Supplementary Materials and methods:

Cell culture

Primary bone marrow–derived macrophages (BMDMs) were grown for 7 days in DMEM (Gibco) supplemented with 10% FBS (Atlanta Biologicals), 30% L929 conditioned media and 1% penicillin and streptomycin (Sigma). BMDMs were seeded in antibiotic–free media at a concentration of 1×10^6 cells onto 12-well plates and incubated overnight.

Fibroblasts were generated from pinnae of adult mice. Pinnae were minced and digested with 100mg/ml collagenase type IV (Worthington Biochemical Corporation) for 3 h, followed by filtration through 70-µm strainers to obtain fibroblasts. Cells were cultured in 50% FBS (Millipore) in DMEM (Gibco) supplemented with HEPES, 1% penicillin and streptomycin, L-glutamine, sodium pyruvate, non-essential amino acids and β-mercaptoethanol for the first 3-4 days. Cells were then sub-cultured in 10% FBS in DMEM supplemented with 1% penicillin and streptomycin. All primary fibroblasts were used before to reaching sixth passage. Fibroblasts were seeded onto six-well plates at a density of 2×10^5 cells per well and incubated overnight.

***In vitro* infection and stimulation**

Francisella novicida strain U112 was grown in BBL™ Trypticase™ Soy Broth (TSB) (211768, BD) supplemented with 0.2% L-cysteine (Fisher) overnight under aerobic conditions at 37°C. Bacteria were subcultured (1:10) in fresh TSB supplemented with 0.2% L-cysteine for 4 h and resuspended in PBS. *S.* Typhimurium SL1344, *Citrobacter rodentium* (ATCC 51459) and *E. coli* (ATCC 11775) were inoculated into LB broth and incubated overnight under aerobic conditions at 37°C. *S.* Typhimurium SL1344 was subcultured (1:10) into fresh LB broth for 3 h at 37°C to generate log-phase grown bacteria. The following conditions were used for bacterial infection: *F. novicida* (MOI 100

and 20 h for caspase-1 activation), *S.* Typhimurium (MOI 1, 4 h), *C. rodentium* (MOI 20 for 20 h) and *E. coli* (MOI 20 for 20 h). Gentamicin (50 µg/ml, Gibco) was added after 2 h (*S.* Typhimurium), 4 h (*C. rodentium* and *E. coli*), and 8 h (*F. novicida*) post-infection to kill extracellular bacteria. Poly(dA:dT) and poly(I:C) (Invivogen) were used at the concentrations indicated in each figure. IAV genomes or cellular mRNA controls were purified from viral stocks or mouse BMDMs using Trizol (Invitrogen). Nucleic acids were transfected using Xfect (Clontech) or Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. To activate the canonical NLRP3 inflammasome, BMDMs were primed using 500 ng/ml ultrapure LPS from *Salmonella minnesota* R595 (InvivoGen) for 4 h and stimulated with 5 mM ATP (Sigma) for 30 min. Levels of lactate dehydrogenase released by cells were determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions (Promega). IncuCyte and SYTOX green nucleic acid staining were performed according to the manufacturer's instructions (Essen Bioscience). Cell culture supernatants were collected for ELISA and LDH assay.

Microarray analysis

RNA was extracted from BMDMs infected with PR8 virus 9h pi (MOI 25) using TRIzol (Life Technologies). Transcript profiling was performed using two biological replicate samples. 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).

Real time qPCR analysis

RNA was extracted from BMDMs infected with PR8 virus 9h pi (MOI 25) using TRIzol. Isolated RNA was reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Life Technologies). Real-time qPCR was performed on an ABI 7500 real-time PCR instrument with 2× SYBR Green (Applied Biosystems). Sequences for qRT-PCR primers are listed in Table S1.

Immunoblotting analysis

For caspase-1 immunoblotting, BMDMs and supernatant were lysed in cell lysis buffer. For immunoblotting other proteins, cells were lysed in RIPA buffer and sample loading buffer containing SDS and 100 mM DTT after washing with cold PBS.

Nuclear and cytoplasmic fractions were separated using NE-PER nuclear and cytoplasmic extraction reagent following manufacturer's protocol (Thermo- Fisher Scientific).

Proteins were separated on 8-12% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked in 5% skim milk followed by incubation with primary antibodies and secondary HRP antibodies. Images were acquired using GE Amersham Imager 600 or Carestream Image station 4000MMPRO.

The antibodies used for immunoblotting are Caspase-1 (AG-20B-0042, 1:3,000 dilution, Adipogen), caspase-8 (#1492, 1:1,000 dilution, CST), cleaved caspase-8 (#8592, 1:1,000 dilution, CST), caspase-3 (#9662, 1:1,000 dilution, CST), cleaved caspase-3 (#9661, 1:1,000 dilution, CST), caspase-7 (#9492, 1:1,000 dilution, CST), cleaved caspase-7 (#9491, 1:1,000 dilution, CST), ZBP-1 (AG-20B-0010-C100, 1:3,000 dilution,

Adipogen), ZBP1 (sc-67258, 1:1000, Santa Cruz Biotechnology), Influenza A virus M1 (#ab20910, 1:2,000 dilution, Abcam), Influenza A virus NS1 (NS1-23-1: sc-130568, 1:2,000 dilution, Santa Cruz Biotechnology), PB1 (sc-17601, 1:500, Santa Cruz Biotechnology), HA (sc-52025, 1:500, Santa Cruz Biotechnology), NP (sc-80481, 1:500, Santa Cruz Biotechnology), HA-tag (PAI-985, 1:1000, Thermo Fisher Scientific), HA-tag (05-904, 1:1000, Millipore), GAPDH (#5174, 1:10,000 dilution, CST) and HRP antibodies (1:1,000 dilution; Jackson Immuno Research Laboratories).

Immunofluorescence staining

BMDMs were infected for 16 h with PR8 virus (MOI 25). Cells were washed and fixed in 4% paraformaldehyde for 15 min at room temperature, followed by blocking in 10% normal goat serum (Dako) supplemented with 0.1% saponin (Sigma) for 1 h. Cells were incubated with a mouse anti-ZBP1 antibody (AG-20B-0010-C100, 1:500 dilution, AdipoGen) for 50 min at 37°C followed by Alexa Fluor 568 anti-mouse IgG (A-11004, 1:250 dilution, ThermoFisher Scientific). Cells were counterstained in DAPI mounting medium (Vecta Labs). Images were acquired using a Nikon C2 confocal microscope.

