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The IFN-inducible GTPase IRGB10 regulates viral replication and inflammasome activation during influenza A virus infection in mice

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Abstract

The upregulation of interferon (IFN)-inducible GTPases in response to pathogenic insults is vital to host defense against many bacterial, fungal, and viral pathogens. Several IFN-inducible GTPases play key roles in mediating inflammasome activation and providing host protection after bacterial or fungal infections, though their role in inflammasome activation after viral infection is less clear. Among the IFN-inducible GTPases, the expression of immunity-related GTPases (IRGs) varies widely across species for unknown reasons. Here, we report that IRGB10, but not IRGM1, IRGM2, or IRGM3, is required for NLRP3 inflammasome activation in response to influenza A virus (IAV) infection. While IRGB10 functions to release inflammasome ligands in the context of bacterial and fungal infections, we found that IRGB10 facilitates endosomal maturation and nuclear translocation and viral replication of IAV. Corresponding with our *in vitro* results, we found that *Irgb10*$^{-/-}$ mice were more resistant to IAV-induced mortality than wild-type mice. The results of our study demonstrate a detrimental role of IRGB10 in host immunity in response to IAV and a novel function of IRGB10, but not IRGMs, in promoting viral translocation into the nucleus.

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Author contributions

T-D.K. conceptualized the study. S.C., D.P., M.Z., B.B., performed the experiments and conducted analysis. S.C. and T-D.K. wrote the manuscript with input from all authors. T-D.K. acquired the funding and provided overall supervision.

Conflict of interests

The authors declare no commercial or financial conflict of interest.

Ethics Statement

Studies were conducted under protocols approved by St. Jude Children's Research Hospital Institutional Committee on the Use and Care of Animals, protocol number 482. It is the policy of the St. Jude Children's Research Hospital Animal Care and Use Committee that all research involving animals be conducted according to the highest possible professional, ethical, and scientific standards and that all animals be housed, maintained, and handled in compliance with the standards set forth by the Animal Welfare Act of 1966 (9 CFR Part 3 as amended); the National Research Council 1996 "Guide for the Care and Use of Laboratory Animals"; the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised September 1986); the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the report of the American College of Laboratory Animal Medicine on Adequate Veterinary Care in Research, Testing, and Teaching; the 2007 Guidelines on Euthanasia; and all other applicable federal, state, and local laws, regulations, and policies. St. Jude Children's Research Hospital is committed to maintaining full accreditation status by AAALAC International.

Keywords

Inflammasome; pyroptosis; IRGB10; GTPases; Influenza A virus; NLRP3

Introduction

Interferon (IFN) signaling and interferon-stimulated genes (ISGs) are vital mediators of cell autonomous immunity, or the ability of a cell to defend itself. IFN expression is induced in response to many viruses and bacteria [1], resulting in the upregulation of a number of effector proteins, including a family of guanylate triphosphatases (GTPases) with important roles in host defense [2–4]. The IFN-inducible GTPases of this family, which includes the immunity-related GTPases (IRGs), perform a number of multi-faceted functions in facilitating the clearance of pathogens and the induction of host responses. IFN-inducible GTPases target pathogen-containing vacuoles, mediate the recruitment of other host proteins, and restrict microbial replication [3,5–11]. In addition, guanylate binding proteins (GBPs) and members of the IRG subfamily, IRGB10 and IRGM proteins, have been found to play key roles in regulating the activation of multi-protein innate immune complexes called inflammasomes [5,9,12–15].

Inflammasomes are signaling complexes that initiate the inflammatory cell death process known as pyroptosis [16]. The basic inflammasome complex is typically comprised of sensor, adaptor, and effector proteins. An inflammasome sensor recognizes a pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP) and recruits the adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) through homotypic interactions, resulting in ASC polymerization and the formation of an ASC speck [17]. Caspase-1 (CASP1) is then recruited to the ASC speck through interactions between the caspase recruitment domains (CARDs) of ASC and CASP1, leading to CASP1 self-activation through proximity-based autoproteolysis [16,18,19]. Active CASP1 then cleaves the downstream substrates pro–interleukin-1β(IL-1β), pro–interleukin-18 (IL-18), and gasdermin D (GSDMD) [20–22]. Cleavage of GSDMD results in the oligomerization of its N-terminal domain and the formation of pores within the membrane of the cell, resulting in the release of bioactive IL-1β and IL-18 and the execution of pyroptotic cell death [22–25]. Recent studies have determined a vital role for a number of IFN-inducible GTPases in the activation of inflammasome complexes [3,5,8,9,11–15,26,27]. GBPs and IRGB10 are regulated by IFN-alpha receptor 1 (IFNAR1) and IFN-regulatory factor 1 (IRF1) and mediate the release of inflammasome-activating ligands after bacterial infection, including lipopolysaccharide (LPS) after *Escherichia coli* infection and double-stranded DNA after *Francisella novicida* infection, through lysis of intracellular host and pathogen membranes [5,11,26,27]. IRGB10, but not GBPs, have also been found to directly target the *Aspergillus* cell wall and facilitate the release of fungal ligands for inflammasome sensing [9]. Conversely, GBPs, but not IRGB10, were shown to control bacterial replication and restrict bacteria-mediated actin polymerization and host cell fusion after *Burkholderia thailandensis* infection [12]. Furthermore, IRGB10 and GBPs, along with IRGM proteins, play roles in the murine IFN response to *Chlamydia* [6,10,15,28]. The roles of these IFN-inducible GTPases in host

defense vary, depending on the infectious context. Additionally, the expression of different IFN-inducible GTPases is highly variable between mammalian species, particularly in the case of members of the IRG family [3,39]. Further, loss and restoration of the expression of different members of this family is dynamic over time in response to evolutionary pressure [29].

