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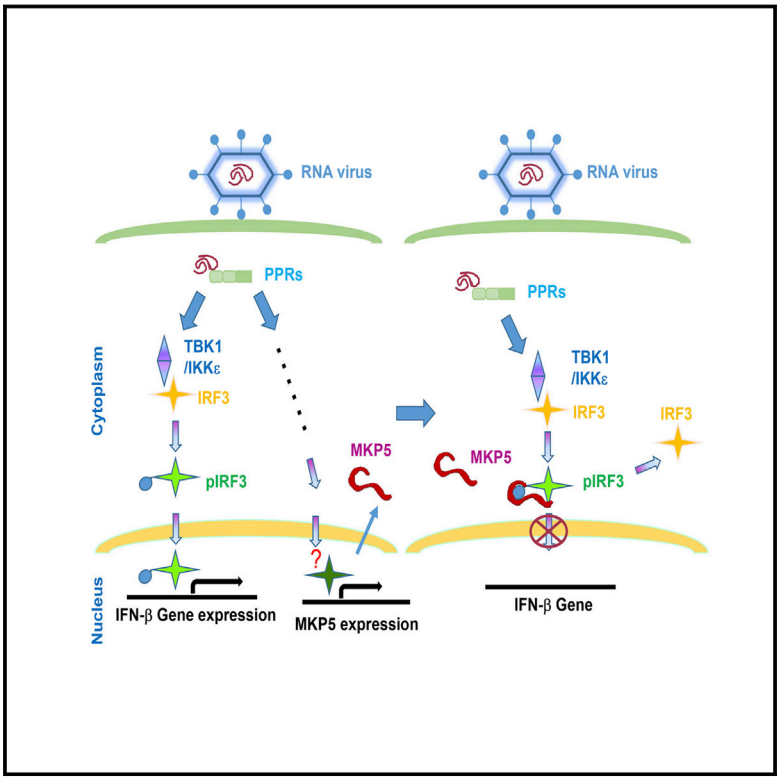
Graphical Abstract



Highlights

- The expression of MKP5 is induced by influenza and other RNA virus infection
- MKP5 directly binds to and inactivates IRF3 to inhibit type I interferon response
- MKP5-deficient mice are resistant to influenza infection compared with WT mice

Authors

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In Brief

James et al. demonstrate that RNA viruses, including influenza virus, VSV, and SeV, induce MKP5 expression to negatively regulate IRF3 activation, thereby suppressing type I interferon response.

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MAPK Phosphatase 5 Expression Induced by Influenza and Other RNA Virus Infection Negatively Regulates IRF3 Activation and Type I Interferon Response

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SUMMARY

The type I interferon system is essential for antiviral immune response and is a primary target of viral immune evasion strategies. Here, we show that virus infection induces the expression of MAPK phosphatase 5 (MKP5), a dual-specificity phosphatase (DUSP), in host cells. Mice deficient in MKP5 were resistant to H1N1 influenza infection, which is associated with increased IRF3 activation and type I interferon expression in comparison with WT mice. Increased type I interferon responses were also observed in MKP5-deficient cells and animals upon other RNA virus infection, including vesicular stomatitis virus and sendai virus. These observations were attributed to the ability of MKP5 to interact with and dephosphorylate IRF3. Our study reveals a critical function of a DUSP in negative regulation of IRF3 activity and demonstrates a mechanism by which influenza and other RNA viruses inhibit type I interferon response in the host through MKP5.

INTRODUCTION

Influenza viruses are respiratory pathogens that pose a major burden to global health and consistently pose risk of epidemic and pandemic outbreaks by newly emerged strains with the most recent example being the emergence of H7N9 influenza strain in China (Li et al., 2013). Influenza viruses are negative-stranded RNA viruses classified as influenza A, B, and C types (Julkunen et al., 2001). Type A influenza viruses are the most pathogenic for humans and are responsible for both pandemic and seasonal flu. Influenza virus generally invades and replicates within epithelial cells but is also capable of infecting macrophages and other leukocytes as well (Hofmann et al., 1997; Julkunen et al., 2001). Newly emerging influenza A strains pose a significant public health challenge due to potential lack of herd immunity in human population and their potential for resistance to current antiviral therapeutics.

The innate immune system is the first line of defense against influenza and other viral infection. Upon infection, influenza single-stranded RNA can be recognized by the cytoplasmic RNA helicase retinoic acid-inducible gene-I (RIG-I) (Pichlmair et al., 2006) or by Toll-like receptor 7 (TLR7) in the endocytic compartment (Diebold et al., 2004) to activate antiviral responses in innate cells. Cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), type I interferons (IFNs), including IFNα and IFNβ, as well as chemokines like IL-8 are produced by various cell types in the lung upon influenza infection. These cytokines are critical for the initiation of immune responses important to control virus replication and activate the adaptive immune response (Julkunen et al., 2001; Michaelis et al., 2009). Type I IFNs are crucial in limiting viral replication and mounting a robust host immune responses against the infection (González-Navajas et al., 2012; Sadler and Williams, 2008). Type I IFNs trigger a multitude of events to inhibit viral replication, such as promoting apoptosis of infected cells, inhibiting protein synthesis to inhibit viral replication, and inducing transcription of many genes, collectively termed interferon-stimulated genes (ISGs), which work in synergy to inhibit viral replication (González-Navajas et al., 2012; Sadler and Williams, 2008).

RIG-I and TLR7 serves as intracellular receptors for pathogens such as influenza virus, vesicular stomatitis virus (VSV), and sendai virus (SeV). TLR7 and RIG-I signal through MyD88 and MAVS (also called as VISA, Cardiff, and IPS-1), respectively, to activate various transcriptional factors, including MAP kinases (MAPKs), nuclear factor-κB (NF-κB), IFN regulatory factor 3 (IRF3), and IRF7 to regulate the expression of genes, including type I IFNs

(Diebold et al., 2004; Kawai et al., 2005; Pichlmair et al., 2006; Tamura et al., 2008). IRF3 is phosphorylated by the kinases TANK-binding kinases 1 (TBK1) and inhibitor of κB kinase ε (IKKε), which leads to its nuclear translocation and subsequent regulation of expression of type I IFNs in response to virus infection (Kawai and Akira, 2008). It is believed that IRF3 or IRF3-IRF7 dimers work concomitantly with other transcriptional factors, including NF-κB and ATF2/c-Jun, to form an enhanceosome for IFNβ gene transcription (Sato et al., 2000; Zhong et al., 2008). The increased susceptibility to viral infection of the IRF3-deficient mice and the impaired production of type I IFNs by IRF3-deficient cells upon viral infection demonstrates the essential role of IRF3 in regulating type I IFN expression (Sato et al., 2000).

MAPKs play critical roles in regulating the expression of various proinflammatory cytokines, including IL-6 and TNF-α in response to microbial infection. The magnitude and duration of MAPK activation in immune responses are controlled by the MAPK phosphatases (MKPs), which are also known as dual-specificity phosphatases (DUSPs) (Liu et al., 2007). Previous work has demonstrated that MKPs/DUSPs are specifically involved in the immune response against influenza infection, where ablation of the MKP1 gene prevents viral clearance. Mechanistically, ablation of MKP1 led to defective $CD8^+$ T cell activation and effector function important for virus clearance (Zhang et al., 2009b). There is also evidence that type I IFN expression may be regulated by MKPs. IFNβ expression was increased in MKP5 KO macrophages compared with WT cells in response to LPS (Zhang et al., 2004). Overexpression of MKP2 and MKP5 inhibited IFNβ promoter activity in response to LPS or poly (I:C) stimulation (McCoy et al., 2008). However, how MKPs/DUSPs regulate type I IFN expression in vivo in response to microbial infection is unclear.

In this study, we investigated the regulation of immune responses to RNA virus infection by MKP5. We demonstrate that the deficiency of MKP5 results in reduced influenza virus replication in the lung, which is associated with increased type I IFN production. We further observed increased IRF3-type I IFN responses in MKP5 KO cells/animals in response to VSV and SeV. Importantly, we found that MKP5 inhibits IRF3 phosphorylation in a mechanism that leads to reduced IRF3 nuclear accumulation to downregulation of type I IFN expression. Overall, our findings provide a mechanism that viruses may utilize to evade host immunity where MKP5 may represent a viable target for new therapeutics aimed at eradicating RNA viruses.

