RESEARCH REPORT

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Interferon Regulatory Factor 3 Exacerbates the Severity of COVID-19 in Mice

Wendy E. Walker, PhD[1,2]
Luiz F. Garcia, MS[2]
Pedro M. Palermo, PhD[3]
Nawar Hakim, MD[4]
Dinesh G. Goswami, PhD[2]
Alok K. Dwivedi, PhD[2]
Douglas M. Watts, PhD[3]

CONTEXT: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019, causing the COVID-19 pandemic. While most infected people experienced mild illness, others progressed to severe disease, characterized by hyperinflammation and respiratory distress. There is still much to learn about the innate immune response to this virus. Interferon regulatory factor 3 (IRF3) is a transcription factor that is activated when pattern recognition receptors detect viruses. Upon activation, IRF3 induces the expression of interferon beta (IFN-β) and interferon-stimulated genes, which protect the host from viral infection. However, coronaviruses antagonize this pathway, delaying type 1 IFN production. It is, therefore, unclear how IRF3 influences COVID-19 disease. Our prior reports showed that IRF3 promotes harmful inflammation during bacterial sepsis in mice.

HYPOTHESIS: We hypothesized that IRF3 cannot effectively control the SARS-CoV-2 viral load and instead promotes harmful inflammation during severe COVID-19.

METHODS AND MODELS: We used mice transgenic for the human angiotensin converting-enzyme 2 transgene, driven by the keratin 18 promoter (K18-ACE2 mice) that were IRF3 deficient or IRF3 sufficient to test how IRF3 influences COVID-19 disease.

RESULTS: Upon infection with SARS-CoV-2, K18-ACE2 mice showed a dose-dependent disease, characterized by mortality, lethargy, weight loss, and lung pathology, reminiscent of clinical COVID-19. However, K18-ACE2 mice lacking IRF3 were protected from severe disease with reduced mortality (84.6% vs. 100%) and disease score. We found that IRF3 promoted IFN-β production in the lungs and reprogrammed the cytokine profile, while viral load in the lungs was similar in the presence or absence of IRF3.

INTERPRETATIONS AND CONCLUSIONS: These data indicated that IRF3 played a detrimental role in murine COVID-19 associated with changes in IFN-β and inflammatory cytokines.

KEYWORDS: COVID-19; cytokines; interferon regulatory factor 3; interferons; human angiotensin-converting enzyme 2 transgenic mice; K18-ACE2 mice

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection induces a range of disease phenotypes, from asymptomatic to severe acute respiratory infection, characteristic of COVID-19. During early infection, the virus triggers multiple pattern recognition receptors (PRRs), including Toll-like receptor (TLR) 2, TLR4, TLR7, TLR9, and RIG-I-like receptors (RLRs) (1–7). Several of these pathways are known to activate interferon regulatory factor 3 (IRF3), a transcription factor that induces interferon (IFN)-β to suppress viral replication (8). SARS-CoV-2, however, manipulates this pathway, delaying IFN-β production (9–12). Therefore, it is unclear if IRF3 and IFN-β can effectively limit viral infection and how they influence the course of disease. We previously showed that that IRF3 promotes inflammation

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KEY POINTS

Question: This study used a mouse model to investigate how interferon regulatory factor 3 (IRF3) influences COVID-19 disease.

Findings: Animals lacking IRF3 were protected from COVID-19 disease with reduced mortality and disease score. Their lungs showed an altered inflammatory profile but little difference in viremia.

Meaning: Our study demonstrates that IRF3 plays a detrimental role in COVID-19 disease in mice associated with altered inflammation rather than viremia.

and lethal disease in mouse models of bacterial sepsis (13–17). We hypothesized that IRF3 may play a similar, detrimental role in COVID-19 disease, promoting inflammation and mortality. In this study, we recapitulated the K18-ACE2 mouse model of severe COVID-19 disease, and used IRF3-deficient mice to test how this transcription factor influenced the pathogenesis of severe COVID-19.

