P58IPK: A Novel “CIHD” Member of the Host Innate Defense Response against Pathogenic Virus Infection

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Abstract

To support their replication, viruses take advantage of numerous cellular factors and processes. Recent large-scale screens have identified hundreds of such factors, yet little is known about how viruses exploit any of these. Influenza virus infection post-translationally activates P58IPK, a cellular inhibitor of the interferon-induced, dsRNA-activated eIF2α kinase, PKR. Here, we report that infection of P58IPK knockout mice with influenza virus resulted in increased lung pathology, immune cell apoptosis, PKR activation, and mortality. Analysis of lung transcriptional profiles, including those induced by the reconstructed 1918 pandemic virus, revealed increased expression of genes associated with the cell death, immune, and inflammatory responses. These experiments represent the first use of a mammalian infection model to demonstrate the role of P58IPK in the antiviral response. Our results suggest that P58IPK represents a new class of molecule, a cellular inhibitor of the host defense (CIHD), as P58IPK is activated during virus infection to inhibit virus-induced apoptosis and inflammation to prolong host survival, even while prolonging viral replication.

Introduction

Through a series of signaling mechanisms, the mammalian innate immune system recognizes and responds to pathogens to protect the host during infection. The response is initiated when pathogen-associated molecular patterns (PAMPs), present in microbial proteins or RNAs, engage cellular pathogen-recognition receptors (PRRs) such as RIG-I, MDA5, or the toll-like receptors [1]. Upon engagement, these PRRs activate proteins such as IRF3, IRF7, or the dsRNA-dependent activated protein kinase, PKR. Here, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

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P58IPK is a cellular inhibitor of PKR that is activated at the post-translational level in response to influenza virus infection [7]. The activation of P58IPK results in reduced levels of PKR-mediated eIF2α phosphorylation, which has long been thought to benefit influenza virus by maintaining a high rate of viral protein translation [8,9]. P58IPK is also activated at the transcriptional level in response to endoplasmic reticulum (ER) stress. During ER stress, P58IPK inhibits another eIF2α kinase, PERK, which functions to regulate protein synthesis during the unfolded protein response [10,11]. P58IPK also plays a larger role in the protein processing efficiency of the ER by binding to misfolded proteins and acting as a co-chaperone [12–14].

Recently, we showed that influenza virus infection of mouse embryonic fibroblasts (MEFs) lacking P58IPK results in increased eIF2α phosphorylation and decreased viral mRNA translation [8]. This effect was due to P58IPK functioning through a PKR-dependent mechanism that is independent of PERK. We also showed that P58IPK functions similarly during infection with vesicular stomatitis virus (VSV) or reovirus. These findings again suggested that influenza virus benefits from P58IPK activation, presumably to the detriment of the host.

Here, we sought to determine for the first time the role of P58IPK using an in vivo virus infection model. To this end, we infected mice lacking P58IPK with either the mouse-adapted A/PR/8/34 (PR8) strain or the reconstructed 1918 (r1918) pandemic influenza virus. We then examined viral replication, lung pathology, PKR and eIF2α phosphorylation, cytokine expression, and global gene transcriptional profiles. We found that influenza virus infections were more lethal in mice lacking P58IPK, due to...
increased lung pathology and inflammation. Therefore, just as P58IPK plays a role in restoring homeostasis during ER stress through its interaction with PERK, P58IPK may function during virus infection to restore homeostasis during the antiviral and inflammatory response through its interaction with PKR. Our findings suggest that P58IPK represents a new class of molecule that is activated during virus infection to help regulate the host antiviral response. While a number of host proteins exist which are crucial in determining the antiviral response, P58IPK is the first to be identified in a mammalian system which inhibits the defense response for proper development of the antiviral state. We coin this new class of regulators “Cellular Inhibitors of the Host Defense” (CIHDs).

Results

P58IPK−/− mice exhibit a higher influenza virus-induced mortality rate

To determine the role of P58IPK using a bona fide mammalian infection model, we infected P58IPK−/− and wild-type mice with a series of doses (10^1 to 10^5 PFU) of the PR8 strain of influenza virus. Although P58IPK−/− and wild-type mice exhibited similar weight loss (morbidity) over time (data not shown), mortality was markedly increased in P58IPK−/− mice. This phenotype was particularly pronounced at lower infectious doses (Figure 1A). When infected with 10^2 PFU, all P58IPK−/− mice died by day 5, whereas only 33% of wild-type mice died by day 6, and the others recovered. This corresponded to a fifty percent lethal dose (LD_{50}) of 10^1.3 PFU for P58IPK−/− mice and 10^2.0 PFU for wild-type mice. The increased mortality in P58IPK−/− mice was not due to increased viral load, since viral titers in the lungs of all infected mice were similar at each time point (Figure 1B). Furthermore, mortality was not caused by viral attachment or initial infection alone, since experiments with a replication-deficient virus (PR8ΔNS1) [15] did not produce disease in either mouse genotype (data not shown).