In addition to its role during bacterial and fungal infections, IFN signaling is a hallmark of viral infection, and a number of ISGs are crucial to antiviral defenses [30]. For instance, upregulation of Z-DNA-binding protein 1 (ZBP1) through IFNAR and IRF1 signaling in response to IAV infection regulates the activation of the NLRP3 inflammasome and PANoptosis, an inflammatory programmed cell death pathway regulated by the PANoptosome complex with key features of pyroptosis, apoptosis, and/or necroptosis [31–49]. Additionally, myxovirus resistance proteins (Mx), ISGs belonging to another branch of the family of IFN-inducible GTPases, play a number of antiviral roles including direct interference with IAV replication and inflammasome activation [50–52]. Furthermore, IRGs and GBPs interfere with the replication complex of positive-sense viruses, thereby restricting viral replication [7,53]. However, in contrast to their clearly described functions in response to bacterial and fungal infections, the role of IRGs and GBPs in regulating inflammasome activation after viral infection is not well understood.

Here, we show that IRGB10, a member of the IRG family found in mice, but not humans, plays a crucial role in mediating IAV-induced inflammasome activation and host susceptibility by facilitating IAV replication. Consistent with these results, we found that *Irgb10*$^{-/-}$ mice are more resistant to IAV infection compared to wild-type mice. Conversely, we found no role for the three murine paralogues of human IRGM in mediating inflammasome activation or viral translocation after IAV infection. In contrast to the reported beneficial activity of IRGs in response to bacterial or fungal infections, our results suggest a detrimental role of IRGB10 during IAV infection.

Results

IRGB10, but not IRGMs, regulates IAV-induced inflammasome activation

Previously, we performed a microarray analysis of wild-type and IFNAR1- and IRF1-deficient bone marrow-derived macrophages (BMDMs) infected with IAV (GSE77611 and GSE103059) [31,54]. As IFNAR1 and IRF1 have been shown to be crucial in the upregulation of IFN-inducible GTPases after bacterial infection, we further analyzed the results of our microarray data to examine the role of IFNAR1 and IRF1 in regulating IRGs and GBPs after IAV infection. Further analysis revealed the impaired transcriptional expression of a number of IFN-inducible GTPases, including members of the IRG family, after genetic deletion of *Ifnar1* or *Irf1* (Fig. 1A). Consistent with our microarray findings, we observed lower transcript abundance of *Irgb10* and *Irgm3* after IAV infection in *Ifnar1*$^{-/-}$ and *Irf1*$^{-/-}$ BMDMs (Fig. 1B). Expression of the *Irgm1* transcript was significantly reduced after IFNAR1 deletion, but was not significantly decreased in BMDMs lacking IRF1, suggesting alternative mechanisms of IFNAR1-dependent regulation of this IRG. These findings demonstrate that the IFN response after IAV infection leads to the upregulation

of IFN-inducible GTPases, including IRGB10 and IRGMs through IFNAR1-, and partially through IRF1-, dependent signaling.

To determine the role of these IRGs in IAV-induced inflammasome activation, we infected BMDMs lacking IRGB10 or IRGMs with IAV and monitored cleavage of CASP1 (Fig. 1C). BMDMs lacking IRGB10 exhibited a dramatic reduction in the processing of CASP1 and a subsequent decrease in the release of IL-1β and IL-18 (Fig. 1D). However, loss of IRGB10 did not impair NLRP3 activation in response to ligands mimicking viral infection; priming of BMDMs with the TLR7/8 agonist R848 followed by ATP treatment or transfection of poly(I:C) resulted in similar CASP1 activation in *Irgb10*$^{-/-}$ BMDMs compared to wild-type BMDMs (Fig. S1), which demonstrates that NLRP3 can still be activated by ligands mimicking viral infections without IRGB10. In contrast to the loss of inflammasome activation observed during IAV infection in *Irgb10*$^{-/-}$ BMDMs, loss of all three murine IRGM paralogues (*Irgm1/2/3*$^{-/-}$) did not markedly reduce CASP1 activation or significantly reduce the release of IL-1β and IL-18 (Figs. 1C and 1D). Taken together, these data demonstrate that IRGB10 and IRGM proteins are upregulated through IFNAR1 and IRF1 signaling after IAV infection and that IRGB10, but not IRGMs, plays a key role in mediating NLRP3 inflammasome activation after IAV infection.

IRGB10 mediates NLRP3 inflammasome priming and viral replication

Canonical activation of the NLRP3 inflammasome is dependent on both a priming and an activation step. Priming of the NLRP3 inflammasome involves signaling through innate immune sensors, such as TLRs. This results in the activation of a number of cellular pathways, including the NF-κB signaling pathway, leading to the upregulation of pyroptotic molecules such as NLRP3 and IL-1β [55,56]. We therefore sought to determine the effect of IRGB10 deficiency on the priming of the NLRP3 inflammasome. Loss of IRGB10 resulted in a decrease in IκBα phosphorylation (plκBα) early in infection (Fig. 2A), indicating that NLRP3 inflammasome priming is impaired in these macrophages. Correspondingly, NLRP3 expression was decreased in *Irgb10*$^{-/-}$ BMDMs after IAV infection by immunoblot analysis. Consistent with these results, the transcript abundance of both *Nlrp3* and *Il1b* was significantly decreased in IRGB10-deficient macrophages after IAV infection (Fig. 2B). These results contrast with reports of normal NLRP3 priming and expression observed in IRGB10-deficient cells during bacterial and fungal infections [5,9], suggesting that this GTPase plays a unique role during IAV infection. Additional signaling pathways, including MAPK signaling, interferon signaling, and production of *Il6* and *Tnfa*, were also impaired in the *Irgb10*$^{-/-}$ BMDMs after IAV infection (Figs. 2A and 2B), suggesting that loss of IRGB10 has a more widespread impact on the cell during IAV infection than just impairment of inflammasome-specific priming and activation.

Reduction in innate immune signaling during infection could be due to a number of factors, including impaired entry of the virus into the cell, decreased viral replication, or defects in host cell signaling. To determine whether signaling was altered due to decreased viral load within the cells, we measured the expression of influenza-specific proteins in *Irgb10*$^{-/-}$ BMDMs after IAV infection *in vitro*. Protein levels of the IAV non-structural protein 1 (NS1) and nucleoprotein (NP) were reduced in the IRGB10-deficient BMDMs

following IAV infection (Fig. 3A), suggesting that the decreased cellular signaling and attenuated inflammasome activation may be due to impaired viral entry or replication. Furthermore, transcript levels of influenza proteins M1 and NP were lower in *Irgb10*$^{-/-}$ BMDMs compared to wild-type after infection (Fig. 3B). This was further reflected in decreased viral titers in the supernatant of infected BMDMs 8 h after infection (Fig. 3C). Confocal microscopy of IAV-infected BMDMs demonstrated that loss of IRGB10 resulted in a decrease in IAV nucleoprotein-positive (NP^{+}) BMDMs 4 h post-infection (Figs. S2A and S2B), indicating that IRGB10 plays a role in the early stages of IAV infection.