METHODS

This study used mice transgenic for the human angiotensin-converting enzyme 2, driven by the keratin 18 promoter (K18-ACE2 mice) and IRF3 knockout x K18-ACE2 mice. Experimental animals were 56.4% female, 43.6% male, and 24.27 ± 3.89 weeks old (mean ± SD). All experiments with SARS-CoV-2 were performed in Biosafety Level-3 containment. SARS-CoV-2 (USA-Washington State A1/2020 SARS-CoV-2 isolate) was propagated in Vero-E6 cells. Mice were anesthetized, and inoculated with 2.5×10^3, 2.5×10^4, or 2.5×10^5 plaque forming units (PFUs) of SARS-CoV-2, administered intranasally in 50 µL volume. Controls received 50 µL of saline solution. We monitored animal mortality for 14 days using humane endpoints, as well as weight and disease score (lethargy), per our prior reports (14–18). Additional mice were euthanized to collect lung lobes; these were homogenized and the infectivity titer was determined by plaque assay, per our prior report (19). We used LEGENDplex (BioLegend, San Diego, CA) bead immunoassays to measure cytokines in lung homogenates and standardized these to the protein content. Data were compared with nonparametric analyses, and validated with a nonparametric bootstrap *t* test (20). Furthermore, we determined the clustering between cytokine expression levels at day 2 using a variable cluster analysis (21). A *p* value of less than 5% was considered significant. For details, see **Supplemental Methods** (http://links.lww.com/CCX/B479).

RESULTS

We first recapitulated the K18-ACE2 mouse model of severe COVID-19 (22–26). K18-ACE2 mice were infected with 2.5×10^3, 2.5×10^4, and 2.5×10^5 PFU of SARS-CoV-2 or administered saline (controls). We observed dose-dependent animal mortality (**Fig. S1*A***, http://links.lww.com/CCX/B479), disease score (**Fig S1*B***, http://links.lww.com/CCX/B479) and weight loss (**Fig. S1*C***, http://links.lww.com/CCX/B479) in SARS-CoV-2-infected animals, while saline controls remained healthy. Lung histology revealed that SARS-CoV-2-infected mice had a remarkable perivascular inflammatory infiltrate predominated by lymphocytes and mononuclear leukocytes, as well as pulmonary edema at 2 days post-infection, similar to clinical COVID-19 (**Fig. S1*D***, http://links.lww.com/CCX/B479).

To investigate the impact of IRF3 on severe COVID-19 disease in this model, we crossed K18-ACE2 mice to IRF3 knockout mice and compared these animals to IRF3-sufficient K18-ACE2 mice (referred to hereafter as IRF3 knockout and wild-type mice, for brevity). Animals were challenged with 2.5×10^4 PFU SARS-CoV-2 or saline (controls). We found that IRF3 knockout mice were protected from severe COVID-19 disease, exhibiting significantly reduced mortality rates (84.6%) vs. wild-type mice (100%; **Fig. 1*A***). Additionally, IRF3 knockout mice showed a significantly attenuated disease score vs. wild-type mice (**Fig. 1*B***). We observed a slight trend toward attenuated weight loss in IRF3 knockout vs. wild-type mice (**Fig. 1*C***). Saline controls remained healthy.

To determine the effect of IRF3 on viremia and cytokine production, we challenged additional cohorts of IRF3 knockout and wild-type mice with 2.5×10^4 PFU SARS-CoV-2, and euthanized the animals to obtain lung homogenates at day 2 and day 4 post-infection. We observed a similar viral load in the lungs