While both P58IPK−/− and wild-type mice exhibited great lung pathology when infected with high doses of PR8, microscopic examination revealed increased lung pathology in P58IPK−/− mice, as compared to wild-type mice, infected with a lower dose of PR8. This was marked by moderate to severe alveolitis and peribronchiolitis, macrophage and neutrophil infiltration, and associated hemorrhage and edema (Figure 2A). By contrast, wild-type mice had mild alveolitis and peribronchiolitis with lower levels of neutrophils, where infiltration of inflammatory cells is scant (Figure 2B). Alveolitis was observed as early as day 1 post infection in P58IPK−/− mice and inflammation increased in severity until day 5 (Figure S1). Other histological features which were more pronounced in P58IPK−/− mice were multifocal intraluminal inflammation with necrotic debris and the formation of hyaline membranes adjacent to alveolar walls (Figure 2C–D). Staining for the macrophage marker F4/80 revealed the presence of greatly increased numbers of macrophages in the lungs of infected P58IPK−/− mice as compared with wild-type mice (Figure 2E–F). The presence of macrophages was observed by day 1 post infection in P58IPK−/− mice, whereas their presence was not detected until day 3 post infection in wild-type mice (Figure S2).

Even though there was increased pathology in the lungs of infected P58IPK−/− mice, a similar number of cells stained positive for influenza virus NP in both P58IPK−/− and wild-type mice (Figure 2G–H) throughout the course of infection (Figure S3). Thus, differences in pathology did not appear to be due to differences in the amount of virus present in lung cells.

Influenza virus infection can induce apoptosis [16], and more specifically, the caspase cascade can be activated through the
expression of IFN [17]. Therefore, to determine if apoptosis was induced in the lungs of P58IPK$^{-/-}$ mice, we looked for the presence of cleaved caspase 3. Caspase 3 is downstream of cleaved caspase 8, and it cleaves other caspases as well as poly(ADP) ribose polymerase [18]. This analysis revealed increased staining of cleaved caspase 3 in the lungs of P58IPK$^{-/-}$ mice compared with that detected in wild-type mice (Figure 2I–J), but no cleaved caspase 3 was observed in the lungs of mock-infected animals (Figure S4). Increased apoptosis in the lungs of P58 IPK$^{-/-}$ mice is consistent with our previous findings that P58 IPK has anti-apoptotic properties [19]. Our observation of increased apoptosis and mortality in mice lacking P58 IPK is also consistent with previous reports of increased apoptosis being a marker for fatal influenza virus infection [20].

P58IPK$^{-/-}$ mice exhibit increased levels of eIF2α and PKR phosphorylation in response to infection

Influenza virus infection of MEFs devoid of P58IPK results in increased PKR activation and eIF2α phosphorylation relative to wild-type MEFs [8]. In the present study, we determined the amount of eIF2α and PKR phosphorylation in the lungs of P58IPK$^{-/-}$ and wild-type mice infected with PR8. We observed increased eIF2α phosphorylation in the lungs of influenza virus-infected P58IPK$^{-/-}$ mice throughout infection (Figure 3A).
Quantification of the results showed that eIF2α phosphorylation was significantly increased in P58IPK−/− mouse lungs at days 1 and 3 post infection (Figure 3B). Increased and prolonged eIF2α phosphorylation is reported to lead to apoptosis [21], which is consistent with our findings of increased levels of caspase 3 activation and mortality in mice lacking P58IPK. Further investigation revealed that there was increased PKR phosphorylation (Figure 3C–D), but not PERK phosphorylation (data not shown), in the absence of P58IPK, resulting in increased eIF2α phosphorylation.

P58IPK−/− mice exhibit increased levels of inflammatory and immune response genes early after infection

Our traditional virology, histology, and biochemical approaches indicated that influenza virus infection of P58IPK−/− mice was associated with increased lung pathology characterized by macrophage infiltration, apoptosis, and increased levels of eIF2α phosphorylation. Each of these effects have been associated with severe influenza virus infection [20,22,23]. In order to discover potentially new mechanisms that might contribute to increased mortality in mice lacking P58IPK, we used oligonucleotide microarrays to profile the host transcriptional response to infection.

For our microarray analyses, P58IPK−/− and wild-type mice were infected intranasally with 10⁴ PFU of PR8, or mock-infected with PBS alone, and sacrificed at days 1, 3, and 5 post infection. RNA was then isolated from left lung lobes for gene expression profiling. Analyses were performed by comparing RNA isolated from each individual animal against a pool of RNA from genotype-matched mock-infected animals. To perform a direct comparison of gene expression profiles of P58IPK−/− and wild-type mice, we used the re-ratio tool in Rosetta Resolver. This tool creates new ratio experiments from two or more existing ratio experiments that share a common reference, therefore removing the reference and focusing on the difference between two conditions. Next, working with one set of gene expression data at each time point, we used Gene Set Enrichment Analysis (GSEA) [24] to identify sets of genes that were differentially expressed between the P58IPK−/− and wild-type mice.