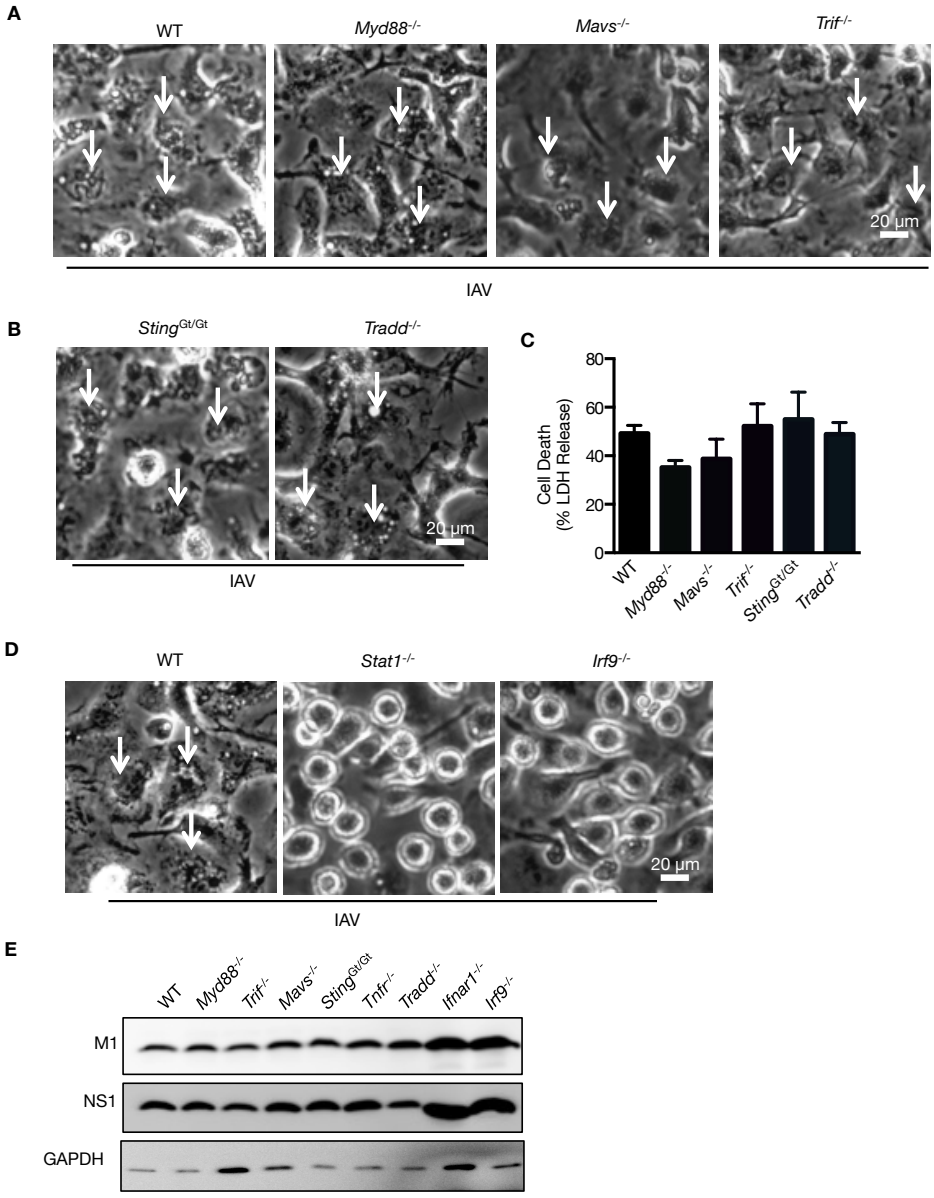


Figure S1: Cell death induced by IAV infection in BMDMs occurs independently of MyD88, MAVS, TRIF, STING and TRADD, but is dependent on the transcription factors STAT1 and IRF9. (A to D) Microscopic analysis and quantification of cell death by LDH release in BMDMs infected with IAV (MOI 25) for 16 h; (n=3). **(E)** Immunoblot analysis of M1 and NS1 proteins in BMDMs infected with IAV; GAPDH was used as loading control; (n=2).

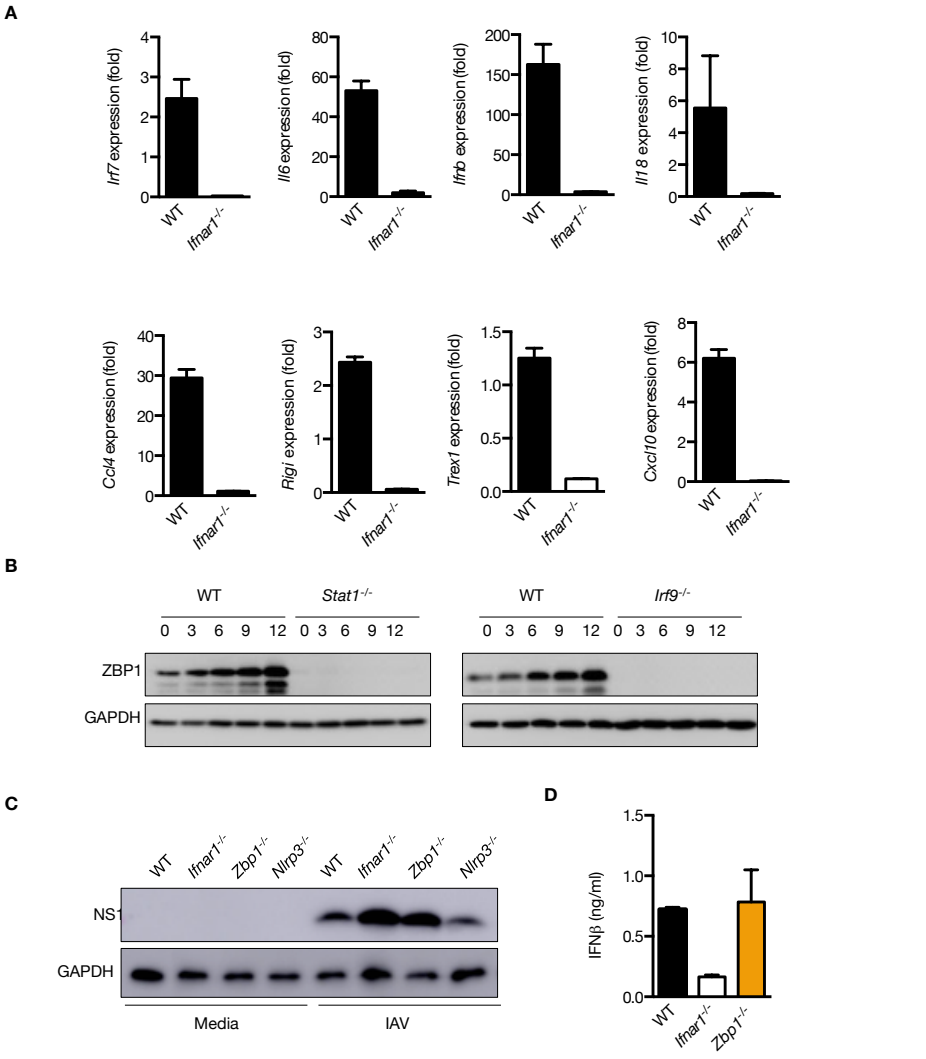


Figure S2: ZBP1 induced via IFN signaling regulates cell death in IAV-infected cells independently of virus replication and IFNβ production. (A) Real-time quantitative RT-PCR analysis of genes differentially regulated in WT and *Ifnar1*$^{-/-}$ BMDMs 9 h after infection with IAV; (n= one experiment with two biological replicates). **(B)** Immunoblot analysis of ZBP1 and GAPDH (loading control) in BMDMs (0-12) h after infection with IAV; (n=2). **(C)** Immunoblot analysis of the IAV NS1 protein and GAPDH (loading control) from BMDMs infected with IAV for 16 h; (n=2). **(D)** Quantification of IFNβ levels in cell culture supernatants from BMDMs infected with IAV for 16 h; (n=2).