Our findings suggest that lower levels of IAV within the IRGB10-deficient BMDMs impair the cellular innate immune response to infection. However, it is unclear if this is due to impaired endocytosis of the virus or attenuated viral replication. To address this, we labeled IAV with rhodamine and monitored uptake of the virus by fluorescence microscopy. Endocytosis of IAV labeled with rhodamine by *Irgb10*$^{-/-}$ BMDMs was comparable to the viral uptake in wild-type BMDMs (Figs. 3D and 3E). These results suggest that the decreased levels of influenza proteins observed in *Irgb10*$^{-/-}$ BMDMs could be due to impaired viral replication, rather than impaired viral entry. It is well-established that IAV replication occurs within the nucleus of infected cells and is dependent on the ability of viral ribonucleoproteins (RNP) components to translocate into the nucleus. We observed that IAV NP remained localized in extranuclear puncta 2 h after infection of *Irgb10*$^{-/-}$ BMDMs, at which time more nuclei in wild-type BMDMs were NP-positive (Figs. 3F and 3G), suggesting that loss of IRGB10 may impair translocation of viral particles into the nucleus.

Nuclear translocation of IAV is dependent on maturation of IAV-containing endosomes. As these compartments mature and acidify, the viral envelope fuses with the endosomal membrane, leading to release of viral core components into the cytosol before translocation to the nucleus for replication [57,58]. As we hypothesized that loss of IRGB10 impaired endosomal maturation and viral release, we monitored acidification of IAV-containing endosomes using IAV labeled with a pH-sensitive rhodamine green fluorophore. As the pH of the environment deceases, the intensity of the probe fluorescence increases. At 2 h post-infection, we observed that the integrated fluorescence intensity of the labeled IAV was significantly lower in *Irgb10*$^{-/-}$ BMDMs compared to wild-type (Figs. 4A and 4B).

Taken together, these results indicate that IRGB10 mediates maturation of IAV-containing endosomes, thereby facilitating the translocation of the influenza RNP complex to the nucleus without directly affecting endocytosis of the virus. This suggests that the observed decrease in NLRP3 inflammasome activation in *Irgb10*$^{-/-}$ BMDMs is the result of impaired viral replication, not viral uptake.

Loss of IRGB10 provides resistance to IAV infection *in vivo*

IRGB10-mediated inflammasome activation has been shown to provide *in vivo* protection after bacterial infection [5]. However, our results demonstrate that loss of IRGB10 impairs IAV replication *in vitro*. These findings suggest that expression of IRGB10 would be detrimental to the host *in vivo* during IAV infection. To address this, we infected wild-type and *Irgb10*$^{-/-}$ mice with mouse-adapted IAV strain PR8 and monitored mice for survival. Consistent with our *in vitro* findings, we found that *Irgb10*$^{-/-}$ mice were less susceptible

to IAV infection (Fig. 5). These results provide the first evidence that IRGB10 may play a detrimental role in host defense during viral infection, as opposed to its beneficial role in bacterial infection.

Discussion

IFN-inducible GTPases are a family of proteins with important functions during host responses to infection. Previous studies have found that IFN-inducible GTPases can restrict microbial growth by directly targeting bacteria, fungi, or the viral replication complexes of positive-sense viruses. In addition, a number of IFN-inducible GTPases play a role in the activation of inflammasome complexes after infection by facilitating the release of pathogenic ligands [5,7,9,11,12,27]. Here, we show that IRGB10, but not IRGM proteins, regulates inflammasome activation after IAV infection, and loss of IRGB10 results in decreased expression of viral proteins. In contrast to the role of IRGB10 in mediating the release of pathogenic ligands in the context of bacterial and fungal infections, we found that the reduced inflammasome activation during IAV infection was due to IRGB10 facilitating endosomal maturation, IAV translocation to the nucleus, and subsequent viral replication. This is consistent with our previous findings that inflammasome activation and PANoptotic cell death occur after the release of viral RNPs into the cytosol [31,32]. The impairment of viral replication due to the loss of IRGB10 led to attenuation in the activation of multiple cellular signaling pathways. It is important to note that while our results demonstrate decreased priming of the NLRP3 inflammasome, the activation and subsequent conformational changes of NLRP3 from its closed to open state may also be impaired. This would explain why such a dramatic impairment of caspase-1 cleavage was observed in *Irgb10*$^{-/-}$ BMDMs, while the decreased NLRP3 expression was more modest in these knockouts. In addition, the results presented here also delineate a novel function of an IRG in facilitating viral replication, rather than inhibiting this process as other IFN-inducible GTPases do [7,50,51]. We further found that mice lacking IRGB10 were less susceptible to IAV infection compared with wild-type mice. This implies that expression of IRGB10 can be detrimental in the context of influenza infection, suggesting that loss of this protein may provide a genetic benefit to organisms exposed to influenza infection.

Although IRGs play numerous roles in the defense of rodents against infectious microbes, the expression of IRGs across species is highly variable and dynamic within a species over time [3,29,59]. The variability in the number of IRGs expressed between species and loss and restoration of members of this family due to evolutionary pressure, along with differences in their regulation by IFNs, suggests that this family of proteins may have an evolutionary cost in response to some selective pressure [3,29,59]. Our results here provide evidence that while IRGs normally play positive roles in cell-autonomous defense, expression of IRGB10 has a detrimental effect in response to influenza virus, a pathogen well-documented to cause pandemics capable of providing a strong selective pressure on the human genome [60]. Correspondingly, IRGB10 is not expressed in humans. Consistent with this context, we found no such detrimental function of the murine IRGM proteins, and IRGM is one of the two remaining IRGs expressed in humans [3,59]. The mechanism by which IRGB10 leads to nuclear translocation of IAV particles is not known. However, here we provide evidence that IRGB10 facilitates maturation of endosomes containing IAV, a

step crucial to the viral translocation process. Structurally and biochemically, IFN-inducible GTPases are considered “dynamin-like”. Dynamin, a GTPase involved in membrane fission and clathrin-mediated endocytosis [61,62], has been shown to play a similar role in facilitating nuclear translocation of IAV in mink lung cells [63]. Further, recent structural insights into IRGB10 suggest that this GTPase may function mechanistically similarly to other members of the dynamin family, such as Alastatin-1, in mediating membrane dynamics [64].