RESULTS

Reduced Influenza Virus Replication and Increased Type I IFN Expression in the Lung of MKP5 KO Mice upon Influenza Infection

To examine the possible involvement of MKP5 in immune response to influenza virus infection, mouse bone-marrow-derived macrophages (BMDMs) were infected with A/Puerto Rico/8/34 (PR8, H1N1) and A/Aichi/2/68 (H3N2) viruses. We found that the mRNA expression of MKP5 was significantly increased in BMDMs in response to both H1N1 and H3N2 virus infection (Figures 1A and S1A), and MKP5 protein expression was induced optimally at 3-hr post-infection (P.I.) (Figures 1B and S1A). Thus, to further examine the function of MKP5 in innate immune responses against influenza infection, WT and MKP5 KO mice were infected with PR8 virus by intranasal (i.n.) inoculation. We found that the viral titers in the lung of MKP5 KO mice were significantly lower compared with WT mice on days 2, 3, and 5 P.I. (Figure 1C), indicating that deficiency of MKP5 resulted in reduced viral replication. We next analyzed IFNβ, an essential component of host antiviral defense (González-Navajas et al., 2012; Sadler and Williams, 2008). We found that IFNβ production in the bronchoalveolar lavage (BAL) and mRNA expression in the lung from MKP5 KO mice were significantly increased on days 2, 3, and 5 P.I. compared with WT mice (Figure 1D). The concentrations of IFNα and IL-6 were increased in the homogenates of lungs from MKP5 KO mice as well, whereas TNF-α expression was comparable (Figure 1E). We also found increased expression of IFNα and IFNβ in the lung from MKP5 knockout (KO) mice upon H3N2 virus infection compared with WT mice (Figure S1B).

Influenza virus infection leads to massive infiltration of immune cells which contribute to inflammation, activation of immune responses, as well as lung injury (Maines et al., 2008; Perrone et al., 2008). However, total infiltrated cell numbers in the lung were comparable between WT and KO mice (Figure S1C). The percentages of the neutrophils, macrophages, and dendritic cells (DCs) were comparable as well (Figures S1D and S1E), indicating that the increased total IFNα and IFNβ levels in the lung of MKP5 KO mice were due to enhanced expression of IFNα and IFNβ on a per cell basis.

IRF3 is essential for the expression of type I IFNs. Therefore, we examined the activation of IRF3 in the lung from WT and KO mice on day 3 P.I. Increased IRF3 activation was observed in KO lungs compared with WT lungs (Figure 1F). Furthermore, significantly increased expression of the ISGs, ISG-15, 2′-5′-oligo (A) synthetase (OAS), RANTES/CCL5, and viperin, was found in the lungs from KO as well (Figure 1G). Conversely, the expression of viral-specific protein polymerase 2 (PB2) was decreased in the lungs from KO mice (Figure 1F). Therefore, deficiency of MKP5 resulted in increased IRF3 activation and type I IFN expression in the lung, which is associated with decreased viral replication in response to influenza infection compared with WT mice.

Deficiency of MKP5 Results in Reduced Disease Severity upon Influenza Infection

To determine the impact of the MKP5 deficiency and enhanced type I IFN expression on influenza pathogenicity, we infected WT and MKP5 KO mice with a sublethal dose of PR8 virus. Both WT and KO mice started to lose weight on day 5 P.I. and reached the maximum weight loss on day 9 P.I. followed by recovery (Figure 1H). However, MKP5 KO mice exhibited substantially less weight loss compared with WT counterparts. Moreover, the expression of HA and NA genes was significantly lower in the lung of KO mice on day 11 P.I. compared with WT mice (Figure S1F), indicating that deficiency of MKP5 resulted in enhanced viral clearance. To determine the impact of MKP5 deficiency on adaptive immune response, influenza-specific $CD4^+$ and $CD8^+$ cells from the lung were analyzed using

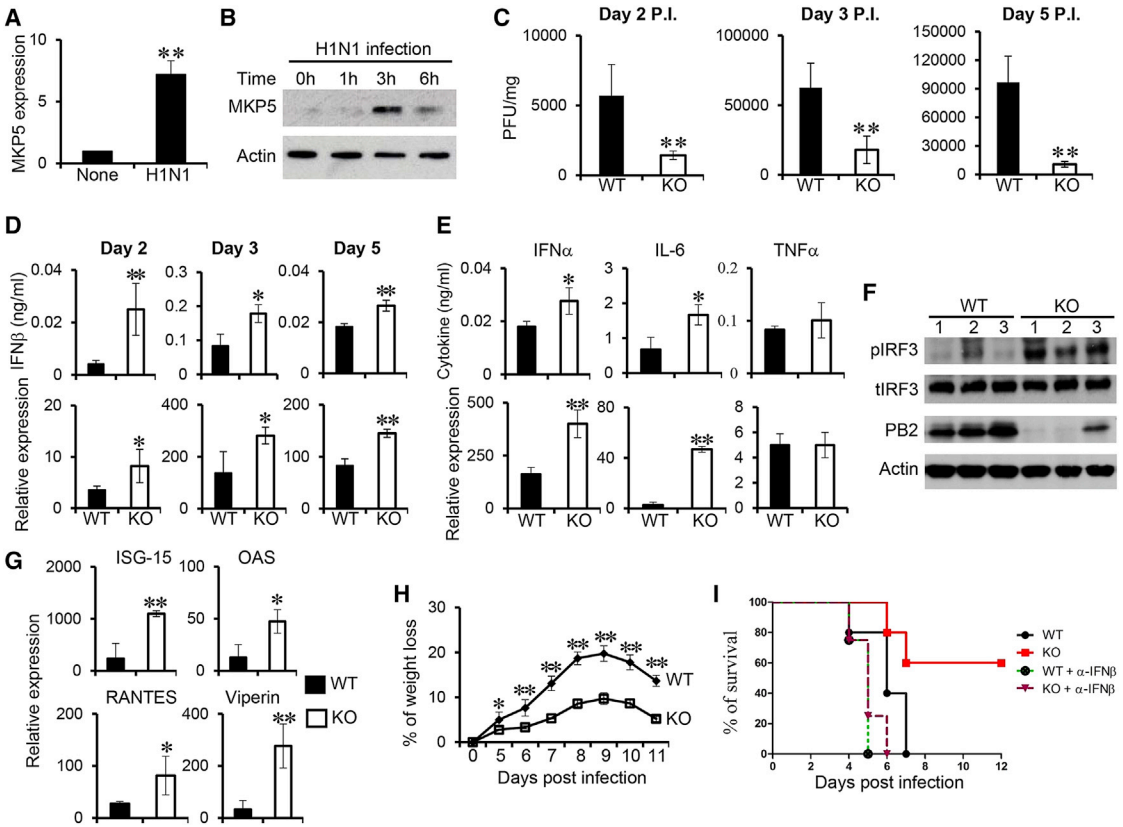

Figure 1. Deficiency of MKP5 Inhibited Influenza Virus Replication in the Lung

(A) BMDMs were infection with or without H1N1 (PR8) influenza virus, and MKP5 expression was determined by quantitative real-time PCR (qPCR).

(B) MKP5 protein expression in BMDMs upon PR8 infection was determined by western blot analysis.

(C) Viral titers in the lung of MKP5 WT and KO mice (four to five mice per group) on days 2, 3, and 5 P.I. were determined by plaque assay.

(D) Protein concentrations in BAL and mRNA expression in the lung of IFNβ on days 2, 3, and 5 P.I. were determined by ELISA and qPCR, respectively.

(E) Protein concentrations in lung homogenates and mRNA expression in the lung of IFNα, IL-6, and TNF-α on day 3 P.I. were determined by ELISA and qPCR, respectively.

(F) IRF3 Ser396 phosphorylation and the expression of PR8 polymerase 2 (PB2) in the lung on day 3 P.I. were determined.

(G) Indicated ISG expression in the lung on day 3 P.I. was analyzed by qPCR.

(H) WT and KO mice (four to five mice in each group) were infected with 50 pfus of PR8 influenza virus. Changes in body weight of WT and KO mice P.I. were monitored daily.

(I) WT and KO mice (five mice in each group) were infected with 3,000 pfus of PR8 virus with or without daily administration of IFNβ neutralizing antibody. The survival of the mice was monitored for up to 12 days. The data shown are representative of two to three independent experiments with similar results. Data are presented as mean ± SEM, *$p < 0.05$, **$p < 0.01$.

$NP_{311-325}/IA^b$ ($CD4^+$ T cells) and D^bNP_{366} ($CD8^+$ T cells) tetramers. A comparable percentage of antigen-specific $CD4^+$ T cells was observed between WT and KO mice (Figure S1G). However, the total number of tetramer $CD4^+$ T cells was lower in the lung from MKP5 KO mice than that from WT mice. Furthermore, both the percentage and total cell number of antigen-specific $CD8^+$ T cells were significantly lower in MKP5 KO (Figure S1H). Together, these results demonstrate that despite the reduced influenza-specific T cell responses, MKP5 KO mice developed less severe disease in response to influenza infection.

To further examine the robust protection observed for MKP5 KO mice against influenza infection, we infected both WT and KO mice with a lethal dose of PR8 virus. We found that all the WT mice were dead on day 7 P.I., whereas about 60% of the KO mice survived (Figure 1I). To address the contribution of the enhanced type I IFN response observed in MKP5 KO mice during influenza infection (Figures 1D and 1E), we administrated IFNβ neutralizing antibody i.n. daily after a lethal dose of PR8 virus infection in WT and KO. We observed that neutralization of IFNβ resulted in the loss of protection observed in MKP5 KO mice (Figure 1I), suggesting that the increased IFNβ expression in MKP5 KO mice is important for the increased resistance to influenza infection.