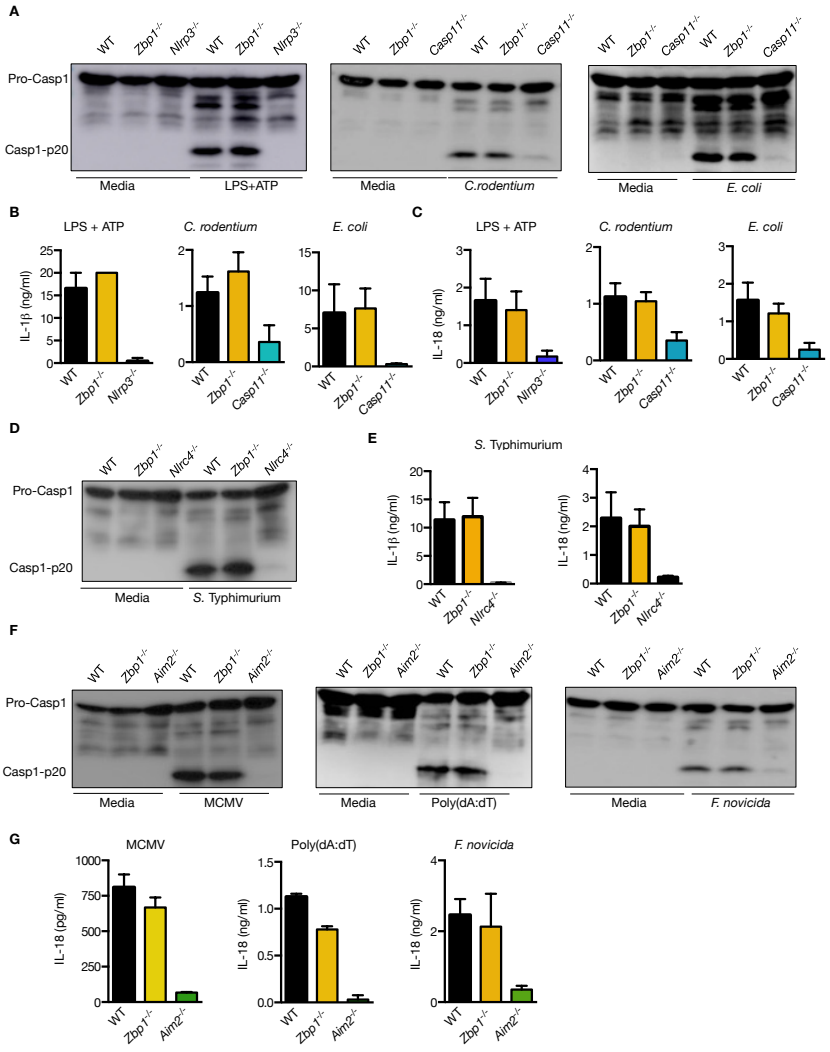


Figure S3: ZBP1 is dispensable for activation of canonical and non-canonical NLRP3, NLRC4 and AIM2 inflammasomes. (A) Immunoblot analysis of pro-caspase-1 and caspase-1 subunit p20 and **(B, C)** levels of IL-1β or IL-18 in LPS-primed BMDMs stimulated with ATP or unprimed BMDMs infected with *C. rodentium* (MOI 20) or *E. coli* (MOI 20) 20 h after infection; (n=3). **(D)** Immunoblot analysis of pro-caspase-1 and caspase-1 subunit p20 and **(E)** levels of IL-1β or IL-18 in BMDMs infected with *S.* Typhimurium grown to log phase (MOI 1) for 3 h; (n=3). **(F)** Immunoblot analysis of pro-caspase-1 and caspase-1 subunit p20 and **(G)** levels of IL-18 in BMDMs infected with

MCMV (MOI 100) for 12 h, transfected with 1 μg/ml poly(dA:dT) for 6 h or infected with *F. novicida* (MOI 100) for 20 h; (n=3).

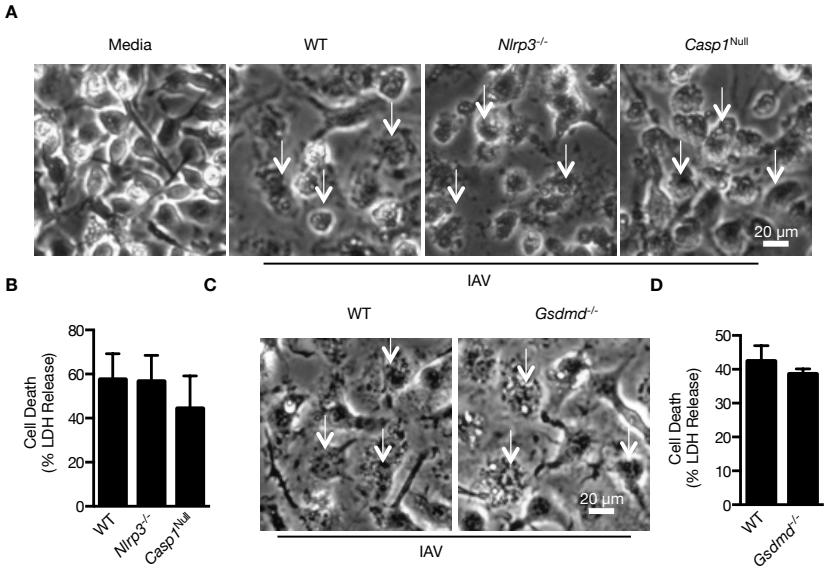


Figure S4: IAV-induced cell death is not prevented by the absence of the NLRP3, caspase-1 or gasderminD. (A to D) Microscopic analysis and quantification of cell death by LDH release in BMDMs infected with IAV for 16 h; (n=3).

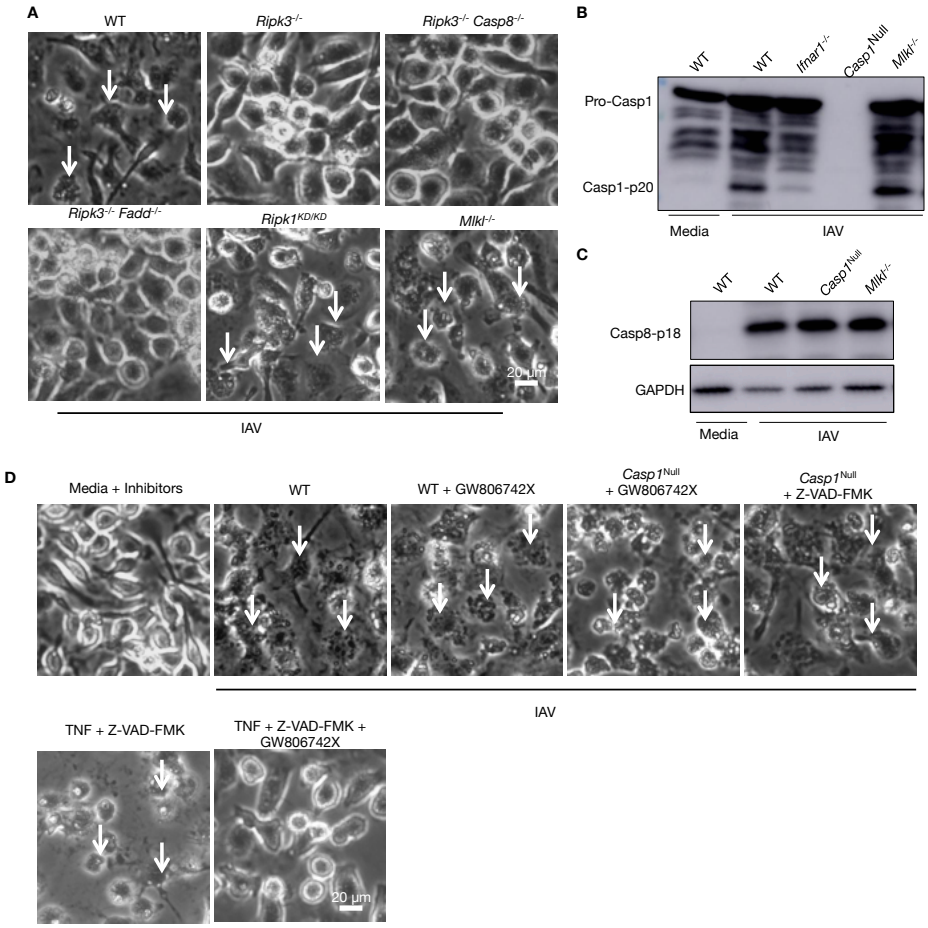


Figure S5: ZBP1 drives activation of complementary cell death pathways during IAV infection. (A) Microscopic analysis of BMDMs infected with IAV (MOI 25) for 16 h; (n=3). **(B to C)** Immunoblot analysis of caspase-1 (A) and caspase-8 and GAPDH (B) in BMDMs 16 h after infection with IAV; (n=2). **(C)** Microscopic analysis of BMDMs infected with IAV (MOI 25) for 16 h in the absence or presence of inhibitors or stimulated with TNF plus Z-VAD-FMK for 16 h in the presence or absence of GW806742X; (n=3).

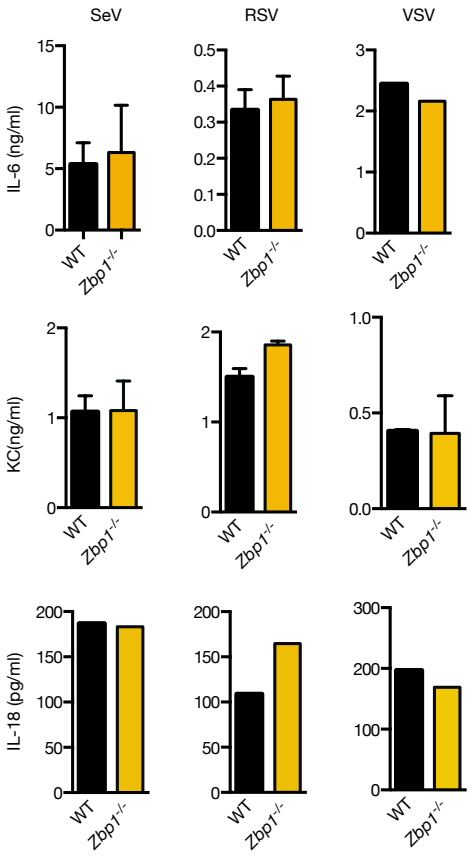


Figure S6: ZBP1 is dispensable for proinflammatory cytokine production in response to other RNA viruses. Levels of IL-6, KC and IL-18 in cell culture supernatants 16 h after infection with SeV (MOI 8), respiratory syncytial virus (RSV MOI 10) and Vesicular stomatitis virus (VSV MOI 10); (n=3).