It is not yet clear how IRGB10 functions to facilitate viral translocation after IAV infection, while IRGM proteins did not appear to function in a similar capacity. Further study will be needed to determine whether IRGB10 plays a dynamin-like role during clathrin-mediated endocytosis, participates in dynamin recruitment during this process, or plays a novel role in the maturation of IAV-containing endosomes. Our results suggest the possibility that IRGB10 expression in humans may have been lost as a casualty in the arms race between host and virus, prompting viruses to adapt by utilizing alternative methods of entry. While a homologue of IRGB10 has not yet been found in humans, future studies to elucidate the precise mechanism of IRGB10-regulated IAV cytosolic release may allow for the identification of novel influenza risk factors through the discovery of functional homologues of the immunity-related GTPases that have been lost in humans.

Materials and Methods

Mice

Wild-type (C57BL6/J), *Irf1*$^{-/-}$ [65], *Ifnar1*$^{-/-}$ [66], *Irgb10*$^{-/-}$ [5], and *Irgm1/2/3*$^{-/-}$ [Dr. Masahiro Yamamoto] mice have been previously described and were backcrossed to the C57/BL6 background. Mice in this study were used between 6 and 8 weeks of age and bred at St. Jude Children’s Research Hospital. The studies described here were conducted under protocols approved by St. Jude Children’s Research Hospital Institutional Committee on the Use and Care of Animals.

Influenza A virus culture

IAV (A/Puerto Rico/8/34, H1N1 [PR8]) was generated by reverse genetics as previously described [67]. Propagation of viral stocks was achieved by inoculating the allantoic cavity of 9- to 11-day old embryonated chicken eggs with seed virus. Titer of the virus was measured in MDCK cells using plaque assay.

Cell culture and stimulation

Primary BMDMs were differentiated in IMDM media supplemented with 10% fetal bovine serum, 30% L929 conditioned media, 1% non-essential amino acids, and 1% penicillin/streptomycin. A total of 1×10^6 BMDMs were seeded into a 12-well plate and rested overnight for infection or stimulation. BMDMs were infected with PR8 virus at a multiplicity of infection of 25 in high glucose DMEM without FBS for two hours. After two hours, high glucose DMEM containing 20% FBS was added to each well to achieve a final FBS concentration of 10%. Samples were then collected for analysis at the indicated time points. For cell stimulations, BMDMs were stimulated with 1 μg/ml R848

(Invivogen) for 4 h followed by 0.5 mM ATP (Roche) for 20 minutes or transfected with 2 μg poly(I:C) (Invivogen) using the Xfect polymer transfection kit (Clontech Laboratories) according to the manufacturer's instructions. For infection with labeled IAV virus, influenza A virus was labeled using NHS-Rhodamine labeling kit (Thermo Fisher Scientific, 46406) or NHS-pHRodo green labeling kit (Thermo Fisher Scientific, P35369) according to the manufacturer's instructions. Dialysis of the tagged virus was performed to remove unincorporated label.

ELISA analysis

For measurement of IL-18 and IL-1β, the mouse uncoated IL-1β ELISA kit (Invitrogen, 88-703-88) and mouse IL-18 ELISA kit (Invitrogen, BMS618–3TEN) were used according to the manufacturer's instructions.

Immunoblotting analysis

For CASP1 immunoblotting, supernatant and lysate were collected together after BMDMs were lysed in cell lysis buffer (5% v/v NP-40, 10 mM DTT) containing protease inhibitors (Roche). For immunoblotting analysis of other proteins, cells were washed with PBS at the indicated time point and lysed with 1× RIPA lysis buffer containing protease and phosphatase inhibitors (Roche). Samples were subjected to SDS-PAGE on 10% or 12% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes, before blocking in 5% milk. Membranes were then incubated overnight with the following primary antibodies: caspase-1 (1:1000 dilution, AdipoGen, G-20B-0042); pIκBx (1:1000 dilution, CST, 2859); IκBα (1:1000 dilution, CST, 9242); NLRP3 (1:1000 dilution, AdipoGen, AG-20B-0014); GAPDH (1:2000 dilution, CST, 5174); IAV NS1 (1:1000 dilution, Santa Cruz, sc130568); IAV NP (1:1000 dilution, Thermo Fisher Scientific, PA5–32242); pERK (1:1000 dilution, CST, 9101); ERK (1:1000 dilution, CST, 9102); pSTAT1 (1:1000 dilution, CST, 7649); STAT1 (1:1000 dilution, CST, 14994); IRF1 (1:1000 dilution, CST, 8478); IL-1β (1:1000 dilution, CST, 12507); β-actin (1:1000 dilution, Proteintech, 66009–1-IG). After the overnight incubation in the primary antibody, membranes were washed and incubated with the appropriate secondary HRP antibody (1:5000 dilution, Jackson ImmunoResearch Laboratories) or anti-mouse fluorescent secondary antibody (1:5000 dilution, Amersham CyDye700 goat anti-mouse, 29360784). Immunoblots were developed and visualized using a GE Amersham Imager 600 or BioRad Imager.

Quantitative real time RT-PCR analysis

RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) before being reverse transcribed using a first-strand cDNA synthesis kit (Applied Biosystems). Real-time quantitative PCR was performed using 2× SYBR Green (Applied Biosciences). Transcript levels for mRNAs of interest were normalized to GAPDH or actin levels. For host transcripts, expression was normalized to that of untreated controls. For viral transcripts, expression was normalized to viral transcript levels 3 h post-infection. Primer sequences can be found in Supplemental Table 1.