Increased Type I IFN Expression and IRF3 Activation in MKP5 KO Macrophages upon Influenza Infection

Type I IFNs play a critical role in inhibiting influenza virus replication (García-Sastre et al., 1998); however, lung epithelial cells are poor producers of type I IFNs and proinflammatory cytokines in response to influenza viruses, particularly to NS1-expressing

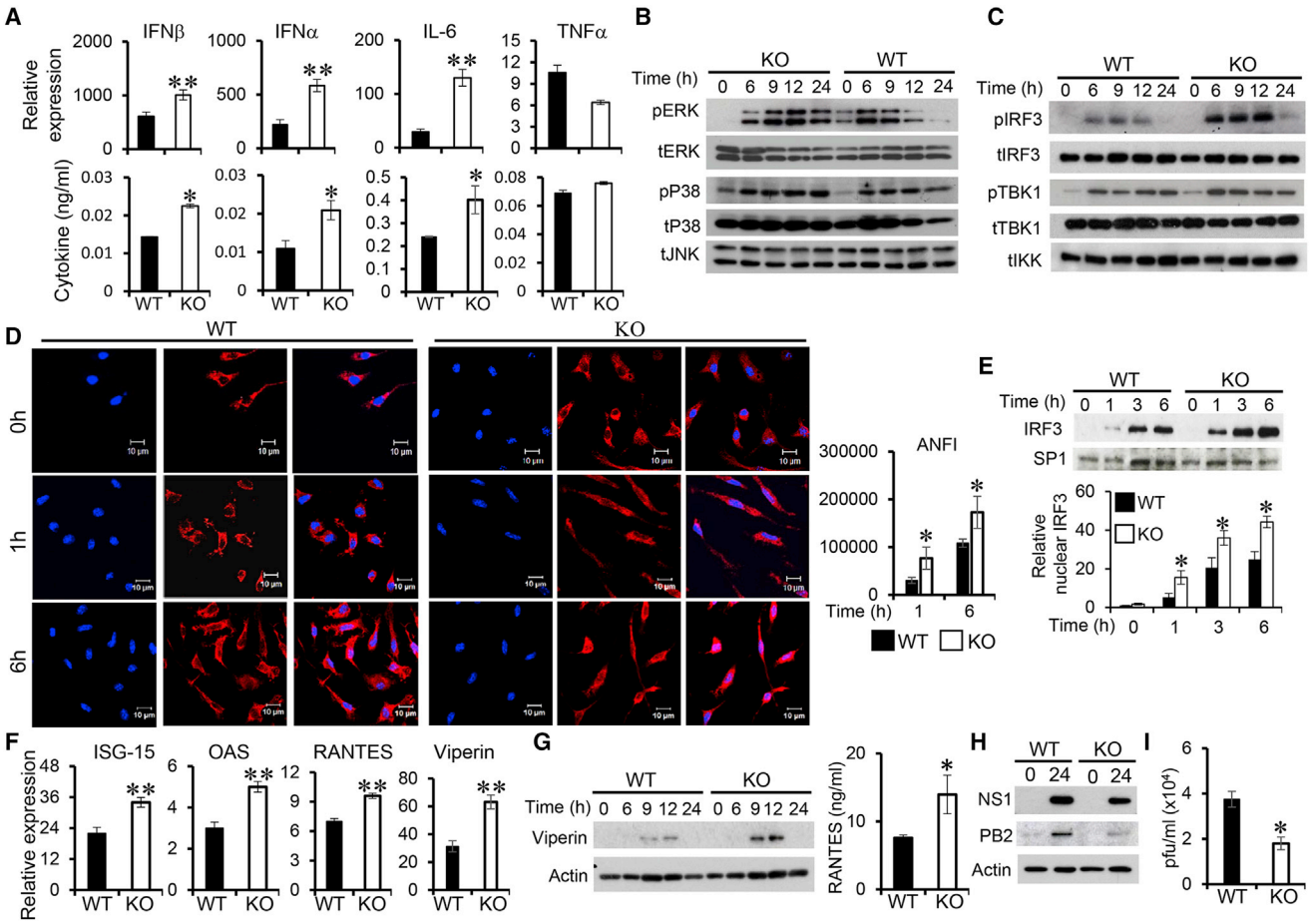

Figure 2. Increased IRF3 Phosphorylation and Nuclear Translocation in MKP5 KO Macrophages in Response to Influenza Infection

(A) WT and KO BMDMs were infected with PR8 virus for 6 hr. Expression of IFNβ, IFNα, IL-6, and TNF-α was determined by qPCR. Cytokine concentrations in culture supernatants 24-hr P.I. were measured by ELISA. WT and KO BMDMs were infected with PR8 virus at MOI of 2 for the indicated period of time.

(B and C) MAP kinase activation (B) and IRF3 and TBK1 phosphorylation (C) were determined.

(D) BMDMs infected with or without PR8 virus at MOI of 2. Subcellular localization of IRF3 was determined by IRF3 intracellular staining, and confocal fluorescence images were captured. Average nuclear fluorescent intensity (ANFI) of 100 cells was quantified using Image J. Scale bar is 10 μM.

(E) Nuclear fractions from WT and KO BMDMs infected with PR8 virus were isolated to determine IRF3 nuclear accumulation.

(F) WT and KO BMDMs were infected with PR8 virus for 6 hr. The expression of ISG-15, 2′-5′-oligo (A) synthetase (OAS), RANTES, and viperin was determined by qPCR.

(G) WT and KO BMDMs were infected with PR8 virus to determine the expression of viperin by western blot and of RANTES by ELISA.

(H) The expression of PR8 non-structure protein 1 (NS1) in WT and KO BMDMs 24h post PR8 infection was determined.

(I) PR8 replication in WT and KO BMDMs at 24-hr P.I. was determined by plaque assay. The data shown are representative of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$.

viruses (Kallfass et al., 2013; Ronni et al., 1997). Macrophages, on the other hand, are also susceptible to influenza virus infection (Geiler et al., 2011; Maines et al., 2008) and produce copious amounts of proinflammatory cytokines and type I IFNs, which are thought to be the critical determinants for disease severity (Hui et al., 2009; Kallfass et al., 2013; Michaelis et al., 2009). Thus, WT and MKP5 KO BMDMs were infected with PR8 virus to examine the expression of IFNα, IFNβ, and proinflammatory cytokines IL-6 and TNF-α. The expression of IFNα and IFNβ in KO macrophages was significantly increased at both the mRNA and protein level (Figure 2A). The expression of IL-6, but not TNF-α, was also increased in KO BMDMs upon PR8 infection compared with WT cells (Figure 2A).

The expression of IFNα and IFNβ in response to viral infection is regulated by various transcriptional factors including NF-κB, IRF3, IRF7, as well as MAPKs (González-Navajas et al., 2012; Taylor and Mossman, 2013). We found that the activation of extracellular signal-regulated kinase (ERK) was unchanged at 6- and 9-hr P.I., but that increased ERK activation was observed in KO cells at 12- and 24-hr P.I. compared with WT cells (Figure 2B). The activation of p38 in WT and KO cells was comparable (Figure 2B), whereas the activation of JNK was undetectable upon PR8 virus infection, suggesting that the increased type I interferon expression observed in MKP5 KO macrophages was not due to increased activation of MAP kinases. IRF3 plays a major role in regulation of type I IFN expression in response to viral

infection where phosphorylation of Ser396 in IRF3 is a critical step in IRF3 activation (McCoy et al., 2008; Servant et al., 2003). We examined the phosphorylation of Ser396 in WT and MKP5 KO BMDMs in response to PR8 virus infection and found that the Ser396 phosphorylation was greatly increased at 6-, 9-, and 12-hr P.I. in KO cells compared with WT cells (Figure 2C). Phosphorylation of IRF3 is mediated by TBK1 and IKKε (Tamura et al., 2008). Comparable activation of TBK1 was detected between WT and KO cells, and phosphorylation of IKKε was not detectable in both WT and KO cells (Figure 2C). These results indicated that the increased IRF3 phosphorylation in MKP5 KO macrophages was not due to increased activation of TBK1 and IKKε.

Upon phosphorylation, IRF3 translocates into nucleus to regulate the expression of genes, including IFNα and IFNβ. To examine whether the increased IRF3 phosphorylation observed in MKP5 KO macrophages leads to increased IRF3 nuclear translocation, WT and KO BMDMs with or without PR8 virus infection were analyzed for IRF3 localization by microscopy. Without infection, IRF3 was predominantly located in the cytoplasm in both WT and MKP5 KO cells (Figure 2D). Upon influenza virus infection, IRF3 nuclear localization was significantly increased in both WT and KO cells. Importantly, there was significantly increased IRF3 nuclear localization in MKP5 KO cells compared with WT cells after infection (Figure 2D). To substantiate this finding, we isolated nuclear fractions from WT and MKP5 KO cells at different time points P.I. to examine IRF3 nuclear accumulation. Significantly increased IRF3 nuclear accumulation was observed at 1-, 3-, and 6-hr P.I. in the KO macrophages (Figure 1E). Concordantly, we found increased expression of various ISGs, including ISG-15, OAS, RANTES/CCL5, and viperin (Figure 2F). In addition, increased RANTES/CCL5 and viperin protein expression was detected in MKP5 KO cells compared with WT cells (Figure 2G). Conversely, decreased expression of influenza nonstructure protein 1 (NS1) and PB2 protein was observed in the KO cells (Figure 2H), which was accompanied by reduced viral replication in MKP5 KO cells compared with WT cells (Figure 2I). Together, these results demonstrated that deficiency of MKP5 in macrophages resulted in increased IRF3-type I IFN responses and reduced influenza virus replication.