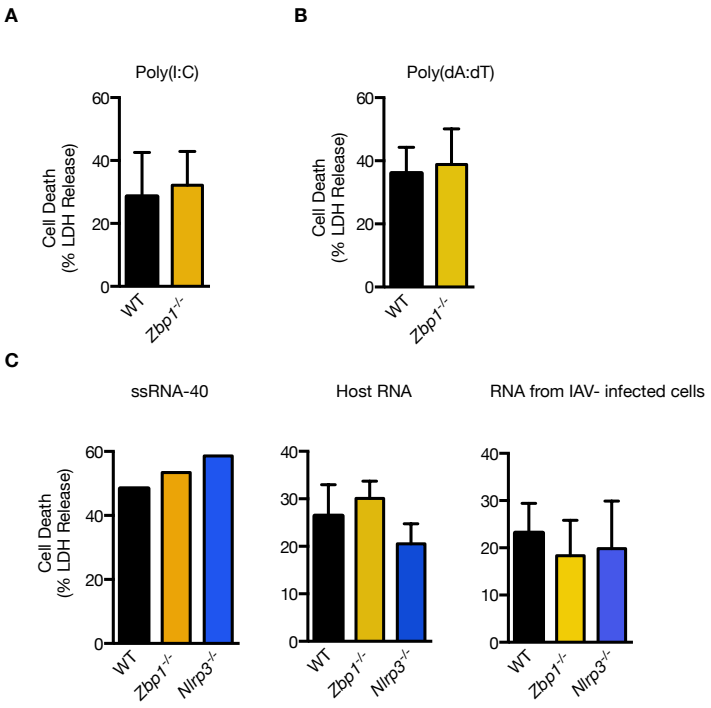


Figure S7: ZBP1 is dispensable for cell death in response to transfected RNA and ds(DNA) ligands. (**A to C**) Quantification of cell death by LDH release in BMDMs transfected with 1 μg/ml poly(I:C) for 6 h, or 1 μg/ml poly(dA:dT) for 6 h, or 5 μg/ml ssRNA-40, host RNA (mouse BMDM RNA) or RNA extracted from IAV-infected WT BMDMs for 24 h; (n=2).

Table S1: Real-time qPCR primer sequences

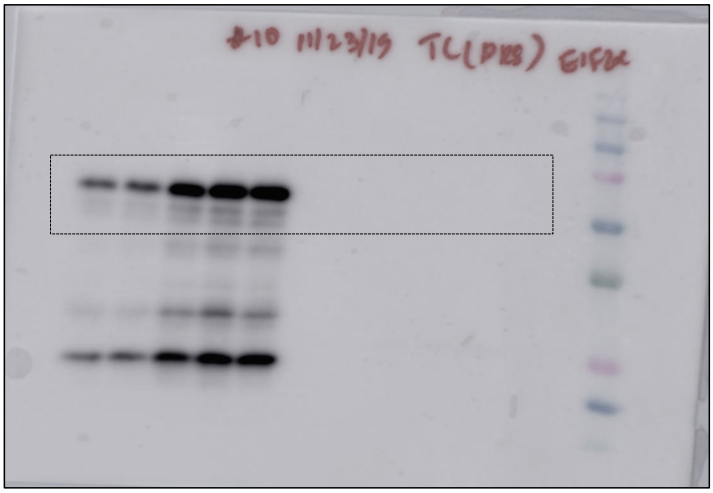
Target	Primer sequence
Il18	Forward: 5'-TGG ATC CAT TTC CTC AAA GG-3'
	Reverse: 5'-TGG ATC CAT TTC CTC AAA GG-3'
Trex1	Forward: 5'-CGT CAA CGC TTC GAT GAC A-3'
	Reverse: 5'-AGT CAT AGC GGT CAC CGT TGT-3'
Cxcl10	Forward: 5'-CCA AGT GCT GCC GTC ATT TTC-3'
	Reverse: 5'-GGC TCG CAG GGA TGA TTT CAA-3'
Ccl4	Forward: 5'-TTC CTG CTG TTT CTC TTA CAC CT-3'
	Reverse: 5'- CTG TCT GCC TCT TTT GGT CAG-3'
Il6	Forward: 5'-TCC AGT TGC CTT CTT GGG AC-3'
	Reverse: 5'-GTA CTC CAG AAG ACC AGA GG-3'
Irf7	Forward: 5'-GAG ACT GGC TAT TGG GGG AG-3'
	Reverse: 5'-GAC CGA AAT GCT TCC AGG G-3'
Ddx58	Forward: 5'-AAG AGC CAG AGT GTC AGA ATC T-3'
	Reverse: 5'-AGC TCC AGT TGG TAA TTT CTT GG-3'
Zbp1	Forward: 5'-GAC GAC AGC CAA AGA AGT GA-3'
	Reverse: 5'-GAG CTA TGT CTT GGC CTT CC-3'
Ifnb	Forward: 5'-GCC TTT GCC ATC CAA GAG ATG C-3'
	Reverse: 5'-ACA CTG TCT GCT GGT GGA GTT C-3'
β-actin	Forward: 5'-CAG CTT CTT TGC AGC TCC TT-3'
	Reverse: 5'-CAC GAT GGA GGG GAA TAC AG-3'
Influenza M1	Forward: 5'-TGA GTC TTC TAA CCG AGG TC-3'
	Reverse: 5'-GGT CTT GTC TTT AGC CAT TCC-3'
Influenza NP	Forward: 5'-CTC GTC GCT TAT GAC AAA GAA G-3'
	Reverse: 5'-AGA TCA TCA TGT GAG TCA GAC-3'

