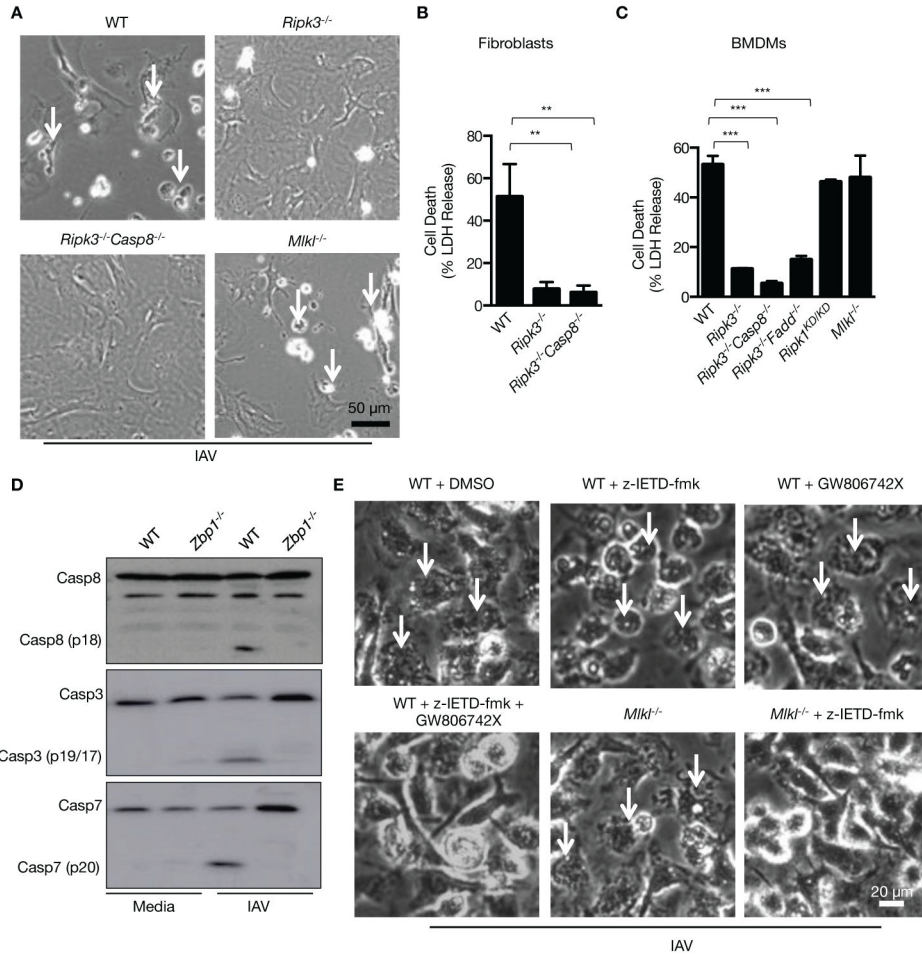


Figure 3. ZBP1 mediates RIPK3-dependent induction of apoptotic and necroptotic cell death pathways during IAV infection

(A and B) Microscopic analysis and quantification of cell death by LDH release in fibroblasts infected with IAV 16 h pi; (p=0.0016; n=3; one-way ANOVA)..(C) Quantification of cell death by LDH release in BMDMs infected with IAV 16 h pi (p=0.0001; n=4; one-way ANOVA)..(D) Immunoblot analysis of the pro- and cleaved-forms of caspase-8, caspase-3 and caspase-7 in BMDMs 16 h pi with IAV (n=3)..(E) Microscopic analysis of BMDMs infected with IAV for 16 h in the absence or presence of inhibitors (n=3).