Increased Type I IFN Expression and IRF3 Activation in MKP5 KO DCs upon Influenza Infection

DCs play an important role in anti-influenza virus infection and also produce both type I IFNs and proinflammatory cytokines (Waithman and Mintern, 2012). Similar to our findings in macrophages, we observed increased expression of IFNα and IFNβ with a further enhancement of TNF-α in MKP5 KO bone marrow-derived DCs (BMDCs) compared with WT cells in response to PR8 infection (Figures 3A and 3B). In addition, increased expression of various ISGs was detected in KO cells (Figure 3C). Increased IRF3 phosphorylation was detected in MKP5 KO DCs at 6-, 9-, and 12-hr P.I. (Figure 3D). Activation of MAP kinases in BMDCs appears to be earlier compared with that in BMDMs (Figures 2B and 3E). ERK activation in MKP5 KO DCs in response to PR8 virus infection appeared to be slightly increased at 2- and 3-hr P.I. compared with that in WT cells. Interestingly, increased activation of p38 and to a lesser extent JNK was detected in MKP5 KO DCs compared with WT cells (Figure 3E). These results suggested MAPK activation by MKP5 is in part differentially regulated in macrophages and DCs during influenza infection.

MKP5 protein is found to be constitutively expressed in human lung epithelial cell line A549 and PR8 infection increased its expression (Figure S2A). To further examine the role of MKP5 in regulation of IRF3-type I IFN response, A549 cells overexpressing MKP5 were generated (Figure S2B). Upon PR8 infection, overexpression of MKP5 inhibited IRF3 phosphorylation and mRNA expression of both IFNα and IFNβ (Figures S2B and S2C). Furthermore, protein expression of IFNβ was significantly lower in MKP5 overexpressing cells compared to that in control cells (Figure S2D). Together, these results demonstrate that MKP5 inhibits IRF3 activation and type I IFN expression in lung epithelial cells in response to influenza infection.

Regulation of Type I IFN Expression in Macrophages by MKP5 Is Independent of Influenza NS1

NS1 of influenza A virus is a multifunctional viral protein with activity that antagonizes type I IFN-mediated antiviral responses (Hale et al., 2008). To address whether the negative regulation of type I IFN expression by MKP5 is associated with NS1, murine macrophages (RAW264.7) were transfected with NS1 genes from H1N1 and H3N2 influenza viruses. We found that overexpression of both H1N1 and H3N2 influenza NS1 genes induced the expression of MKP5 (Figure S2E). In addition, overexpression of NS1 in A549 cells induced the expression of MKP5 as well (Figure S2F). NS1, but not matrix protein 2 (M2) from PR8, induced both mRNA and protein expression of MKP5 in RAW264.7 cells (Figures S2G and S2H). Furthermore, PR8 virus deficient in NS1 ($PR8^{-NS1}$) is impaired in the induction of MKP5 in BMDMs (Figure S2I). Next, we infected WT and MKP5 KO BMDMs with PR8 or $PR8^{-NS1}$ viruses to examine the expression of IFNα and IFNβ. As expected, $PR8^{-NS1}$ virus infection caused increases in the expression of IFNα and IFNβ in both WT and KO macrophages compared with cells infected with PR8 virus (Figure S2J). However, the expression of IFNα and IFNβ in MKP5 KO cells remained higher than those in WT cells upon $PR8^{-NS1}$ virus infection. Moreover, overexpression of MKP5 in macrophages inhibited IFNα and IFNβ expression in response to $PR8^{-NS1}$ virus infection (Figure S2K). These results indicate that the inhibition of MKP5 on type I IFN expression is independent of NS1.

Increased IRF3 Activation and Type I IFN Expression in MKP5 KO Macrophages upon RIG-I and TLR3 Activation

Influenza virions can be detected by RIG-I (Kato et al., 2006; Pichlmair et al., 2006). To further understand the regulation of IRF3 by MKP5, we stimulated WT BMDMs with 5′ triphosphate double-stranded RNA (5′ppp-dsRNA), a synthetic ligand for RIG-I. We found that MKP5 expression was induced in BMDMs at both the mRNA and protein levels in response to 5′ppp-dsRNA stimulation (Figure 4A). WT and KO BMDMs were then stimulated with 5′ppp-dsRNA to examine IRF3 activation. We found increased IRF3 phosphorylation in KO cells compared with WT cells (Figure 4B). Furthermore, MKP5 KO BMDMs had increased type I IFNs and ISGs compared with WT cells (Figures 4C and 4D).

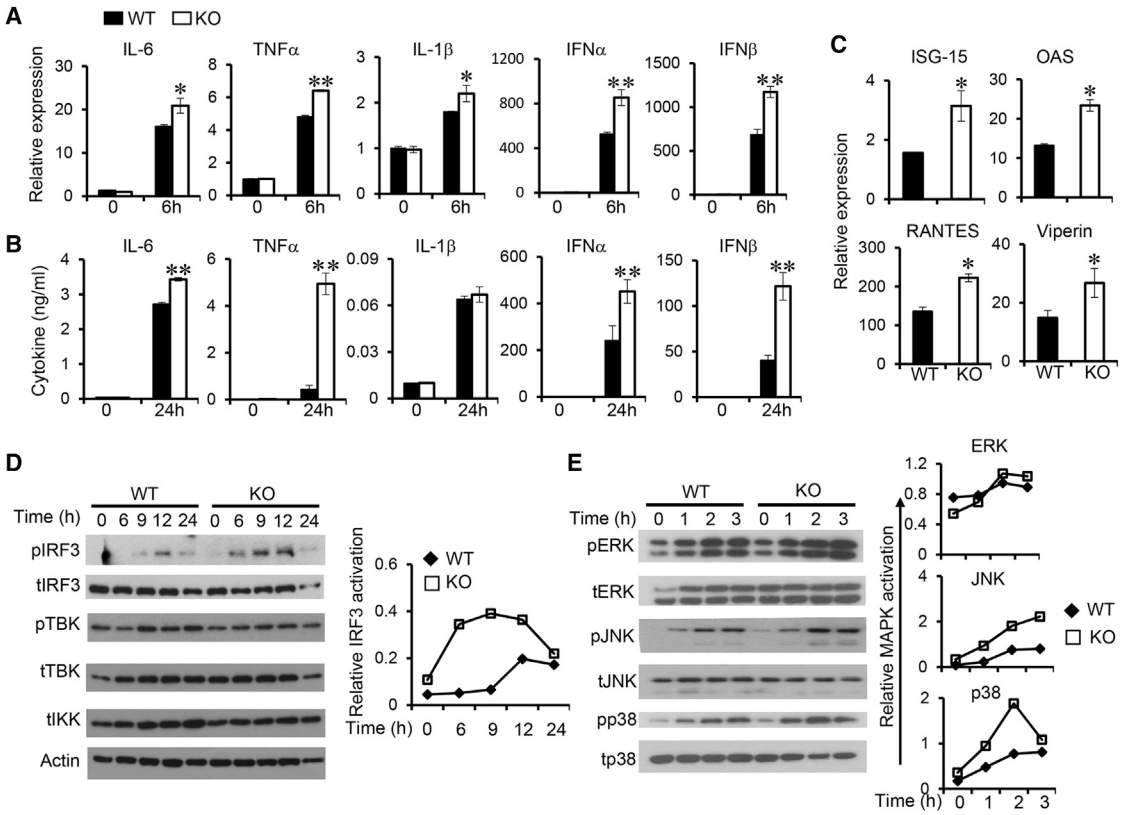

Figure 3. Increased Type I IFN Expression and IRF3 Phosphorylation in MKP5 KO DCs

(A and B) WT and MKP5 KO BMDCs were infected with PR8 virus. mRNA and protein expression of IL-6, TNF-α, IL-1β, IFNα, and IFNβ were determined by qPCR and ELISA, respectively.

(C) WT and KO BMDCs were infected with PR8 virus for 6 hr. ISG expression was examined by qPCR.

(D and E) IRF3 and TBK1 phosphorylation (D) and MAP kinase activation (E) were examined. The data shown are representative of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$.

TLR3 is involved in innate immune response to influenza infection (Iwasaki and Pillai, 2014). We also found that TLR3 activation caused by stimulation with poly (I:C) in BMDMs induced the expression of MKP5 (Figure 4E). MKP5 KO macrophages exhibited increased IRF3 activation (Figure 4F), significantly increased expression of type I IFNs (Figure 4G) and ISGs (Figure 4H) in response to TLR3 activation compared with WT cells.