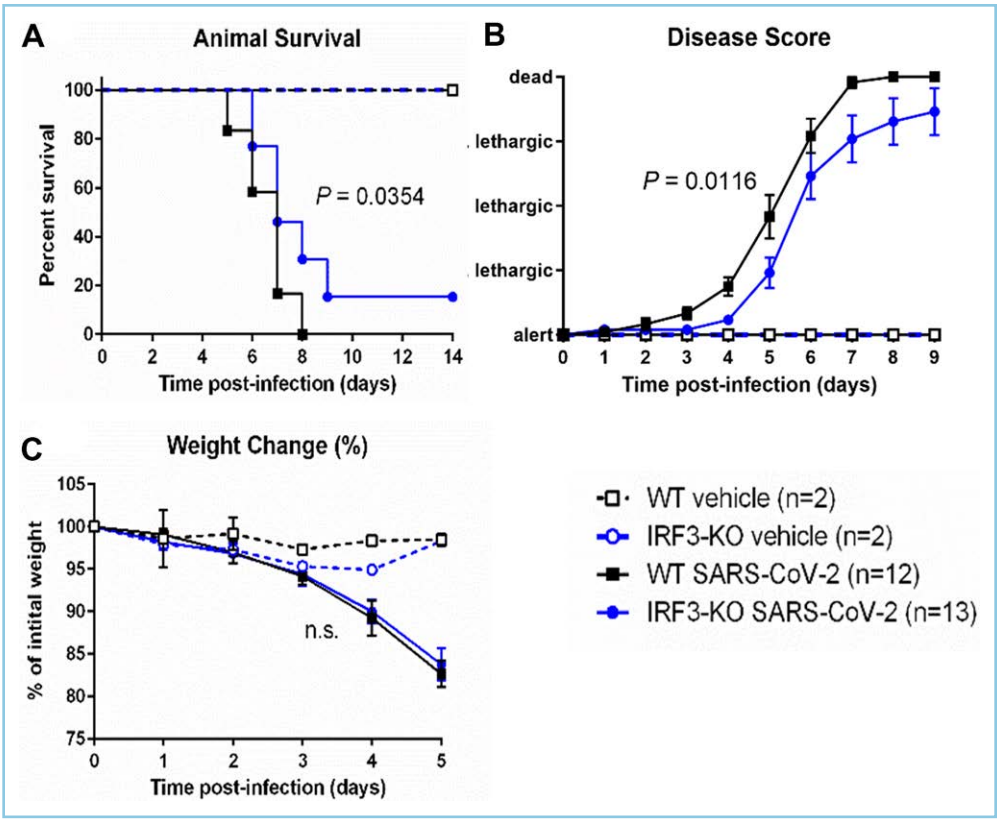

Figure 1. Interferon regulatory factor 3 (IRF3)-knockout (KO) mice are protected from severe COVID-19 disease. IRF3-KO and wild-type (WT) mice (both carrying the human angiotensin converting-enzyme 2 transgene, driven by the keratin 18 promoter [K18-ACE2]) were infected with 2.5×10^4 plaque forming units of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or saline vehicle as a control. Graphs show animal survival (**A**), animal disease score (**B**), and percent weight change (**C**), relative to the initial animal weight. *p* values show the results of a log-rank test (animal survival), and a two-way analysis of variance on the rank data, reflecting the group difference (for disease score and weight change). n.s. = not significant.

of wild-type vs. IRF3 knockout mice at both time points (**Fig. 2*A***). Animals administered saline showed no viral plaques. We also measured cytokines in the lung homogenates. We found that IFN-β was significantly lower in the lungs of IRF3 knockout vs. wild-type mice at day 2 post-infection and also found lower levels (borderline significance) at day 4 post-infection (**Fig. 2*B***). Additionally, the levels of interleukin (IL)-12p70 (**Fig. 2*C***) and IL-10 (**Fig. 2*D***) were significantly lower in IRF3 knockout vs. wild-type mice at day 2 post-infection. We also observed a trend toward lower levels of IL-1α, tumor necrosis factor alpha (TNF-α), IL-23, and IL-27 in IRF3 knockout vs. wild-type mice at day 2 post-infection and for granulocyte-macrophage colony-stimulating factor (GM-CSF) at day 2 and day 4 post-infection (**Fig. S2*A–E***, http://links.lww.com/CCX/B479). The levels of IFN-α, IFN-γ, IL-1β, IL-6, IL-17A, C-X-C motif chemokine ligand (CXCL)-9, CXCL-10, and monocyte chemoattractant protein-1 (MCP-1) showed minimal differences in wild-type vs. IRF3 knockout mice (**Fig. S2*F–M***, http://links.lww.com/CCX/B479). A bootstrap test supported these results (**Fig. S4**, http://links.lww.com/CCX/B479).

We next performed a clustering analysis of the inflammatory cytokines on day 2 to identify cytokines that were consistently grouped together based on their expression levels. We found four clusters among 16 cytokines, explaining 74% of the variability (**Fig. S3**, ***A*** and ***B***, http://links.lww.com/CCX/B479). Cluster 1 included CXCL-10, IFN-α, IFN-β, IL-6, MCP-1, TNF-α, IFN-γ, and IL-1α explaining 43% of the variability; cluster 2 included GM-CSF, IL-10, IL-1β, and IL-12p70 explaining 15% of the variability; cluster 3 included CXCL-9 and IL-17A; and cluster 4 included IL-27 and IL-23. These results were confirmed with a bootstrap test (Fig. S4, http://links.lww.com/CCX/B479).