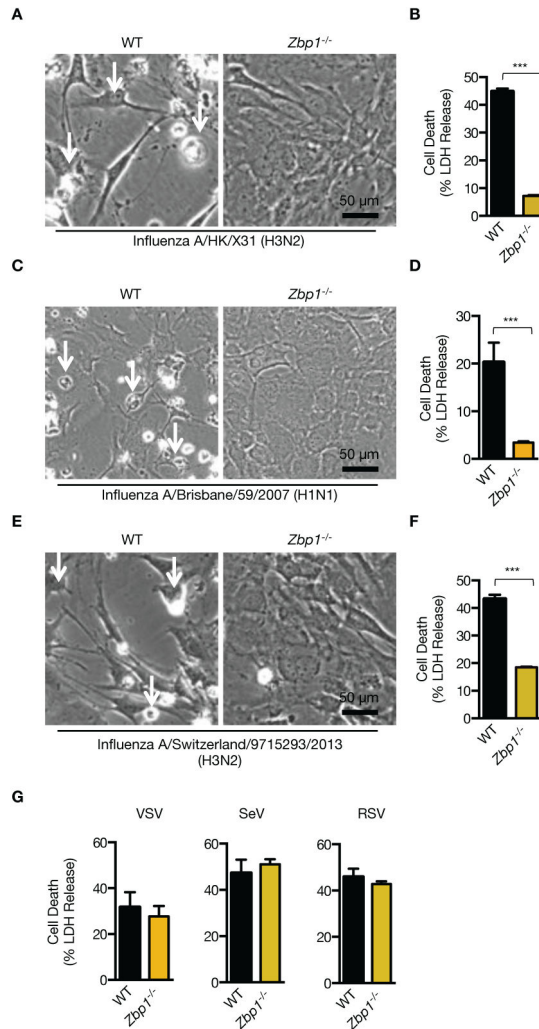


Figure 4. ZBP1 regulates cell death in response to both mouse-adapted and human strains of IAV, but not in response to RNA viruses belonging to Paramyxoviridae and Rhabdoviridae family

(A to F) Microscopic analysis and quantification of cell death by LDH release in fibroblasts infected with mouse adapted Influenza A/X31 (H3N2), Influenza A/Brisbane/59/2007 (H1N1) and A/Switzerland/9715293/2013 (H3N2) 16h pi ($p=4.5184 \times 10^{-5}$ (X31); $p=0.02523$ (A/Brisbane); $p=0.00671454$ (A/Switzerland); $n=4$; two-tailed *t* test). (G) Quantification of cell death by LDH release in BMDMs infected with VSV (MOI 10), SeV (MOI 8) and RSV (MOI 10) 16 h pi ($n=3$).