GSEA determines how two distinct phenotypes differ in their gene expression profiles by ranking the significance of the gene ontology (GO) sets identified. Given a defined set of genes annotated with a certain GO function, GSEA determines whether the members of that set are randomly distributed throughout the ranked list or if they are found primarily at the top or bottom of that list. Those sets at the top or bottom of the list describe the phenotypic distinction between the two sets [24]. While traditional GO analysis uses a preselected set of genes created using an arbitrary fold-change or P-value cutoff, GSEA examines the entire gene set [25].

We found that GO categories at the top of the day 3 list were characterized by the negative regulation of metabolic processes, whereas those at the top of the day 5 list included a random assortment of biochemical processes (Figure S5 and Figure S6). The top of the GSEA list for day 1 included many processes...
associated with the immune and inflammatory responses (Figure S7). A subset of GO categories, highlighted in Figure S7, is shown in Figure 4, and these categories are connected by edges, the thickness of which corresponds to the number of overlapping genes in each category. The gene expression data showed a dramatic increase in chemokine expression in P58IPK−/− mice, which likely resulted in an exaggerated inflammatory response [26]. Although some gene expression values were low, even small changes in gene expression can have a drastic effect on biological function [27]. In addition, GSEA indicated that the GO categories were significantly perturbed, even if the genes within the categories were not highly regulated. Together, our gene expression analyses showed that the absence of P58IPK during influenza virus infection caused an increase in inflammatory, immune response, and cell death-related genes at early times post infection, likely prognostic of the eventual increased lung pathology and ultimately death.

P58IPK−/− mice exhibit increased levels of IL-6 and IFNβ in response to infection

Since genomic profiling indicated an increased inflammatory response in P58IPK−/− mice, we next evaluated whether mice lacking P58IPK exhibited increased cytokine abundance at the protein level. One important cytokine that has been shown to be over-expressed during fatal influenza virus infection is interleukin-6 (IL-6) [28]. Using an enzyme-linked immunosorbent assay (ELISA), we found that IL-6 levels were increased in the lungs of P58IPK−/− mice compared with wild-type animals throughout the course of infection (Figure 5A). Furthermore, we detected increased levels of IL-6 in the serum of infected P58IPK−/− mice at day 5 post infection (Figure 5B). This increase in IL-6 protein in the serum and lungs of infected P58IPK−/− mice was accompanied by a drastic increase in IL-6 mRNA (Figure 5C). Furthermore, levels of IFNβ mRNA were significantly increased in the lungs of P58IPK−/− mice at day 3 post infection (Figure 5D). IFNβ induction results in the increased production of IL-6 [29], and together, the expression of these two cytokines suggests that a greater IFN and cytokine response contributes to the increased lung pathology and higher mortality rate associated with influenza virus infection of P58IPK−/− mice.

Pandemic influenza virus infection results in an amplified inflammatory response in P58IPK−/− mice

In addition to using P58IPK−/− mice to determine the role of P58IPK during influenza virus infection, we were also interested in determining whether these mice could provide new insight into the unusually high virulence of the virus responsible for the 1918 influenza pandemic. Infection with 5 × 10^6 PFU of r1918 resulted in the death of all P58IPK−/− mice by day 4 post infection and the death of all wild-type animals by day 7 (Figure 6A). This corresponded to an LD50 of 101.8 PFU for P58IPK−/− mice and 102.5 PFU for wild-type mice. Similar to PR8, wild-type mice required five times the dose of r1918 to achieve fifty percent mortality, as compared to P58IPK−/− mice. The increased mortality of P58 IPK−/− mice was again not due to differences in

![Figure 4. Inflammatory response genes are more up-regulated in influenza virus-infected P58IPK−/− mice at 1 day post infection.](image-url)

Gene Set Enrichment Analysis (GSEA) analysis was performed on a sample set derived from P58IPK−/− and wild-type mice infected with the PR8 strain of influenza virus in triplicate at 1 day post infection. All infected samples were compared to genotype-matched mock-infected samples via microarrays analysis. Replicate samples were then in silico error-weighted pooled and re-ratioed to compare P58IPK−/− gene expression to wild-type gene expression. Following GSEA analysis using only those genes which were significantly regulated, the top gene ontology categories related to the immune response were selected (see Figure S7 for entire table). An edge is placed between gene ontology categories if they share common genes, and the edge’s thickness increases as the number of common genes increases. The log10 ratio of P58IPK to wild-type gene regulation is noted for selected gene ontology categories.
viral load, since viral titers were nearly identical in P58IPK^2/2 and wild-type mice (Figure 6B). Microscopic examination revealed lung pathology similar to that reported previously [22,30].