Unmodified Western blots

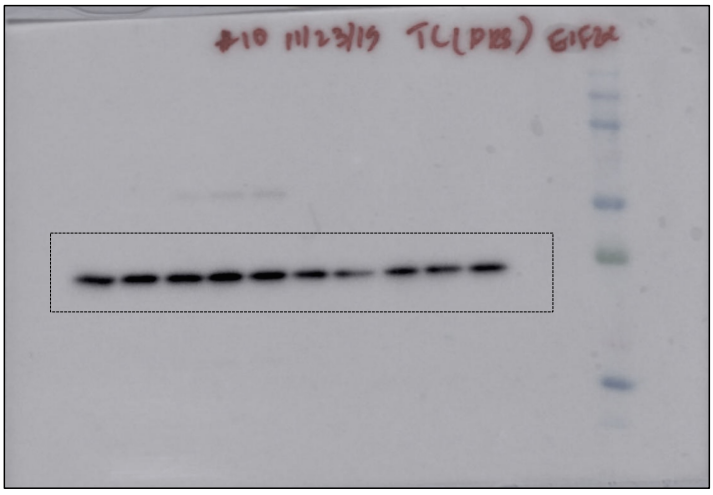
Western blots associated with Fig.1

Fig. 1G

ZBP1



GAPDH



Western blots associated with Fig.2

Fig. 2A Casp1

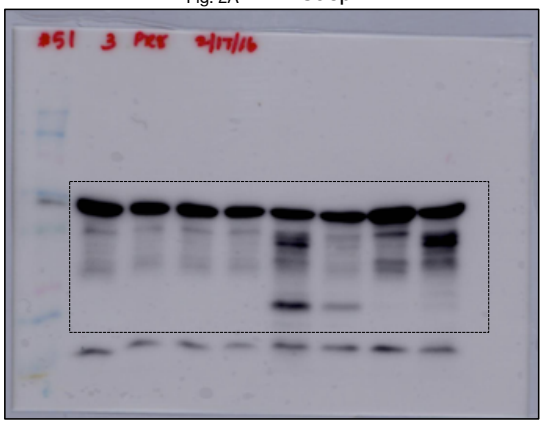


Fig. 2D Casp1

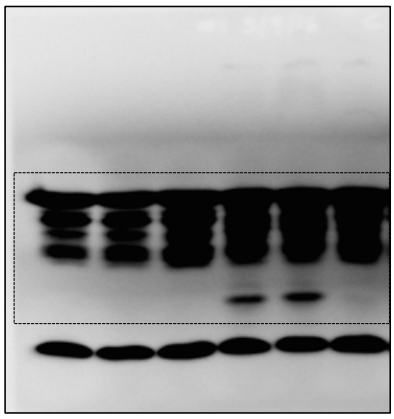


Fig. 2E Casp1

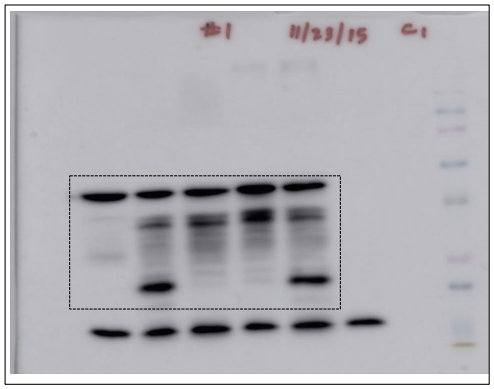


Fig. 2F

ZBP1

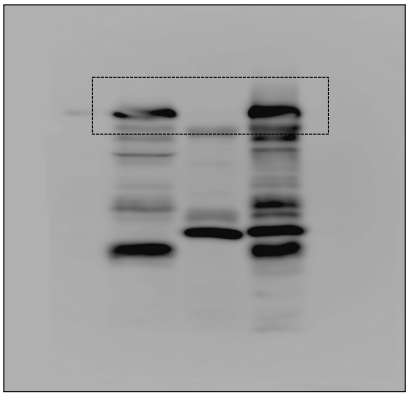
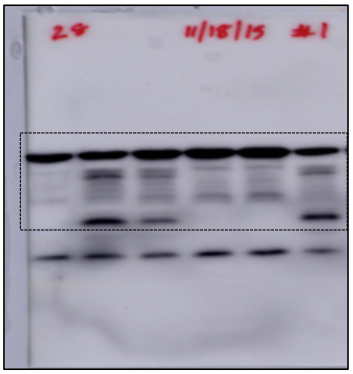
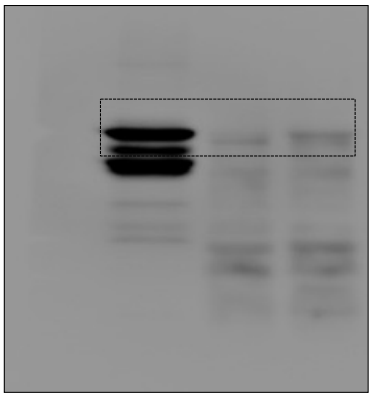


Fig. 2G Casp1

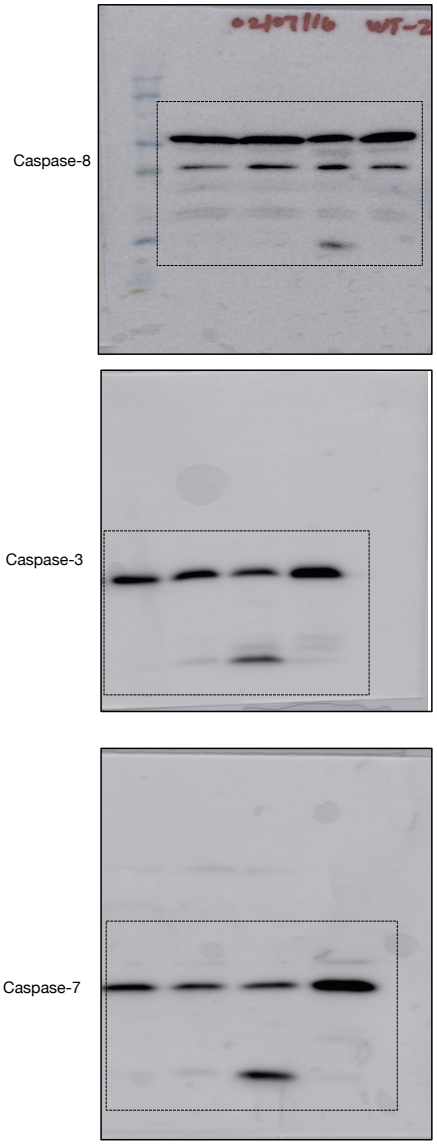


RIPK3



Western blots associated with Fig.3

Fig.3D



Western blots associated with Fig. 5

Fig.5A

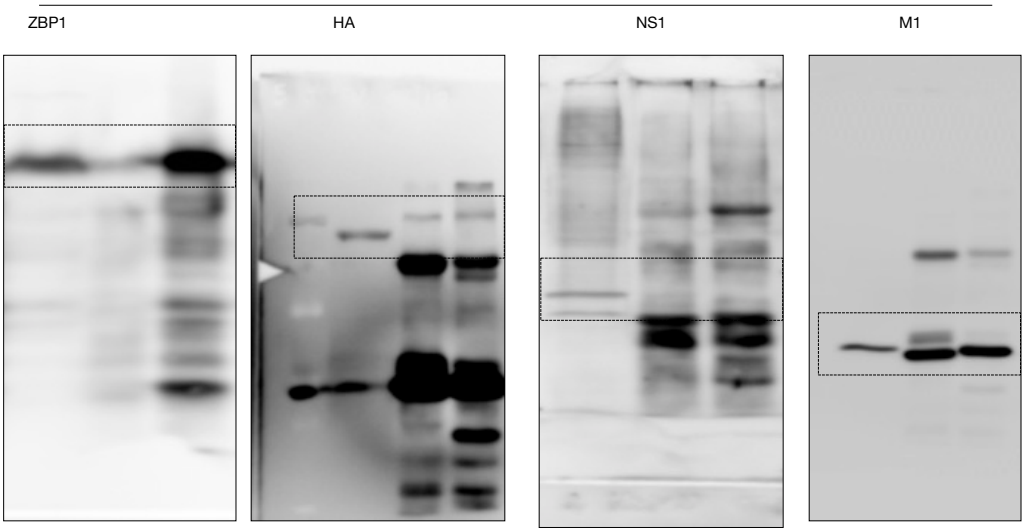
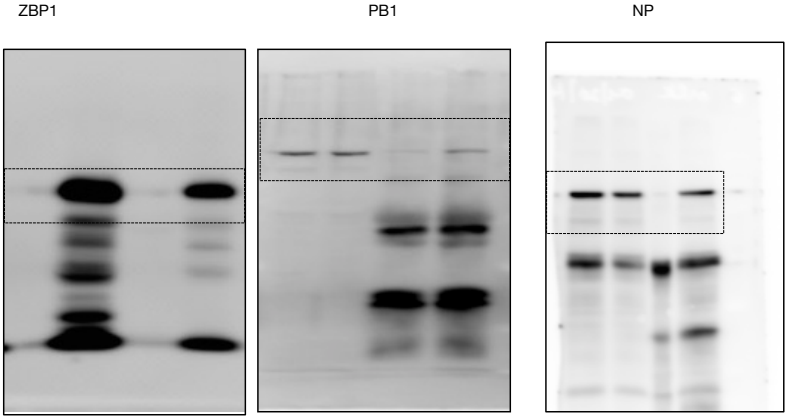