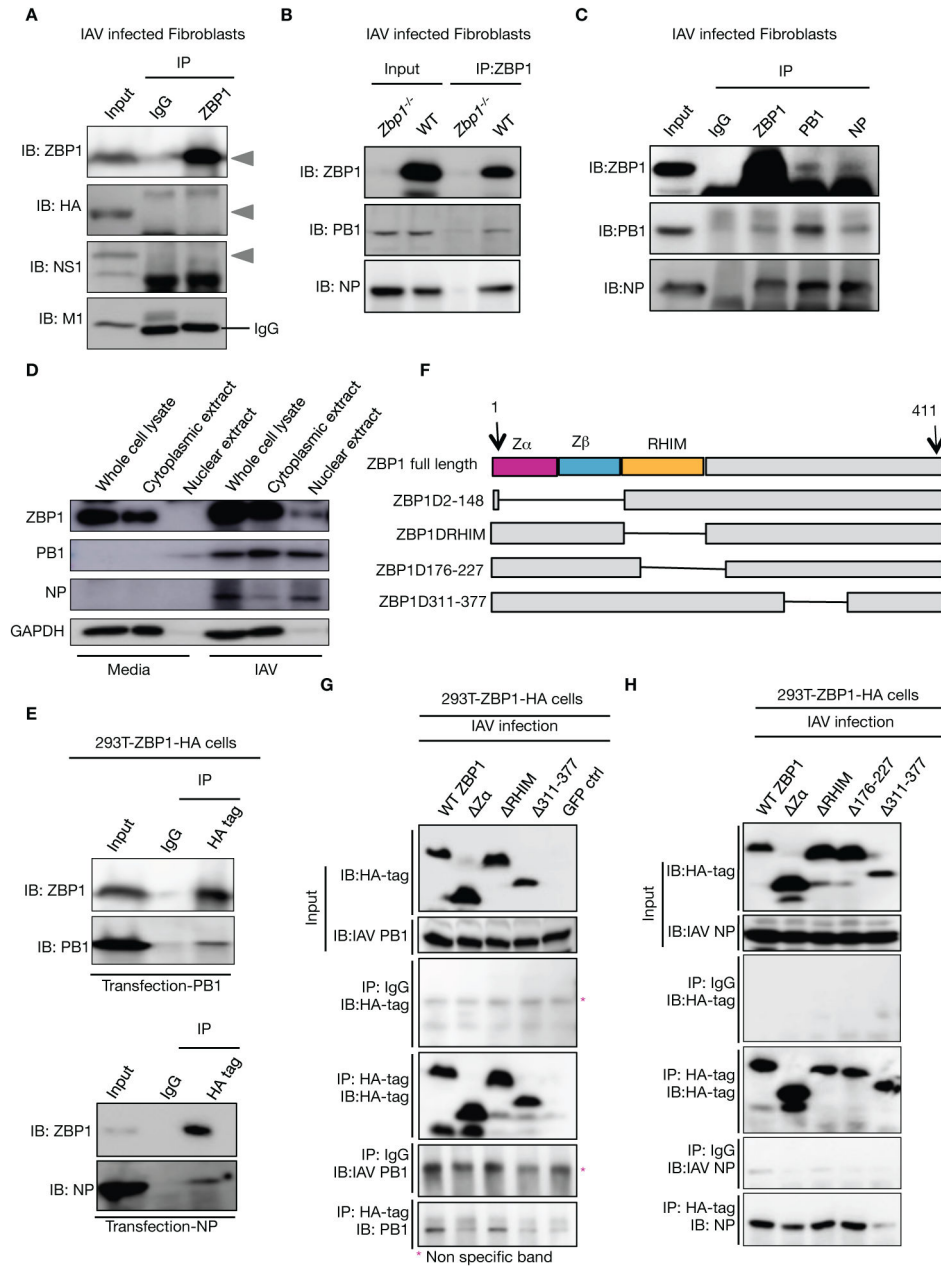


Figure 5. ZBP1 functions as a sensor of IAV infection by interacting with IAV PB1 and NP proteins

(A) Immunoprecipitation of endogenous ZBP1 from lysates of WT BMDMs infected with IAV for 16 h, and immunoblotted for ZBP1, M1, NS1 and HA; (n= 4)..(B) Immunoprecipitation of endogenous ZBP1 from lysates of WT or *Zbp1*^{-/-} fibroblasts infected with IAV for 16 h, and immunoblotted for ZBP1, PB1 and NP; (n= 3)..(C) Immunoprecipitation of ZBP1, PB1 and NP from lysates of WT fibroblasts infected with IAV for 16 h, and immunoblotted for ZBP1, PB1 and NP; (n= 3)..(D) Immunoblot analysis of ZBP1, PB1, NP and GAPDH from whole cell lysates, nuclear and cytoplasmic fractions of WT fibroblasts infected with IAV for 8 h; (n=3)..(E) Immunoprecipitation of HA-tagged

ZBP1 from lysates of 239T cells expressing HA-tagged ZBP1 transfected with plasmids expressing PB1 or NP, and immunoblotted for ZBP1, PB1 and NP; (n= 2)..(F) Domain architecture of murine ZBP1 and schematics of the constructs used in this study..(G and H) Immunoprecipitation of HA-tagged ZBP1 from lysates of 239T cells expressing HA-tagged ZBP1 constructs infected with IAV for 16 h, and immunoblotted for the IAV proteins PB1, HA and NP, and HA tag; (n= 2).