Deficiency of MKP5 Leads to Increased IRF3-Type I IFN Responses upon VSV and SeV Infection

TLR3 and RIG-I are important in host sensing of viral RNAs. Thus, to examine the importance of MKP5 in immune responses to other RNA viruses, we examined the expression of MKP5 in BMDMs upon VSV and SeV infection. Both VSV and SeV infection induced the expression of MKP5 (Figures 5A and 5G). Next, we examined the activation of IRF3 and the expression of type I IFNs in WT and MKP5 KO BMDMs in response to VSV and SeV infection. Phosphorylation of IRF3 was detected at 3 hr after both VSV and SeV infection in WT and KO BMDMs (Figures 5B and 5H). However, IRF3 phosphorylation was found enhanced by MKP5 KO cells. Moreover, increased expression of IFNα and IFNβ was observed in KO cells compared with WT cells (Figures 5C and 5I). In addition, the expression of ISG-15, PKR, RANTES and viperin was also increased in KO cells (Figures 5D and 5J). Next, we infected WT and MKP5 KO mice with a sublethal dose of VSV i.n. and serum levels of IFNβ were determined on day 3 P.I. We found increased serum IFNβ in MKP5 KO mice compared with WT mice (Figure 5E). Significantly less body weight reduction upon VSV infection was observed in MKP5 KO mice (Figure 5F), suggesting that the KO mice developed less severe disease. Together, these results demonstrate that in addition to influenza viruses, other RNA viruses including VSV and SeV can induce the expression of MKP5 which in turn suppresses IRF3-mediated type I IFN responses.

MKP5 Interacts with and Dephosphorylates IRF3

To further understand how MKP5 regulates IRF3 activity, we examined whether MKP5 directly interacts with IRF3 by transfecting Flag-tagged MKP5 cDNA into RAW264.7 cells for co-immunoprecipitation. We found that endogenous IRF3 readily co-immunoprecipitates with MKP5 (Figure 6A). Similar results were obtained in 293 T cells transfected with both MKP5 and IRF3 (Figure S3A). We also found that MKP5 interacts with

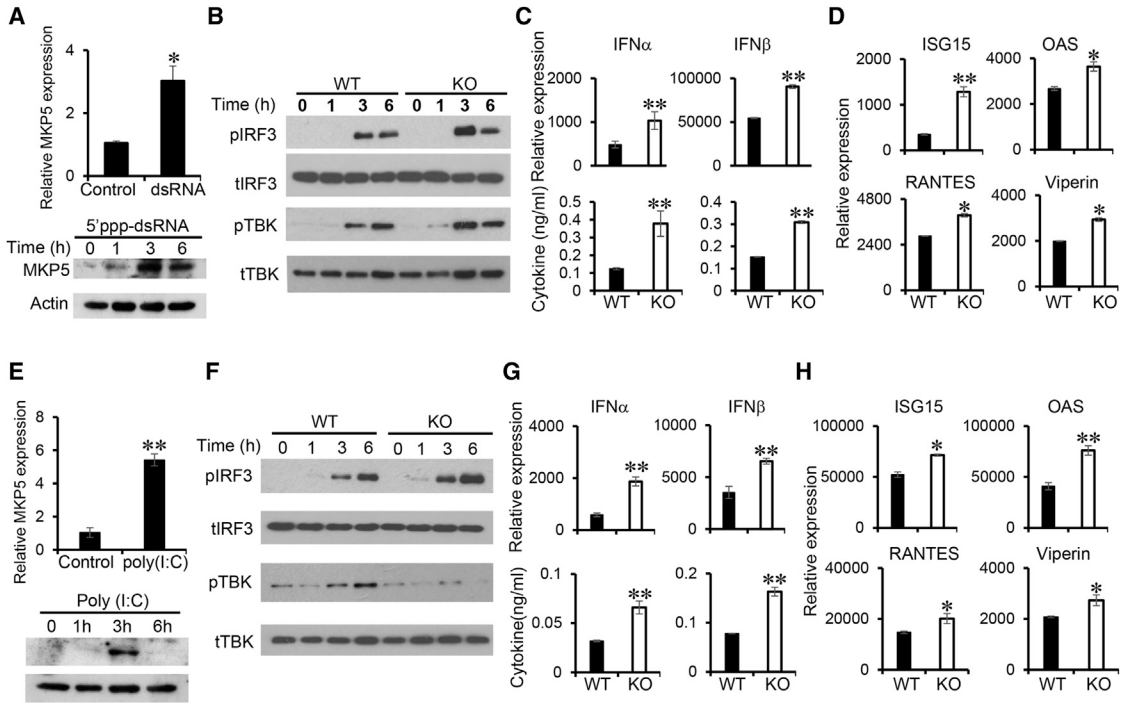

Figure 4. Increased IRF3 Activation and Type I IFN Expression in MKP5 KO Macrophages in Response to RIG-I and TLR3 Stimulation

(A) WT BMDMs were transfected with 5′ppp-dsRNA using Lipofectamine LTX reagent (Invitrogen). MKP5 mRNA expression was determined at 6 hr after by qPCR. MKP5 protein expression was examined.

(B) WT and KO BMDMs were stimulated with 5′ppp-dsRNA to examine the activation of IRF3 and TBK1.

(C) mRNA and protein expression of IFNα and INFβ after 5′ppp-dsRNA stimulation was determined.

(D) mRNA expression of ISGs at 3 hr after 5′ppp-dsRNA transfection was analyzed by qPCR.

(E) WT BMDMs were transfected with poly (I:C) to examine MKP5 mRNA and protein expression as in (A).

(F–H) BMDMs were stimulated with poly (I:C) to examine IRF3 and TBK1 activation (F), type I IFN (G), and indicated ISG expression (H).

The data shown are representative of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$.

both TBK1 and IKKε (Figure S3B). To substantiate the interaction between IRF3 and MKP5, we infected RAW264.7 macrophages with PR8 virus. Anti-IRF3 antibody was used to pull down interacting proteins. As shown in Figure 6B, obvious MKP5 protein was pulled down at 6-hr P.I., demonstrating that IRF3 interacts with MKP5 in macrophages in an infection-inducible fashion. To further validate the interaction between MKP5 and IRF3, we synthesized mouse MKP5 and IRF3 proteins using wheat embryo extract-mediated cell-free protein synthesis system (Takai et al., 2010) and performed AlphaScreen assay, a luminescence-based assay (Ullman et al., 1994). Co-incubating MKP5 with IRF3 generated a signal that was far greater than signals generated from MKP5 incubated with bacterial protein DHFR or IRF3 incubated with DHFR (Figure 6C), which confirmed the direct and specific interaction between MKP5 and IRF3.

To further elucidate the interaction between MKP5 and IRF3, various constructs of MKP5 and IRF3 were generated that incorporated deletions of various DNA regions encoding a specific protein domain/region (Figure S3C). To identify the IRF3-interaction region on MKP5, coimmunoprecipitations of Flag-MKP5 deletion mutants and HA-IRF3 were carried out. Deletion of N-terminal region NT1 from amino acid 1 to 150 (aa 1–150, ΔNT1) slightly decreased the interaction with IRF3 (Figure 6D, panel 1, lane 4), while deletion of the C-terminal region (aa 406–483, ΔCT1), including its catalytic domain (Tanoue et al., 1999), had no effect (lane 6). Further deletion of the C-terminal region of MKP5 from aa 406 to aa 290 (ΔCT2) greatly reduced its interaction with IRF3 (lane 7), whereas deletion of both the NT1 and CT2 regions from MKP5 almost completely abrogated its interaction with IRF3 (lane 10). Conversely, expressing the NT1 region alone showed a weakened binding to IRF3 compared with that of WT MKP5 (lanes 8 and 3, respectively). The CT2 region alone showed greater binding to IRF3 than the full-length MKP5 (lanes 9 and 3). Collectively, these results demonstrated a complex 3D interaction whereby two regions in MKP5, the NT1 and CT2 regions, may be important for its interaction with IRF3, while the CT2 region plays a dominant role in MKP5 binding to IRF3.