Fig.5B



Western blots associated with Fig. 5

Fig.5C

ZBP1 PB1 NP

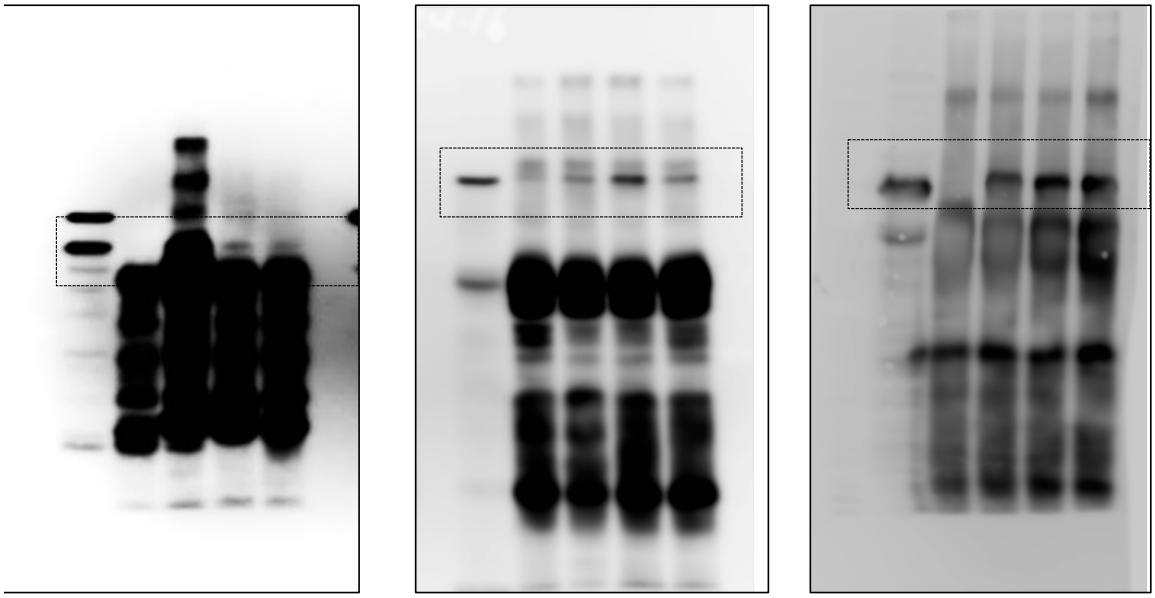
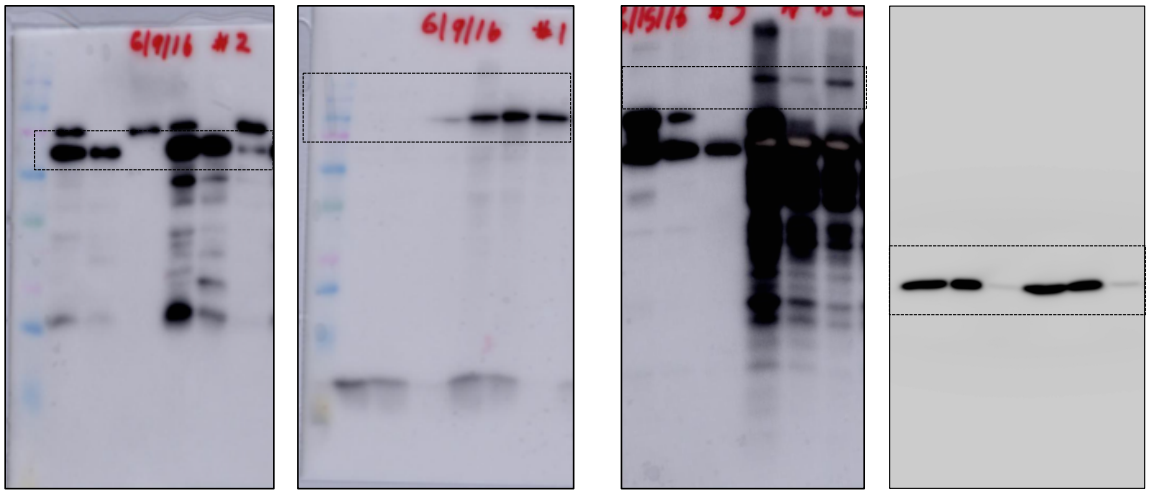