We then used global gene expression profiling to determine the transcriptional response of P58IPK^2/2 mice to infection with r1918 and to evaluate similarities and differences in the host response to r1918 and PR8. We began our analysis by comparing lung gene expression profiles of r1918-infected and mock-infected P58IPK^2/2 mice and selecting for genes that were expressed at a higher level during virus infection. We then determined which genes among this group were expressed at a lower level in r1918-infected wild-type mice. Because these genes were induced in P58IPK^2/2 mice in response to virus infection, but were expressed at a lower level in virus-infected wild-type mice, the infection-induced expression of these genes appears to be impacted by the presence or absence of P58IPK.

This same analysis path was then used to analyze the gene expression data generated from the PR8 infections described above, again resulting in a set of infection-induced genes, the expression of which appears to be impacted by the presence or absence of P58IPK. Of the annotated genes in this set, 65 were also present in the final set of genes identified from the r1918 virus infection data. The majority of these genes (47 of 65) are associated with inflammatory, immune response, and cell-death pathways and their expression patterns in P58IPK^2/2 and wild-type mice are shown in Figure 7A. Because these genes were all expressed at a higher level in P58IPK^2/2 mice, they may therefore be associated with the increased pathology and mortality rate observed in these animals. However, of particular interest are the set of genes present in the middle panel of this Figure, which were induced in P58IPK^2/2 mice in response to infection with either PR8 or r1918, but which were induced in wild-type mice only in response to the r1918 virus. To evaluate the functional relationships of these genes more closely, we used Ingenuity Pathways Analysis (IPA) to create a network of the set of genes in the middle of Figure 7A, showing direct or indirect interactions reported for these cell-death- and inflammatory-response-related genes (Figure 7B). The network is centered around tumor necrosis factor (Tnf), which is interesting because activation of Tnf-related pathways has been shown to be associated with H5N1 influenza viral infections of macrophages [31]. This pattern of expression suggests that these genes may also contribute to the exceptional virulence of the r1918 virus, but,
reports, we demonstrated that P58IPK is activated upon infection to protect the host during influenza virus infection. In previous studies, we also showed that cells devoid of P58 IPK exhibit a heightened inflammatory response marked by increased lung pathology and apoptosis. Furthermore, at the lowest doses of influenza virus, wild-type mice recovered from infection, suggesting that P58IPK benefits the host during infection.

Our findings provide the first direct evidence that P58IPK is a regulator of the host innate immune response repertoire and that activation of P58IPK results in decreased lung pathology and host mortality. A model describing these results is presented in Figure 6, which is divided into two categories: the apoptotic and inflammatory responses. P58IPK functions through each via its inhibition of PKR. P58IPK inhibits the apoptotic response via its inhibition of PKR-mediated caspase activation and eIF2α phosphorylation. P58IPK also inhibits the inflammatory response via its inhibition of PKR-mediated NF-kB activation, which ultimately activates IFN-stimulated genes. Therefore, P58IPK is necessary to counterbalance the activities of PKR, and together, the over-activation of pathways downstream of PKR results in increased host pathology and mortality [20,29]. While our analysis revealed that neither PERK nor its downstream targets were activated during influenza virus infection, we regard these negative results as inconclusive due to the difficulty in performing these experiments, especially with regard to sample preparation [34].

Since influenza virus hemagglutinin has been shown to cause ER stress [35], we are performing experiments with more sensitive assays to look for markers of ER stress in influenza virus-infected mice.

There is evidence of a connection between PKR and proteins involved in apoptosis, namely FADD and caspase 8 [36,37], and PKR may engage a death receptor that is upstream of caspase 8 activation and apoptosis. Although a definite connection between PKR and FADD-mediated caspase 8 activation remains to be determined, apoptosis is induced by the over-expression of PKR [38]. Additionally, caspase 8 activation results in caspase 3 activation, resulting in apoptosis [39]. Fas ligand (FasL), Tnf, and caspases 1 and 7, which activate caspases 8 and 3 [40–43], were identified in our microarray analysis as being more up-regulated in P58IPK−/− mice during influenza virus infection. We also previously demonstrated that P58IPK is anti-apoptotic, as nude mice injected with cells over-expressing P58IPK undergo malignant transformation [19]. Finally, P58IPK−/− mice, which have basal levels of ER stress [12,13], exhibit increased caspase 3 activation and apoptosis of β-cells in the pancreas [44]. We have now shown that the stimulus of influenza virus infection in lung tissue contributes to increased apoptosis in the absence of P58IPK. This was marked by increased and prolonged eIF2α phosphorylation [21] and increased caspase 3 activation in the lungs of these mice.