Immunofluorescence staining and imaging

After infection, BMDMs were fixed in 4% paraformaldehyde and blocked in 10% goat serum (Life Technologies) in PBS. Cells were incubated with an antibody against IAV NP (1:250 dilution, Thermo Fisher Scientific, PA5–32242) overnight at 4°C, followed by incubation with AlexaFluor 555-conjugated anti-mouse IgG (1:250 dilution, Invitrogen, A-21236) for 1 hour at room temperature and counterstained with DAPI. Images were obtained using a Lecia SP8 confocal microscope. Quantification of NP^+ nuclei was performed using FIJI software. Nuclei were segmented using Otsu segmentation, and nuclei colocalized with NP staining were counted. Live-cell fluorescence of cells infected with labeled IAV was monitored using the Incucyte SX5 or Marianas system configured on a Zeiss Axio Observer microscope. Quantification of rhodamine-positive BMDMs and integrated intensity of the pH-rhodamine probe was analyzed using the Sartorius Incucyte SX5 Live-Cell Analysis System.

In vivo IAV infection

Female wild-type (C57BL6/J) and *Irgb10*$^{-/-}$ mice aged 6- to 9-weeks were infected intranasally with 50 PFU IAV (A/Puerto Rico/8/34, H1N1 [PR8]) in 50 μL PBS after avertin (150 mg/kg) anesthetization. Mice were monitored for 14 days for survival. Mice were co-housed for 7 days prior to infection and throughout the 14 day survival study.

Quantification and statistical analysis

Statistical analysis was performed using GraphPad v8 software. Data are shown as mean ± SEM. Statistical significance was determined using a two-tailed unpaired *t* test where $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

All relevant data are within the paper and its Supporting Information files. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Corresponding Author, Thirumala-Devi Kanneganti (thirumala-devi.kanneganti@stjude.org).

Abbreviations:

IRG	immunity related GTPase
IFN	interferon
IFNAR	interferon-alpha receptor
IRF1	interferon regulatory factor 1
NLRP3	NOD-like receptor pyrin domain-containing 3
IAV	Influenza A virus
ISG	interferon-stimulated gene

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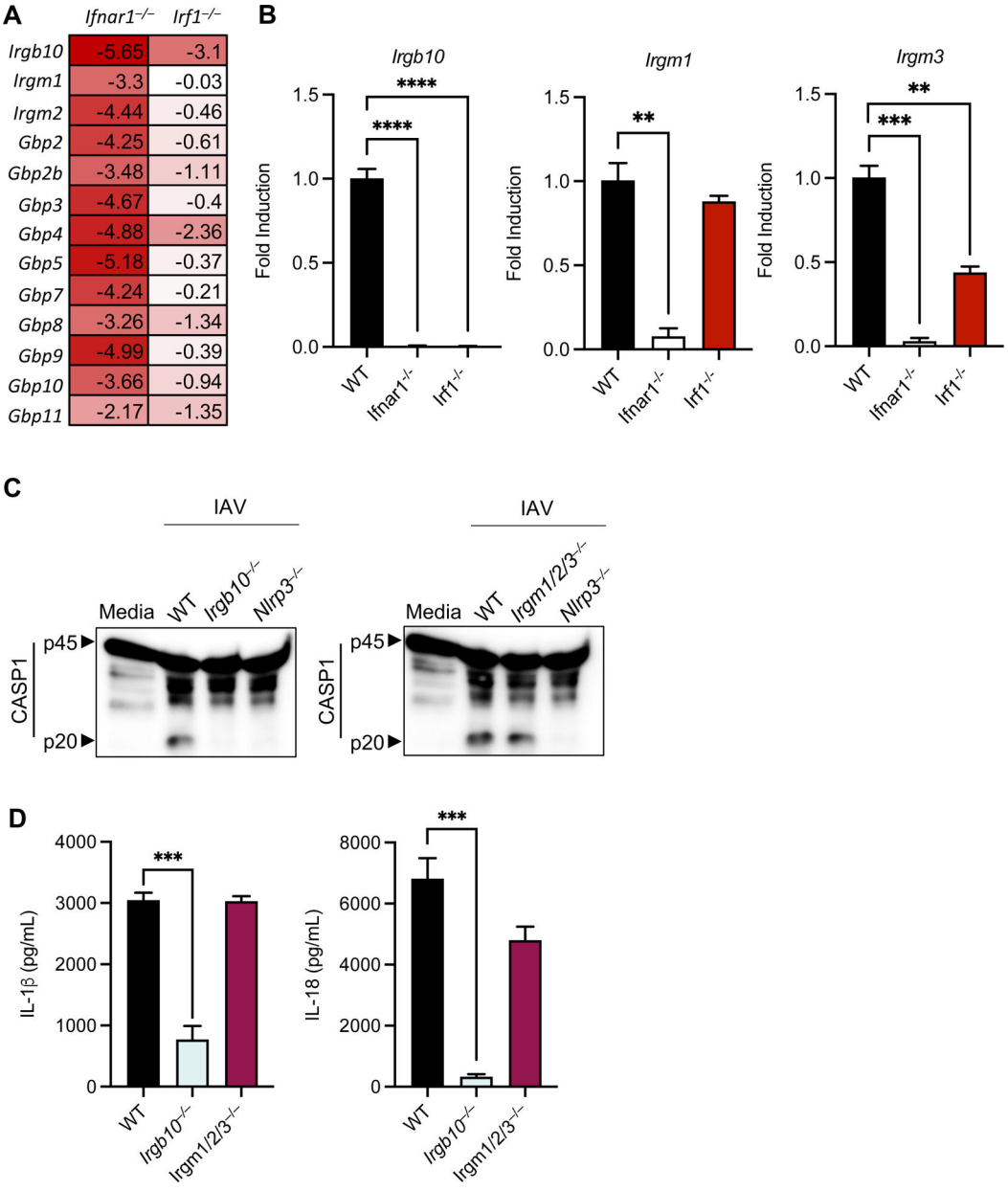

Fig. 1.