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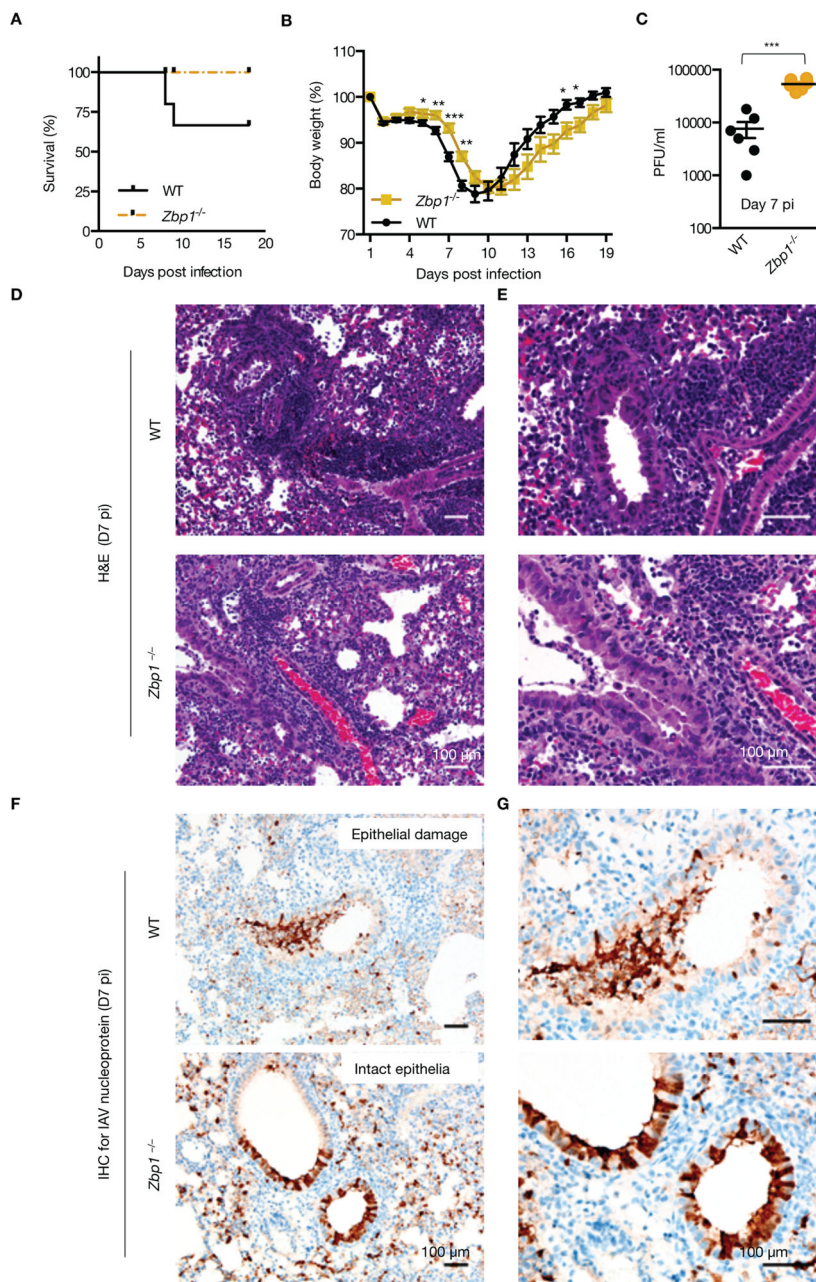


Figure 6. ZBP1 promotes inflammatory responses and epithelial damage during IAV infection *in vivo*.

(A) Survival analysis of female WT and *Zbp1*^{-/-} mice infected with 1000pfu (approximately one LD₅₀) of IAV; (p=0.0819; n = 15 (WT) and 9 (*Zbp1*^{-/-}) mice; Mantel-Cox test)..(B) Body weight (%) of WT and *Zbp1*^{-/-} mice 0–18 d after infection compared to pre-infection body weight (set as 100%); (p=0.02743813 (D5); 0.00103121 (D6); 0.00081354 (D7); 0.05424982 (D14); 0.0231683 (D15); 0.02152296 (D16); WT (n = 21) and *Zbp1*^{-/-} (n = 15); two-tailed *t* test)..(C) Lung viral titers in WT and *Zbp1*^{-/-} mice infected with IAV for 7 days; (p=0.0022; n=6; Mann Whitney test). H &E staining (D and E; E is an inset of D)

and immunohistochemistry analysis for IAV NP protein (**F and G; G is an inset of F**) of lungs from WT and *Zbp1*^{-/-} mice infected with IAV for 7 days; (n=6).

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