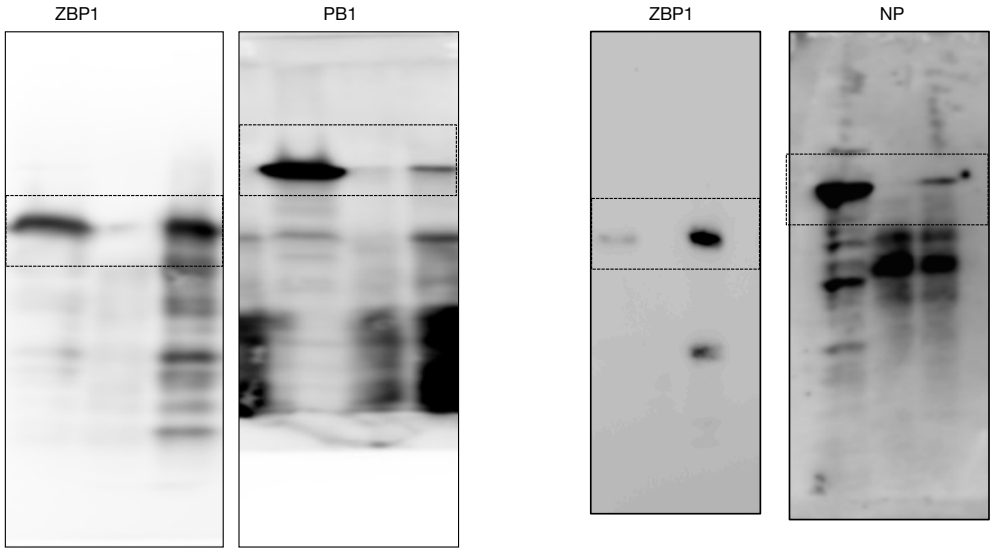
Fig.5D

ZBP1 PB1 NP GAPDH



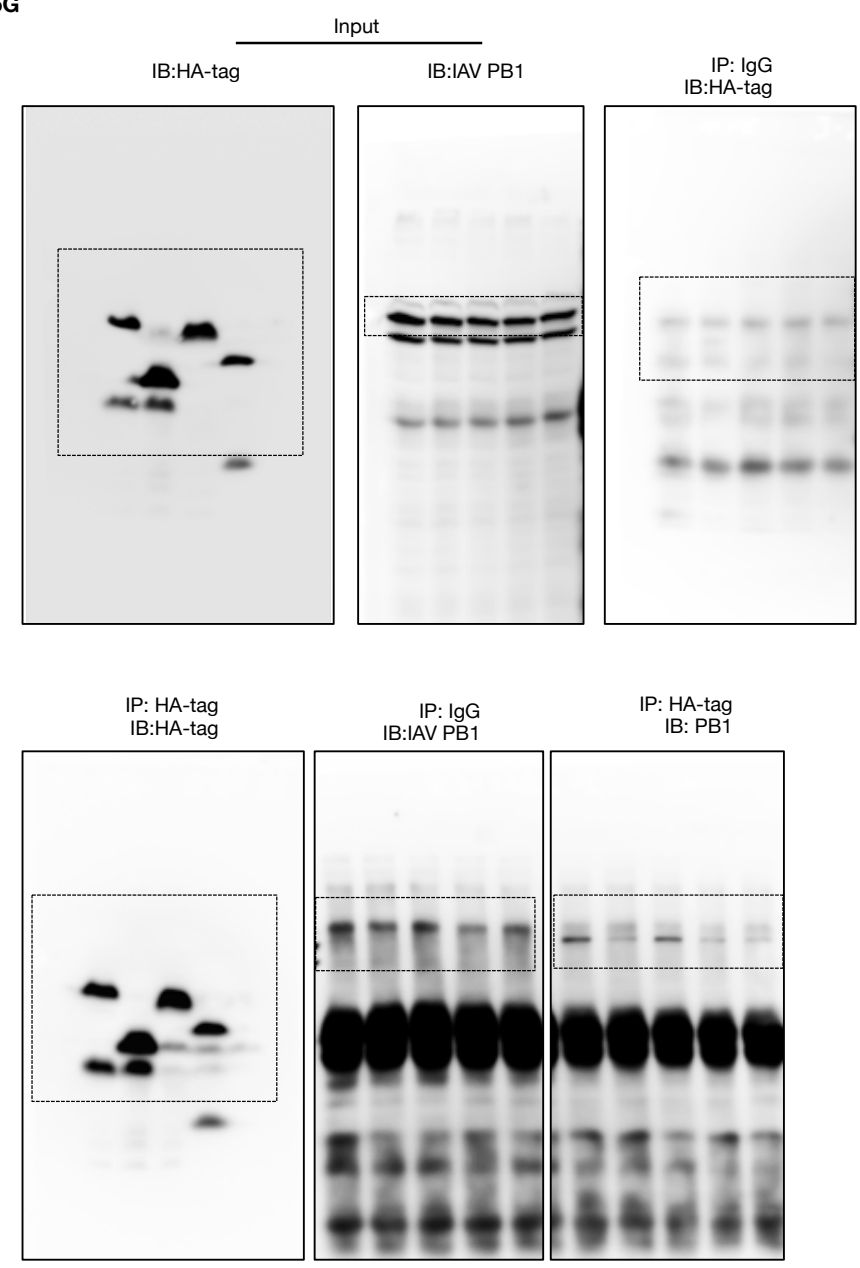
Western blots associated with Fig. 5

Fig.5E



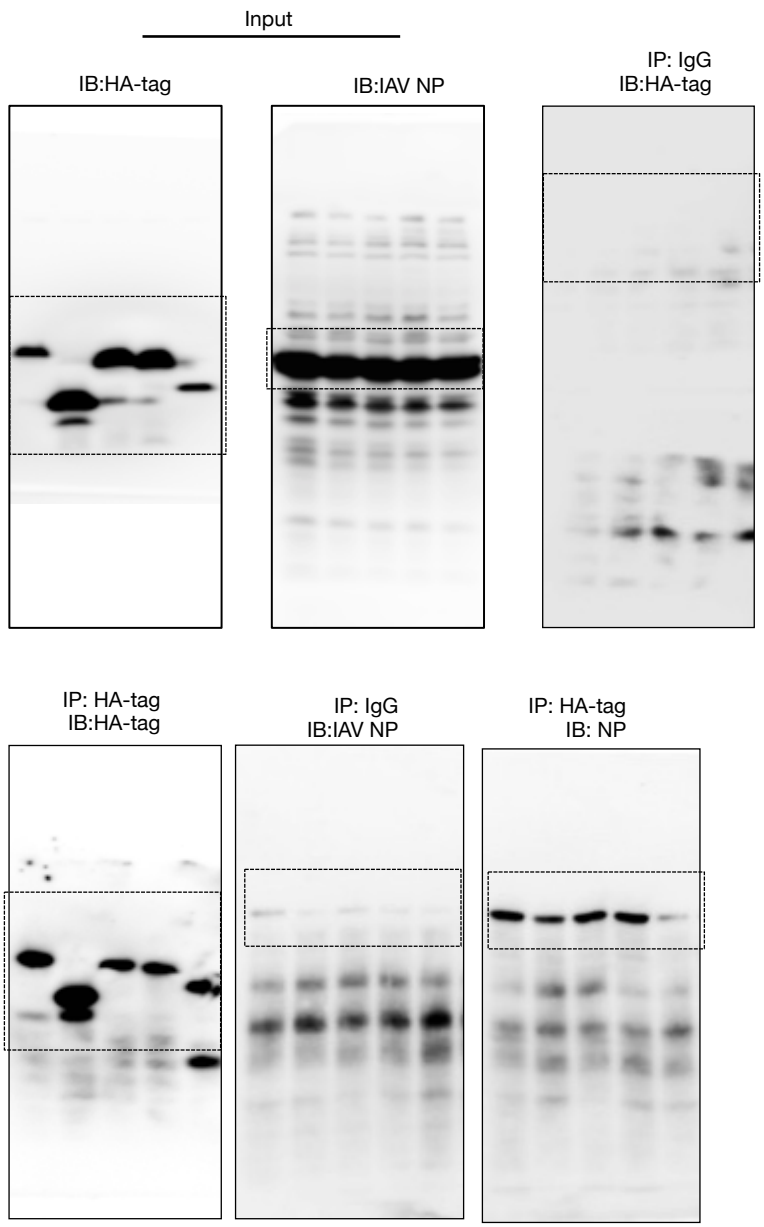
Western blots associated with Fig. 5

Fig.5G



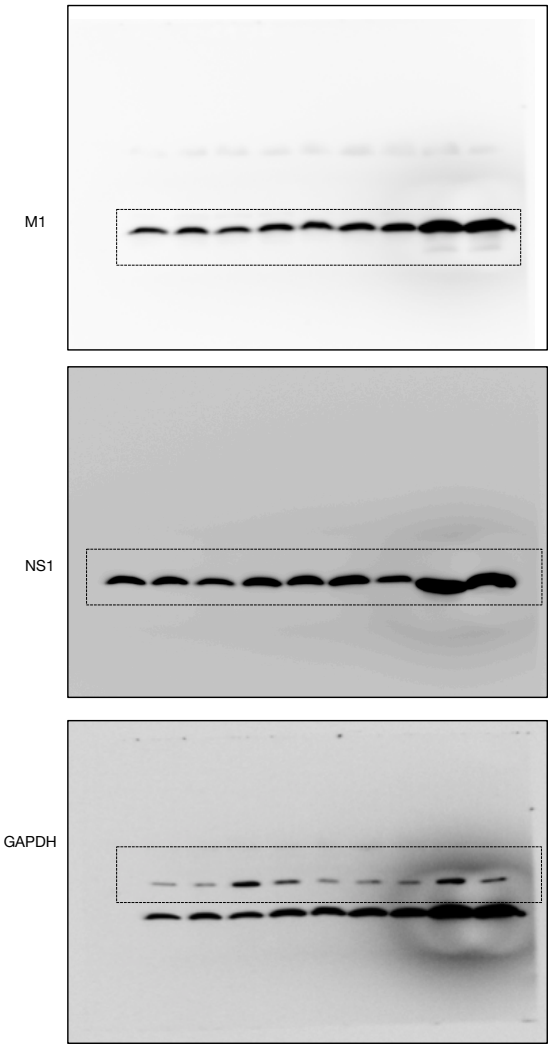
Western **blots** associated with Fig. 5

Fig.5H



Western blots associated with Fig. S1

Fig.S1E



Western blots associated with Fig. S2

Fig.S2B

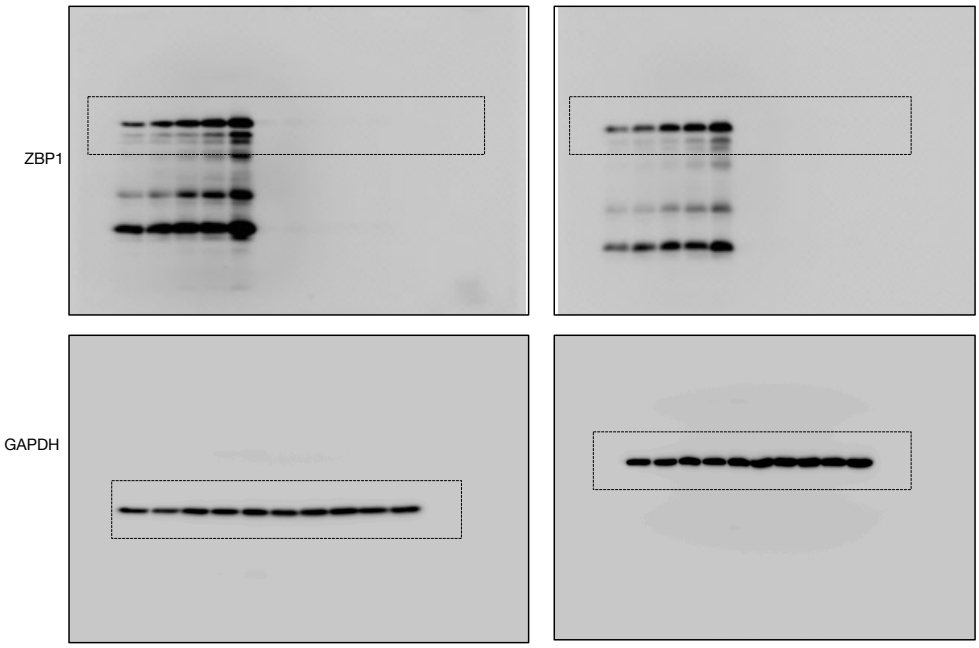
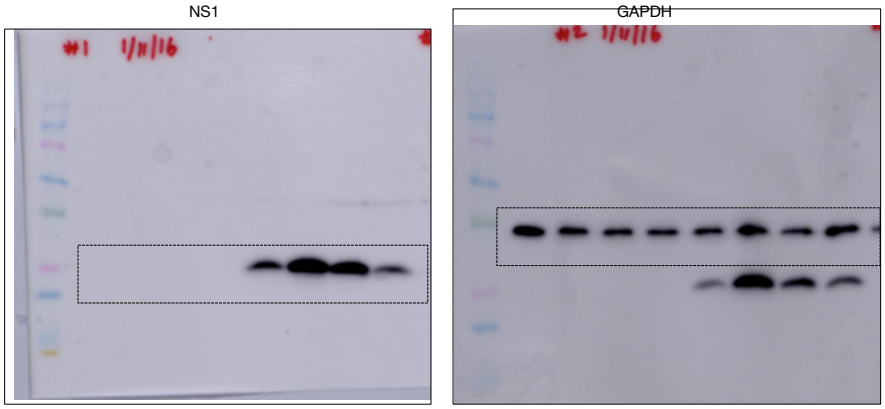