IRGB10 facilitates NLRP3 inflammasome activation during IAV infection, while IRGMs are dispensable

(A) Microarray gene expression analysis of IFN-stimulated genes in *Ifnar1*$^{-/-}$ and *Irf1*$^{-/-}$ BMDMs after 9 h of IAV infection [31,54]. (B) mRNA expression of IFN-inducible GTPases 9 h after IAV infection in wild-type, *Ifnar1*$^{-/-}$, and *Irf1*$^{-/-}$ BMDMs. (C) Immunoblot analysis of CASP1 cleavage after overnight (16 h) IAV infection in BMDMs lacking IRGB10 or IRGM1/2/3, or NLRP3 as a negative control. (D) ELISA analysis of IL-1β and IL-18 levels in the supernatant of IAV-infected BMDMs 16 hpi. Data are shown as mean + SEM, and significance was determined by a two-tailed unpaired *t* test (B), ns, not

significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data shown are from one experiment and are representative of at least three independent experiments.

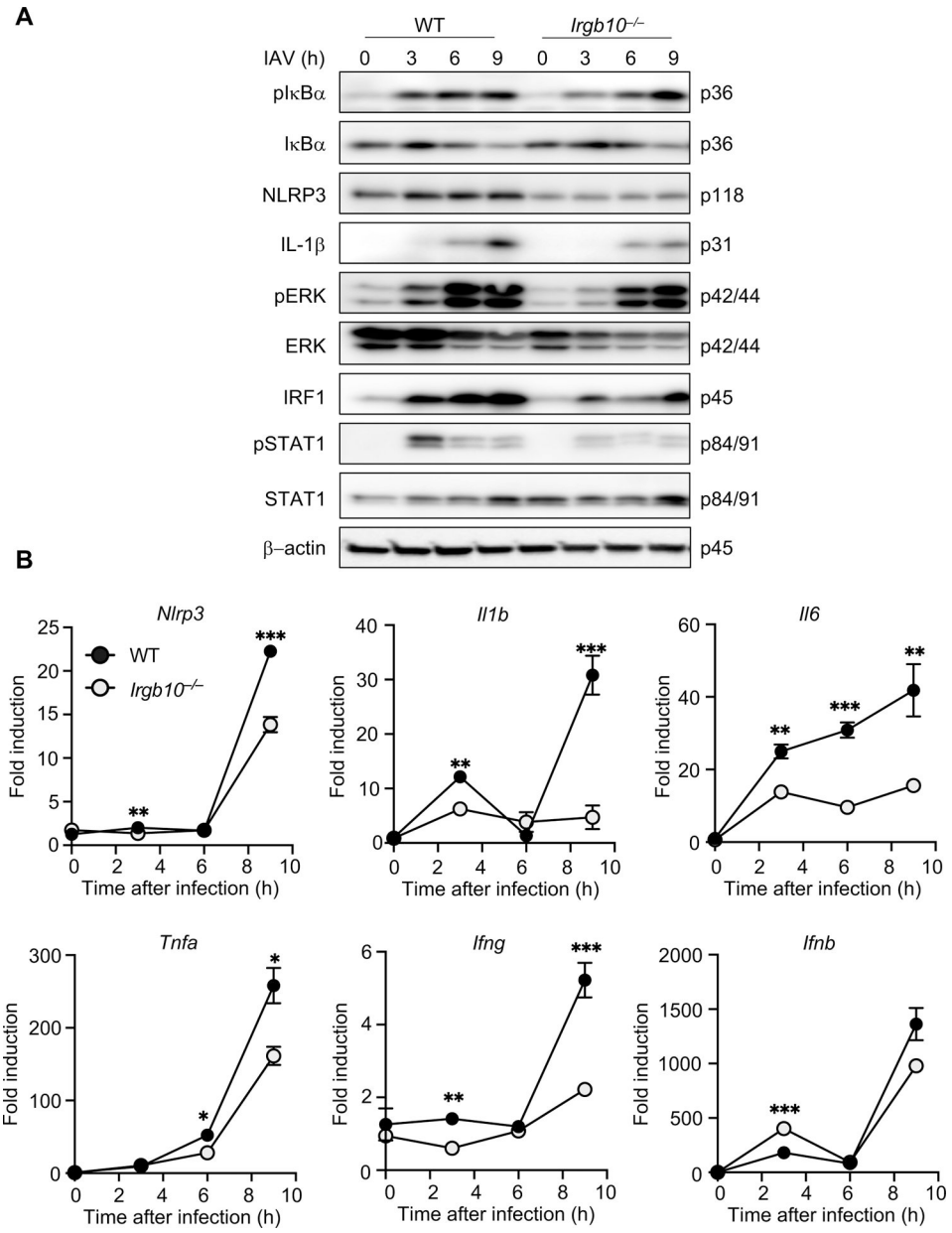

Fig. 2.

IRGB10 mediates NLRP3 priming and innate signaling

(A) Immunoblot analysis of phosphorylated and total IκBα, phosphorylated and total ERK, IRF1, phosphorylated and total STAT1, NLRP3, pro–IL-1β, and β-actin after IAV infection in wild-type and *Irgb10*$^{-/-}$ BMDMs. (B) mRNA expression of *Nlrp3*, pro-*Il1b*, *Il6*, *Tnfa,* *Ifng,* and *Ifnb* after IAV infection in wild-type and *Irgb10*$^{-/-}$ BMDMs. Data are shown as mean ± SEM, n = 4 and significance was determined by a two-tailed unpaired *t* test (B) **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Data shown are from one experiment and are representative of at least three independent experiments.