Similarly, to identify the MKP5-interaction region on IRF3, coimmunoprecipitations of IRF3 mutants and MKP5 were carried out. The deletion of C-terminal region comprising the IRF association domain (IAD) and tail region (ΔIAD) or C-terminal region beyond IAD domain on IRF3 (ΔTail) completely abolished its interaction with MKP5 (Figure 6E, panel 1, lanes 5 and 7), whereas deletion of its DNA binding domain (DBD), nuclear export signal (NES), PRO domains, or internal deletion of IAD

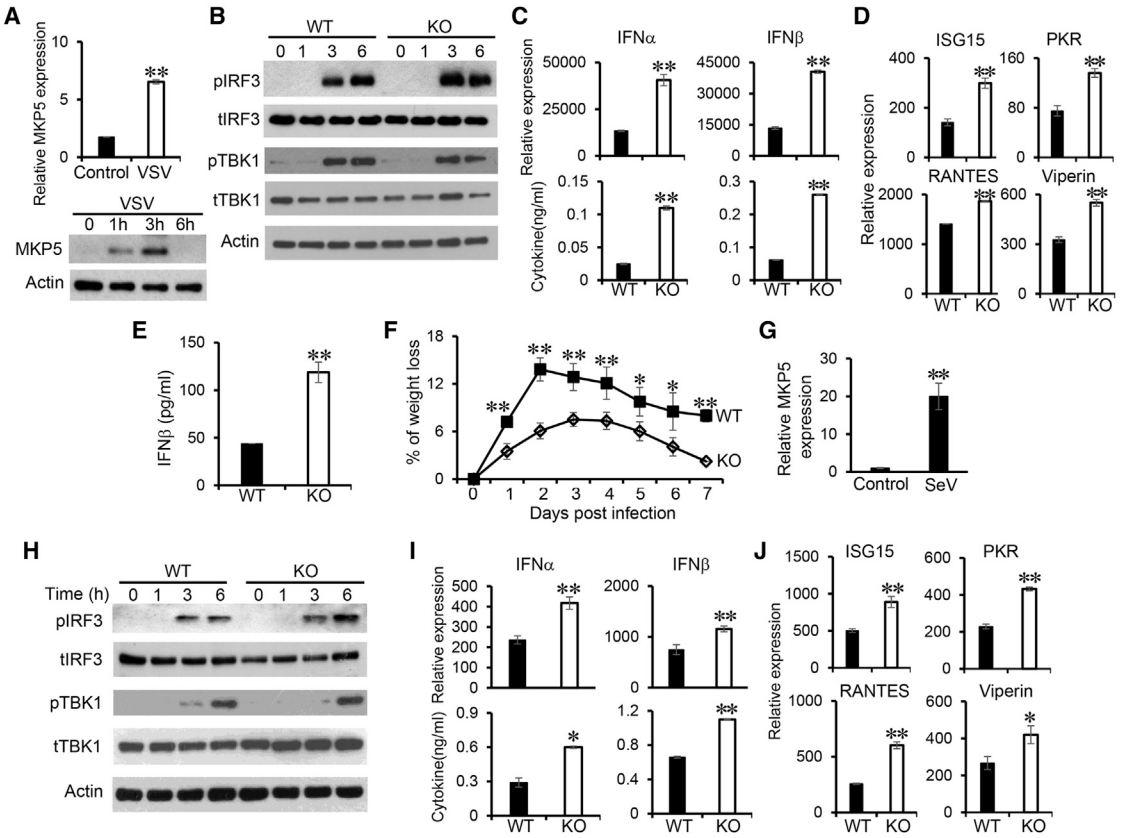

Figure 5. MKP5 Deficiency Resulted in Increased IRF3 Activation and Type I IFN Responses in Macrophages upon VSV and SeV Infection

(A) WT BMDMs were infected with VSV for 3 hr to examine MKP5 mRNA expression. MKP5 protein expression is at the indicated time points after VSV infection was determined.

(B) WT and MKP5 KO BMDMs were infected with VSV to examine IRF3 and TBK1 activation.

(C and D) IFNα and IFNβ expression (C), as well as the expression of ISGs (D) in WT and KO BMDMs in response to VSV infection were analyzed.

(E) WT and MKP5 KO mice (n = 5) were infected with a sublethal dose of VSV intranasally. Serum levels of IFNβ on day 3 P.I. were examined by ELISA.

(F) Changes in body weight of the mice P.I. were monitored daily.

(G) WT BMDMs were infected with SeV for 3 hr to examine MKP5 mRNA expression.

(H–J) IRF3 and TBK1 activation (H), type I IFN expression (I), as well as the expression of the indicated ISGs (J) in WT and KO BMDMs in response to SeV infection were analyzed.

The data shown are representative of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$.

domain (ΔID-IAD) did not, demonstrating that the tail region of IRF3 is essential for its interaction with MKP5. In an AlphaScreen assay, IRF3-ΔIAD showed similar binding activity to MKP5 as DHFR, further confirming the essential role of the tail region in the interaction between IRF3 and MKP5 (Figure 6C).

To test whether MKP5 dephosphorylates IRF3, we prepared phosphorylated IRF3 and incubated it with recombinant MKP5 or MKP5 phosphatase inactive mutant ($MKP5^{mut}$) (Zhang et al., 2004). MKP5, but not $MKP5^{mut}$, dephosphorylated Ser396 and Ser386 of IRF3 (Figures 6F and S4A). In addition, we found that neither MKP5 nor $MKP5^{mut}$ incubated with phosphorylated TBK1 resulted in dephosphorylation (Figure S4B). Furthermore, MKP5 inhibited TBK1 overexpression-induced IRF3 phosphorylation in 293T cells in a phosphatase activity-dependent manner (Figure S4C). Therefore, MKP5 dephosphorylates IRF3 at Ser396 and Ser386, which is dependent on its phosphatase activity.

Interaction of MKP5 with IRF3 Is Independent of MAP Kinases

Consistent with previous findings (Nomura et al., 2012), we found that MKP5 interacts with ERK, JNK, and p38 (Figure S5A). To address whether the binding of MKP5 to ERK, p38, and JNK could affects its interaction with IRF3, we generated MKP5 deletion mutants in which the binding regions of MKP5 for ERK (MKP5 ΔERK) or JNK/p38 (MKP5 ΔJNK/p38) or both (MKP5 ΔMAPK) (Tanoue et al., 2002; Theodosiou et al., 1999) were deleted (Figure S3C). These deletion mutants were tested for their ability to bind IRF3. We found that the deletion of the ERK binding region (ΔERK) or the JNK/p38 binding sites (ΔJNK/p38) or both (MKP5 ΔMAPK) from MKP5 abolished its binding to ERK or JNK/p38, or ERK/JNK/p38, respectively (Figures S5B, S5C, and 7A). However, these deletion mutants retained their full ability to bind IRF3 (Figure S5D, panel 1, lanes 3 and 4, and Figure 7A, panel 1, lane 5). Together, these results

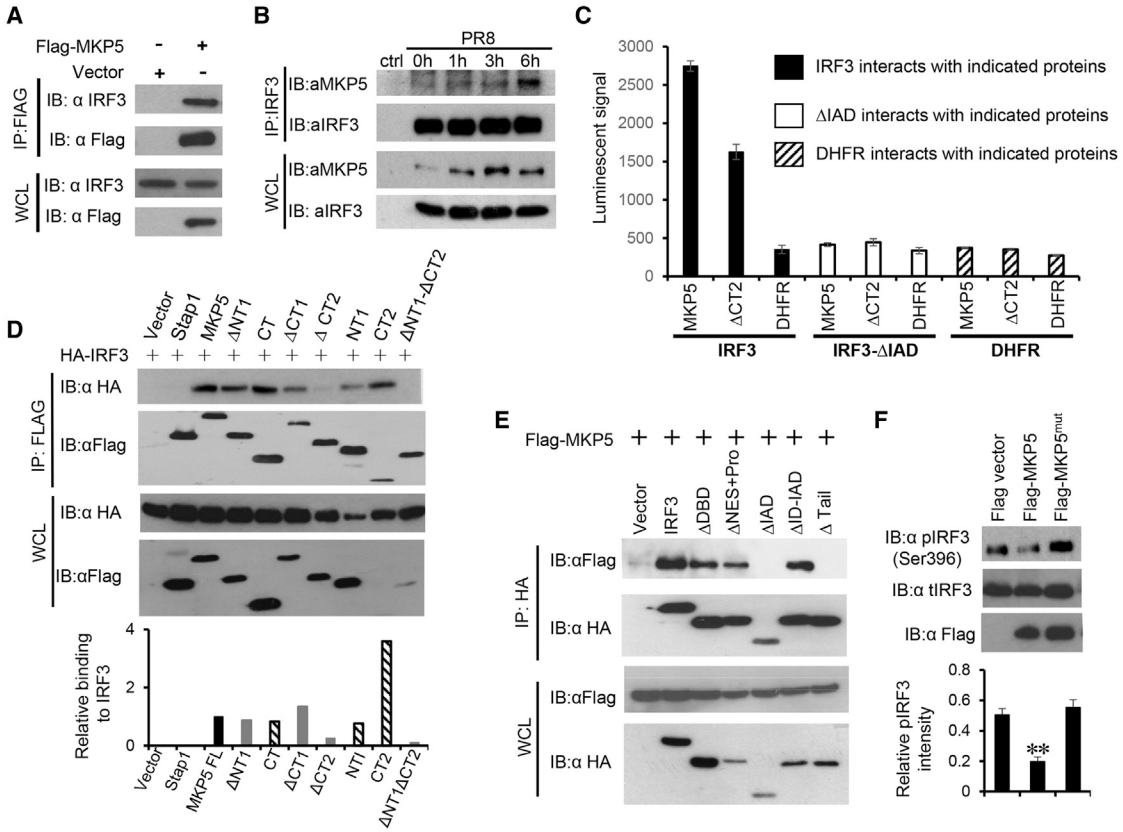

Figure 6. MKP5 Directly Interacts with and Dephosphorylates IRF3

(A) RAW264.7 macrophages were transfected with flag-MKP5. Interaction between MKP5 and endogenous IRF3 was examined by immunoprecipitation (IP).