DISCUSSION

PRRs play a key role in host defense against viruses through the induction of IFN-β. However, the inflammation induced by these pathways can damage host tissues. Our prior reports showed that IRF3 plays a detrimental role in mouse models of sepsis and systemic inflammation (13, 14, 16, 17). In this study, we recapitulated a mouse model of COVID-19 in K18-ACE2 mice. Mice developed lethal disease after SARS-CoV-2 infection, with dose-dependent mortality, lethargy and weight loss, and inflammatory lung pathology consistent with clinical severe COVID-19 (Fig. S1, http://links.lww.com/CCX/B479), consistent with reports by

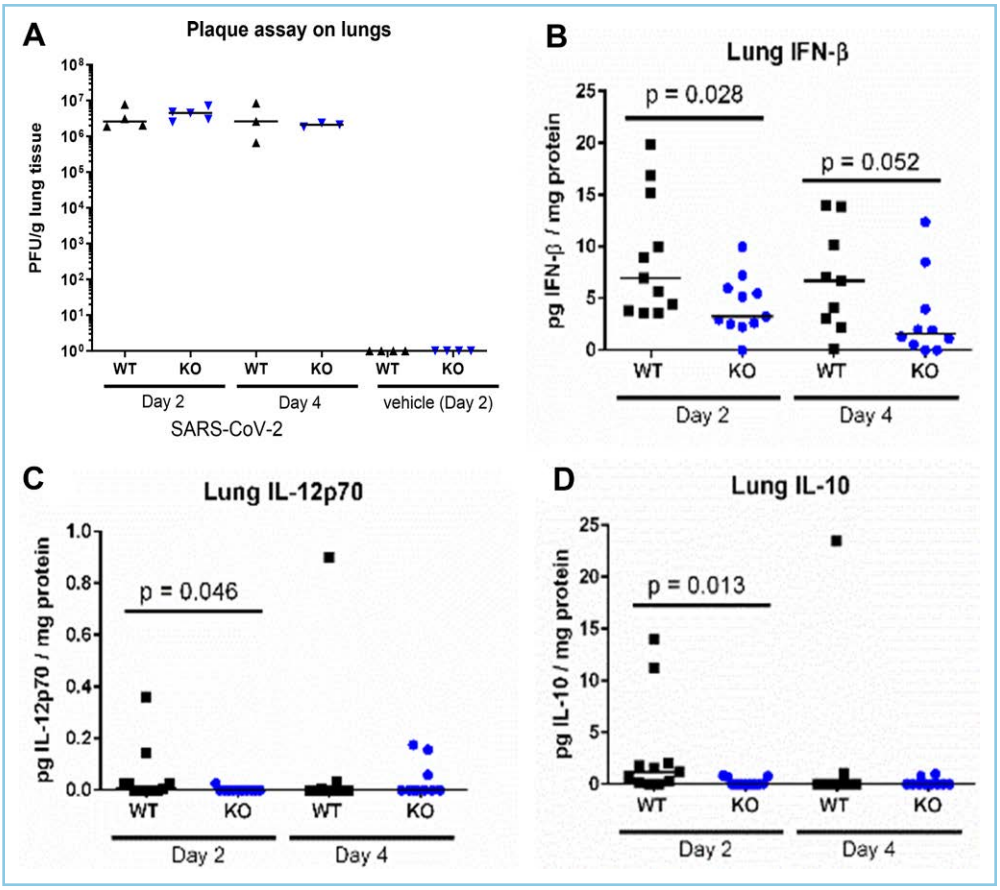

Figure 2. Interferon regulatory factor 3 (IRF3) alters the inflammatory cytokine profile in the lungs after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. IRF3-knockout (KO) and wild-type (WT) mice (both carrying the human angiotensin converting-enzyme 2 transgene driven by the keratin 18 promoter [K18-ACE2]) were infected with 2.5×10^4 plaque forming units of SARS-CoV-2 or saline vehicle as a control. Cohorts of animals were euthanized and their lung lobes collected at day 2 and day 4. *Graphs* show the levels of viral load (**A**), normalized to lung weight, and interferon beta (IFN-β) (**B**), interleukin (IL)-12p70 (**C**), and IL-10 (**D**), normalized to protein content. *Each point* represents the individual value for a single mouse lung lobe, and the *line* indicates the median in each group. *p* values show the result of a Wilcoxon rank-sum test.

others (22–26). We next investigated if IRF3 affected COVID-19 disease in this model. IRF3 knockout mice carrying the K18-ACE2 transgene had improved survival and a lower disease score vs. wild-type mice (**Fig. 1**). These results demonstrate a novel, detrimental role for IRF3 in COVID-19 pathogenesis in mice, akin to its role in sepsis (14, 16, 17). Our study represents a new key finding demonstrating how innate immunity influences severe COVID-19 disease.