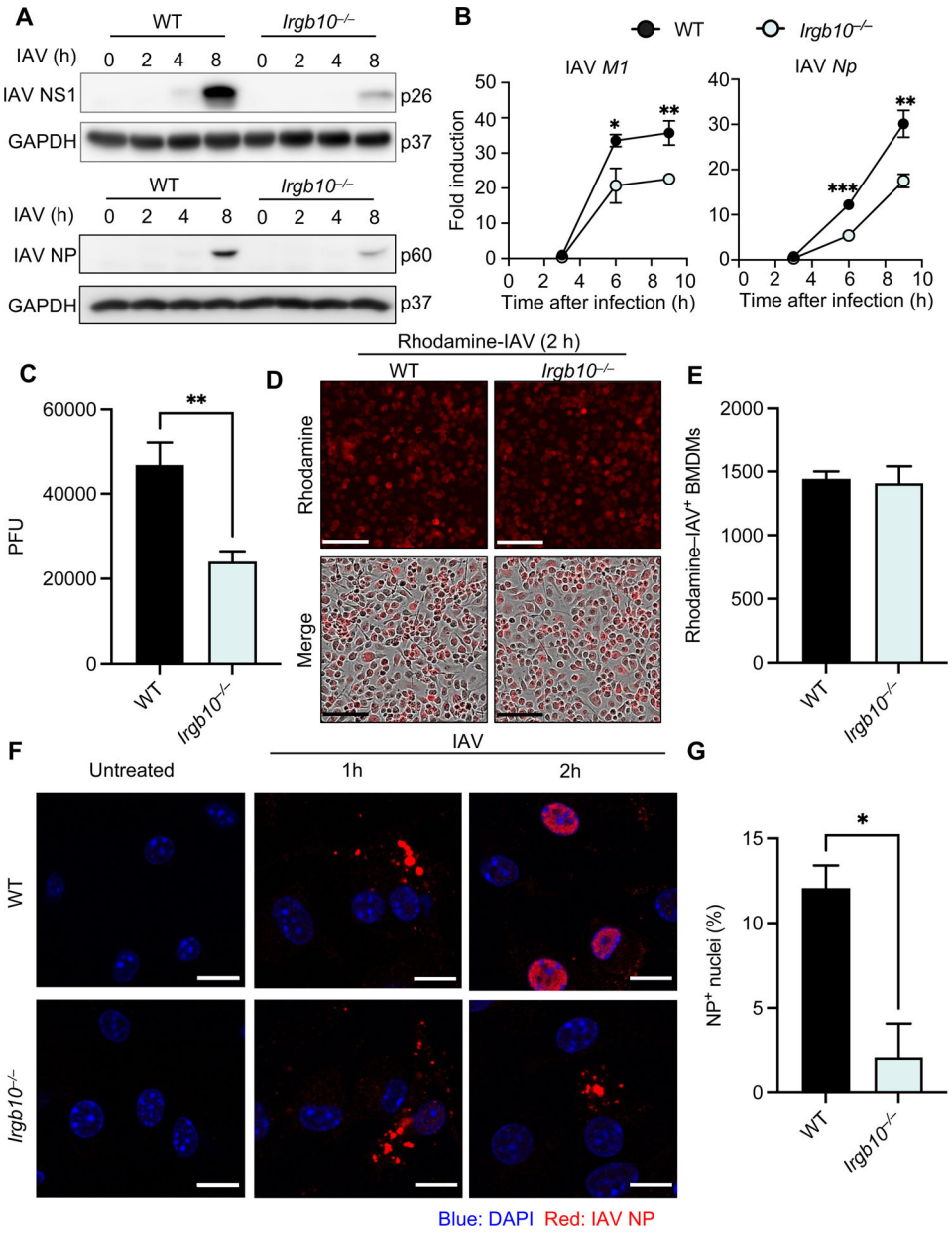

Fig. 3.

IRGB10 facilitates viral replication and trafficking of IAV to the nucleus

(A) Immunoblot analysis of protein expression of IAV NS1 or IAV NP proteins in wild-type and *Irgb10*$^{-/-}$ BMDMs. (B) mRNA expression of viral *M1* and *Np* proteins in wild-type and *Irgb10*$^{-/-}$ BMDMs after IAV infection. (C) Plaque-forming units from the supernatant of wild-type and *Irgb10*$^{-/-}$ BMDMs 8 h after infection. (D) Uptake of rhodamine-labeled IAV by wild-type and *Irgb10*$^{-/-}$BMDMs. Scale bars indicate 100 μm. (E) Quantification of rhodamine-IAV^{+} BMDMs in wild-type and *Irgb10*$^{-/-}$ BMDM 2 h after infection. (F) Localization of IAV NP in wild-type and *Irgb10*$^{-/-}$ BMDMs at the indicated timepoints post-infection with IAV. Scale bars indicate 10 μm. (G) Quantification of NP^{+} nuclei in wild-type and *Irgb10*$^{-/-}$ BMDM 2 h after infection. Data are shown as mean ± SEM, and

significance was determined by a two-tailed unpaired *t* test (B, C, E, G), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data shown are from one experiment and are representative of at least three independent experiments.