(B) WT BMDMs were infected with PR8 virus. Cell lysates were precleared with A/G magnetic beads (Pierce) for 1 hr followed by incubation with anti-IRF3 antibody (Cell Signaling) overnight. After incubation, A/G beads were added to pull-down IRF3 interacting proteins followed by western blot analysis to detect MKP5.

(C) Purified in vitro synthesized MKP5, MKP5 C-terminal deletion mutant (ΔCT2), IRF3, IRF3 C-terminal mutant (ΔIAD) proteins were incubated with streptavidin-coated donor beads or protein A-conjugated acceptor beads (AlphaScreen IgG detection kit, Perkin Elmer) at 16°C for 1 hr. Interaction of proteins was analyzed by an EnSpire Alpha microplate reader (Perkin Elmer).

(D) HEK293T cells transfected with HA-tagged IRF3 together with various MKP5 deletion mutants indicated for IP to examine the ability of these MKP5 mutants to interact with IRF3.

(E) Lysates from HEK293T expressing Flag-MKP5 together with various HA-tagged IRF3 constructs IP with anti-HA beads were subjected to western blot analysis to examine their interaction ability with MKP5.

(F) Purified phosphorylated IRF3 was incubated with recombinant MKP5 or MKP5^{mut} in phosphatase assay buffer for 2 hr at 37°C. Ser396 level was determined by western blot analysis. The data shown are representative of three independent experiments with similar results.

demonstrate that the interaction of MKP5 with IRF3 is independent of its interaction with MAP kinases.

MKP5 Suppresses IRF3 Nuclear Accumulation in Response to Influenza Infection

To further understand the MKP5-dependent mechanistic regulation of IRF3, we investigated the change in subcellular distribution of IRF3 in the presence or absence of MKP5 during influenza infection using confocal microscopy. To this end, we overexpressed IRF3 (tagged with mCherry) with or without MKP5 (GFP-tagged) in macrophages. Without influenza infection, IRF3 was mainly localized in the cytoplasm (Figure S6A), whereas MKP5 was localized in both cytoplasm and nucleus, as shown before (Tanoue et al., 1999). Six hours after influenza infection, substantial localization of IRF3 in the nucleus was observed (Figures 7B, panel 3, and S6B). Conversely, cells co-expressing MKP5 and IRF3 showed substantial reduction in IRF3 nuclear accumulation compared with cells overexpressing IRF3 alone (Figures 7B, panel 4, and S6B). In addition, deletion of MAP kinase binding sites from MKP5 (MKP5ΔMAPK) did not impair its ability to retain IRF3 in cytoplasm in response to influenza infection (Figures 7C and S6C), whereas inactivation of MKP5 phosphatase activity (MKP5^{mut}) or deletion of IRF3-binding regions from MKP5 (MKP5$^{\Delta NT1/\Delta CT2}$) resulted in the loss of retaining IRF3 in cytoplasm (Figure 7C).

To substantiate this result, we co-transfected TBK1/IRF3, IFNβ promoter luciferase construct (IFNβ-Luc) together with MKP5, MKP5^{mut}, or MKP5$^{\Delta NT1/\Delta CT2}$ into 293T cells to examine IFNβ promoter activities. MKP5 suppressed IFNβ transcriptional activity, and MKP5^{mut} or MKP5$^{\Delta NT1/\Delta CT2}$ lost the suppressive

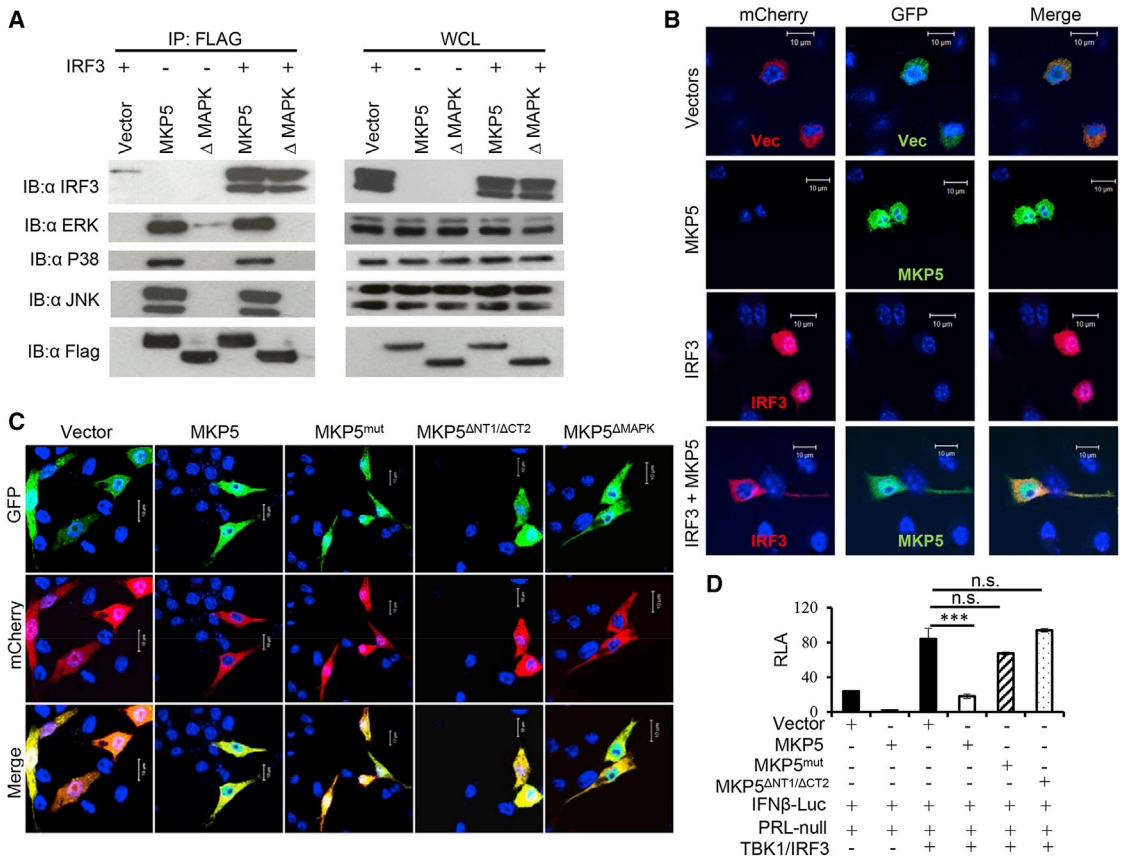

Figure 7. MKP5 Suppresses IRF3 Nuclear Accumulation

(A) HEK293T cells were transfected with HA-IRF3 together with either Flag-vector, MKP5, or MKP5ΔMAPK. Binding ability of MKP5 deletion mutants with IRF3 were examined by IP and western blot.

(B) Raw 264.7 macrophages were transfected with either mCherry-IRF3, GFP-MKP5, or both, along with vector controls and allowed to express the protein of interest for 20 hr. Samples were processed and analyzed by confocal microscopy following 6-hr virus infection. Scale bar is 10 μM.

(C) Raw 264.7 cells were transfected with mCherry-IRF3 together with WT MKP5, MKP5 phosphatase-deficient mutant ($MKP5^{mut}$), MKP5 IRF3-binding mutant ($MKP5^{\Delta NT1/\Delta CT2}$), or MKP5 MAPK-binding mutant ($MKP5^{\Delta MAPK}$) followed by infection with $PR8^{-NS1}$ viruses for 6 hr. Subcellular localization of MKP5 and IRF3 was analyzed by confocal microscopy. Scale bar is 10 μM.

(D) IFNβ promoter luciferase construct (IFNβ-Luc) was transfected into 293T cells together with indicated plasmids, including WT MKP5, $MKP5^{mut}$, $MKP5^{\Delta NT1/\Delta CT2}$ to examine IFNβ promoter activity triggered by TBK1-mediated IRF3 activation using dual-luciferase assay. The data shown are representative of three independent experiments with similar results.

function on IFNβ transcriptional activity (Figure 7D). In addition, macrophages stably overexpressing MKP5 inhibited IRF3 phosphorylation and greatly reduced the expression of type I IFNs (Figures S7A–S7C). Furthermore, the expression of ISG-15, OAS, and RANTES was also reduced when MKP5 was overexpressed (Figure S7D). Protein expression of RANTES by MKP5 overexpressing cells was significantly reduced compared with control cells as well (Figure S7E). Collectively, these results demonstrate that MKP5 inhibits type I IFN and other IRF3-target gene expression in response to influenza virus infection by suppressing IRF3 activation and nuclear accumulation.