Regarding the upstream pathways that activate IRF3, prior studies determined that SARS-CoV-2 activates TLR2, TLR4, TLR7, TLR9, and RLRs (1–7). These pathways converge upon myeloid differentiation primary response 88, IRF3, and IRF7 to induce an innate immune response (8). Our data also suggest the downstream mechanism whereby IRF3 exacerbates severe COVID-19. The IRF3 knockout mice exhibited lower levels of IFN-β, IL-12p70, and IL-10 vs. their wild-type counterparts (**Fig. 2**), and a trend toward lower levels of IL-1α, TNF-α, IL-23, IL-27, and GM-CSF (Fig. S2, http://links.lww.com/CCX/B479). Furthermore, cytokines were co-expressed in four distinct clusters based on a variable cluster analysis, suggesting hierarchical co-regulation by IRF3 (Fig. S3, http://links.lww.com/CCX/B479). We therefore predict that IRF3 exerts its effects by altering the cytokine network in a hierarchical fashion, and thereby promotes harmful inflammation, akin to its role in bacterial sepsis (17).

We observed a similar viral load in wild-type vs. IRF3 knockout mice (Fig. 2*A*), which may seem surprising given the classic role of IRF3 and IFN-β is to suppress viral infection. However, prior reports showed that SARS-CoV-2 manipulates IFN production, via cleavage of IRF3 and other mechanisms (9–12). We speculate that, due to manipulation of the host response by the virus, IFN-β is not avidly produced by infected cells. In contrast, we predict that once the viral load has built up, IRF3 becomes activated in noninfected cells that sense SARS-CoV-2 components, where the virus is unable to suppress IRF3 activation. Hence, IFN-β is produced at 2 days post-infection, at a time point that is too late to suppress viremia, and instead promotes harmful inflammation. This notion is consistent with a prior study in a non-lethal mouse model of SARS-CoV-2 infection, which

found that mice lacking the receptor for type 1 interferons (IFNAR) or IRF3/7 showed little difference in viral load, and reduced inflammation in the lungs (27). Our results are consistent with this prior report and go further in showing the effects of IRF3 on animal mortality.

Our study may help to explain the mixed results of clinical trials that administered type 1 IFN to patients with COVID-19. An early clinical trial suggested that IFN-β could shorten the time to negative culture when administered to COVID-19 patients in combination with antiviral therapy (28). In another small randomized clinical trial, IFN-β did not change the time to clinical response; however, early administration of IFN-β reduced mortality (29). The subsequent Solidarity trial showed that IFN had little effect on mortality, ventilation, or length of hospital stay, when used alone or in combination with lopinavir (30). Also, in the Adaptive COVID-19 Treatment trial, administration of IFN-β + remdesivir did not improve time to recovery vs. placebo + remdesivir (31). Furthermore, for patients who already required high-flow oxygen at enrollment, the group administered IFN-β had more serious adverse events vs. the placebo group (31). These clinical studies support the notion that IFN-β may exhibit both beneficial early effects and harmful late effects during COVID-19 disease.

CONCLUSIONS

This report showed that IRF3 plays a detrimental role in a mouse model of severe COVID-19. Further research is required to expand our understanding of the helpful and harmful effects of innate immunity on COVID-19 disease and reveal new therapeutic targets.

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1 *Department of Biomedical Sciences, Mercer University School of Medicine, Columbus, GA.*

2 *Department of Molecular and Translational Medicine, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center (TTUHSC) El Paso, El Paso, TX.*

3 *Department of Biological Sciences, University of Texas at El Paso (UTEP), El Paso, TX.*

4 *Department of Pathology, TTUHSC El Paso, El Paso, TX.*

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This work represents a collaboration between all the authors. Dr. Walker conceived the study, designed and performed experiments, and analyzed data. Drs. Garcia, Palermo, and Goswami performed experiments and analyzed data. Dr. Hakim analyzed lung histology. Dr. Dwivedi performed statistical and data analysis. Dr. Watts contributed to the conception and experimental design, performed experiments, and analyzed data. Dr. Walker wrote the article with contributions from all authors.

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For information regarding this article, E-mail: walker_w@mercer.edu

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