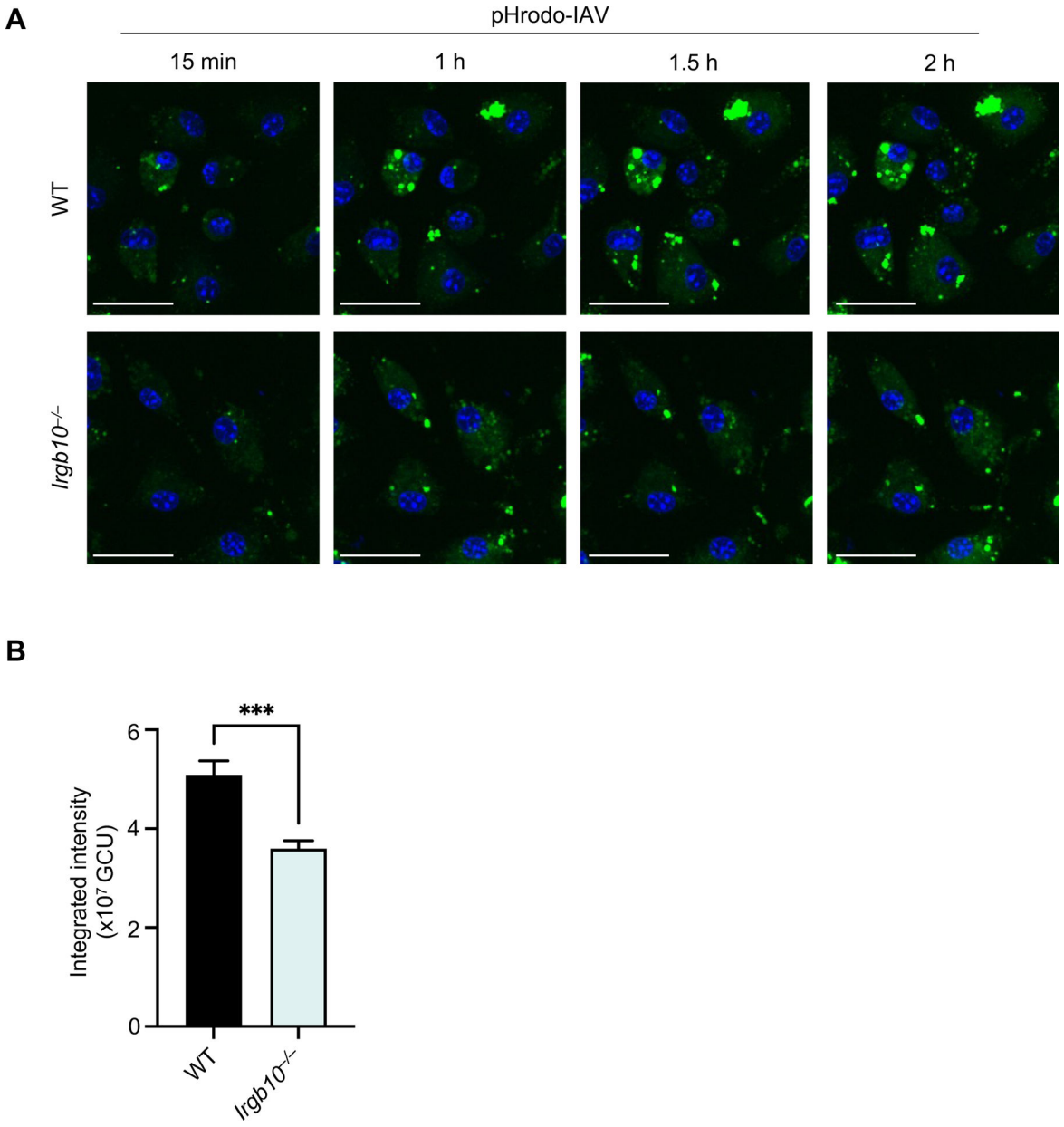

Fig. 4.

Loss of IRGB10 impairs maturation of IAV-containing endosomes

(A) Acidification of pH-rhodamine (pHrodo)-labeled IAV in wild-type or *Irgb10*$^{-/-}$ BMDMs. Scale bars indicate 30 μm. (B) Quantification of integrated intensity of pHrodo-labeled IAV 2 h post-infection. Data are shown as mean + SEM, and significance was determined by a two-tailed unpaired *t* test, ***$P < 0.001$. Data shown are from one experiment and are representative of at least three independent experiments.

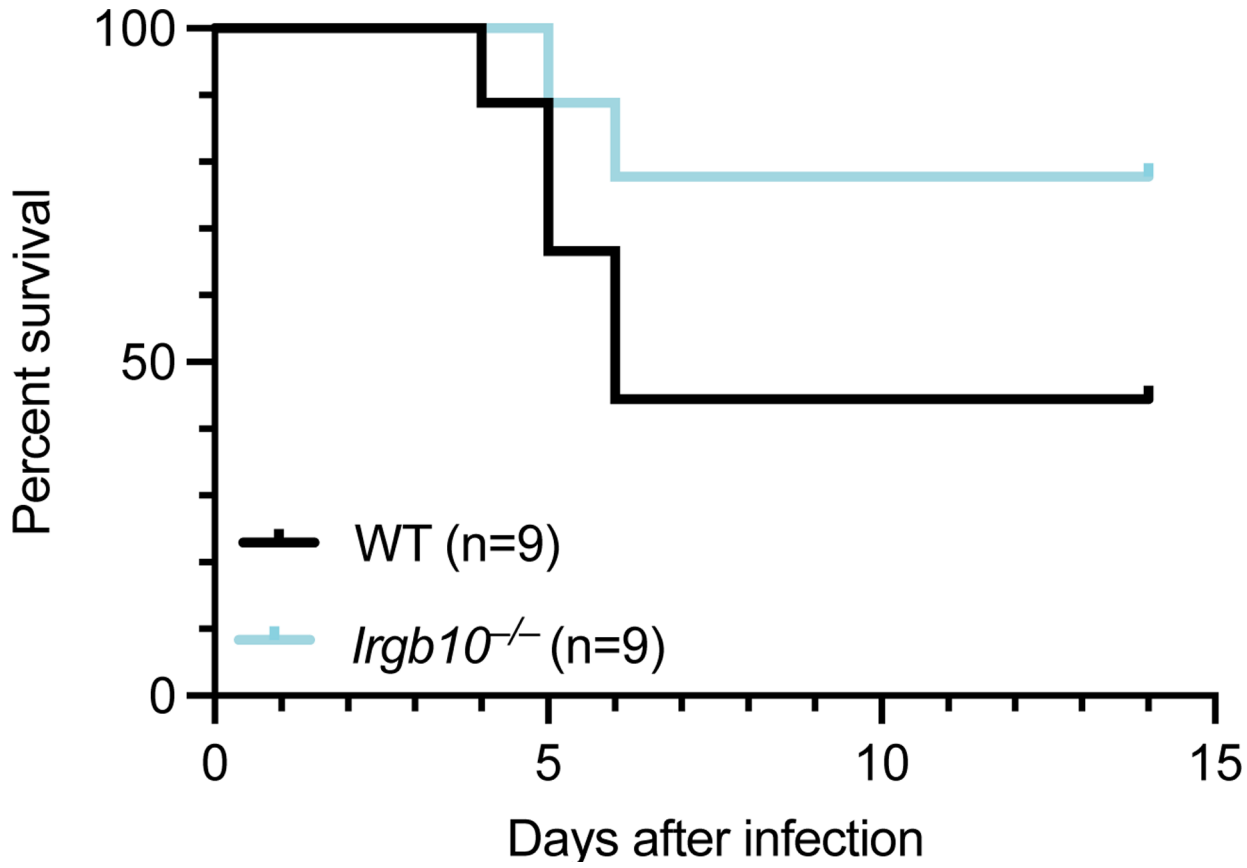

Fig. 5.

IRGB10 enhances susceptibility to IAV infection *in vivo*

Survival of wild-type and *Irgb10*$^{-/-}$ mice after IAV infection. Data are pooled from two independent experiments.