With respect to the inflammatory response arm of the model, PKR activates NF-κB, which in turn activates Fosp3, IFNβ, and IFNγ, and a number of chemokines [18,45,46]. Each of the downstream NF-κB responses shown in Figure 8, among many others, was up-regulated in P58IPK−/− mice as compared with wild-type mice. Again, this is likely due to uncontrolled activation of PKR in the absence of P58IPK. Treatment of bronchial epithelial cells with dsRNA activates both PKR and NF-κB, which results in the secretion of pro-inflammatory interleukins and chemokines [47]. This response could lead to increased lung pathogenesis during a viral infection, which was a striking difference between influenza virus-infected P58IPK−/− and wild-type mice. While only one lung lobe was analyzed for each mouse, and neither step sectioning nor bronchoalveolar lavages were performed, pathogenesis in P58IPK−/− mice was marked by increased macrophage infiltration and hyaline membrane formation. Hyaline membranes obstruct the exchange of oxygen across the alveolar walls and is characteristic of diffuse alveolar damage [48]. It is also a characteristic sign of lethal influenza virus

**Discussion**

In this study, we demonstrate that P58IPK plays a novel role in regulating the antiviral and inflammatory responses to influenza virus infection. Here, we have specifically shown, through detailed high-throughput and biochemical analysis, how P58IPK may protect the host during influenza virus infection. In previous reports, we demonstrated that P58IPK is activated upon infection with influenza virus [32] and that P58IPK binds to and inhibits PKR [7,33]. We also showed that cells devoid of P58IPK exhibit higher levels PKR activation and eIF2α phosphorylation, yet lower levels of viral mRNA translation during influenza virus infection [8]. Together, these results suggest that P58IPK activation is required for more efficient influenza virus replication in vitro and that the virus usurps P58IPK activation to the detriment of the host. However, our current study has expanded and changed our understanding of how the activation of P58IPK impacts viral replication since we have now shown that the absence of P58IPK did not affect levels of viral load in vivo, although we did not examine rates of viral replication. Nevertheless, influenza virus infection in P58IPK−/− mice resulted in increased mortality due to a heightened inflammatory response marked by increased lung

![Figure 6. P58IPK−/− mice exhibit increased mortality to pandemic influenza virus infection yet similar levels of virus replication.](Image)

(A) Three P58IPK−/− and wild-type mice were infected with 10^5 or 5 × 10^5 PFU of the r1918 strain of influenza virus. The number of surviving mice, as determined by having at least 20% of starting weight, at each day is shown. (B) Three P58IPK−/− and wild-type mice infected with 10^6 PFU of r1918 were sacrificed at 3 days post infection. Levels of infectious virions in diaphragmatic lung homogenates were determined by triplicate plaque assay on MDCK cells. The results represent the mean activity of 3 independent samples ± standard deviation. doi:10.1371/journal.ppat.1000438.g006

more importantly, they may contribute to the virus’ exceptional virulence in animals lacking P58IPK.
Both PR8 and r1918 infections result in an exaggerated inflammatory response in infected P58IPK<sup>2/2</sup> mice. (A) Venn diagram analysis was performed on a sample set derived from P58IPK<sup>2/2</sup> and wild-type (WT) mice infected with the PR8 or r1918 strains of influenza virus in triplicate at 1 day post infection. All infected samples were compared to genotype-matched mock-infected samples via microarrays analysis. Replicate samples were then pooled and error-weighted <i>in silico</i>. For each virus, a gene set was isolated which included genes which were up-regulated in P58IPK<sup>2/2</sup> mice as compared to mock and as compared to wild-type mice. The intersection of the sets for both PR8 and r1918 infection was isolated for IPA. Of the 113 genes in this set, 65 had annotation, and 47 of those had functions related to the inflammatory and cell death responses; log<sub>10</sub> ratio regulation of these genes in P58IPK<sup>2/2</sup> and wild-type mice as compared to mock is represented for both viral infections. The bar graph represents the log<sub>10</sub> ratio of P58IPK<sup>2/2</sup> to wild-type gene regulation, showing that each gene is more up-regulated in P58IPK<sup>2/2</sup> mice. (B) IPA network analysis of the middle set of genes in (A) highlights a subset of genes that are up-regulated in wild-type mice during r1918 infection but not PR8 infection. This diagram shows the direct or indirect interactions reported for these cell-death- (blue shading) and inflammatory-response-related (yellow shading) genes. Genes shaded orange fit into both categories.

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infections in cynomolgus monkeys [49]. In culture, influenza virus infection of macrophages stimulates the release of IL-6 [50], and we observed an increase in IL-6 mRNA and protein levels in the lungs of virus-infected P58IPK \( \frac{-}{-} \) mice due to increased macrophage infiltration in the lungs of these animals. Increased levels of IL-6 have also been associated with fatal influenza virus infections [51,52], which is consistent with the increased mortality observed in P58IPK \( \frac{-}{-} \) mice. This is especially significant because it was at day 5 post infection, when IL-6 levels were highest, that mice lacking P58 IPK died from infection. IFN-\( \beta \) expression has been shown to be correlated with IL-6 expression during influenza virus infection and severe acute respiratory syndrome (SARS)-coronavirus infection [53,54], and IFN-\( \beta \) expression was also elevated in influenza virus-infected P58IPK \( \frac{-}{-} \) mice.