DISCUSSION

Targeting type I IFNs is the major mechanism employed by viruses to evade host immune defense. Viruses have developed an impressive diversity of strategies to circumvent the potent antiviral mechanism mediated by the type I IFN system (Taylor and Mossman, 2013). Here we demonstrated a type I IFN evading mechanism by influenza viruses and other RNA viruses including VSV and SeV through the induction of MKP5 (DUSP10), a member of the MKP/DUSP protein family.

Reversible protein phosphorylation regulates almost all aspects of cell life where pathogens may exert to evade immunity by altering the phosphorylation states of host proteins (Cohen, 2001). Phosphatases produced by microbial pathogens play a key role in pathogenesis (Subramaniam et al., 2013; Viboud and Bliska, 2005). For instance, the protein tyrosine phosphatase YopH from *Yersinia* can enter human cells causing uncontrolled dephosphorylation of many tyrosines to break down signal transduction mechanisms, which could lead to fatal outcome of the host (Viboud and Bliska, 2005). On the other

hand, microbial pathogens are able to modulate host protein kinases and phosphatases to control host responses to their infection. For example, the oncogenic Marek's disease virus produces a bZIP transcription factor, Meq, to activate major kinases in the ERK pathway and simultaneously to repress phosphatases such as PPP2CB and DUSP4 for oncogenesis (Subramaniam et al., 2013). MKPs/DUSPs are protein tyrosine phosphatases that contain a catalytic domain with significant sequence similarity to the corresponding region of the protein tyrosine phosphatase VH-1 from *vaccinia* virus and are able to dephosphorylate both tyrosine and serine/threonine residues within one substrate (Keyse, 2000). Their function in immune responses to microbial infection, especially in regulating proinflammatory cytokine and chemokine expression by controlling MAP kinase activation, is well documented (Al-Mutairi et al., 2010; Chi et al., 2006; Zhang et al., 2004; Zhao et al., 2006). The regulation of IRF3-type I IFNs by MKP5 unveiled in this study represents a novel function of MKPs/DUSPs in innate immunity. On the other hand, type I IFNs induced by self-DNA/RNA or microbial infection have been linked with several autoimmune diseases such as psoriasis, systemic lupus erythematosus (SLE) and celiac disease by various mechanisms including promoting the recruitment and activation of inflammatory cells, and facilitating autoantibody production (González-Navajas et al., 2012; Tamura et al., 2008). Therefore, the negative regulation of IRF3-type I IFNs by MKP5 could be beneficial for the host in type I IFN-mediated immunopathogenesis, which needs to be further clarified. Our findings on MKP5 induced by RNA viruses to inhibit IRF3-type I IFNs has thus shed new light on the role of MKPs/DUSPs in microbial-host interaction.

Recently, a role of protein phosphatase 2A (PP2A) in regulation of IRF3 activation was reported (Long et al., 2014). We found that in response to influenza infection, MKP5 mRNA was more highly induced compared with that of PP2A (Figure S7F). Both PP2A and MKP5 are able to dephosphorylate phospho (p)-IRF3 (Figure S7G). However, they appear to negatively regulate IRF3 activation by different mechanisms. PP2A does not directly interact with IRF3 and the adaptor protein RACK1 is necessary to mediate its interaction with IRF3 (Long et al., 2014). It was proposed that PP2A keeps IRF3 in an inactive state in cytoplasm during resting and dephosphorylates pIRF3 exported from the nucleus after activation (Long et al., 2014). These processes are achieved through the formation of PP2A-RACK1-IRF3 complex. On the other hand, MKP5 expression is induced upon recognition of virus infection. This protein is evenly distributed in the cytoplasm and the nucleus where it directly binds to IRF3 (Figures 6 and 7). In addition, RACK1 binds to the IAD domain of IRF3, whereas MKP5 binds to the tail region outside of the IAD domain at the C terminus of IRF3. Therefore, MKP5 and PP2A could work independently or cooperatively in the cytoplasm to control the activation of IRF3 following virus infection, which will require further investigation.

NS1 protein of influenza A viruses plays a major role in inhibiting type I IFN-mediated antiviral effects through multiple mechanisms, including limitation of pretranscription of IFNβ by interfering with RIG-I, inhibition of posttranscription of IFNβ production, and blocking the function of OAS/RNase L and PKR (Hale et al., 2008). In this study, we found that NS1 of both H1N1 and H3N2 influenza viruses expressed in macrophages induced the expression of MKP5 to suppress the production of type I IFNs via IRF3. The mechanism on how NS1 induce the expression of MKP5 is unclear. NS1 contains nuclear localization sequences (NLSs) mediating its binding to importin-α and rapid nuclear importing (Hale et al., 2008). It is possible that such nuclear localization can be sensed by the cells which leads to the induction of MKP5. In addition to importin-α, NS1 is able to bind to other host proteins, including nucleolin, eIF4GI, and RIG-I. It is also possible that the binding of NS1 to those proteins activates unknown mechanisms leading to MKP5 expression. The inhibitory function of MKP5 on IFN production is independent of NS1 since the $PR8^{-NS1}$ virus induced significantly higher levels of type I IFNs from MKP5 KO cells than WT cells and overexpression of MKP5 in macrophages inhibited type I IFN expression in response to $PR8^{-NS1}$ virus infection (Figure S2). Therefore, these findings advance our understanding on the function of NS1 in antagonizing host immune responses.

MAPKs are the primary substrates of MKPs/DUSPs. However, proteins other than MAPKs have been identified as substrates of MKPs/DUSPs. For instance, MKP1 is able to dephosphorylate histone H3 at Ser10 (Kinney et al., 2009). MKP-3 can dephosphorylate FOXO1 at Ser256 to promote its nuclear translocation (Wu et al., 2010). Here, we found that MKP5 directly interacts with IRF3 to dephosphorylate Ser396 and Ser386 to inhibit its activation and subsequent nuclear translocation (Figures 6, 7, and S4A). Our finding that MKP5 retains IRF3 in cytoplasm in response to influenza infection (Figures 7 and S6) indicates that a possible scaffolding function of MKP5 could contribute to its negative regulation of IRF3, which will be the subject of future investigation.

It has been shown that ERK and JNK were able to phosphorylate IRF3 at Ser171 and Ser173, respectively (Moore and Petro, 2013; Zhang et al., 2009a). Inhibition of p38 suppressed IFNβ expression in response to H5N1 influenza infection (Börgeling et al., 2014). The minimal change of MAP kinase activation caused by the deficiency of MKP5, together with the direct MKP5-IRF3 interaction and IRF3 dephosphorylation by MKP5, leads us to believe that increased IRF3-type I IFN response in MKP5 KO cells and animals in response to RNA virus infection is mainly due to direct regulation of IRF3 by MKP5. Nevertheless, we did not exclude the possible contribution of MAP kinases to the increased IRF3 activity observed in MKP5 KO cells in this study.

Finally, we found that the increased innate immune responses caused by the deficiency of MKP5 resulted in less severe disease upon influenza virus infection and protected the mice from influenza lethal infection (Figure 1), despite the reduced antigen-specific $CD4^+$ and $CD8^+$ T cell responses. The reduced antigen-specific $CD4^+$ and $CD8^+$ T cell responses in MKP5 KO mice could be due to an intrinsic defect of T cells caused by MKP5 deficiency as we showed previously (Zhang et al., 2004) or reduced virus replication due to enhanced type I IFN responses or both. The function of MKP/DUSP proteins in immune regulation of RNA viral disease may represent a pathway to be further explored for therapeutic purposes.

EXPERIMENTAL PROCEDURES

Mice and Viruses

MKP5 KO mice generated previously (Zhang et al., 2004) were crossed with C57BL/6 mice for 12 generations. WT and KO pups from the same heterozygous breeders (MKP5+/− × MKP5+/−) were used to establish the MKP5 WT and KO colonies and the two colonies were maintained separately. All animals were housed in ABSL2 conventional clean animal facility. The animal experiments were performed in accordance with the Singapore National Advisory Committee for Laboratory Animal Research Guidelines. The protocol was reviewed and approved by the National University of Singapore Institutional Animal Care and Use Committee (identification number: 2013-05890).

The influenza virus A H1N1 strain A/Puerto Rico/8/34 (PR8), NS1-deficient PR8 (PR8-NS1), and the mouse adapted influenza A H3N2 virus strain A/Aichi/2/68 (Narasaraju et al., 2009) were propagated by allantoic inoculation of 10-day embryonated chicken eggs. Virus titers were determined as PFU on MDCK monolayers.

Statistical Analysis

Statistical significance was determined using two-tailed Student's unpaired t test. Data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$.

More experimental procedures can be found in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.02.030.

AUTHOR CONTRIBUTIONS

Y.Z. conceived and designed the study and wrote the manuscript. S.J.J., H.J., and H.T. performed the experiments and data analysis. H.T. Y.S., T.S., and N.Y. performed AlphaScreen assay and analysis. C.W.P. and M.C.P. contributed to the study on influenza NS1-related work. H.X., V.T.K., J.M.R., R.A.F., and C.D. contributed reagents/analytic tools and critical review of the manuscript.

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