Fig.S2C



Western blots associated with Fig. S3

Fig.S3A

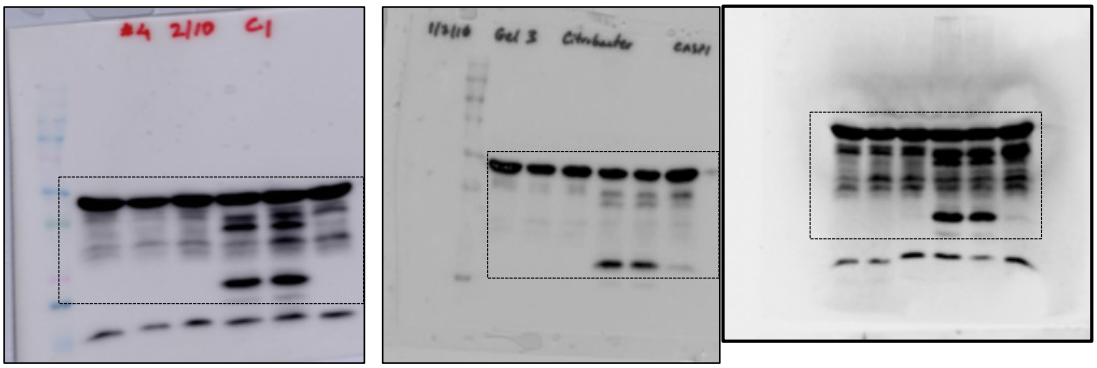


Fig.S3D

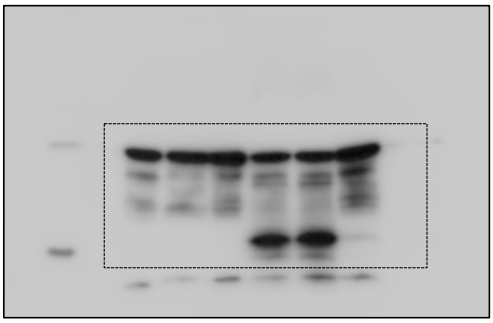
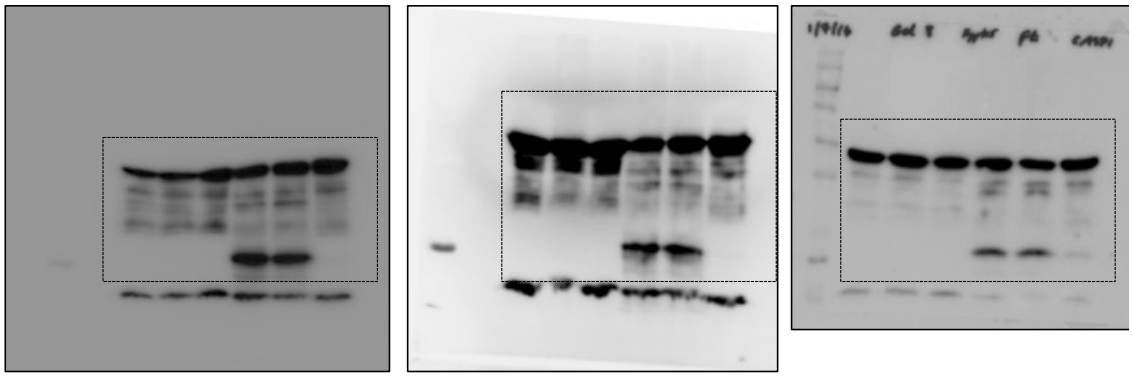


Fig.S3F



Western blots associated with Fig. S5

Fig.S5B

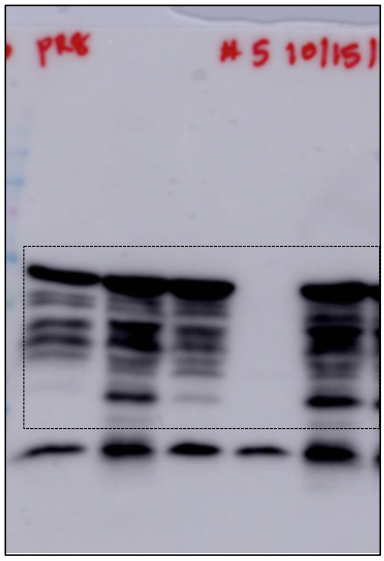
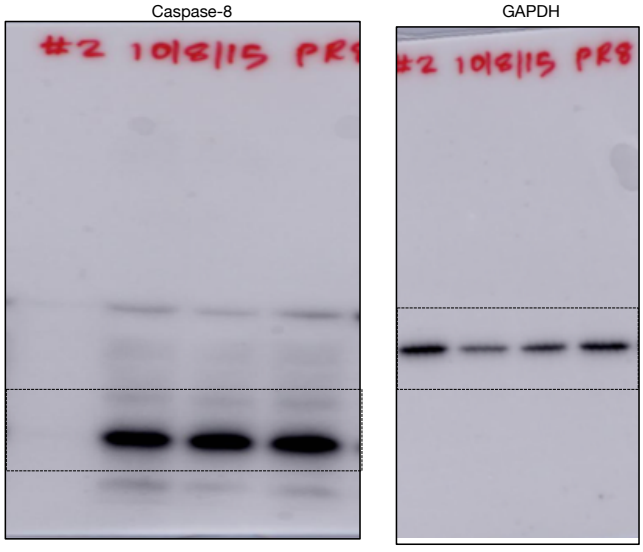
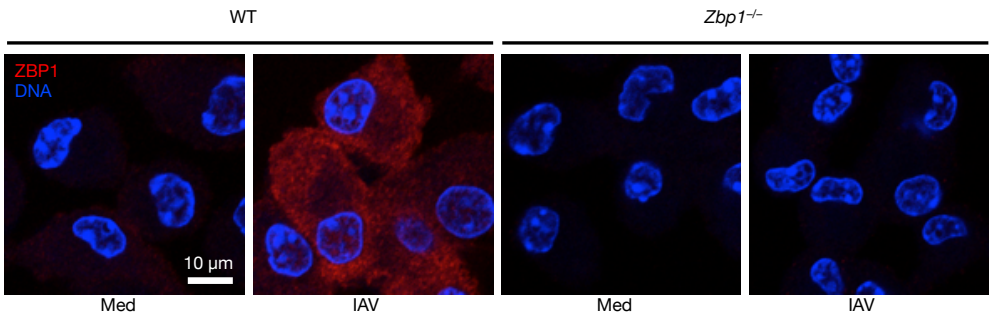


Fig.S5c



Staining controls for Fig. 1H



Source data for *in vivo* infections

Fig.S6A

Survival study			
WT		*Zbp1*$^{-/-}$	
No. Infected	No. Dead	No. Infected	No. Dead
15	5	9	0

Fig.S6c

Lung Viral titers (pfu/ml)	
WT	*Zbp1*$^{-/-}$
1000	57000
3000	65000
18000	43000
12000	37000
5000	69000
7000	51000