Antiviral and inflammatory responses are typically necessary to clear the virus and to recover from infection [55]. However, during infection with a virus such as Ebola virus, the marked overexpression of genes associated with the immune and inflammatory response may contribute to disease pathology and death [56,57]. We have also shown that infection of mice or macaques with H5N1 avian influenza viruses, or the 1918 pandemic virus, results in rapid disease and death, most likely due to an early and dysregulated host inflammatory response, which is associated with severe lung disease and mortality [22,31,52,58,59]. Our current genomic profiling data suggest a similar phenomenon is occurring in P58IPK \( \frac{-}{-} \) mice infected with influenza virus, where the significant expression of immune response-related genes at 1 day post infection was correlated with eventual death of the animal. In contrast, the expression profiles from wild-type mice exhibited increased expression of inflammatory response genes, but not until later times post infection, which was correlated with prolonged survival. Not only did these observations hold for the mouse-adapted PR8 virus, but also for the human 1918 pandemic strain. This is noteworthy, because two strains of influenza virus, each with different species specificity, produced the same disease phenotype in P58IPK \( \frac{-}{-} \) mice via the same pathway. Furthermore, we identified a subset of genes, focused largely around Tnf and matrix metallopeptidase 3 (Mmp3), which were up-regulated in wild-type mice only during 1918 infection and not PR8 infection. Although it has been shown that Tnf activation increases the expression of Mmp3 [60], and that matrix metallopeptidases are up-regulated in airway epithelium disease in which macrophages and eosinophils are highly present [61], it has not previously been shown that Mmp3 activation is a function of influenza virus infection. This suggests that even by 1 day post infection, lung cells are undergoing a remodeling process which will ultimately lead to pathogenesis and mortality, by a potentially novel pathway, during pandemic influenza virus infection.

Together, our results show that P58IPK plays an important role in regulating the innate immune response, as its presence results in decreased apoptotic and inflammatory responses, while its absence results in increased apoptotic and inflammatory responses and increased mortality.
a more controlled response to influenza virus infection. While P58IPK benefits the host through reduced lung pathology and prolonged survival, it also provides the virus with additional time to replicate and spread among other hosts. The results of our study are therefore somewhat different than those observed in plant virus infection models, where plants lacking P58IPK also exhibited increased host death. Since plants lacking P58IPK exhibit lower levels of viral replication, it was concluded that P58IPK is required for virulence and acts as a susceptibility factor [9]. However, unlike animals, plants do not invoke cell death as part of their immune response to virus infection. Since we have shown that in wild-type mice P58IPK functions to modulate the innate immune, inflammatory, and cell death responses during viral infection, resulting in decreased pathology and a lower mortality rate, activation of mammalian P58IPK actually functions to reduce the virulence of influenza virus infection. Thus, we have identified P58IPK as a new class of molecule that is activated during virus infection to inhibit the over-activation of inflammatory and cell death responses in order to prolong host survival. Further studies as to how to modulate the P58IPK pathway, and thus the host’s antiviral and inflammatory response, may lead to the discovery of novel therapeutics for targeted intervention during a pandemic influenza virus infection.

Materials and Methods

**P58IPK−/− mice, viruses, and cells**
P58IPK−/− mice were 100% C57BL/6 (from a C57BL/6 embryonic stem cell line) and were maintained on the C57BL/6 background by alternate generation backcrossing as previously described [44]. Mice were maintained in a specific pathogen-free barrier facility with standard rodent diet and 12 h alternating light and dark cycles. The PR8 strain of influenza virus was grown in 10-day-old embryonated chicken eggs [62]. The r1918 influenza virus was generated as previously described [22,30]. Plaque assays were performed using Madin–Darby canine kidney (MDCK) cells grown as monolayers in high glucose Dulbecco’s modified Eagle’s medium supplemented to contain 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin G (50 units/ml), and streptomycin sulfate (50 μg/ml).

**Mouse infections**
Ten- to twelve-week-old P58IPK−/− or wild-type C57BL/6 mice (Charles River Laboratories) were anesthetized with isoflurane and infected intranasally with 106 to 107 plaque-forming units (PFU) of the PR8 strain of influenza virus. On days one, three, and five post infection, three mock-infected and three P58IPK−/− or wild-type mice infected with 106 or 104 PFU were sacrificed. Remaining animals were weighed each day for ten days post infection and sacrificed when they lost at least 20% of their starting body weight. Blood and lung tissue was collected from each mouse at the time of sacrifice. All experiments were performed in a specially separated negative-pressure HEPA (high-efficiency particulate air) filtered biosafety level 2 laboratory. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the University of Washington Institutional Animal Care and Use Committee. Infections of P58IPK−/− and wild-type C57BL/6 mice with r1918 was performed as previously described [22].

**Plaque assay and protein analysis**
Diaphragmatic lung lobes from each animal were weighed, homogenized in PBS, and samples were then assayed in triplicate for viral yield by standard plaque assay on MDCK cells. Viral yields were calculated according to the formula: \( \text{yield}_{t=x} = \left( \frac{\log_{10} \text{PFU/ml}_{t=0}}{\log_{10} \text{PFU/ml}_{t=x}} \right) \), where \( t \) is time and \( x \) is the time post infection.

**Histopathological, immunohistochemical, and statistical analysis**
Cardiac lung lobes were excised, perfused, and fixed in >10 volumes of 10% neutral-buffered formalin (Fisher Scientific) for 48 h. Paraffin embedding, sectioning, and staining were performed by the Experimental Histopathology Shared Resources at the Fred Hutchinson Cancer Research Center (Seattle, WA). Four micron sections of lung were stained with H&E and PAS by standard methods. Sections were also immunostained for cleaved caspase 3 and macrophage marker F4/80 as described [63]. Influenza virus NP was detected with a rabbit polyclonal antibody (a kind gift from Adolfo García-Sastre) at 1:8000 and using the Quantikine assay kit (R&D Systems) as described by their protocol.

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P58IPK−/− mice were 100% C57BL/6 (from a C57BL/6 embryonic stem cell line) and were maintained on the C57BL/6 background by alternate generation backcrossing as previously described [44]. Mice were maintained in a specific pathogen-free barrier facility with standard rodent diet and 12 h alternating light and dark cycles. The PR8 strain of influenza virus was grown in 10-day-old embryonated chicken eggs [62]. The r1918 influenza virus was generated as previously described [22,30]. Plaque assays were performed using Madin–Darby canine kidney (MDCK) cells grown as monolayers in high glucose Dulbecco’s modified Eagle’s medium supplemented to contain 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin G (50 units/ml), and streptomycin sulfate (50 μg/ml).

**Mouse infections**
Ten- to twelve-week-old P58IPK−/− or wild-type C57BL/6 mice (Charles River Laboratories) were anesthetized with isoflurane and infected intranasally with 106 to 107 plaque-forming units (PFU) of the PR8 strain of influenza virus. On days one, three, and five post infection, three mock-infected and three P58IPK−/− or wild-type mice infected with 106 or 104 PFU were sacrificed. Remaining animals were weighed each day for ten days post infection and sacrificed when they lost at least 20% of their starting body weight. Blood and lung tissue was collected from each mouse at the time of sacrifice. All experiments were performed in a specially separated negative-pressure HEPA (high-efficiency particulate air) filtered biosafety level 2 laboratory. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the University of Washington Institutional Animal Care and Use Committee. Infections of P58IPK−/− and wild-type C57BL/6 mice with r1918 was performed as previously described [22].

**Plaque assay and protein analysis**
Diaphragmatic lung lobes from each animal were weighed, homogenized in PBS, and samples were then assayed in triplicate for viral yield by standard plaque assay on MDCK cells. Viral yields were calculated according to the formula: \( \text{yield}_{t=x} = \left( \frac{\log_{10} \text{PFU/ml}_{t=0}}{\log_{10} \text{PFU/ml}_{t=x}} \right) \), where \( t \) is time and \( x \) is the time post infection.

**Histopathological, immunohistochemical, and statistical analysis**
Cardiac lung lobes were excised, perfused, and fixed in >10 volumes of 10% neutral-buffered formalin (Fisher Scientific) for 48 h. Paraffin embedding, sectioning, and staining were performed by the Experimental Histopathology Shared Resources at the Fred Hutchinson Cancer Research Center (Seattle, WA). Four micron sections of lung were stained with H&E and PAS by standard methods. Sections were also immunostained for cleaved caspase 3 and macrophage marker F4/80 as described [63]. Influenza virus NP was detected with a rabbit polyclonal antibody (a kind gift from Adolfo García-Sastre) at 1:8000 and using the protocol described for cleaved caspase 3 without antigen retrieval.

To score lung inflammation and damage, a semi-quantitative scoring system was used; for this, the entire cardiac lobe surface was analyzed with respect to the following parameters: alveolitis, peribronchiolitis, and perivasculitis. Each parameter was graded on a scale of 0–4 with 0 as “absent,” 1 as “slight,” 2 as “mild,” 3 as “moderate,” and 4 as “severe.” The total “lung inflammation score” for each mouse lung lobe was determined as the sum of the scores for each parameter, the maximum being 12. The average, standard deviation, and P-values from a two-tailed t-test assuming non-equal variance were determined for each set of triplicate mice for each genotype. Representative micrographs from the most significant doses are presented for both genotypes.
To score immunohistochemical staining, the same method described above was used to score the entire cardiac lobe surface, except that a scoring system of 0–3 was used with 0 as “none,” 1 as “infrequent,” 2 as “common,” and 3 as “widespread.”

**Quantitative RT-PCR**

Following animal sacrifice, the left lung lobe was homogenized in Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol), and total RNA was isolated using RNeasy (Qiagen). The quantity of total RNA was determined by spectrophotometry using a NanoDrop ND-1000 Fluorospectrometer. Contaminating DNA was removed by treating samples with RNase-free DNase and removal reagents (Ambion). Reverse transcription was performed using TaqMan reverse transcription reagents (Applied Biosystems). RT-PCR was performed as previously described [64]. Each target was run in quadruplicate, with 100 ng of sample in reaction volumes of TaqMan 2× PCR Universal Master Mix (Applied Biosystems). Genome copy numbers were normalized to β-actin values determined in parallel using Taqman gene expression assay endogenous control primer-probe sets (Applied Biosystems). Quantification of each gene, relative to the calibrator, was calculated by the instrument, using the equation 2^(-ΔΔCt) within the Applied Biosystems Sequence Detection Software version 1.3. The minor groove binding probe and primer sets for each gene were part of an Applied Biosystems assay set as follows: Mouse IL6: Mm00446190_m1; Mouse IFNβ: Mm00439546_s1.

**Expression microarray analysis and bioinformatics**

Amplification of mRNA was performed as described previously using equal masses of total RNA isolated from left lung lobes of infected mice [57]. An equal-mass pool of mRNA isolated from the lungs of five individual mock-infected mice was prepared as a reference sample. Microarray slide hybridization was performed using mouse oligonucleotide genome CGH arrays (G4426B; Agilent Technologies). For each infection group, expression oligonucleotide array analysis was performed using RNA isolated from lung tissue from three individual animals. The data presented are the error-weighted average changes in expression calculated from four technical replicate arrays performed on three individual mice. All data were entered into a custom-designed relational database and subsequently uploaded into Rosetta Resolver System 7.1 (Rosetta Biosoftware), Spotfire Decision Site 9.1 (Spotfire/Tibco), or Ingenuity Pathways Analysis (Ingenuity Systems, Inc.). Primary microarray data are available at the Fred Hutchinson Cancer Research Center for expert advice and histological and immunohistochemical services.

**Supporting Information**

**Figure S1** Pathology increases at a greater rate in mice lacking P58IPK, P58IPK−/− and wild-type mice were mock infected or infected with 10^3 PFU of the PR8 strain of influenza virus. At 1, 3, and 5 days post infection, cardiac lung lobes were excised and fixed in 10% neutral-buffered formalin. Lobes were paraffin embedded, sectioned, and stained for hematoxalin and eosin. Bar = 50 μm.

**Figure S2** Macrophage infiltration occurs at a greater rate in mice lacking P58IPK, P58IPK−/− and wild-type mice were mock infected or infected with 10^3 PFU of the PR8 strain of influenza virus. At 1, 3, and 5 days post infection, cardiac lung lobes were excised and fixed in 10% neutral-buffered formalin. Lobes were paraffin embedded, sectioned, and stained for the macrophage marker, F4/80. Bar = 50 μm.

**Figure S3** Mice lacking P58IPK do not exhibit increased levels of viral protein throughout infection. P58IPK−/− and wild-type mice were mock infected or infected with 10^3 PFU of the PR8 strain of influenza virus. At 1, 3, and 5 days post infection, cardiac lung lobes were excised and fixed in 10% neutral-buffered formalin. Lobes were paraffin embedded, sectioned, and stained for influenza virus NP. Bar = 50 μm.

**Figure S4** Caspase 3 is activated in mice lacking P58IPK during influenza virus infection. P58IPK−/− and wild-type mice were mock infected or infected with 10^3 PFU of the PR8 strain of influenza virus. At 1, 3, and 5 days post infection, cardiac lung lobes were excised and fixed in 10% neutral-buffered formalin. Lobes were paraffin embedded, sectioned, and stained for cleaved caspase 3. Bar = 50 μm.

**Figure S5** Day 3 Gene Set Enrichment Analysis (GSEA) table. Gene ontology categories are shown if there are 3 or more genes in the category. Categories highlighted in grey represent those discussed in the text.

**Figure S6** Day 5 Gene Set Enrichment Analysis (GSEA) table. Gene ontology categories are shown if there are 3 or more genes in the category.

**Figure S7** Day 1 Gene Set Enrichment Analysis (GSEA) table. GSEA table from the analysis described in Figure 4. Gene ontology categories are shown if there are 3 or more genes in the category. Categories highlighted in grey are used for the analysis in Figure 4.

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**Author Contributions**

Conceived and designed the experiments: AGG JLF GRM LAP TMT. Performed the experiments: AGG JLF GRM LAP VSC TMT. Analyzed the data: AGG JLF GRM LAP XP MDD SCP SEK TMT. Contributed reagents/materials/analysis tools: GRM LAP JAN TMT. Wrote the paper: AGG MJK MGK.

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